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Tumor-Targeted Drug Delivery and Sensitization by MMP2-responsive Polymeric Micelles

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Abstract

Low tumor specificity and multidrug resistance (MDR) remain challenging for many anticancer drugs. In this study, the micelles assembled by a matrix metalloproteinase 2 (MMP2)-sensitive self-assembling efflux inhibitor (PEG2k-pp-PE) were developed and evaluated in various cancer models. In vitro, the PEG2k-pp-PE micelles enhanced the cellular uptake and tissue penetration and sensitized the cancers to drug treatments in MDR cancer cells and their three-dimensional multicellular spheroids. Their efflux inhibitory capability was comparable to those of the well-known small-molecule P-glycoprotein (P-gp) inhibitor and polymeric P-gp inhibitor. In vivo, the PEG2k-pp-PE micelles could specifically and effectively deliver the loaded cargoes to the tumor, as evidenced by the enhanced drug accumulation and prolonged drug retention in the tumor tissue, resulting in the improved anticancer activity. Our results suggest that the PEG2k-pp-PE micelles may have great potential to be a simple but multifunctional nanocarrier for concurrent tumor-targeted drug delivery and sensitization of resistant cancers.

Graphical abstract

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No conflict of interest.

Supplementary data

Supplementary data to this article can be found online at

A simple, multifunctional micellar nanoparticle assembled by a matrix metalloproteinase 2-sensitive polymeric efflux inhibitor, PEG2k-pp-PE, can simultaneously deliver the drug, target the tumor, and sensitize the resistant cancer to drug treatment. The assembled PEG2k-pp-PE micelles significantly improve tumor targetability and decrease drug efflux/clearance of the loaded drugs, resulting in the enhanced anticancer activity.

Keywords

stimuli-responsive drug delivery; tumor targeting; multidrug resistance; polymeric micelle; nanoparticle

Introduction

Over the past few decades, nanoparticle-based systems have showed promise in drug delivery, as evidenced by several marketed nanomedicines, while “passive” tumor targeting and slow drug release compromise their clinical outcomes. Matrix metalloproteinase 2 (MMP2), a major extracellular enzyme involving in cancer initiation, growth and metastasis, is upregulated in numerous cancers.¹ MMP2 has been a biomarker and therapeutic target for cancer diagnosis and treatment.^{2, 3} Recently, MMP2 has been used as a tumoral stimulus for “on-demand” drug delivery and tumor targeting.⁴

Though a lot of effort has been made, multidrug resistance (MDR) remains challenging in cancer treatment. A well-established cause of the MDR involves the overexpression of the ATP binding cassette (ABC) transporters.⁵ Among them, the P-glycoprotein (P-gp, also known as ABCB1) induced MDR greatly jeopardizes the clinical outcome of anticancer drugs and is one major reason responsible for the failure of chemotherapy and molecularly targeted therapy.⁵

Combination drug therapy is currently considered the most effective approach to circumvent drug resistance.⁵ Concurrent use of anticancer drugs and P-gp inhibitors has been investigated to reduce the MDR. Although various small molecule P-gp inhibitors have been discovered,⁶ concerns exist regarding their toxicity, poor physicochemical properties, low tumor specificity, and undesirable influence on the pharmacokinetic (PK) of their P-gp substrates, i.e. the co-administered drugs.⁷ Furthermore, co-delivery of drugs and P-gp

inhibitors is complicated and may cause additional side effects.^{7, 8} Recent studies showed that some pharmaceutical polymers could influence the drug efflux. In particular, the D- α -tocopherol polyethylene glycol (1k) succinate (TPGS),⁹ and Pluronic copolymers¹⁰ have been investigated as the excipients in drug formulations and nanoparticles to overcome the P-gp induced drug resistance. However, due to the absence of “active” targeting mechanisms, these nanomaterials target the tumor merely via the enhanced permeability and retention (EPR) effect.

We have developed various MMP-sensitive copolymers or conjugates and found their potential as the nanocarriers for stimuli-sensitive drug delivery and tumor targeting.^{11–14} Recently, we synthesized a series of homologous analogues of polyethylene glycol - MMP2-cleavable peptide - phosphatidylethanolamine (PEG-pp-PE) copolymers and revealed the relationship between their chemical structure – activity against the P-gp-induced drug efflux in MDR cancer cells.¹⁵ However, most of the previous studies were focused on the nanoparticle preparation and in vitro evaluation. To be a nanocarrier, whether the PEG-pp-PE copolymers can specifically deliver drugs to the tumor and overcome the MDR at the same time is unknown. In this study, the MMP2-sensitive polymer (PEG2k-pp-PE), the most effective one regarding efflux inhibitory capability¹⁵ was used to construct the micellar nanoparticles. The PEG2k-pp-PE micelles were expected to have three roles, the drug delivery, tumor targeting, and sensitization of resistant cancer cells to drug treatments. Here, both the chemotherapy drug, doxorubicin hydrochloride (DOX, as a water-soluble fluorescent indicator), and molecularly targeted therapy drug, dasatinib (DSB, a water-insoluble molecule that can be loaded to micelles), were used as the model drugs and P-gp substrates.^{12, 14, 16} In addition, the commercially available nonsensitive polymer (PEG2k-PE), small molecule P-gp inhibitor (verapamil, VRP), and polymeric P-gp inhibitor (TPGS) were used as the controls. The cellular uptake, tissue penetration, and cytotoxicity of the PEG2k-pp-PE micelles were evaluated in various cancer cells and their 3D multicellular spheroids. The in vivo biodistribution, tumor targeting, and anticancer activity of the micelles were evaluated on the tumor-bearing mice.

Methods

Materials, cell cultures and animals, micelle characterization, establishment of cell spheroids and animal model, and statistical analysis are described in Supplementary Data.

Preparation of the DSB-loaded micelles.

PEG2k-pp-PE was synthesized and characterized as our previous reports.^{13, 15} The drug-loaded

PEG2k-pp-PE micelles (PEG2k-pp-PE/DSB) were prepared by a thin-film hydration method. Briefly, DSB and PEG2k-pp-PE were co-dissolved in methanol and then dried to obtain a thin drug-polymer film under nitrogen flow, followed by hydration with HBSS. The untrapped DSB was removed by a 0.45 μ m syringe filter.^{12, 17, 18} The entrapped DSB was quantitated by HPLC.¹²

Cellular uptake in cancer cell monolayers.

The MDR (NCI/ADR-RES and MES-SA/Dx5) or sensitive (4T1) cancer cells were seeded in 24-well plates (1×10^5 cells/well) 24 h before the treatment. The cells were pre-incubated with polymers or VRP for 0.5 h, followed by incubation with $2 \mu\text{g} \cdot \text{mL}^{-1}$ DOX for additional 2 h. For the fluorescence-activated cell sorting (FACS), the cells were trypsinized and harvested by centrifugation (2000 rpm for 4 min). The cells were suspended in 200 μL of PBS and analyzed on a BD Accuri™ C6 flow cytometer. For confocal microscopy, the cells were fixed by 4 % paraformaldehyde and stained with $2 \mu\text{M}$ of Hoechst 33258 for 1 min, followed by observation on a Nikon eclipse 80i confocal microscope system.

To study the cellular process, PEG2k-pp-PE was labeled by FITC at the PEG terminus and mixed with unlabeled PEG2k-pp-PE for use. To study the effect of the MMP2 cleavage on the efflux inhibition, the polymers were pretreated with MMP2 at 37°C overnight to ensure the complete cleavage.

DSB uptake in cancer cell monolayers.

The NCI/ADR-RES cells were seeded in 12-well plates (2×10^5 cells/well) 24 h prior to the treatment. The cells were pre-incubated with polymers or VRP for 0.5 h, followed by incubation with $10 \mu\text{M}$ DSB for additional 2 h. For the PEG2k-pp-PE/DSB micelles, the cells were only incubated with the micelles for 2 h. After treatments, the cells were washed with PBS for four times to remove any extracellular DSB. Then, the cells were lysed by 1% Triton X-100 and 150 μL of the cell lysate was mixed with 350 μL DMSO/acetonitrile (1/2, v/v) by sonication for drug extraction and protein precipitation. The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was measured by HPLC.¹²

Inhibition of drug efflux in cancer cell monolayers.

The NCI/ADR-RES cells were seeded in 24-well plates (1×10^5 cells/well) 24 h prior to the treatment. The cells were pre-incubated with high concentration ($20 \mu\text{g} \cdot \text{mL}^{-1}$) of DOX for 4 h. Then, the DOX-internalized cells were incubated with polymers or VRP for additional 24 h, followed by the FACS.

Cytotoxicity in cancer cell monolayers.

The NCI/ADR-RES, MES-SA/Dx5, and 4T1 cells were seeded in 96-well plates (3×10^3 cells/well) 24 h before the treatment. The free DSB, mixture (PEG2k-PE+DSB), mixture (PEG2k-pp-PE+DSB), PEG2k-pp-PE/DSB micelles, and mixture (VRP+DSB) were incubated with the cells for 48h. The cell viability was determined by the MTT assay.

Drug penetration through cancer cell spheroids.

The 4–5 day NCI/ADR-RES or 4T1 spheroids were pre-incubated with polymers or VRP for 0.5 h, followed by incubation with $10 \mu\text{g} \cdot \text{mL}^{-1}$ DOX for additional 2 h. After treatments, the spheroids were removed by pipet and gently washed with PBS. Then, the spheroids were observed under a confocal microscope. Z-stack images were obtained at an interval of 12.5 μm .

The fluorescence intensity was analyzed by ImageJ software. The mean fluorescence intensity was plotted against the distance from the spheroid periphery.

DSB uptake in cancer cell spheroids.

The NCI/ADR-RES spheroids were pre-incubated with polymers or VRP for 0.5 h, followed by incubation with 10 μ M DSB for additional 2 h. For the PEG2k-pp-PE/DSB micelles, the cells were only incubated with the micelles for 2 h. After treatments, the spheroids were gently washed with PBS three times. Then, the spheroids were lysed and the drug was extracted for HPLC quantitation by the same protocols used for monolayer cells.

Cytotoxicity in cancer cell spheroids.

The free DSB, PEG2k-PE+DSB, PEG2k-pp-PE+DSB, PEG2k-pp-PE/DSB, and VRP+DSB were incubated with the spheroids for 48h, respectively. The cell viability was determined by CellTiter-Blue[®] Cell Viability Assay (Promega).^{12, 19} Briefly, 20 μ L of reagent was diluted with 200 μ L of fresh medium and incubated with the cells at 37°C for 12 h. Thereafter, the fluorescence intensity was recorded at λ_{ex} 560 nm and λ_{em} 590 nm on a microplate reader.

Cancer spheroid growth inhibition.

In this experiment, the NCI/ADR-RES spheroids were established with a low initial cell density (5×10^3 cells/well). On day 3, the DSB formulations were added to the spheroids and incubated for additional 12 days. The morphology and size of the spheroids were observed under a confocal microscope. To have an accurate measurement, the area rather than diameter of the spheroids in the micrographs was measured.²⁰

Drug retention in the tumor.

When the 4T1 tumor volume was around 300 mm³, 100 μ L of DOX solution (5 mg·kg⁻¹) or mixture of DOX and polymers was injected directly into the tumor site (intratumoral injection, i.t.). The images (DOX fluorescence) were determined at 12 and 24 h post-injection by the Carestream image station system FX Pro.

Biodistribution and tumor targeting.

The DiR was used to substitute drug for near infrared (NIR) fluorescence imaging. When the 4T1 tumor reached around 300 mm³, 100 μ L of DiR (0.5 mg·kg⁻¹) or DiR-loaded micelles were intravenously (i.v.) injected via the mouse tail vein. The whole-body NIR images were determined at 2, 4, 8, 12, and 24 h post-injection. Then, the mice were sacrificed and the images of the major organs and tumors were monitored.

In vivo anticancer activity study.

As the 4T1 tumor reached around 100 mm³, the saline, DSB solution, or DSB-loaded micelles were injected intravenously via the tail vein at a dose of 5 mg·kg⁻¹ every other day for 5 days (3 injections in total). The tumor size and mouse body weight were monitored. On day 12 after the first injection, the mice were sacrificed and the major organs and tumors were collected. The tissue sections were prepared for the hematoxylin-eosin (H&E) staining.

All animal experiments were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University.

Results

Preparation and characterization of the PEG2k-pp-PE micelles.

Due to the excellent self-assembly capability,¹⁵ the PEG2k-pp-PE polymers self-assembled to a 100 nm micelle with a spherical shape and smooth surface, as evidenced by the dynamic light scattering (DLS) and transmission electron microscopy (TEM) results (Figures 1A–B). DSB was readily loaded into the PEG2k-pp-PE micelles, with the drug loading and encapsulation efficiency of 3.8 ± 0.1 % (w/w) and 76.4 ± 1.6 %, respectively. The drug encapsulation did not significantly change the micelles' particle size. The PEG2k-pp-PE micelles were near neutral (Figure 1C) due to the outer PEG corona. The fluorescence resonance energy transfer (FRET) results showed that the PEG2k-pp-PE micelles were stable in PBS while they were dissociated in the presence of MMP2 (Figures 1D), indicating that the stability of the PEG2k-pp-PE micelles was MMP2-dependent.¹⁵ The micelle dissociation was also confirmed by the micelle size in the presence of MMP2 (Figure 1A). In PBS, the PEG2k-pp-PE micelles could sustainably release the loaded DSB (65% at 24h vs. 100% at 4 h of free DSB). However, in the presence of MMP2, the drug release was significantly increased to around 88% at 24h (Figure 1E), in consistent with the micelle stability data. The mechanism of the MMP2-responsive micelle dissociation and drug release was described in Figure S1.

Drug uptake and efflux in cancer cell monolayers.

Both DSB and DOX·HCl (a water-soluble salt, only used as a fluorescent probe) are well-studied anticancer drugs as well as P-gp substrates.^{16, 21} The cellular uptake, efflux inhibition and cytotoxicity of the PEG2k-pp-PE micelles were determined in the MDR cancer cells (NCI/ADR-RES and MES-SA/Dx5)¹⁹ (Figure S2) as well as drug-sensitive cancer cells (4T1).²² To better evaluate the efflux inhibition effect, the nonsensitive polymer (PEG2k-PE) and well-known P-gp inhibitors, VRP (small-molecule inhibitor)²³ and TPGS (polymeric inhibitor),⁹ were used as the controls.

To study the effect of PEG2k-pp-PE on the DOX uptake, the cells were incubated with the polymers, followed by incubation with DOX. In the MDR cells, the free DOX or PEG2k-PE +DOX showed low cellular uptake, while the P-gp inhibitors (VRP, TPGS, and PEG2k-pp-PE) showed much stronger fluorescence, indicating that the P-gp inhibitors facilitated the DOX uptake (Figure 2 A–B and S3A). However, in the 4T1 cells which express low level of P-gp and are sensitive to DOX,²² no significant difference in the DOX uptake was observed among all treated groups (Figure S3B). After cell internalization, DOX was accumulated in cell nuclei (Figure 2A). In addition, the tested P-gp inhibitors could increase the DSB uptake in the NCI/ADR-RES cells (Figure 2C). It is worth noting that the drug-loaded micelles (PEG2k-pp-PE/DSB) had the similar drug uptake compared to their physical mixture (PEG2k-pp-PE+DSB), indicating that the drug encapsulation in the micelles didn't influence the polymers' efflux inhibitory capability.

Though the aforementioned drug “uptake” study is widely used to evaluate drug delivery systems, it tests the overall/combined effect of the drug efflux and uptake. To clarify the polymer’s efflux inhibitory capability, the DOX-internalized NCI/ADR-RES cells were used as the model (Figure 2D–E). After 4 h DOX pre-incubation, the cells (0 h) obtained high fluorescence intensity (~42000). Further incubation with PBS substantially lowered cellular fluorescence (~12000, at 24 h), indicating the DOX efflux. PEG2k-PE could not inhibit the DOX efflux and showed the similar result as PBS, while both PEG2k-pp-PE and VRP considerably inhibited the DOX efflux (~28000 and ~36000, respectively, at 24 h). Here, TPGS was not used as a control because of its toxicity after the long-term incubation (Figure S4A).

To further understand the cellular process, the PEG-pp-PE was labeled with FITC at the PEG terminus. We found that FITC-PEG-pp-PE could enter the MDR cells and inhibited the DOX efflux, as evidenced by the intracellular green (of polymers) and red (of DOX) fluorescence, while after the complete cleavage by the exogenous MMP2, both green and red fluorescence were gone (Figure 2F). Here, the endogenous MMP2 secreted by cancer cells was not sufficient to cleave all PEG2k-pp-PE polymers in a short time, but the action was able to trigger the rapid micelle dissociation and release of the remaining, intact polymers from the micelles (Figure 1F), ensuring the polymers’ uptake and function. In contrast, the overnight incubation at high MMP2 concentration could completely cleave the polymers, resulting in the loss of their efflux inhibitory capability. The data suggested that the intact polymer structure (PEG-pp-PE) is key to manipulate the drug efflux¹⁵ and the polymer most likely entered the cells to exert this action.

Cytotoxicity in cancer cell monolayers.

Since the TPGS alone showed a significant toxicity (Figure S4A–C), the combination of DSB with TPGS was not tested in the cytotoxicity study. In the MDR cell monolayers, the VRP+DSB, PEG2k-pp-PE+DSB, and PEG2k-pp-PE/DSB showed higher cytotoxicity compared to the free DSB and PEG2k-PE+DSB, while no much difference in the IC₅₀ was observed between free DSB and PEG2k-PE+DSB (Table 1). However, in the sensitive cells (4T1), all DSB treatments had the comparable IC₅₀ values. The drug-loaded micelles had the similar cytotoxicity as the mixture of drugs and polymers, suggesting that the PEG2k-pp-PE micelles might be capable of concurrent drug delivery and sensitization of cancer cells.

Drug penetration and uptake in 3D cancer cell spheroids.

The drug penetration through the cell spheroids was evaluated using DOX as a fluorescent probe. In the NCI/ADR-RES spheroids (Figure 3A), much stronger fluorescence was observed in the P-gp inhibitor-treated groups compared to the groups treated with free DOX or PEG2k-PE+DOX. The P-gp inhibitors including PEG2k-pp-PE markedly improved the DOX penetration, as evidenced by the overall DOX uptake (area under curve) and penetration depth [the height of peak and the distance from the periphery (0 μ m) to the peak] (Figure 3B). The penetration data were also confirmed by the DSB uptake in the spheroids (Figure 3C). Interestingly, PEG2k-pp-PE and other P-gp inhibitors significantly enhanced the DOX penetration in the 4T1 spheroids, compared to the free DOX and PEG2k-PE+DOX (Figure S5), suggesting that once forming the 3D culture, the sensitive 4T1 cells became

resistant to the DOX treatment,²² which is so called “multicellular resistance”.^{24, 25} The results indicated that the P-gp inhibitors including PEG2k-pp-PE could inhibit the drug efflux/resistance in the cancer multicellular spheroids.

Cytotoxicity in 3D cancer cell spheroids.

TPGS was not evaluated in cell spheroids due to its high toxicity (Figure S4D–F). The free DSB showed much lower cytotoxicity in the spheroids than in the monolayers, confirming the increased drug resistance of the 3D cultures.²⁵ In particular, the 4T1 cells were sensitive to DSB with an IC₅₀ of < 35 nM (Table 1), while the 4T1 spheroids were resistant with a ~75% cell viability at 1 μM DSB (Figure S6). The PEG2k-PE didn't influence the performance of DSB, while the PEG2k-pp-PE formulations (as either a mixture or micelle) significantly enhanced DSB cytotoxicity (Figures 3D and S6). The effect of PEG2k-pp-PE were comparable with that of VRP in all tested spheroids.

Inhibition of cancer spheroid growth.

In the absence of DSB, the cell spheroids were tightly organized and grew rapidly, while the obvious growth inhibition was observed in all DSB-treated groups, confirming the DSB's tumor growth inhibition capability (Figure 3E). Particularly, on day 15, the growth of all DSB-treated spheroids were retarded (with around 70% – 107% of their initial sizes), while the sizes of the untreated spheroids were almost doubled (Figure 3F). Among them, the DSB combining with VRP or PEG2k-pp-PE showed the greatest growth inhibition effects.

In vivo drug clearance from the tumor.

To study if PEG2k-pp-PE could inhibit the in vivo drug efflux, the DOX clearance from the 4T1 tumor site was analyzed upon the intratumoral (i.t.) injection (Figure 4A–C). The fluorescence of free DOX could be only observed in the tumor site within 12 h and was completely gone after 24 h. In contrast, the polymers inhibited DOX clearance from the tumor. The fluorescence intensity of PEG2k-pp-PE+DOX was ~30% stronger at 12h and ~100% stronger at 24h than those of PEG2k-PE+DOX, respectively. Moreover, PEG2k-pp-PE effectively inhibited the DOX clearance from the tumor site and its DOX signal at 24 h was almost as strong as that at 12 h. The results clearly indicated that PEG2k-pp-PE could inhibit not only the drug efflux of cancer cells and spheroids but also the drug clearance from the tumor tissues under the vivo condition.

In vivo biodistribution and tumor targeting.

The DiR (a NIR dye)-loaded micelles were injected via the mouse tail vein in the tumor-bearing mice and analyzed by the animal imaging. The free DiR was rapidly cleared from the body and showed negligible signal even at 2 h upon injection. In contrast, the polymeric micelles (PEG2k-PE/DiR or PEG2k-pp-PE/DiR) showed much stronger overall fluorescence, probably due to the PEG-mediated prolonged blood circulation.¹² Compared with the PEG2k-PE micelles, the PEG2k-pp-PE micelles were rapidly and preferentially accumulated in the tumor site (Figure 4D). The ex-vivo images showed that the PEG2k-pp-PE micelles had higher tumor targeting capability than the free DiR or PEG2k-PE micelles (Figure 4E–F).

In vivo anticancer activity.

To evaluate the in vivo anticancer activity, the DSB-loaded micelles were injected intravenously at $5 \text{ mg} \cdot \text{kg}^{-1}$ DSB for three times in the 4T1 tumor-bearing mice. All DSB formulations could inhibit the tumor growth (Figure 5A–C). Compared to the free DSB, the polymeric micelles exhibited much stronger tumor growth inhibition effect and the strongest antitumor activity was observed with the PEG2k-pp-PE/DSB micelles. No significant changes in the body weight among the treated mice were observed (Figure 5D), confirming that DSB, as a targeted therapy drug, had higher tolerance than chemotherapy drugs.²⁶ The H&E staining (Figure 5E) indicated that the vital organs except the liver had no histological change after treatments, while the DSB formulations caused significant cell death/necrosis in the tumor sections. In the control (saline) group, the s.c. 4T1 tumor generated metastatic foci in the liver, one of the major organs of 4T1 tumor metastasis.²⁷ The free DSB or PEG2k-PE/DSB decreased the number of tumor metastasis, but they could not completely inhibit the metastasis. In contrast, the metastatic foci were completely disappeared after the PEG2k-pp-PE/DSB treatment.

Discussion

Due to the MMP2 overexpression in numerous cancers, the MMP2-responsive approach may be a great strategy for “pan-cancer” targeting.⁴ The P-gp inhibitors have been investigated in combination with anticancer drugs to treat the MDR cancer. However, low tumor specificity, undesirable side effects, and the complexity of delivery systems may impair drug development and clinical outcome.^{7, 8} In our recent studies, a series of MMP2-sensitive efflux inhibitors, PEG-pp-PE, have been synthesized. Among them, PEG2k-pp-PE was the most effective one^{13, 15} and was used to construct the polymeric micelles in this study.

DSB, a hydrophobic tyrosine kinase inhibitor, was approved for treating leukemia and was also investigated for other types of cancer. Currently, DSB is given via oral route and no tumor-targeted DSB nanomedicines are available. Recent reports indicated that DSB was a substrate of various efflux transporters, including P-gp, and underwent drug resistance.^{12, 16} In our design, the MMP2-responsive PEG2k-pp-PE micelles ensured both the long circulation of the DSB-loaded micelles in the bloodstream at a low MMP2 level²⁸ and the drug accumulation and release in the tumor at high MMP2 level.

The PEG2k-pp-PE could effectively inhibit the drug efflux and improved drug efficacy in MDR cells when used as a co-administered efflux inhibitor or as a micellar drug nanocarrier. The efflux inhibitory capability of PEG2k-pp-PE was comparable to those of VRP and TPGS. Among the tested polymers, interestingly, the PEG2k-PE somehow inhibited drug uptake (Figure 2B–C). It might be understandable. At high polymer concentration, more PEG2k-PE polymers were able to anchor on the cell surface with the protruding PEG2k, instead of complete internalization, compromising cell internalization.²⁹ In contrast, the MMP2-sensitive sheddable PEG of PEG2k-pp-PE^{13, 15} could minimize its negative impact on cell functions.

The influence of the tumor microenvironment on drug resistance and cancer treatment has been acknowledged.^{24, 25} The distance for a drug molecule to travel in the tumor tissue with multiple layers of cells is much shorter than in the cell monolayer. In addition, the architecture heterogeneity, nutrient and oxygen gradients, and cell-cell/cell-matrix interactions of the tumor are highly associated with the tumor's aggressive behavior, including the MDR.^{24, 25} In this study, we found that, regardless of the mechanisms of resistance, in both cell monolayers and spheroids, the PEG2k-pp-PE polymers and micelles could inhibit drug (DOX or DSB) resistance as effectively as the commercial P-gp inhibitors, ensuring the anticancer activity.

To study the in vivo performance, we developed the s.c. syngeneic 4T1 tumor in the BALB/c mouse because (i) the BALB/c mouse is immunocompetent and allows us to study the influence of the immune system on drug delivery;³⁰ (ii) the 4T1 cells overexpress MMP2,^{12, 31} and (iii) the drug resistance in 4T1 cells is inducible via the formation of the tumor (Figure S5). In this model, the PEG2k-pp-PE micelles had high tumor targeting capability compared to nonsensitive PEG2k-PE micelles. The tumor targeting effect might be a result of (i) the PEG's stealth effect; (ii) the EPR effect of the 4T1 solid tumor;^{32, 33} (iii) the MMP2-responsive micelle dissociation and drug release (Figure 1); and (iv) the inhibited drug clearance from the tumor (Figure 4A–C).

It has been reported that DSB given either orally or intravenously only moderately inhibited the 4T1 tumor growth.¹⁸ The recent Phase II clinical trials of DSB also confirmed that DSB used as a single agent failed to exhibit significant anticancer activity against the metastatic breast cancer.³⁴ In addition, the acquired DSB resistance might be another reason of low drug efficacy.^{12, 16} In this study, after loading of DSB to PEG2k-pp-PE micelles, the DSB's in vivo efficacy was dramatically improved, as evidenced by the increased tumor growth inhibition and inhibited tumor metastasis. Taken all together, the PEG2k-pp-PE micelles could deliver the loaded drug to the tumor and sensitize the tumor to the drug treatment at the same time, resulting in strong anticancer activity.

In conclusion, the drug delivery, tumor targeting, and anticancer activity of MMP2-sensitive PEG2k-pp-PE polymers and their self-assembled micelles were evaluated on cancer cells, multicellular spheroids, and tumor-bearing mice. The results indicated that the PEG2k-pp-PE polymers and micelles could inhibit the drug efflux, facilitate cellular uptake and penetration, and increase drugs' tumor targeting and retention, leading to the improved anticancer activity. The PEG2k-pp-PE micelles might have great potential to be a multifunctional nanocarrier for effective cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MDR	multidrug resistance
MMP2	matrix metalloproteinase 2
P-gp	P-glycoprotein
ABC	ATP binding cassette
PK	pharmacokinetic
TPGS	D- α -tocopherol polyethylene glycol (1k) succinate
EPR	enhanced permeability and retention
PEG-pp-PE	polyethylene glycol - MMP2-cleavable peptide – phosphatidylethanolamine
DOX	doxorubicin hydrochloride
DSB	dasatinib
VRP	verapamil
FACS	fluorescence-activated cell sorting
H&E	hematoxylin-eosin
DLS	dynamic light scattering
TEM	transmission electron microscopy
FRET	fluorescence resonance energy transfer

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Figure 1.

Physicochemical properties, stability and drug release of the PEG2k-pp-PE micelles. (A) Particle size (DLS), (B) morphology (TEM), and (C) zeta potential of the micelles ($1 \text{ mg} \cdot \text{mL}^{-1}$); (D) Micelle stability determined by the FRET; (E) In vitro accumulative drug release at 37°C . ($n=3$)

Figure 2.

Drug uptake and efflux in the NCI/ADR-RES cell monolayer. DOX·HCl (a soluble salt) uptake determined by (A) confocal microscopy and (B) flow cytometry. (C) DSB uptake determined by HPLC. Inhibition of DOX efflux determined by (D) confocal microscopy and (E) flow cytometry. (F) Cellular uptake of FITC-PEG2k-pp-PE and DOX. To completely cleave the FITC-PEG2k-pp-PE, the polymer was pretreated with MMP2 at 37 °C overnight. Panels A, B, C and F: Cells were incubated with polymers or VRP for 0.5h, followed by incubation with DOX for 2 h. Panels D and E: Cells were incubated with DOX for 4 h, followed by incubation with polymers or VRP. The scale bar represents 50 μm . Data were expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (n=3)

Figure 3.

Drug penetration and efficacy in the NCI/ADR-RES cell spheroids. (A) Z-stack images (confocal microscopy) of the spheroids and (B) the fluorescence intensity vs. penetration distance curves after 2 h incubation with DOX formulations. (C) DSB uptake after 2 h incubation with DSB formulations. (D) Cytotoxicity of DSB formulations. (E) Micrographs and (F) growth profile of the cell spheroids after adding 2 μM of DSB formulations on day 3. The scale bar represents 200 μm . Data were expressed as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (n=3)

Figure 4.

In vivo drug clearance and tumor targeting in the 4T1 tumor-bearing mice. (A) Whole-body imaging, (B) ex-vivo tumor imaging, and (C) quantitation of ex-vivo images upon intratumoral (i.t.) injection of the DOX formulations. (D) Real-time whole-body, (E) ex-vivo imaging (at 24 h), and (F) quantitation of ex-vivo images (at 24 h) upon intravenous (i.v.) injection of the DiR-loaded micelles through the mouse tail vein. The hair on mouse back including the tumor site was shaved to decrease its interference with the fluorescence signal. Data were expressed as mean $\pm$ SD. *p < 0.05; ***p < 0.001. (n=3)

Figure 5.

In vivo anticancer activity of the DSB-loaded micelles in 4T1 tumor-bearing mice. (A) Tumor growth curves. (B) Images and (C) weights of the excised tumors on day 12. (D) Mouse body weights. (E) H&E staining of the vital organs and tumors. The tumor metastatic foci were indicated in the liver sections. The necrotic areas were indicated in the tumor sections. Data were expressed as mean $\pm$ SD. *** $p < 0.001$. (n=5)

Table 1.

	NCI/ADR-RES (μM)	MES-SA/DX5 (μM)	4T1 (nM)
DSB	4.16±0.21	14.72±0.15	25.2111.73
PEG2k-PE+DSB	4.49±0.49	12.56±0.24	26.92±1.70
PEG2k-pp-PE+DSB	1.02±0.32	1.23±0.02	24.91±1.03
PEG2k-pp-PE/DSB	1.02±0.03	1.03±0.09	33.03±2.05
VRP+DSB	1.28±0.13	1.30±0.10	27.21±1.30