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Research Article

Development, Characterization of Hydroxyl Terminated Dendritic Macromolecules as Prospective Drug Carriers

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Abstract

The principal aspiration is to develop triazine dendrimers as potential drug carriers for sustained release. Triazine based dendrimer was synthesized by divergent method evading protection/ deprotection or functional group interconversion. Synthesized dendrimers were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR and ESI-Mass spectrometry. Synthesized full generation dendrimers G1, G2 and G3 were applied as solubility enhancers of hydrophobic drug ketoprofen. Ketoprofen was loaded by G3 dendrimer by inclusion complex method. Sustained release of ketoprofen from ketoprofen loaded dendrimer was studied and compared to of free ketoprofen. Cytotoxicity and hemolytic assay of dendrimer was studied to evaluate toxicity of dendrimer as drug vehicle.

Keywords: Triazine based dendrimer; ketoprofen; sustained release; cytotoxicity; hemolysis

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1. Introduction

Formulation of hydrophobic or poorly water soluble drugs of one of the most demanding issues in pharmaceutical research and development [1] as poor solubility of hydrophobic drugs leads to decreased bioavailability [2]. At present, liposomes and polymeric drug delivery systems are used for drug delivery of hydrophobic drugs. However, liposomal drug delivery suffers from limitations such as low stability, difficult to target specific tissues, toxicity and adverse side effects [3]. Whereas polydispersity of linear polymers is a limitation of polymeric drug delivery systems [4].

Dendrimers are nano-sized macromolecules blessed with many exceptional properties such as high terminal functionality, narrow polydispersity index, presence of internal voids and compact globular structure [5, 6]. They are attractive aspirants for many applications the field of drug delivery [7, 8], water remediation [9, 10], sensors [11], blood substitution [12] etc.

Dendrimers based on triazine have grown in last two decades [13]. Due to temperature controlled nucleophillic substitution of triazine trichloride, triazine dendrimer can be synthesized by facile route without protection/deprotection or manipulation of functional group. So, limitations associated with synthesis of other classes of dendrimers can be avoided [14, 15]. Previously it was reported that triazine dendrimers with hydroxyl groups on periphery and aromatic core solubilizes and encapsulate hydrophobic drugs due to hydrophobic interaction and hydrogen boding [14, 16]. It was also reported that hydroxyl- or methoxy-terminated dendrimers based on a polyester scaffold were less toxic both *in vivo* and *in vitro* [17]. This prompted us to design dendritic architecture with similar features as prospective drug carrier.

In this paper, we have reported synthesis generation 3 dendrimer based on N,N'-bis(4,6-dichloro-1,3,5-triazin-2-yl)-[1,1'-biphenyl]-4,4'-diamine as core [15]. Synthesized dendrimer generations were characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR and ESI-Mass spectrometry. Full generation dendrimer G1, G2 and G3 were used as solubility enhancers of ketoprofen. Effect of pH, generation and concentration of dendrimer on ketoprofen solubilisation was studied. Ketoprofen was loaded by dendrimers using inclusion complex technique. Sustained release study of ketoprofen loaded dendrimer was carried out and compared with free ketoprofen. Cytotoxicity and hemolytic assay was carried out to evaluate toxicity of dendrimers.

2. Experiment

2.1 Materials

Triazine trichloride, 4,4’-diaminobiphenyl, acetone, dichloromethane (DCM) and methanol were purchased from Sigma-Aldrich Ltd. Sodium hydroxide was purchased from Merck Ltd. All the reagents and solvents for the synthesis and analysis were used as received. Ketoprofen was generously provided by A.R. College of Pharmacy, Vallabh Vidhyanagar. Acid phthalate buffer (pH 4.0), Borate alkaline buffer (pH 10.0) and Phosphate buffer saline (pH 7.4) were prepared according to Indian Pharmacopoeia (1996)

2.2 Analytical Techniques

FTIR was carried out in the range of 250–4000 cm^{-1} using Perkin Elmer-Spectrum RX-FTIR spectrometer instrument through KBr disc and pellet method for solid samples or nujol mull method for liquid samples. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 400 MHz in Brucker Avance II 400 (Germany) using TMS as internal standard using either D_2O or DMSO-d6 as solvents. Mass spectra were recorded on Waters Micromass Q-ToF Micro (USA) instrument equipped with electrospray ionization. Shimadzu UV-1800 spectrophotometer was used to measure absorbance of ketoprofen at its characteristic wavelength of 260nm.

2.3 Methods

2.3.1 Synthesis of N,N'-bis(4,6-dichloro-1,3,5-triazin-2-yl)-[1,1'-biphenyl]-4,4'-diamine (core)

Cyanuric Chloride (0.02mmol) was dissolved in dichloromethane and kept in an ice bath. A solution of a 4,4'-diaminobiphenyl (0.01mmol) containing sodium hydroxide (0.02 mmol) in water was added drop wise in the solution of cyanuric chloride at 0-5 ℃ with stirring. The solution was stirred at 0-5 ℃ for 2 hrs. Then the solution was filtered, washed with methanol and acetone and dried under vacuum: A white colored solid was formed.

Yield: 75 %.; FTIR (KBr, cm^{-1}) *v*: 3261 (Secondary NH- Stretching), 3011, 2963 (Aromatic C-H stretching), 1723, (Aromatic C=N stretching), 796 (C-Cl stretching).; ^{1}H-NMR (400 MHz, DMSO) δ ppm: 7.2902-7.3250 (d, 4H, Ar-H-), 7.5160-7.6165 (d, 4H, Ar-H-) ^{13}C-NMR (75 MHz, DMSO) δ ppm: 116.94 (Ar-C-), 128.10 (Ar-CH), 136.23(Ar-CH), 149.70 (Ar-C-NH Carbon), 163.49 (triazine C-N), 167.97 (triazine C-Cl); ESI-Mass: Calculated Weight 480; Obtained (m/z); 481 (M+1), 482 (M+2).

2.3.2 Synthesis of Generation 1 Dendrimer (G1)

N,N'-bis(4,6-dichloro-1,3,5-triazin-2-yl)-[1,1'-biphenyl]-4,4'-diamine (0.01mmol) was dissolved in an excess of diethanolamine (0.04mmol) which was used as both solvent and reactant. The resulting mixture was refluxed for 2 hrs. After cooling, it was dispersed and washed by acetone repeatedly to give generation 1 dendrimer which was light brown colored with honey like consistency.

Yield: 68%.; FTIR (Nujol, cm^{-1}) *v*: 3391 (O-H stretching), 3086, 2954 (Aromatic C-H stretching), 1728, 1643 (aromatic C=N stretching), 1045 (C-O stretching).; ^{1}H-NMR (400 MHz, D_2O) δ ppm: 3.6144-3.7006 (16H, -N-CH_2-CH_2-OH), 3.8114-3.8958 (t, 16H, -N-CH_2-CH_2-OH), 7.3285-7.3609 (t, 4H, Ar-H), 7.4651-7.4910 (t, 4H, Ar-H); ^{13}C-NMR (75 MHz, D_2O) δ ppm: 49.90 (-N-CH_2-CH_2-OH), 60.12(-N-CH_2-CH_2-OH), 118.26 (Ar-C-), 128.81 (Ar-CH-), 136.41 (Ar-CH-), 149.84 (Ar-C-NH), 163.63 (triazine C-N), 167.69; ESI-Mass: Calculated molecular weight 754; Obtained 755 (M+1).

2.3.3 Synthesis of Generation 1.5 Dendrimer (G1.5)

Cyanuric chloride (0.08mmol) was dissolved in dichloromethane and kept in an ice bath. A solution of G1 dendrimer (0.01mmol) containing sodium hydroxide (0.08 mmol) in water was added drop wise in the solution of cyanuric chloride at 0-5 ℃ with stirring. The solution was stirred at 0-5 ℃ for 2 hrs and

refluxed for 6 hrs. Then the solution was filtered, washed with methanol and acetone and dried under vacuum: A white colored solid was formed.

Yield: 78%.; FTIR (KBr, cm^{-1}) *v*: 3214 (Secondary N-H stretching), 3053, 2884(aromatic C-H stretching), 1717, 1753 (aromatic C=N stretching), 1051 (C-O stretching), 786 (C-Cl stretching); ^{1}H-NMR (400 MHz, DMSO) δ ppm: 3.9612-4.0236 (m, 16H, -N-CH_2-CH_2-O-tri), 4.1437-4.1901 (m, 16H, -N-CH_2-CH_2-O-tri), 7.4589-7.4889 (t, 4H, Ar-H), 7.5285-7.5710 (t, 4H, Ar-H); ^{13}C-NMR (75 MHz, DMSO) δ ppm: 60.12 (-N-CH_2-CH_2-O-tri), 68.96(-N-CH_2-CH_2-O-tri), 115.50 (Ar-C-), 129.00 (Ar-CH-), 136.10 (Ar-CH), 149.77 (Ar-C-NH), 163.37 (Triazine C-N), 167.11 (Triazine C-0), 169.49 (Triazine C-Cl); ESI-Mass: Calculated Molecular weight: 1938; Found 1939 [M+1].

2.3.4 Synthesis of Generation 2 dendrimer (G2)

G1.5 dendrimer (0.01mmol) was dissolved in an excess of diethanolamine (0.16 mmol) which was used as both solvent and reactant. The resulting mixture was refluxed for 2 hrs. After cooling, it was dispersed and washed by acetone repeatedly to give generation 2 dendrimer which was light brown colored with honey like consistency.

Yield: 68 %.; FTIR (Nujol, cm-^{1}) *v*: 3366 (O-H stretching), 2942, 2881 (aromatic C-H Stretching), 1642 (aromatic C=N stretching), 1044 (C-O stretching);^{1}H-NMR (400 MHz, D_2O) δ ppm: 3.6268-3.7180 (t, 64H, -N-CH_2-CH_2-OH), 3.8232-3.8905 (t, 64H, -N-CH_2-CH_2-OH), 3.9920- 4.0894 (m,16H,-N-CH_2-CH_2-O-tri), 4.1455-4.2222(m, 16H,-N-CH_2-CH_2-O-tri), 7.5141-7.5291 (d, 4H, Ar-H), 7.5942-7.6071 (d, 4H, Ar-H); ^{13}C-NMR (75 MHz, D_2O) δ ppm: 48.50(-N-CH_2-CH_2-OH), 50.04 (-N-CH_2-CH_2-O-tri), 60.60 (-N-CH_2-CH_2-OH), 67.50(-N-CH_2-CH_2-O-tri), 115.00 (Ar-C-), 128.11 (Ar-CH), 136.10 (Ar-CH), 145.21 (Ar-C-NH), 167.70 (triazine C-N), 169.45 triazine (C-N-(CH2-CH2-O-)$_2$), 170.80 (Triazine C-O), 179.10 (Triazine C-N(CH2-CH2-OH)$_2$ ESI-Mass: Calculated Molecular Weight: 3037; Found 3038 (M+1)

2.3.5 Synthesis of Generation 2.5 Dendrimer (G2.5)

Cyanuric Chloride (0.32mmol) was dissolved in dichloromethane and kept in an ice bath. A solution of G2 dendrimer (0.01mmol) containing sodium hydroxide (0.32 mmol) in water was added drop wise in the solution of cyanuric chloride at 0-5 ℃ with stirring. The solution was stirred at 0-5 ℃ for 2 hrs and refluxed for 6 hrs. Then the solution was filtered, washed with methanol and acetone and dried under vacuum: A white colored solid was formed.

Yield: 78%.; FTIR (KBr, cm^{-1}) *v*: 3210 (Sec. N-H stretching), 3118, 3030 (Aromatic C-H), 1680, 1710 (Aromatic C=N stretching), 1054 (C-O stretching), 773, 753 (C-Cl); ^{1}H-NMR (400 MHz, DMSO) δ ppm: 3.9910-4.0340 (m, 80H, -N-CH_2-CH_2-O-tri), 4.1268-4.2023 (m, 80H, -N-CH_2-CH_2-O-tri), 7.5018-7.5129 (d, 4H, Ar-H), 7.5620-7.5708 (d, 4H, Ar-H); ^{13}C-NMR (75 MHz, DMSO) δ ppm: 49.12 (outer -N-CH_2-CH_2-O-tri), 51.00 (inner -N-CH_2-CH_2-O-tri), 60.04 (outer-N-CH_2-CH_2-O-tri) 67.15(inner -N-CH_2-CH_2-O-tri), 117.11 (Ar-C), 129.00 (Ar-CH-), 138.10 (Ar-CH), 147.15 (Ar-C-NH), 168.20 (triazine C-N), 169.15 (inner C-N-(CH_2-CH_2-O-)$_2$,) 175.86 (outer C-N-(CH_2-CH_2-O-)$_2$), 178.40 (outer C-Cl), 180.51 (inner triazine C-O); ESI-Mass: Calculate Mole. Wt.; 7771; Found: 7772 (M+1), 7773 (M+2).

2.3.6 Synthesis of Generation 3 Dendrimer (G3)

Generation 2.5 dendrimer (0.01mmol) was dissolved in an excess of diethanolamine (0.64 mmol) which was used as both solvent and reactant. The resulting mixture was refluxed for 2 hrs. After cooling, it was dispersed and washed by acetone repeatedly to give generation 3 dendrimer which was light brown colored with honey like consistency.

Yield: 70 %.;FTIR (Nujol, cm^{-1}) *v*: 3364 (O-H stretching), 2941 (Aromatic C-H stretching frequency), 1619, 1671 (Aromatic C=N stretching), 1068 (C-O stretching);^{1}H-NMR (400 MHz, D_2O) δ ppm: 3.5718-3.6871 (m, 264H, -N-C$\underline{H_2}$-CH_2-OH), 3.8220-3.9356 (t, 264H, -N-CH_2-C$\underline{H_2}$-OH), 4.0420-4.1261(m, 80H,-N-C$\underline{H_2}$-CH_2-O-tri),4.2001-4.2581(m, 16H,-N-CH_2-C$\underline{H_2}$-O-tri), 7.5178-7.5217 (t, 4H, Ar-H), 7.5757-7.5897 (t, 4H, Ar-H);^{13}C-NMR (75 MHz, D_2O) δ ppm: 49.55 (outer -N-$\underline{C}H_2$-CH_2-O-H), 51.74 (inner -N-$\underline{C}H_2$-CH_2-O-tri), 61.74 (outer-N-CH_2-$\underline{C}H_2$-O-H), 67.68 (inner -N-CH_2-$\underline{C}H_2$-O-tri), 116.07 (Ar-$\underline{C}$-), 128.01 (Ar-$\underline{C}$H-), 135.90 (Ar-$\underline{C}$-H), 144.79 (Ar-$\underline{C}$-NH), 168.68 (Triazine $\underline{C}$-N), 169.16 (inner $\underline{C}$-N-(CH_2-CH_2-O-)$_2$), 175.15 (outer Triazine $\underline{C}$-O), 178.02 (outer $\underline{C}$-N-(CH_2-CH_2-O-)$_2$), 180.20;ESI-Mass: Calculated Molecular Weight: 12166 Found: 12167 (M+1)

2.3.7 Solubility Study

Solubility study was carried out according to the method described by Higuchi and Connors [18]. Excess of ketoprofen was added to screw-capped vials containing different concentrations (0.6 mmol to 3 mmol) of dendrimer generations in buffers of 4.0, 7.4 and 10 pH. Vials were shaken for 48 h at 37 ℃ in shaking water bath. The vials were centrifuged to remove undissolved ketoprofen and absorbance of ketoprofen were measured at its characteristic wavelength 260 nm using Shimadzu UV-1800 spectrophotometer.

2.3.8 Drug loading

Generally there are two approached for drug loading in dendrimer either by inclusion complex or by conjugation. In present approach we have utilized inclusion complex technique. Drug loading was performed by reported methods with little modifications [14, 19]. A known amount of ketoprofen was added to generation 3 dendrimer G3$(OH)_{128}$ (3 mmol in 10 ml of distilled water) solution. The mixture was stirred for 72 hours at room temperature. The mixture was then filtered and 5 ml of methanol was passed through five times through the filter to remove excess of ketoprofen. Excess Ketoprofen from filter and each fraction of methanol was analyzed by UV spectrophotometer to determine amount of encapsulated drug indirectly.

2.3.9 *In vitro* drug release

Pure ketoprofen was dissolved in methanol (2 mg/ml) and used as control. The prepared ketoprofen loaded dendrimer was dissolved in distilled water at a concentration of 2 mg/ml (the same concentration of ketoprofen as 2 mg/ml pure drug solution). This solution (2 ml in volume) was transferred to a dialysis bag (size cut off = 2.5 nm) immediately. The dialysis bag was placed in a 50 ml-beaker containing 40 ml distilled water. The outer phase was stirred continuously. After a scheduled interval of time for 0.5 hours,

100 µl of sample was withdrawn from the outer phase, and the outer phase was again replenished with 100 µl distilled water. The absorbance of the outer phase was monitored at 260 nm using a spectrophotometer in order to characterize the concentration of ketoprofen.

2.3.10 Hemolysis study [20]

About 5 ml of the human blood from healthy individual was collected in a tube containing heparin. The blood was centrifuged at 1500 RPM for 3 minutes. The supernatant (Erythrocyte) was collected and plasma was discarded. The pellet was washed for 3 times using 0.75% NaCl and centrifuged at 1500 RPM for 5 mins. The cells were resuspended in normal saline to 0.5%. Washed erythrocytes were stored at 4 ℃ and used within 6 hours for the haemolysis assay. To 0.5 ml of cell suspension, 0.5 ml of different concentration of test sample (40, 60, 80 and 100 µg/mL in phosphate buffer saline (pH 7.2)) was added and incubated for 1 hr. After centrifugation, the supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 540 nm. The phosphate buffer saline and distilled water were used as minimal and maximum hemolytic control.

2.3.11 Cytotoxicity Study [20]

The monolayer cell culture was trypsinized and the cell count was adjusted to 3 Lac cells/ ml using medium containing 10% fetal bovine serum. Pre incubate cells at a concentration of 1 × 106 cells/ml in culture medium for 3 hours at 37 ℃ and 5% CO_2. The cells were seeded at a concentration of 5 × 104 cells/well in 100 µl culture medium and incubated at 37ºC in 5 % CO_2 incubator for 24 hrs. After 24 hours, when the monolayer formed, the supernatant was flicked off and added previously diluted with media of 100µl of different concentrations of test extract in microtitre plates and kept for incubation at 37 ℃ in 5 % CO_2 incubator for 48 hours and cells were periodically checked for granularity, shrinkage, swelling. After 48 hours, the sample solution in the wells was flicked off and 10µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37 ℃ in 5% CO_2 incubator. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 570 nm.

2.3.12 Statistical analysis

Data are expressed as the mean, standard deviation (SD) of obtaining results. The statistical analysis of data was performed using analysis of variance (ANOVA) (Graphpad, Version 2.01, San Diego, CA). A value of $p < 0.05$ was considered as statistically significant.

3. Result and Discussion

3.1 Synthesis and Characterization

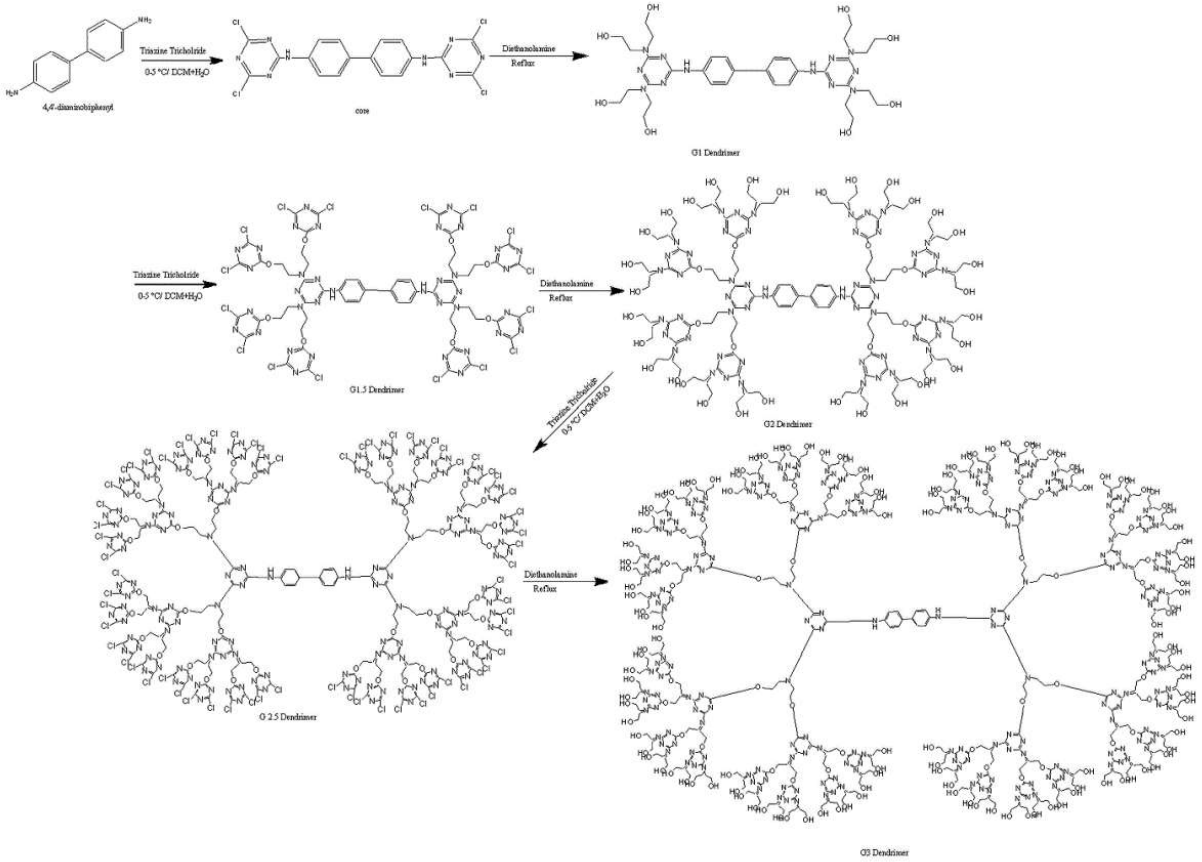

Figure 1 Synthesis of Generation 3 Triazine based Dendrimer

Synthetic route to generation 3 dendrimers is outlined in Figure 1. Temperature controlled nucleophillic substitution of chlorine triazine trichloride and its selectivity towards the hydroxyl to secondary amino group in nucleophile was used throughout [15, 21]. In the first step, one mole of 4,4'-diaminobiphenyl was reacted with two moles of triazine trichoride at low temperature to give *N,N'-bis(4,6-dichloro-1,3,5-triazin-2-yl)-[1,1'-biphenyl]-4,4'-diamine* as core for dendrimer synthesis. Core compound was isolated and purified by washing with acetone and methanol. In the second step, core was reacted with diethanolamine at refluxing temperature to give G1 dendrimer. Full generation dendrimer was purified by dispersing in dichloromethane and acetone. Similar to first step, G1 dendrimer was again reacted with cyanuric chloride to give G1.5 dendrimer. G1.5 dendrimer was then reacted with diethanolamine at reflux to give G2 dendrimer. The above two steps were iterated until G2.5 and G3 dendrimers were obtained. Synthesis was facile, free from protection/ deprotection or interconversion of functional groups with high yields [15, 21].

Half and full generations dendrimer were differ in their solubility and appearance (Table 1). It was observed that hydroxyl terminated dendrimers were freely soluble in water and were light brown liquids.

Whereas chlorine terminated half generation dendrimers were insoluble in water and were white solids. Half generation dendrimers were terminated by hydrophobic triazine rings which may have accounted for their poor water solubility. On the contrary, full generation dendrimers were terminated by hydroxyl groups on the periphery which might have contributed to its good water solubility [24].

Table 1 Physical Description of Dendrimer Generations

Compound	Molecular formula	Appearance	Solubility in water	Surface groups (number)
Core	$C_{18}H_{10}Cl_4N_8$	White solid	Insoluble	Cl (4)
G1	$C_{34}H_{50}N_{12}O_8$	Brown liquid	Soluble	OH (8)
G1.5	$C_{58}H_{42}Cl_{16}N_{36}O_8$	White solid	Insoluble	Cl(16)
G2	$C_{122}H_{202}N_{52}O_{40}$	Brown liquid	Soluble	OH(32)
G2.5	$C_{218}H_{170}Cl_{64}N_{148}O_{40}$	White solid	Insoluble	Cl(64)
G3	$C_{474}H_{810}N_{212}O_{168}$	Brown liquid	Soluble	OH (128)

Table 2 Prominent peaks in Infrared spectrums of Dendrimer Generations

Compound	IR absorption band(cm^{-1}) for functional group		
	O-H	C-Cl	C-O
Core	---	796	---
G1	3391	---	1045
G1.5	---	786	1051
G2	3366	---	1044
G2.5	---	753	1054
G3	3364	---	1068

Infrared spectrum of core showed absorption bands at 3261 cm^{-1} for secondary NH- stretching, 3011, 2963 cm^{-1} for aromatic C-H stretching, 1723 cm^{-1} for C=N stretching of triazine ring and 796 cm^{-1} for stretching of terminal C-Cl groups. As shown in Table 2., Infrared spectrums of full generation dendrimer G1, G2 and G3 dendrimers displayed absorption bands in the range of 3350-3400 cm^{-1}, along with bands in the range of 1500-1700 cm^{-1} for C=N stretching of triazine ring, 3000-2800 cm^{-1} for C-H stretching and 1040-1060 cm^{-1} for C-O stretching which confirmed presence of hydroxyl group. It was noted that absorption band for C-Cl stretching were absent in FTIR spectrums of full generation

dendrimers. Infrared spectrums of chlorine terminated dendrimers showed absorption bands for C-Cl stretching in the range of 700- 800 cm^{-1} , 1500-1700 cm^{-1} for C=N stretching of triazine ring, 3000-2800 cm^{-1} for C-H stretching and 1040-1060 cm^{-1} for C-O stretching. IR spectrums of half generation dendrimers showed absence of bands for O-H stretching. It was also noted that IR spectrums of both full and half generation dendrimers showed absorption bands in the range of 1030-1070 cm^{-1} for C-O stretching and absorption band for C-O stretching was absent in infrared spectrums of core.

^{1}H-NMR spectrum for core [Figure 2 A.] showed two doublets at δ 7.2902-7.3250 ppm and 7.5160-7.6165 ppm for aromatic protons of biphenyl core moiety. Apart for the above peaks for core moiety, ^{1}H-NMR spectrum [Figure 2 B] of G1 dendrimer displayed two additional triplets at δ 3.6144-3.7006 and 3.8114-3.8958 ppm for methylene protons of diethanolamine moiety. In ^{1}H-NMR spectrum of G1.5 dendrimer [Figure 2 C] , the above two triplets were appeared in the downfield region at δ 3.9612-4.0236 and 4.1437-4.1901 ppm as G1.5 dendrimer was terminated by triazine rings. ^{1}H-NMR spectrum of G2 dendrimer[Figure 2 D] showed triplets at δ 3.6268-3.7180, 3.8232-3.8905 for outer diethanolamine moiety, triplets at δ 3.9920- 4.0894 4.1455-4.2222 ppm for inner diethanolamine moiety and peaks at δ 7.5141-7.5291, 7.5942-7.6071 ppm for aromatic protons. ^{1}H-NMR spectrum of G2.5 dendrimer [Figure 2 E] showed two triplets at δ 3.9910-4.0340 and 4.1268-4.2023 ppm as both inner and outer diethanolamine groups were terminated by triazine rings. ^{1}H-NMR spectrum of G3 dendrimer [Figure 2 F] showed triplets at δ 3.5718-3.6871, 3.8220-3.9356 ppm for outer diethanolamine moiety, triplets at δ 4.0420- 4.1261, 4.2001-4.2581 ppm for inner diethanolamine moiety and peaks at δ 7.5178-7.5217, 7.5757-7.5897 ppm for aromatic protons.

^{13}C-NMR spectrum of core [Figure 3. A] showed peaks δ 116.94, 128.10, 136.23, 149.70 ppm for aromatic carbons and δ 163.49, 167.97 ppm for triazine carbons. ^{13}C-NMR of G1 dendrimer [Figure 3. B] showed peaks at δ 49.90, 60.12 ppm for diethanolamine moiety, peaks at δ 118.26, 128.81, 136.41, 149.84 ppm for aromatic carbons and peaks at δ 163.63, 167.69 ppm for triazine carbons. ^{13}C-NMR of G1.5 dendrimer [Figure 3. C] showed peaks at δ 60.12, 68.96 ppm for diethanolamine moiety, peaks at δ 115.50, 129.00, 136.10, 149.77 ppm for aromatic carbon and δ 163.37, 167.11, 169.49 ppm for triazine carbon. ^{13}C-NMR spectrum of G2 [Figure 3. D] dendrimer showed peaks at δ 48.50, 60.60 ppm for outer diethanolamine moiety, peaks at δ 50.0, 67.50 ppm for inner diethanolamine moiety, peaks at δ 115.00, 128.11, 136.10, 145.21 ppm for aromatic carbons and peaks at δ 167.70, 169.45, 170.80 180.90 ppm for triazine carbons. ^{13}C-NMR spectrum [Figure 3. E] of G2.5 dendrimer showed peaks at δ 49.12, 60.04 ppm for outer diethanolamine moiety, peaks at δ 51.00, 67.15 ppm for inner diethanolamine moiety, and peaks at δ 117.11, 129.00, 138.10, 147.15 for aromatic carbons and peaks at δ 168.20, 169.15, 175.86, 178.40, 180.51 for triazine carbons. ^{13}C-NMR spectrum of G3 dendrimer [Figure 3. F] showed peaks at δ 116.07, 128.01, 135.90, 144.79 ppm for aromatic carbons, peaks at δ 51.75, 67.68 ppm for inner diethanolamine moiety, peaks at δ 49.55, 61.74 for outer diethanolamine moiety and peaks at δ 168.68, 169.16, 175. 15, 178.02, 180.20 ppm to triazine carbons.

All the dendrimer generations were characterized by ESI-Mass spectrometry. It was revealed that all molecular ion peaks of dendrimer generations corresponds to their calculated molecular weight [Figure 4 A-F].

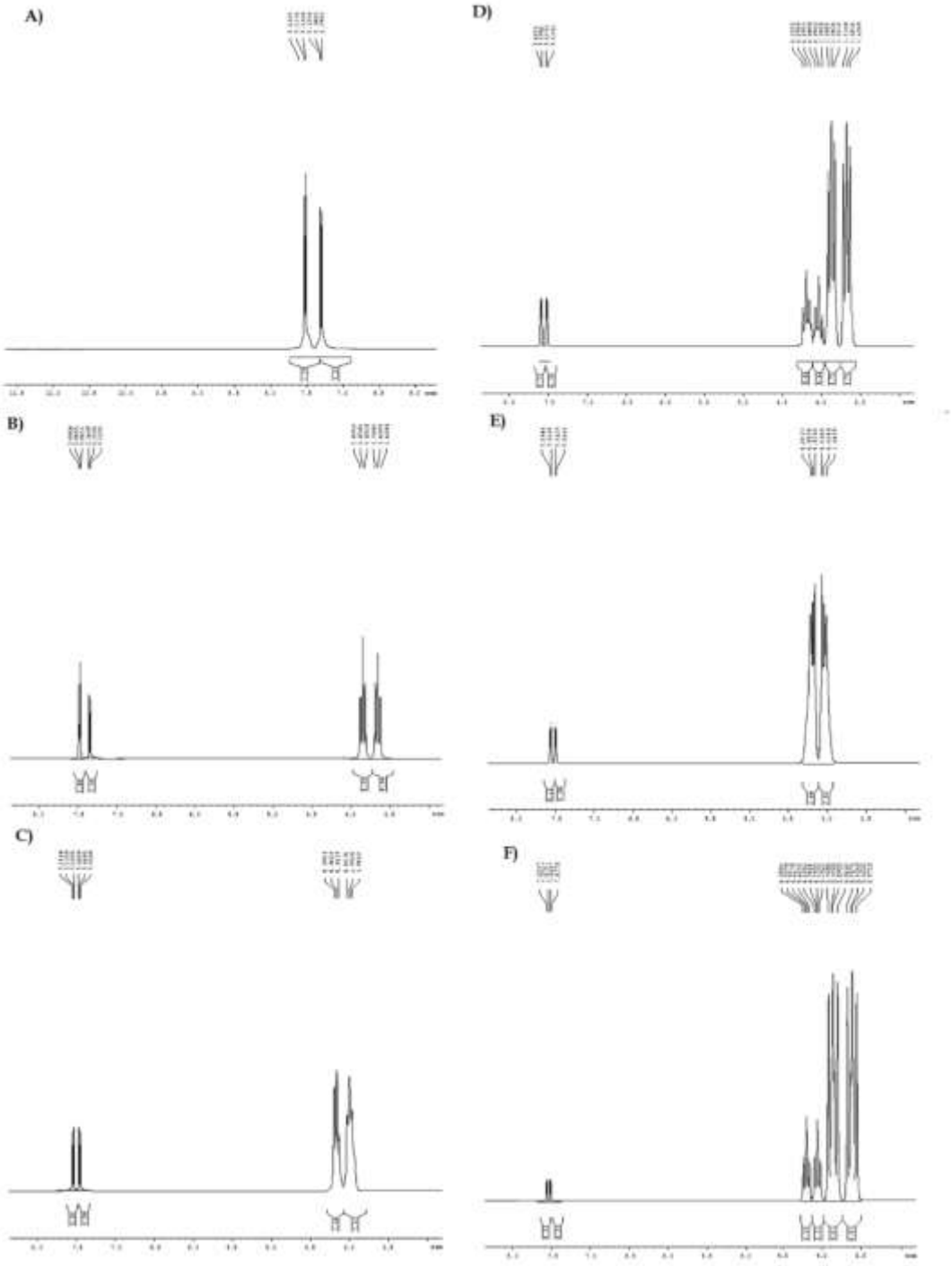

Figure 2 ^{1}H-NMR Spectrum of A) Core, B) G1 dendrimer, C) G1.5 Dendrimer, D) G2 dendrimer, E) G2.5 Dendrimer and F) G3 dendrimer

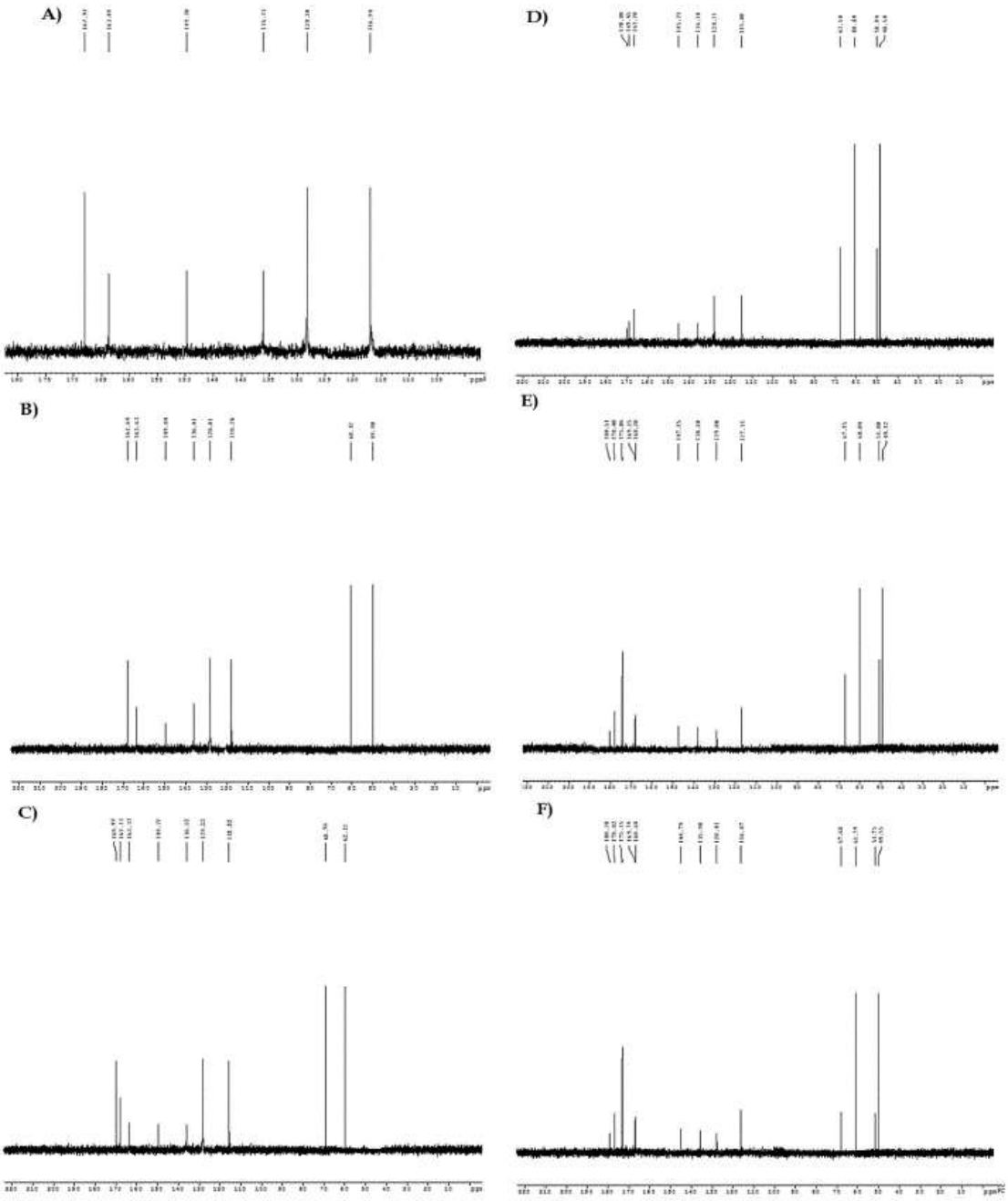

Figure 3 ^{13}C-NMR Spectrum of A) Core, B) G1 dendrimer, C) G1.5 Dendrimer, D) G2 dendrimer, E) G2.5 Dendrimer and F) G3 dendrimer

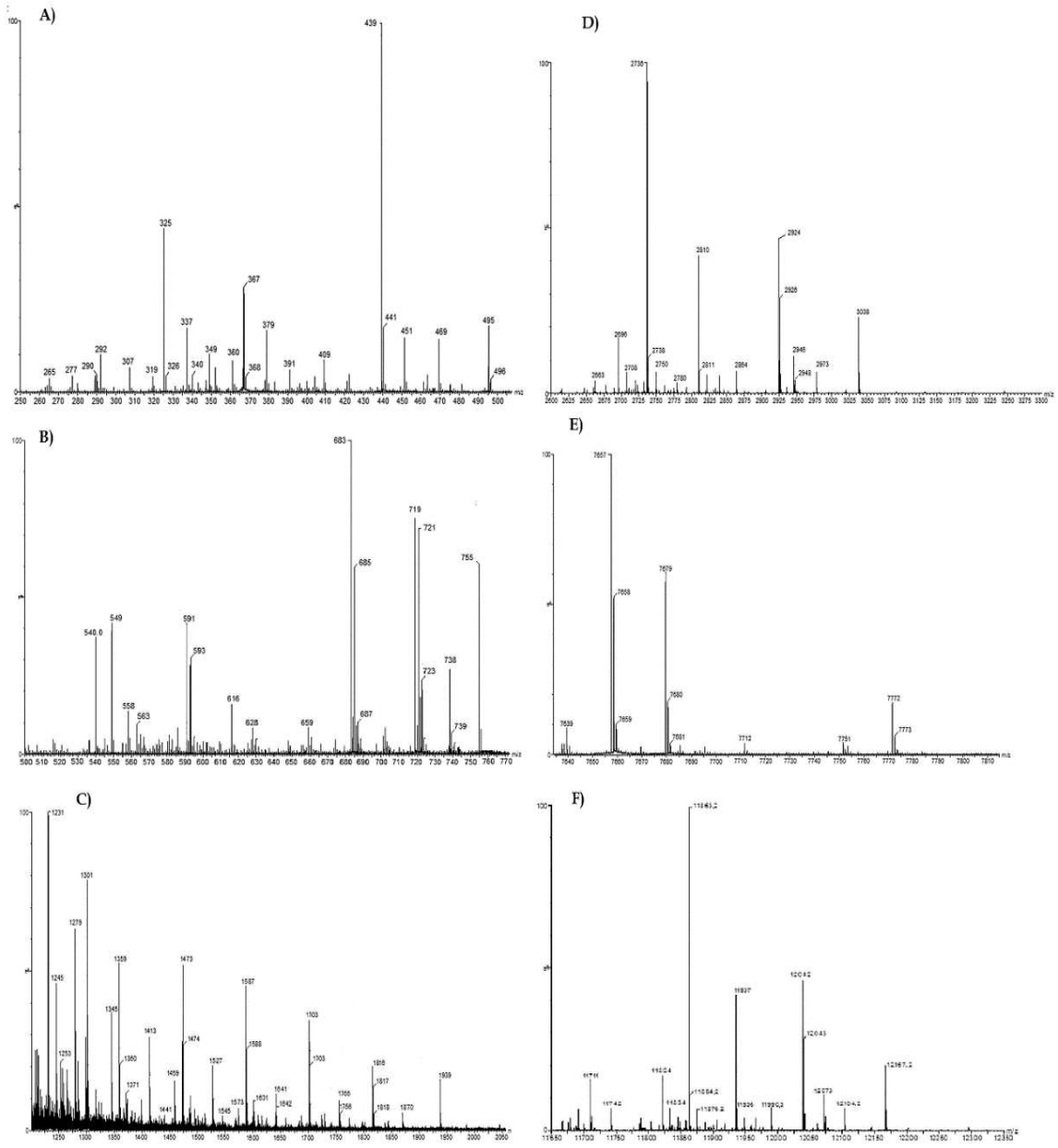

Figure 4 ESI-Mass spectrums of A) Core, B) G1 dendrimer, C) G1.5 Dendrimer, D) G2 dendrimer, E) G2.5 Dendrimer and F) G3 dendrimer

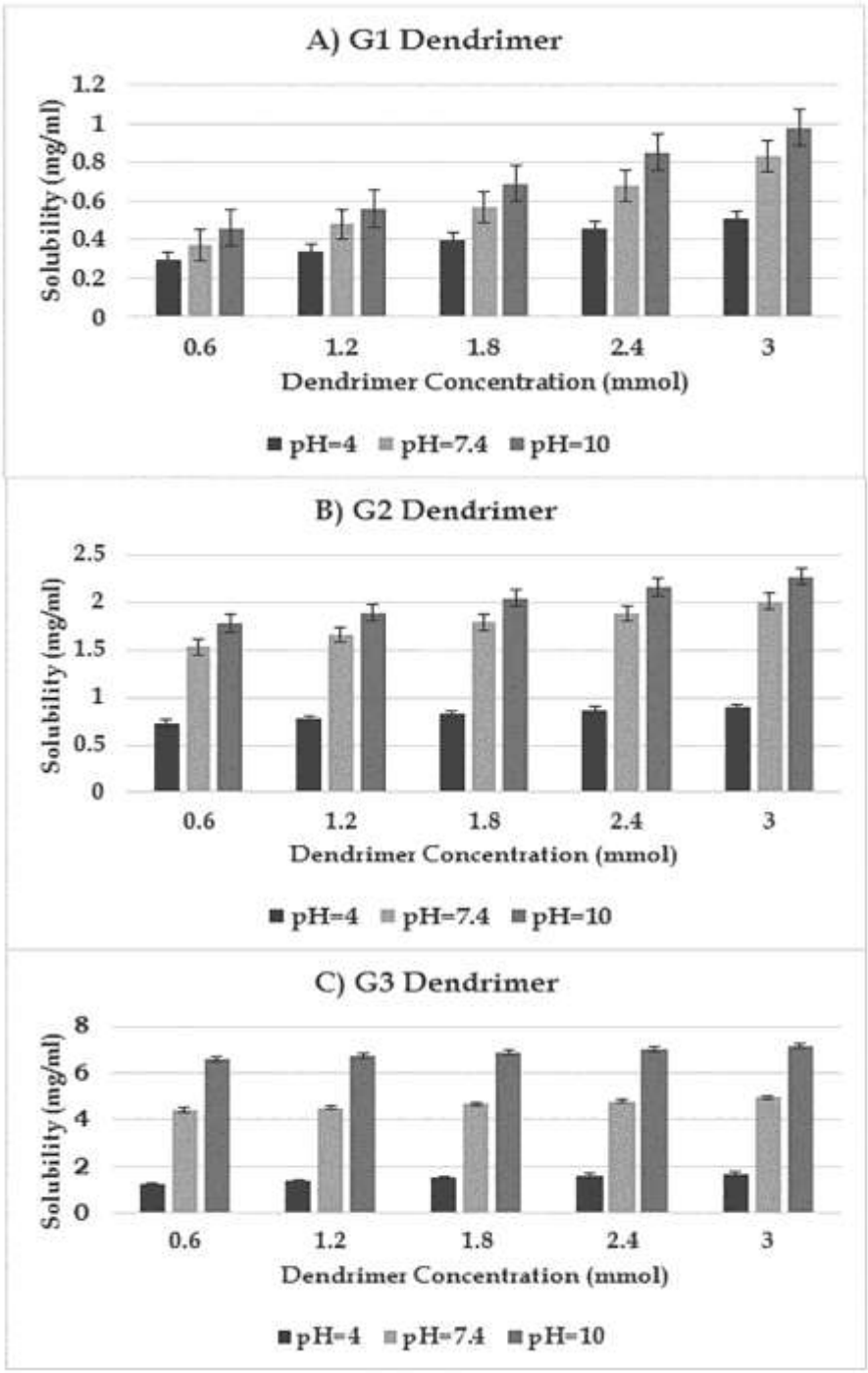

Figure 5 Effect of the generations of triazine dendrimers and pH on aqueous solubilisation of Ketoprofen (n = 3)

3.2 Solubility Study

A series of solubility studies for ketoprofen by dendrimer generations were carried out using different concentrations (0.6 mmol to 3 mmol) of dendrimer generations at pH 4.0, 7.4 and 10.0 [Figure. 5]. Solubility of practically water insoluble drug ketoprofen was improved approximately up to 4 mg/ml by generation 3 dendrimer at pH 7.4. It was also witnessed that with increase in concentration of dendrimer generations, solubility of ketoprofen was increased in a linear manner. It was proposed that as a dendrimer contains a hydrophobic triazine ring in interior regions which may impart hydrophobic interaction and the hydroxyl groups in the exterior, which may impart hydrogen bonding so, thus mechanism for enhanced solubility of ketoprofen by dendrimer could be either hydrophilic interaction or hydrogen bonding or both [16]. It was revealed that with increased in pH generation number of dendrimer. With every increase in generation of dendrimer there was significant increase in surface area, terminal hydroxyl groups and size of dendrimer so, the ability of dendrimer to interact with drug molecule was significantly increased. Hence, solubility of drugs were significantly increased with increase in dendrimer generation [16].

3.3 Sustained Release Study

As maximum drug solubilisation was observed for G3 dendrimer so, it was further explored as carrier for drug delivery. Ketoprofen was loaded into dendrimer by inclusion complex method [14]. Drug loaded dendrimer further characterized by UV spectrophotometer which revealed that about 24.60% of ketoprofen was loaded by dendrimer.

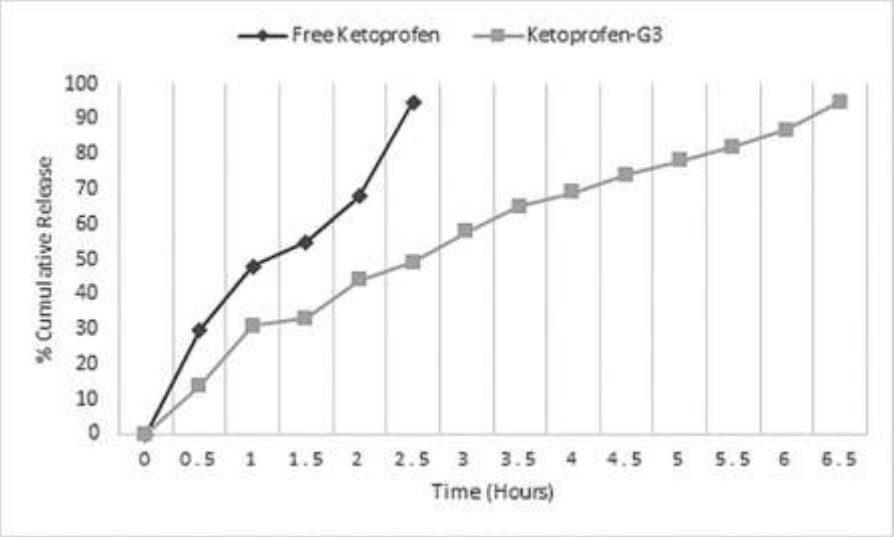

Figure 6 % Cumulative release profile ketoprofen from free ketoprofen and ketoprofen containing G3 dendrimer (n=3)

As shown in Figure 6, about 95% of Ketoprofen was released within 2.5 hours from free ketoprofen. Whereas same quantity of drug was released after 6.5 hours from ketoprofen loaded dendrimers. So, Ketoprofen loaded dendrimer releases ketoprofen slowly compared to free ketoprofen.

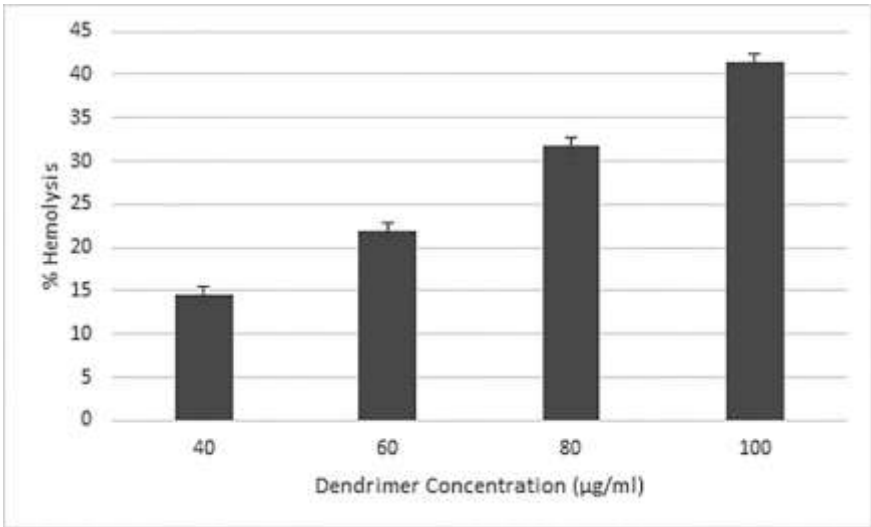

Figure 7 % Hemolysis of Red blood cells by G3 dendrimer after 1 hour of incubation (n=3)

3.4 Hemolysis

Hemolysis assay gives quantitative estimation about hemoglobin release when red blood cells are treated with dendrimers. It was observed that all G3 dendrimer showed concentration dependent hemolysis. However, triazine based G3 dendrimer were less hemolysis compared to in-practice PAMAM dendrimer [Figure 7.][22]. Positively charged amine terminated PAMAM dendrimer interacts with negatively charged surfaces of red blood cells and caused hemolysis [23]. In comparison, G3 dendrimers have less toxicity due to hydroxyl end groups at periphery.

3.5 Cytotoxicity

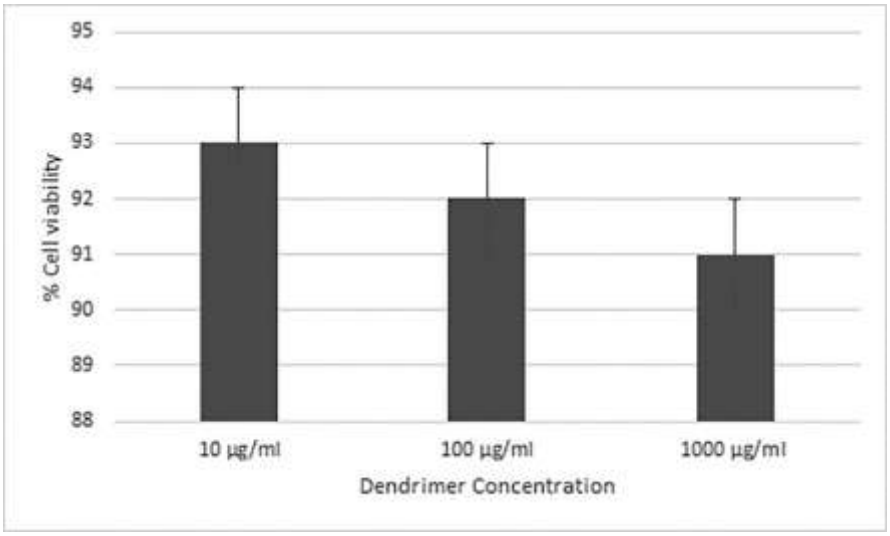

Figure 8 Cytotoxicity of AG3, BG3 and CG3 dendrimers on A-549 cell lines after 48 hours of incubation

Cellular toxicity of G3 dendrimers on A-549 cell lines were investigated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay technique. It is water soluble, yellow colored dye. Living cells are able to converted MTT into water insoluble, blue colored formazan crystals by reductive cleavage of tetrazoluim ring. Formazan crystals extracted by organic solvents and measured at 550 nm, and the result is correlated with living cells to measure cell viability. Our results [Figure 8] displayed that dendrimers showed more than 90% cell viability at concentration levels ranging from 10 µg/ml to 1000 µg/ml. So, synthesized dendrimers having less cytotoxic compare to PAMAM dendrimers.

4. Conclusion

Dendrimer generations were synthesized by facile route obviating protection/deprotection or manipulation of functional groups. Full generation dendrimers has significantly enhanced aqueous solubility of practically insoluble ketoprofen. Sustained release study has revealed that dendrimer released from ketoprofen loaded dendrimers slowly compared to free ketoprofen. Cytotoxicity and hemolytic assay has revealed that dendrimers were less toxic and can be further explored as drug carriers in future

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