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Surface modified dendrimers: Synthesis and characterization for cancer targeted drug delivery

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ABSTRACT

Dendrimers represents a highly branched three-dimensional structure that provides a high degree of surface functionality and versatility. PAMAM dendrimers are used as well-defined nanocontainers to conjugate, complex or encapsulate therapeutic drugs or imaging moieties. Star-burst [PAMAM] dendrimers represent a superior carrier platform for drug delivery. The present study was aimed at synthesis of a surface modified dendrimer for cancer targeted drug delivery system. For this 4.0 G PAMAM dendrimer was conjugated with Gallic acid [GA] and characterized through UV, IR, ^{1}H NMR and mass spectroscopy. Cytotoxicity study of dendrimer conjugate was carried out against MCF-7 cell line using MTT assay. The study revealed that the conjugate is active against MCF-7 cell line and might act synergistically with anti-cancer drug and gallic acid–dendrimer conjugate might be a promising nano-platform for cancer targeting and cancer diagnosis.

1. Introduction

Cancer is uncontrolled proliferation of cells. The dynamic characteristics of cancer and tumor growth create difficulty in treating and delivering drugs to only the specific tissue type.[1] Mostly, current anti-cancer agents do not greatly differentiate between cancerous and normal cells, this leads to systemic toxicity and adverse effects. In addition, rapid elimination and widespread distribution into non-targeted organs and tissues requires the administration of a drug in large quantities, which is uneconomical.[2] Targeted drug delivery to cancer cells or tumor vasculature is an attractive approach to fight against cancer. Macromolecular carriers have shown promise as an ideal targeted drug delivery system.[3] Targeted cancer therapies interfere with cancer cell division (proliferation) and spread in different ways. Many of these therapies focus on proteins that are involved in cell signaling pathways, which form a complex communication system that governs basic cellular functions and activities, such as, cell division, cell movement, how a cell responds to specific external stimuli, and even cell death. By blocking signals by which cancer cells grow and divide uncontrollably, targeted cancer therapies can help to stop cancer progression and may induce cancer cell death through a process known as apoptosis. Other targeted therapies can cause cancer cell death directly, by specifically inducing apoptosis or indirectly by stimulating the immune system to recognize and destroy cancer cells and/or by delivering toxic substances to them.

In last decade dendrimers have emerged as one of the most promising nano-particulate carrier system that has greatly attracted the scientific community. Dendrimer drug delivery systems have the possibility of revolutionizing the cancer specific drugs and treatments to be localized at the cancerous tumor site.[4] Sinek et al. have reported that nanoconstructs having size range from 1 to 10 nm are capable of diffusing directly into tumor cells.[5] Significantly PAMAM dendrimers have size range of 2.3 nm in generation-2 (G-2) to 5.3 nm in G-5.[6,7] In this regard dendrimers can prove to be an important carrier for the delivery of anti-cancer drugs. Dendrimers are unimolecular polymeric systems synthesized in a re-iterative manner. At the same time their synthesis can be optimized to control their size, shape, molecular mass, composition and reactivity. As the relationships between dendrimer architecture, biocompatibility, retention and delivery have become better elucidated, unique dendrimer derivatives have been synthesized for greater specificity and functionality, particularly with regards to pharmacokinetics and targeted delivery.[8] Typically, dendrimers are used as well-defined scaffolding or nanocontainers to conjugate, complex or encapsulate therapeutic drugs or imaging moieties.[9] Dendrimers have hyper-branched structure with precisely placed functional groups that bear important role in controlling the properties of therapeutic moieties that are encapsulated or complexed with it. Large numbers of surface functional groups on dendrimer's outer shell can be modified or conjugated with a variety of guest molecules.[10]

Gallic acid is a polyhydroxyphenolic compound which can be found in various natural products, such as green tea, gallnuts, oak bark, green tea, apple-peels, grapes, strawberries, pineapples,

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bananas and many other fruits.[11] Various biological activities of gallic acid have been reported, including anti-bacterial, anti-viral and anti-inflammatory.[12–14] The major interest in gallic acid is related to its antitumoral activity. In fact, anti-cancer activity of gallic acid has been reported in various cancer cells, such as leukemia, prostate cancer, lung cancer, gastric, colon, breast, cervical, and esophageal cancer.[15–18] Gallic acid and its derivatives, show selective cytotoxicity against a variety of tumor cells with a higher activity than that shown against normal cells.[19–21]

Gallic acid was reported as a free radical scavenger and as an inducer of differentiation and apoptosis in leukemia, lung cancer, and colon adenocarcinoma cell lines as well as in normal lymphocyte cells.[22–24] Apoptosis induced by gallic acid is associated with oxidative stresses derived from reactive oxygen species (ROS), mitochondrial dysfunction and an increase in intracellular Ca_2^+ level.[25,26] GA is a strong natural antioxidant to scavenge ROS.[27–30]

Polyphenols are promising classes of cancer chemopreventive agents, possibly due to their strong anti-tumor-promoting potential.[31–34]The natural antioxidant gallic acid (GA) has demonstrated a significant inhibition of cell proliferation and induction of apoptosis in a series of cancer cell lines.[35] Gallic acid inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ATM–Chk2 activation, leading to cell cycle arrest, and induces apoptosis in human prostate carcinoma DU145 cells.[17] Activation of ROS-dependent ATM/p53 signaling as a critical mechanism of gallic acid-induced cell death. Gallic acid exerts anticarcinogenic activity via the NK cell-mediated necrosis and apoptosis.[36]

GA induces apoptosis by activating a preexisting apoptotic pathway.[37] Indeed, GA induces the activation of PARP, caspase-9 and caspase-3, which preceded the onset of apoptosis. GA also shows an increase in pro-apoptotic Bax expression and a decrease in anti-apoptotic Bcl-xL expression. Furthermore, it is observed that GA blocks the cancer cell proliferation by arresting cells at G2/M phase, by down-regulating activities of cyclin B1, Cdc2 and Cdc25C which were required for G2/M transition and thus had a selective cytotoxicity on cells enriched at G2/M phase (Fig. 1).

2. Materials and methods

2.1. Materials

Gallic acid, ethylenediamine [EDA], methyl acrylate [MA] and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride [EDAC] were purchased from Loba Chemie. All other reagents and organic solvents used were of the highest quality available and were purchased from the regular source. Doubly distilled and deionized water was used throughout the work.

2.2. Procedures for synthesis of 4.0 G PAMAM–GA conjugate

The overall procedures for synthesis of 4.0 G PAMAM–GA conjugate are presented in Scheme 1 and 2. The final product was characterized by FTIR, ^{1}H NMR spectroscopy and ES–MS.

2.2.1. Synthesis of 4.0 G PAMAM dendrimer

Ethylenediame (EDA) core PAMAM dendrimers were synthesized using Tomalia's divergent growth approach.[38] The synthesis involves two consecutive steps—the Michael addition of primary amine (EDA in the first step) to double bond of methyl acrylate (MA) followed by amidation of the resulting multiester (tetraester at the very beginning), with EDA.[39] Michael addition of methyl acrylate to ethylenediamine in methanol gives the ester terminated half generation dendrimers designated, G*n*.5. The exhaustive amidation reaction of ester terminated dendrimers with large excess of ethylenediamine in methanol produce amine terminated full generation dendrimers referred to as G*n*. The reiteration of this two-step procedure leads to next higher dendrimer generations (Scheme 1). The percentage yield of half generation dendrimers varies between 70 to 85%, while for full generations varies between 85 to 95%. The overall yield of 4.0 G PAMAM dendrimers is approximately 26.2%.

2.2.2. Synthesis of 4.0 G PAMAM–GA conjugate

To a solution of gallic acid (0.38 g, 2.235 mM) in water/ethanol (1:1, 20 mL), EDAC (0.43 g, 2.250 mM) in 20 mL water was added with constant stirring. 4.0 G PAMAM dendrimers (1 g, 0.070 mM) was dissolved in 20 mL distilled water and added slowly to the reaction mixture with constant stirring in an ice bath for 30 min., stirring was continue for another 24 h at rt. After completion of the reaction, reaction mixture was dialyzed against distilled water to remove the byproducts and unreacted gallic acid (Scheme 2). The percentage yield of 4.0 G PAMAM–GA conjugate is 79.5%.

2.3. Characterization of dendrimer and PAMAM–GA conjugate

IR spectra of 4.0 G PAMAM dendrimer and PAMAM–GA conjugate were taken using FTIR-2000A, ABB Spectrophotometer. ^{1}H NMR spectra of 4.0 G PAMAM dendrimer and PAMAM–GA conjugate were recorded on Bruker DRX-300 (300 MHz) spectrometer. The samples were solubilized in methanol [MeOD]. To confirm the molecular weight of 4.0 G PAMAM dendrimer and PAMAM–GA conjugate, mass spectral analysis of the dendrimer was performed. The electrospray mass spectra were recorded on a THERMO Finnigan LCQ Advantage max ion trap mass spectrometer. The samples were dissolved in solvent such as methanol/acetonitrile/water.

2.4. Cytotoxicity assay

Cytotoxicity study of dendrimer conjugate was carried out against MCF-7 cell line using MTT assay. The compound was dissolved in DMSO and serially diluted to get final dilution in the range of 0.01–100 μg/mL. DMSO concentration was kept less than 0.1% in all the samples. Cell lines were seeded in 96 well plates and treated with different concentrations of the test samples and incubated at 37 °C, 5% CO_2 for 96 h. MTT reagent (40 μL/mL) was added to the wells and incubated for 4 h. 200 μL DMSO was added per well, absorbance was determined at 550 nm. Percentage inhibitions were calculated and plotted with the concentrations used to calculate the IC_{50} values.

3. Results and discussion

3.1. FT-IR analysis

The dendrimer modification process was proven by comparison of FT-IR spectra of the 4.0 G PAMAM dendrimer and 4.0 G PAMAM–GA conjugate as shown in Fig. 2. It can be seen from Fig. 2 that, compared with the 4.0 G sample, the 4.0 G PAMAM–GA conjugate possess absorption bands broadening at 3314 cm^{-1} due to stretching vibration of the O–H bond, bands at 1564 and 1469 cm^{-1} due to the aromatic (C=C) stretching vibration, strong bands at 1642 cm^{-1} due to the aromatic amide (–$CONH_2$) and shifting of the C–H stretching from 2919 and 2852 to 2973 and 2884 cm^{-1}. All of these reveal the existence of PAMAM–GA conjugate.

3.2. ^{1}H NMR analysis

The NMR spectra and chemical shift value of conjugated dendrimer as compared to plain dendrimer provide the proof of conjugation of gallic acid with 4.0 G PAMAM dendrimer. Significant shifts

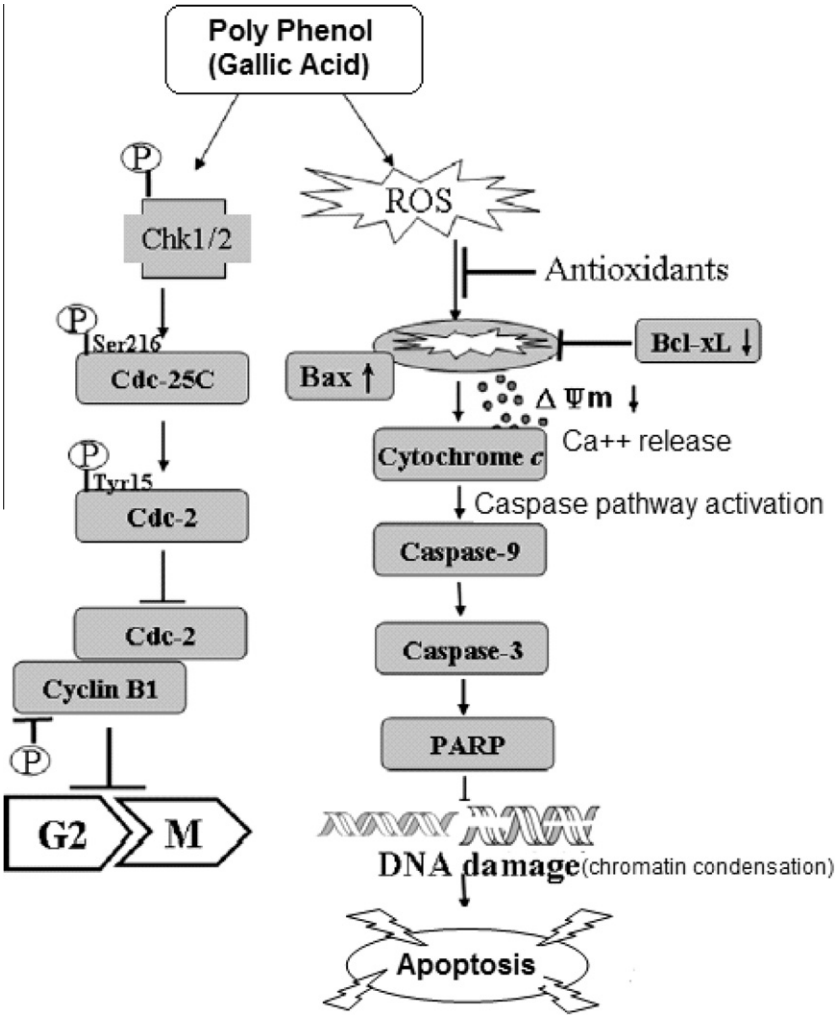
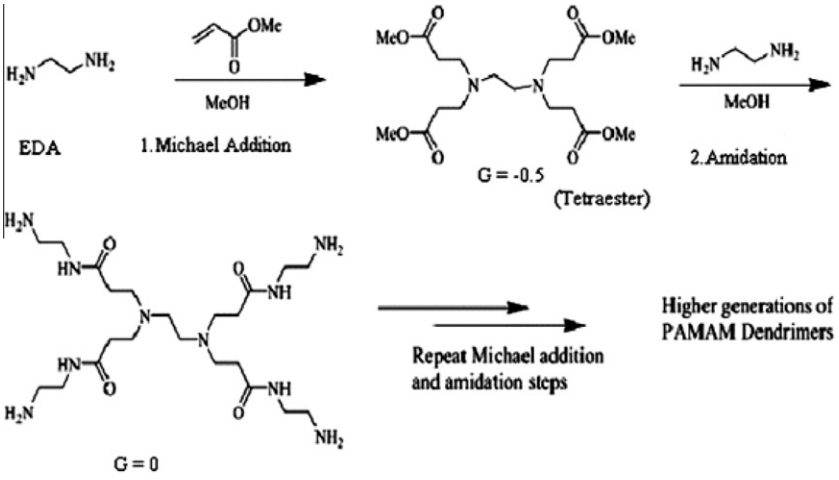


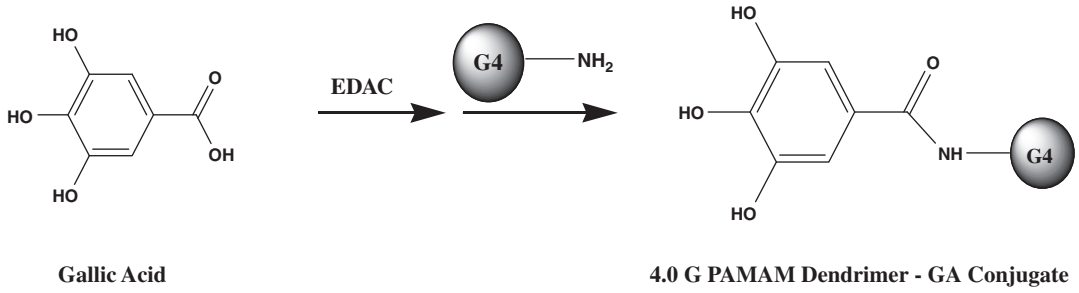
Figure 1. Schematic representation of biological activities of gallic acid.



Scheme 1. General synthesis of PAMAM dendrimer.

of secondary $-CH_2$ group was noticed on conjugation. Similarly, appearance of newer peaks of amide linkage [–NHCO–GA] at 8.13 ppm and 8.53–8.87 ppm of Ar-H, confirms the formation of conjugate (Fig. 3 and 4).

1. *4.0 G PAMAM dendrimer*: ^{1}H NMR (300 MHz, MeOD): δ 2.93 (–NCH_2CH_2N–); δ 2.98 (–NCH_2CH$_2$CO–); δ 2.91 (–NCH$_2$CH_2 CO–); δ 3.30 (–CONHCH_2CH$_2$N–); δ 2.95 (–CONHCH$_2$CH_2N–); δ 3.46 (–CONHCH_2CH$_2$NH$_2$); δ 2.99 (–CONHCH$_2$CH_2NH$_2$).



Scheme 2. Synthesis of PAMAM–GA conjugate.

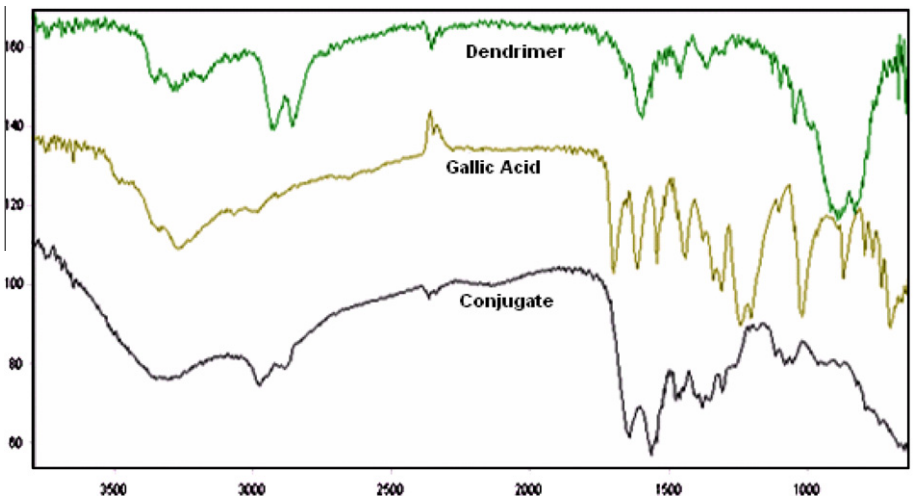


Figure 2. Overlay IR spectra of 4.0 G PAMAM, gallic acid and PAMAM–GA conjugate.

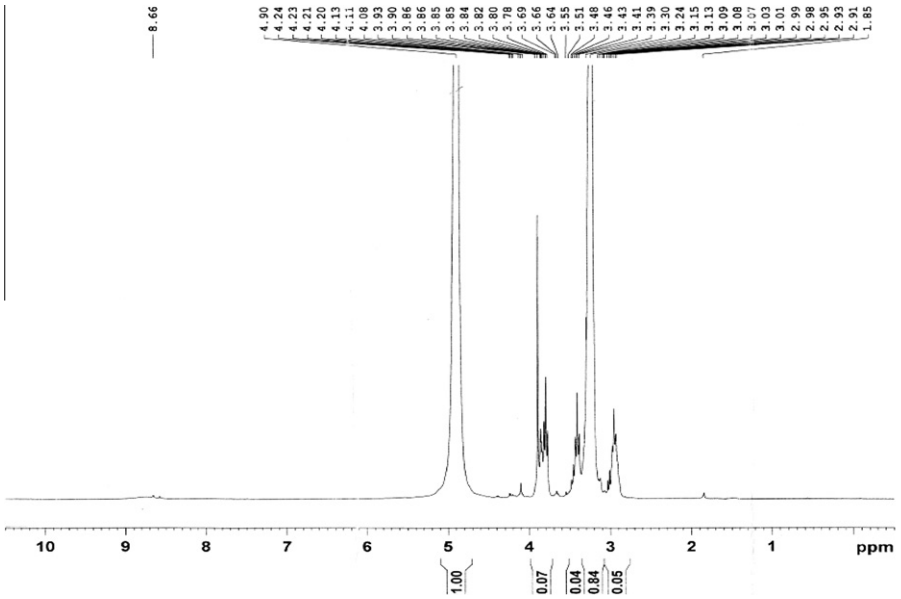


Figure 3. NMR spectra of 4.0 G PAMAM dendrimers.

2. *4.0 G PAMAM–GA conjugate*: ^{1}H NMR (300 MHz, MeOD) δ 2.92 (–NCH_2CH_2N–); δ 3.15 (–NCH_2CH_2CO–); δ 2.63 (–NCH_2CH_2CO–); δ 3.30, 3.37 (–CONHCH_2CH_2N–); δ 3.148, 3.32 (–CONHCH_2CH_2N–); δ 8.13 (–NHCO–GA); δ 8.53–8.87(Ar-H).

3.3. ESI-MS analysis

ESI mass spectral analysis indicates the change in the molecular weight of 4.0 G PAMAM dendrimer and PAMAM–GA conjugate. The mass of 4.0 G PAMAM dendrimers was found to be 14483.

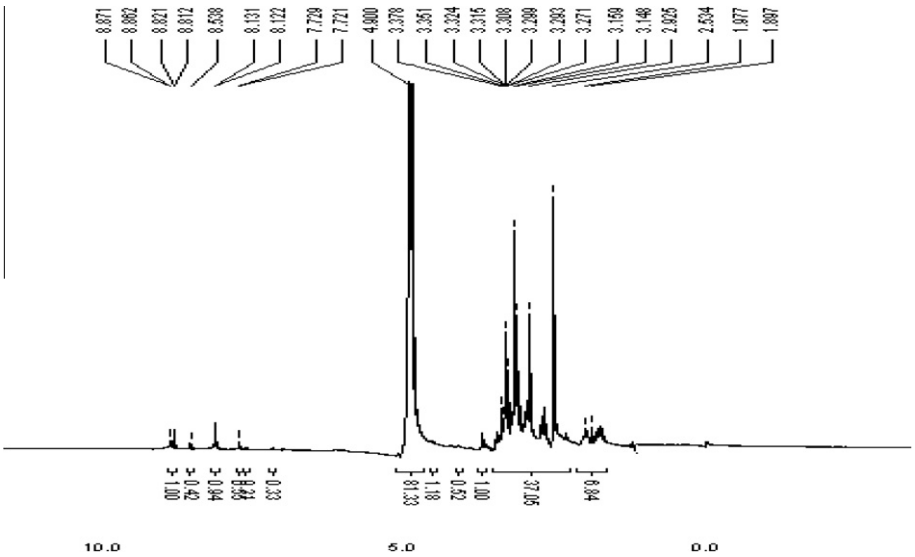


Figure 4. NMR spectra of 4.0 G PAMAM–GA conjugate.

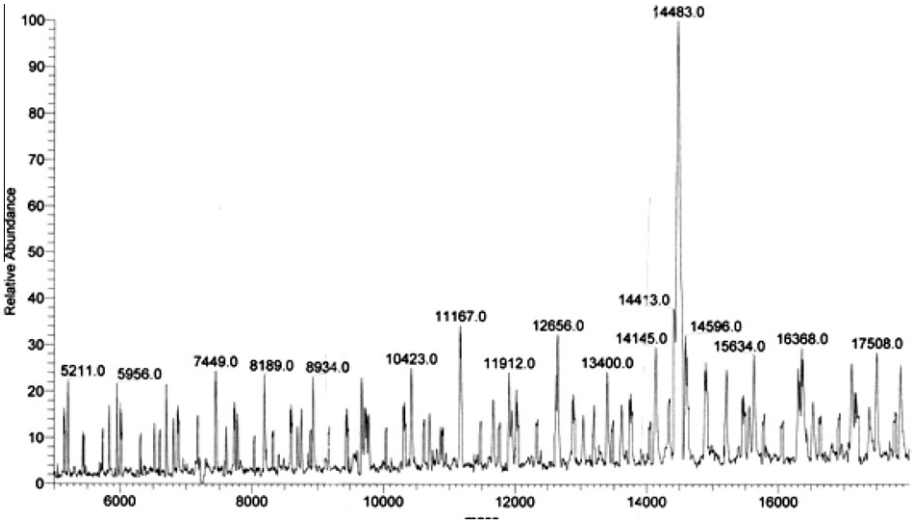


Figure 5. Mass spectra of 4.0 G PAMAM dendrimer.

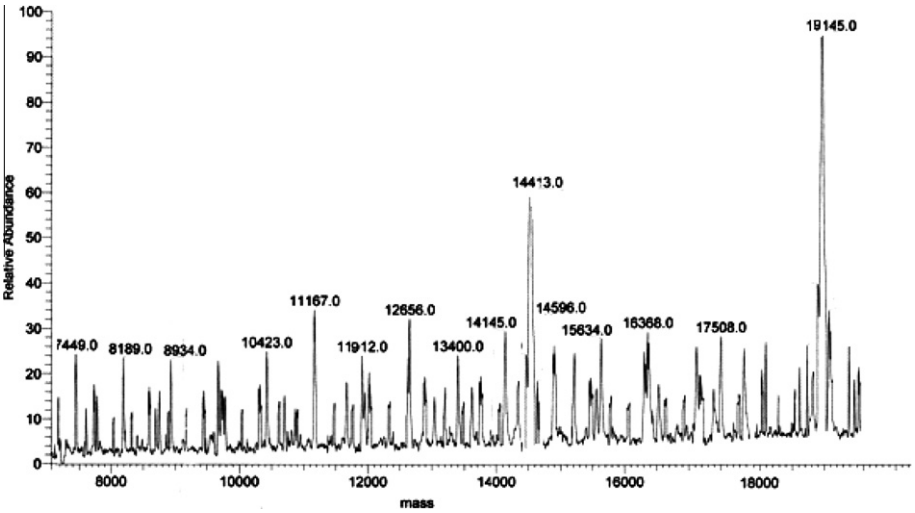


Figure 6. Mass spectra of 4.0 G PAMAM–GA conjugate.

The mass spectra of conjugated system revealed the mass of 19145 Daltons. Increase in mass further confirmed the conjugation of gallic acid molecules to 4.0 G PAMAM dendrimer. The mass spectra of 4.0 G PAMAM dendrimer and PAMAM–GA conjugate are shown in Fig. 5 and 6.

3.4. Cytotoxicity assay

Cytotoxicity study of 4.0 G PAMAM dendrimers, GA and 4.0 G PAMAM–GA conjugate were carried out against MCF-7 cell line using MTT assay. The 4.0 G PAMAM–GA conjugate was also tested against normal cell line. The cytotoxicity activity (IC_{50} value) of 4.0 G PAMAM dendrimers and gallic acid against MCF-7 cell line were found to be 81 μg/mL (≈5.6 μM) and 36.2 μg/mL (≈216.6 μM), respectively. The cytotoxicity activity (IC_{50} value) of 4.0 G PAMAM–GA conjugate against MCF-7 cell line and normal cell line were found to be 25 μg/mL (≈1.3 μM) and more than 100 μg/mL (>5.22 μM), respectively. Cytotoxicity study revealed that the conjugate is active against MCF-7 cell line as well as having four time selectivity over normal cell line. 4.0 G PAMAM–GA conjugate might act synergistically with anti-cancer drug.

4. Conclusion

Dendrimer–gallic acid [PAMAM–GA] conjugate was synthesized and characterized through IR, NMR and Mass techniques. Subsequently conjugate was tested for cytotoxicity, the in vitro results gained for their cytotoxicity, is hoped that this conjugate would offer synergistic potency with anti-cancer drug in vivo and retain the anti-cancer drug at the surface of the conjugates in the physiologic plasma condition. The present study has resulted in identification of new dendrimer conjugate, which might prohibit the unwanted release of free anti-cancer drug before reaching the target site, that is, tumor cells; to prevent the undesirable systemic toxicity in comparison with free drug.

Our results indicated that gallic acid–dendrimer conjugate might be a promising nano-platform for cancer targeting and cancer diagnosis. Current work of using gallic acid–dendrimer conjugates as scaffolds for preparing cancer therapeutics is limited to 'proof of concept' studies and a long road lies ahead to actually use these nanodevices in clinical practice.

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