Dendrimer-based nanocarriers demonstrating a high efficiency for loading and releasing anticancer drugs against cancer cells in vitro and in vivo

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Abstract
Dendrimer, a new class of hyper-branched polymer with predetermined molecular weight and well-controlled size, has received much attention in nanobiomedical applications such as drug carrier, gene therapy, disease diagnosis, etc. In this study, pegylated polyamidoamine (PAMAM) dendrimer at generation 3.0 (G 3.0) and carboxylated PAMAM dendrimer G 2.5 were prepared for loading anticancer drugs. For loading cisplatin, carboxylated dendrimer could carry 26.64 wt/wt% of cisplatin. The nanocomplexes have size ranging from 10 to 30 nm in diameter. The drug nanocarrier showed activity against NCI-H460 lung cancer cell line with half maximal inhibitory (IC50) of 23.11 ± 2.08 µg ml⁻¹. Pegylated PAMAM dendrimers (G 3.0) were synthesized below 40 nm in diameter for carrying 5-fluorouracil (5-FU). For 5-FU encapsulation, pegylated dendrimer showed a high drug-loading efficiency of the drug and a slow release profile of 5-FU. The drug nanocarrier system exhibited an antiproliferative activity against MCF-7 cells (breast cancer cell) with a half maximal inhibitory (IC50) of 9.92 ± 0.19 µg ml⁻¹. In vivo tumor xenograft study showed that the 5-FU encapsulated pegylation of dendrimer exhibited a significant decrement in volume of tumor which was generated by MCF-7 cancer cells. These positive results from our studies could pave the ways for further research of drugs dendrimer nanocarriers toward cancer chemotherapy.

Keywords: dendrimer, 5-FU, cisplatin, drug delivery, xenograft assay

Classification number: 2.05

1. Introduction
Dendrimer is one of the most studied nanopolymers containing internal cavities which can be utilized as a novel nanocarrier for drug, protein and genes delivery. Moreover, dendrimer externally exposed amine or carboxylic groups could be decorated with targeting or/and drug molecules [1, 2]. Several reports indicated that anticancer drugs (camptothecin, 6-mercaptopurin, methotrexate, adriamycin, 5-fluorouracil and paclitaxel) encapsulated into the pegylated polyamidoamine (PAMAM) dendrimer exhibit a significant enhancement of its water solubility, storage stability, reduction of side-effects and anti-tumor activity [3–8].

However, there are a few disadvantages accompanied with PAMAM dendrimer drug-delivery system including hemolytic toxicity and cell lysis due to a strong interaction of the positively charged dendrimer and the negatively
charged cell membrane resulting in disruption of the cellular membrane [9–11].

Pegylation or alkylation of the dendrimer can help to reduce toxicity by preventing the contact between terminal protonated amine groups with cell membranes, resulting in improving their biocompatibility. The conjugation may lead to the increase of the inner space of dendrimers that contributes to increment of drug-loading capacity [12].

It is well-known that cisplatin and 5-fluorouracil (5-FU) are highly effective drugs for chemotherapy but they have caused side-effects for patients due to their non-specific interaction with abnormal and normal cells. It was expected that these drugs loaded in dendrimers or nanoparticles could have reduced side-effects due to their controlled release [13]. Moreover, the use of nanocarriers could prolong the residence time of the drug in plasma circulation and enhance its accumulation in cancer tumors via enhanced permeability-retention (EPR) effect.

This work studies dendrimer-based drug delivery systems which were prepared from the pegylated PAMAM dendrimer G3.0 carrying 5-FU anticancer drug or complexation of cisplatin and carboxylated dendrimer (G2.5). The cisplatin-loaded nanocarrier’s capability to release cisplatin and activity against NCI-H460 lung cancer cell were evaluated. The in vitro and in vivo effectiveness against human breast cancer MCF-7 cell line of this 5-FU loaded system was assessed using sulforhodamine B colorimetric assay and xenograft technique, respectively.

2. Materials and methods

2.1. Materials

5-FU and cisplatin were purchased from Merck Chemicals. Monomethoxy polyethylene glycol 5000 (MPEG-5000) was obtained from Sigma-Aldrich. p-Nitrophenyl chloroformate (NPC) was purchased from Acros Organics. PAMAM dendrimers at generation 3.0 (G 3.0) and carboxylated PAMAM G2.5 (hydrolyzed from PAMAM G2.5, Mw 5800) were prepared in Department of Materials and Pharmacy Chemistry (Institute of Applied Materials Science (VAST) [14] following the procedure reported by Tomalia et al [15]. Regenerated cellulose MWCO 3500–5000D and 10000D dialysis bags were purchased from Spectrum Laboratories Inc.

2.2. Synthesis of dendrimer-based nanocarriers and drug loading methods

2.2.1. Carboxylated PAMAM dendrimer G 2.5 and cisplatin-contained nanocomplex. PAMAM dendrimer G2.5 was synthesized from PAMAM dendrimer G 2.0 following our previous study. Two hundred milligrams of G2.5 (0.035 mmol) was hydrolyzed completely with 15 ml of NaOH 0.1 M in 2 h to obtain carboxylated PAMAM dendrimer. The product was dialyzed (MWCO 3500D) in 48 h and lyophilized. The carboxylated PAMAM dendrimer was characterized and used for loading cisplatin drug.

Hydrolysis of cisplatin: 200 mg of cisplatin (0.66 mmol) was fully dissolved in water, then 14 ml of AgNO3 0.1 M was dropped slowly into the cisplatinum solution and stirred for 24 h at room temperature in N2 gas; the reactor was carefully wrapped to prevent the decomposition of the light. AgCl precipitate was removed by centrifugation to obtain an aquated species of cisplatin (figure 1).

Preparation of cisplatin-contained nanocomplex: 5 ml solution of G2.5 (100 mg, 0.0175 mmol) was added dropwise to the aquated cisplatin (with molar ratio of G2.5 to cisplatin of 1:24) under stirring for 24 h in N2 atmosphere. Then the solution was transferred to dialysis bags of 3.500 Da and dialyzed for 40 min. The reaction mixture was lyophilized, the nanocomplex was obtained in lightly yellow powder form. The structure and properties of nanocomplex was determined by UV–Vis spectroscopy, 13C-nuclear magnetic resonance (13C-NMR) and transmission electron microscopy (TEM). Drug release and anticancer cells behavior of the nanocomplex were evaluated.

2.2.2. Pegylated PAMAM dendrimer G3.0 and 5-FU-loaded pegylated dendrimer. Five grams of dried MPEG 5000 (1.0 mmol) were completely dissolved in dimethylformamide
(DMF) at 40 °C and then reacted with NPC (2.0 mmol) in the presence of triethylene amine under N₂ atmosphere. The mixture was stirred overnight. The product was precipitated in excess of diethyl ether to obtain a white powder of the activated MPEG. The product was then dried and used for further synthesis. Activated MPEG-5000 (4.63 g, 896.0 µmol) in 10 ml DMF solution was added dropwise to PAMAM G3.0 dendrimer (155.224 mg, 22.4 µmol) in 20 ml of DMF with stirring under N₂ atmosphere for 48 h (figure 2). The crude product was dialyzed (MWCO 10 000D) in 48 h. The product was then lyophilized and used for loading 5-FU drug.

For drug loading, a 5-FU excess was added to the pegylated dendrimer solution 0.75 mM under slow stirring (50 rpm) for 24 h. The mixture was centrifugated (5000 rpm) to remove the amount of insoluble 5-FU. This solution was twice dialyzed under strict sink conditions in 20 min to remove free drug from the formulation, which was then estimated spectrophotometrically (λ = 265.5 nm) to determine indirectly the amount of drug loaded within the system. The dialyzed formulation was lyophilized and used for further studies. Drug-loading (DL%) in pegylated dendrimer were weight ratio of 5-FU in nanocarrier and 5-FU plus nanocarrier [16].

2.3. In vitro drug release evaluation

2.3.1. Cisplatin release study. One hundred and five milligrams of G2.5-cisplatin complex was dissolved in 5 ml phosphate buffer solutions at pH 7.4 or acetate buffer solution at pH 5.5. Then the solutions added to the dialysis bag 3500 Da and dialyzed with 500 ml phosphate buffer saline (PBS). At a predetermined time interval, 50 ml of dialyzed solution was drawn to determine cisplatin release by inductively coupled plasma atomic emission spectroscopy (ICP-AES) measurement and another 50 ml of deionized water was added to the dialyzed solution to compensate for the withdrawn volume.
2.3.2. 5-FU release study. The 5-FU-loaded pegylated dendrimer (260.6 mg) and 10 ml deionized water were added to dialyzer membrane (MWCO 3500D) and dialyzed against 1000 ml deionized water. At a predetermined time interval, 10 ml of dialyzed solution was drawn to determine 5-FU release by absorbance measurement at wavelength 265.5 nm and another 10 ml of deionized water was added to the dialyzed solution to compensate the withdrawn volume. The similar concentration of dendritic solution without drug loading was dialyzed in the same condition to serve as control.

2.4. Cytotoxicity assays

The inhibition capabilities of NCI-H460 cell growth of cisplatin, carboxylated dendrimer and its complex was estimated at the screening concentration of 100 µg ml⁻¹. For IC50 evaluation, cell viability was analyzed at different material concentrations using sulforhodamine colorimetric assay. Based on the dose–response curve between the compound concentration and growth inhibition percentage, the IC50 values were subsequently determined using regression analysis. Experiments were performed in triplicates for each compound. The values were expressed as means ± standard deviation (STD).

The inhibition capability of MCF-7 cell growth of PAMAM, pegylated PAMAM, free 5-FU and pegylated PAMAM dendrimer 5-FU complex was done at the same protocol.

2.5. In vivo tumor xenograft

The study was conducted at Stem Cell Research and Application Laboratory, University of Science in Ho Chi Minh City. Briefly, tumors were created in 30 Swiss mice of similar weights and sizes by subcutaneous injection of MCF-7 into the mouse’s thigh at the same cell density (10⁷ cells per animal). After 2 weeks, the animals (n = 16) with tumors that reached the required and less changed volumes were divided into four individual groups which served as control, administered with 10 mg free 5-FU per kg per day, pegylated PAMAM dendrimer–drug complex with an equivalent amount of 10 mg kg⁻¹ 5-FU per day and 5 mg 5-FU per kg per day. The animals were treated in 10 days and the changes of the tumor volumes were recorded during the treatment. The existence of MCF-7 cells in tumors was observed by hematoxylin and eosin stain.

2.6. Characterizations

NMR data was collected using a Bruker AC 500 MHz spectrometer. The average molecular weights of pegylated polymer were calculated from gel permeation chromatography (GPC) technique using Agilent 1100-GPC system. Deionized water was used as a fluent at a flow rate of 1 ml min⁻¹ through an ultrahydrogel column. Platinum content was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, association of analytical communities (AOAC) 990.08 method). For transmission electron microscopy (TEM), pegylated dendrimer was dissolved in methanol at a certain concentration, placed on 300 mesh carbon-coated copper grid, and then air-dried for several hours. TEM images were obtained at 100 kV by a JEM-1400 (JEOL) with magnifications up to ×100 000.

3. Result and discussion

3.1. Characterizations of carboxylated PAMAM dendrimer G2.5

Complexation of the aquated cisplatin and carboxylated dendrimer G2.5 could be well-defined with NMR and UV–Vis spectra. The complex formation caused the chemical shift change of carbon of carbonyl and methylene binding with carboxylate toward the lower magnetic field compared with carbon of PAMAM dendrimer (figure 3(a)). In UV–Vis absorbance spectrum, the wavelength of maximum absorbance of the complex is shifted to the ultraviolet area farther than the peak of cisplatin (data not shown here). It demonstrates that the ligand exchange of cisplatin occurred. TEM image result shows that complexation between PAMAM G2.5 formed nanoparticles below 30 nm in diameter (figure 3(b)). The nanoparticles with diameter of more than 30 nm could be formed due to crosslinking formation of several nanocomplexes at which aquated cisplatin played a role as a crosslinker (figure 3(c)).
3.2. Characterizations of pegylated PAMAM dendrimer G3.0

Pegylation of PAMAM could be confirmed by $^1$H-NMR in which the signal of MPEG methylene protons next to the previously activated group shifted from 4.45 to 4.18 ppm (reported in our previous study) [16]. The obtained GPC result indicated pegylation degree was about 30% as estimated between molecular weight of PAMAM (6909 g mol$^{-1}$) and its pegylation (57 800 g mol$^{-1}$, PDI = 1.4). Ten amine groups had been pegylated among a total of 32 amine groups [16]. The pegylation of dendrimer could be well-defined by TEM in which its morphology is sphere-shaped and diameter is ranging from 30 to 40 nm (Figure 4). There is a significant size increment of the pegylated PAMAM dendrimer as compared to the PAMAM dendrimer G3.0 (diameter below 5 nm).

3.3. Drug loading efficiency and in vitro release profiles

3.3.1. Cisplatin carried dendrimer. In preparation of drug nanocarrier system, understanding of physiological tumor is significant to design and modulate drug delivery-release toward a high targeting efficacy and minimal leakage of the drug. The drug-loaded nanocarrier could be more preferential in which most of the drug is still loaded in the system at normal tissue with pH 7.4 and released at the tumor site with lower pH (5.5–6.5). For loading cisplatin, carboxylated dendrimer could carry 26.64% wt/wt of cisplatin. Cisplatin release profile showed that the released drug is higher at pH 5.5 than that at pH 7.4 and the drug sustainedly released from the system (Figure 5). It can be explained that the complex is more stable at pH 7.4. This is significant to apply in body condition when the drug could prolong in plasma circulation and then accumulate in cancer tumors via EPR effect.

3.3.2. 5-FU loaded pegylated dendrimer. The loading efficiency was about 30% wt/wt calculating approximately 30 drug molecules that were encapsulated within each pegylated PAMAM dendrimer. Moreover, the optimal drug loading was also determined around 35%. Entrapment efficiency of 5-FU in the pegylated dendrimer did not significantly increase when 5-FU excess was used. 5-FU was physically entrapped with hydrogen bondings inside pegylated PAMAM dendrimer cavities [16]. Release profile of the encapsulated drug molecules is shown in Figure 6. The drug slowly releases from the system and reaches more than 84% being released at 24h. This behavior is very significant to prolong drug bioavailability because 5-FU anticancer drug was reported to have a short remaining time in blood circulation [17].

3.4. Cytotoxicity assays and IC50

For cisplatin-loaded carboxylated PAMAM, the results showed that carboxylated PAMAM was cytocompatible and cisplatin was highly cytotoxic. However, cisplatin-loaded carboxylated can reduce the cytotoxic ability of cisplatin more than three times. The result could be attractive to studies on the drug nanocarrier and its utilization for many toxic anticancer drugs (Table 1).

For 5-FU encapsulation, the obtained result showed that PAMAM (100 µg ml$^{-1}$) inhibited 14.06 ± 1.42% cell growth and pegylated PAMAM (100 µg ml$^{-1}$) inhibited...
3.5. Activity of drug-loaded dendrimer against cancer cell in vivo

In vivo tumor xenograft study showed that cancer tumor was formed as shown in figure 7(a) after 2 weeks post-injection of MCF-7 cells into mice. Figure 7(b) shows interface region of normal and tumor tissue in which a high density of the located MCF-7 cells in the tumor region was observed by using H&E stain. Figure 7(c) shows antitumor activity of free 5-FU, drug-loaded dendrimer and peglated dendrimer within 10 days. The 5-FU encapsulated pegylated dendrimer at dose of 5 mg 5-FU per kg mice exhibited a significant decrement in volume of tumors as compared to dose of 10 mg 5-FU per kg mice. This could be explained by the fact that 5-FU has a short half-life and its structure could be changed via metabolism in animals [18]. Therefore, its efficacy was lower than the cases with pegylated dendrimer loading 5-FU at which the drug release could be protected and controlled. Cases of control and pegylated dendrimer administration did not cause decrement in tumor volume. These positive results from our study could pave ways for further research on dendrimer nanocarriers and anticancer drugs toward cancer therapy.

4. Conclusion

This paper demonstrated a high efficiency for loading and releasing anticancer drugs against cancer cells in vitro and in vivo of dendrimer-based drugs delivery systems which was prepared from the pegylated PAMAM dendrimer G3.0 carrying 5-FU anticancer drug or complexation of cisplatin and carboxylated dendrimer. The drug-loaded dendrimer nanocarriers could reduce drug’s toxicity and maintain substantial antiproliferative activity toward cancer cells in vitro and in vivo. The preliminary results show the potential of dendrimer-based nanocarriers in chemotherapy.

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