Low Generation Triazine-based Dendrimers - Synthesis, Characterization and *in Vitro* Biological Activity

MASTER DISSERTATION

Xupeng Zhang
MASTER IN NANOCHEMISTRY AND NANOMATERIALS

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Dissertation submitted to the University of Madeira in fulfillment of the requirements for the degree of Master in Nanochemistry and Nanomaterials

by Xupeng Zhang

Work developed under the supervision of

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Funchal – Portugal

September 2015

DECLARATION

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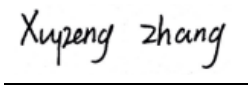

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ABSTRACT

In the present study, two low generation triazine-based dendrimers, $G1.0(Cl)_4$ dendrimer and $G1.5(OH)_8$ dendrimer, were synthesized and their cytotoxicity were tested by using the NIH 3T3 and the A2780 cell lines. In the synthesis process of the $G1.0(Cl)_4$ dendrimer, cyanuric chloride (CAC) which has high reactivity chlorine atom was connected to the terminal of triethylene glycol (TEG) via nucleophilic substitution by controlling temperature. The prepared $G1.0(Cl)_4$ dendrimer was purified by silica gel column chromatography. Then the four chlorine atoms in the $G1.0(Cl)_4$ dendrimer were substituted by diethanolamine (DEA) to give dendrimer with the hydroxyl terminal group $G1.5(OH)_8$.

The starting materials, CAC, $G1.0(Cl)_4$ dendrimer and $G1.5(OH)_8$ dendrimer were analyzed by one-dimensional NMR, FTIR and MS techniques. The two dendrimers, $G1.0(Cl)_4$ and $G1.5(OH)_8$, showed perfect stability in the air environment at room temperature. However, $G1.0(Cl)_4$ is not soluble in water while the $G1.5(OH)_8$ dendrimer is a water soluble compound. Furthermore, cell biological evaluation at the studied concentrations showed that the CAC, as well as the prepared $G1.0(Cl)_4$ and $G1.5(OH)_8$ dendrimers, have no cytotoxicity towards the NIH 3T3 and A2780 cell lines.

Keywords: triazines; dendrimers; cyanuric chloride; cytotoxicity.

RESUMO

No presente estudo, dois dendrímeros baseados em triazinas de baixa geração, G1.0(Cl)$_4$ e G1.5(OH)$_8$ foram sintetizados e a sua citotoxicidade foi testada através da utilização das linhas celulares NIH 3T3 e A2780.

No processo de síntese do dendrímero G1.0(Cl)$_4$, o cloreto cianúrico, que possui uma elevada reactividade no átomo de cloro, foi ligado aos grupos terminais do trietilenoglicol através da substituição nucleofílica por controlo da temperatura. O dendrímero G1.0(Cl)$_4$ preparado foi purificado por coluna cromatográfica em sílica gel.

Posteriormente, os quatro átomos de cloro do dendrímero G1.0(Cl)$_4$ foram substituídos por dietanolamina, de modo a obter-se o dendrímero G1.5(OH)$_8$ com grupos hidroxilo terminais.

O material de partida cloreto cianúrico e os dendrímeros G1.0(Cl)$_4$ e G1.5(OH)$_8$ foram analisados pelas técnicas de RMN unidimensional, FTIR e MS. Verificou-se que os dois dendrímeros G1.0(Cl)$_4$ e G1.5(OH)$_8$ são estáveis ao ar, à temperatura ambiente. Contudo, o dendrímero G1.0(Cl)$_4$ não é solúvel em água, enquanto o dendrímero G1.5(OH)$_8$ é solúvel em água. Além disso, a avaliação biológica celular nas concentrações estudadas, evidenciaram que o cloreto cianúrico bem como os dendrímeros preparados G1.0(Cl)$_4$ e G1.5(OH)$_8$ não apresentam citotoxicidade para as linhas celulares NIH 3T3 e A2780.

Palavras-chave: triazinas, dendrímeros, cloreto cianúrico, citotoxicidade.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	Antibiotic-Antimycotic
APCI	Atmospheric Pressure Chemical Ionization
CAC	Cyanuric Chloride
DEA	Diethanolamine
DIPEA	Diisopropylethylamine
DMEM	Dulbecco’s Modified Eagle Medium
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
ED	Ethylenediamine
EDTA	Ethylene Diamine Tetraacetic Acid
EPR	Enhanced Permeability and Retention
ESI	Electrospray Ionization
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy
HIV	Human Immunodeficiency Virus
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PAMAM	Polyamidoamine Dendrimer
PEG	Poly(ethylene glycol)
PETIM	Ploy Propyl Ether Imine
PGA	Penicillin-G-Amidase
PLL	Poly-L-Lysine
PPI	Polypropylenimine Dendrimer
RPMI-1640	Roswell Park Memorial Institute
RSV	Respiratory System Virus
SDBS	Spectral Database for Organic Compounds

TEG	Triethylene glycol
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran

CHAPTER 1-INTRODUCTION

1.1 Dendrimers

1.1.1 The concept and the structural characteristics of dendrimers

Nowadays, nanotechnology shows a very rich attraction to the scientists and researchers, because materials associated with this technology present completely different properties. Several research groups working in the field of nanotechnology are interested in dendrimers, a particular type of molecule with regular high structure (*1*).

Dendrimers have a regular and symmetrical structure that is obtained by progressively repeated reactions and are the three-dimensional monodisperse type of macromolecules (*2, 3*). In 1978, Vogtle and coworkers were the first to try the use of "cascade" method in the preparation of dendrimers (*4*). Tomalia and coworkers were the first to synthesize a significantly active polyamidoamine (PAMAM) dendrimer family (*5*) which was coined as "starburst polymers". Dendrimers have showed a wide range of potential applications such as in photoelectric sensors (*6, 7*), liquid crystals, catalysts (*8, 9*), pharmaceutical formulations (*10, 11*), gene delivery (*12*), surfactants, nano composite materials (*13*) and as film materials (*14*).

In general, dendrimers mean that the macromolecule has a perfect dendritic spherical structure (Figure 1). (i) A molecule that has more than two identical chemical functions is used as a core; (ii) Starting from the core, branches are grown, consisting of repeated units, including at least one point of junction (this repetition follows some rules that result in different and radially concentric layers called "generations"); Nowadays the generation of dendrimers normally reach around 1-10 generations, and the molecular weight may correspond to thousands or even millions of daltons; and (iii) Different types of terminal groups, usually located on the surface of dendrimers; these surface groups are imperative to give special properties to dendrimers (*15*).

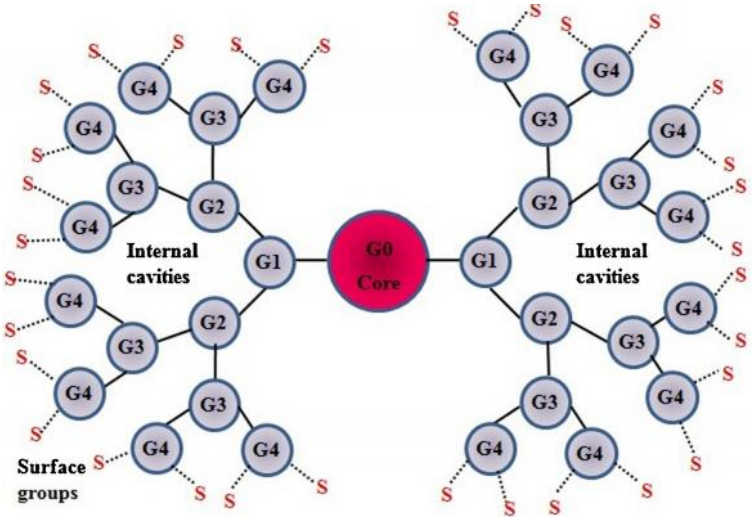
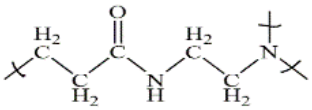
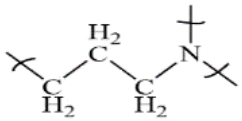
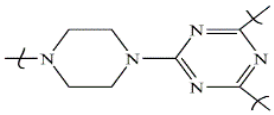
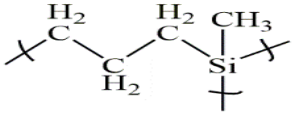
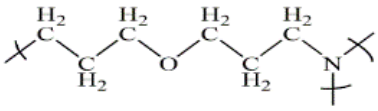
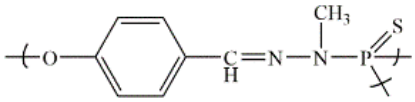
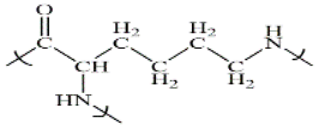
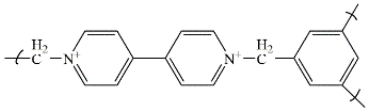


Figure 1: Outline showing the general structure of dendrimers (*15*).

Until now, hundreds of different types of dendrimers are published in the literature, but only a few of them are commercially available. In Table 1, some most frequent dendrimers used in research are presented.

Table 1: Most relevant families of dendrimers with their repeated units.

Name	**Repeated units**	**Ref.;**
Cationic PAMAM dendrimer		(*5*, *16*)
Polypropylenimine (PPI) dendrimer		(*4*, *17*)
Triazine dendrimer		(*18*, *19*)
Carbosilane dendrimer		(*20*, *21*)
Poly(PropylEtherImine) (PETIM) dendrimer		(*22*, *23*)

Phosphorus dendrimer		(*24*, *25*)
Poly-L-Lysine (PLL) dendrimer		(*26*, *27*)
Viologen dendrimer		(*28*, *29*)

1.1.2 The properties of dendrimers and their unique characteristics

Dendrimers have many features such as regular structure, high symmetry, high density of terminal groups, controlled relative molecular mass, intramolecular adjustable cavities, good hydromechanical properties, and low melt viscosity (*30*). These properties give to this kind of molecules a series of unusual characteristics such as a reactive behavior (their surface is easily modifiable to endow the dendrimer with multi-functionality), an easy film formation, and good mechanical properties (*30*). Dendrimers also are widely used for drug delivery, bioimaging, and as antiviral and transfecting agents (*31*, *32*).

1.1.3 Toxicity and safety of dendrimers

To be used in biomedical applications, dendrimers should present low toxicity, be non-immunogenic (*33*) and also preferably biodegradable (*34*). For example, cationic dendrimers can cause cell lysis when they interact with the negative charges of biological membranes (*35*). Because the dendrimers are nanosized, they can easily interact with the cellular components (*36*).

The cytotoxicity of dendrimers is related to the functional groups located on the core, branches and surface. However, the most strong influence on the cytotoxicity of the dendrimers comes from the terminal groups (*34*). According with several studies, dendrimers with cationic groups such as amine, guanidine, carboxylate, sulfonate,

phosphonate were more cytotoxic than the dendrimers with anionic groups (*37*). For that reason, PAMAM dendrimers modified with –OH groups, showed lower cytotoxicity than PAMAM-NH_2 because the –OH groups are neutral at the physiological pH (*38*). Another way to reduce the cytotoxicity of cationic dendrimers is by modifying the surface of cationic dendrimers with poly(ethylene glycol) (PEG) or pyrrolidone (*33*).

1.2 Dendrimers for biomedical applications

As we know, PAMAM dendrimers are interesting in the biomedical area (*39*). PAMAMs have been reported to allow several cell types for high-efficiency transfection (*40*). Additionally, the use of PEG chains in dendrimer surface is good for dendrimers' biocompatibility (*12*, *41*). The work of Calabretta *et al.* shows that PAMAM dendrimers partially modified with PEG present high cytotoxicity against the Gram-negative bacteria *Pseudomonas aeruginosa*, while it is not cytotoxic to human corneal epithelial cells at the same concentration (*42*).

1.2.1 As antimicrobials

Nowadays, there is a huge market demand for new antimicrobials. Dendrimers with cationic and amphiphilic properties can disrupt the membrane of mammalian cells, bacteria, and fungi, thus providing new directions in the search for new antimicrobial agents (*43*).

Chen *et al.* have reported a family of PPI dendrimers (Figure 2) whose antibacterial properties were analyzed by using a bioluminescence method. The results showed that the antibacterial properties are related to the size of the dendrimers, and the hydrophobic chain's length (*44*).

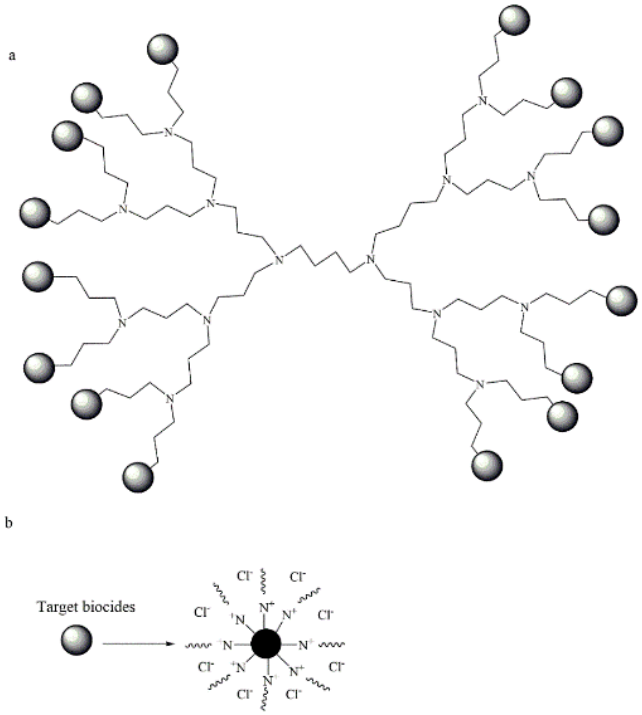


Figure 2: Example of a PPI dendrimer with antimicrobial properties synthesized by Chen *et al. (44)*.

1.2.2 Dendrimers for drug delivery applications

Dendrimers have a branched structure that offers internal cavities, which can help in the solubilization of medicines (*45*). Once most of the chemotherapeutic drugs present hydrophobicity, their delivery by using intravenous or intraperitoneal injections is limited. Also, when free drugs exist in the blood and do not enter cells, the kidneys can filter and remove them quickly, implying the need for multiple treatments sessions. Dendrimers are expected to help to overcome some of the obstacles presented before. Firstly, dendrimers can work like unimolecular micelles to encapsulate and solubilize drugs inside of their cavities (*45*). Secondly, if a dendrimer that carries a drug is larger than 5nm, the carrier's size exceeds the renal threshold for clearance and then is less likely to be removed by the kidneys. So the drug can stay in the patient's body longer (*45*). Finally, because in the normal tissues the microvascular endothelial gap is compact, and its structure is intact, it is not easy for the dendrimers to pass through the blood vessel walls when they have a size larger than 5 nm (*45*). In the tumor tissues, the vascular wall gaps are wider, the structure integrity of the vessels is poor, and the

lymphatic drainage is lacking. All these properties endow the tumor tissues with high selective permeability and retention for macromolecular substances. This phenomenon is called the high permeability and retention effect of solid tumor tissues referred to as the enhanced permeability and retention (EPR) effect. Therefore, the use of dendrimers is a passive form to target the tumor tissues and deliver chemotherapeutic drugs. Researchers such as Fox *et al.* (*46*) describe how dendrimer structure is related to the EPR effect in a very interesting review.

There is an interesting bio-application of dendrimers called enzymatic activation of low generation dendritic prodrugs. These dendrimers can disassemble triggered by special enzymatic conditions (*47*). As illustrated in Figure 3, the red part is penicillin-G-amidase (PGA) (which release is enzymatically triggered), the blue part is 4-nitroaniline, and the purple part are two acetylene functional groups which were used to connect various PEG-azide units. When this dendrimer was modified using PEG tails, the aqueous solubility of this dendrimer increased.

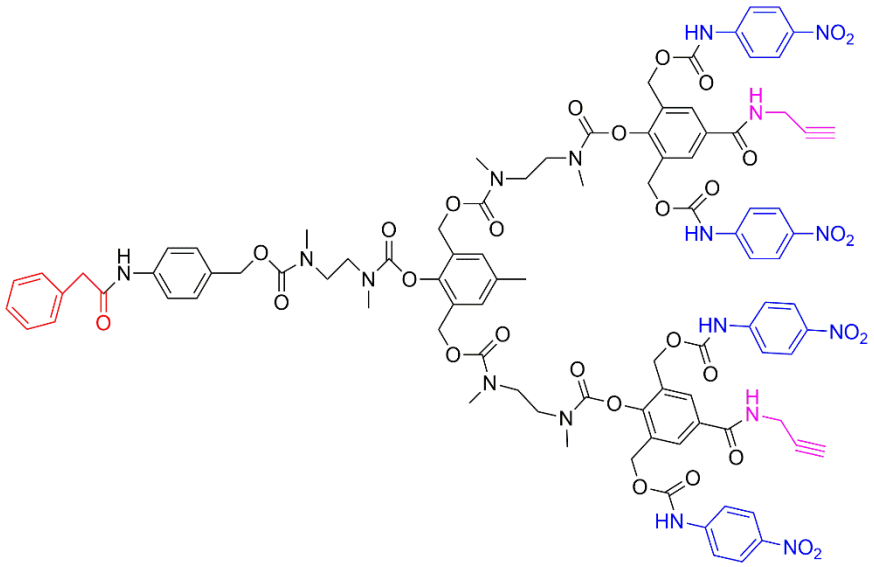


Figure 3: Structure of a dendritic molecule as a drug delivery system (*47*).

1.2.3 Dendrimers for gene delivery applications

A wide variety of viral gene delivery systems has been presented to deliver genes into different cells. As the most capable carriers, viral vectors have their

limitations that consist in high carcinogenicity and immunogenicity *in vivo* (*48*). Non-viral vectors, in general, show lower efficiency, but they offer safety and flexibility (*49, 50*).

Because of their monodispersity, a large number of terminal groups, and regular structure, dendrimers systems can be used with advantage for gene delivery (*51, 52*). For instance, the capacity of PAMAM dendrimers as gene delivery systems was demonstrated because they possess a huge number of cationic amines at the surface (*40, 53, 54*). The positive charge of these dendrimers allow them to bind to negatively charged nucleic acids and, also, favor the interactions with the cell membrane (*50, 55*). Also, transfection efficiency can be improved by modifying the dendrimers with different groups such as alkyl chains, and membrane targeting peptides (*56, 57*).

Figure 4 schematically illustrates the cells uptake of dendriplexes. In order to transfer the nucleic acid to cells, the dendrimer is first mixed with the nucleic acid resulting in charge neutralization and compaction of the DNA. After, the dendriplex interacts with the cell membrane that is negatively charged. Then an endosome is formed by cell endocytosis. Finally, the endosome suffers lysis (that is helped by the proton sponge effect exerted by the dendrimer inside the endosome), and the DNA is released in the cell interior (*48*).

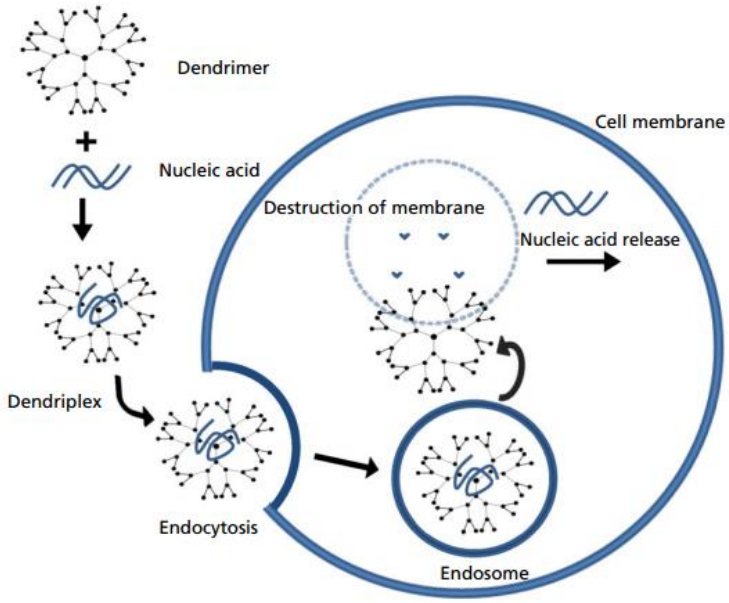


Figure 4: The process of using dendrimers to transport nucleic acids into the cells (*48*).

1.3 Triazine-based dendrimers

The dendrimers which use 1,3,5-triazine ring as a branching unit or core are called triazine-based dendrimers. The triazine unit has good electron affinity, and may perform as an electron transport component (*58*). The triazine dendrimer branches start from the triazine ring that is usually obtained from the commercially available cyanuric chloride (CAC) compound. Different types of diamines are used to connect triazines. Due to diverse characteristics such as the length and the flexibility of diamines, triazine dendrimers may exhibit multiple properties (*59*).

Triazine dendrimers show a wide compositional variety and can be synthesized to exhibit orthogonally functional surfaces appropriated for drug delivery applications (*60*). The process of triazine dendrimer synthesis can be designed (using reactions that are fast, chemoselectivity and with high yields) to improve the solubility of dendrimers and to obtain the adequate structure-activity relation (*61*).

1.3.1 The properties of triazine ring as starting material in the preparation of dendrimers

Triazine ring is the six-membered heterocyclic ring containing nitrogen, is chemically very stable, and can be decomposed by heating in concentrated sulfuric acid at 150 ℃ (*62*). Due to its good stability (at a range of pH and thermal conditions), it can pass through a variety of harsh reaction conditions without being destroyed/ decomposed. Triazine ring (especially melamine) can be used in the supramolecular area by recognizing molecules through hydrogen bonding interaction (Figure 5). One system based on the interaction between melamine and CAC showed significance in the self-assembly of linear and cyclic hydrogen-bonded assemblies (Figure 6) (*63*). Between atoms N to N, N to O, O to O, different types of hydrogen bond were formed. Due to this property, it is possible to increase the water solubility of triazine dendrimers which have O or N atoms coupled on the triazine ring.

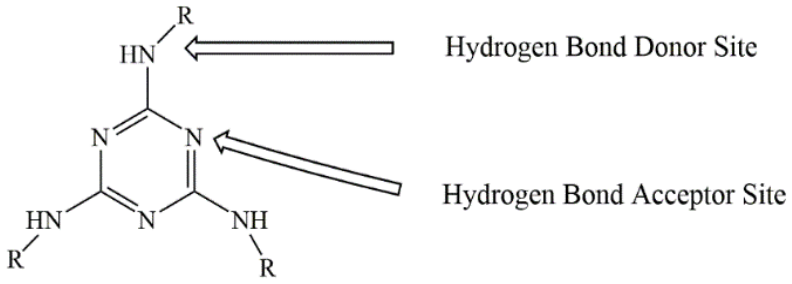


Figure 5: Sites of triazine/melamine derivatives that can act as donor or acceptor (*62*).

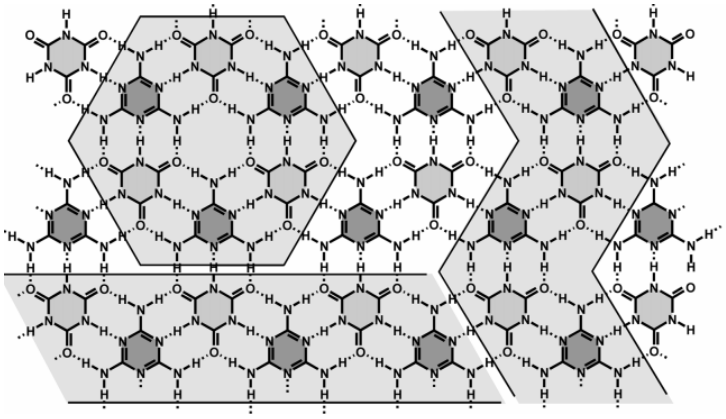


Figure 6: Complementary hydrogen bond formation between cyanuric acid and melamine (*63*).

The way to synthesize triazine dendrimer relies on the nucleophilic substitution reaction of trichlorotriazine (*60*). The main materials to supply the triazine ring are CAC, melamine, and cyanuric acid. In terms of industrial production, CAC is an inexpensive reagent. In the structure of CAC, three carbons connect respectively, three nitrogen atoms. Due to the effect of the C=N unsaturated bond, the reactivity of chlorine is high, and it is easy to occur nucleophilic substitution, but the active extent of three chlorines is different (Figure 7). The chlorine can be substituted by functional groups like OH, NH_2, SH, NHR and form new materials with different characteristics and applications. When we only need to replace one chloride, the temperature of reaction should be controlled at temperatures of -15 to 5^{o} C and the reaction should be under the attendance of an acid-binding agent; for the removal of the second chlorine, the temperature should be increased to 40-60 ℃ and the acid-binding agent is necessary; the removal of the third chlorine can be achieved by keeping reaction temperature at

temperature 90-100° C and the acid-binding agent presence (*64*).

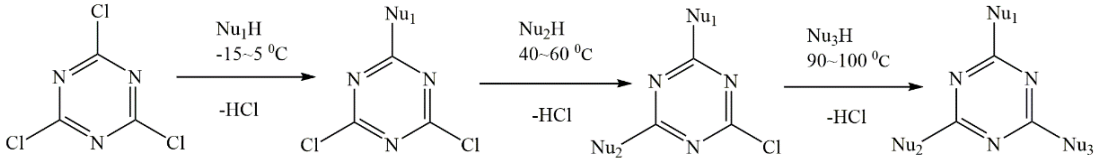


Figure 7: Chemoselective reactivity of CAC *(60).*

1.3.2 The applications of triazine derivatives

Triazine derivatives refer to the chemical compounds containing triazine structures. These derivatives are antibacterial, antitumor and have good optical properties. They are widely used in pesticides, medicine, fluorescent whitening agents, antioxidants, lubricants, paper treatment agents, rubber and in textile auxiliaries areas (*37*, *65*).

Kukla *et al.* reported the ability of triazine against the human immunodeficiency virus (HIV) (*66*). Menicagli *et al.* were among the first to show the potential of the antitumor properties of triazine derivatives, by studying the *in vitro* antitumor properties of a series of 2-alkyl-(alk-1′-ynyl,aryl)-4,6-dialkoxy-1,3,5 -triazines derivatives (*65*) (Figure 8). Their findings showed that the presence of saturated or unsaturated moiety bonded to the heterocyclic ring via a C-C bond, as well as the type of the structure of the carbon-carbon bonded residue, seem to play a significant role in the observed cytotoxic activity.

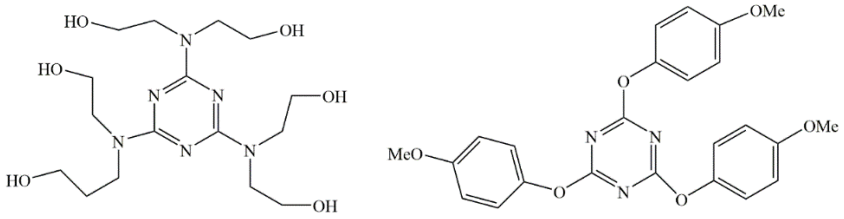


Figure 8: Triazine derivatives with antitumor properties synthesized by Menicagli *et al*. (*65*).

1.3.3 Synthesis of triazine-based dendrimers

Nowadays, dendrimers are usually synthesized by two methods called divergent (1) and convergent (2). In the divergent method, the dendrimers are started from the

core. More procedures are needed to achieve successive generations. In the convergent method, the branches/dendrons are synthesized separately and then connected to the core. Accordingly, the theoretical output is related to the amount of branches that are initially used (*67*). Also, other methods are used to synthesize triazine dendrimers such as solid phase synthesis (*68*).

(1) Convergent method

The convergent method for triazine-based dendrimers generally presents three steps: firstly, utilize the selectivity of CAC to get AB_2 monomer; secondly, repeat the first step to growth AB_2 and arrive to dendron; finally, connect the dendron to a compatible core to obtain the triazine-based dendrimer.

The nucleophilic aromatic substitution reaction of the chloride (which are on the triazine rings) with phenolates was used to synthesize triazine dendrimers. To improve this substitution reaction, a poor electron group such as heterocyclic or the groups, which are connected on the heterocyclic structure are needed. Specially, it is more efficient to substitute the chlorine that is on the heterocyclic structure (like CAC) (*69*).

Verheyde *et al.* (*70*) used the convergent method to synthesize a triazine-based dendrimer containing multiple triazine rings (Figure 9). First, through the Grignard reaction the AB_2 monomer was obtained. The two chlorine atoms of the AB_2 monomer reacted with two 3,5-bis-t-butylphenol that is inexpensive. Then the $–CH_3$ of **1** (Figure 9) was converted to –H with the presence of boron tribromide. After, two groups of **1** were connected to AB_2 (Figure 9) and the dendron was obtained. After repeating the above reaction to getting branches, the triazine-based dendrimer was obtained by choosing a suitable core like CAC (*70*).

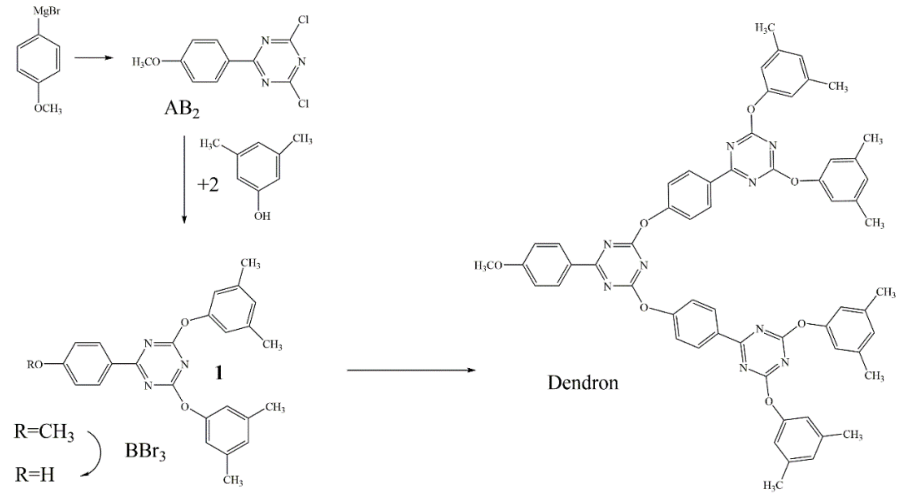


Figure 9: The convergent method used by Verheyde *et al.* in the preparation of triazine-based dendrimer (*70*).

Takagi *et al.* used CAC and *p*-nitrophenol as raw materials, and got the AB_2 monomer (Figure 10). Then compound **1** was prepared for the terminal groups of the dendrimer. To connect the $–NO_2$ of compound 1, a reduction step was necessary. Under the presence of $Fe/FeSO_4 \cdot 7H_2O$, the nitro group was reduced to amino-group (Figure 10). Then the amino-group reacted easily with the chlorine atom of the triazine core by removal of HCl. N-generation of dendron was obtained by repeating the reaction. Finally, the triazine-based dendrimer was got using the CAC as the core (*71*).

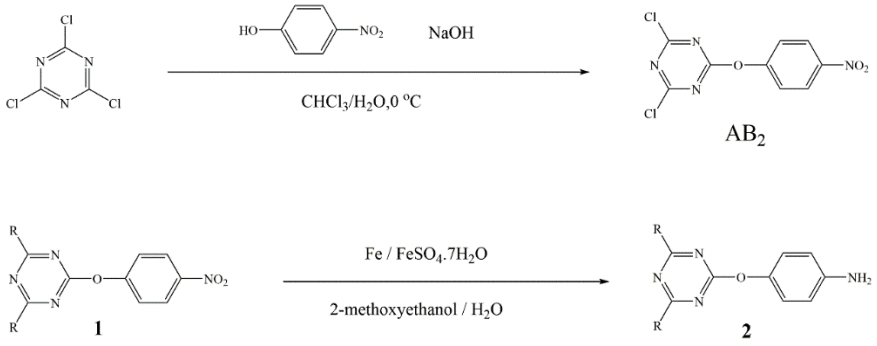


Figure 10: AB_2 monomer in the preparation of triazine-based dendrimers synthesized by Takagi *et al.* (*71*).

Recently, Enciso *et al.* synthesized a low generation triazine dendrimer in high yields by using microwave assisted reactions (*72*). The main advantage is that the nucleophilic substitution reactions on the CAC progress more rapidly with the

assistance of microwave, when compared to the traditional reaction approach.

In the first reaction, to ensure the complete conversion of CAC, more than 2 equivalents of diamine were used (Figure 11). When this reaction was finished, monosubstitution compounds were not discovered. The whole reaction was under the presence of microwave at 60 ℃, and only 10 min was needed to finish this reaction. Finally, a clear oil product was obtained with 95% yield (*72*).

For the synthesis of C2, harsher conditions were needed. 10 equivalents of diamine were used to reduce the product of the undesired dimer (Figure 12). This reaction needed more time (30 min) than the synthesis reaction of C1, and a high temperature (95 ℃) was also needed. Finally, a clear oil product was obtained with 87% yield (*72*).

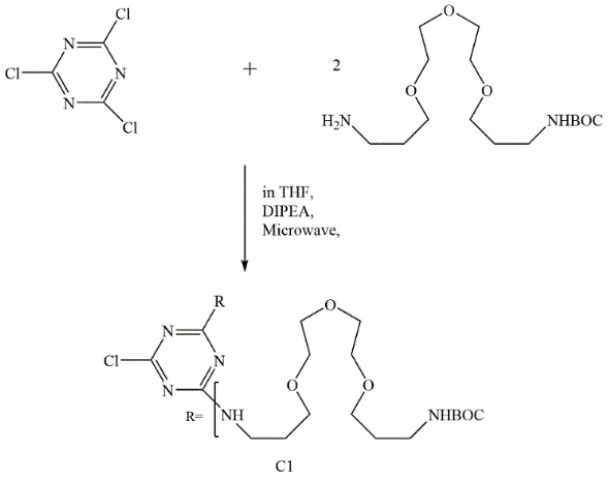


Figure 11: Synthesis of low generation triazine dendrimer, C1, with the help of microwave (*72*).

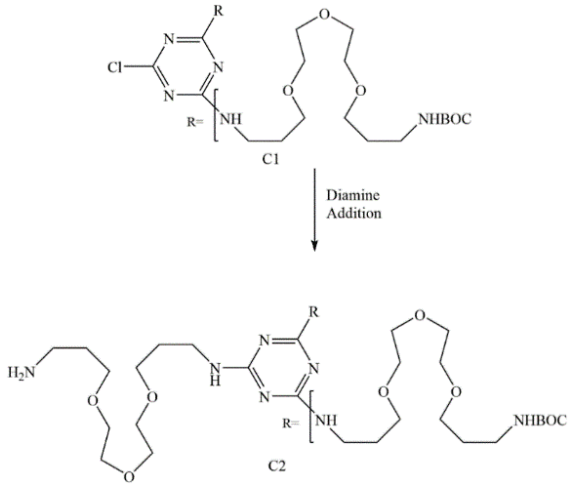


Figure 12: Synthesis of low generation triazine dendrimer, C2, with the help of microwave (*72*).

For the high generation of triazine dendrimers, an expected yield is not obtained, and traditional thermal conditions are still required. However, the microwave-assisted synthesis method can reduce the time for preparing low generation dendrimers and provides more time for the studies of high generation dendrimers (*72*). Depending on the synthesis steps, the same authors synthesized the generation 9 of this family of dendrimers using a macromonomer (Figure 13) (*73*).

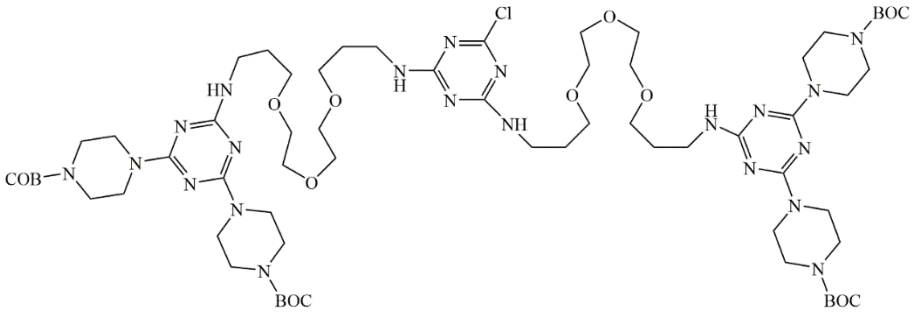


Figure 13: The macromonomer was used by Enciso *et al.* in which the BOC-piperazine is used to protect the $–NH_2$ groups (*73*).

(2) Divergent method

In the divergent method, one step is needed to protect the functional group. However, this approach revealed to be experimentally too complicated. CAC was widely used as a core to synthesize triazine dendrimers due to its selective chemical property of three chlorine atoms (*60*).

Takagi *et al.* (*71*) used CAC and *p*-nitroaniline as starting material to synthesize compound **1**. After the reduction of $–NO_2$ groups on compound **1** (Figure 14), compound **2** and compound **3** were connected to give a higher generation dendrimer. Finally, these reactions were repeated to achieve the final triazine dendrimer.

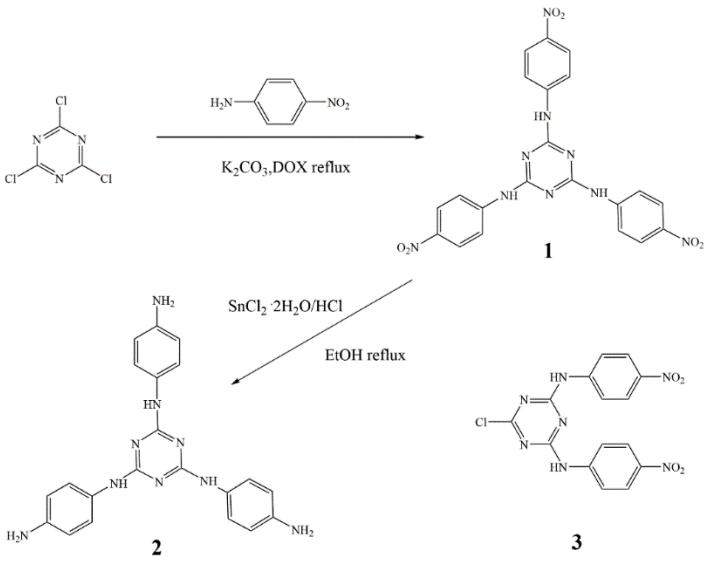


Figure 14: The divergent method used by Takagi to synthesize a triazine-based dendrimer (*71*).

Namazi and Adeli (*74, 75*) reported the use of PEG as the core and CAC as the main material to synthesize triazine-based dendrimers (G1.0, G1.5,G2.0) through the divergent method. The synthetic route is showed in Figure 15. In the reaction *i*, the PEG and CAC was mixed in dichloromethane, using as the base NaOH. The yield of the product G1.0 is 100%. In the reaction *ii*, excess diethanolamine (DEA) and G1.0 was mixed at room temperature. After refluxing for 4 h, the product G1.5 was obtained with 100% yield. In step *iii*, the CAC reacted first with phenol. Then the product 4 of this reaction was mixed with G1.5 in dried dichloromethane under argon, using NaOH as the base. The yield of the obtained product generation 2 (G.2) was 70%.

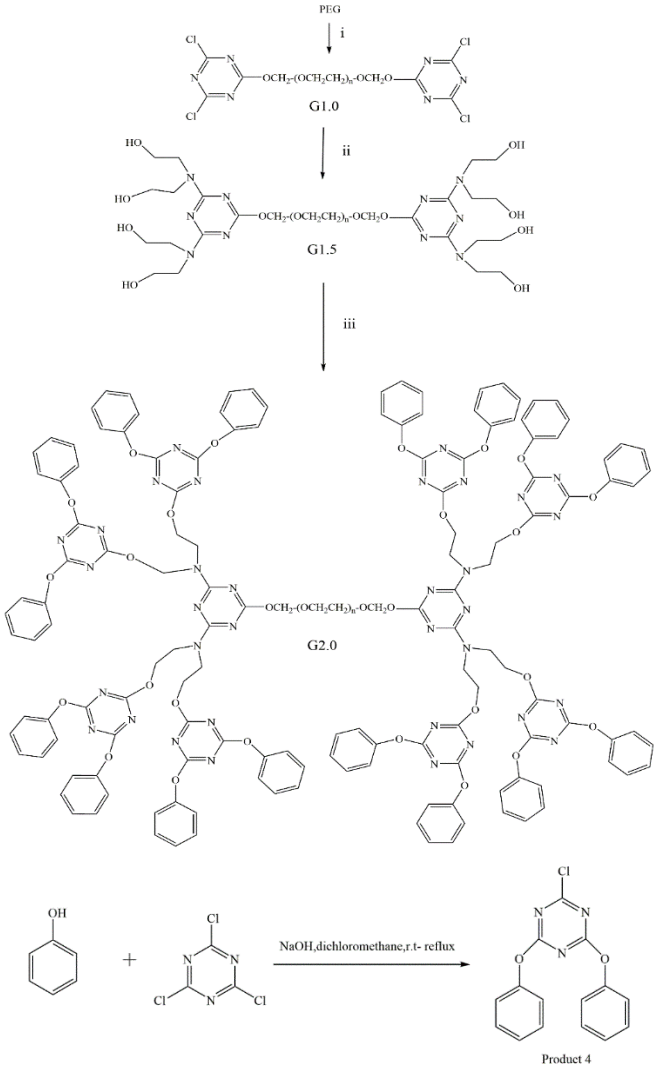


Figure 15: The divergent method used by Namazi and Adeli (*75*) for the preparation of a PEG triazine-based dendrimer.

(3) Solid phase synthesis

Dilly *et al.* (*68*) introduced a new way to prepare triazine-based dendrimers using solid phase synthesis method. These kinds of dendrimers were separated from solid phase under the action of trifluoroacetic acid (TFA). The synthesis route is showed in Figure 16. First, diisopropylethylamine (DIPEA) was used as a base. The product 1 was obtained by the reaction of CAC with the supporting material. Then the product 1 reacted with ethylenediamine (ED) at 80-100 ℃ under the action of DIPEA, and the product 2 was obtained. Product 3 was achieved by the reaction of product 2 and CAC. Yoo *et al.* also used solid phase approach and divergent method to synthesize other triazine-based dendrimers (*76*).

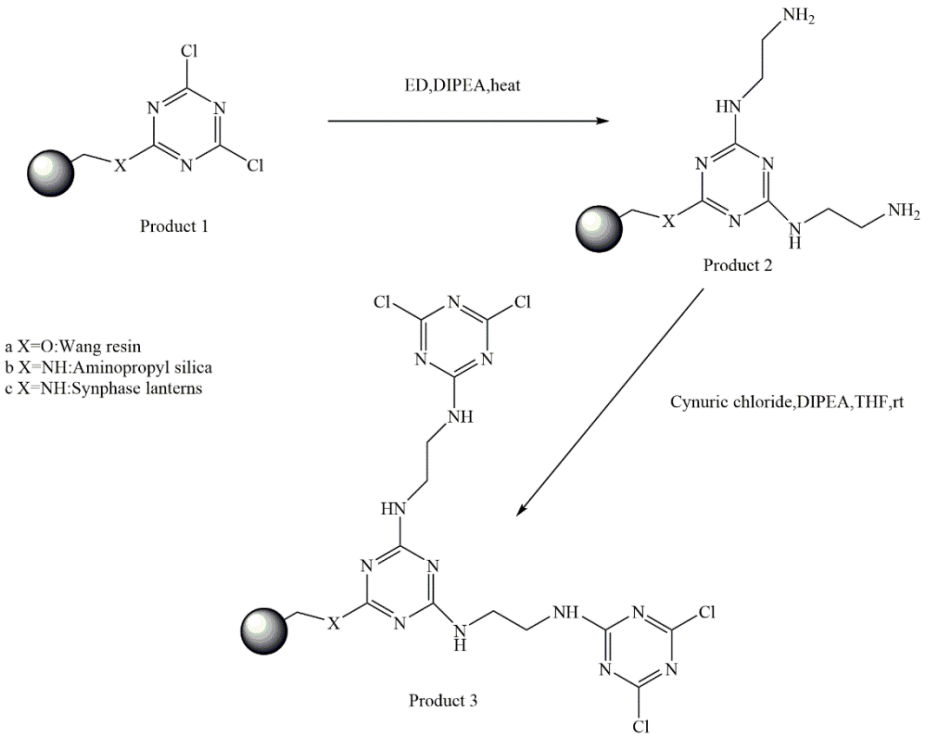


Figure 16: The solid-phase method used by Dilly *et al.* (*68*).

1.3.4. The properties and applications of triazine-based dendrimers

In general, the solubility of the triazine-based dendrimers is poor, but it can be improved by modifying the terminal groups or linkers of the dendrimers. The triazine-based dendrimers normally have good thermal oxidative stability. Kraus and Louw *(77)* used a convergent method to synthesize the triazine-based dendrimers by using the trimeric acid (1,3,5-benzenetricarboxylic acid) as the core and the silicon phthalocyanine as the functional group. The obtained triazine-based dendrimers were stable in the external environment and, according to the authors, can be useful for applications in photosynthetic materials (*77*).

Due to the unique biological activity of the triazine-based dendrimers, they can be used in the research of antibiotics, anticancer drugs and in the drug delivery field. Biphenyl drugs are effective inhibitors of respiratory system virus (RSV). Nikitenko *et al.* (*78*) reported a kind of biphenyl compound which contains triazine ring (compound **1** in Figure 17). This compound presented a better inhibitory activity than the biphenyl

compound that contains a pyrimidine analog (compound **2** in Figure 17).

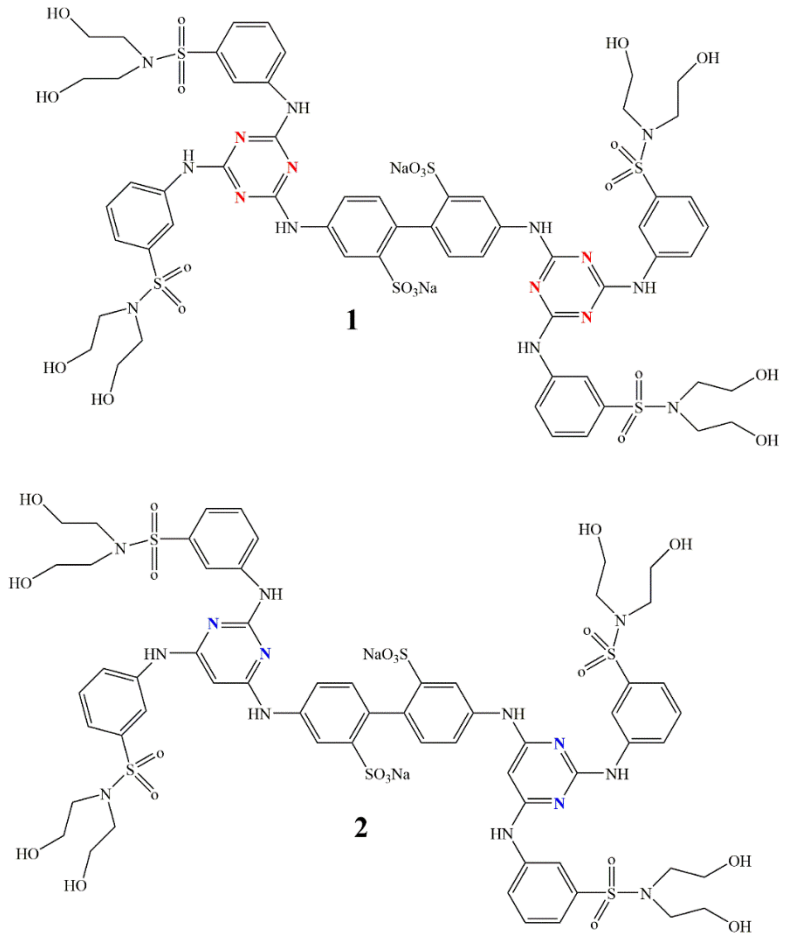


Figure 17: The triazine compound **1** and the pyrimidine analog **2** (*78*).

Simanek's group studies (*79*) showed that the triazine-based dendrimers can reduce the toxicity of drugs when they are used as drug carriers. They synthesized a triazine-based dendrimer molecule that can decrease the toxicity of anticancer drugs (methylamine purine, 6-mercaptopurine) using the injected peritoneal injection method (*80*). By mixing these drugs with triazine-based dendrimers, a good increase on the solubility of these drugs was observed. The mice study showed that the use of triazine-based dendrimers can reduce the toxicity of anticancer drugs and allow the use of increased doses (*79, 81, 82*).

A series of aryl- triazine-based dendrimers that have conjugated systems was synthesized by Kim's team (*83, 84*). This dendrimer family has electroluminescence properties and can be hopefully applied in organic light-emitting diodes.

Compared with the other families of dendrimers, triazine-based dendrimers are less studied and, despite the obtained achievements, is a promising field of study, particularly for the preparation of metallodendrimers. The stability and biological and optical activities of triazine-based dendrimers provide them with a broad prospect of applications.

1.4 Objectives and General Strategy of the Project

The objective of this project was to synthesize low generation triazine-based dendrimers by using, as starting materials, CAC and triethylene glycol (TEG), and test the cytotoxicity of the prepared compounds. Our interest in the triazine dendrimers is related with our previous experience in the field of the preparation of metallodendrimers for non-linear applications (*85*) and on the use of PAMAM (*57*) and PPI dendrimers as scaffolds for the preparation of low-generation ruthenium based metallodendrimers (*86*) for drug and gene delivery. The abundant possibilities to synthesize triazine-based dendrimers with expected solubility in aqueous environments and low cytotoxicity made us start a preliminary study on the preparation of these type of dendrimers for biological applications (*e.g.* drug delivery).

As showed in Figure 18, the main synthesis process depends on the chemoselectivity of three different chloride atoms from the starting aromatic material, CAC. This molecule was connected to the core, the TEG, by a nucleophilic reaction to get $G1.0(Cl)_4$. The product was purified by silica chromatography. Then the DEA was used to modify the $G1.0(Cl)_4$ to get $G1.5(OH)_8$. The products were characterized by nuclear magnetic resonance (NMR) techniques, Mass Spectrometry (MS), Fourier Transform Infrared Spectroscopy (FTIR). Finally, the cytotoxicity of these compounds was investigated *in vitro* using the NIH 3T3 fibroblast cell line of mouse (used as a model of normal cells) and the A2780 human ovarian carcinoma cell line (used as a model of cancer cells).

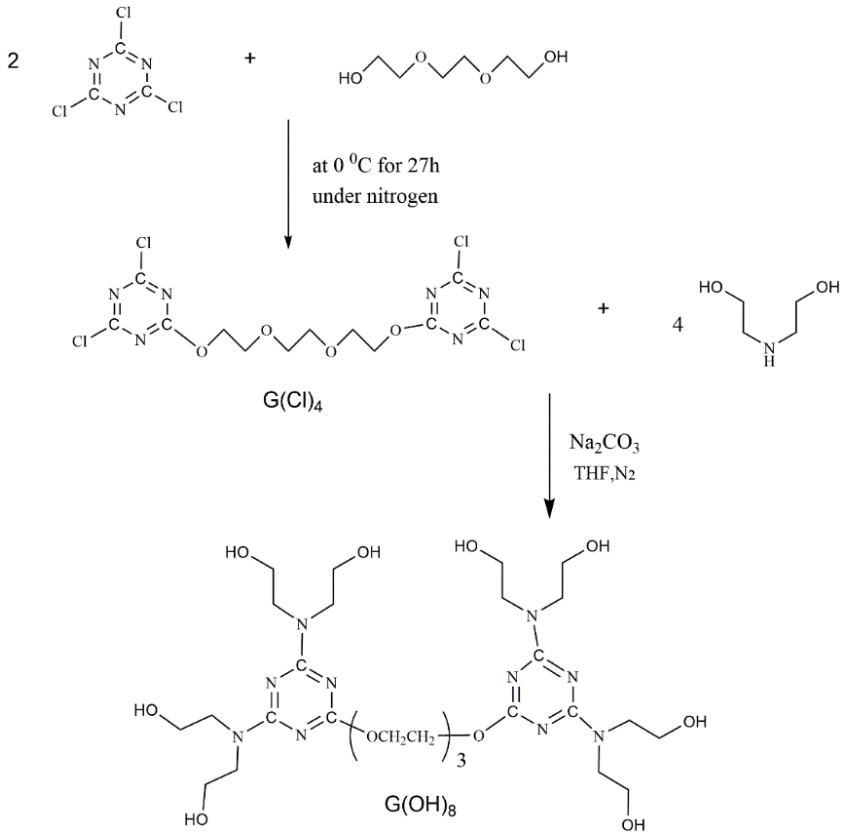


Figure 18: Synthesis process of $G(OH)_8$ dendrimer.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Reagents and equipment

CAC (purity > 99%) was purchased from ACROS ORGANICS (New Jersey, USA). Sodium hydroxide, chloroform, *n*-hexane, tetrahydrofuran (THF), 1,4-dioxane, TEG were bought from Thermo Fisher Scientific (MA, USA). Ethyl acetate was obtained from VWR (PA, USA). DEA was purchased from Sigma-Aldrich (MO, USA). Deuterated chloroform, deuterated dimethyl sulfoxide (DMSO), and diisopropylamine were bought from MERCK (Darmstadt, Germany).

In the cytotoxicity studies, two types of cell lines were used, NIH 3T3 and A2780. The Dulbecco's Modified Eagle Medium (DMEM) was acquired from Sigma-Aldrich (MO, USA) and to this was added 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (AA) and this medium was used for the NIH 3T3 cells. The Roswell Park Memorial Institute media (RPMI-1640) was acquired from Sigma-Aldrich (MO, USA) and was also used with 10% FBS and 1% AA and this medium was used for the A2780 cells. These media were used to culture the cells. The trypsin-EDTA solution was used to detach the cells from the bottom of the plates.

NMR Spectroscopy

Acquisition of ^{1}H and ^{13}C spectra was made on an NMR Spectrometer of 400 MHz from Bruker (UltraShield^{TM} 400 Plus ULTRA LONG HOLD) Console AVANCE 400 II+.

MS Spectroscopy

A Mass Spectrometer type Ion Trap Multipolar LC-MSMS (Bruker Esquire 6000 Mass Spectrometer) with Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) sources and MSn capacity was used in the mass spectrometry studies.

FTIR spectroscopy

The FTIR spectra were acquired using KBr pellets on a Perkin Elmer Spectrum Two spectrometer.

2.2 Synthesis of the G1.0$(Cl)_4$ dendrimer

Under nitrogen atmosphere, a solution of CAC (17.08g, 92.6mmol) in 50ml of dry THF (Solution 1) was prepared and a solution of TEG (6.95g,46.3mmol) with N,N-DIPEA (16.1ml, 46.3mmol) in 6ml of dry THF at 0 ℃ (a water solution with salt and ice was used to control the temperature) (Solution 2). Then, these two solutions were mixed as showed in Figure A1 in the ANNEX, at 0℃, under a nitrogen atmosphere. After being stirred for 27 h at 0℃, the mixture was filtered, and the obtained white solid was washed with 20 ml of THF. The excess solvent was evaporated using a rotary evaporator system. The white solid was purified by silica gel column chromatography using a mixture of hexane and ethyl acetate with a ratio of 4:1. Finally, the product (which was from silica gel column) was washed with hexane to remove the ethyl acetate. Finally 5.5g of G1.0$(Cl)_4$ was obtained as a white solid (yield: 26.6%). ^{1}H NMR ($CDCl_3$, 400 MHz) [δ: 4.627, 4.634, 4.638, 4.642, 4.650(m, 4H); 3.843, 3.851, 3.854, 3.858, 3.866 (m, 4H); 3.683 (s, 4H)].^{13}C NMR ($CDCl_3$, 400 MHz) [δ: 60.55, 68.69, 69.46, 71.02, 172.721]. IR (KBr) ν: 2920 (-CH_2), 1384 (-C-Cl) cm^{-1}.

2.3 Synthesis of the G1.5$(OH)_8$ dendrimer

Because 1,4-dioxane combines with atmospheric oxygen upon prolonged exposure to air, forming potentially explosive peroxides, this reaction was processed in a three neck flask (250mL) under nitrogen.

In a flask, 25 mL of 1, 4-dioxane was added to 2.5g of G1.0 $(Cl)_4$ (5.6 mmol) under stirring. Then, the solution was moved under a nitrogen atmosphere to a three neck flask.

We added DEA（2.7mL，28mmol）to the solution of G1.0(Cl)$_4$ drop wise. Then sodium hydroxide solution (0,9g, 22.4mmol NaOH in 1 ml water) was added as a base (its molar ratio to G1.0(Cl)$_4$ was 4:1). This mixture was stirred for 0.5h at room temperature, then the temperature was increased to 80 ℃ and refluxed for 4.0h. At the end of the reaction, the mixture was filtered by using a vacuum filter funnel. The excess of 1,4-dioxane was removed using a rotary evaporator (under 17mbar, at 37 ℃ conditions), the filtrate was dispersed and washed with THF and acetone, filtered again, and the filter cake was dried using the rotary evaporator. Finally, the product was washed with acetone to remove the impurities:1.24 g of G1.5(OH)$_8$ was obtained as a white oil (yield: 33.3%). ^{1}H NMR (DMSO-d_6, 400 MHz) [δ:4.683, 4.694, 4.706, 4.724, 4.736, 4.749 (m,8H); 4.272, 4.284, 4.295 (t,4H); 3.672, 3.661, 3.684 (tri, 4H); 3.568(s, 36H)]. IR (KBr) ν: 3382 (-OH), 2948 (-CH_2), 2879 (-CH_2), 1583 (-C=N-), 1520 (-C=N-), 1342 (-CH_2) cm^{-1}. MS (ESI) [M+H]$^+$ m/z: calcd. for $C_{28}N_{10}O_{12}H_{52}$, 720.77; found, 721.4.

2.4 Cell Biological Evaluation

The NIH 3T3 and A2780 cell lines were used to evaluate the cytotoxicity of CAC, G1.0(Cl)$_4$ and G1.5(OH)$_8$. First, NIH 3T3 fibroblasts and A2780 cells were plated in 96-well plates. After 24h, the old media were removed and complete medium and the test solutions (10μL of DMSO, CAC-DMSO, G1.0(Cl)$_4$-DMSO and G1.5(OH)$_8$-DMSO solutions) were added to the cell culture wells and then incubated for 48 h, at 37 ℃. Medium and DMSO were used as controls.

The resazurin reduction assay was chosen to measure the cellular metabolic activity to conclude about the cell viability. Then the medium was removed; the wells were filled with fresh medium containing 10% of resazurin, and the cells were kept in the incubator for 3 h. After 3 h, the medium with resazurin was transferred to 96-well white opaque plates and the resorufin fluorescence (λ_{ex}=530nm, λ_{em}=590 nm) was read by using a microplate reader (model Victor3 1420, PerkinElmer).

One-way ANOVA statistical analyzes with Tukey's test was chosen to evaluate the statistical significance of experimental data. All results are reported as mean ± SD. 0.05 was selected as the significance level, and the data was indicated with (**) for $p < 0.01$ and (***) for $p < 0.001$, respectively.

CHAPTER 3 – RESULTS AND DISCUSSION

3.1 Synthesis and characterization of G1.0(Cl)$_4$ dendrimer

Due to the chemoselectivity and by controlling temperature, CAC is used to synthesize a large number of triazine-based dendrimers.

In this work, two equivalents of CAC were connected to TEG as a core. The oxygen atoms in the chain of TEG can improve the polarity of the final dendrimer and form H-bonding with H_2O, which can increase the solubility of the final dendrimer in aqueous solution.

The ^{1}H NMR spectrum of G1.0(Cl)$_4$ in $CDCl_3$ is shown in Figure 19. The peak shift at 7.260 ppm is from $CDCl_3$. The solvent impurities originating from column chromatography are depicted as (d) and (e) in Figure 19.

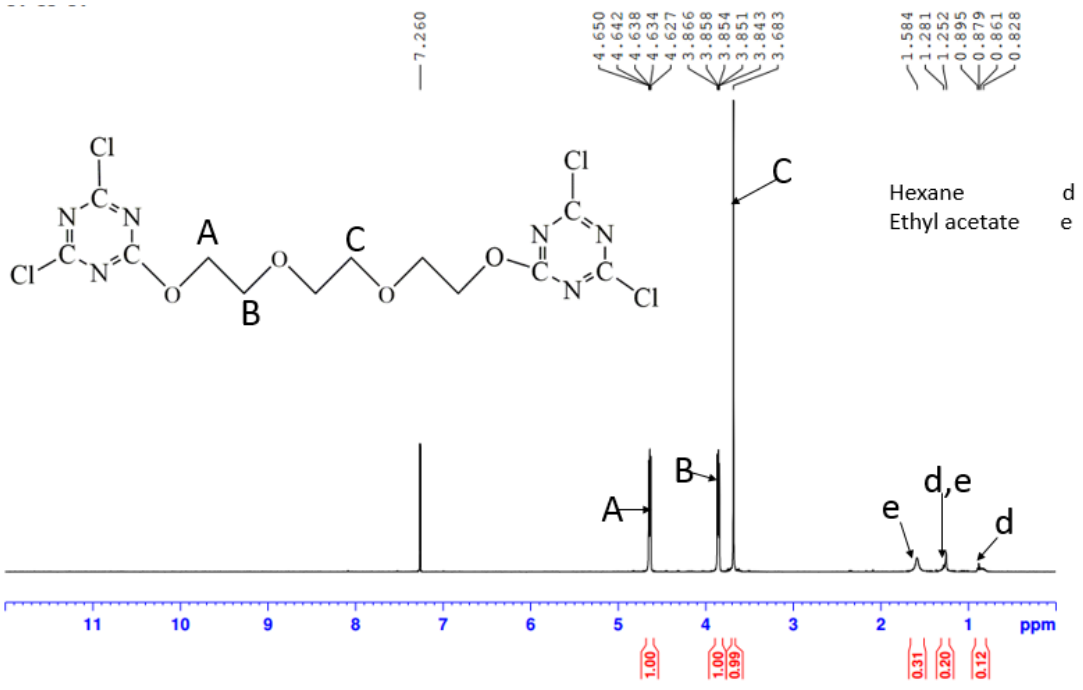


Figure 19: ^{1}H NMR spectrum of G1.0(Cl)$_4$ in $CDCl_3$.

In Figure A2 in the ANNEX, we have the ^{1}H NMR of hexane prepared in $CDCl_3$ acquired from the Spectral Database for Organic Compounds (SDBS). In the ^{1}H NMR spectrum of G1.0$(Cl)_4$ in Figure 19, signals at δ: 0.890, 1.271, 1.280 correspond to hexane. Compared with the ^{1}H NMR of ethyl acetate in $CDCl_3$ obtained from the SDBS (see ANNEX, Figure A3), the signals indicated at (e) in the ^{1}H NMR spectrum of G1.0$(Cl)_4$ were from ethyl acetate.

In the ^{1}H NMR spectrum of TEG in $CDCl_3$ (see ANNEX, Figure A4) there are peaks at 3.721, 3.711 and 3.700 ppm (these peaks are labeled as A in the Figure); 3.652 ppm (this peak is labeled as C in the Figure); 3.599, 3.587 and 3.577 ppm (these peaks are labeled as B in the Figure). In Figure 19, a single peak at C which has a chemical shift at 3.683 ppm (s, 4H) is related with the single peak at 3.652 ppm (s,4H) observed in the SDBS spectrum for the TEG starting material. Due to the position of the carbon in the middle of G1.0$(Cl)_4$, which is indicated as C in the chemical structure in Figure 19, the chemical shift of the proton at this carbon C was not influenced by the atoms from the G1.0$(Cl)_4$ termini.

When the oxygen atom from the -OH group of TEG was connected with CAC, the protons at carbon A in the G1.0$(Cl)_4$ chemical structure (see Figure 19) were less shielded. So the signal of the protons at carbon A in G1.0$(Cl)_4$ moved to the left. The signals of the protons at carbon A should be [δ: 4.627, 4.634, 4.638, 4.642 and 4.650 ppm (m, 4H)] and the peaks of the protons at carbon B [δ: 3.843, 3.851, 3.854, 3.858 and 3.866 ppm (m, 4H)].

The ^{13}C NMR spectrum of ethyl acetate has four signals at 171.080, 60.440, 21.000 and 14.280 ppm (SDBS). Looking at the ^{13}C NMR spectrum of G1.0$(Cl)_4$, in Figure 20, the observed peaks at 14.348, 20.299, 21.182, and 171.233 ppm correspond to ethyl acetate.

The ^{13}C NMR spectra in Figure 20 also revealed a relevant peak with a chemical shift at 172.721 ppm for both CAC and G1.0$(Cl)_4$. This means that there is a triazine ring in the G1.0$(Cl)_4$ product.

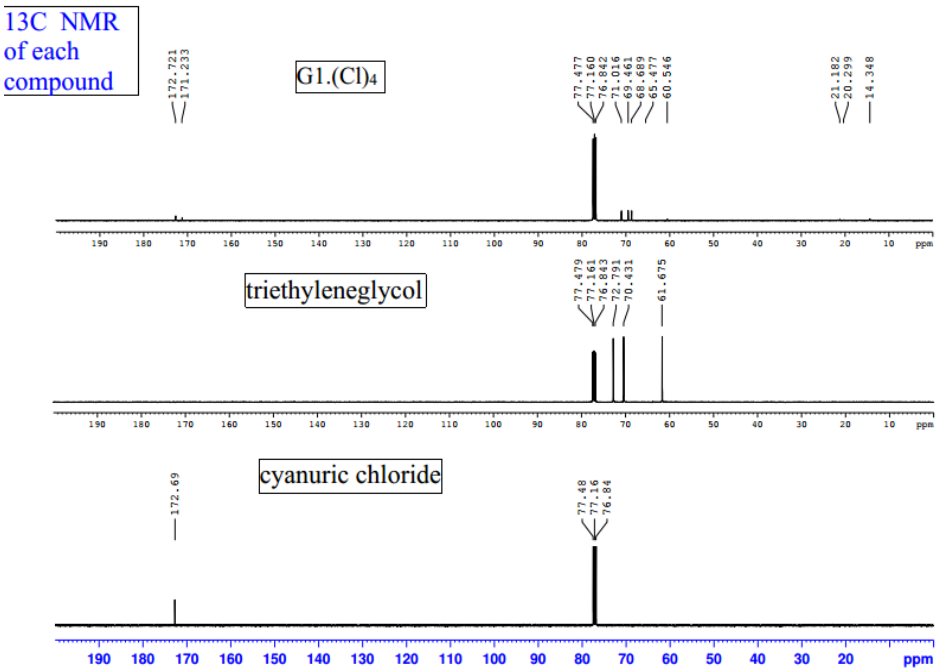


Figure 20: ^{13}C NMR of G1.0$(Cl)_4$, TEG and CAC in $CDCl_3$.

In the ^{13}C NMR spectrum of TEG, there are 3 signals at 72.791, 70.431, and 61.675ppm. The ^{13}C NMR spectrum of G1.0$(Cl)_4$ presents peaks at 71.016, 69.461, 68.689 and 60.546 ppm that belong to the TEG core, confirming that the connection between TEG and CAC was successful.

The ^{1}H NMR spectrum of the G1.0$(Cl)_4$ compound acquired at room temperature after two weeks confirmed its stability.

3.2 Synthesis and characterization of the G1.5$(OH)_8$ dendrimer

As presented in Figure 21, DEA was used to increase the generation and the solubility of the dendrimer in solution.

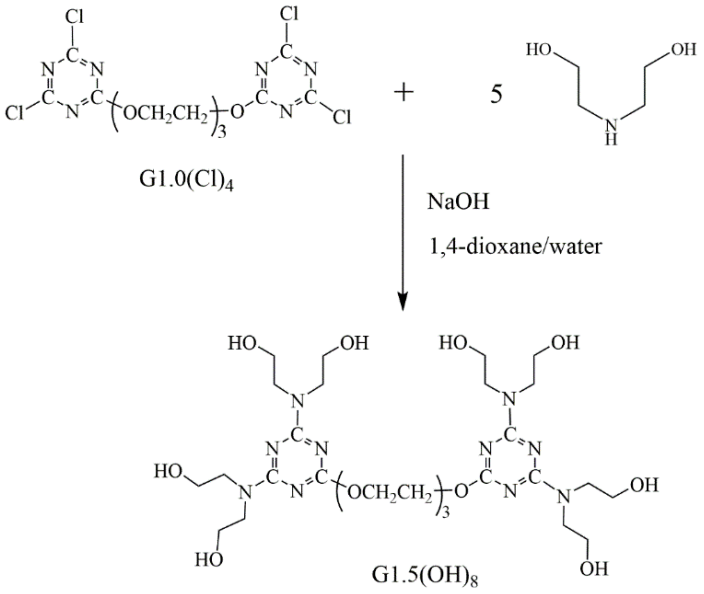


Figure 21: Synthesis of $G1.5(OH)_8$.

The results of the solubility test for $G1.5(OH)_8$ in solvents with different polarity are presented in Table 2. Except DMSO, water, dimethyl formamide (DMF) and methanol, the other pure solvents cannot dissolve the product even at 60 ℃. The table shows that only solvents with higher polarity (except acetonitrile) and a mixture of dioxane:methanol (9:3) can dissolve $G1.5(OH)_8$ totally.

Table 2: Solubility of $G1.5(OH)_8$ in selected organic solvents or mixture of solvents at room temperature.

Solvent	**Polarity***	**Solubility at Room Temperature**
Methylene chloride	3.10	No obvious dissolution
Tetrahydrofuran	4.00	No obvious dissolution
Chloroform	4.10	No obvious dissolution
Ethyl acetate	4.40	No obvious dissolution
Dioxane	4.80	No obvious dissolution
Acetone	5.10	No obvious dissolution
Methanol	5.10	Well dissolved
Acetonitrile	5.80	No obvious dissolution
DMF	6.40	Well dissolved
DMSO	7.20	Well dissolved
Water	10.20	Well dissolved
Dioxane:Methanol=9:3		Well dissolved

*The polarity index of the different solvents was obtained from LSU Macromolecular Studies Group.

Comparing the ^{1}H NMR spectra of $G1.5(OH)_8$ and $G1.0(Cl)_4$, the most relevant difference corresponds to peak D in Figure 22, which can be attributed to the proton of the –OH groups from the $G1.5(OH)_8$ termini. As the spectrum showed, the shape of this signal is two triplets instead of the expected wide single peak of the –OH. Probably, the main reason for the observed effect on the proton peaks (8H) comes from the fact that DMSO may be bound to one or two –OH groups depending on the concentration (*87*).As to peaks A, B and C of $G1.5(OH)_8$, it is similar to $G1.0(Cl)_4$ according to compare Figure 19 with Figure 22.

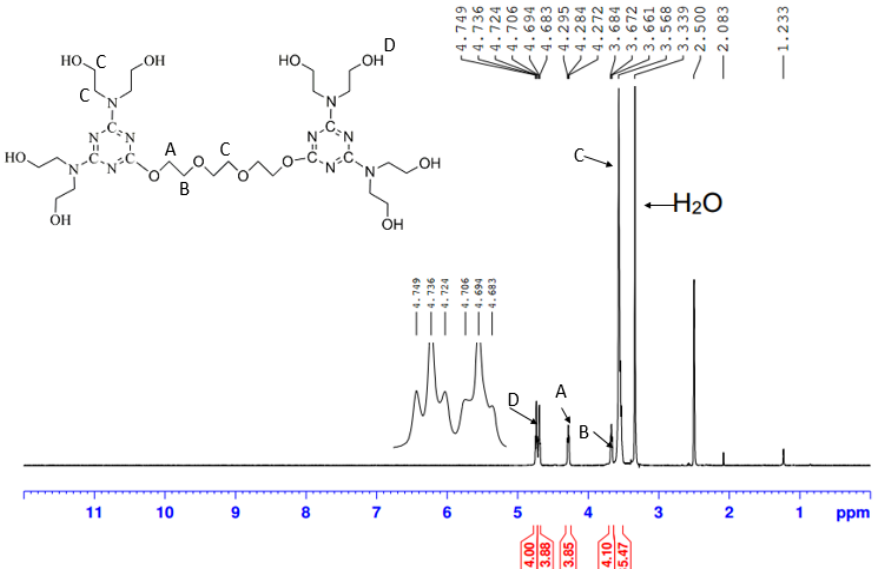


Figure 22: ^{1}H NMR spectrum of $G1.5(OH)_8$ in DMSO.

3.3 The MS spectrum of $G1.5(OH)_8$ dendrimer

The mass spectrum of the $G1.5(OH)_8$ dendrimer in MeOH (Figure 23) presents two special fragments. One fragment was the molecular peak M=721.4 that is in accordance with the molecular weight of $G1.5(OH)_8$ dendrimer and the other one at M=436.1 corresponds to the half of $G1.5(OH)_8$ dendrimer. The peak 743.4 is probably formed by the reaction between of $G1.5(OH)_8$ with Na^{+}.

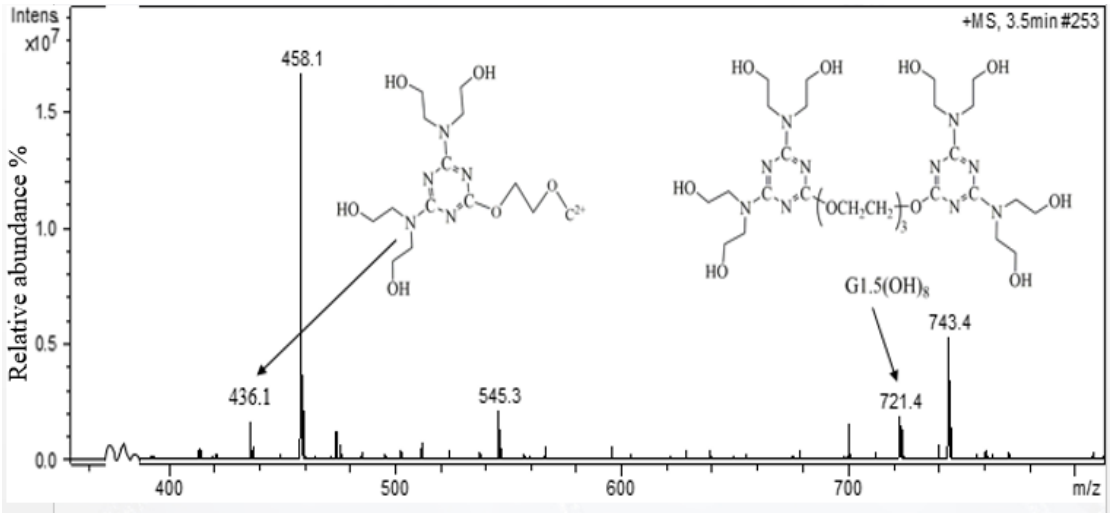


Figure 23: The mass spectrum of $G1.5(OH)_8$ in MeOH.

3.4 The FTIR spectrum of CAC, $G1.0(Cl)_4$ dendrimer and $G1.5(OH)_8$ dendrimer

The characteristic vibrations of the chemical bonds in the functional groups of any prepared compound will display special bands in the FTIR spectrum (*88*). So we can use FTIR spectroscopy to compare different molecules to find the relationship between different compounds. In Table 3 the IR spectrum vibrational bands obtained for the materials prepared in this work are compared with those obtained from Bio-Rad database.

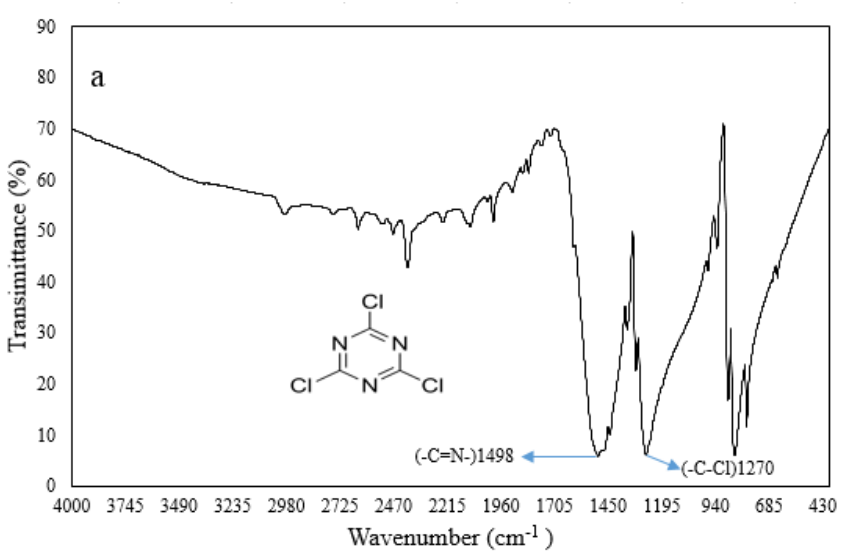
Table 3: Comparison of IR spectrum stretching vibrations of the materials in this work and those obtained from the database.

Bonds	**Data from this work**	**Stretching vibration***
C-Cl	1270, 1384 cm^{-1}	1200-1000 cm^{-1}
C=N	1498, 1583, 1520 cm^{-1}	1500 cm^{-1}
-OH	3463, 3382 cm^{-1}	3650-3320 cm^{-1}
CH_2	2920, 2948, 2879, 1342 cm^{-1}	2936-2843 cm^{-1} 1485-1445 cm^{-1}

*Stretching vibration values obtained from the Bio-Rad data base (http://www.bio-rad.com/)

In Figure 24a, the peak (ν_{as} = 1498 cm^{-1}) is from the –C=N- of CAC. This

band also appeared in the spectrum of Figure 24c, which wavenumbers are ν_{as} = 1583 cm^{-1} and ν_{as} = 1520 cm^{-1}, respectively. In the case of spectrum 24b, the stretching vibration of –C=N- is unclear. The peaks at ν_{as} = 1270 cm^{-1} in Figure 24a and ν_{as} = 1384 cm^{-1} in Figure 24b are from –C-Cl of CAC. In Figure 24b and 24c, the maximum at ν_{as} = 1342 cm^{-1}, ν_{as} = 2879 cm^{-1}, ν_{as} = 2920 cm^{-1} and ν_{as} = 3382 cm^{-1} are attributed to the $–CH_2$ bonds. In Figure 24c, the stretching vibration –OH correspond to ν_{as} = 3382 cm^{-1}. In Figure 24b, there is a wide peak (ν_{as} = 3463 cm^{-1}). Considering that KBr was used to prepare the FTIR sample, maybe the type and amount of sample in the KBr, the preparation of the FTIR sample and the quantity of moisture in the KBr matrix affect the resolution of the IR analysis. Comparing the achieved results with the NMR and MS data (in the case of $G1.5(OH)_8$), we can confirm the successful preparation of the desired dendrimers.



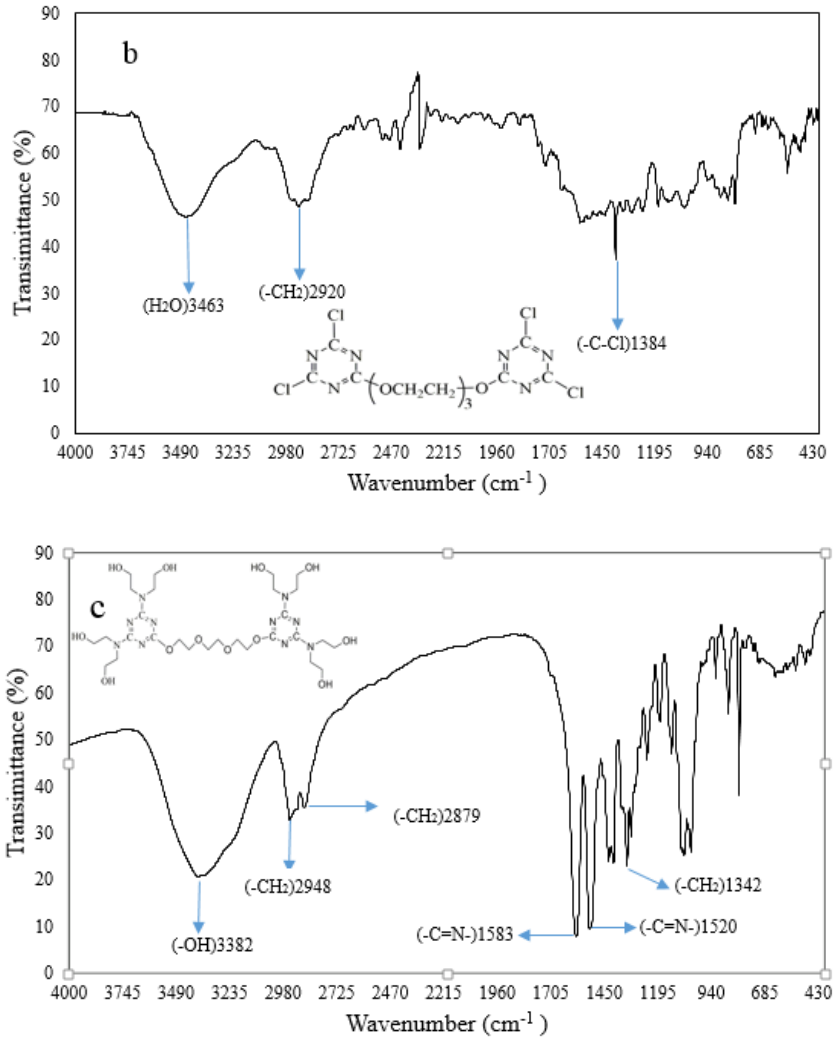


Figure 24: The FTIR spectrum of CAC (a), G1.0$(Cl)_4$ dendrimer (b) and G1.5$(OH)_8$ dendrimer (c).

3.5 *In Vitro* Cytotoxicity of CAC, G1.0$(Cl)_4$ dendrimer and G1.5$(OH)_8$ dendrimer

The application of triazine-based dendrimers in the biological field depends on their inherent toxicities, which can hinder or limit their biological applications. The *in vitro* cytotoxicity of CAC , G1.0$(Cl)_4$ dendrimer and G1.5$(OH)_8$ dendrimer were tested by using NIH 3T3 and A2780 cell lines. The resazurin reduction assay showed the results of cell viability (see Figures 25 and 26). The wells that only had medium were used as a control. A second control used a mixture of medium (either DMEM or RPMI-1640) with DMSO since this solvent was used to dissolve the tested compounds.

As shown in Figures 25 and 26, the viability of cells in the presence of CAC, G1.0(Cl)$_4$ dendrimer and G1.5(OH)$_8$ dendrimer exceeded 80% in the concentration range 1 μM to 100 μM for both cell lines. As such, the compounds present very low toxicity within the selected concentration range.

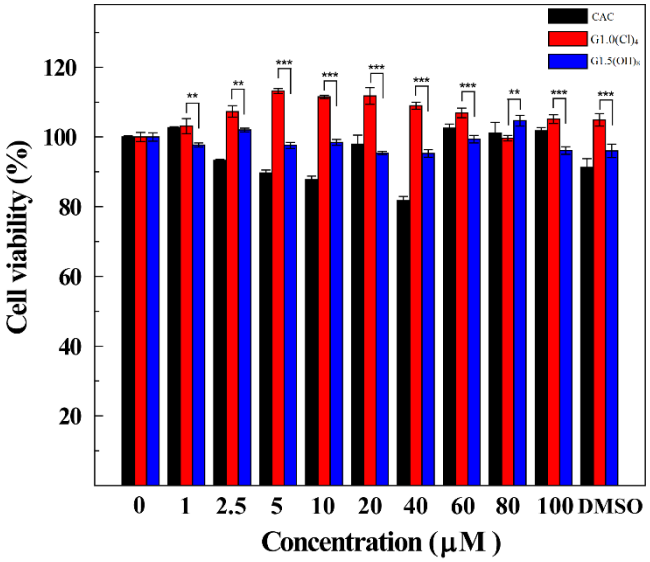


Figure 25: Cytotoxicity evaluation of CAC, G1.0(Cl)$_4$ dendrimer and G1.5(OH)$_8$ dendrimer at different concentrations for 48 h and for NIH 3T3 cells using resazurin reduction assay. All results were expressed as the mean ±SD, n =3. One-way ANOVA with Tukey's test was used to assess the statistical difference between the group means (**p <0.01, *** p <0.001).

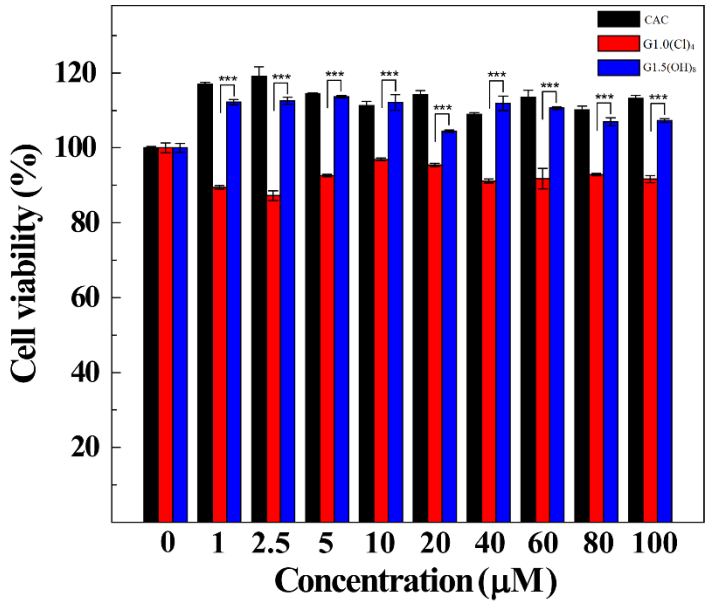


Figure 26: Cytotoxicity evaluation of CAC, G1.0(Cl)$_4$ dendrimer and G1.5(OH)$_8$ dendrimer at different concentrations for 48 h and for A2780 cells using resazurin reduction assay. All results were expressed as the mean ±SD, n =3. One-way ANOVA with Tukey's test was used to assess the statistical difference between the group means (**p <0.01, *** p <0.001).

Figures 27 and 28 make a comparison between the cytotoxicity of $G1.0(Cl)_4$ dendrimer and $G1.5(OH)_8$ dendrimer for increasing compound concentrations and for NIH 3T3 cells and A2780 cells. $G1.0(Cl)_4$ and $G1.5(OH)_8$ have respectively significant difference on the toxicity of these two kinds of cells.

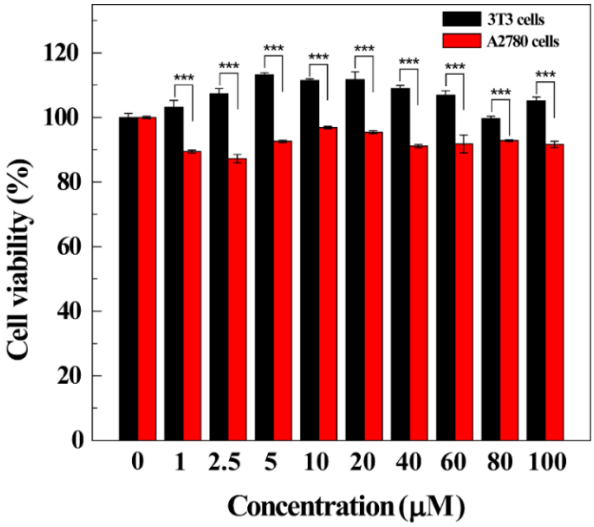


Figure 27: Comparison of the cytotoxicity of $G1.0(Cl)_4$ dendrimer at different concentrations between NIH 3T3 cells and A2780 cells after 48h. All results were expressed as the mean ±SD, n =3. One-way ANOVA with Tukey's test was used to assess the statistical difference between the group means (**p <0.01, *** p <0.001).

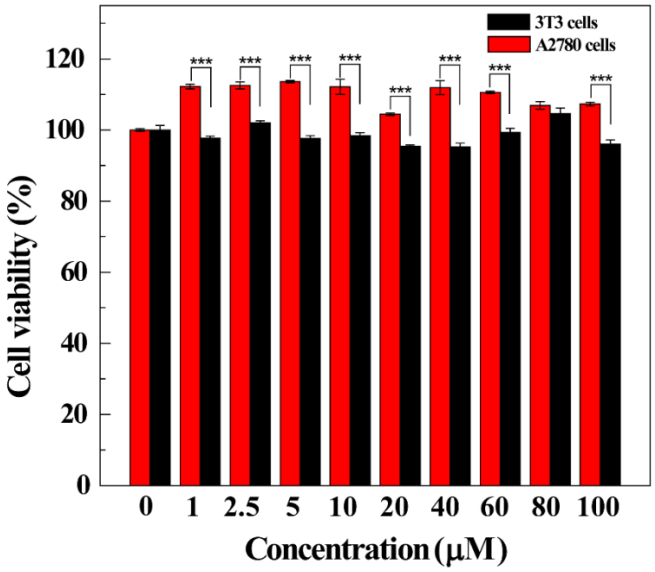


Figure 28: Comparison of the cytotoxicity of $G1.5(OH)_8$ dendrimer at different concentrations between NIH 3T3 cells and A2780 cells after 48h. All results were expressed as the mean ±SD, n =3. One-way ANOVA with Tukey's test was used to assess the statistical difference between the group means (**p <0.01, *** p <0.001).

The inherent toxicity of dendrimers hinder their application in pharmaceutical field (*89*) and, compared with the interior groups, the surface groups is much more active to influence the toxicity of dendrimers (*60*). In general, when the surface charge of dendrimers is cationic, the dendrimers show high toxicity. The main reason for that behavior is related with the fact that cationic charges can interact with the biological membranes that are negatively charged. As a result of this interaction, the cell membrane can be disrupted due to the creation of nanoholes (*89*). The terminal atoms of the dendrimer G1.0$(Cl)_4$ are chloride atoms that are very electronegative (*90*). So, the surface of G1.0$(Cl)_4$ is rich in electrons and presents a negative character. Oxygen is also an electronegative atom. Once the dendrimer G1.5$(OH)_8$ has 8 –OH terminal groups, its surface also has a strong negative character. For these reasons the toxicity of our dendrimers is low.

GENERAL CONCLUSIONS

In summary, the G1.0$(Cl)_4$ dendrimer and the G1.5$(OH)_8$ dendrimer were synthesized and characterized by using common synthesis techniques. These two dendrimers showed good stability on the atmospheric air at room temperature. Importantly, once the G1.5$(OH)_8$ dendrimer displays a good solubility in water (and DMSO), it is possible to foresee their use in biological applications. CAC, G1.0$(Cl)_4$ and G1.5$(OH)_8$ dendrimer do not have cytotoxicity according to the results of the resazurin reduction assay with 0-100 μM concentrations.

During this work, special techniques of organic chemistry were used, as well as common and advanced synthesis techniques, to confirm the preparation of the reported triazine-based dendrimers. Despite the small number of results obtained in the time available, it is expected that they can open the way for the preparation of higher dendrimer generations for biological applications, particularly for drug delivery.

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ANNEX

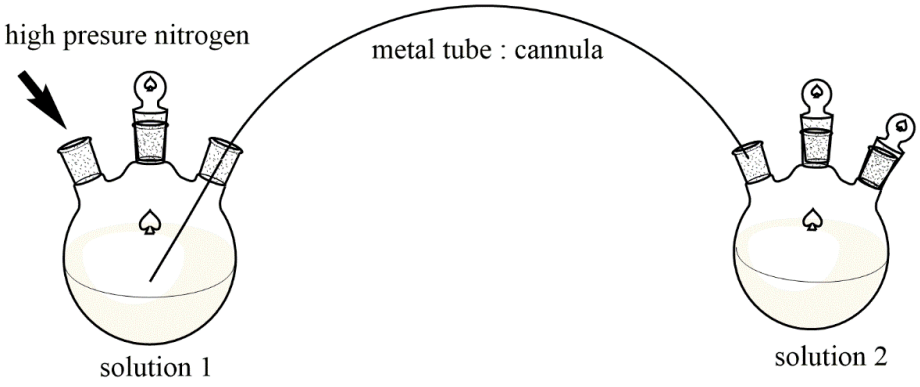

Figure A1: The way to transfer one solution to another one under nitrogen condition.

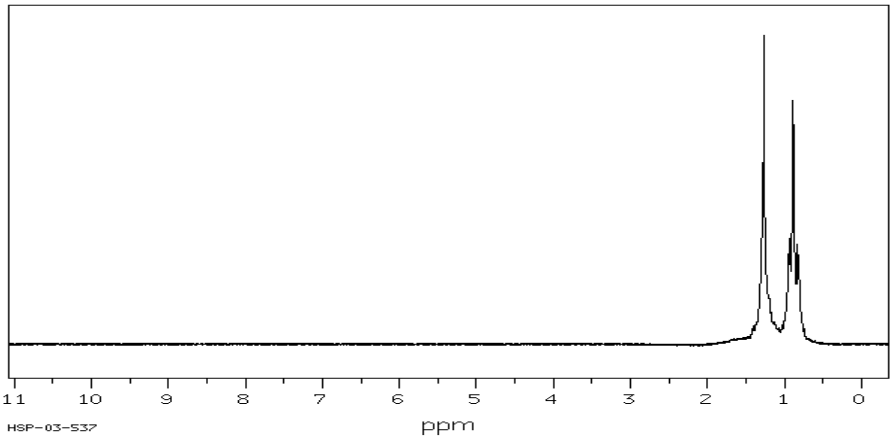

Figure A2: ^{1}H NMR spectrum of hexane in $CDCl_3$.

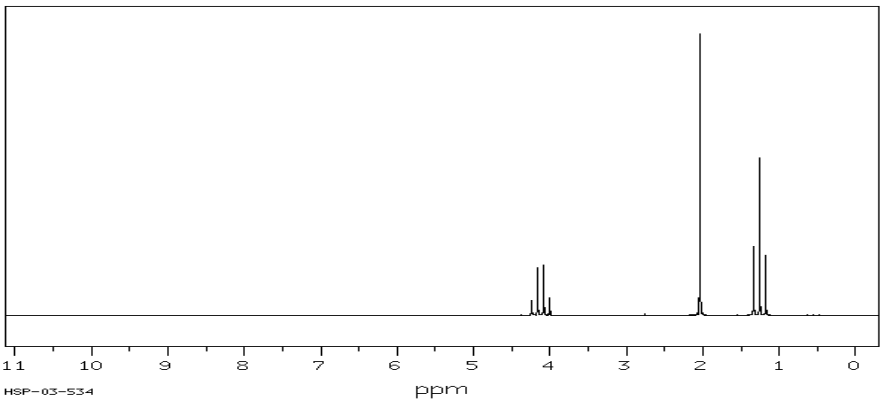

Figure A3: ^{1}H NMR spectrum of ethyl acetate in $CDCl_3$.

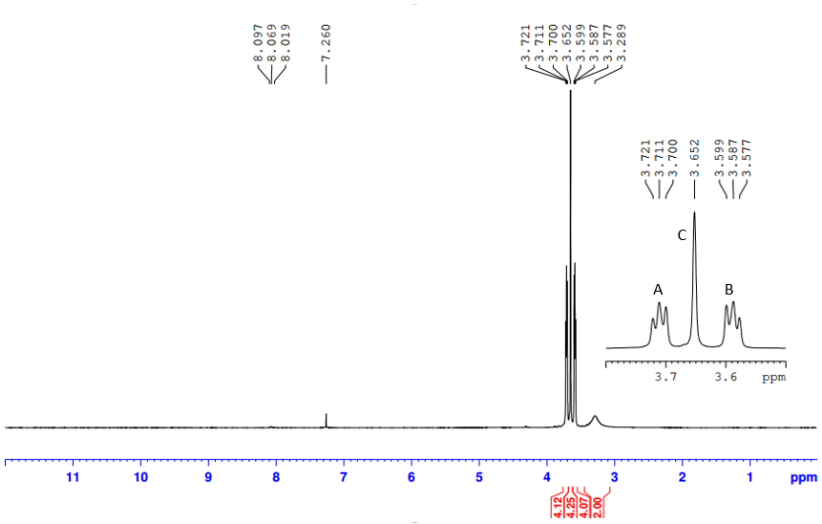

Figure A4: ^{1}H NMR spectrum of TEG in $CDCl_3$.

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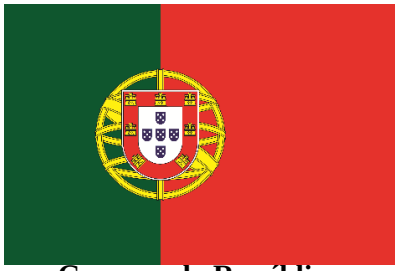

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