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Original Article

NOVEL HYDROXYL TERMINATED DENDRIMERS AS POTENTIAL DRUG CARRIERS: SUSTAINED RELEASE, HEMOLYSIS AND CYTOTOXICITY STUDY

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ABSTRACT

Objective: Potential of novel hydroxyl terminated dendrimer generations $G1(OH)_8$, $G2(OH)_{32}$ and $G3(OH)_{128}$ as solubility enhancers of model drug ketoprofen was evaluated. $G3(OH)_{128}$ dendrimer was further explored as the novel carrier for sustained release of ketoprofen. Cytotoxicity and hemolytic potential of $G3(OH)_{128}$ dendrimer were studied to evaluate toxicity of dendrimer.

Methods: Higuchi and Connors method was employed to evaluate improved solubility of ketoprofen at different pH and dendrimer generation. Ketoprofen was loaded into $G3(OH)_{128}$ dendrimer by inclusion complex method. Ketoprofen loaded dendrimer was characterized by Flourier Transform infrared spectroscopy. Sustained release of ketoprofen from ketoprofen loaded dendrimers was studied and compared to that of free ketoprofen. Cytotoxicity of dendrimers on A-549 cell lines were studied by MTT assay technique. Hemolytic potential of G3 dendrimer was also studied.

Results: Solubility of practically insoluble ketoprofen was improved up to 0.77-4.89 mg/ml by dendrimer generations. Solubility of ketoprofen was increased with increase in pH, concentrationand generation number of dendrimer. Ketoprofen was released relatively slowly from ketoprofen loaded dendrimer compared to free ketoprofen. Cytotoxicity and hemolytic assay revealed that dendrimers were less toxic compared to PAMAM dendrimers.

Conclusion: Improved solubility of ketoprofen by dendrimer generations, its slow release from $G3(OH)_{128}$ dendrimer and cytotoxicity and hemolytic assay showed dendrimers have potential as drug carriers.

Keywords: Triazine Based Dendrimer, Sustained Release, Cytotoxicity, Hemolysis, Ketoprofen, Encapsulation.

INTRODUCTION

It is well known that low aqueous solubility of the new pharmaceutical active agent is an issue for the pharmaceutical development process which should be addressed early on during compound development [1]. Most part of the body is made up of water, therefore low water solubility of these drugs causes early elimination from gastrointestinal tract which results in poor bioavailability of drugs [2, 3]. Additionally, membrane permeability is the second important factor affecting bioavailability of poorly water soluble drugs. Discovery of new drugs has always been time consuming and costly process. Hence, delivery of poorly water soluble drugs is one of the major challenges in pharmaceutical research and development [4]. Currently, liposomes and polymeric drug delivery systems are used for drug delivery of poorly water soluble drugs. However, liposomal drug delivery suffers from limitations such as low stability, difficult to target specific tissues, toxicity and adverse side effects [5]. Whereas poly dispersity of linear polymers is a limitation of polymeric drug delivery systems [6].

Dendrimers are a fourth new architectural class of polymers with several unique properties such as nanoscale size, monodisperse molecular weight distribution, scaffolding properties, large number of functional groups and dimensions that mimics biomolecules like proteins [7]. Dendrimers possess hydrophobic core and hydrophilic exterior and they have shown to exhibit micelle like behavior and container like properties in solution [8]. In addition, dendrimers have an enhanced permeability and retention effect that allows them to target tumor cells more effectively than small molecules [9]. Dendrimer based drug delivery systems have been a subject of many reviews [10, 11].

We have already reported the synthesis, properties and various applications of hydroxyl terminated dendritic macromolecules [12-17]. Our dendritic architecture has hydroxyl groups on the periphery and previously hydroxyl-or methoxy-terminated dendrimers based on a polyester scaffold were shown to be nontoxic both *in vitro* and *in vivo* [18]. This motivated us to evaluate our dendritic architecture as a carrier for sustained release of poorly water soluble drugs.

In the present work, we have developed full generation triazine based dendrimer $G1(OH)_8$, $G2(OH)_{32}$ and $G3(OH)_{128}$ with 8, 32 and 128 hydroxyl groups on the periphery as a solubility enhancer and drug carrier for poorly water soluble drug ketoprofen. Effect of certain parameters such as pH, concentration and the generation on solubilisation of ketoprofen by dendrimer was studied. Ketoprofen was loaded in $G3(OH)_{128}$ dendrimer by the inclusion complex technique and release of Ketoprofen from ketoprofen loaded dendrimer was measured and compared with free ketoprofen. Ketoprofen loaded dendrimer was characterized by FTIR. Cytotoxicity and hemolysis assay of $G3(OH)_{128}$ dendrimer was carried out in order to evaluate toxicity and biocompatibility of dendrimer.

MATERIALS AND METHODS

Chemicals and instrumentation

Ketoprofen was generously provided by A. R. College of Pharmacy, Vallabh Vidhyanagar as gift sample. Triazine trichloride (cyanuric chloride), piperazine, acetone, dichloromethane and methanol were purchased from Sigma-Aldrich (India) Ltd. 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). All the reagents and solvents for the synthesis and analysis were used as received. Absorbance was measured on Shimadzu UV-1800 spectrophotometer. Double distilled water was used for solubility studies. FTIR was carried out in the range of 250–4000 cm^{-1} using Perkin Elmer-Spectrum RX-FTIR spectrometer instrument.

Methods

Synthesis and characterization of triazine based dendrimer

Triazine based dendrimer was synthesized as per reported procedure [12]. Triazine trichloride (0.02 mmol) was reacted with

piperazine (0.01 mmol) at 0-5 C to give 1, 4-bis(4, 6-dichloro-1, 3, 5-triazin-2-yl) piperazine as core for dendrimer synthesis. 1, 4-bis (4, 6-dichloro-1, 3, 5-triazin-2-yl)piperazine was purified by washing with acetone and methanol. 1, 4-bis (4, 6-dichloro-1, 3, 5-triazin-2-yl)piperazine (0.01 mmol) was reacted with diethanolamine (0.04 mmol) to give hydroxyl terminated $G1(OH)_8$ dendrimer. $G1(OH)_8$ dendrimer was purified by washing and dispersing in dichloromethane. Similar to the first step, $G1(OH)_8$ dendrimer (0.01 mmol) was reacted with triazine trichloride (0.08 mmol) at 0-5° C to give chlorine terminated G1.5 dendrimer (G1.5). Similar to the second step, chlorine terminated half generation dendrimer (G1.5) (0.01 mmol) was reacted with diethanolamine (0.16 mmol) to give $G2(OH)_{32}$ dendrimer. The above two steps were repeated to give half generation G2.5 and third generation G3 $(OH)_{128}$ dendrimers respectively. Synthesized core and all dendrimer generations were fully characterized by spectral analysis such as, FT-IR, ^{1}H-NMR, ^{13}C-NMR and ESI-Mass spectrometry [12].

Solubility study

Solubility study was carried out according to the method described by Higuchi and Connors [19]. 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 °C in shaking water bath. The vials were centrifuged to remove un dissolved ketoprofen and absorbance of ketoprofen was measured at its characteristic wavelength 260 nm using Shimadzu UV-1800 spectrophotometer.

Drug encapsulation

Generally there are two approached for drug loading in dendrimer either by inclusion complex or by conjugation. In the present approach, we have utilized inclusion complex technique. Drug loading was performed by reported methods with little modifications [20, 22]. 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. Access Ketoprofen from filter and each fraction of methanol was analyzed by UV spectrophotometer to determine amount of encapsulated drug indirectly.

***In vitro* drug release [22]**

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 waters. 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 waters. The absorbance of the outer phase was monitored at 260 nm using a spectrophotometer in order to characterize the concentration of ketoprofen.

Hemolysis study [21]

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 °C 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.

Cytotoxicity study [21]

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 °C and 5% CO_2. The cells were seeded at a concentration of 5× 104 cells/well in 100 µl culture medium and incubated at 37 °C 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 °C 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 °C 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.

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.

RESULTS AND DISCUSSION

Synthesis of dendrimers

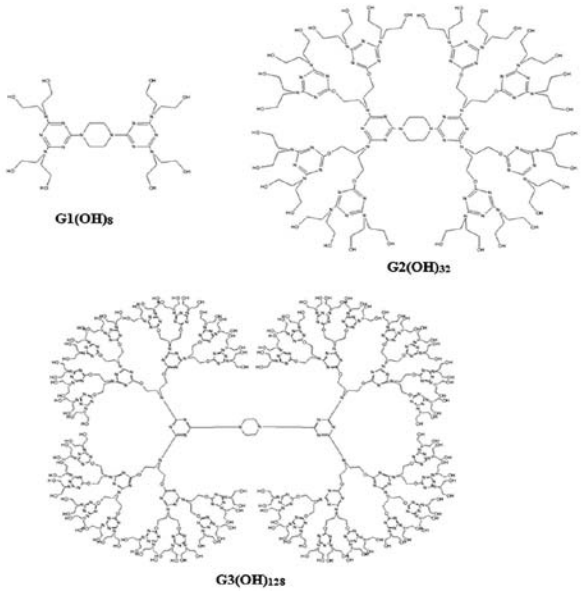

Fig. 1: Structures of: i) G1 $(OH)_8$, ii) G2$(OH)_{32}$ and c) G3$(OH)_{128}$ Dendrimer

Table 1: Physical description of dendrimer generations

Compound	Molecular formula	Appearance	Solubility in water	Theoretical surface groups
Core	$C_{10}H_8Cl_4N_8$	White solid	Insoluble	Cl (4)
$G1(OH)_8$	$C_{26}H_{48}N_{12}O_8$	Brown liquid	Soluble	OH (8)
G1.5	$C_{50}H_{40}Cl_{16}N_{36}O_8$	White solid	Insoluble	Cl(16)
G $2(OH)_{32}$	$C_{114}H_{200}N_{52}O_{40}$	Brown liquid	Soluble	OH(32)
G2.5	$C_{210}H_{168}Cl_{64}N_{148}O_{40}$	White solid	Insoluble	Cl(64)
$G3(OH)_{128}$	$C_{466}H_{808}N_{212}O_{168}$	Brown liquid	Soluble	OH (128)

Synthesis and characterization of s-triazine based dendritic generation G1$(OH)_8$ b) G2$(OH)_{32}$ and G3$(OH)_{128}$ [fig. 1] based on piperazine was already reported [12]. As shown in table 1, only full generation dendrimers G1$(OH)_8$, G2$(OH)_{32}$ and G3$(OH)_{128}$ were water soluble whereas half generation dendrimers and core compound were water insoluble. Therefore, only full generation dendrimers G1$(OH)_8$, G2$(OH)_{32}$ and G3$(OH)_{128}$ were utilized for drug solubilisation and drug delivery.

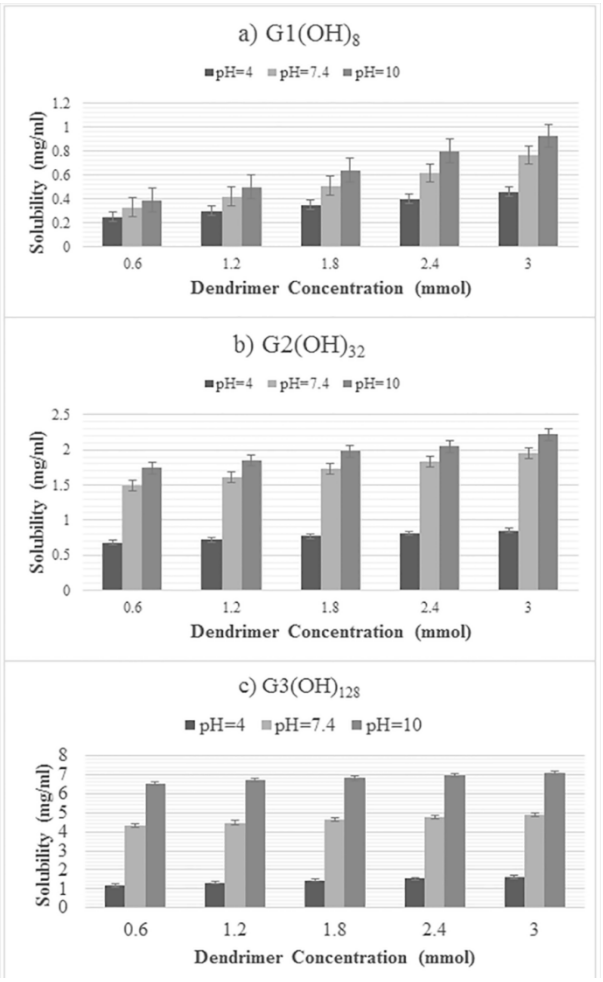

Fig. 2: Effect of the generations of triazine dendrimers and pH on aqueous solubilisation of Ketoprofen (n = 3)

Drug solubilisation

A series of solubility experiments for ketoprofen by dendrimer generation was carried out using different concentrations (0.6 mmol to 3 mmol) of dendrimer generations at pH 4.0, 7.4 and 10.0. Solubility results are furnished in fig. 2. It was observed that dendrimer generation significantly enhances solubility of practically insoluble drug ketoprofen in the range of 0.77 to 4.89 mg/ml by dendrimer generations. It was also observed that with an 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, 17]. It was also observed that with increased in pH generation number of dendrimer, solubility of ketoprofen was increased. It was also observed that with the increase in generation number, solubility of ketoprofen was also increased because surface area, the number of surface hydroxyl groups and the size of dendrimer was increased with increase in generation number [16, 17].

Drug encapsulation and Characterization of ketoprofen loaded dendrimer

G3$(OH)_{128}$ dendrimershowed maximum solubilisation of ketoprofen, therefore sustained release study was carried out by using G3$(OH)_{128}$ dendrimer. Ketoprofen drug was loaded with G3$(OH)_{128}$ dendrimer by inclusion complex technique [20, 22]. It was observed from UV spectrometer that about 24.28% ketoprofen was loaded in G3$(OH)_{128}$ dendrimer. Ketoprofen loaded dendrimer was further characterized by FT-IR spectroscopy and compared with FT-IR spectrums of pure G3$(OH)_{128}$ and ketoprofen drug. FT-IR spectrum of pure G3$(OH)_{128}$ dendrimer [fig. 3a] showed characteristic absorption bands 3356 cm^{-1} for O-H stretching for hydroxyl groups, 1064 cm^{-1} for C-O stretching of ether linkages. FT-IR spectrum of pure ketoprofen [fig. 3b] showed characteristic absorption bands at 3010 cm^{-1}, 2895 cm^{-1} for aromatic C-H stretching, 1665, 1735 cm^{-1} for carbonyl stretching. FT-IR spectrum of ketoprofen loaded dendrimer [fig. 3c] showed absorption band at 3377 cm^{-1} for O-H stretching, at 2885, 2810 cm^{-1} for C-H stretching, at 1785, 1615 cm^{-1} for carbonyl stretching and at 1055 cm^{-1} for C-O stretching. So, a little shift in bands for O-H stretching and carbonyl stretching was observed and other characteristic bands for both G3$(OH)_{128}$ dendrimer and ketoprofen remained unchanged. So, 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, dendrimer may have enhanced solubility of ketoprofen and their encapsulation by either hydrophilic interaction or hydrogen bonding or both [16, 17, 22].

Sustained release

It was noted that about 95% of ketoprofen were released within 2.5 hours from free ketoprofen. Whereas the same quantity of the drug was released after 6.5 hours from ketoprofen loaded dendrimer [fig. 4.]. So, Ketoprofen loaded dendrimer releases ketoprofen slowly compared to free ketoprofen. However, release of ketoprofen was comparatively faster than PAMAM dendrimer which released about 76% ketoprofen in 10 hours [25].

Hemolytic potential

Hemolysis assay gives quantitative estimation about hemoglobin release when red blood cells are treated with dendrimers. The data obtained in such assay also give a qualitative indication of potential damage to RBC's of dendrimer administered. Hemolytic assay of dendrimer at different concentration 40, 60, 80, 100 µg/ml was carried out. The results are displayed in fig. 5. It was observed that G3$(OH)_{128}$ dendrimer showed concentration dependent hemolysis. However, triazine based G3$(OH)_{128}$ dendrimer were significantly less hemolysis compared to PAMAM dendrimer [23]. Positively charged amine groups of PAMAM dendrimer interacts with negatively charged surfaces of red blood cells and caused hemolysis [24]. In comparison, G3$(OH)_{128}$ dendrimers has an ionic hydroxyl groups on the surface which may have minimized interaction with red blood cells and displayed significantly less toxicity.

Cytotoxicity

Cytotoxicity of G3$(OH)_{128}$ dendrimer on A-549 cells was evaluated by MTT assay technique. The results are displayed in fig. 6. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)is a yellow, tetrazolium salt. Metabolically active cells convert MTT into the dark blue water soluble formazan as a result of cleavage of tetrazolium ring. Formazan can be dissolved in a solvent and can be measured quantitatively. Our results [fig. 6] displayed that G3$(OH)_{128}$ dendrimer displayed more that 90% cell viability at concentration levels ranging from 10 µg/ml to 1000 µg/ml. So, G3$(OH)_{128}$ dendrimer was significantly less cytotoxic. Microscopic images [fig. 7. a-d] displays morphology of A-549 cell lines on when treated with control and different concentration of dendrimers is displayed, which showed a decrease in cell density with increase in dendrimer concentration from 10 µg/ml to 1000 µg/ml [26].

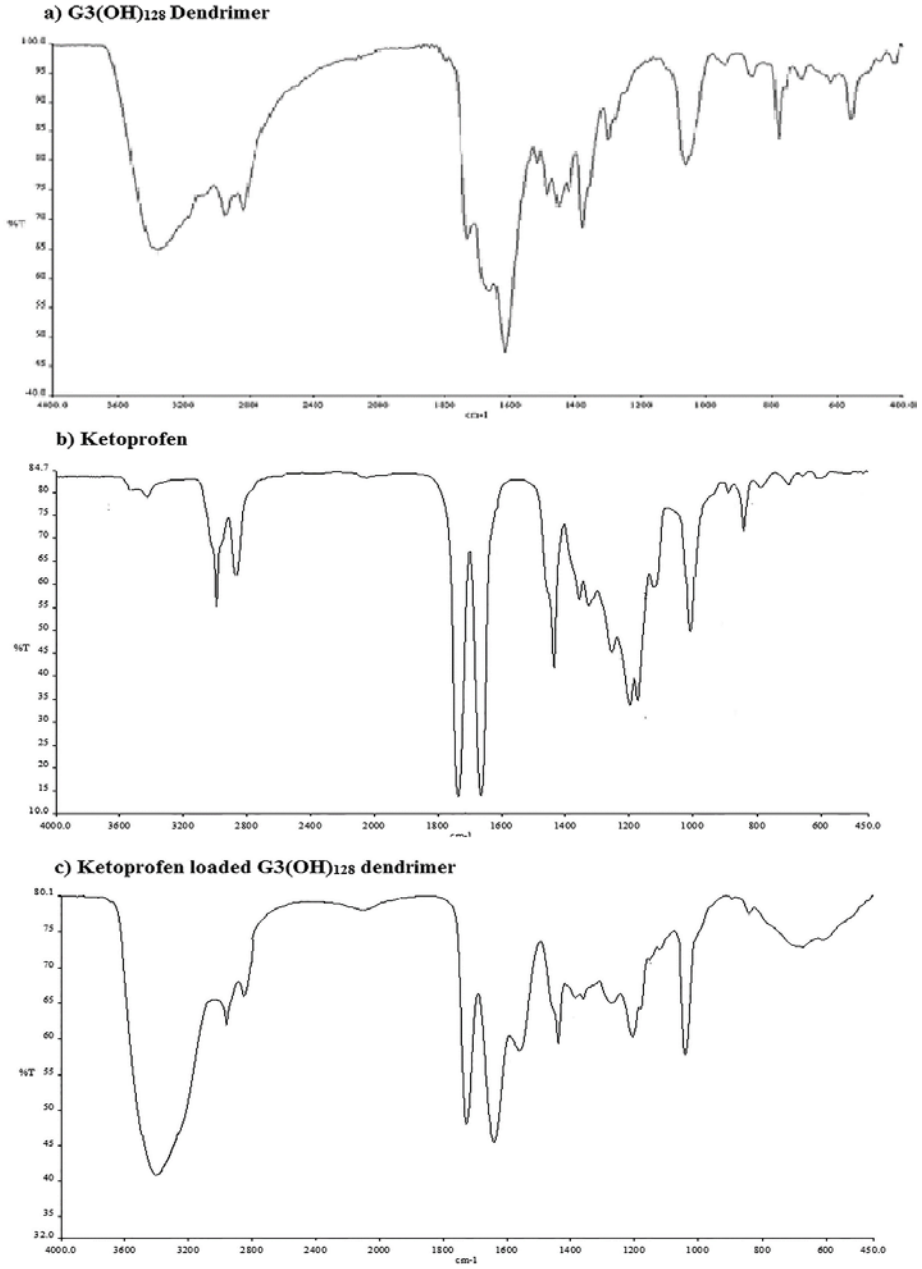

Fig. 3: a) IR spectrum of G3(OH)$_{128}$ dendrimer, b) FT-IR spectrum of ketoprofen and c) FT-IR spectrum of ketoprofen loaded G3(OH)$_{128}$ dendrimer

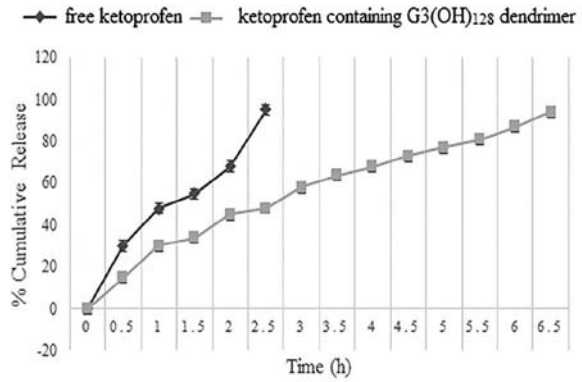

Fig. 4: % Cumulative release profile ketoprofen from free ketoprofen and ketoprofen containing G3(OH)$_{128}$ dendrimer (n=3)

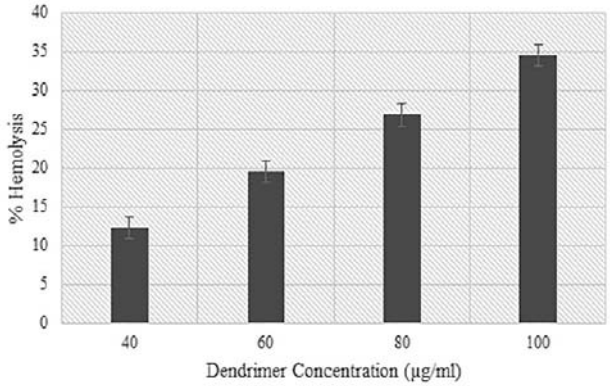

Fig. 5: %Hemolysis of Red blood cells by G3(OH)$_{128}$ dendrimer after 1 hour of incubation (n=3)

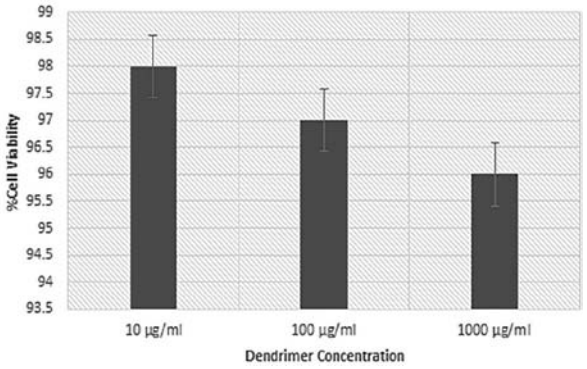

Fig. 6: Cytotoxicity of G3(OH)$_{128}$ dendrimers on A-549 cell lines after 48 hours of incubation (n=3)

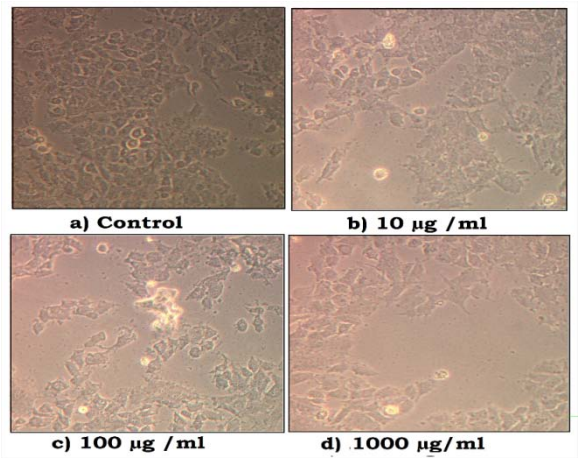

Fig. 7: Microscopic images of A-549 cell lines treated with a) Control; b) 10 µg/ml of G3(OH)$_{128}$ Dendrimer; c) 100 µg/ml of G3(OH)$_{128}$ Dendrimer and d) 1000µg/ml of G3(OH)$_{128}$ Dendrimer

CONCLUSION

The limitations associated with liposomes and polymeric drug delivery systems such as low stability, polydispersity can be overcome by dendrimer drug delivery systems. Hydroxyl terminated dendrimer generations have significantly enhanced solubility of ketoprofen. G3 (OH) $_{128}$ dendrimer has successfully encapsulated ketoprofen and sustained release study has displayed its sustained release potential. Cytotoxicity and hemolytic assay have revealed that dendrimers have low toxicity and biocompatibility.

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CONFLICT OF INTERESTS

Declared None

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