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Cytotoxicity Enhancement of Doxorubicin in Conjugation with PAMAM G4.5 Dendrimer Containing Gold Nanoparticles

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Abstract : Poly(amidoamine) dendrimer (PAMAM) encapsulated gold nanoparticles (AuNPs) were prepared by reduction of gold salts with $NaBH_4$. The dendrimers used were PAMAM generation 4.5 having carboxyl groups surface. After incorporation of AuNPs into PAMAM, doxorubicin molecules were attached to PAMAM surface groups via ester linkage using dicyclohexylcarbodiimide coupling reaction. The conjugates obtained were characterized by UV-Vis spectroscopy, infrared spectroscopy, dynamic light scattering and transmission electron microscopy. We also evaluated cytotoxicity of doxorubicin before and after conjugation with PAMAM-AuNPs.As the result, we could prepare conjugate of doxorubicin-PAMAM-AuNPs (DOX-PAMAM-AuNPs) with particle size of 25.92 ± 7.99 nm (using TEM) and 147.88 nm (using DLS method). From this research, we found that DOX-PAMAM-AuNPs conjugate could reduce doxorubicin binding to human serum albumin, from 60.71 ± 0.99 % to 47.12 ± 12.39 %. Cytotoxicity assay of DOX-PAMAM-AuNPs conjugate against MCF-7 cell line gave IC_{50} value at 0.035 ± 0.039 µg/mL, while free doxorubicin had larger IC_{50} value, which was0.868 ± 0.235 µg/mL.
Keywords : Doxorubicin, Gold Nanoparticles, PAMAM G4.5 Dendrimer.

Introduction

Doxorubicin is a cytotoxic agent that has been widely used in cancer chemotherapy. It kills tumor cells by inhibiting topoisomerase II and intercalating DNA chain thus inhibits DNA transcription and replication[1]. However, biodistribution and therapeutic effects of doxorubicin are not good enough due to poor ability to target tumor cells[2]. Doxorubicin also has high toxicity, such as cardiomiopathy[3]. To overcome these problems, many strategies have been developed to improve the selectivity of doxorubicin to tumor cells, one is using targeted drug delivery system applications.

Dendrimers have played a key role in drug delivery due to a number of surface functional groups and cavity that can facilitate the delivery process of anticancer drugs. Those functional groups can be attached by targeting molecules, drugs and imaging molecules[4]. They have the ability to control the release of drugs, prolong distribution of drug in the body and increase drug solubility. They also can increase accumulation of anticancer drug in tumor tissues as the result of enhanced permeability and retention (EPR) effect[5].

Gold (Au) in the form of nanoparticles has also been widely used in the treatment of cancer by photothermal effect produced when Au particles were irradiated by near infrared light or laser[6]. The heat generated by that method could induced cell apoptosis. AuNPs surface can be functionalized, especially via thiol and amine groups, so that other molecules can be attached to them. Furthermore, gold nanoparticles can be used as contrast agent for CT-scan modality.

In this research, we combined doxorubicin (DOX) and gold nanoparticles (AuNPs) to enhance the ability to kill cancerous cells. By utilizing the EPR effect, PAMAM dendrimer which brings DOX and AuNPs can be accumulated in tumor tissues. One molecule of PAMAM dendrimer can attach some DOX molecules so that the concentration of the drug in the site of action will be higher and the cytotoxic effects will be more optimal. Furthermore, PAMAM dendrimer is expected to reduce interaction of DOX and plasma protein via steric hindrance. PAMAM dendrimer can also be used as a barrier to prevent AuNPs aggregation by incorporating them in the cavity of PAMAM dendrimer.

The aim of this research is to prepare and characterize a conjugate consists of doxorubicin, PAMAM G4.5 dendrimer and gold nanoparticles. We further call this conjugate as DOX-PAMAM-AuNPs to make it simple. Doxorubicin was linked to surface functional groups of PAMAM G4.5 dendrimer via ester bond, while gold nanoparticles was incorporated in the PAMAM dendrimer cavity. We also evaluated in vitro cytotoxicity of the conjugate. This conjugate system is expected to enhance therapetic and minimize side effects of doxorubicin.

Experimental

Materials

Chloroauric acid ($HAuCl_4$) and PAMAM G4.5 Dendrimer were purchased from Sigma-Aldrich, USA. Doxorubicin HCl were obtained from PT. SanbeFarma, Indonesia in production of RPG Life Sciences Limited, India. Other reagents were obtained from vendors in the highest quality available and include N,N-dicyclohexylcarbodiimide (Sigma-Aldrich, USA), *4-(dimethylamino) pyridine* (Sigma-Aldrich, USA), dimethyl sulfoxideanhidrat, $NaBH_4$ (Aldrich, USA), human serum albumin (Sigma-Aldrich, USA). Human breast adenocarcinoma cell line (MCF-7) were obtained from LAPTIAB, BPPT, Indonesia.

Methods

Preparation of PAMAM G4.5 Dendrimer Encapsulated Gold Nanoparticles (PAMAM-AuNPs)

Freshly prepared $HAuCl_4$ solution (100 µL; 0,01mol/L) in DMSO was added to PAMAM G4.5 dendrimer solution (46,5 µL; 0,0015 mol/L) in DMSO. The mixture was stirred for 15 minutes at room temperature, protected from light, to complete the formation of the Au^{3+}–PAMAM complex. Then 75 µL of $NaBH_4$ in aqueous solution (0,02mol/L) was quickly added during vigorous stirring to reduce Au^{3+} to Au(0). A reddish brown solution was formed. The final molar ratios of [PAMAM G4.5]/[Au^{3+}] were 0,07 and 1, respectively. The mixture was stirred for 1 hour at room temperature, protected from light, to complete the reduction reaction.

Preparation of Doxorubicin-PAMAM G4.5-Gold Nanoparticles Conjugates (DOX-PAMAM-AuNPs)

500 µL solution of dicyclohexylcarboodiimide (DCC) 0,0196 mol/L and 50 µL solution of dimethylaminopiridine (DMAP) 0,0178 mol/L in DMSO were added to PAMAM-AuNPssolution obtained from previous step. The mixture was stirred for 30 minutes. Then, 100 µL solution of DOX HCl 0,0111 mol/L in DMSO was added during vigorous stirring. Molar ratio of DOX and PAMAM G4.5 was respectively 16 and 1. The reaction mixture was stirred for 48 hours at room temperature, protected from light. After 48 hours, it was filtered to remove dicyclohexyl urea (DCU), and the filtrate was purified using ultrafiltration tube Vivaspin (Sartorius, Germany) with molecular weight cut-off 10000 Da. The assay of doxorubicin in conjugate was measured by UV-Vis spectroscopy in λ = 481 nm, while gold nanoparticles was measured by atomic absorption spectrometry in λ = 242,8 nm.

Characterization of Doxorubicin-PAMAM G4.5-Gold Nanoparticles Conjugates

The conjugates were characterized using UV-Visspectrophotometer (Shimadzu UV-1601), fourier transformation infra red (Shimadzu FTIR Type 8400S), particle size analyzer (Cordouan Technologies), transmission electron microscope (JEM 1400).

Evaluation of DOX-PAMAM-AuNPs conjugate binding to human serum albumin

Protein binding test was done using centrifugation method. 100 µL solution of conjugate (80 µg/mL, listed in DOX equivalent) was placed in centrifugation tube which contained 100 µL solution of human serum albumin (HSA) 5% and 1 mL of saline solution. The mixture was vortexed for 1 minute and incubated at 37ºC for 10 minutes. 1 mL solution of trichloracetic acid (TCA) 5% was added to the previous mixture and then vortexed. The final mixture was centrifugated for 15 minutes (3000 rpm). Supernatant was separated and

measured quantitatively using UV-Vis spectroscopy in λ=481 nm. Protein binding test was also done to free doxorubicin as comparison. Percentage of DOX-HSA binding was calculated by following equation:

$$\text{Protein plasma binding}(\%) = \frac{D_t - D_f}{D_t} \times 100$$

Where:
D_t = doxorubicin in solution used (conjugate or free)
D_f = doxorubicinin supernatant (unbound DOX)

In Vitro Cytotoxicity Assay

Anti-cancer effect of DOX-PAMAM-AuNPs conjugate, PAMAM-AuNPs conjugate and free DOX was determined by MTT-based cytotoxicity assay using human breast adenocarcinoma cell line (MCF-7). The assay was assessed by direct counting using a hemacytometer. Cells were seeded in 96-well plates at a cell density of 5×10^4 cells/well and cultivated in culture media at 37°C in 5% CO_2 atmosphere. After incubated over 24 hours, serial dilutions of either free DOX or conjugated DOX were added to each well. All experiments were done at least in triplicate. After incubation for 24 hours, cell culture medium was removed and washed with phosphate buffer saline (PBS). After that, 100 µL MTT solution (0.5 mg/mL) was added to each well, including control medium (without cells) and negative control (non-treated cells). After incubation for 4 h at 37°C, 100 µL of a solubilizing buffer (10% sodium dodecyl sulfate in 0.1 N HCl) was added. Absorbance of each well was measured at 570 nm, and relative cytotoxicities were obtained using a non-treated cell as a control.

Results& Discussion

Synthesis and characterization of PAMAM-AuNPs

We have reduced 0.01 mol/L solutions of $HAuCl_4$ by addition of $NaBH_4$ in the presence of PAMAM G4.5 dendrimers in DMSO medium (Figure 1). We used DMSO as AuNPs synthesis medium because the next synthesis step (synthesis of DOX-PAMAM-AuNPs) must be applied in non-aqueous medium. If we used aqueous medium in PAMAM-AuNPssynthesis, we should remove the water before starting the synthesis of DOX-PAMAM-AuNPs. Removing the water will increase the risk of AuNPs aggregation. Thus, we preferred to directly use DMSO as PAMAM-AuNPs synthesis medium.

Figure 1. Color of solution of (a) $HAuCl_4$and PAMAM G4.5 shortly before mixed (clear pale yellow), (b) $HAuCl_4$and PAMAM G4.5 compex before addition of $NaBH_4$ (colorless), (c) AuNPs encapsulated PAMAM (reddish brown)

The UV-Vis spectra characteristic of PAMAM-AuNPscan be seen in Figure 2. $HAuCl_4$ solution in DMSO showed a strong absorption band at λ 326 nm (Figure 2a). This showed a different result from literature in which $HAuCl_4$ solution showed a strong absorption at λ 220 nm and shoulder at λ 290 nm due to charge transfer between Au ion and chloroligan [7]. This difference may be caused bythe difference in solvent used. The different solvent used may result in different hydrogen bond arrangement between solute and solvent, thus give different absorption band. PAMAM G4.5 dendrimer solution showed weak absorption band at λ 285 nm (Figure 2b).

The absorbance band of $HAuCl_4$ solution was shifted from λ 326 nm to 277 nm when adding PAMAM G4.5 dendrimer (Figure 2c), probably due to the formation of ion complex between $AuCl_4^-$ and tertiary amine of PAMAM G4.5 dendrimer in its interior structure. After reduction with $NaBH_4$, peak absorption at λ 281 nm and 516 nm were appeared (Figure 2d).Absorption at 281 nm shows particular gold cluster while absorption at 516

nm shows surface plasmon resonance characteristic of gold nanoparticles [7]. These results showed that gold nanoparticles were already formed and encapsulated in PAMAM G4.5 dendrimer cavity.

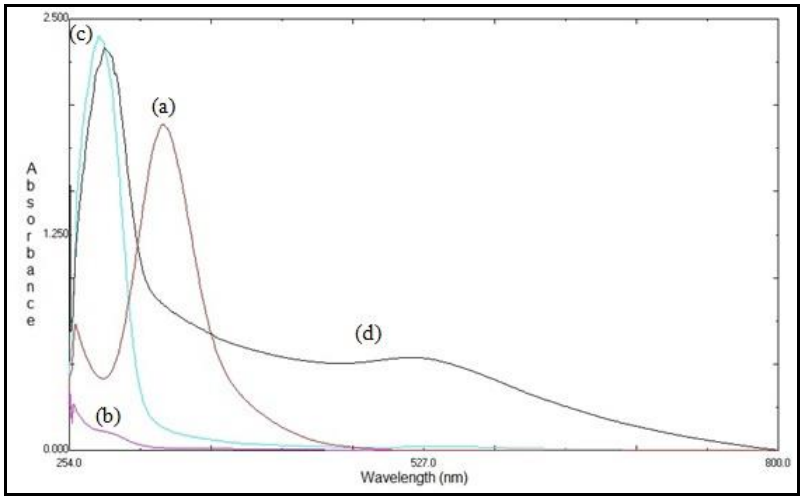

Figure 2.UV-Vis spectra of (a)$HAuCl_4$ solution, (b) PAMAM G4.5 dendrimer solution, (c) $HAuCl_4$-PAMAM before reduction and (d) PAMAM-AuNPs solution in DMSO

Synthesis and characterization of DOX-PAMAM-AuNPs

DOX was conjugated to PAMAM G4.5 dendrimer using DCC as a coupling agent and DMAP as a catalyst in one-pot reaction synthesis scheme (Figure 3). The conjugation reaction involved terminal –COOH groups of PAMAM G4.5 dendrimer and C14 –OH group of DOX, forming ester linkage. UV-Vis spectra of doxorubicin in DMSO alone showed peak absorption at λ 481 nm, while DOX-PAMAM-AuNPsconjugates also showed peak absorption at λ 481 nm and absorption band from λ 582 nm to 800 nm which results from AuNPs (Figure 4).

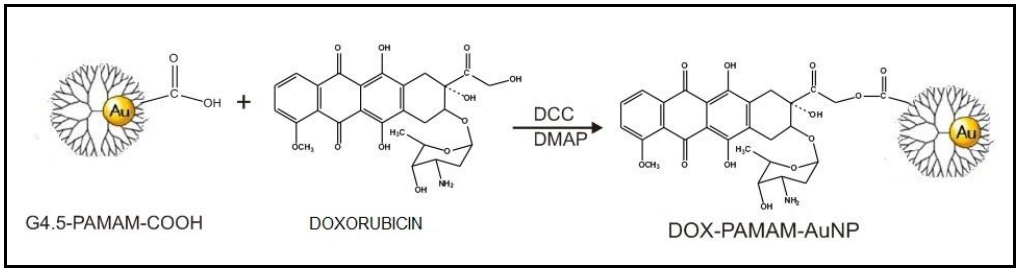

Figure 3. Conjugation scheme of doxorubicin and PAMAM G4.5 dendrimer

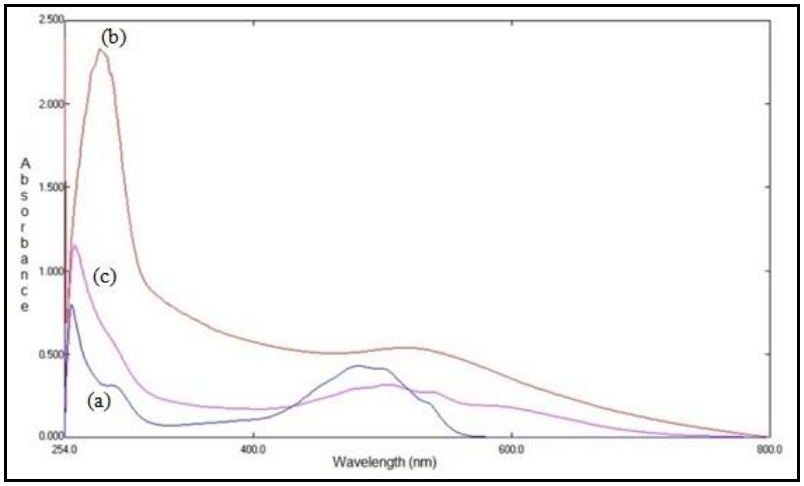

Figure 4.UV-Vis spectra of (a) doxorubicin HCl, (b) PAMAM-AuNPs and (c) DOX-PAMAM-AuNPs in DMSO

Infrared (IR) spectra of free DOXHClshowed strong peak at 3100-2800 cm^{-1}which results from C–H and –OH alcoholic vibration (Figure 5). Peak at 3317.67 cm^{-1}shows primary amine ($–NH_2$) from doxorubicin. Peak at 3028.34 $cm^{-1}$and 2935.76 cm^{-1}, respectively, showsaromatic C–H boundandalkyl C–H. C–C bound in aromatic ring was showedby peak at 1413.87 cm^{-1}. Carbonil groups (C=O) were showed bypeak at 1732.13 $cm^{-1}$and 1583.61 cm^{-1}.

IR spectra of DOX-PAMAM-AuNPs conjugates showed peak at wavenumber 1585.54 cm^{-1}dan 1045.45 cm^{-1} which shows C–O stretch vibration of carbonyl group (C=O). C–O stretch vibration at 1585.54 cm^{-1} is not seen in IR spectra of PAMAM-AuNPs as well as DOX. It gives the possibility that the C–O stretch vibration in referred peak is produced by the carbonyl group of the ester bond between DOX and PAMAM G4.5 dendrimer.

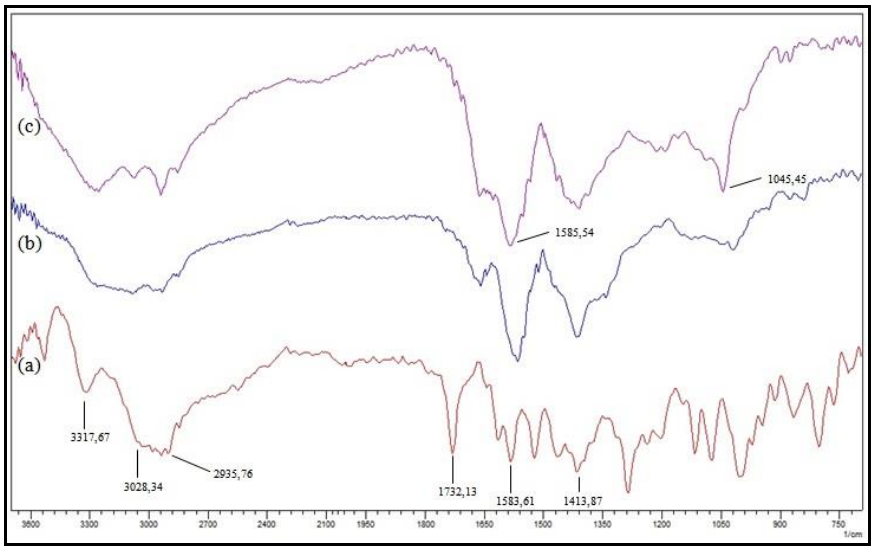

Figure 5. IR spectra of (a) doxorubicin, (b) PAMAM-AuNPs and (c) DOX-PAMAM-AuNPs conjugates

From the assay results, we obtained approximately 16.50 ± 1.14% gold nanoparticles encapsulated in PAMAM G4.5 dendrimer. This low yield is probably due to the use of DMSO as AuNPs synthesis medium. Thus the formation of complex ions between $AuCl_4^-$ with amine groups of PAMAM G4.5 were not optimum. As a result, many gold ions were not encapsulated on the dendrimer PAMAM G4.5 and separated from DOX-PAMAM-AuNPs conjugated uring purification.

DOX payload in conjugate was approximately 62.86 ± 6.88%. Gurdag, Khandare, Stapels, Matherly, and Kannan (2006) prepared methotrexate conjugate to PAMAM G2.5 and G3[8]. The result was only 22.4% of methotrexate can be conjugated to the PAMAM dendrimer. The higher drug payload in our research is probably due to higher dendrimer generation used in our study, thus more terminal groups can be conjugated to doxorubicin.

Particle size, particle size distribution and polydispersity index

Figure 6 shows TEM micrographs and size distribution histogram of PAMAM-AuNPs and DOX-PAMAM-AuNPs. It can be seen that PAMAM-AuNPs have particle size of 23.97 ± 4.19 nm before conjugation with DOX and 25.92 ± 7.99 nm after conjugation process. From this result, it can be concluded that the conjugation reaction did not influence the particle size of AuNPs encapsulated PAMAM G4.5 dendrimer. It is probably because PAMAM G4.5 dendrimer can effectively restrict the formation and growth of larger gold particles. Particle size of PAMAM-AuNPs and DOX-PAMAM-AuNPs were also determined by dynamic light scattering (DLS) method. From DLS results, PAMAM-AuNPs have $Z_{average}$ = 79.38 nm while DOX-PAMAM-AuNPs conjugates have $Z_{average}$ = 147.88 nm. The result by DLS method is summarized in Table 1.

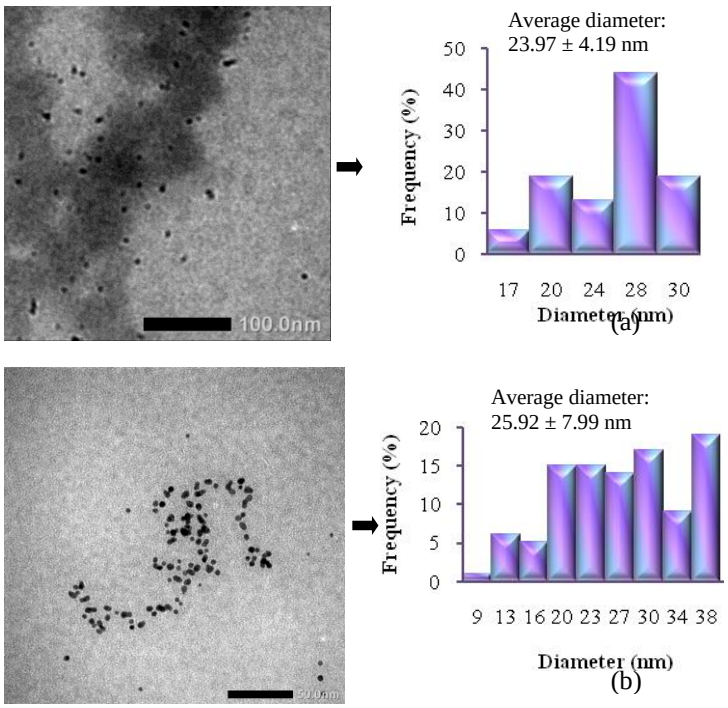

Figure 6.TEM images of (a) PAMAM-AuNPs and(b) DOX-PAMAM-AuNPs conjugates

Particle size below 150 nm can prolong residence time in circulation and defined as stealth or macrophage evading nanoparticles[9]. Thus, the DOX-PAMAM-AuNPs conjugate produced in this research has the potency for passive targeting drug delivery via enhanced permeability and retention (EPR) effect. It is because intercellular junction of tumor endothelium have large size, which is 1,2-2 µm, thus allow particles below that size to pass by[10].

Table 1. Summary of particle size, particle size distribution and polydispersity index determination using DLS method

Parameter	PAMAM-AuNPs	DOX-PAMAM-AuNPs
Dv10	17.79 nm	102.36 nm
Dv50	35.49 nm	141.29 nm
Dv90	77.65 nm	213.85 nm
D_{mean} intensity	99.12 nm	152.31 nm
D_{mean} volume	45.79 nm	150.57 nm
D_{mean}number	19.86 nm	119.38 nm
$Z_{average}$	79.38 nm	147.88 nm
Polidispersity index	0.3370	0.0560

Evaluation of DOX-PAMAM-AuNPs conjugate binding to human serum albumin

From the experiment, we found that as many as 60.71 ± 0.99% of unconjugated DOX bound to human serum albumin (HSA) (Table 2 and Figure 7). Protein binding of DOX was reduced when DOX is conjugated to PAMAM-AuNPs. We found that only as many as 47.12 ± 12.39% of DOX-PAMAM-AuNPs conjugate were bound to HSA. These results indicate that conjugation of doxorubicin to PAMAM-AuNPs can reduce the plasma protein binding of doxorubicin, in this case albumin. This is probably because one of the –OH group of doxorubicin already bound to PAMAM G4.5 thus reducing the number of groups that can bind to albumin. In addition, the binding of doxorubicin with PAMAM can provide steric hindrance that can prevent the bond between doxorubicin and albumin.

Table 2.Summary of protein binding assay of unconjugated DOX and DOX-PAMAM-AuNPs conjugates

Material	D_T (µg)	D_F (µg)	D_B (µg)	% D_B	Drug-HSA binding (%)
Unconjugated doxorubicin	225	90.07	134.93	59.97	60.71 ± 0.99
	225	85.86	139.14	61.84	
	225	89.26	135.74	60.33	
DOX-PAMAM-AuNPs conjugates	8	4.55	3.45	43.15	47.12 ± 12.39
	8	3.12	4.88	61.01	
	8	5.02	2.98	37.20	

D_T = amount of initial drug
D_F = amount of drug present in supernatant (unbound drug)
D_B = amount of drug bound to HSA

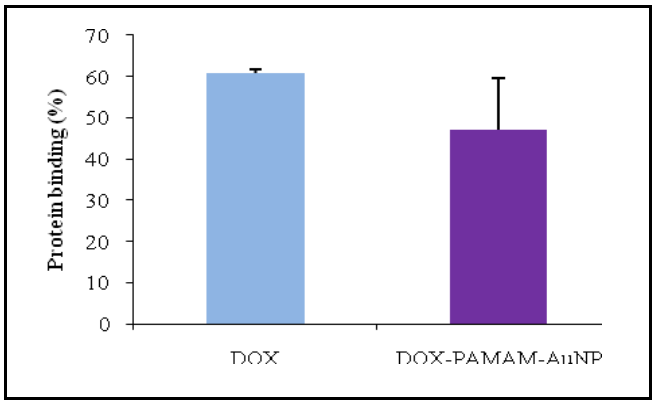

Figure 7.Comparison of unconjugated doxorubicin (DOX) and DOX-PAMAM-AuNPs conjugates binding to human serum albumin

In Vitro Cytotoxicity Assay

The in vitro cytotoxicity of DOX and DOX-PAMAM-AuNPs conjugates were tested in terms of growth inhibition of human breast adenocarcinoma cell lines (MCF-7). Figure 8 shows the response of MCF-7 cell lines to both free DOX and DOX-PAMAM-AuNPs conjugate. From the curve we can see that at low concentration, that is 0.3125 µg/mL dan 0.625 µg/mL, DOX-PAMAM-AuNPs conjugate could inhibit MCF-7 cell lines proliferation more effectively than unconjugated DOX. While at higher concentration, both unconjugated DOX and DOX-PAMAM AuNPs conjugate did not give significant difference in proliferation inhibition.

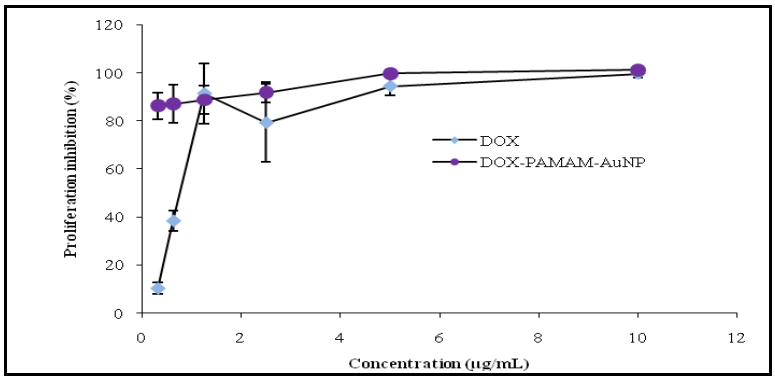

Figure 8.Comparative growth inhibition profiles of unconjugated doxorubicin (DOX) and DOX-PAMAM-AuNPs conjugates in MCF-7 cell lines

IC_{50} value of unconjugated DOX was approximately 0.868 ± 0.235 µg/mL. The result is different from previous research, where IC_{50}value of free DOXagainst MCF-7 was 0.417 µg/mL[11].IC_{50}value of DOX-PAMAM-AuNPsconjugate was 0.035 ± 0.039 µg/mL (listed in DOX equivalents). From this result we can conclude that DOX-PAMAM-AuNPs conjugates were 23 times more potent than uncojugated DOX (Figure 9).

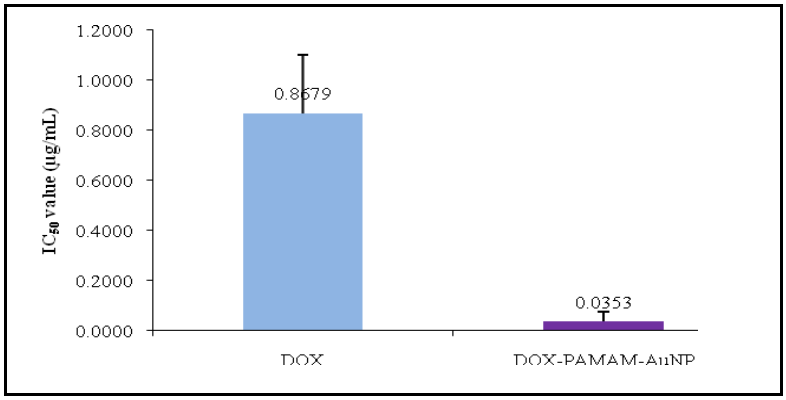

Figure 9. IC_{50}value comparison of unconjugated DOX and DOX-PAMAM-AuNPs conjugate

Conjugation DOX with dendrimer or other carrier can inhibit its cytotoxicity, depending on the groups used as binding site and also the characteristics of the carrier. For example, research conducted by Lai, et al. (2007) showed that the dendrimer conjugate of doxorubicin with PAMAM through amide bond significantly decreased cytotoxicity[12]. That is because amide bond is too strong that doxorubicin can not be released from the dendrimer and ultimately can not produce cytotoxic effects.

In this study, the cytotoxicity of doxorubicin in DOX-PAMAM-AuNPs conjugate was increased up to 23-fold compared with unconjugated doxorubicin. This is presumably because the PAMAM G4.5 dendrimer used as a carrier can increase the efficiency of drug uptake by the cells (cellular uptake)[13]. Increasing in efficiency of cell uptake causes increasing DOX amount that can enter tumor cells, thus increase the cytotoxic activity of DOX. Another hypothesis regarding the reason of increasing cytotoxicity effect of DOX in conjugate with PAMAM-AuNPs is because DOX was conjugated to PAMAM via ester bond. This ester bond is more susceptible to hydrolysis when subjected to internalization into cancer cells, thus free DOX can be easily released from conjugate. After released from conjugate, DOX can enter the nucleus and perform the activity.

Gold nanoparticles in DOX-PAMAM-AuNPs conjugate was expected to play a role in increasing cytotoxicity of the conjugate. Pan, et al. (2009) mentioned that AuNPscould induce necrosis via oxidative stress and mitochondrial destruction[14]. Cytotoxicity of AuNPs depends on particle size, in which the smaller particle size can lead to increasing cytotoxicity. PAMAM G4.5 dendrimer as carrier is also thought to have a role in promoting the entry of cancer cells into the AuNPs.

Conclusions

Conjugation of DOX to dendrimers which encapsulate gold nanoparticles via ester linkage, yielded conjugates with different characteristics in terms of protein binding and cytotoxicity. The significantly improved activity (up to 23-foldimprovement in the IC_{50} values) of the DOX-PAMAM-AuNPs conjugates is hypothesized as result from the distinct intracellular release profiles of the drug from the conjugates. DOX release profile from conjugates may be required to confirm this hypothesis. To enhance the cytotoxic activtity of the conjugates, the use of targeting agents such as folic acid may further selectively enhance the level of internalized DOX due to specific accumulation of the nanodevice in tumor tissues.

Acknowledgement

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