RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

RSC Publishing

ARTICLE

Enhanced Therapeutic Efficacy and Cytotoxicity of Doxorubicin-loaded Vitamin E-Pluronic Micelles against Liver Cancer

Y.J. Li,* M. Dong, F.M. Kong, J.P. Zhou

Cite this: DOI: 10.1039/x0xx00000x

Received 00th November 2014,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

In this study, a new polymeric micelle delivery system was developed to increase the therapeutic efficacy of doxorubicin (DOX) and to reduce the associated side effects. For this purpose, DOX-loaded pluronic-alpha-tocopheryl succinate polymeric micelles (P/TOS-DOX) were successfully prepared towards the therapeutic treatment of hepatocellular carcinoma. We have showed that drug-loaded micelles exhibits typical pH-dependent and sustained drug release profile. These micelles were nanosized around ~100 nm with spherical morphology. The micelles were predominantly distributed in the cytoplasmic region that will facilitate the cancer killing potency of chemotherapeutic drug. The IC50 value of DOX and P/TOS-DOX was remained at 1.18 µg/ml and 0.72 µg/ml, respectively, indicating the significant inhibition of cancer cell proliferation by the micellar carrier. Subsequently, micellar incorporation of drug effectively shielded from elimination and subsequently prolonged the blood circulation and half-life of anticancer drug. P/TOS-DOX accumulated preferentially in the tumor immediately after systemic administration and significant proportion of drug was observed by the end of 24h. Especially, P/TOS-DOX exhibited superior tumor growth inhibition comparing to that of free DOX treated group. More importantly, the side effects of DOX were effectively decreased by the administration of micellar carriers. Overall, our results suggest that P/TOS-DOX could be an attractive carrier (therapeutic approach) for the hepatocellular carcinoma therapy.

.

Introduction

Liver cancer or hepatocellular carcinoma (HCC) is one of the most popular and lethal cancers in the world and third most common cause of cancer related death.[1] With every year, number of death due to HCC is kept increasing especially in developing country like China. In United States alone, nearly 20000 patients died with 25000 new cases identified in the year 2012.[2,3] At present, chemotherapy-based single or dual drug regimen is the main treatment option. However, conventional chemotherapy often suffers from non-specific targeting; non-specific mode of action, and high adverse effects, often hinders its clinical success rate.[4] Besides, one of the major problems associated with cancer chemotherapy is to maintain the therapeutic concentration of drug in the tumor site for the desired period of time.[5,6] To overcome the limitations associated with chemotherapeutic drugs, various drug delivery systems have been developed to improve the delivery and to reduce the side effects.[7] The nanoparticulate delivery system could potentially avoid reticuloendothelial system (RES) and help prolong the half of drug in the body and blood circulations. Moreover, nanoparticle of size less than 200 nm could passively target cancer tissues via enhanced permeability and retention (EPR) effect.[8-10] Although many polymeric nanoparticles have been designed, a quest to prepare novel nanocarriers always remains.

In this regard, amphiphilic block copolymers-based polymeric micelles have attracted significant attention of researchers worldwide.[11] The prime advantage with polymeric micelles are small particle size, high drug loading, long blood circulation, good biodistribution, and lower side effects.[12] Especially, pluronic block copolymers which consists of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) has favorable properties.[13] Pluronic polymers are reported to recuperate multidrug resistance (MDR) and increase the potency of anticancer drugs. Besides, hydrophilic PEO could effectively prolong the blood circulation time by virtue of its antifouling characteristics.[14] However, low drug loading capacity and instability of pluronic micelles in the systemic environment or circulations (lack of structural integrity) limits its further use.[15] Alpha-tocopheryl succinate (α-TOS) which is a well-known Vitamin E analogue could be used as hydrophobic segment.[16] Earlier, α-TOS has been reported to possess typical anticancer property against multiple cancer cells. We expect that when lipophilic portion of TOS is conjugated with pluronic, it would allow better drug solubilization and improve the systemic stability. The high drug encapsulation and improved systemic stability might improve the cancer cell mortality.[17,18]

Doxorubicin (DOX), an anthracycline anticancer drug is indicated in the treatment of multiple cancers including breast, ovarian, prostate, advanced or recurrent liver cancers.[19] Despite its potent therapeutic

effect, clinical application of DOX is hindered by its high toxicity towards normal tissue and severe side effects such as cardiotoxicity. In the present study therefore, an alternative attempt has been made to improve the chemotherapeutic efficacy of DOX and at the same time to reduce its side effects.[20,21]

In this study, amine group of pluronic123 was chemically conjugated with the carboxylic group of TOS in the terminal portion. We developed the pluronic-TOS based polymeric micelles to increase the intracellular concentration of DOX in the liver cancer cells. The main goal of this study is to improve the cancer chemotherapy towards liver cancers. The DOX-loaded polymeric micelles were prepared by the self-assembly of P/TOS and anticancer drugs. Physicochemical characterizations were performed to ascertain its size and shape parameters. The cytotoxic effect of P/TOS-DOX was studied against HepG2 liver cancer cells. The cellular uptake and intracellular localization of P/TOS-DOX was studied by means of flow cytometer and confocal microscopy. Biodistribution of drug loaded polymeric micelles was studied in tumor model to ascertain the concentration of drugs in the vital organs. Finally, in vivo anticancer efficacy studied was performed in HepG2 cancer cells bearing tumor xenograft nude mice.

Results and Discussion

Preparation of self-assembled polymeric micelles

The encapsulation of anticancer drugs in polymeric micelles could not only improve the solubility of drugs but also decreases the drug-related side effects. In the present study, PEO-PPO-PEO block copolymer (pluronic) was conjugated with tocopheryl succinate (TOS) to improve the drug solubilization and to improve the systemic stability (Figure 1). The micelles will stably protect the drug in the systemic circulation and controls the release of drugs.

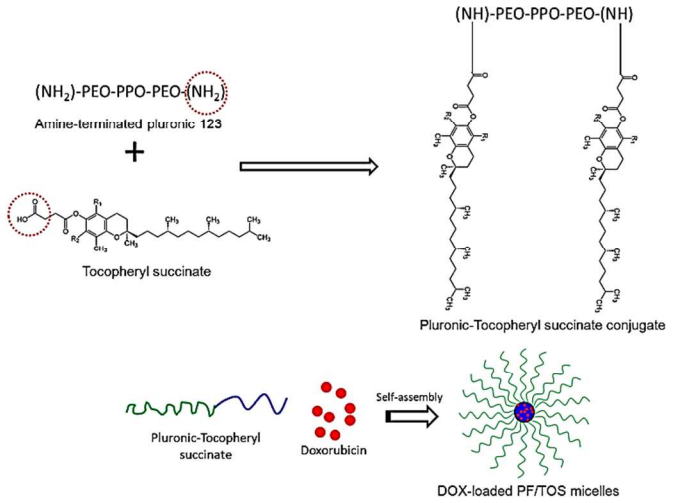


Figure 1: Schematic illustration of preparation of pluronic-tocopheryl succinate block copolymer and formation of doxorubicin loaded polymeric micelles.

The P/TOS-DOX micelles were formed by self-assembly process. The block copolymer and DOX base spontaneously self-aggregate to form drug-loaded polymeric micelles. Various inter- and intra-molecular physical forces were involved during the formation of micelles. The core of the polymer micellar delivery system serves as a reservoir that accommodates drug molecules through a combination of hydrophobic, electrostatic interactions, hydrogen bonding or via chemical conjugation of the drug to the core-forming block of the copolymer. The P-TOS conjugate composed of hydrophilic PEO and hydrophobic tocopheryl succinate self-aggregate in the aqueous media and form a typical core-shell nanoparticles. The critical micellar concentration (CMC) of so-formed P/TOS-DOX micelles was 25 µg/ml. The average size of P/TOS-DOX was found to be around ~100 nm with an excellent dispersity index of 0.15 (PDI) (Figure 2a). It has been reported that particle size is an important requirement for the biodistribution and long blood circulation profile. Additionally, smaller particle size of <200 nm could evade phagocytosis system and could passively target cancer tissues via enhanced permeation retention (EPR) effect.[22] The particle size was further confirmed in dried state by means of TEM imaging. The particles were spherical in nature with perfect boundary surrounding the each particle (~80 nm). The particles were uniformly dispersed in the copper grid (Figure 2b). Moreover the size of the nanoparticles measured from TEM was consistent with the DLS observation although state of the particles was different in these two methods.

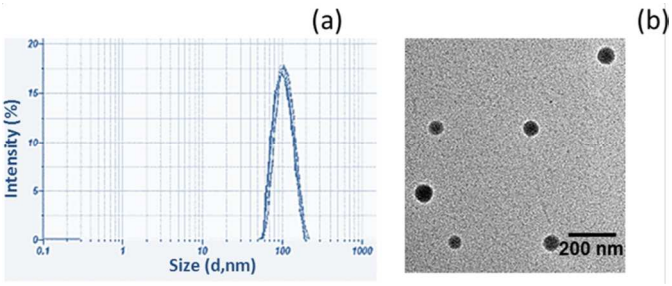


Figure 2: (a) Size distribution of P/TOS-DOX micelles determined by dynamic light scattering technique (b) transmission electron microscope of P/TOS-DOX micelles.

The drug loading capacity (LC) of NP was evaluated in order to demonstrate its suitability for the systemic administration. The LC of P/TOS-DOX was observed to be 21.2±2.65% indicating its ability to retain high amount of drug in the core of the micelles. In contrast, Pluronic-DOX showed a very low LC of 8.5±3.1% due to the lack of sufficient hydrophobicity.

In vitro drug release

The release of DOX from P/TOS-DOX micelles was studied in 3 different pH conditions (pH 7.4, pH 6.8, and pH 5.0). As demonstrated in Figure 4, micelles showed a typical pH-responsive drug release pattern with high release rate in lower acidic pH conditions. It can be seen that nearly ~15%, ~28%, ~50% of DOX released in pH 7.4, pH 6.8, and pH 5.0 conditions, respectively. By 48h, almost 100% of drug released in pH 5.0 conditions, whereas >60% of drug is still entrapped in the micelles in pH 7.4 conditions (Figure 3). As clearly seen in Figure, higher release rate of DOX was achieved at lower pH, with the present system. Because of the basic nature of doxorubicin (pKa = 8.3), it has higher solubility at lower pH. Therefore, the entrapped DOX in the micelles has a greater tendency to go into the release medium of lower pH. The favored release in acidic medium would result in higher release rate of doxorubicin in tumor cells, adding therapeutic efficiency to the delivery system. It is worth noting that no initial burst release of drug was observed in any pH conditions indicating that drug was well incorporated in the hydrophobic core of the micelles.[23] The particle size of P/TOS-DOX micelles after 1h of release study was observed to be ~110 nm, while, size decreased to ~70 nm after 24h

of the release study. The decrease in particle size of micelles could be due to the release of drug from the bulkier core of the system. Overall, a sustained release of DOX was observed from the micelles which may be due to the better solubilization of drug in the hydrophobic core. Such sustained and controlled release of anticancer drug might be advantageous to the cancer targeting.

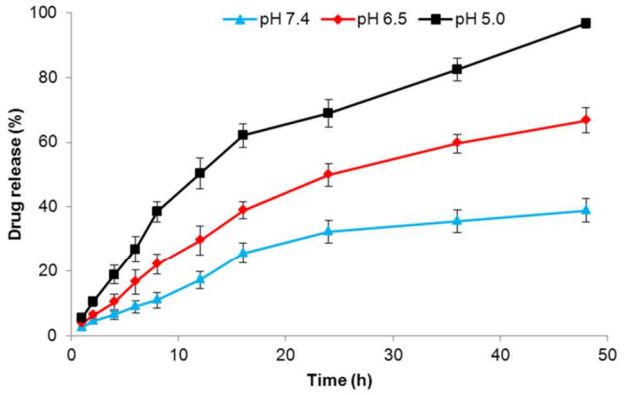


Figure 3: In vitro release profile of DOX from P/TOS-DOX micelles. The release study was performed in pH 7.4, pH 6.8, and pH 5.0 conditions. Results are expressed as means + standard deviation from three independent experiments.

Cellular uptake

Confocal laser scanning microscope was used to study the cellular uptake mechanism of P/TOS-DOX in HepG2 cancer cells. To visualize the cellular uptake and associated mechanism, lysosome was stained with Lysotracker green and nuclei were stained with DAPI. The confocal microscopy images showed that red fluorescence (originated from P/TOS-DOX) was mainly located in the cytoplasmic region rather than the entire cell (Figure 4a). The high intensity of DOX fluorescence in the perinuclear region suggests that much of drug was released from the micelles and available in the free form. It could be anticipated that acidic conditions of lysosome might provoked the release of DOX from the micellar delivery systems. These results corroborate the fact that micelles after endocytosis uptake, destabilize in the cytoplasmic region, resulting in the release of drug which travels to the nuclear region. It has been reported by many authors that micelles release the therapeutic load in the cytosol and perinuclear region of cells.[24,25]

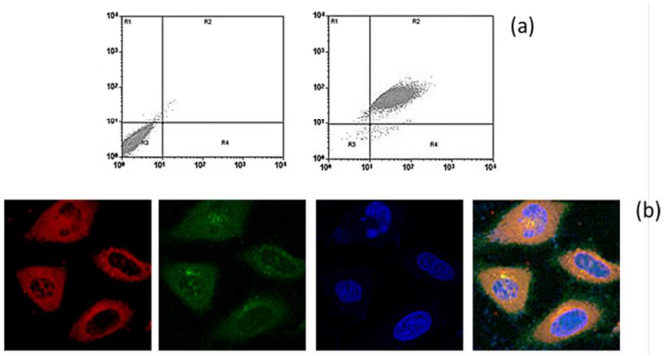


Figure 4: (a) Qualitative cellular uptake analysis of P/TOS-DOX micelles in HepG2 cancer cells. The fluorescence visualization was carried out using confocal laser scanning microscopy. The concentration of DOX used as 10 µg/ml (b) Flow cytometer analysis of P/TOS-DOX micelles after 3h incubation.

Flow cytometer analysis was carried out to quantitate the micelles internalization. The flow cytometer sorting showed that cells were predominantly present in the double-positive chamber (quadrant) whereas control cells were largely present in the lower quadrant (Figure 4b). The results therefore clearly suggest the fact that micelles were present in the cytoplasmic region, consistent with the confocal microscopy images. The distribution of micelles in the cytoplasmic region will improve the cancer killing potency of chemotherapeutic drug.

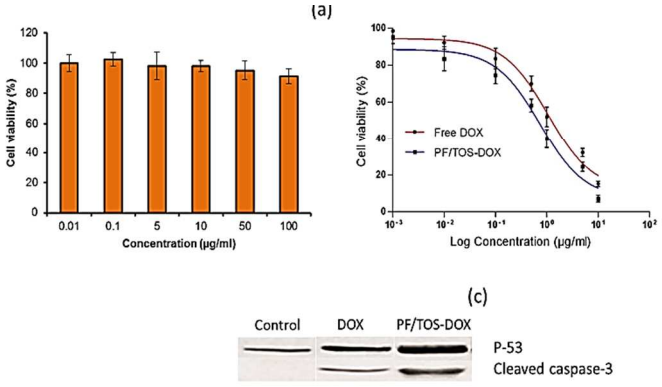


Figure 5: (a) Biocompatibility profile of P/TOS blank polymeric micelles studied against HepG2 cancer cells (b) cell viability of HepG2 cancer cells upon incubation with free DOX and P/TOS-DOX micelles after 24h incubation. The cell cytotoxicity was evaluated by means of MTT assay.

Cytotoxic effect of blank and drug-loaded micelles

The biocompatibility and cytotoxic effect of polymeric micelles was evaluated by means of MTT assay. The biocompatibility of nanomaterials employed for the delivery carrier is utmost important in order to ensure the high success rate of cancer delivery system. The blank micelles were exposed to HepG2 cancer cells at different concentration to maximum of 100 µg/ml. It has been observed that micelles maintained excellent biocompatibility profile throughout all the concentrations tested. Especially, more than >90% cell viability was observed when exposed with 100 µg/ml at 24h (Figure 5a). The results therefore suggest that block polymers as well as micelles were safe and could be potentially used for the systemic targeting.

The anticancer effect of drug loaded micelles was studied in order to evaluate its suitability as efficient carrier in liver cancer treatment. The cytotoxic effect of DOX and P/TOS-DOX was studied in the HepG2 cancer cells. As shown in Figure 5b, DOX and P/TOS-DOX showed a typical concentration-dependent cytotoxicity whereas drug loaded micelles showed superior cytotoxic effect than comparing to that of free DOX. The IC50 value was calculated to estimate its cytotoxic effect in quantitative manner. The IC50 value of DOX and P/TOS-DOX was remained at 1.18 µg/ml and 0.72 µg/ml, respectively, indicating the significant inhibition of cancer cell proliferation by the micellar carrier. The difference in the cytotoxic effect of free drug and drug loaded carrier might be due to the nature of cellular uptake and intracellular mechanism. It has been reported that free DOX due to its small molecular weight and hydrophilic nature could directly diffuse into the cancer cell while at the same time it could be pumped out. In case of P/TOS-DOX, endocytosis-mediated cellular uptake might allow the slow and continuous

release of drug in the intracellular environment, resulting in higher cell cytotoxicity and cell death.[26] DOX localized in the cell nuclei is likely intercalated into DNA strands; therefore, it shows its toxicity against tumor cells.

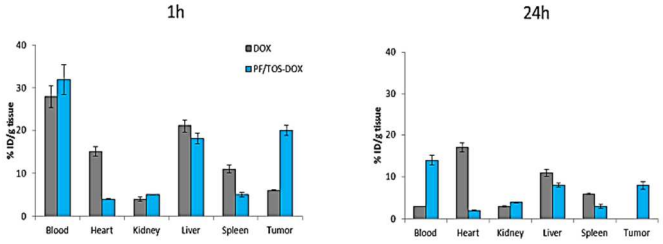


Figure 6: Biodistribution profile of DOX from P/TOS-DOX micelles after intravenous administration to tumor bearing nude mice, (a) 1h (b) 24h. The formulations were administered via tail vein injection.

The over expression of P53 and caspase-3 are regarded as a hallmark of cell apoptosis or cancer cell death. These apoptosis markers are highly expressed in tumor suppressive conditions. The result was consistent with the cytotoxic assay as the nanoformulations showed high expression of both P53 and caspase-3.

In vivo biodistribution studies

The biodistribution ability of free DOX and P/TOS-DOX was studied in HepG2 cancer cell bearing tumor model. It can be seen that both free DOX and P/TOS-DOX attained high concentration in blood after 1h of intravenous administration, while less than <2% of free drug was detected at the end of the study period. On the other hand, drug-loaded micelles maintained significantly higher concentration even at the end of 24h (Figure 6). The data clearly reflect that micellar incorporation of drug effectively shielded from elimination and subsequently prolonged the blood circulation and half-life of anticancer drug. Such prolonged blood circulation profile will effectively facilitate the passive targeting of drug in the tumor tissues via EPR effect. Subsequently, P/TOS-DOX accumulated preferentially in the tumor immediately after systemic administration and significant proportion of drug was observed by the end of 24h. Whereas, only ~5% could be found in tumor from free drug administered group after 1h and no drug was found after 24h indicating the rapid elimination of drug from the systemic circulation. One more potential observation from the biodistribution study is that nearly ~15% of DOX was observed in heart following the administration of free drug and significant level of drug was still present by the end of 24h. On the contrary, less than <5% of drug accumulated in the heart tissue from P/TOS-DOX indicating its safety profile. The biodistribution profile clearly suggests that micellar carrier extended the blood circulation profile while at the same time reduced the organ related toxicity.

Antitumor efficacy studies

The in vivo antitumor study was performed in HepG2 hepatic cancer cell bearing tumor mouse model. The antitumor efficacy was carried out to evaluate the effect of micellar carrier on the tumor suppression rate. As shown in Figure 7a, tumor volume rapidly increased in the control group reaching over 2500 mm^3 at the end of 18^{th} day. The free DOX and P/TOS-DOX treated group effectively suppressed the tumor growth. Especially, P/TOS-DOX exhibited superior tumor growth inhibition comparing to that of free DOX treated group. On day 18, free DOX treated group showed a tumor volume of ~1200mm^3 whereas P/TOS-DOX treated group showed a tumor volume of only ~600mm^3. A combination of multiple factors could explain the superior antitumor effect of P/TOS-DOX in tumor mice. Especially, prolonged blood circulation profile of P/TOS-DOX effectively facilitated the passive tumor targeting via EPR effect. The passive targeting of micelles might increase the intracellular concentration of anticancer drugs. Moreover, a pH-responsive release pattern of P/TOS-DOX might trigger the liberation of drug at the target site in the nucleus.[27,28]

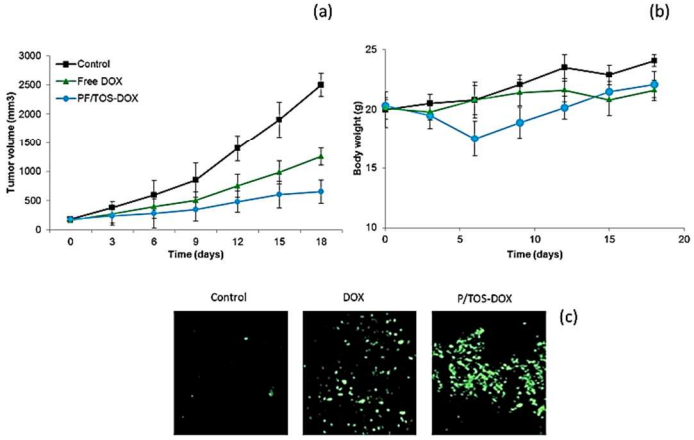


Figure 7: In vivo antitumor efficacy evaluation in HepG2 tumor bearing mouse model treated with free DOX and P/TOS-DOX micelles. Untreated mice group was observed as control. (a) Tumor volume variations (b) variation of body weight (c) TUNEL assay of extracted tumor section.

Body weight was monitored to observe the safety profile of administered formulations. As seen from Figure 7b, free DOX treated group lost more than 10% of body weight indicating its severe systemic toxicity. The P/TOS-DOX treated group however did not show any sign of weight loss indicating its excellent safety profile.

The anticancer efficacy of formulations was further studied by means of TUNEL assay (Figure 7c). P/TOS-DOX treated group showed significantly higher TUNEL-positive tumor cells indicating the superior apoptosis effect of micellar carriers. The data clearly reflect that drug loaded micellar formulations remarkably increased the apoptosis cells and decrease the number of proliferating tumor cells.

CONCLUSION

In conclusion, doxorubicin-loaded pluronic-alpha-tocopheryl succinate polymeric micelles (P/TOS-DOX) were successfully prepared towards the therapeutic treatment of hepatocellular carcinoma. We have showed that drug-loaded micelles exhibits typical pH-dependent and sustained drug release profile. These micelles were nanosize around ~100 nm with spherical morphology. The micelles were predominantly distributed in the cytoplasmic region that will facilitate the cancer killing potency of chemotherapeutic drug. The IC50 value of DOX and P/TOS-DOX was remained at 1.18 µg/ml and 0.72 µg/ml, respectively, indicating the significant inhibition of cancer cell proliferation by the micellar carrier. Subsequently, micellar incorporation of drug effectively shielded from elimination and subsequently prolonged the blood circulation and half-life of anticancer drug. P/TOS-DOX accumulated preferentially in the tumor immediately after systemic administration and significant proportion of drug was observed by the end of 24h. Especially, P/TOS-DOX exhibited superior tumor

growth inhibition comparing to that of free DOX treated group. More importantly, the side effects of DOX were effectively decreased by the administration of micellar carriers. Overall, our results suggest that P/TOS-DOX could be an attractive carrier (therapeutic approach) for the hepatocellular carcinoma therapy.

MATERIALS AND METHODS

Materials

Pluronic P123, tocopheryl succinate, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) were purchased from Sigma-Aldrich, China. Doxorubicin hydrochloride was procured from Zhejiang Hisun pharmaceutical Co., Ltd. (Taizhou, China). Unless and otherwise stated, all other chemicals were of reagent grade and used without further purifications.

Preparation of doxorubicin-loaded pluronic123-tocopheryl succinate micelles

Pluronic 123 was conjugated with tocopheryl succinate as reported earlier. Briefly amino group of pluronic 123 (P123) was chemical conjugated with the carboxyl group of tocopheryl succinate (TOC). For this, 500 mg of P123 (amine terminated), 76 mg of TOC, and 108 mg of EDC was dissolved in 5 ml of DMSO. The entire mixture was stirred in an inert atmosphere for 40h. The resulting chemical conjugate was dialyzed (MW 3000 cut off) using a dialysis bag for 1 week with frequent replacement of distilled water. By this process, unreacted or unconjugated TOC or P123 was removed and the final product was carefully freeze-dried.

Prior to the preparation of drug-loaded micelles, doxorubicin.HCL (DOX) was converted into the base form by the addition of excess of trimethylamine. The DOX base was used for the further entrapment process. 5 mg of DOX and 50 mg of P123-TOS conjugate (P/TOS) was dissolved in acetonitrile and stirred for 1h. The organic mixture was then packed in dialysis bag and dialyzed against distilled water for 12-24h. The organic solvent and un-entrapped DOX was removed by dialysis bag and final DOX loaded P/TOS (P/TOS-DOX) was collected and freeze dried and used for further experiments.

Characterization of P/TOC-DOX micelles

The surface charge and hydrodynamic diameter and distribution index of P/TOC-DOX was evaluated by dynamic light scattering technique. Malvern Zetasizer Nano ZS, UK was used to determine the size and charge of the micelles. The samples were suitably diluted with distilled water and performed at a fixed angle of 173° at 25°C.

The morphology of micelles was determined by means of transmission electron microscope (TEM; JEOL, Japan). A dilute solution was prepared and dropped onto a carbon coated copper grid. The samples were then stained with phosphotungistic acid (PTA) and allowed to stay for 15 min. The samples were then dried and observed under TEM.

Evaluation of drug content

The entrapment efficiency (EE) and drug loading capacity (DLC) was estimated by spectroscopy method. Briefly, DOX-loaded micelles (freeze dried) was dissolved in water and methanol was added in the ratio of 1/50 fold. The mixture was centrifuged and supernatant was evaluated for the amount of DOX incorporated. The amount of DOX was quantified by UV–vis spectroscopy at 482 nm. EE and DLC was calculated using the following formulas:

$$EE\ (\%) = (\text{amount of DOX entrapped/total DOX added initially}) \times 100$$

$$DLC\ (\%) = (\text{amount of DOX entrapped/total weight of nanoparticles}) \times 100$$

In vitro drug release

The drug release study was carried out using dialysis method. For this purpose, freeze dried drug loaded polymeric micelles were dissolved in measured quantity of distilled water such that the final concentration remains at 1 mg/ml of dispersion. 1ml of nanoparticle dispersion was put into dialysis membrane (MW 3500Da) and both the ends were tightly sealed. The dispersion loaded dialysis membrane was immersed in a Falcon tube containing 25 ml of release media. Phosphate buffered saline (PBS, pH 7.4) and acetate buffered saline (ABS, pH 5.0) was used a respective release mediums. At specific time points, 1 ml of release media was withdrawn and replaced with equal volume of fresh release medium. The amount of drug released in the release media was quantified using UV-Vis spectrophotometer at 482 nm.

Cell culture

Human hepatocarcinoma cell line HepG2 was purchased from Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China). HepG2 cells were cultured in DMEM growth medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture. The cells were maintained at ambient conditions of 5% CO_2/95% air atmosphere at 37°C.

Cytotoxicity assay

The anticancer effect of free DOX and P/TOS-DOX was tested in HepG2 cancer cells by MTT assay. The cells were grown in fully supplemented DMEM medium and plated in a 96-well plate at a seeding density of 1×10^4 cells per well. Cells were allowed to attach for 20h, after which it is treated with respective formulations at different concentrations and incubated for further 24h. The media was removed and washed twice with PBS. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) solution in PBS (20µL, 5.0 mg/mL) was added and incubated for further 4h. The supernatant was carefully removed and formazan was extracted by means of DMSO (100 µL). The absorbance was measured at 490 nm using a microplate reader. The cell viability was compared in relation with control cells which were untreated. The cytotoxicity of bare micelles of polymeric materials was investigated in the same manner with the exception of drug.

Flow cytometric analysis

The cells were seeded in a 6-well plate and allowed to attach for 20h. The cells were treated with P/TOS-DOX and incubated for 4h. The cells were extracted by means of 0.25% Trypsin. The cell suspension was centrifuged, washed twice and reconstituted in PBS. The cellular uptake was observed in BD FACSCalibur flow cytometer (Beckton Dickinson, U.S.A.) and analyzed using Cell Quest software.

Cellular uptake by confocal microscopy

The cellular uptake of P/TOC-DOX micelles was further studied by confocal microscopy. Briefly, HepG2 cells at a seeding density of 1×10^5 cells per well was added to the 6-well plate and incubated for 24h. The cells were then exposed with P/TOC-DOX micelles and incubated for 3h at 37°C. The medium was removed and washed twice with PBS carefully. The lysosome was stained with Lsyotracker Green and nuclei were stained with DAPI, respectively. Cells were again washed and fixed with 4% (w/v) paraformaldehyde. The samples (cover slips) were then observed under confocal microscope. Confocal laser scanning microscopy was performed on a Leica TCS SP5 II equipped with a63×oil immersion objective lens.

In vivo antitumor study

The in vivo antitumor study was carries out in human liver hepatocellular carcinoma (HepG2) cells bearing tumor nude mice. The xenograft model was created by injecting HepG2 cell suspension (1×10^6) into the right flank of the 5-week old nude mice. The experiments were started when the tumor volume reached approximately ~100 mm^3. Following which, mice were divided into 3 groups with eight mice in each group. Each group received DOX and P/TOS-DOX at an equivalent dose of 5 mg/kg and the third group was maintained as control. The tail vein injections were carried out 3 times with an interval of 3 days. The tumor volume and body weight of mice were monitored during the entire study period. Especially, tumor volume was measured using Vernier caliper and the tumor size was calculated using the equations: $V=a \times b^2/2$, whereas, 'a' and 'b' represents the longest and shortest diameter.

In vivo TUNEL assay

After the antitumor study, tumors were surgically removed and fixed with 10% formalin and kept at 4°C. TUNEL assay was performed as per the manufacturer's protocol of cell death detection kit (Roche (Mannheim, Germany). The sections were placed in the glass slide and deparaffinized and proteinase K was added to the slides. The slide was incubated for 40 min and rinsed twice with PBS buffer and then incubated with TUNEL reaction mixture for further 45 min. The slides were washed and incubated with reaction mixture (50μL TUNEL reaction mixtures+2μl enzyme solution+48μl label solution). The slides were washed and viewed under confocal laser scanning microscopy (Leica TCS SP5 II equipped with a63×oil immersion objective lens). Extent of green fluorescence was interpreted in terms of apoptotic cells.

Biodistribution study

For the biodistribution study, tumor model as developed as mentioned in the above section. The xenograft model was created by injecting HepG2 cell suspension (1×10^6) into the right flank of the 5-week old nude mice. The experiments were started when the tumor volume reached approximately ~100 mm^3. Each mice group was administered with DOX and P/TOS-DOX via tail vein injections. Specific amount of animals were selected at 1h and 24h of experimental time point. Animals were given a high dose of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) IP to induce anesthesia. First blood was collected, heparinized, and stored. Individual organs including tumor, liver, spleen, kidney, and heart were collected, washed quickly with PBS. The organs were collected in pre-weighed EP tubes and assayed for DOX content. 1ml of PBS was added to pre-weighed organs and sonicated by 30 cycles of Bioruptor ultrasonic treatment, active every 15 s for a 15s duration at 200 W, in an ice bath. The homogenate was processed by the above mentioned extraction process for sample preparation and amount of drug in each organ was estimated by means of HPLC.

Statistical analysis

Data were presented as mean+SD. Differences between more than two groups were assessed by one-way ANOVA using SPSS 17.0.

ACKNOWLEDGEMENT

The work was supported from the research grant of China Medical University, China.

NOTES and REFERENCES

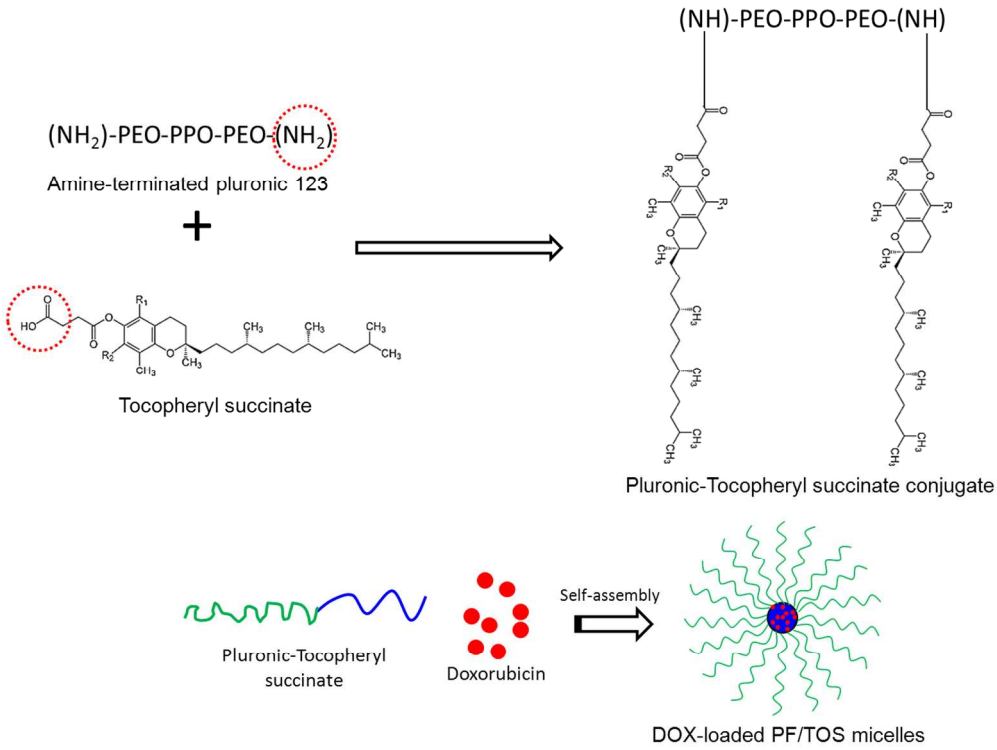
Yu-Ji Li*, Ming Dong, Fan-Min Kong, Jian-Ping Zhou

Department of General Surgery，the First Affiliated Hospital，China Medical University．Shenyang, Liaoning 110001, China.

Yu-Ji Li: liyuji182@gmail.com

1. R. Siegel, C. DeSantis, K. Virgo, et al, *CA Cancer J Clin*. 2012, **62**, 220.
2. M. Marra, I. M. Sordelli, A. Lombardi, et al, *J Transl Med*. 2011, **9**, 171.
3. M. Caraglia, G. Giuberti, M. Marra, et al, *Cell Death Dis*. 2011, **2**, 150
4. G.J. Kim, S. Nie, *Materials Today* 2005, **8**, 28.
5. R.K. Jain, *J Control Rel* 1998, **53**, 49.
6. J.L.S. Au, S.H. Jang, J. Zheng, C.T. Chen, S. Song, L. Hu, M.G. Wientjes, *J. Control. Rel* 2001, **74**, 31.
7. M.L. Etheridge, S.A. Campbell, A.G. Erdman, C.L. Haynes, S.M. Wolf, J. McCullough, *Nanomedicine*. 2013, **9**, 1.
8. V.P. Torchilin, *Nat Rev Drug Discov*. 2005, **4**, 145.
9. K. Kataoka, A. Harada, Y. Nagasaki, *Adv Drug Deliv Rev*. 2001, **47**, 113.
10. H. Maeda, *Bioconjug Chem*. 2010, **21**, 797.
11. N. Suthiwangcharoen, T. Li, L. Wu, H.B. Reno, P. Thompson, Q. Wang, *Biomacromolecules* 2014, **15,** 948.
12. T. Li, L. Wu, N. Suthiwangcharoen, M.A. Bruckman, D. Cash, J.S. Hudson, S. Ghoshroy, Q. Wang, *Chemical Communications* 2009, **28**, 2869.
13. Z. Wei, S. Yuan, Y.Z. Chen, S.Y. Yu, J.G. Hao, J.Q. Luo, X.Y. Sha, X.L. Fang, *Eur J Pharm Biopharm*. 2010, **75**, 341.
14. A.V. Kabanov, E.V. Batrakova, V.Y. Alakhov, *Adv Drug Deliv Rev*. 2002, **54** , 759.
15. W. Zhang, Y. Shi, Y. Chen, J. Ye, X. Sha, X. Fang, *Biomaterials* 2011, **32**, 2894.
16. M. Birringer, J.H. EyTina, B.A. Salvatore, J. Neuzil, *Br J Cancer* 2003, **88**, 1948.
17. J. Neuzil, *Br J Cancer* 2003, **89**, 1822.
18. Y.W. Won, S. M. Yoon, C.H. Sonn, K.M. Lee, Y.H. Kim, *ACS Nano* 2011, **5**, 3839.
19. V. Voliani, G. Signore, O. Vittorio, P. Faraci, S. Luin, J. Peréz-Prieto, F. Beltram, *J. Mater. Chem. B*, 2013, **1**, 4225.
20. D.G. Ahn, J. Lee, S.Y. Park, Y.J. Kwark, K.Y. Lee, *ACS Appl Mater Interfaces*, 2014 **6**, 22069.
21. N. Suthiwangcharoen, T. Li, K. Li, P. Thompson, S. You, Q. Wang, *Nano Research*, 2011, **5**, 483.
22. V.P. Torchilin, *Eur J Pharm Biopharm*. 2009, **71**, 431.
23. H. Yuan, L.J. Lu, Y.Z. Du, F.Q. Hu, *Mol Pharm*. 2011, **8**, 225.

24. T. Ramasamy, H. B. Ruttala, J. Y. Choi, T. H. Tran, J. H. Kim, S. K. Ku, H. G. Choi, C. S. Yong, J. O. Kim, *Chem. Comm*. 2015. DOI: 10.1039/c5cc00482a.
25. J.L. Markman, A. Rekechenetskiy, E. Holler, J.Y. Ljubimova, *Adv Drug Deliv Rev*, 2013, 65, 1866.
26. Q. Tian, X.H. Wang, W. Wang, C.N. Zhang, P. Wang, Z. Yuan, *Nanomedicine* 2012, **8**, 870.
27. D.E. Owens III, N.A. Peppas, *Int J Pharm*. 2006, **307**, 93.
28. Y. Tao, M. Ning, H. Dou, *Nanomed Nanotechnol Biol Med*. 2012



Graphical Abstract
238x179mm (300 x 300 DPI)