Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin–PEG–folate conjugate

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Abstract

For folate-receptor-targeted anti-cancer therapy, doxorubicin aggregates in a nano-scale size were produced employing doxorubicin–polyethylene glycol–folate (DOX–PEG–FOL) conjugate. Doxorubicin and folate were respectively conjugated to α- and ω-terminal end group of a PEG chain. The conjugates assisted to form doxorubicin nano-aggregates with an average size of 200 nm in diameter when combined with an excess amount of deprotonated doxorubicin in an aqueous phase. Hydrophobically deprotonated doxorubicin molecules were aggregated within the core, while the DOX–PEG–FOL conjugates stabilized the aggregates with exposing folate moieties on the surface. The doxorubicin nano-aggregates showed a greater extent of intracellular uptake against folate-receptor-positive cancer cells than folate-receptor-negative cells, indicating that the cellular uptake occurred via a folate-receptor-mediated endocytosis mechanism. They also exhibited more potent cytotoxic effect on KB cells than free doxorubicin. In a human tumor xenograft nude mouse model, folate-targeted doxorubicin nano-aggregates significantly reduced the tumor volume compared to non-targeted doxorubicin aggregates or free doxorubicin. These results suggested that folate-targeted doxorubicin nano-aggregates could be a potentially useful delivery system for folate-receptor-positive cancer cells.

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1. Introduction

Recently, nano-particulate drug delivery systems containing anti-cancer agents have received much attention due to their unique accumulation behavior at the tumor site [1–3]. Enhanced permeation and retention (EPR) effect is now considered as a major mechanism for their unique bio-distribution profile in the tumor tissue [4,5]. Various nano-particulate carriers such as polymer conjugates, polymeric micelles, nanoparticles, and liposomes are utilized to selectively deliver various anti-cancer agents at the tumor in a passive targeting manner [6,7]. However, a more effective and active targeting system was further needed to enhance intracellular uptake of drug containing nano-carriers within cancerous cells at the
Various targeting moieties or ligands against tumor-cell-specific receptors have been immobilized on the surface of nano-particulate carriers to deliver them within cells via receptor-mediated endocytosis. Among them, vitamin folic acid (folate) has been widely employed as a targeting moiety for various anti-cancer drugs [9–13]. Folate binding protein, a glycosylphosphatidylinositol (GPI) anchored cell surface receptor for folate, has been known to be overexpressed in several human tumors including ovarian and breast cancers, while it is highly restricted in normal tissues [14]. For this reason, folic acid has been covalently conjugated to anti-cancer drugs and liposomes for the purpose of selective targeting against tumors [12,13]. A folate group covalently attached to phospholipids or cholesterol was used to form doxorubicin encapsulated liposomes. These liposomes conjugated with folic acid showed selective targeting effect on human carcinoma with reducing cardiac toxicity of doxorubicin [13]. Antisense oligonucleotide targeted to epidermal growth factor receptor (EGFR) was also successfully targeted to cancer cells using folate-conjugated liposomes [15].

More recently, we developed folate-targeted biodegradable polymeric micellar system for doxorubicin [16]. Doxorubicin (DOX) and folate (FOL) were separately conjugated to a di-block copolymer of poly(1,1-lactide-co-glycolic acid)-poly(ethylene glycol) [PLGA–PEG]. DOX–PLGA–PEG and PLGA–PEG–FOL were mixed with deprotonated DOX under a basic aqueous condition to produce mixed DOX micelles entrapping DOX aggregates within the core, while exposing FOL on the surface. It was found that the above folate-targeted polymeric micelles exhibited enhanced and selective targeting behaviors against folate-positive cancer cells in vitro. In vivo animal study also showed significant tumor suppression effect for a human tumor xenograft nude mouse model.

In this study, FOL and DOX were separately conjugated at α- and ω-terminal end of a PEG chain to produce FOL–PEG–DOX. The FOL–PEG–DOX conjugate has a targeting moiety at one end and an anti-cancer drug moiety at the other end in a single flexible PEG chain structure. It was hypothesized that FOL–PEG–DOX could sterically stabilize deprotonated and hydrophobic DOX nano-aggregates in an aqueous solution by anchoring the conjugated DOX moiety to DOX aggregates while exposing the more hydrophilic FOL moiety outside. This is schematically shown in Fig. 1. Stabilized DOX nano-aggregates surrounded with PEG chains having FOL targeting moieties at their ends were used to comparatively evaluate their in vitro cellular uptake against FOL-overexpressed and deficient cell lines and to examine tumor suppression effect for a human tumor xenograft nude mouse model.

2. Materials and methods

2.1. Materials

Bi-functional poly(ethylene glycol) [NH2–PEG–COOH] (weight average MW: 3400) was purchased.
from Nektar Therapeutics (San Carlos, CA). N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), folate, doxorubicin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Human squamous carcinoma cell line, KB cell (folate receptor (+)) and A549 cell (folate receptor (−)) were obtained from the Korea Cell Line Bank (Seoul, Korea). RPMI1640 medium without folic acid was purchased from Invitrogen technologies (Carlsbad, CA). All other chemicals were of analytical grade.

2.2. Preparation of FOL–PEG–DOX conjugate

The carboxylate group of folic acid was activated by NHS and DCC as described earlier with a minor modification [16]. Briefly, 1 g of folic acid dissolved in 20 ml of dimethylsulfoxide (DMSO) was reacted with 0.9 g of NHS and 0.5 g of DCC under nitrogen atmosphere at room temperature for 12 h (folic acid/NHS/DCC molar ratio=1:2:2). The activated folic acid (0.65 g) was reacted with 0.5 g of NH2–PEG–COOH dissolved in 5 ml of DMSO. The reaction was performed under nitrogen atmosphere at room temperature for 4 h. The resultant solution was diluted with 45 ml of acetone (non-solvent for folic acid) and centrifuged. The supernatant was diluted with deionized water and dialyzed against deionized water three times (SpectraPor 6, MW cutoff=1000) and freeze-dried. The resultant FOL–PEG–COOH was activated and then reacted with the amine group of doxorubicin. Briefly, 0.35 g of FOL–PEG–COOH dissolved in DMSO was reacted with 53 mg of doxorubicin in the presence of 23 mg of NHS, 13 mg of DCC, and 11 mg of triethylamine (TEA) (FOL–PEG–COOH/doxorubicin/TEA molar ratio=1:1:1.2). The reaction was performed under nitrogen atmosphere at room temperature for 4 h. The reaction mixture was extensively dialyzed against deionized water three times (SpectraPor 6, MW cutoff=1000) and freeze-dried. The resultant FOL–PEG–DOX was activated and then reacted with the amine group of doxorubicin. Briefly, 0.35 g of FOL–PEG–COOH dissolved in DMSO was reacted with 53 mg of doxorubicin in the presence of 23 mg of NHS, 13 mg of DCC, and 11 mg of triethylamine (TEA) (FOL–PEG–COOH/doxorubicin/TEA molar ratio=1:1:1.2). The reaction was performed under nitrogen atmosphere at room temperature for 4 h. The reaction mixture was extensively dialyzed against deionized water and freeze-dried under vacuum to obtain FOL–PEG–DOX conjugate. A reversed phase chromatography result showed a single peak, suggesting that other side products were not formed (data not shown). In order to synthesize PEG–DOX conjugate without folate, methoxy-poly(ethylene glycol)–NHS (mPEG–NHS) was used instead of NH2–PEG–COOH. The conjugation percentages of DOX and FOL were calculated by determining the amount of DOX and FOL conjugated in FOL–PEG–DOX or PEG–DOX in DMSO at 480 and 365 nm, respectively. A synthetic scheme is described in Fig. 2.

2.3. Preparation and characterization of DOX nano-aggregates using FOL–PEG–DOX conjugate

Ten milligrams of FOL–PEG–DOX or PEG–DOX and 2 mg of free doxorubicin dissolved in 1 ml of acetone were slowly added into 10 ml of deionized water containing TEA (molar ratio of TEA to doxorubicin=2:1) were mixed to prepare DOX/FOL nano-aggregates (DOX nano-aggregates prepared by using FOL–PEG–DOX) and DOX nano-aggregates (DOX nano-aggregates prepared by using PEG–DOX), respectively. DOX nano-aggregates were spontaneously formed by directly dispersing the organic phase into the aqueous phase. After the organic phase was completely evaporated with gentle stirring, unencapsulated doxorubicin and TEA were removed by extensive dialysis against deionized water (SpectraPor 6, MW cutoff=10,000). The size of DOX aggregates was measured using a laser light scattering technique (ZetaPlus, Brookhaven Instrument, USA). To determine the loading amount of DOX, a fixed amount of DOX nano-aggregates was centrifuged, separated, and dissolved in DMSO. DOX concentration was then determined by measuring absorbance at 480 nm using a DOX calibration curve in DMSO.

2.4. Cytotoxicity assay

MTT-based in vitro cytotoxicity assay was performed to compare anti-cancer effects of DOX nano-aggregates against KB and A549 cell lines according to the previously established method [17]. KB and A549 cell lines were maintained in RPMI1640 medium without folic acid, and with 10% fetal bovine serum (FBS) at 37 °C in 5% CO2 atmosphere. Each cell harvested in a logarithmic growth phase was seeded on a 96 well plate at a cell density of 5×10^4 cells/ml. After incubating the cells in a logarithmic phase with various concentrations of DOX/FOL nano-aggregates, DOX nano-aggregates, or free doxorubicin for 48 h, 20 μl of MTT dye (5 mg/ml) was added to each well. After 4 h of incubation at 37 °C, the percentage of cell viability was determined at 570 nm relative to non-treated cells.
2.5. Evaluation of cellular uptake of DOX/FOL nano-aggregates

In order to investigate selective cellular uptake of DOX/FOL nano-aggregates via folate-receptor-mediated endocytosis, KB cells and A549 cells were incubated with DOX/FOL nano-aggregates in the presence or absence of folic acid in the culture medium. For flow cytometry analysis, cells were incubated with doxorubicin nano-aggregates (equivalent doxorubicin concentration: 20 \( \mu \)M) in RPMI1640 supplemented with 10% FBS in the presence of folic acid (2 mM) or absence of folic acid. After 3 h of incubation, the cells were washed three times with PBS and then harvested for further analysis. In order to quantify the amount of doxorubicin within the cell, the cells (1 \( \times \) 10^4 counts) were analyzed by a flow cytometry (FACSCalibur, USA), where the gate was arbitrary set for the detection of green fluorescence with a forward scattering (FSC) range between 200 and 600 in a linear scale. In order to visualize cellular uptake of doxorubicin nano-aggregates by cancer cells, the cells were examined by a confocal microscopy (Carl Zeiss LSM510, Germany) with an excitation wavelength of 488 nm and an emitting wavelength at 515 nm.

2.6. Animal study

Female athymic nude mice (body weight=20–25 g) were subcutaneously implanted with human epidermal carcinoma KB cells (1 \( \times \) 10^7 cells/animal). Following the implantation, tumors were allowed to grow another 21 days until well established, at which time dosing with DOX/FOL nano-aggregates, DOX nano-aggregates, or free doxorubicin were performed. DOX/FOL nano-aggregates, DOX nano-aggregates, or free doxorubicin (equivalent dose of doxorubicin=5 mg/kg) suspended in PBS were injected to tail veins of animals at day 0 and day 7. At predetermined time, a major axis...
and a minor axis of tumors were measured using a caliper. Tumor volume was then determined. All animals were accommodated in a pathogen-free laboratory environment throughout the experiments.

2.7. Statistical analysis

All data were processed and analyzed by Sigma-Plot 8.0 software (SPSS, IL). The statistical significances were evaluated by t-test of the software and \( p < 0.05 \) was considered significant.

3. Results and discussion

DOX–PEG–FOL conjugate was synthesized as shown in Fig. 2. The conjugation process was monitored by a gel permeation chromatography (GPC) at 480 and 365 nm for the detection of doxorubicin and folate, respectively. The GPC results at each conjugation step confirmed that doxorubicin and folate were successfully conjugated at \( \alpha \)- and \( \omega \)-terminal end group of a PEG chain (data not shown). The molar conjugation percent of DOX and FOL to the PEG chain was 69.1% and 69.3%, respectively. It should be noted that folate has \( \alpha \)- and \( \gamma \)-carboxylic acid and both of them could be activated when using the DCC/NHS chemistry. Although it is known that the \( \gamma \)-carboxylic acid was primarily conjugated due to its higher reactivity [18,19], it cannot be ruled out the possibility that \( \alpha \)-carboxylic acid conjugate was also produced. Additionally, in the second conjugation step of doxorubicin to FOL–PEG–COOH using the DCC/NHS agents, unreacted \( \alpha \)-carboxylic acid in the conjugated FOL could be activated, producing DOX–FOL–PEG–FOL as a by-product. However, a reversed-phase HPLC result, detected at 365 nm for folate residue, showed a single peak, suggesting that the conjugate was homogeneous without producing undesirable side products. Doxorubicin nano-aggregates stabilized with DOX–PEG–FOL conjugate were produced by slowly dropping an acetone solution of DOX–PEG–FOL or DOX–PEG into an aqueous solution containing TEA, resulting in the spontaneous formation of DOX/FOL nano-aggregates or DOX nano-aggregates after solvent evaporation. The basic aqueous condition rendered protonated DOX to be unprotonated, thereby resulting in the formation of hydrophobic DOX nano-aggregates surrounded by DOX–PEG–FOL conjugate. The size of DOX/FOL nano-aggregates was approximately 200±11.8 nm in diameter, which was confirmed by a dynamic light scattering in water. No significant change in particle size was observed in PBS. Conventionally, polymeric micelles have been made of AB-type block copolymers composed of a hydrophilic segment and a hydrophobic segment [1,2,16]. These polymers self-assemble to form polymeric micelles in an aqueous phase with a unique core-shell structure. Biodegradable di-block copolymers have been used to solubilize hydrophobic drugs inside the core, such as paclitaxel [20]. We herein synthesized a DOX–PEG–FOL conjugate having DOX and FOL at the two terminal ends of a PEG chain to solubilize unprotonated and hydrophobic DOX molecules. In aqueous solution, the DOX–PEG–FOL conjugate could stabilize DOX nano-aggregates by anchoring the DOX moiety to the inner core of DOX nano-aggregates. The spontaneous formation of stabilized DOX nano-aggregates could be attributed to the binding of a DOX moiety in the DOX–PEG–FOL conjugate on the surface of DOX nano-aggregates. It was reported that DOX having an anthraycline ring structure forms a dimer in an aqueous solution due to \( \pi–\pi \) interaction between the planar, aromatic anthracycline rings [21–24]. Thus, it is reasonable to postulate that the DOX–PEG–FOL conjugate stabilized the DOX nano-aggregates by preferential interactions of the DOX moiety in the conjugate onto the surface of DOX inner core while the remaining PEG–FOL part in the conjugate was oriented towards the aqueous bulk phase. As a result, surrounding PEG chains non-covalently tethered on the DOX nano-aggregates play a critical role in producing nano-scale DOX particles by a steric stabilization mechanism. In this way, a targeting moiety, FOL, can be exposed on the surface for cell recognition.

Unprotonated DOX molecules produced under the basic condition were precipitated to form DOX nano-aggregates in the presence of DOX–PEG–FOL conjugate. Two molar excess of TEA to DOX was optimal in the maximum DOX loading within the core of DOX nano-aggregates, which is consistent with the previous report [22]. The size of DOX nano-aggregates was almost uniform regardless of the amount of DOX loading amount as listed in Table 1. At the
initial DOX loading amount of 11.7%, most DOX molecules could be loaded within DOX nano-aggregates. The loading efficiency of free doxorubicin gradually decreased as the initial loading of doxorubicin increased. However, it should be noted that a relatively large amount of DOX could be physically loaded into the DOX nano-aggregates in a more facile way, compared to conventional polymeric micelles [1,2,6].

In order to investigate selective targeting ability of DOX/FOL nano-aggregates against FOL receptors on the cell, KB cells and A549 cells were employed as FOL receptor (+) cancer cells and FOL receptor (−) cancer cells, respectively. As shown in Fig. 3, flow cytometry results show that a greater fraction of DOX/FOL nano-aggregates was taken up within KB cells than A549 cells, when folic acid was absent in the culture medium (Fig. 3, left). There was no difference in the extent of cellular uptake of DOX between KB cells and A549 cells, when 2 mM of folic acid was present in the culture medium (Fig. 3, right), indicating that the presence of folic acid in the culture medium competitively inhibited the binding of DOX/FOL nano-aggregates to FOL-receptor-positive KB cells, but not to FOL-receptor-negative A549 cells. The above results suggest that DOX/FOL nano-aggregates were transported within cells by a FOL-receptor-mediated endocytosis process.

Table 1

<table>
<thead>
<tr>
<th>NA</th>
<th>Initial loadinga (% w/w)</th>
<th>Effective diameterb (nm)</th>
<th>Loading amountb (% w/w)</th>
<th>Loading efficiencyb (%)</th>
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<tr>
<td>A</td>
<td>11.7</td>
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<td>97.2±8.2</td>
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<tr>
<td>B</td>
<td>39.8</td>
<td>204.7±11.1</td>
<td>23.6±3.9</td>
<td>59.2±9.9</td>
</tr>
<tr>
<td>C</td>
<td>56.9</td>
<td>214.3±18.7</td>
<td>25.9±5.1</td>
<td>45.5±8.9</td>
</tr>
</tbody>
</table>

*a Initial loading is a weight ratio of initially added free doxorubicin to doxorubicin nano-aggregates.

*b Values were measured three times and the standard deviations were obtained.

Cellular uptakes of DOX, DOX nano-aggregates, and DOX/FOL nano-aggregates by KB cells were comparatively estimated by a laser scanning confocal microscope as shown in Fig. 4. For the confocal microscopic observation, FOL was not added in the medium. When free DOX was incubated with KB cells, DOX was mainly present within the nucleus. This was mostly due to a multi-drug resistance effect. Free DOX molecules transported into the cytoplasm of the cell, in a passive diffusion manner, were effluxed out by P-glycoprotein pumps, while some of them could reach into the nucleus and bind to DNA. In general, DOX-loaded nano-particles, such as liposomes, micelles, polymer nanoparticles, have been known to be taken up by cells through an endocytic pathway, thereby making them escape from the effect of P-glycoprotein pumps [25]. The endocytic delivery of DOX within cells by using nano-particles maintains the intracellular DOX concentration to be high in the cytoplasm region. This can be
seen in Fig. 4. Cellular uptake extent of DOX/FOL nano-aggregates was significantly higher than those of free DOX and DOX nano-aggregates under the same condition. In addition, it can be visualized that DOX/FOL nano-aggregates were primarily located on the surface of cell membrane, due to their preferential binding to FOL receptors on the membrane. The confocal results directly indicate that DOX/FOL nano-aggregates were taken up by a FOL-receptor-mediated endocytosis process, in accordance with the FACS results. In contrast to free DOX, DOX/FOL and DOX nano-aggregates were mainly distributed in the cytoplasm without exhibiting much accumulation in the nucleus. Since the confocal image was obtained after 3 h of incubation, most DOX molecules transported inside the cells were likely to be still in an aggregated state in the cytoplasm region with little chance to be solubilized in the cytoplasmic fluid. The aggregated DOX molecules could have much lower fluorescence intensity than an individual DOX molecule in the intracellular environment because they were self-quenched [21]. Hence it is reasonable to say that the concentration of molecularly dissolved DOX in the cytoplasm was not sufficient enough in the early incubation period to be partitioned to the nucleus. This also could be the reason for the observed modest difference of cellular uptake between KB and A549 cells in the flow cytometric analysis as shown in Fig. 3. The flow cytometric analysis was performed after 3 h of incubation, not enough for the dissolution of DOX molecules in the cytoplasm. In Fig. 4C, it is of interest to note that intracellularly transported DOX molecules are densely located around the nucleus membrane, but they are sparsely distributed in the cytoplasm region near the cell membrane. This also supports the fact that free and solubilized DOX molecules tended to diffuse towards the nucleus, while some part of them present near the inner cell membrane was pumped out via P-glycoprotein pumps. The DOX molecules in the cytoplasm were later observed to slowly migrate into the nucleus over a prolonged incubation period (24 h) and they were eventually located in the nucleus.

Cell cytotoxicities of DOX, DOX nano-aggregates, and DOX/FOL nano-aggregates for KB and A549 cells were investigated as shown in Fig. 5. For KB cells, DOX/FOL nano-aggregates (IC$_{50}$=5.6 μM) exhibit superior cytotoxic activities to DOX nano-aggregates (IC$_{50}$=9.8 μM) (Fig. 5A). On the other hand, for A549 cells, IC$_{50}$ values of DOX/FOL nano-aggregates (IC$_{50}$=9.0 μM) and DOX nano-aggregates (IC$_{50}$=9.2 μM) were similar (Fig. 5B). This reveals that FOL moieties in DOX/FOL nano-aggregates played an important role in enhancing cytotoxic effect by binding of DOX/FOL nano-aggregates with FOL receptors on KB cells, and subsequently increasing their intracellular uptake as a result of the receptor-mediated endocytosis. Free DOX shows less cytotoxicity against both cell lines compared to DOX/FOL and DOX nano-aggregates, resulting from the reduced cellular uptake of DOX. KB cells are known to have much higher IC$_{50}$ value than any other cancer cell lines because of their severe multi-drug resistance (MDR) effect [2,4,5]. As seen in Fig. 4, free DOX in the cytoplasm was rapidly eliminated by the action of P-glycoprotein pumps. Therefore, FOL-receptor-mediated intracellular delivery approach of DOX by using FOL decorated DOX nano-aggregates is an attractive way in circumventing the MDR effect as well as in increasing the cytotoxic effect. It should be
noted that a relatively short incubation period used in the MTT-based cytotoxicity assay (48 h) was not enough to determine a long-term cytotoxicity of DOX/FOL nano-aggregates. Because the intracellular DOX nano-aggregates present in the cytoplasm must be solubilized in a molecularly dissolved state prior to reaching into the nucleus, a more prolonged incubation time would be required to fully exert the cytotoxicity effect of DOX. We previously observed similar cytotoxic results for doxorubicin encapsulated PLGA nanoparticles and PLGA–PEG micelles when the MTT-based cytotoxicity assay was used [16].

Human tumor xenograft nude mice were used to determine in vivo anti-tumor activities of DOX/FOL nano-aggregates. An equivalent DOX amount of DOX, DOX nano-aggregates, or DOX/FOL nano-aggregates was systemically administered through tail veins. Fig. 6 shows that DOX/FOL nano-aggregates
have a superior anti-tumor activity to DOX nano-aggregates and free DOX, where the difference was statistically considered significant ($p < 0.05$). After 23 days, tumor volumes for mice treated with DOX/FOL nano-aggregates was about 40% less than those treated with DOX. The enhanced anti-tumor effect of DOX/FOL nano-aggregates was attributed to two factors: (1) passive targeting of DOX/FOL nano-aggregates to loosened vascular endothelial junctions in the vicinity of solid tumor (enhanced permeation and retention effect) and (2) active targeting of DOX/FOL nano-aggregates to tumor cells overexpressing FOL receptors (receptor-mediated endocytosis). The passive targeting allowed DOX/FOL nano-aggregates in the tumor site, while the active targeting permitted them to be readily taken up by tumor cells at the site. Thus the combined passive and active targeting effects were likely to act synergistically, and they were mainly responsible for the observed delayed tumor volume growth. Since DOX molecules were slowly released out in an aqueous phase from DOX/FOL nano-aggregates for 2 weeks in an in vitro experiment (data not shown), it is conceivable that a small amount of DOX was continuously released out at the tumor site or within tumor cells during the animal study (23 days). This sustained release effect of DOX/FOL nano-aggregates at the target site would be an additional advantage in reducing the tumor volume. Moreover, because DOX/FOL nano-aggregates were decorated with PEG chains on the surface, their circulation time in the blood stream was greatly prolonged similar to pegylated liposomes containing DOX [26]. The present study suggests that DOX–PEG–FOL conjugate could serve as a novel “PEGylated doxorubicin” surfactant to stabilize unprotonated DOX aggregates in a nano-scale particulate suitable for targeting to the tumor site, enhancing cellular uptake, and achieving sustained release within cells. Further detailed dose-optimization studies will be required for better understanding in vivo pharmacokinetic and bio-distribution behaviors.

4. Conclusions

This study demonstrates that DOX–PEG–FOL conjugate assisted to form and stabilize DOX nano-aggregates. The DOX moiety in the conjugate interacted with hydrophobic DOX aggregates, while the other FOL moiety in the PEG chain was oriented outside for enabling the binding of them with FOL receptors on tumor cells. DOX/FOL nano-aggregates could contain a large amount of hydrophobic and unprotonated DOX in the inner core. The DOX/FOL nano-aggregates showed enhanced cellular uptake, increased targeting capacity, and increased cytotoxicity against KB cells overexpressing FOL receptors. In vivo experiment employing a human tumor xenograft animal also confirmed a superior anti-tumor effect of FOL-targeted doxorubicin nano-aggregates.

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References


