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Development and Characterization of Triazine Based Dendrimers for Delivery of Antitumor Agent

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In the present study we developed the novel kind of triazine dendrimers by utilizing differential reactivity of the cyanuric chloride (triazine trichloride) which overcome the limitations associated with the others classes of dendrimers like toxicity, low yield, high synthesis cost etc. Triazine dendrimers were synthesized by divergent method using triazine trichloride as core and diethanolamine as branching unit to avoid the use of protecting group and functional group interconversion up to third generation. These hydroxyl terminated dendrimers were characterized by FTIR, ^{1}HNMR, ^{13}CNMR, ES mass spectroscopy, and by Elemental analysis. The yield of pure G3 dendrimers was 63%. This novel dendrimers increases the aqueous solubility of hydrophobic drug Paclitaxel up to 0.562 mg/ml as well as showed control release behavior. Hemolytic and toxicology studies of this dendrimer in mice showed no adverse toxicity to the kidneys and the liver up to 200 mg/kg dose (i.p). Triazine being a hydrophobic compound, the core of this dendrimer is hydrophobic and supposed to easily incorporate the hydrophobic guest while presence of hydroxyl group on periphery increases its water solubility and reduces its toxicity; and thus it is useful in various fields like gene delivery, MRI contrasting agents, vaccines or as solubilization tool.

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Keywords: Dendrimers, Paclitaxel, Triazine Trichloride, Diethanolamine, Solubilization.

1. INTRODUCTION

Dendrimers are versatile, derivatisable, monodispersed, highly branched, well-defined, compartmentalized polymers with sizes and physicochemical properties resembling those of biomolecules like proteins.[1] The structure of these materials has great impact on their physical and chemical properties.[2] Due to their unique behavior, dendritic molecules have made significant contributions in many fields including but not limited to drug delivery,[3] protein mimicry,[4] gene transfection,[5] quantum dots and nano dots,[6] sensors and detectors,[7] image contrast agents,[8] catalysis[9] and separations.[10] Dendrimers bearing more than one type of peripheral as well as branching groups can be well prepared, but many times their synthesis, requires numerous steps, which is sometimes challenging.[11]

Because of the wide range of applicability of this macromolecule, efforts are continuing to improve the efficiency of dendrimers in the drug delivery and to reduce its synthesis cost. Although, polyamidoamine (PAMAM) and polypropylene imine (PPI) dendrimers are most researched category of dendrimers, however, this offers some limitations. The synthesis up to higher generation is necessary to achieve highly branched globular structures,[12] which is probably difficult and time consuming. These also provides less hydrophobic interior compared to dendrimers having aromatic core.[13] However, in spite of their broad applicability, toxicity associated due to the terminal $-NH_2$ groups (cationic charge), limits these candidatures for their clinical applications.[14] Fréchet and Hawker[15] developed less toxic dendrimers using polyaryl ether, but they have poor water solubility. Several research groups routinely synthesized new dendrimers or modified the previously established class of dendrimers to overcome these limitations.[16]

Approaches to develop a simple and less toxic dendrimers for drug delivery is an attractive and promising area of research in dendrimer chemistry. Number of cores and branching units has been earlier utilized for the development of new dendrimers. Some important cores used include diaminobutane, ethylenediamine, ammonia, amino acid, bis(4-fluorophenyl) sulfone, carbosilane etc. however, this list can never be completed.

1,3,5-Triazine derivatives have been known for a long period of time.[17] Triazine and its derivatives found widespread applications in the pharmaceutical, textile, plastic, and rubber industries, and have successfully been used as pesticides, dyestuffs, optical bleaches, explosives,

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and surface active agents.[18] The chemistry of this group of compounds has been studied intensively and well reviewed.[19] Triazine trichloride is one of the versatile derivatives of triazine family, first prepared in 18th century. It is readily obtained though the cyclotrimerization of cyanogen chloride using activated charcoal as the catalyst.[20] Because of its versatility it can be the promising core used in dendritic synthesis.

Triazine trichloride as a "core" adding the new era in dendrimer synthesis[21] because the branching necessary for dendrimer growth results from direct substitution onto the core, rather than through functional groups appended to the core as well as exhibits the aromatic nature and temperature dependent substitution. Triazine trichloride has become the molecule of choice for synthesis of novel class of dendrimers as it possesses numerous advantages including its ability to diversify the chemical functionality without using the protecting groups and the nucleophilic aromatic substitution on its ring occurs sequentially in temperature dependent manner thus no need of functional groups manipulation required. The aromatic interior of these molecules makes them significantly more hydrophobic likely to favor noncovalent sequestration of hydrophobic guests.[13] The synthetic versatility of this system suggests that more "composition space" can be examined with these molecules.[22]

The differential reactivity of triazine trichloride is manipulated in two ways, i.e., temperature and nucleophilicity (Scheme 1). Steffensen and Simanek[23] demonstrated differential reactivity of triazine trichloride with various amines. Many types of triazine based dendrimers have been reported in the literatures[24] out of which melamine dendrimers reported by Simanek group was extensively explored for its drug delivery ability.[25]

Dreyer et al. synthesized triazine dendrimers, by utilizing dialkylamine as linker molecule. However, number of other molecules can be used as linker molecules like diethanolamines, p-aminobenzylamine etc.[26] for synthesis. Further, Simanek and Hollink[27] described synthesis of functionally diverse macromolecules, using Boc-protected $NH(CH_2CH_2NH_2)_2$ as a linker by utilizing nucleophilic aromatic substitution reactions.

Paclitaxel (Taxol®) is a promising anti-tumor agent with poor aqueous solubility used widely in the treatment of ovarian and breast cancer. Intravenous administration of Paclitaxel formulation containing non-aqueous vehicle cremophor EL causes allergic reactions and precipitation on aqueous dilution. Paclitaxel lacks functional groups that are ionisable in a pharmaceutically useful range and therefore manipulation in pH does not enhance its solubility. Furthermore, common approaches to improve solubility like addition of charged complexing agents or by producing alternate salts of the drug are not feasible in case of paclitaxel.[28] Moreover, the extensive clinical use of this drug is somewhat hampered due to lack of appropriate delivery vehicles. This clearly establishes the need for the development of alternate formulation of Paclitaxel (PTX) having good aqueous solubility and at the same time free from any side effects. Various approaches and novel carriers like cosolvents, emulsions, micelles, liposomes, nanoparticles, cyclodextrins, microspheres and prodrug are employed to resolve the problems associated with the PTX delivery.

Dendrimers are also the carrier of choice to deliver PTX safely because of its unique characteristics and therefore many research groups worked extensively to establish the ability of dendrimers for PTX delivery.[29]

In the present study, we have synthesized and characterized triazine trichloride "core" based novel dendrimers having diethanolamine and triazine trichloride both, as branching units in their structural framework for improved delivery of Paclitaxel. In this sequence earlier Namazi and Adeli[30] synthesized dendritic triblock copolymer using triazine trichloride and diethanolamine as A and B block. The reaction conditions in the reported study were simple, excluding protection and deprotection steps with high yield.

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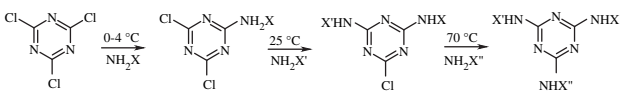

Scheme 1. Temperature dependent nucleophilic aromatic substitution of triazine trichloride where *X*, *X′*, *X″* denotes alkyl, halogens, or any substituting group.

2. EXPERIMENTAL DETAILS

2.1. Materials

Triazine trichloride was purchased from Sigma Aldrich Chemicals Pvt. Ltd. (India) and used as received. Acetone, Tetrahydrofuran (THF) methanol was purchased from Qualigens Fine Chemicals (India), 1-4 Dioxane was purchased from Loba Chemie Pvt. Ltd. (India) and diethanolamine and sodium bicarbonate was from Central Drug House (P) Ltd. (India). All the reagents and solvents for the synthesis and analysis were used as received. FTIR studies were carried out in the range of 600–4000 cm^{-1} using FTIR-470, Jasco (UK), instrument through KBr disc and pellet method. ^1H NMR and ^{13}C NMR spectra were recorded at 400 MHz in Brucker Avance II 400 (Germany). The electrospray (ES) mass spectra were recorded on Micromass Quattro II triple quadrupole mass spectrometer.

2.2. Synthesis Method

2.2.1. Generation 1

Cyanuric chloride (4 g, 21.69 mmol) was dissolved in 50 mL of tetrahydrofuran (THF) containing sodium bicarbonate ($NaHCO_3$; 65.07 mmol) and placed on ice bath.

Diethanolamine (6.5 mL, 65.07 mmol) in excess was added drop wise in the solution of cyanuric chloride at 0 °C with stirring. Solution was stirred at 0 °C for 1.5 hr; for 12 hr at room temperature, and then refluxed for 28 hr at 100 °C. Product was washed with acetone and methanol and dried in vacuum to get the G1 dendrimers which was dark brown in color and honey like in consistency with 7.8 g yield.

IR: 3364 cm^{-1} (νO–H), 2963 cm^{-1} (νC–H), 1738, 1621 cm^{-1} (νC=N), 1064 cm^{-1} (νC–O).

^{1}*H NMR (In* D_2O*)*: δ 3.21–3.25 (12H) (Triazine-N-CH_2), δ 3.86–3.89 (12H) (CH_2–OH).

2.2.2. Generation 1.5

Generation 1 dendrimers (7 g, 17.94 mmol) was dissolved in 40 mL of methanol containing $NaHCO_3$ (122.83 mmol) and kept in ice bath. Cyanuric chloride (19.81 g, 107.47 mmol) was dissolved in 200 mL of THF. The G1 dendrimers solution was added in solution of cyanuric chloride with stirring at 0 °C. The solution was stirred for 1.5 hr at 0 °C and for 1 hr at room temperature, and then refluxed for 6 hr, filtered, washed with acetone and dried under vacuum; a white colored solid product was formed with 12.4 g yield.

IR: 2779 cm^{-1} (νC–H), 1716, 1621 cm^{-1} (νC=N), 1053 cm^{-1} (νC–O), 767 cm^{-1} (νC–Cl).

^{1}*H NMR (In DMSO-d6)*: δ 3.06–3.08 (12H) (Triazine-N-CH_2), δ 3.75–3.78 (12H) (CH_2–O-Triazine).

2.2.3. Generation 2

Generation 1.5 dendrimers (8 g, 6.25 mmol) was dissolved in 100 mL of 1,4 dioxane containing $NaHCO_3$ (75.10 mmol). To this solution diethanolamine (7.25 mL, 75.10 mmol) was added in excess, drop wise, with stirring. The solution was stirred at room temperature for 6 hr. A clear solution was formed which was then refluxed for 28 hr at 100 °C. After refluxing the solution was cooled, filtered, and solvent was evaporated in vacuum to get the product, which was washed with acetone and methanol and dried in vacuum. The product was light brown color powder with 7.2 g yield.

IR: 3405 cm^{-1} (νO–H), 2876 cm^{-1} (νC–H), 1641, 1487 cm^{-1} (νC=N), 1056 cm^{-1} (νC–O)

^{1}*H NMR (In* D_2O*)*: δ 3.50–3.53 (12H) (Triazine-N-CH_2), δ 3.99–4.02 (12H) (CH_2–O-Traizine), δ 3.19–3.22 (48H) (Triazine-N-CH_2), δ 3.82–3.83 (48H) (CH_2–OH).

2.2.4. Generation 2.5

Generation 2 dendrimers (6 g, 2.85 mmol) was dissolved in 50 mL of methanol containing $NaHCO_3$ (68.5 mmol) and placed on ice bath at 0 °C. Cyanuric chloride (12.63 g, 68.5 mmol) was dissolved in 100 mL of THF and to this solution, G2 dendrimers was added slowly with stirring at 0 °C. The solution was stirred for 1.5 hr at 0 °C and for 1 hr at room temperature, and then refluxed for 6 hr, filtered washed with acetone and dried under vacuum; a white solid product was formed with 10.6 g yield.

IR: 2779 cm^{-1} (νC–H), 1777, 1716, 1647 cm^{-1} (νC=N), 1052 cm^{-1} (νC–O), 766 cm^{-1} (νC–Cl).

^{1}*H NMR (In DMSO-d6)*: δ 3.06–3.08 (60H) (Triazine-N-CH_2), δ 3.70–3.72 (60H) (CH_2–O-Traizine).

2.2.5. Generation 3

Generation 2.5 dendrimers (8 g, 1.41 mmol) was dissolved in the 100 mL of 1,4 dioxane containing $NaHCO_3$ (67.94 mmol). To this solution diethanolamine (6.56 mL, 67.94 mmol) was added in excess drop wise with stirring. The solution was stirred at room temperature for 6 hr. A clear solution was formed which was then refluxed for 28 hr at 100 °C. After refluxing, the solution was cooled; filtered and evaporated the solvent in vacuum to get the product that was washed with acetone and methanol, purified by column chromatography to get the pure dendrimers, which was light brown in color with 8 g yield.

FTIR: 3413 cm^{-1} (νO–H), 2872 cm^{-1} (νC–H), 1611, 1487 cm^{-1} (νC=N), 1061 cm^{-1} (νC–O).

^{1}*H NMR (In* D_2O*)*: δ 3.53–3.56 (60H) (Triazine-N-CH_2), δ 3.90–3.94 (60H) (CH_2–O-Traizine), δ 3.42–3.44 (192H) (Triazine-N-CH_2), δ 3.66–3.69 (192H) (CH_2–OH).

^{13}*C NMR* (*In* D_2O*)*: 48 ppm (Triazine-N-CH_2), 59 ppm (CH_2–O-Traizine), 56 ppm (CH_2–OH), 158 ppm (Triazine Portion).

Calcd: $[M]^+$ $C_{345}H_{600}N_{156}O_{126}$ mass = 8949.5, Found: ESI: $[M+H]^+$ mass = 8950.

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2.3. Solubilization Studies

Solubilization of Paclitaxel using triazine dendrimers was done according to the phase solubility studies performed by Higuchi and Connors.[31] Briefly, known amount of drug (in excess) was added separately into 12 screw-capped vials (USP Type I glass) (10 ml) containing different concentrations (0.55×10^{-2}, 1.11×10^{-2}, 1.67×10^{-2}, and 2.23×10^{-2} mM) of triazine dendrimers (1.0, 2.0, 3.0 G) in distilled water (4 vials for each generation). Separately known amount of drug (in excess) was also added in a vial containing only distilled water as control. Vials were shaken for 48 hr in a metabolic shaker (Indian Equipment Corporation, Mumbai, India) at room temperature and allowed to stand for 24 hr to attain equilibrium. Solutions were filtered using nylon membrane filter of pore size 0.45 μm (Pall Gelman Sciences, USA) and methanol (5 ml × 5 times) was passed through the same membrane filter to remove out insoluble Paclitaxel from filter and each fraction of methanol was analyzed by HPLC,[32] performed on reverse phase Zorbax® C18 column (25 cm × 0.45 cm) at λ_{max} 227 nm (UV detector) after proper dilution to determine indirectly the concentration of PTX in

dendrimers solution i.e., solubility. To establish the effect of pH on solubilization ability of dendrimers, solvent of different pH (4, 7.4 and 10) was used instead of distilled water.

2.4. *In Vitro* Studies

2.4.1. *Drug Loading and Release*

Generally two approaches have been followed for loading of drug within dendritic construct i.e., by inclusion complex or by conjugation. In the present study we used inclusion complex approach for loading of PTX because it is a hydrophobic drug and hence can be physically entrapped in the hydrophobic region of the triazine dendrimers. Drug (PTX) was loaded in dendrimers by reported procedure with minor modifications.[33] Briefly, known amount of drug (in excess) was added to the dendrimer solution (2.23×10^{-2} mM in 10 ml of distilled water) and the mixture was stirred for 72 hr at room temperature. This mixture was then filtered with 0.45 μm (Pall Gelman Sciences, USA) membrane filter, and methanol (5 ml × 5) was passed through the same membrane filter to remove out unencapsulate Paclitaxel from filter and each fraction of methanol was analyzed by HPLC to determine indirectly the amount of drug encapsulated within dendrimers. The filtrate was lyophilized and used for further studies.

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Drug release from known amounts of PTX loaded triazine dendrimers was determined using modified dissolution method[34] with slight modification. The release medium used was the PBS (pH 7.4) having Tween 80 (0.1%). The dialysis bag (MWCO 3000 Da) was filled with the solution of PTX loaded triazine dendrimers (50 mg in 1 ml of distilled water) and placed in 50 ml of release medium at 37 °C with slow magnetic stirring under sink conditions. Aliquots of 1 ml were withdrawn from the release medium and replenished with the same volume of fresh medium to maintain perfect sink condition. The aliquots were analyzed after proper dilution in HPLC at 227 nm to know the amount of drug release from the system.

2.5. Toxicity Studies

2.5.1. *Hemolysis Study*

The RBC suspension was obtained by following reported procedure for hemolytic studies.[35] Briefly, the human blood was collected in HiAnticlot blood collection vials (Himedia Labs, India). The RBCs were separated out from the blood by centrifugation and diluted with saline to make the RBC suspension. Dendrimer solution (in saline) of concentration 0.5, 1.0, 1.5, 2.0 and 5.0 mg/mL (0.5 mL) was added to the RBC suspension (4.5 mL) and incubated for 1 hr and 24 hr separately. After centrifugation, supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 540 nm (λ_{max} of Hb).

2.5.2. *Acute In Vivo Toxicity Study*

The BALB/c mice of uniform weight (20 ± 2 g) and 3–4 weeks old were cared under a protocol approved by the university animal ethical committee of Dr. H. S. Gour University, Sagar (M.P). BALB/c mice were housed at constant temperature (22 ± 2 °C) and humidity (50–60%) and with 12 hr light/dark cycle, and fed a standard diet *ad libitum*. The mice were assigned in 6 groups, each group containing 6 animals. First five groups were administered the 40, 60, 100, 200 and 500 mg/kg of dendrimers solution (in saline) via I.P route, respectively. The last group served as control and was administered only saline solution. After 48 hr blood was collected from each mice by retro orbital plexus and analyzed for blood urea nitrogen (BUN) level and for serum glutamine pyruvate transaminase (SGPT) level in local pathology laboratory.

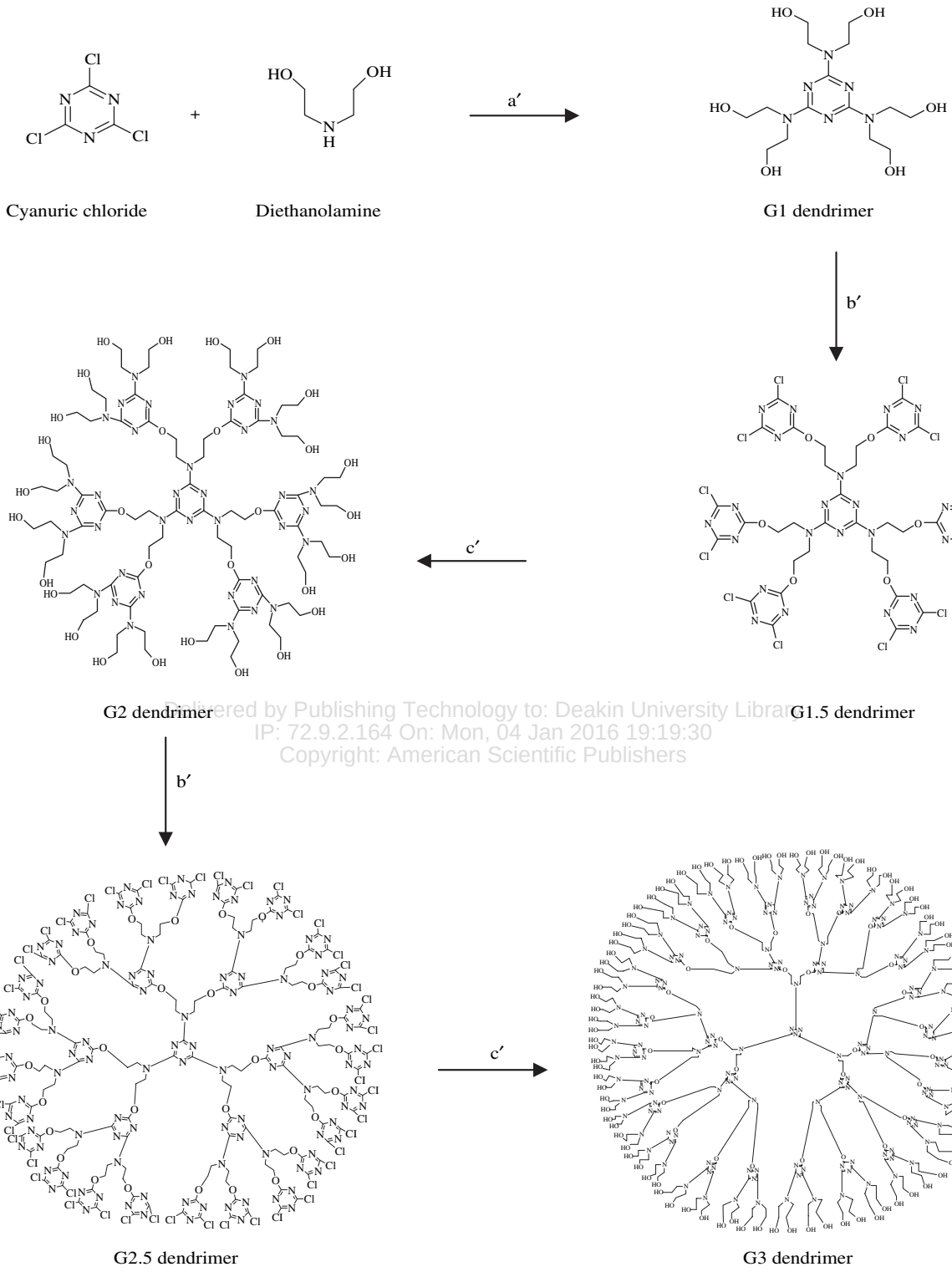
Data Analysis and Statistics: Statistical analysis was performed using Student's *t*-test with Prism 4 software (GraphPad Software, San Diego, CA). For all statistical analysis, the difference was considered significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Synthesis

The chemoselectivity of triazine trichloride for different amines are taken into account for the synthesis of this novel triazine based dendrimers. Nucleophilic aromatic substitution reaction took place when triazine trichloride was allowed to react with diethanolamine with the release of HCl. The proposed dendrimers synthesis has been exclusively developed for the first time in our laboratory. Since the secondary amine present on diethanolamine is more nucleophilic as compared to hydroxyl group therefore the dendrimer growth proceeds in chemoselective fashion (Scheme 2). One equiv of triazine trichloride was allowed to react with 3 equiv of diethanolamine to get generation 1 (G1) dendrimers which was dark brown in color and honey like consistency. The substitution on triazine ring takes place similarly as shown in Scheme 1. To increase the generation, G1 dendrimers was allowed to react with triazine trichloride at 0 °C, and at room temperature, giving generation 1.5 (G1.5) dendrimers which was white powder, insoluble in water having chlorine group on the periphery. Similarly diethanolamine, triazine trichloride and again diethanolamine were attached step-by-step respectively to the half and full generation of dendrimers to develop dendrimers up to third generation (G3) with 63% yield. $NaHCO_3$ was used in each synthesis step to neutralize the released HCl. The full practical descriptions of G1–G3 dendrimers was given in Table I.

In each reaction step, simply washing the reaction mixture with acetone can remove both the excess triazine trichloride and diethanolamine because both are soluble in



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Scheme 2. Synthetic Strategy of Triazine Dendrimers. Reagent and conditions: (a′) THF, $NaHCO_3$, 0–2 °C → 1.5 h, RT (room temperature) 25 ± 2 °C → 12 h, 100 ± 2 °C → 28 h (b′) triazine trihloride, THF, Methanol, $NaHCO_3$, 0–2 °C → 1.5 h, RT → 1 h, 60 ± 2 °C → 6 h (c′) Diethanolamine, Dioxane, $NaHCO_3$, RT → 6 h, 100 ± 2 °C → 28 h.

acetone whereas dendrimers (half and full generations) are insoluble in acetone thus minimizing the chances of formation of side product in further steps. Full generation dendrimers was washed with methanol to removes sodium chloride formed during neutralization as salt was insoluble in methanol. Additionally, G2 and G3 dendrimers were purified by column chromatography using silica gel to obtained product free from impurities.

Table I. Physical description of triazine dendrimers.

Dendrimer generation	Molecular formula	Appearance color/form	Theoretical molecular weight	Aqueous solubility	Group at periphery (number)
G1	$C_{15}H_{30}N_6O_6$	Dark brown/Semisolid	390.78	Soluble	OH (6)
G1.5	$C_{33}H_{24}Cl_{12}N_{24}O_6$	White/Solid	1278.24	Insoluble	Cl (12)
G2	$C_{81}H_{144}N_{36}O_{30}$	Brown/Solid	2101.92	Soluble	OH (24)
G2.5	$C_{153}H_{120}Cl_{48}N_{108}O_{30}$	White/Solid	5651.76	Insoluble	Cl (48)
G3	$C_{345}H_{600}N_{156}O_{126}$	Light brown/Solid	8949.50	Soluble	OH (96)

3.2. Characterization

During synthesis the growth of dendrimer is preliminarily detected by its water solubility, color and by TLC. G1, G2 and G3 dendrimers were freely soluble in water and dark brown to light brown in color. On the other hand G1.5 and G2.5 dendrimers were insoluble in water and white in color. TLC plates were run for identifying the consumption of reactants and formation of products.

In G1, G2 and G3 Dendrimers, hydroxyl groups were present on periphery in contrast to chlorine groups on the periphery of G1.5 and G2.5 dendrimers. This is the basic difference in the structure of different generation of dendrimers. Thus in FTIR spectroscopy G1, G2 and

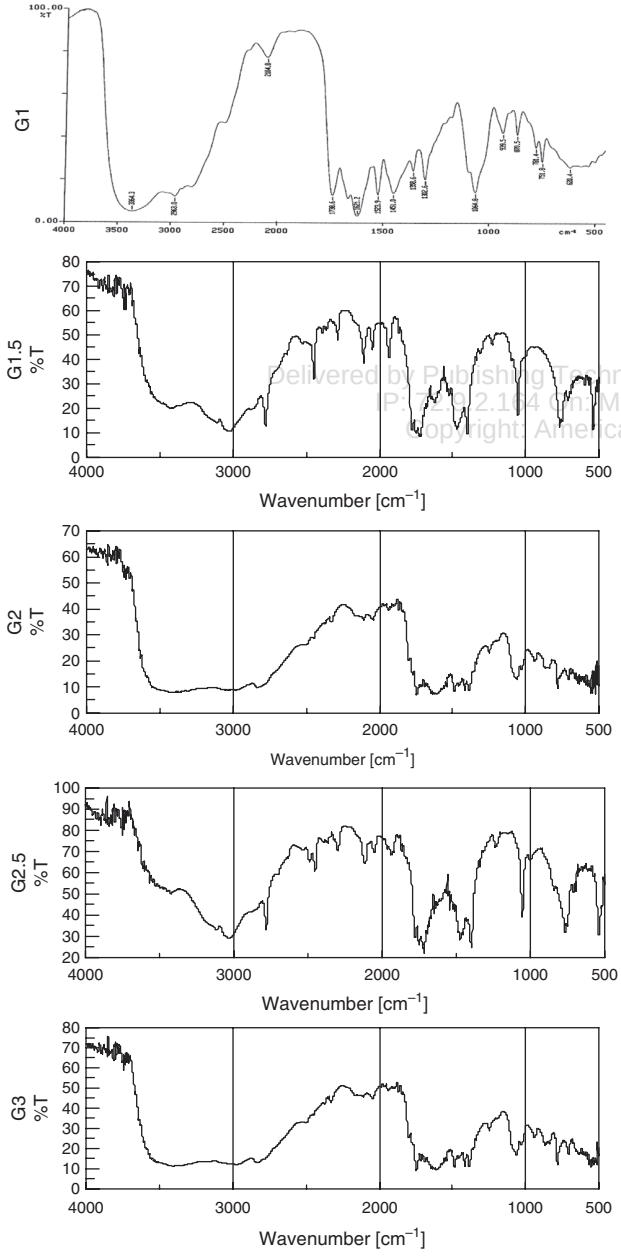

Fig. 1. FTIR spectra of triazine dendrimers.

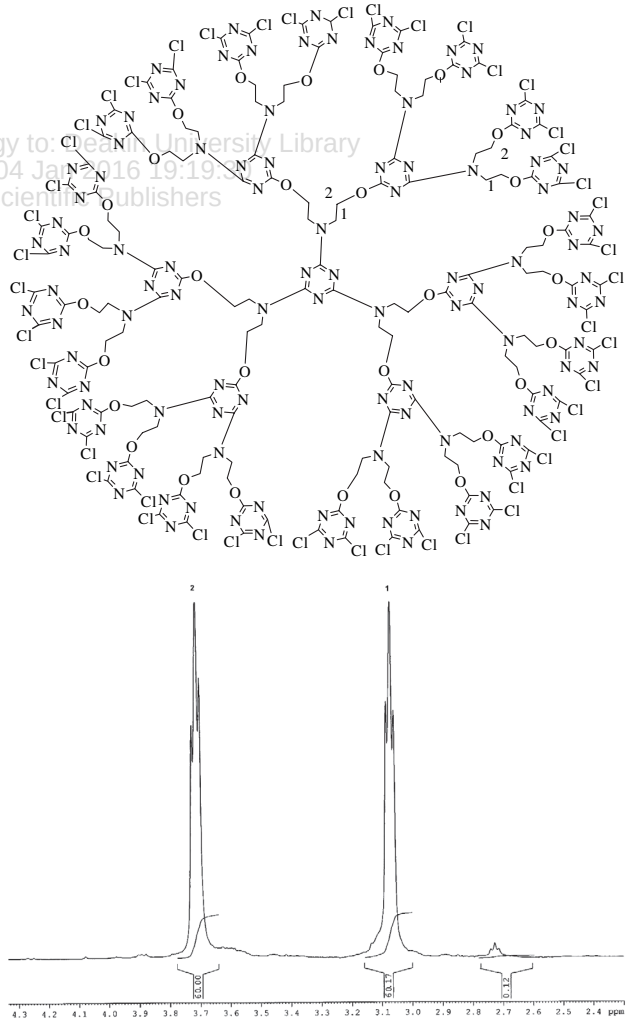

Fig. 2. ^{1}H NMR Investigation of G2.5 Dendrimers.

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G3 dendrimers showed O–H streching at 3364, 3405, and 3413 cm^{-1} and C–Cl stretching was absent whereas in G1.5 and G2.5 O–H stretching was not detected and C–Cl stretching was detected at 767 and 766 cm^{-1} (Fig. 1).

In ^1H NMR spectroscopy two triplets of methylene groups were observed in G1, G1.5 and G2.5 dendrimers due to the presence of CH_2 groups in two different environments (Fig. 2). In G2 and G3 dendrimers four triplets were detected because CH_2 groups were present in four different environments (Fig. 3). In the structure of dendrimer it was clearly seen that protons were present in the form of methylene group hence triplets were detected by NMR spectroscopy. The instrument detected single triplet for the numbers of methylene groups present in the same environment whereas chemical shifts of the peaks were changed. The number of protons detected in NMR was matching with the theoretical numbers. Absence of side products in ^1H NMR during these experiments should be conspicuously noted.

Additionally we characterized G3 triazine dendrimers by ^{13}C NMR and by ESI mass spectroscopy for confirming carbon atoms present in the structure of dendrimer and for determining its exact mass respectively. Carbon atoms in triazine ring are all in same environment and therefore single peak for triazine ring was detected at 158 ppm. Carbon of CH_2 groups are present in three different environment i.e., in first and second ring N–CH_2, CH_2-Triazine whereas in third ring first methylene group was present in same environment i.e., N–CH_2 but another carbon was in different environment i.e., CH_2–OH. Therefore three peaks were detected by NMR spectroscopy for the carbon of methylene groups present in the G3 triazine dendrimers. By ESI mass spectra exact mass was found to be 8950 Dalton (Fig. 4).

The results of elemental (CHN) analysis showed that the experimental percent of elements were matching with the theoretical percentage (Table II). By this result we concluded that the generation of dendrimers increases because in even generation the percent hydrogen is more (because of addition of diethanolamine) compared with the odd generation (because of addition of cyanuric chloride). All the data suggests that the synthesis of dendrimers up to third generation is feasible in step-by-step manner.

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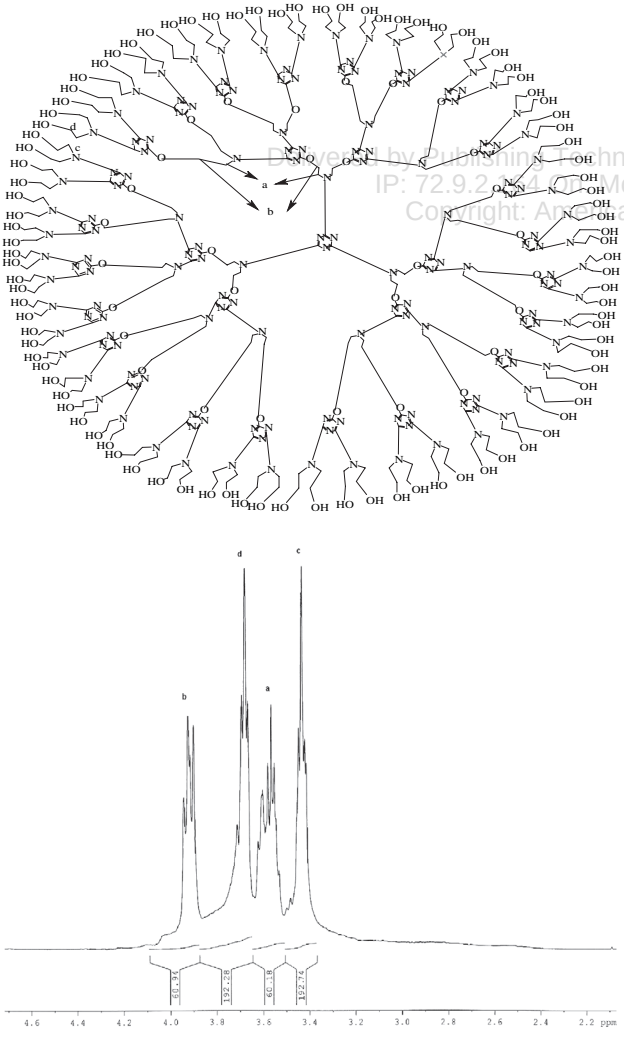

Fig. 3. ^1H NMR investigation of G3 dendrimers.

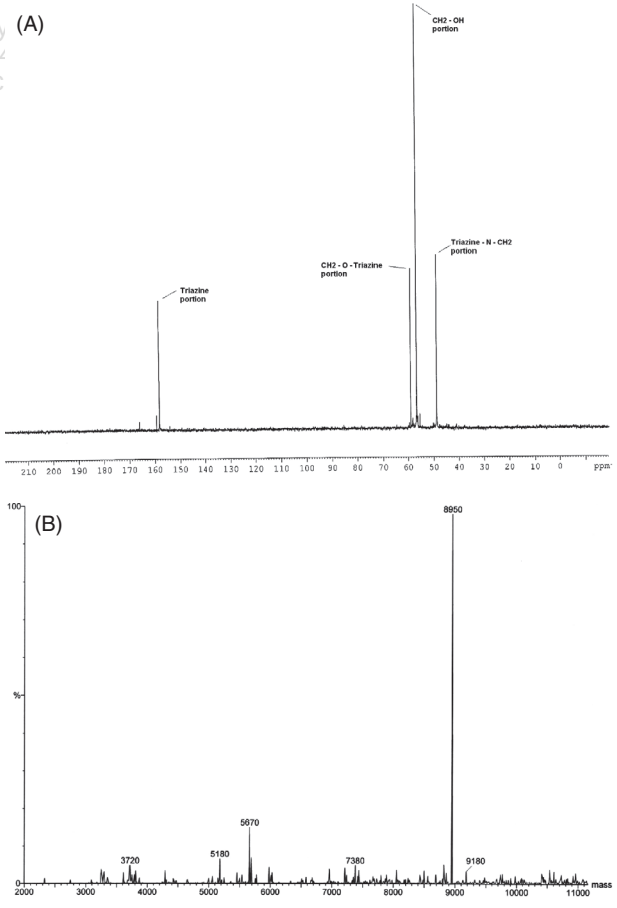

Fig. 4. ^{13}C NMR (A) and ESI mass spectra (B) of G3 Dendrimers.

Table II. Elemental analysis data of triazine dendrimers.

Dendrimers	Percentage of element present					
	Theoretical			Experimental		
Generation	C	H	N	C	H	N
G1.0	46.14	7.74	21.52	46.12	7.72	21.50
G1.5	31.01	1.89	26.30	31.03	1.86	26.30
G2.0	46.28	6.90	23.99	46.28	6.89	24.00
G2.5	32.51	2.14	26.76	32.50	2.12	26.76
G3.0	46.30	6.76	24.41	46.30	6.75	24.40

3.3. Solubilization Efficiency of Dendrimers

Dendrimers as unimolecular micelles are extensively used as a solubilizing tool to improve the aqueous solubility of poorly soluble agents thus increase their bioavailability as well as reduced toxicity. Novel triazine dendrimers have internal hydrophobic region (triazine rings) and external hydrophilic region (hydroxyl groups) thus act as micelles and use to enhance the solubility. Paclitaxel aqueous solubility was found to be enhanced with the use of different generations and concentrations of triazine dendrimers. Aqueous solubility of PTX increases with increase in dendrimers concentration. Solubility of PTX in water (0.0003 mg/ml) was enhanced up to 0.562 mg/ml with the use of triazine G3 (2.23×10^{-2} Mm concentration) dendrimers. It was shown that increasing dendrimers generation increased the aqueous solubility of PTX (Fig. 5). Thus, for further solubilization studies only G3 triazine dendrimers was used. Triazine ring imparts hydrophobic interaction whereas hydroxyl groups present on the periphery take part in hydrogen bonding thus, the mechanism involved in the solubility enhancement could be either hydrophobic interaction or hydrogen bonding or both.

While performing the solubility study of PTX at different pH, it was concluded that at pH 4 triazine dendrimers displayed maximum enhancement in aqueous solubility compared to pH 7.4 and 10 (Fig. 6).

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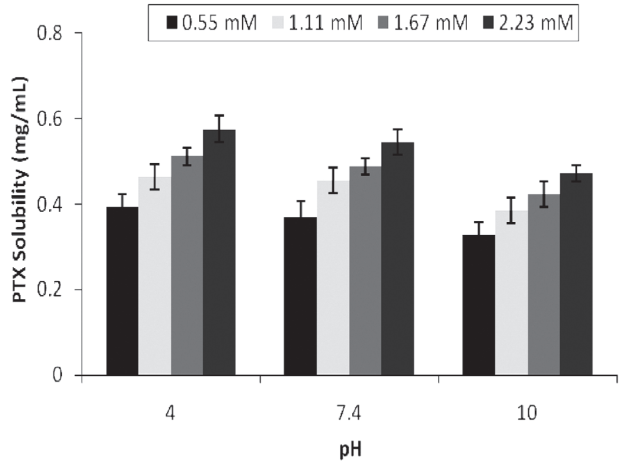

Fig. 6. Effect of pH of solvent on solubilizing ability of triazine dendrimers ($n = 3$).

The order of the ability of triazine dendrimers to solubilize PTX at different pH was pH 4.0 > pH 7.4 > pH 10.0. The reason behind this order might be due to degradation of drug at higher pH.[36]

3.4. Encapsulation Efficiency and *In Vitro* Release Profile

Novel triazine dendrimers showed 24.34 wt% of drug loading (PTX) inside its hydrophobic region. PTX loaded in dendrimers showed 81% of drug release after 24 hr, whereas nearly similar quantity of free PTX (85%) was released only after 4 hr, suggesting the controlled release property of dendrimers (Fig. 7). The release behavior of PTX from triazine dendrimers exhibited a non-linear release profile by showing initial faster release followed by slower release. The reason behind this phenomenon was possibly the $\pi-\pi$ interaction between aromatic triazine ring and PTX.

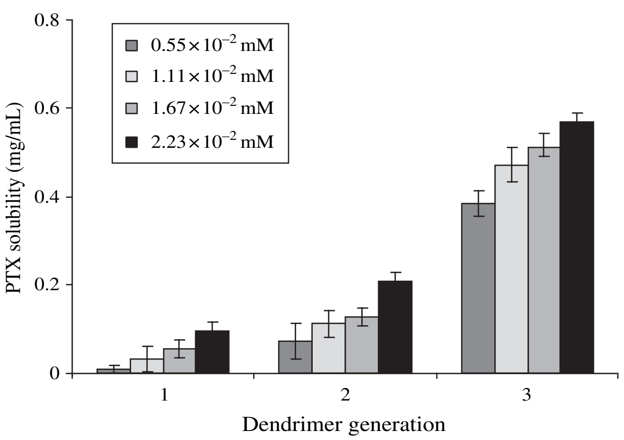

Fig. 5. Effect of the generations of triazine dendrimers on aqueous solubilization of PTX ($n = 3$).

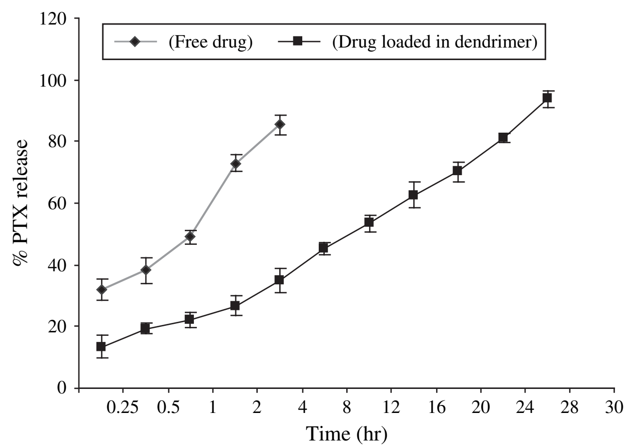

Fig. 7. Cumulative release patterns of free PTX and PTX loaded in dendrimers ($n = 3$).

3.5. Toxicity Studies

Cationic macromolecules in general cause destabilization of the cell membrane resulting in cell lysis. Thus amino-terminated dendrimers were shown to be generally cytotoxic comparatively anionic and neutral dendrimers. Previously hydroxyl- or methoxy-terminated dendrimers based on a polyester scaffold were shown to be non toxic both *in vitro* and *in vivo*.[37] Hemolytic study gives quantitative measure of haemoglobin (Hb) release from RBCs and the data obtained in such assays gives a qualitative indication of potential damage to RBCs on dendrimers administration. Many researchers's used this study as a preliminary tool to determine the membrane polymer interaction. The novel triazine dendrimer was found to be non-hemolytic up to concentration of 5 mg/mL after 1 hr incubation but hemolytic after 24 hr showed concentration-dependent and time dependent hemolysis (Fig. 8). This may be due to the aromatic interior of dendrimers which causes haemolysis through hydrophobic membrane contact. In contrast, toxicity study on melamine dendrimers-derivatives showed that the cationic dendrimers were more haemolytic (at both 1 and 24 h) than anionic or PEGylated melamine dendrimers.[38] In acute toxicity study effects of triazine dendrimers on renal function were evaluated by change in BUN level, all the groups of mice (except group 5) did not show any significant change in BUN level when compared to the control group after 48 hr of injection. The effects of triazine dendrimers on hepatic function were evaluated by changes in SGPT activity in serum. None of the groups of mice (except group 5) showed any significant change in SGPT activity when compared to the control group after 48 hr of injection (Fig. 9). However, a statistically significant increase in BUN and SGPT level was observed in those mice which are in group 5th after 48 hr of injection. The results showed by novel triazine dendrimers in toxicity study suggested that this dendrimer was safe in BALB/c mice up to dose of 200 mg/kg via IP route. The inherent toxicity of this dendrimers was less compared to Simanek's cationic triazine dendrimers[13] which concluded that synthesized triazine dendrimers may also be a possible candidate for biomedical and clinical applications. We concluded that the *in vivo* toxicity of this molecule to liver and kidneys is low enough to justify that this dendrimer is suitable for tumor inhibition studies.

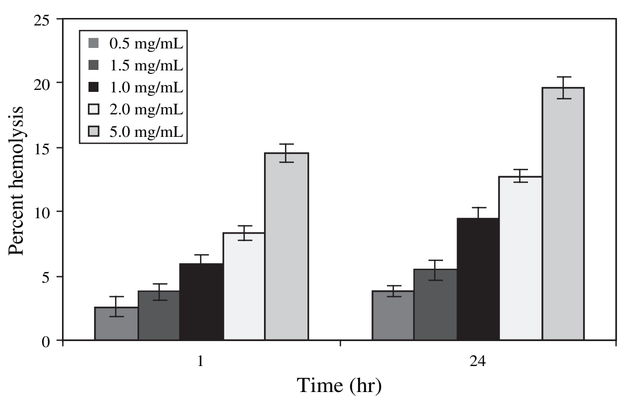

Fig. 8. Percent hemolysis by 3.0G triazine dendrimers of different concentration on human RBCs after 1 and 24 hr exposure ($n = 3$).

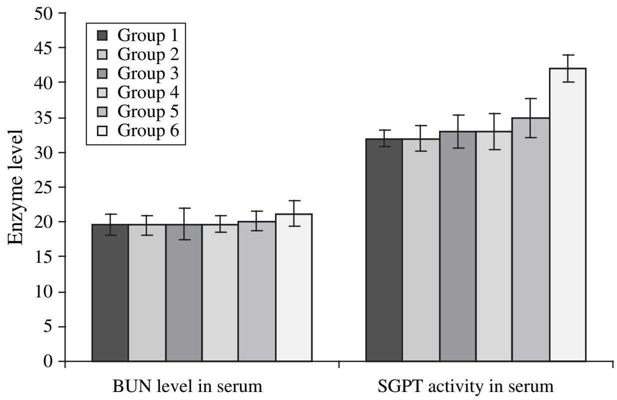

Fig. 9. Change in serum enzyme level post dosing in mice ($n = 3$).

4. CONCLUSION

Our laboratory is constantly engaged in exploring dendrimers potentiality as drug delivery vehicle. By taking this background in our mind we synthesized cost effective triazine based dendrimer up to third generation with 63% yield using divergent method because the convergent method of dendrimer synthesis is not feasible for large scale synthesis. This triazine dendrimer can be synthesized without using protecting groups thus it must be somewhat economical and easy to synthesize compared to other existing triazine dendrimers. The synthesis was accomplished in five steps from commercially available materials, requiring extraction, evaporation or recrystallization to purify the intermediate dendrimer generations. Thus this synthetic strategy will overcome the problems associated with the synthesis of other classes of dendrimers like tedious and lengthy method of preparation, low yield, high synthesis cost and cytotoxicity associated with cationic dendrimers because the proposed triazine dendrimers have hydroxyl group on the surface so it may be biocompatible and thus this carrier system may be useful in various clinical applications. Further exploration of this novel class of dendrimer is underway and its further possible utility in drug delivery applications is being examined in our laboratory.

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