**Original Research Paper**

**Dendritic macromolecules as nano-scale drug carriers: Phase solubility, in vitro drug release, hemolysis and cytotoxicity study**

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**Abstract**

Potential of nanoscale triazine based dendritic macromolecules G1, G2 and G3 as solubility enhancers of drug was investigated. Effect of pH, concentration and generation of synthesized dendritic macromolecules on solubility of ketoprofen was studied. G3 dendrimer was further exploited as carrier for sustained release. Ketoprofen was encapsulated by inclusion complex method and also characterized by Flourier Transform Infrared spectroscopy. Sustained release study of ketoprofen from ketoprofen loaded dendrimer was carried out and compared with free ketoprofen. Hemolytic potential and Cytotoxicity assay using A-549 lung cancer cell lines revealed that synthesized triazine based dendritic macromolecules having more potential that commercially available PAMAM dendrimer.

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**1. Introduction**

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely prescribed medications in the world. Their main benefit originates from their anti-inflammatory and analgesic properties [1]. These drugs are commonly employed for the conditions associated with osteoarthritis and other chronic musculoskeletal conditions [2,3]. Ketoprofen is a member of nonsteroidal anti-inflammatory drug, also used as an inhibitor of prostaglandin synthetase [4], administered orally, three or four times per day [5]. Several side effects are related with ketoprofen such as gastrointestinal side effects, renal side effects and additional side effects such as
hypersensitivity limits its application [6,7]. It was advised that the use of ketoprofen in the parenteral application could control these critical side effects. However, low water solubility of ketoprofen leads to poor bioavailability pose a great challenge for the formulation of drug [7] for use in topical and parenteral applications. Many methodologies have been applied to improve solubility and reduce side effects of ketoprofen for example use of liposomes, synthetic polymer as drug delivery agents [8,9]. Ketoprofen has often employed as model drug for such studies [10].

Dendrimers are three dimensional, nanosized macromolecules having monodisperse molecular weight distribution obtained by repetitive sequence of reactions [11]. Dendrimer have several unique properties such as nano-scale monodispersity, scaffolding properties, amplifiable and functionable surface groups and dimensions that mimics biomolecules such as protein [12]. Therefore dendrimers are often used in biomedical applications such as drug solubilization [13], drug delivery [14], MRI contrast agents [15] etc. Application of dendrimer as a vehicle for drug delivery has been of great interest [16].

Dendrimers based on triazine are well known. Synthesis of triazine dendrimer is facile since it does not require functional group manipulations compared to other classes of dendrimers such as PAMAM dendrimer [17]. Recently, dendrimers based on triazine have been used in a wide range of applications such as in optics [18,19], for the delivery of anti-tumor agents [20], in molecular recognition [21], catalytic supports [22] etc.

Previously, synthesis and characterization and application of s-triazine based dendrimer for water remediation has been reported [23–25]. Synthesized dendrimer has hydroxyl groups on periphery and previously it has been reported that hydroxyl terminated dendrimers are less cytotoxic than amine terminated PAMAM dendrimers [26] and recent biomedical applications on triazine based dendrimers [26–28] motivating us to investigate triazine based dendrimer as a carrier of sustained release using ketoprofen as a model drug.

In the present investigation, potential of triazine based dendrimer as potential drug carrier of ketoprofen was evaluated. Ketoprofen was loaded to G3 dendrimer by inclusion complex method. Ketoprofen loaded dendrimer was further investigated by Infrared spectroscopy. Release of Ketoprofen from Ketoprofen-dendrimer complex in dialysis bag was measured and compared with that of free Ketoprofen. Cytotoxicity and hemolysis was carried out to evaluate toxicity and biocompatibility of the dendrimer.

2. Materials and methods

2.1. Materials

Ketoprofen was generously provided by A.R. College of Pharmacy, Vallabh Vidhyanagar as gift sample. Triazine trichloride (cyanuric chloride), 1,4-butanediamine, acetone, dichloromethane and methanol were purchased from Sigma-Aldrich (India) Ltd. 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. Carl Zeiss-Primovert inverted microscope was used for microscopic images of A-549 cell lines.

2.2. Synthesis and characterization of triazine based dendrimer

Triazine based dendrimer was synthesized by following method. Triazine trichloride (0.02 mmol) was reacted with 1,4-butanediamine (0.01 mmol) at 0–5 °C to give N,N-bis(4,6-dichloro-1,3,5-triazin-2-yl)butane-1,4-diamine as core for dendrimer synthesis. N,N-bis(4,6-dichloro-1,3,5-triazin-2-yl)butane-1,4-diamine was purified by washing with Acetone and Methanol. N,N-bis(4,6-dichloro-1,3,5-triazin-2-yl)butane-1,4-diamine (0.01 mmol) was reacted with diethanolamine (0.04 mmol) to give hydroxyl terminated generation 1 (G1) dendrimer. G1 dendrimer was purified by washing and dispersing in dichloromethane. Similar to first step, G1 dendrimer (0.01 mmol) was reacted with triazine trichloride (0.08 mmol) at 0–5 °C to give chlorine terminated half generation G1.5 dendrimer (G1.5). Similar to second step, chlorine terminated half generation dendrimer (G1.5) (0.01 mmol) was reacted with diethanolamine (0.16 mmol) to give full generation hydroxyl terminated dendrimer (G2) [23]. The above two steps were repeated to give half generation G2.5 and full generation G3 dendrimers respectively. Synthesized core and all dendrimer generations were fully characterized by spectral analysis such as FT-IR, 1H-NMR, 13C-NMR and ESI-Mass Spectrometry [23].

2.3. Solubility study

Solubility study was carried out according to the method described by Higuchi and Connors (1965). Excess of ketoprofen was added to screw-capped vials containing different concentrations (0.6 mmol–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 undissolved ketoprofen and absorbance of ketoprofen were measured at its characteristic wavelength 260 nm using Shimadzu UV-1800 spectrophotometer.

2.4. Drug encapsulation

Drug loading was performed by reported methods with little modifications [26,29]. Known amount of ketoprofen was added to a solution containing G3 dendrimer (3 mmol in 10 ml of distilled water). The mixture was stirred for 72 h 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.

2.5. 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 (MW cut off = 3000 Da) 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 h, 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.

2.6. Hemolysis study [30]

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 min. The supernatant (Erythrocyte) was collected and plasma was discarded. The pellet was washed for 3 times using 0.75% NaCl and centrifuged at 419

Fig. 1 – Structure of A) G1 dendrimer, B) G2 dendrimer, C) G3 dendrimer.
1500 rpm for 5 min. The cells were resuspended in normal saline to 0.5%. Washed erythrocytes were stored at 4°C and used within 6 h for the hemolysis 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 h. After centrifugation, 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 was used as minimal and maximum hemolytic control.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Appearance</th>
<th>Solubility in water</th>
<th>Surface groups (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0.5 C_{10}H_{10}Cl_{4}N_{8}</td>
<td>White solid</td>
<td>Insoluble</td>
<td>Cl (4)</td>
<td></td>
</tr>
<tr>
<td>G1 C_{26}H_{50}N_{12}O_{8}</td>
<td>Brown liquid</td>
<td>Soluble</td>
<td>OH (8)</td>
<td></td>
</tr>
<tr>
<td>G1.5 C_{50}H_{42}Cl_{16}N_{36}O_{8}</td>
<td>White solid</td>
<td>Insoluble</td>
<td>Cl(16)</td>
<td></td>
</tr>
<tr>
<td>G2 C_{114}H_{204}N_{52}O_{40}</td>
<td>Brown liquid</td>
<td>Soluble</td>
<td>OH(32)</td>
<td></td>
</tr>
<tr>
<td>G2.5 C_{210}H_{170}N_{148}O_{40}Cl_{64}</td>
<td>White solid</td>
<td>Insoluble</td>
<td>Cl(64)</td>
<td></td>
</tr>
<tr>
<td>G3 C_{466}H_{810}N_{212}O_{168}</td>
<td>Brown liquid</td>
<td>Soluble</td>
<td>OH(128)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 – Effect of the generations A) G1 dendrimer, B) G2 dendrimer, C) G3 dendrimer and pH on aqueous solubilization of Ketoprofen (n = 3).
2.7. Cytotoxicity study [30]

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 $1 \times 10^6$ cells/ml in culture medium for 3 h at 37°C and 5% CO₂. The cells were seeded at a concentration of $5 \times 10^4$ cells/well in 100 µl culture medium and incubated at 37 °C in 5% CO₂ incubator for 24 h. After 24 h, when the monolayer formed, the supernatant was flicked off and added previously diluted with media of 100 µl of different concentrations of test extract in microtitre plates and kept for incubation at 37 °C in 5% CO₂ incubator for 48 h and cells were periodically checked for granularity, shrinkage, swelling. After 48 h, the sample solution in the wells was flicked off and 10 µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 h at 37 °C in 5% CO₂ 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.

2.8. Statistical analysis

Data are expressed as the mean standard deviation (SD) of obtained 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. Results and discussion

3.1. Preparation mechanism

Synthesis and characterization of s-triazine based dendritic generation G1(OH)$_8$, G2(OH)$_{32}$ and G3(OH)$_{128}$ [Fig. 1] based on 1,4-butanediamine was already reported [23]. 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 solubilization and drug delivery.

3.2. Drug solubilization

As Shown in Fig. 2 a series of solubility experiments for ketoprofen by dendrimer generation were carried out using different concentrations (0.6 mmol–3 mmol) of dendrimer generations at pH 4.0, 7.4 and 10.0. Solubility results are furnished in Fig. 2. It was witnessed that dendrimer generation significantly enhances solubility of practically insoluble drug ketoprofen in the range of 0.77–4.89 mg/ml by dendrimer generations. It was also revealed that with an increase in concentration of dendrimer, solubility of ketoprofen was increased in a linear manner. It was proposed that as 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 could be either hydrophilic interaction or hydrogen bonding or both [26,27]. It was also observed that with increased in pH and generation number of dendrimer, solubility of ketoprofen was increased. With the increase in generation number, surface area and terminal hydroxyl groups of dendrimer was increased hence solubility of dendrimer was increased.

3.3. Drug loading and in vitro release

Ketoprofen loaded dendrimer was prepared as per reported method [26] which was further characterized by UV/Vis spectrophotometer. It was observed that 24.55% of ketoprofen was encapsulated in dendrimer. Ketoprofen loaded dendrimer was further investigated by infrared spectroscopy. As Shown in Fig. 3A, FT-IR spectrum of pure G3 dendrimer showed
absorption bands $3389$ cm$^{-1}$ for O–H stretching for hydroxyl groups, $1068$ cm$^{-1}$ for C–O stretching of ether linkages. FT-IR spectrum of ketoprofen showed absorption bands at $3010$ cm$^{-1}$, $2895$ cm$^{-1}$ for aliphatic C–H stretching, $1665$, $1735$ cm$^{-1}$ for carbonyl stretching showed in Fig. 3B. FT-IR spectrum of ketoprofen loaded G3-dendrimer showed absorption band at $3410$ 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 showed in Fig. 3C. So, overall characteristic bands for both G3 dendrimer and ketoprofen remained unchanged in IR spectrum of ketoprofen loaded dendrimer. So it can be understood that dendrimer contains 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 [26].

The in vitro drug release of Ketoprofen from ketoprofen loaded dendrimer was examined in distilled water at room temperature showed in Fig. 4. 95% of Ketoprofen was released within $2.5$ h from free ketoprofen. Whereas same quantity of drug was released after $7$ h from ketoprofen loaded dendrimers. So, Ketoprofen loaded dendrimer releases ketoprofen slowly compared to free ketoprofen.

3.4. Hemolytic potential

It was observed that G3 dendrimer showed Fig. 5 concentration dependent hemolysis. However, triazine based G3 dendrimer were significantly less hemolysis compared to PAMAM dendrimer [31]. Positively charged amine groups of PAMAM dendrimer interacts with negatively charged surfaces of red blood cells and caused hemolysis [32]. In comparison, G3 dendrimers have anionic hydroxyl groups on the surface which minimized interaction with red blood cells and displayed significantly less toxicity.

3.5. Cytotoxicity

Cytotoxicity [Fig. 6] displayed that G3 dendrimer displayed more that $90\%$ cell viability at concentration levels ranging from $10$ μg/ml to $1000$ μg/ml. So, G3 dendrimer was significantly less cytotoxic. Microscopic images showed Fig. 7. A–D, 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 [33].

4. Conclusion

Dendrimer generations have improved aqueous solubility of practically insoluble drug ketoprofen. Ketoprofen was released gradually from ketoprofen loaded dendrimer compared to free ketoprofen. Cytotoxicity and hemolytic assay exhibited that the dendrimer was significantly less toxicity and biocompatible which displayed utility of dendrimer as potential drug carrier system.

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