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Impact of the amount of PEG on prodrug nanoassemblies for efficient cancer therapy

Yaqiao Li, Lingxiao Li, Qianhui Jin, Tian Liu, Jin Sun, Yongjun Wang, Zhijun Yang, Zhonggui He, Bingjun Sun

a WuYa College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China
b School of Chinese Medicine, Hong Kong Baptist University, Hong Kong 999077, China

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A B S T R A C T
PEGylation has been widely used to improve the pharmacokinetic properties of prodrug self-assembled nanoparticles (prodrug-SANPs). However, the impacts of the amount of PEG on the self-assemble stability, cellular uptake, pharmacokinetics, and antitumor efficacy of prodrug-SANPs are still unknown. Herein, selenoether bond bridged docetaxel dimeric prodrug was synthesized as the model prodrug. Five prodrug-SANPs were designed by using different mass ratios of prodrugs to PEG (W_prodrug/W_DSPPE=PEG2000 = 10:0, 9:1, 8:2, 7:3 and 6:4), and defined as Pure drug NPs, 9:1NPs, 8:2NPs, 7:3 NPs and 6:4 NPs, respectively. Interestingly, 8:2 NPs formed the most compact nanostructure, thus improving the self-assemble stability and pharmacokinetics behavior. In addition, the difference of these prodrug-SANPs in cellular uptake was investigated, and the influence of PEG on cytotoxicity and antitumor efficacy was also clarified in details. The 8:2 NPs exhibited much better antitumor efficacy than other prodrug-SANPs and even commercial product. Our findings demonstrated the pivotal role of the amount of PEG on prodrug-SANPs.

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1. Introduction
Chemotherapy is still the mainstay in the therapy of cancer clinically [1]. However, the clinical application of most chemotherapeutics (such as docetaxel, DTX) is limited by poor delivery efficiency [2]. Many strategies have been used to improve the delivery efficiency of chemotherapeutics including prodrug strategies [3], nanoparticulate drug delivery system (nano-DDS) [4–8]. Prodrug can change the physics and chemistry character of drug such as lack of stability, low solubility and nonselective toxicity by modifying the structure of the parent drug [9]. Due to enhanced permeability and retention (EPR) effect, nano-DDS facilitates the high accumulation of drug in tumor [10]. In addition, prodrug self-assembled nanoparticles (prodrug-SANPs), which inheriting the benefits of prodrug and nano-DDS, have become a hotspot...
of drug delivery [9]. Since the prodrug is both the carrier and the drug, prodrug-SANPs possess extremely high drug loading and relative low exipient associated side effects [11].

Most nanomedicines, including prodrug-SANPs, generally require hydrophilic materials to modify the surface of the nanoparticles to improve their pharmacokinetic properties [12]. Polyethylene glycol (PEG), a commonly used surface modification material, is one of the few synthetic polymers approved by the FDA [8,13]. PEG is formed by the polymerization of ethylene oxide, and has the advantages of neutral pH, high hydrophilicity [14]. Moreover, PEG possesses the advantages of non-toxicity, non-immunogenicity, and good biocompatibility [15–17]. Modification of prodrug-SANPs with hydrophilic PEG can prevent or minimize the surface adsorption of proteins [18] thus repelling opsonization [19]. Therefore, coating with PEG, a process called PEGylation, can increase the circulating half-life of prodrug-SANPs by avoiding phagocytosis of reticuloendothelial system (RES) [12,20–23]. One example is the first FDA approved anticancer liposome (Doxil®). PEGylation of liposomes using DSPE-PEG2000 reduces the phagocytosis of RES, thereby increasing the half-life and circulation time of Doxil® [20–22].

PEG not only affect the systemic circulation time of prodrug-SANPs, but also affect the stability and cellular uptake of prodrug-SANPs [20]. Especially, for prodrug-SANPs, which the main body of the nanostructure is the prodrug molecule [23], the addition of PEG and the amount of PEG are possible to impact the assembly ability and assembly stability of the prodrug, thereby affect its in vivo fate. In addition, the modification of hydrophilic PEG can provide a hydration shell on the surface of the prodrug-SANPs, which will impact the cellular uptake of the prodrug-SANPs [24]. Therefore, the amount of PEG materials on prodrug-SANPs demands comprehensive consideration and design, taking into account the stability, cellular uptake and pharmacokinetics behavior of prodrug-SANPs. However, none of the correlational research has been reported to our knowledge.

In this study, we designed the selenoether linker bridged docetaxel dimeric prodrug as a model drug to systematically explore the effect of the amount of PEG on the drug delivery efficiency of the prodrug-SANPs [11,25]. Five prodrug-SANPs were developed by using different ratios of prodrugs to PEG \( W_{\text{prodrug}}/W_{\text{DSPE-mPEG2000}} = \{10.0, 9.1, 8.2, 7.3 \text{ and } 6.4\} \), and defined as Pure drug NPs, 9:1NPs, 8:2NPs, 7:3 NPs and 6:4 NPs, respectively. We clarified the impacts of the amount of PEG on the self-assembly stability, pharmacokinetics, cellular uptake and antitumor efficacy of prodrug-SANPs.

2. Materials and methods

2.1. Materials

DiR, Docetaxel, hydrogen peroxide \((H_2O_2)\), fetal bovine serum \((FBS)\), Trypsin, Dulbecco's modified eagle medium \((DMEM, \text{high glucose})\), Roswell Park Memorial Institute \((RPMI-1640)\) and 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide \((MTT)\) were bought from Meilun Biotech \((Dalian, \text{China})\). Bromoacetic acid, selenium, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride \((EDCI)\), 3-bromopropionic acid, sodium borohydride \((NaBH_4)\), 4-dimethylaminopyridine \((DMAP)\), 4-bromobutyric acid were all bought from Aladdin \((Shanghai, \text{China})\). 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-methyl \((\text{polyethylene glycol})–2000 \((\text{DSPE-mPEG}_{2000})\) were obtained from Shanghai Advanced Vehicle Technology Pharmaceutical Ltd. \((Shanghai, \text{China})\). Ki67 cell proliferation kit and TUNEL apoptosis detection kit were obtained from Beijing Solarbio Science & Technology Co., Ltd. Cell culture materials were provided by NEST Biotechnology. All reagents mentioned in this work were of analytical grade.

2.2. Synthesis of selenodiacetic acid linker

The synthesis of selenodiacetic acid linker resembles our previous research [11]. All the reactions were under nitrogen atmosphere. Selenium powder, pure water and NaBH4 solution was mixed. The above mixture was first reacted at 0°C for 30 min, and then at 105°C for 1 h. At last, the mixture was added the bromoacetic aqueous solution and stirred at 25°C for 12 h, then removed the impurities from reaction mixture. Using diluted hydrochloric acid regulated pH of the filtrate to 3.0–4.0. Then extracting the product by using ethyl acetate. The precipitation was recrystallized in ethyl acetate and dried to obtain the product selenodiacetic acid [11].

2.3. Synthesis and characterization of dtx dimeric prodrug \((\text{DTX-Se-DTX})\)

DTX and selenodiacetic acid linker was added in \(\text{CH}_2\text{Cl}_2\), and used DMAP and EDCI as catalysts for the reaction. Preparative liquid chromatography and Nuclear magnetic resonance spectroscopy \((\text{600 MHz } ^1\text{H NMR, Bruker AV-400})\) was used to purified and characterized prodrug, respectively. High-resolution mass spectrometry \((\text{Agilent 1100 Series LC/MSD Trap})\) and High Performance Liquid Chromatography \((\text{HPLC})\) were also used in this research.

2.4. Preparation of prodrug-SANPs

2 mg DTX-Se-DTX and 0.22 (9:1), 0.50 (8:2), 0.86 (7:3), 1.33 (6:4) mg DSPE-mPEG_{2000} were well dissolved in 400 \(\mu\)l ethanol. The preparation of different ratios of PEGylated prodrug-SANPs by dropping the mixture ethanol solution into 2ml deionized water while stirring rapidly. The preparation of non-PEGylated prodrug-SANPs was the same procedure but without adding PEG. Finally, ethanol was removed by using vacuum distillation. All the concentration of prodrug-SANPs were 1 mg/ml. The drug loading of all prodrug-SANPs was calculated as:

\[
\text{Drug Loading (%)} = \frac{\text{Weight of } \text{DTX}}{\text{Weight of prodrug}} \times 100\%
\]

2.5. Molecular simulations

The molecular dynamics simulations were utilized to illustrate the self-assembly mechanism of PEG and prodrug binding in different proportions. In brief, using Discovery
Studio Client [26] to construct the prodrug molecules and the DSPE-mPEG2000 molecules. Quantum mechanics method was used to optimize the small ligand molecule and obtain the stable structure. After the stable configuration was obtained, the Packmol program [27] was used to structure the four prodrug-SANPs. Molecular dynamics simulations were then performed for the complex. The molecular RESP charge [28] parameters were fitted and GAFF field [29] parameters were constructed based on antechamber program in AmTools [28]. The TIP3P water box model side length is set to 1 nm. Energy minimization was performed at 5000 steps, followed by short-term 100 ps simulation under NVT and NPT ensemble respectively, and finally 30 ns equilibrium simulation was generated. The truncated radius was set at 0.8 nm, the time step was set at 2 fs, and the temperature and pressure were set at 298 K and 1 bar, respectively. The configurations are saved every 10 ps for subsequent analysis. All molecular simulations were calculated by Gromacs2018 program [30]. Combined free-energy calculations are implemented using the g_mmpbsa tool [31].

2.6. Stability of prodrug-SANPs

The four prodrug-SANPs were stored at 4 °C and 25 °C 14 days to test the storage stability [32]. Further, 10% FBS and 90% FBS (pH 7.4) was used to simulate the in vivo environment, and investigated the in vivo stability of different ratios of PEGylated prodrug-SANPs for 24 h [33]. The interactions between prodrug-prodrug molecules or prodrug-PEG molecules were test by treating four prodrug-SANPs with of urea, SDS or NaCl. All of the above medium were shaken in 37 °C. All the concentration of prodrug-SANPs in above medium were 0.05 mg/mL at scheduled time points, the diameter was determined (n = 3 for each group).

2.7. In vitro drug release

Mixture of 70% PBS (pH 7.4) and 30% ethanol (v/v) was used as release medium to measure the in vitro drug release behavior [34]. The different ratios of PEGylated prodrug-SANPs and DTX solution were added in release medium with or without predetermned of H2O2 (equivalent DTX concentrations of 6.67 μg/mL). HPLC was applied to determine the peak area of theoretic DTX and DTX released from prodrug-SANPs at the scheduled time (n = 3 for each group). Drug release rate (%) = Peak area of released DTX / Peak area of theoretic DTX × 100%. All of the above medium shook at 37 °C. Confirming the intermediate by using LC/MSD.

2.8. Cell culture

Mouse mammary carcinoma cells (4T1 cells), mouse prostate cancer cells (RM-1 cells) and mouse fibroblast cells (3T3 cells) were supplied from the cell bank of Type Culture Collection of Chinese Academy of Sciences. The 4T1 cells were cultured in RPMI-1640 medium. And the RM-1 cells and 3T3 cells were cultured in DMEM medium. All cells grew in cell incubator filled with 37 °C, 5% CO2.

2.9. Cytotoxicity assays

4T1 cells, RM-1 cells, 3T3 cells were put in 96-well plates for 1000 cells per well and cultured 12 h. The fresh medium was applied for further incubating 48 h which containing with various concentrations of DTX solution or different ratios of PEGylated prodrug-SANPs, and utilized the cells without treatment as control group. MTT was added to all plates and cultured for 4 h and replaced by dimethyl sulfoxide (DMSO). The survival rates were analyzed by using microplate reader and determined the IC50 values (n = 3 for each group) by Prism 9. The selective index (SI) values were gained from IC50 of 3T3 cells to tumor cell lines.

2.10. Cellular uptake

4T1 cells were cultured in 24-well plates (5 × 104 cells per well) for 24 h. Then incubated for 2 h, 4 h and 12 h at 37 °C by using DTX solution and different ratios of PEGylated prodrug-SANPs (equivalent DTX concentrations of 1000 ng/ml and 2000 ng/ml, n = 3 for each group). After that, were washed thrice by using cold PBS, collected, sonicated and centrifugated. Then, the concentration of free DTX in the cells were measured by UPLC-MS-MS (ACQUITY UPLCTM, Waters Co., Ltd., Milford, MA, USA). Intracellular protein concentration was detected by BCA kit.

2.11. Animal studies

All the animals were provided and approved by the Institutional Animal Ethical Care Committee (IAEC) of Shenyang Pharmaceutical University. The ethics approval number is SYPHU-IACUC-C2021-5–12–106. All experiments about animal were observed the Guidelines for the Care and Use of Laboratory.

2.12. In vivo pharmacokinetic study

Male Sprague–Dawley (SD) rats were used to study the pharmacokinetic profiles of different ratios of PEGylated prodrug-SANPs. The animals were intravenously injected by DTX solution and different ratios of PEGylated prodrug-SANPs (equivalent DTX concentration of 4 mg/kg, n = 3 for each group). Plasma was gotten at the predetermined time intervals and analyzed concentrations of prodrugs and free DTX by using UPLC-MS-MS. AUC and Cmax were calculated using DAS 2.1.1.

2.13. In vivo biodistribution

The tumor model was established by using female BALB/c mice injected by 4T1 cells in the back. When tumor volume reached ~ 400 mm3, tumor-bearing mice were divided into fifteen groups (n = 3 for each group). The mice were injected with DiR solution and DiR-labeled different ratios of PEGylated prodrug-SANPs (equivalent DiR concentrations of 1 mg/kg, equivalent DTX concentrations of 10 mg/kg). The mice of DiR solution and DiR-labeled different ratios of PEGylated prodrug-SANPs were sacrificed at 1 h, 4 h and 12 h. The major
organs including heart, liver, spleen, lung, kidney and tumor of each mouse were isolated for imaging by noninvasive optical imaging system (IVIS). And fluorescence semi-quantitative analysis was performed

2.14. In vivo antitumor efficacy

The tumor model was established by using female BALB/c mice injected by 4T1 cells in the back. The day was regarded as Day 0 for treatment when the tumor size rose to 100 mm³. Then, the mice were treated with saline, DTX solution and the four different ratios of PEGylated produg-SANPs (equivalent DTX concentration of 2 mg/kg, n = 5 for each group) every other day for five times. Caliper has been used to measure the maximum diameter (L) and the minimum diameter (W) of the tumor every day. And the body weight was measured daily. The tumor size was calculated as: Tumor (mm³) = L × W × W × 0.5. At the Day 9, the mice were sacrificed, and the tumors and spleens were weighed after treatment. And the tumor burden was calculated by Wtumor to Wmice. After that, the toxicity of each group was determined. The major organs, and tumor of each group were studied by hematoxylin and eosin (H&E) staining. The lung metastatic nodules were observed after soaking and staining with Bouin’s tissue fixative solution. The serum was investigated by hepatic and renal function analysis. TUNEL assay and Ki-67 immunofluorescence staining were investigated to evaluate the apoptosis and proliferation of tumor. DAPI-positive cells were used to observe the processed slides. Image J was used to calculate quantification of the relative area (%) of apoptosis cells and proliferating cells.

2.15. Statistical analysis

GraphPad Prism 7.0 was used to analyze all data. Student’s t-test (two-tailed) was used to analyze the statistical comparisons. Statistical significance was considered at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

3. Results and discussion

3.1. Synthesis of dtx dimeric prodrugs

The synthesis of DTX-Se-DTX was shown in Fig. S1. The chemical structure of the produg was confirmed by MS and ¹H NMR (Fig. S2). The purity of the produg was over 97%.

3.2. Preparation and characterization of produg-SANPs

In order to research the influence of DSPE-mPEG<sub>2000</sub> on the produg-SANPs, non-PEGylated produg-SANPs and different ratios of PEGylated produg-SANPs were prepared (Fig. 2A). According to the different ratios of produg to PEG (W<sub>produg</sub>/W<sub>DSPE-mPEG<sub>2000</sub></sub> = 10.0, 9.1, 8.2, 7.3, and 6.4), the produg-SANPs were defined as Pure drug NPs, 9:1 NPs, 8:2 NPs, 7:3 NPs and 6:4 NPs respectively. As shown in Table S1 and Fig. 2B-F, pure drug could assemble into nanoparticles with a slightly larger diameter of 175.1 nm. 9:1 NPs, 8:2 NPs and 7:3 NPs exhibited similar hydrodynamic diameter around 90 nm. However, 6:4 NPs showed large and nonuniform particle size (> 300 nm). In addition, Transmission electron microscopy images revealed a uniform spherical morphology of these produg-SANPs except 6:4 NPs (Fig. 2B-2F). This indicated that the amount of DSPE-mPEG<sub>2000</sub> significantly impacted the formation of produg-SANPs. Due to the nonuniform particle size, 6:4 NPs was no longer investigated in the follow-up studies. The drug loading capacity of produg-SANPs were extremely high even for 7:3 NPs (more than 60%, DTX equivalent, w/w).

3.3. Molecular dynamics simulations of produg-SANPs

The molecular dynamics simulations were utilized to elucidate the assembly molecular mechanisms. The conformational variation during molecular dynamics simulations were shown in Fig. 3. As for Pure drug NPs, the conformation of clusters formed loose structure, indicating poor self-assembly ability of Pure drug NPs. In comparison, the conformational variation of PEGylated produg-SANPs was formed into stable and tight nanostructure in a relative short time with the hydrophilic PEG facing outward. However, 9:1 NPs and 7:3 NPs formed voids in the middle of the produg-SANPs and generated relatively loose nanoparticles. Among three PEGylated produg-SANPs, 8:2 NPs assembled into stable nanostructure about 10 ns, demonstrating that 8:2 NPs possessed better self-assembly ability. As for thermodynamics, the binding free energy (∆G<sub>total</sub>) and energy decomposition components of Pure drug NPs, 9:1 NPs, 8:2 NPs, 7:3 NPs were calculated and summarized in Table S2. A negative ∆G<sub>total</sub> indicates favorable system of interactions, and larger absolute values of potential energy expresses better stability. The absolute values of ∆G<sub>total</sub> of 8:2 NPs (−22,953.60 kJ/mol) was much larger than that of Pure drug NPs (−8948.90 kJ/mol), 9:1 NPs (−10,438.10 kJ/mol), and 7:3 NPs (−18,502.40 kJ/mol). And decomposition components of energy also help to make certain of the contribution of detailed interactions. As expected, van der Waals force was the main force for all the nanoparticles, since the hydrophilic force is the dominant force in self-assembly process (Table S2). Electrostatics, polar energy and non-polar energy also played a leading role in the stability of nanoparticles.

The intermolecular interactions of the produg-SANPs were shown in Figure S3A. In addition to the interaction between produg molecules, the produg also formed hydrogen bond interactions with PEG molecules in the presence of PEG. We also evaluated the radius of gyration and solvent accessible surface of nanoparticles. As shown in Fig. S3B- S3C, the variation of radius of gyration and solvent accessible surface of produg-SANPs were followed the order of the 8:2 NPs > 7:3 NPs > 9:1 NPs > Pure drug NPs. The 8:2 NPs, with a maximum change of radius of gyration (about 4 nm) and solvent accessible surface (appropriate 2000 nm²), formed the most compact nanostructure.

3.4. Stability of produg-SANPs

All the produg-SANPs were stable with negligible changes in particle size under storage condition (Fig. 4A-4B), during 14 d at 4 °C and 25 °C. The stability of produg-SANPs was further
investigated in PBS (pH 7.4) including 10% FBS (v/v) to predict the stability of prodrug-SANPs in the body. Among these four prodrug-SANPs, 8:2 NPs showed excellent stability with insignificant change in particle size even after 24 h (Fig. 4C). In contrast, the particle size of Pure drug NPs, 9:1 NPs and 7:3 NPs were increased in different degrees followed the order of Pure drug NPs > 9:1 NPs > 7:3 NPs. In addition, prodrug-SANPs were treated in NaCl, urea or sodium dodecyl sulfate (SDS), which could eliminate electrostatic force, hydrogen bonds, and hydrophobic force, respectively. As shown in Fig. 4D-4F, all the prodrug-SANPs were sensitive to SDS rather than urea. This indicated that hydrophobic force is the dominate force in self-assembly, which was in line with the molecular dynamics simulations results (Table S2). Interestingly, the particle size of Pure drug NPs and 9:1 NPs were increased significantly when incubated with NaCl. This finding suggested that Pure drug
NPs and 9:1 NPs tended to instability under extreme condition. Both molecular dynamics simulations and stability tests indicated that the amount of PEG impacted the stability of prodrug-SANPs. Moderate DSPE-mPEG2000 (8:2) could improve the stability prodrug-SANPs while excess (7:3) or inadequate DSPE-mPEG2000 (9:1 or 10:0) could disturb the self-assemble process. In summary, when the mass ratio of prodrug to PEG was 8:2, the most compact and stable prodrug-SANPs were formed.

3.5. In vitro drug release

In blank media, 9:1 NPs, 8:2 NPs and 7:3 NPs exhibited similar release profile, and could release approximately 20% DTX after 24 h. The proportion of DTX released from Pure drug NPs could reach 40% at 24 h (Fig. 5B). These finding demonstrated that the addition of PEG could improve the stability of prodrug-SANPs, thus prevent premature drug release in the blood circulation. In addition, we further explored the oxidation-responsivity of prodrug-SANPs using hydrogen peroxide (H₂O₂) as model triggers. At low H₂O₂ concentration, the drug release from 9:1 NPs, 8:2 NPs and 7:3 NPs were comparable, while the DTX release of Pure drug NPs were slight faster than PEGylated prodrug-SANPs, which were consist with the results in the blank media (Fig. 5C). At high H₂O₂ concentration, all prodrug-SANPs possessed similar drug release rate, and could release 80% DTX after 24 h incorporation (Fig. 5D). DTX-Se-DTX prodrug was the main body of these nanoparticles, and the release of DTX mainly depended on the oxidation of selenoether bond, thereby these prodrug-SANPs exhibited comparable release rate under the high H₂O₂ concentration. The H₂O₂-triggered drug released mechanism was illustrated in Fig. 5A. The oxidation-responsive selenium atoms were oxidized to hydrophilic selenoxide by H₂O₂, then promoting the hydrolysis of DTX-adjacent ester bond. We next tested the molecular weights of intermediates after oxidation to verify

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**Fig 3** – Molecular dynamics simulations of different ratios of PEGylated prodrug-SANPs. Schematic diagram of the aggregation process of prodrug-SANPs from 0 ns to 30 ns (DTX-Se-DTX as shown as blue, and DSPE-mPEG2000 as purple).

**Fig 4** – Stability of prodrug-SANPs in different environments. Store at 4 °C (A) and 25 °C (B) for 14 d (C) The colloidal stability of prodrug-SANPs in PBS (pH 7.4) including 10% FBS (v/v). Size change curves of prodrug-SANPs incubated with (D) Urea, (E) SDS and (F) NaCl at 37 °C (medium concentration: 0.4M). Data are presented as mean ± SD (n = 3).
the mechanism. The molecular weights of the monoxides of DTX-Se-DTX and the released DTX were showed in Fig. S4.

3.6. Cytotoxicity and cellular uptake

The cytotoxicity assay of prodrug-SANPs was conducted in two tumor cells and one normal cell. As shown in Fig. 6A-6B, Fig. S5A–S5B and Table S3, prodrug-SANPs had weaker cytotoxicity than DTX solution in two kinds of tumor cells, following the order of DTX solution ≈ 8:2 NPs > 9:1 NPs > Pure drug NPs > 7:3 NPs. Abelovementioned in vitro release studies indicated that there was no significant different in these prodrug-SANPs except for Pure drug NPs. Therefore, we speculated that the ratios of PEG modification affected the cellular uptake efficiency of prodrug-SANPs [35], thereby causing the difference of cytotoxicity [36]. The cellular uptake of prodrug-SANPs was further determined in 4T1 cells using UPLC-MS/MS. As shown in Fig. 6D-6E. At 2 h, the cellular uptake of DTX by 8:2 NPs-treated group was significantly higher than that of other prodrug-SANPs and comparable to that of DTX solution. The cellular uptake of these prodrug-SANPs followed the order of DTX solution ≈ 8:2 NPs > 9:1 NPs > Pure drug NPs > 7:3 NPs, which was consistent with the cytotoxicity results. The reason for the high uptake efficiency of DTX solution was that DTX is a lipophilic small molecule drug, which is easy to enter cells through lipophilic cell membrane. Moreover, there are two factors that determine the uptake efficiency of prodrug-SANPs. One is the stability of the prodrug-SANPs, and the other is the amount of PEG on prodrug-SANPs. Pure drug NPs exhibited the poor stability, thereby tended to premature aggregation outside the cells, which limited the efficiency of cellular uptake. Too much modification of PEG on 7:3 NPs not only impacted the stability but also hindered the cellular uptake of prodrug-SANPs. Therefore, 7:3 NPs possessed the poorest cellular uptake efficiency and cytotoxicity. 8:2 NPs formed the most compact and stable prodrug-SANPs, with suitable PEG modification, showing the highest cellular uptake efficiency and the strongest cytotoxicity, which was comparable to DTX solution (Fig. 6F).

As shown in Fig. 6C & 6D and Table S4, all prodrug-SANPs showed lower cytotoxicity than DTX solution on 3T3 normal cells. To compared the cytotoxicity of prodrug-SANPs and DTX solution between tumor cells and normal cells, we next calculated the selectivity index (SI) and displayed in Table S4. The Pure drug NPs, 9:1 NPs, 8:2 NPs and 7:3 NPs displayed much higher SI values than DTX solution. These results indicated that prodrug-SANPs could selectively kill tumor cells due to oxidation-responsivity release of DTX in response to high oxidation state in tumor cells. In comparison, prodrug-SANPs exhibited negligible DTX released in the normal cells, showing good safety.

3.7. In vivo pharmacokinetics and biodistribution

We next used SD rats to investigate the pharmacokinetic profiles of DTX solution and prodrug-SANPs [37]. The pharmacokinetic profiles were shown in Fig. 7 and Table S5. As expected, DTX in DTX solution and Pure drug NPs were cleared quickly in the blood, while the PEGylated prodrug-SANPs increased the area under the curve (AUC) to different degrees. The AUC of total DTX (released DTX plus DTX in prodrug) in the Pure drug NPs, 9:1 NPs, 8:2 NPs and 7:3 NPs groups were approximately 1.31-, 2.94-, 19.66- and 8.79- times higher than DTX solution group (Table S5). These findings showed that the ratio of DSPE-PEG2000 PEGylated prodrug-SANPs significantly impacted the pharmacokinetic profiles. Among four prodrug-SANPs, the 8:2 NPs, which enhanced colloidal stability, exhibited the highest AUC.

We next determined the biodistribution of prodrug-SANPs in 4T1 tumor-bearing BALB/C mice by using IVIS spectrum (Fig. 8). The results of tumor accumulation were well confirmed to the stability and pharmacokinetic behavior. 8:2 NPs, with the best stability and pharmacokinetic behavior,
**Fig. 6** – IC₅₀ values (nmol/L) and cellular uptake of DTX solution and prodrug-SANPs. IC₅₀ values (nmol/L) on (A) 4T1 cells, (B) RM-1 cells, (C) 3T3 cells. The cellular uptake of DTX solution and prodrug-SANPs in 4T1 cells at equivalent DTX concentration of (D) 1000 ng/ml and (E) 2000 ng/ml. (F) The cellular uptake efficiency of prodrug-SANPs in tumor cells. Data are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 by two-tailed Student’s t-test.

**Fig. 7** – Pharmacokinetics of PEGylated prodrug-SANPs. (A) Blood circulation affects stability of prodrug-SANPs. Molar concentration–time curves of (B) the prodrugs, (C) the released DTX, and (D) the sum of prodrugs and the released DTX. Data are presented as mean ± SD (n = 3).
showed the highest accumulation in tumors. The fluorescence of 8:2 NPs in tumor gradually increased from 1 h to 12 h (Fig. 8B-8D). In comparison, DiR solution was mainly concentrated in liver, spleen, and lung, and there was almost no fluorescence in tumor. As we expect, Pure drug NPs and 9:1 NPs also showed almost no distribution in tumor. It’s possible due to the poor colloid stability of Pure drug NPs and 9:1 NPs, which were cleared quickly in the blood. The 8:2 NPs exhibited significantly better tumor accumulation, which also suggested that the 8:2 NPs has the best antitumor efficacy.

3.8. In vivo antitumor efficacy

We further determined the antitumor activity of DTX solution and prodrug-SANPs (Fig. 9A). On Day 9, the volume of some tumors in the saline group has already reached nearly 1500 mm³, which would not meet the ethical standards and animal welfare. Therefore, we could not conduct a longer in vivo anti-tumor investigation (Fig. 9B). As shown in Fig. 9B-9D, the Pure drug NPs showed poor antitumor activity due to their poor stability and pharmacokinetic behavior. The antitumor activity of DTX solution was between that of 9:1 NPs and 7:3 NPs groups. In addition, 8:2 NPs exhibited the best antitumor efficacy, since it possessed the optimal colloidal stability, cytotoxicity, and AUC among these four prodrug-SANPs. We further detected the lung metastasis of 4T1 tumor-bearing mice after five times treatment for each group (Fig. 9E). The visible metastasis sites were circled in white. Among them, 8:2 NPs treated group showed less lung metastasis nodules, and had good anti-metastasis ability. TUNEL assay and Ki-67 assay were used to investigate the cellular apoptosis and the cellular proliferation of tumor tissues (Fig. 9F). 8:2 NPs showed significant higher apoptosis and lower proliferation than other groups (Fig. 9G-9H). TUNEL assay and Ki-67 assay also indicating that 8:2 NPs had the best antitumor efficacy than other groups. Hence, small changes in the ratio of DSPE-mPEG2000 for PEGylated prodrug-SANPs have great influences on the antitumor efficacy.

In addition, we investigated the toxicity of DTX solution and the PEGylated prodrug-SANPs by measuring body weight, hepatorenal function, H&E staining, and spleen size in mice. The body weight of mice injected DTX solution showed significant decline, while there was no change in other mice injected prodrug-SANPs (Fig. 9F). The AST values of DTX solution group were abnormal high than saline group or any other groups (Fig. 9F), which indicated impaired of hepatic function. As shown in Fig. 9F, the lung metastasis in H&E...
staining was found in all groups, while 8:2 NPs treated group showed fewer lung metastasis nodules. Besides, the spleen of DTX solution and Pure drug NPs treated group was shrunken (Fig. 9G). 4T1 tumor acts in an endocrine fashion to stimulate myelopoiesis with the production and export of large numbers of granulocytes. The rapidly evolving granulocytosis would cause splenomegaly by massive granulocytic infiltrates [38]. Therefore, the spleens and granulocytes of our tumor model groups were all much larger than normal. Some research show that spleen weight is positively correlated with numbers of granulocytes in 4T1 tumor-bearing mice [39]. The granulocytes of DTX solution and Pure drug NPs were significantly lower than other groups (Fig. S9) which was consistent with the trend of spleen weight. The low granulocytes indicated that DTX solution and Pure drug NPs could cause myelosuppression [40].

4. Conclusion

To investigate the influence of the amount of PEG on the self-assemble ability and drug delivery efficiency of prodrug-SANPs, a new oxidation sensitive DTX dimeric prodrug was co-assembled with different ratios of PEG ($W_{prodrug}/W_{DSPE-mPEG2000} = 10:0, 9:1, 8:2, \text{ and } 7:3$). We found that the amount of PEGylation significantly impacted the stability of prodrug-SANPs. When the mass ratio of prodrug to PEG was 8:2, the most compact and stable prodrug-SANPs were formed. In addition, 8:2 NPs possessed efficient cellular uptake efficiency and strong cytotoxic activity, which were comparable to DTX solution. The ratios of PEG also significantly impacted the in vivo fate of prodrug-SANPs. The 8:2 NPs exhibited higher AUC and much better antitumor efficacy than other prodrug-SANPs and even commercial product. Our findings demonstrated the pivotal role of the amount of PEG on prodrug-SANPs.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.02.002.

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