ORIGINAL ARTICLE

# Pamam dendrimer - vitamin conjugate for delivery of paclitaxel as anticancer agent

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## Abstract

**Background:** The major issues with anticancer agent are that they randomly attack cancerous as well as healthy cell. These are injurious and its side effects can be reduced by developing a drug delivery vehicle. That is particular to tumor cells and this may be achieved by employing a strategy called active targeting strategy wherein the functionalities that respond to over expressed receptors (e.g., biotin, and folate conjunction on dendrimers surface) on tumor cells are attached to the drug carrier. **Objective:** In the present study, biotin- $G_4$PAMAM dendrimer conjugates were synthesized and structures were characterized. **Materials and Methods:** $G_4$-PAMAM Dendrimers were biotinylated using sulfo-NHS-LC-biotin and structural characterization was performed using $^1$H NMR and transmission electron microscopy. The effect of generation and release rate, hemotoxicity with biotinylated dendrimer was performed. **Results:** The results suggested that biotinylated $G_4$PAMAM dendrimers may be potential drug carriers for paclitaxel targeting to cancer. **Conclusion:** Biotinylated $G_4$-PAMAM dendrimers show potential as nanocarriers in targeted drug delivery. Biotinylation of dendrimer thus reduces the distracted charge-mediated uptake and as well as also rising the *in vivo* biocompatibility, as seen with decrease in hemotoxicity with biotinylated dendrimers.

**Key words:** Paclitaxel, $G_4$Dendrimer, Sulfo-NHS-LC-biotin, Anticancer drug carrier

## INTRODUCTION

Despite the advancements in various field of research; medicinal chemistry, pharmaceutics, nanotechnology, etc., and various detection modalities, still cancer remains a big threat to the mankind. Cancer is one of the leading causes of mortality in all over the world. It is characterized by the uncontrolled proliferation of cells that find their origin in genetic mutations, radiation exposure, carcinogenetic substances, etc. Current clinical management for the cancer incorporates: Surgical interventions, radiation therapy, hormonal therapy, chemotherapy, etc. In chemotherapy, anticancer chemotherapeutic agents are administered as tiny molecule; cytotoxic drugs that have inherent limitations. Limitations of conventional chemotherapy incorporates; anaphylactic hypersensitivity reactions that may be life threatening,[1-3] bio-distribution, and clearance of paclitaxel notably affected duo to its strong affinity to the serum lipoprotein dissociation products,[4-7] paclitaxel formulated in Cremophor EL, enkindles the axonal degeneration, and demyelination that leads to the peripheral neuropathy,[8] cancer cell drug resistance, etc.,[9] and non-specificity to the cancer cells that ultimately strikes to the non-cancerous cells also and causes extensive release of cytotoxic molecules throughout the body and creates significant side effects. Therefore, this is our urgent need to develop; (i) enhanced specificity of existing chemotherapeutic agents to cancerous cells, (ii) reduced toxicity to non-cancerous cells, and (iii) better antitumor efficacy.

At present, anticancer therapy relies heavily on the administration of small molecule cytotoxic drugs that attack both cancerous and non-cancerous cells due to limited selectivity of the drugs and prevalent distribution of the cytotoxic molecules throughout the body. The antitumor efficacy and systemic toxicity of existing chemotherapeutic

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drugs can, however, be improved by employing formulation and particle engineering approaches. Thus, drug delivery systems can be developed that more exclusively target tumor tissue using both reactive (such as the enhanced permeation and retention effect) and active (through the use of cancer targeting ligands) modalities.

Dendrimers are one such system that can be developed with high structural monodispersity, long plasma circulation times, and precise control over surface structure and biodistribution properties. Chemotherapeutic drugs can be associated with dendrimers through covalent conjugation to the surface, or through encapsulation of drugs within the structure. Surface modification of dendrimers using various ligands including small molecule ligands such as vitamins (folic acid, biotin *etc.*), antibodies against tumor associated antigens, and cell penetrating peptides have generated a wide range of target specific nanocarriers.[10]

Biotin is an essential micronutrient for normal cellular functions (e.g., fatty acid biosynthesis, and gluconeogenesis), growth, and development. Humans and other mammals cannot synthesize biotin and thus must obtain it from exogenous sources through intestinal absorption. Rapidly dividing cells such as cancer cells have a voracious appetite for certain vitamins including biotin, Vitamin B12, and folate and biotin levels have been found to be significantly higher in some cancer cells compared to normal tissue.[11] Biotinylation has been used as a strategy to specifically target chemotherapeutic agents to cancer cells. Camptothecin when conjugated with biotinylated polyethylene glycol has shown enhanced cytotoxicity and apoptotic activity by caspase-dependent pathway.[12]

Paclitaxel was selected as anticancer drugs which have been shown experimentally to have antitumor activity[13] by promoting microtubule polymerization, a process which disrupts the normal tubule dynamics essential in cellular division, and leads to cell death by apoptosis.[14] It has been reported to efficacy against ovarian and breast cancer and more recently, against malignant gliomas and brain metastases.[15] In spite of its clinical efficacy, it can be concluded that use of paclitaxel is limited by its poor solubility as well as low permeability.

To quench these exigency, we have applied formulation and particle engineering approach; (i) specificity enhancement of the drug molecules for cancerous cells by increasing permeation and retention effect, (ii) application of cancer targeting ligands to enhance the cancer cell specific binding of drugs and least accumulation in the body, that is, Biotinylation,[16] and (iii) application of such system that can be developed by virtue of; high structural mono-dispersity, long plasma circulation times, and precise control over surface structure and bio-distribution, that is, Dendrimers.

The present study explores the comprehensive delineation of the dendritic system in a controlled release drug delivery. The Biotinylation of $G_4$PAMAM (fourth generation, Poly [amido] amine [Amine terminated Diamino butane cure]) dendrimers in minimum steps and evaluated for encapsulation efficacy and toxicity study in Wister rats for increase safety and efficacy of paclitaxel drugs.

## MATERIALS AND METHODS

### Materials

$G_4$PAMAM dendrimers were obtained from (Nanosynthon USA). Paclitaxel (Sun Pharma), Sulfo-NHS-LC-Biotin (Sun Pharma), Chloroform (Merck), Acetonitrile and Methanol HPLC grade (Rankem), HPLC water (Rankem), Methanol (Merck), HPLC. Instrument (Simadzu), U.V (Systronics), and Bath sonicater (Rolex).

### Synthesis of Biotinylated $G_4$PAMAM Dendrimers

Biotinylation of $G_4$PAMAM dendrimers was carried out as reported method.[17] In this method, 20 mg of $G_4$PAMAM dendrimer was dissolved in 2 ml of 0.1 M phosphate buffer (pH 9.0) and sulfo-NHS-LC-biotin was added at a molar ratio of 1:32 ($G_4$PAMAM: sulfo-NHS-LC-biotin) and the reaction mixture was stirred for 2 h at room temperature. The mixture was then dialyzed (1,000 Da MW cutoff) against de-ionized water to remove unconjugated biotin. The biotin-conjugated dendrimers were lyophilized with the help of Lyopilizer a white product of biotinylated $G_4$PAMAM dendrimer was found [Figure 1].

### Formulation of Paclitaxal using Biotinylated $G_4$PAMAM Dendrimer

A 40 mg of biotinylated $G_4$PAMAM and their unconjugated native counterparts were dissolved in 4 ml of deionized water.

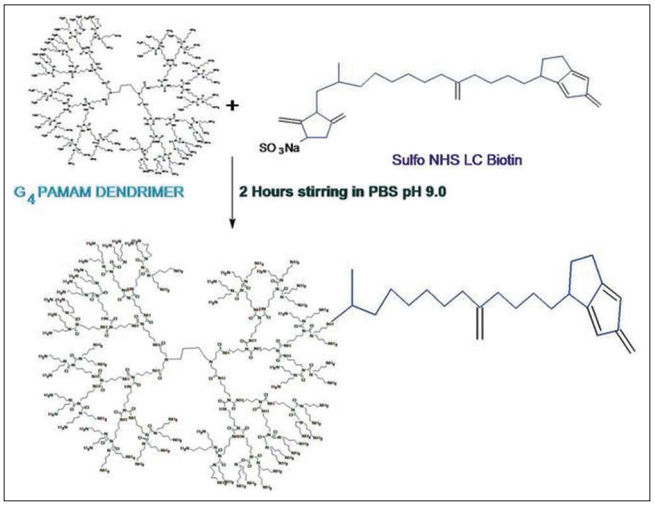


**Figure 1:** Conjugation of biotin with PAMAM dendrimer ($NH_2$ surface)

In a round-bottom flask approximately 28.2 mg (equimolar 32 times of dendrimer) of paclitaxel was dissolved in 15 ml of deionized water. When the paclitaxel was completely dissolved, dendrimer solution was added to paclitaxel solution drop wise under stirring at room temperature. The solution was left to react for 4 h and dialyzed (3.5 kDa MWcut-off) against deionized water for 24 h.

## Formulation Characterization

### *Particle size distribution*

Size distribution was measured on Particle size analyzer (Malvern) in Central Drug Research Institute, Lucknow. The formulation of biotinylated $G_4$PAMAM dendrimer was filled in 15 mL sample cell after initialization, of instrument, stirred, and diluted to the concentration to obscuration >10 and <20. The laser beam is directed through the sample. The light scattered by the particles moving through the leaser focus is recorded in angular region. The intensity distribution are collected by a multichannel analyzer and stored in PC.

### *Transmission electron microscopy (TEM)*

TEM measurement was performed in Indian Institute of Toxicology and Research, Lucknow, with the help of Tecnai G2 Twin spinet Netherland equipped with gratin odious TM CCD camera controller.

### *Procedure*

Sample was taken and sonicate (bath sonicater) for 10 s. Moreover, we have taken the 2 µl of the sample and 10 µl urinal acetate mixed properly. After 5 min, one drop sample was tube and poured in TEM Gold Grid. Grid was dried in overnight (24 h) and further second coating was applied, again dried for 24 h and examined the image under the TEM [Figure 2].

## Estimation of Efficiency of Loading of Paclitaxel Biotinylated$G_4$ PAMAM Dendrimers

Accurately measured quantity of 10 ml of paclitaxel loaded solubilizate of biotinylated $G_4$PAMAM dendrimer vehicles (methanol) were dispersed in the HPLC mobile phase (Acetonitrile:water: 6:4), bath sonicated for 15 min. The sample was filtered using 0.45 µm membrane filter (Millipore), suitably diluted and analyzed by HPLC as reported.

## *In Vitro* Release Rate Studies

A treated dialysis tube was used for *in vitro* release studies. Formulation equivalent to 10 mg drug was introduced into prewashed dialysis tubing and placed in a beaker containing 200 ml freshly prepared PBS (pH 7.4). The sink condition was maintained by constantly stirring the buffer with the help of magnetic stirrer. Sample aliquots (5ml) were withdrawn periodically and replaced with equal volume of fresh PBS. Each sample was analyzed at 227 nm by U.V Spectrophotometer as reported method.[18] The data of release of paclitaxel from biotinylated dendrimer formulation are calculated from standard curve.[19]

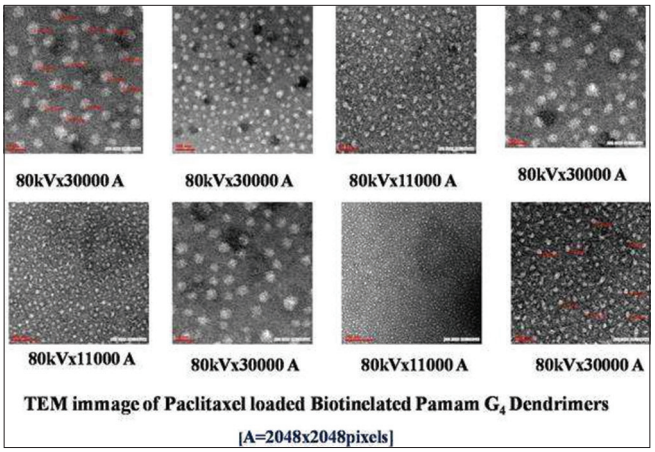


**Figure 2:** Tem images of biotinylated $G_4$ PAMAM dendrimer

## Hemocompatibility Studies

Hemocompatibility studies include hemolytic and hematological (blood count) evaluations for assessing *in vitro* and *in vivo* effects of administered dendrimer correspondingly on blood components. Hemolytic toxicity studies were performed following a slightly modified reported procedure.[20]

Briefly, fresh whole blood from male Wister rats was collected using heparinized capillary in blood collecting vials (HiMedia, India) and centrifuged at 2000 rpm for 15 min in an ultracentrifuge. RBC collected from bottoms were washed with physiological saline (0.9% w/v) until a clear colorless supernatant was obtained above the cell mass. Cells were resuspended in normal saline to obtain 2% erythrocyte concentration and this was further used for hemolytic toxicity studies. To 1.0 mL of RBC suspension, 1.0 mL of physiological saline was added to serve as negative control, while replacing saline with deionized water was considered 100% hemolytic positive control. Dendrimer dispersions with and without paclitaxel were mixed with equal volume of RBC suspension and incubated at 37±0.2$^0$ C for 30 min with the gentle intermittent shaking. After incubation, hemoglobin content was measured spectrophotometrically at λ max 227 nm, of sample against control. The percent hemolysis was calculated for each sample by taking the absorbance of positive control ($Ab_{positive\ control}$) as 100% hemolytic sample, using following equation.

$$\text{Haemolysis} = \frac{\text{Absorbance sample } \lambda \text{ max28}}{\text{Absorbance positive control } \lambda \text{ max228}} \quad 100$$

Hematological studies were performed in male Wister rats. Animals were divided in three treatment groups with three rats in each. Paclitaxel and formulation were administrated intravenously delivering 1.0 mg/kg of paclitaxel to first, second, and third group of animals, respectively. After lapse of 24 h blood from each animal was collected and analyzed for RBC, WBC, and differential counts in CDRI. All animal studies were performed in accordance with the guidelines of CPCSEA (Committee for the purpose of control and Supervision of Experiments on Animal, Ministry of Culture, Government of India) and protocols were duly approved Institutional Animal Ethics Committee, RITM Lucknow.

### Stability Studies

Different formulation (5 ml) and drug were kept in umber colored vials. Properly sealed vials containing biotinylated $G_4$PAMAM dendrimer formulation were kept for stability studies over a month at room temperature and 40°C. All vials were visually observed, and analyzed for drug content at the time intervals of 0, 1, 7, 15, and 30 days. The initial and final pH values were also measured.[21]

### *In Vivo* Evaluation

The *in vivo* performance of a drug delivery system is perhaps the most important criteria in its development as a clinically acceptable dosage form. *In vivo* studies are carried out on a system promising *in vitro* performance on laboratory animals such as rats, mice, hamsters, and monkeys. For a specific site drug delivery system, *in vivo* studies are conducted to ascertain the ability of the system in achieving compartmentalization of the drug in the targeted tissue or non-targeted tissues. The blood levels as well as the urinary excretion of the drug may be monitored[22] have listed various parameters which help in precise preclinical *in vivo* evaluation of a target oriented drug delivery system.

## RESULTS

### Synthesis of Biotinylated $G_4$PAMAM Dendrimers

Biotinylated $G_4$PAMAM dendrimer was resulted in a high yield of fluffy white fibrous solid of melting point and yield 232°C, 13.4 mg, respectively.

### Characterization of Biotinylated $G_4$PAMAM Dendrimers

Product sample were scanned U.V data that show highest peak of $\lambda_{max}$ 227 nm

### Nuclear Magnetic Resonance Spectroscopy

$^1$H NMR data revealed the presence of biotin ring juncture protons which were absent from the parent PAMAM dendrimer; however, the other characteristic peaks of the dendrimer (2.6–3.3 ppm) were observed in biotinylated $G_4$PAMAM dendrimer [Figure 3].

### Mass Spectroscopy

The extent of biotinylation was quantified using mass spectroscopy. For PAMAM$G_4$, mass depicted 14 biotin molecules attached [Figure 4].

### Infrared Spectroscopy

The IR spectrum of biotinylated $G_4$PAMAM dendrimer confirms the formation of biotinylated $G_4$PAMAM dendrimer as peaks of all functional groups.

### TEM

The mean size of biotinylated $G_4$PAMAM dendrimer formulation was in the range of 22–30 nm and was fairly

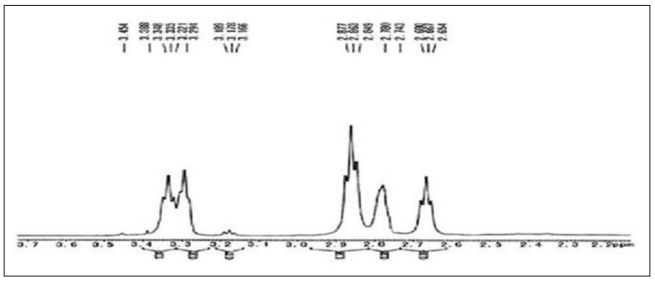

**Figure 3:** $^1$HNMR spectra of biotinylated $G_4$ PAMAM dendrimer

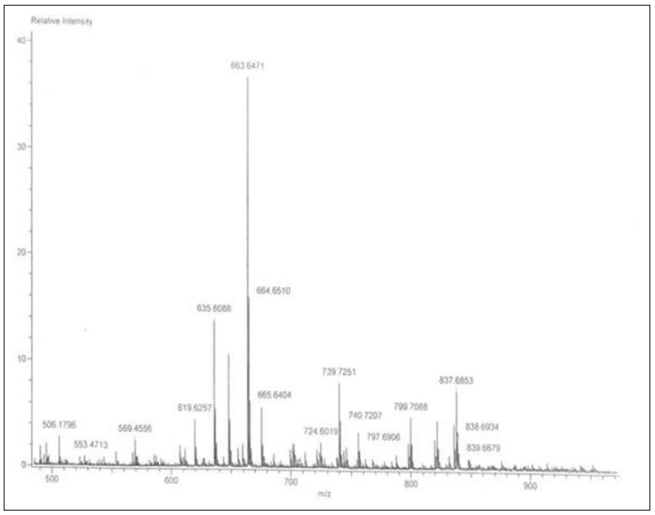


**Figure 4:** Mass spectra of biotinylated $G_4$ PAMAM dendrimer

uniform. The size of paclitaxel containing formulation ranged from 22 to 35 nm [Figure 2].

### Size Distribution

The mean size of biotinylated $G_4$PAMAM dendrimer formulation was in the range of 0.2µm size. The particle sizes reported here were determined instrumentally using a Malvern's Mastersizer. The image is shown in Figure 2.

### Drug-loading Efficiency of Biotinylated Dendrimers

Estimation of paclitaxel loading efficiency of biotinylated $G_4$PAMAM dendrimer by HPLC revealed that the percentage of paclitaxel encapsulated as calculated content 21.10%.

### Release Rate

The release of paclitaxel was about 12.5 ± 0.17% in 24 h. Release pattern is shown in Table 1 and Figure 5.

### Hemocompatibility Studies

The hemocompatibility studies show reduction in hemotoxicity with biotinylated dendrimers.

### Stability Study

Biotinylated $G_4$PAMAM dendrimer formulation was stable in injection form. Degradation was less than 2% at room temperature and about 4%°C in formulation from 30 days. Assay was more than 97.2% and 98% for formulation. The result is shown in Table 2 and Figure 6.

### Toxicity of Dendrimer Conjugates

All mice were observed for the duration of the studies for signs of dehydration, inability to eat or drink, weakness, or change in activity level. No gross toxicity, either acutely or chronically up to 99 days, was observed regardless of whether the dendrimer conjugate contained paclitaxel. The weight was monitored throughout the experiment and no loss of weight was observed; in fact, the animals gained weight. At each time point, a gross examination and histopathology of the liver, spleen, kidney, lung, and heart were done. No morphologic abnormalities were observed on the histopathology examination. No *in vivo* toxicity was noted in any animal group following the dendrimer injection.

## DISCUSSION

This study divulges synthesis of biotinylated $G_4$PAMAM dendrimer. The modification consists of conversion of four

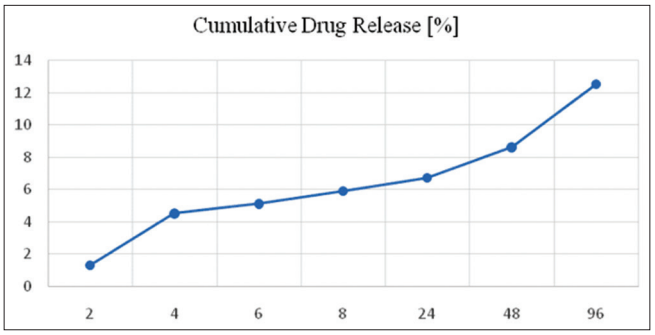


**Figure 5:** Cumulative release of biotinylated $G_4$ PAMAM dendrimer (%)

**Table 1:** Cumulative drug release of paclitaxel biotinylated $G_4$PAMAM dendrimers

| Formulation | Time interval (h) | Cumulative drug release (%) |
|---|---|---|
| Paclitaxel biotinylated PAMAM$G_4$ dendrimers | 2 | 1.3±0.17 |
| | 4 | 4.5±0.23 |
| | 6 | 5.1±0.17 |
| | 8 | 5.9±0.17 |
| | 24 | 6.7±0.21 |
| | 48 | 8.6±0.33 |
| | 96 | 12.5±0.17 |

Mean value±SE, *n*=3

**Table 2:** Stability study of biotinylated $G_4$PAMAM dendrimers

| Time (days) | Assay (% drug remaining) | | | |
|---|---|---|---|---|
| | Pure drug | | Test formulation | |
| | Room temperature | At 40°C | Room temperature | At 40°C |
| 0 | 100±0.49 | 100±0.44 | 100±0.49 | 100±0.49 |
| 1 | 99.9±0.37 | 99.6±0.29 | 99.8±0.32 | 99.7±0.49 |
| 7 | 99.7±0.37 | 99.3±0.31 | 99.5±0.31 | 99.3±0.44 |
| 15 | 99.4±0.48 | 98.7±0.44 | 99.1±0.37 | 98.9±0.57 |
| 30 | 99.1±0.42 | 98.2±0.31 | 98.8±0.17 | 98.5±0.29 |

Mean value±SE, *n*=3

generation 64 primary amines of $G_4$PAMAM dendrimers into Biotinylated $G_4$PAMAM dendrimers. The products were isolated water-soluble partially modified dendrimer. These characteristics made separation and characterization very easy. The water-soluble product was used for further characterization. It was obtained as high yield of fluffy white fibrous solid material, the yield was 81%.

The dendrimer was actually encapsulating in the biotinylated, $G_4$PAMAM dendrimer formulation was proved qualitatively by the shift in absorbance maxima of the copper sulfate solution. The copper sulfate solution has absorption maximum at 267.5 nm. On addition of dendrimer the maximum is shifted

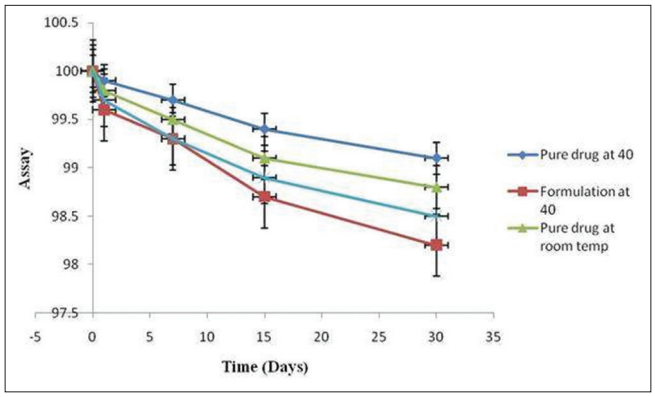


**Figure 6:** Stability study of paclitaxel biotinylated $G_4$PAMAM dendrimers

to 302 nm indicating red shift. This can visually be absorbed by the change in color of copper sulfate solution from blue to violet in high concentrations. This reaction is very sensitive and could detect small amount of dendrimer in solution by shift in absorption maxima of copper sulfate solution. There was no change in absorption maxima of the dialysis solution on addition of copper sulfate indicating that most of the dendrimer is entrapped within the biotin composition due to ionic interaction.

Structural validation of biotinylated $G_4$PAMAMdendrimer was done using $^1$H NMR and $^{13}$C NMR and IR spectroscopy. The interpretation confirms the formation of biotinylated $G_4$PAMAM dendrimer as peaks of all functional groups were obtained in IR. Peaks for respective proton and carbon were obtained in $^1$H NMR and $^{13}$C NMR spectra. The biotin attached to the end of amino groups of dendrimer was determined. The analysis shows that approximately 65% biotin attached to the dendrimer due to stearic hindrance of biotin molecule.

As mentioned earlier, the biotinylated $G_4$PAMAMdendrimer formulation was aggregating on sonication and it was difficult to reduce the size. The particle sizes reported approximately 0.2 µm were determined instrumentally using a Malvern's Mastersizer.

Under TEM, the size of the selected formulation (Biotinylated $G_4$PAMAM) ranged is less than 100nm to few microns in size due to aggregation and fusion. The average size was larger and size and distribution was border than determined from Malvern's Mastersizer. These differences may be due to the differences in samples during measurement: The Mastersizer measure wet sample and TEM measure dry sample. Additional aggregation due to drying effects may be the reason for higher size and size distribution of the formulation particles. Elongated and spherical structures were observed embedded with dendrimer. Based on Malvern's Mastersizer and TEM observation, following structures are proposed for the dendrimer containing formulations; dendrimer enclosing the paclitaxel and aggregate of dendrimer interacted biotin.

The drug entrapment process after the biotinylated $G_4$PAMAM formulation involved increase in temperature that also cause increased aggregation. The entrapment is directly proportional to amount of formulation biotinylated $G_4$PAMAM. The entrapment increased and reached a saturation level with increasing drug concentration. The entrapment of paclitaxel was also directly proportional to the dendrimer incorporated in the formulation. Another possibility may be that the proportion of these dendrimers attach with the surface group was unable to encapsulate drug due to their open structure. The loading values for dendrimer containing biotinylated $G_4$PAMAM include the proportion of the drug that remained associated with surface attached dendrimer. The release was lowered in the biotinylated $G_4$PAMAM formulation in 8 h, due to highly branched structure of dendrimer significantly accelerated the release of water-soluble formulation.

Biotinylated $G_4$PAMAM formulation was stable in aqueous form. Degradation was less than 2% at room temperature and about 4% at 40°C in aqueous form in 30 days. Assay was more than 98.5% and 96.2% for above formulation and drug, respectively. Overall, the stability of the formulation was sufficiently promising for its successful applicability.

## CONCLUSION

In essence, this study reveals that $G_4$PAMAM Dendrimer conjugated with biotin ligand provides a promising tumor tissue specific, drug delivery system for paclitaxel. This amalgamation dispenses not only reduction in distracted charge-mediated uptake but also upturn in *in vivo* biocompatibility. This methodology attributes its novelty by virtue of preservation of the chemical integrity as well as pharmacological properties and slower release of drug-polymer conjugate to free drug that leads to tissue targeting and controlled delivery. Study reveals that the effort on conjugated systems will, therefore, advance the dendrimer-based drug delivery field at a far greater rate, good aqueous solubility of the complex. For the effective treatment by chemotherapeutic approach and to control the pharmacokinetic behavior of drug, this approach may provide a landmark to the advancement of clinical and preclinical managements of disease of interest.

### Highlights

1. Study explores the development of novel drug delivery vehicle for paclitaxel to achieve more targeted anticancer therapeutic response
2. *In vitro* and *in vivo* studies were performed with application of modern analytical modalities to attain the goal of the study
3. Developed novel drug vehicle for paclitaxel conveys reduced distracted charge-mediated uptake as well as elevated *in vivo* biocompatibility.

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## DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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