Enhanced cellular uptake and cytotoxicity of folate decorated doxorubicin loaded PLA-TPGS nanoparticles

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Received 5 January 2015
Accepted for publication 9 January 2015
Published 2 February 2015

Abstract
Doxorubicin (DOX) is one of the most effective anticancer drugs for treating many types of cancer. However, the clinical applications of DOX were hindered because of serious side-effects resulting from the unselective delivery to cancer cell including congestive heart failure, chronic cardiomyopathy and drug resistance. Recently, it has been demonstrated that loading anti-cancer drugs onto drug delivery nanosystems helps to maximize therapeutic efficiency and minimize unwanted side-effects via passive and active targeting mechanisms. In this study we prepared folate decorated DOX loaded PLA-TPGS nanoparticles with the aim of improving the potential as well as reducing the side-effects of DOX. Characteristics of nanoparticles were investigated by field emission scanning electron microscopy (FESEM), dynamic light scattering (DLS) method and Fourier transform infrared spectroscopy (FTIR). Anticancer activity of the nanoparticles was evaluated through cytotoxicity and cellular uptake assays on HeLa and HT29 cancer cell lines. The results showed that prepared drug delivery system had size around 100 nm and exhibited higher cytotoxicity and cellular uptake on both tested HeLa and HT29 cells.

Keywords: doxorubicin, copolymer PLA-TPGS, folic acid (Fol), Fol/DOX/PLA-TPGS NPs, drug delivery nanosystem (DDNS)
Classification numbers: 2.05, 5.08, 5.09

1. Introduction

Doxorubicin (DOX), which is a member of anthracycline family, was ranked among the most effective anti-cancer drugs. It has been clinically used for treating a broad spectrum of cancers, such as leukemias, lymphomas, soft-tissue, osteogenic sarcomas, pediatric malignancies, solid tumors, breast and lung carcinomas [1]. In spite of its potential, DOX induces serious side-effects in dose dependent manner including congestive heart failure, chronic cardiomyopathy and the development of tumor cell resistance. In addition, DOX is also rapidly degraded and eliminated after intravenous administration [2]. Many attempts, therefore, have been made to modify DOX molecule in order to produce new analogues of DOX. However, the achieved results are not the deserving replacements for DOX. DOX still remains the focus in clinical researches aimed at identifying new strategies for better use in cancer treatment.

Using drug delivery nanosystem (DDNS) is a promising strategy for a more controlled and targeted delivery of DOX [3–5]. The small size of drug delivering nanoparticles allows them to escape from the biological attacks of the body and reach the tumor site at higher concentration through the enhanced permeability and retention effect (EPR), known as passive targeting [6]. Moreover, these drug delivery systems could selectively target the tumor through specific bindings.
between targeting moieties attached on the surface of nanoparticles and the receptors which are characteristic for each type of cancer. This mechanism is called the active targeting [7].

The most common studied carrier for delivering DOX is liposome, and Doxil® is the first FDA-approved nano-drug [8]. However, the main limitation of liposome is its intrinsic instability. The dispersion of liposome often has a tendency to flocculate [9]. Among the materials for fabricating DDNS, polymeric micelle composed of amphiphilic copolymers is a promising candidate thanks to its highly structural stability, small size and high drug loading efficiency [10]. In our previous reports, our group demonstrated that polymeric micelle composed of copolymer poly(lactide)-d-α-tocopheryl polyethylene glycol 1000 succinate (PLA-TPGS) is the potential system for loading and delivering anticancer drugs such as paclitaxel and curcumin [11–14]. Therefore, we believe that copolymer PLA-TPGS is also a good solution for delivering DOX. More interestingly, TPGS is not only a good emulsifier but is also able to overcome multidrug resistance, which is one of the side-effects of DOX [15]. Furthermore, folic acid as targeting ligand was introduced to the system in order to enhance the specificity to cancer cells.

In this study we prepared folate decorated DOX loaded PLA-TPGS nanoparticles (Fol/DOX/PLA-TPGS NPs) aimed at fabricating a DOX delivery nanosystem able to selectively target cancer through passive and active targeting mechanisms. The obtained results showed that the Fol/DOX/PLA-TPGS NPs with the size around 100 nm improve the cellular uptake and cytotoxicity on cancer cells.

2. Experimental

2.1. Materials

Copolymer PLA-TPGS was obtained from Laboratory of biomedical nanomaterials, Institute of Materials Science, Vietnam Academy of Science and Technology. Vitamin E TPGS (d-a-tocopheryl polyethylene glycol 1000 succinate) was obtained from Merck. Doxorubicin hydrochloride (DOX.HCl), 1-(ethyl-3-(3-dimethylamino) propylcarbodiimide (EDC), N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), glutaric acid, folic acid, ethylene diamine and triethylamine were obtained from Sigma-Aldrich. All solvents used are HPLC grade, which include toluene, dichloromethane (DCM), methanol and dimethyl sulfoxide (DMSO, anhydrous) from Aldrich. Distilled water was used throughout all experiments. Human cervical carcinoma (HeLa) and human colon adenocarcinoma (HT-29) cell lines were obtained from Lab of Department of Biology, Hanoi University of Science. Solvents and chemicals for bioassays were purchased from Invitrogen.

2.2. Synthesis of TPGS-folate (TPGS-Fol)

Folate was covalently attached to TPGS molecules through a modified process described by Pan and Feng [16]. Firstly, TPGS was activated by reaction with aspartic acid in the presence of DCC and NHS as catalysts at the molar ratio of TPGS/aspartic acid/DCC/NHS of 1/1/1.2/1.2. Aspartic acid was attached to TPGS through the esterification of its carboxyl group with hydroxyl group of TPGS. The reaction was carried out at room temperature for 24 h. The product was filtered to remove unreacted parts and by-product. Secondly, folic acid was animated by reaction with NHS using DCC as catalyst in DMSO solvent at the molar ratio of folic acid/DCC/NHS of 1/1/1.2 and stirred for 6 h at 50 °C. The product was then reacted with ethylene diamine and precipitated with acetonitrile. Finally, activated TPGS was reacted with animated folic acid at a molar ratio of 1/1.2 for 6 h at 37 °C. The product was filled and then precipitated with acetonitrile. The final product was lyophilized to obtain dry TPGS-Fol.

2.3. Preparation of doxorubicin loaded nanoparticles (DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs)

DOX loaded nanoparticles were prepared by an emulsion solvent evaporation method. DOX.HCl (15 mg) was dissolved in dichloromethane (15 ml) and then deprotonated by the addition of triethylamine (1.5 ml). The dichloromethane solution of DOX was stirred in a closed flask for 6 h. Copolymer PLA-TPGS or mixture of PLA-TPGS and TPGS-Fol (9:1, w/w) (40 mg) was dissolved in double distilled water (60 ml). The dichloromethane solution of DOX was added dropwise into the water solution of copolymer under vigorous stirring. The mixture was stirred for 24 h in a closed flask and then dichloromethane was evaporated under vacuum pressure. The obtained mixture was centrifuged at 5600 rpm for 10 min to remove free DOX. The red transparent solution was collected. A half of this solution was lyophilized and stored at 4 °C.

2.4. Characterization methods

Molecular structure of DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs was characterized by Fourier transform infrared spectroscopy (FTIR, SHIMADZU spectro-photometer) using KBr pellets in the wave number region of 400–4000 cm\(^{-1}\). Their morphology was investigated by field emission scanning electron microscopy (FE-SEM) on a Hitachi S-4800 system. Size distribution was measured by dynamic light scattering (DLS) method.

Drug loading content (LC) and drug encapsulation efficiency (EC) were determined with a UV–vis spectrophotometer at 480 nm. A calibration curve was obtained with DOX.HCl/water solution at different concentrations of DOX. The LC and EC were calculated based on the following equations:

\[
LC(\%) = \frac{W_{\text{drug}}}{W_{\text{total}}} \times 100,
\]
In vitro drug release of drug delivery systems were performed in phosphate buffer saline (PBS) solution at pH 7.4 at 37 °C. 5 mg lyophilized nanoparticle was dispersed in 20 ml PBS. After each period of time, a 3 ml sample was taken and 3 ml distilled water was added. The taken sample was centrifuged at 5600 rpm for 10 min to remove released DOX. DOX concentration in obtained solution was determined based on absorbance intensity at 480 nm. DOX release was calculated by following equation:

\[
\text{DOX release (\%)} = \frac{C_{t,\text{DOX}} - C_{0,\text{DOX}}}{C_{0,\text{DOX}}} \times 100
\]

with \( C_{0,\text{DOX}} \) is initial concentration of DOX, \( C_{t,\text{DOX}} \) is concentration of DOX at time \( t \).

2.5. Cell culture

Two cancer cell lines (HT29 and HeLa) were chosen to investigate cytotoxicity and targeting effect of drug delivery systems. Cancer cells were activated and cultured under atmosphere of 5% CO\(_2\) and 95% air at 37 °C. Each cell line was cultured by an appropriate medium. The media were refreshed every 2 days to ensure sufficient nutrients and remove dead cells. After being cultured, cells were placed on a 96-wells plate and kept stable for 48 h.

2.6. In vitro cytotoxicity study

In vitro cytotoxicity of drug delivery systems was examined by MTS assay. A cell was exposed to free DOX, DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs at the concentration of DOX ranging from 0.017 to 5.172 \( \mu \)M. After 48 h incubation with drug formulations, the cell was incubated with the mixture of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in the presence of PMS (phenazinemethosulfate). MTS will be converted to formazan by dyhydrogenase in a viable cell. The absorbance of produced formazan, which correlated with the number of viable cells in each well, was measured by a UV–vis spectrophotometer at 490 nm. The absorbance of treated groups was compared with the control group to obtain cell survival percentage. All experiments were run in triplicate and the results were recorded as mean ± standard deviation.

2.7. In vitro cellular uptake study

In vitro cellular uptake experiments were performed on HT29 cell lines. The cells were exposed to free DOX, DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs at a concentration of 10 \( \mu \)M for 2 h. Cells were washed three times with deionized water, stained with Hoechst 33 342 fluorescent stain for 15 min and washed three times with deionized water. Cell images were taken by laser scanning confocal microscope (LSCM) at 346 and 480 nm.

3. Results and discussion

3.1. Drug loading content and drug encapsulation efficiency

LC and EC were determined through the absorbance of DOX solutions at 480 nm. The calculated LC and EC of DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs were 30.68 ± 1.18, 81.80 ± 4.73% and 25.81 ± 0.80, 68.82 ± 2.13%, respectively. The slight decrease of LC and EC may be due to the reduction in emulsification efficiency of copolymer PLA-TPGS when mixed with TPGS-Fol.
3.2. Chemical structure of DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs

Chemical structures of DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs were investigated by FTIR spectroscopy. Figure 1(a) shows the FTIR spectra of PLA-TPGS, DOX and DOX/PLA-TPGS NPs. After loading DOX, the characteristic bands of PLA-TPGS and DOX were observed to be shifted. Typically, the bands at 2974 and 1756 cm$^{-1}$ are assigned to the C–H and C=O stretching vibrations of PLA-TPGS [11] which, respectively, shifted to 2920 and 1760 cm$^{-1}$ in spectrum of DOX-PLA-TPGS. Besides, characteristic bands at 1630 cm$^{-1}$ corresponding to N–H bending vibrations, at 1427 cm$^{-1}$ corresponding to C–C stretching vibrations, at 1010 cm$^{-1}$ corresponding to C–O stretching vibration of DOX [17] were observed at 1620, 1400 and 1023 cm$^{-1}$ in DOX-PLA-TPGS NPs spectrum. The appearance of these characteristic bands proved the loading of drug into the micelle system of PLA-TPGS.

Figure 1(b) shows the changes in FTIR spectra of DOX/PLA-TPGS NPs, folic acid and Fol/DOX/PLA-TPGS NPs. Characteristic bands of DOX/PLA-TPGS NPs at 2920, 1760, 1620, 1400, 1216 and 1023 cm$^{-1}$ were shifted to 2927, 1703, 1615, 1400 and 1023 cm$^{-1}$, respectively, in the FTIR spectrum of Fol/DOX/PLA-TPGS NPs. The peaks at 1703 and 1615 cm$^{-1}$ were also attributed to the C=O stretching vibrations of folic acid (at 1695 and 1608 cm$^{-1}$). In addition, the presence of peak at 1512 cm$^{-1}$ related to the bending mode of N–H vibration of folic acid [18].

3.3. Morphology and size distribution of DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs

To evaluate the sizes of DOX loaded PLA-TPGS nanoparticles, field emission scanning electron microscope (FESEM) images were taken of DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs and are shown in figure 2. We can see that nanoparticles have spherical shape with a uniform diameter of about 50–60 nm.

The hydrodynamic diameter of the polymeric system was determined by DLS method (figure 3). The obtained results from DLS were slightly larger, about 90 nm for DOX/PLA-TPGS and 110 nm for Fol/DOX/PLA-TPGS. The size distribution followed the standard curve with one peak distribution. The difference in size between the results of FESEM and DLS was because of the different states. Data from FESEM showed the dried state of nanoparticles, while DLS method measured the size of nanoparticles suspended in aqueous environment.

3.4. Drug release

Figure 4 shows the DOX release from DOX.HCl, DOX/PLA-TPGS and Fol/DOX/PLA-TPGS NPs. In the case of DOX.HCl, the drug was absolutely soluble in PBS solution right after dissolving into buffer solution. For both cases of DOX/PLA-TPGS and Fol/DOX/PLA-TPGS NPs, the DOX release from nanoparticles displayed a biphasic release profile. The initial burst associated with the fast release of drug molecules took place in the first 8 h. In the second phase, DOX was progressively released and reached about 60% release. The difference in DOX release rate from DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs was not noticeable.

3.5. In vitro cytotoxicity

Figures 5(a) and (b) show the HT29 and HeLa dose response curve of free DOX, DOX/PLA-TPGS and Fol/DOX/PLA-TPGS NPs, which were determined using MTS assay. For both cancer cell lines we can see that as the DOX concentration increased, the cell survival decreased which indicates that the effect of free drug and loaded drug are dependent on the concentration. From these results, the half maximal inhibitory concentration (IC$_{50}$) was calculated. For HT29 cells, IC$_{50}$ values of free DOX, DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs were 0.64 ± 0.03, 1.39 ± 0.05 and 0.39 ± 0.04 μM, respectively, while for HeLa cells, IC$_{50}$ values of those were 0.46 ± 0.03, 1.22 ± 0.07 and 0.24 ± 0.02 μM, respectively. From these values, we suggest that the DOX loaded nanoparticles with folate decoration significantly decreased the IC$_{50}$ values for both HT29 and HeLa cells while the values of those without folate were much higher compared to free DOX. Similar results were also obtained by quantitatively comparing the cell density from the cytotoxicity images shown in figure 6.
3.6. Cellular uptake

Figure 7 shows the cellular uptake of Free DOX, DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS NPs. Cells after incubation with nanoparticles were stained with nuclei staining Hoechst 33342. The blue fluorescent light of Hoechst (excited at 346 nm) shows the nuclei position of cell in the samples while the red fluorescent light of DOX (excited at 480 nm) expresses the position of drug inside the cells. From these images, we can see that DOX was taken into the nuclei of the cells in all cases. Quantitatively, Fol/DOX/PLA-TPGS NPs exhibited the best cellular uptake via the highest fluorescent intensity. In contrast, it is hard to observe the red fluorescent light in cells incubated with DOX/PLA-TPGS NPs. This shows that the cellular uptake of DOX/PLA-TPGS NPs is lowest.

4. Discussion

In this study we fabricated DOX loaded nanoparticles based on amphiphilic copolymer PLA-TPGS as nanocarrier and folic acid as targeting ligand with the aim of inducing controlled release and targeted delivery of DOX. DOX.HCl after removing HCl becomes a lipophilic molecule which is entrapped in lipophilic core of polymeric micelles composed by amphiphilic copolymer PLA-TPGS. The interaction between drug and micelle was investigated by FT-IR spectra. From these results we can see that there were no new chemical bonds appearing in DOX loaded nanoparticles for both cases, DOX/PLA-TPGS NPs and Fol/DOX/PLA-TPGS, compared to free DOX, PLA-TPGS and Fol. The drug–micelle interactions only made some slight shifts at characteristic peaks of drug and polymer, meaning that these interactions were physical interactions, such as Van Der Waals or hydrophobic interactions. The physical interaction between drug and micelle will not make a change in chemical structure of drug molecules and therefore the remaining potential of the drug.

DDNSs have brought huge advantages in improving therapeutic efficacy and minimizing serious side-effects of anti-cancer drugs. For the case of DOX, although they are approved for use against a wide range of human cancers, their long-term clinical use is compromised by irreversible cardiomyopathy and subsequent congestive heart failure. Improving the therapeutic efficacy and reducing side-effects of DOX by encapsulating it into a nanocarrier is a proven potential strategy [19, 20]. DDNS helps to enhance the elimination half-life ($T_{1/2}$), mean residence time, targeted delivery of DOX to tumor and reduced delivery to heart [21]. The advantages are based on their small size which improves accumulation of drugs at the tumor sites through the well-known EPR effect. In this study, amphiphilic copolymer PLA-TPGS made DOX loaded nanoparticles with very small hydrodynamic diameter of around 100 nm which is the ideal size range for the drug delivery system.

Controlled release of drug from delivery system is an important aspect determining their therapeutic efficacy. Drug controlled release helps to protect the drug from enzymes leading to the drug elimination out of the body. In this study, about 60% of DOX was released from nanoparticles after 48 h for both DOX/PLA-TPGS and Fol/DOX/PLA-TPGS NPs, suggesting that at least about 40% of DOX may be protected from enzyme degradation or hydrolytic degradation (if these things happen). Otherwise, drug release rate also determines
the biological effects of drug after it is located in the tumor. If the DDNSs release their drug content at a rate that is rapid compared to the rate of tissue accumulation, then their therapeutic activity may be compromised. Charrois reported that pegylated liposomal doxorubicin formulations with different drug release rates induced different pharmacokinetics, biodistribution and therapeutic activity murine breast cancer [22]. However, it is hard to determine an appropriate drug release rate which is only based on *in vitro* drug release data. It must be based on clinical data for each kind of drug delivery system and each kind of disease.

Active targeting is a potential strategy to achieve better selective targeting for cancer treatment. Targeting ligands, which are attached to DDNSs, will induce specific bindings to the receptors which are unique and overexpressed on the cell surface of human tumors. In this study, we used folic acid as targeting ligand which has high binding affinity to the folate receptor overexpressed on cell surface of many human tumors [23]. Attaching folic acid on the drug delivery system induced higher cytotoxicity and better cellular uptake on HT29 and HeLa cells compared to free DOX and DOX loaded nanoparticles without folate (DOX/PLA-TPGS NPs). Meanwhile, the cytotoxicity and cellular uptake of DOX/PLA-TPGS NPs was smaller than that of free DOX. The results could be explained based on the cell internalization mechanism. It is reported that cellular uptake of nanoparticles happens via endocytosis process [24] while free DOX in the form of water soluble molecule was internalized in the cell via passive

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**Figure 5.** Dose response curve of free DOX and DOX-loaded nanoparticles against human cancer HT29 cells (a) and HeLa cells (b).

**Figure 6.** *In vitro* cytotoxicity of free DOX and DOX loaded nanoparticles on HeLa (a) and HT29 (b) cell lines at the DOX concentration of 30 μM.
diffusion [25]. Because of existing in the form of single molecule, the entry of DOX.HCl molecules into the cell via passive diffusion may be easier and faster than the endocytosis of DOX/PLA-TPGS NPs. For Fol/DOX/PLA-TPGS NPs, folic acid induced the specific binding to the folate receptor overexpressed on HeLa and HT29 cell surface. This binding facilitated folate receptor-mediated endocytosis resulting in better cellular uptake of Fol/DOX/PLA-TPGS NPs.

5. Conclusion

In this study we successfully fabricated DOX loaded nanoparticles based on amphiphilic copolymer PLA-TPGS with size around 100 nm. Folic acid was covalently attached to TPGS molecules to produce Fol/DOX/PLA-TPGS NPs. The in vitro biological effects of the DOX loaded nanoparticles were evaluated on HeLa and HT29 cell line. The results indicated that Fol/DOX/PLA-TPGS NPs induced noticeably better cytotoxicity and cellular uptake on these cancer cells compared to those of DOX/PLA-TPGS NPs and free DOX. This result suggested Fol/DOX/PLA-TPGS NPs as a promising system for efficient delivery of DOX.

Acknowledgments

The authors are grateful to Academician Nguyen Van Hieu for his encouragement and interest in this research. The authors would like to acknowledge all members of IMS-VAST Key Laboratory for providing the lab facilities.

This work was financially supported by the Vietnam Academy of Science and Technology under Grant No. VAST03.03/13-14 (HPT) and the National Foundation for Science and Technology development of Vietnam-NAFOSTED under Grant No. 106.99-2012.43 (HPT).

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