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Dynamic disordering of liposomal cocktails and the spatio-temporal favorable release of cargoes to circumvent drug resistance

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ABSTRACT

Multidrug resistance (MDR) has been a major impediment to the success of cancer chemotherapy. Extensive efforts have been devoted to the development of drug delivery systems using nanotechnology to reverse MDR in cancer. However, the spontaneous release of drug payloads was always a slow process, which leads to the low intracellular drug concentration resulting in consequent drug insensitivity. To circumvent this limitation, we described a liposomal cocktail (**LMDHV**) constructed by a pH-responsive molecule (i.e., malachite green carbinol base (MG)) and liposome conjugated with Her-2 antibody for codelivery of doxorubicin (DOX) and verapamil (VER) to suppress drug resistance in Her-2 positive breast cancer. MG inserted in the bilayer as pH responders greatly contributed to the destabilization of the vesicle membrane in low pH, followed by the rapid release of the payloads. **LMDHV** showed 6-fold reversal efficiency in DOX resistant breast cancer owing to the efficient tumor targeting delivery and rapid burst release of drug intracellularly. Compared to tumor inhibition ratio of treated groups by free DOX (32.4 ± 7.4%), our designed kinetically favorable drug release system exhibited significantly ($P < 0.01$) enhanced tumor inhibition ratio up to 83.9 ± 12.5%, which is attributed to the remarkably increased drug concentration in cells. The spatio-temporal favorable release of drugs resulted in synergistic inhibition of tumor growth in xenografts. We envision that this new type of liposomal cocktail might be potentially utilized to circumvent drug resistance in the future.

1. Introduction

Multidrug resistance (MDR) remains one of the main challenges in the successful chemotherapy of human cancer [1–4]. Although a combination of chemotherapeutic agents are often used to prevent drug resistance in cancer patients, the ability of the cancer cells to adapt and develop one or more drug resistance pathways ultimately leads to treatment failure in most cancers [5–7]. The common underlying mechanism of MDR has been correlated with the overexpression of the ATP binding cassette (ABC) transporters, particularly P-glycoprotein (P-gp), leading to the efflux of many anticancer drugs with consequent drug insensitivity [8–10]. P-gp, over-expressed in the malignant tissues, is a transmembrane protein functioning as pumps that extrude toxins and drugs out of the cells [11,12]. Much effort has been directed towards developing drugs that either evade efflux or suppress the function of P-gp to reverse MDR and hence restore the sensitivity of resistant cancer cells to multiple anticancer drugs [13–15]. Some small-molecule compounds, such as verapamil (VER) and cyclosporine A, were used as functional P-gp inhibitors [16,17]. Also, it was reported that the chemotherapeutic agents encapsulated into nanoparticles could evade the drug efflux pumps and increase the intracellular drug concentration [18–23].

The physical treatment strategies, e.g., photothermal [24,25], photodynamic [26,27] and hyperthermia [28,29] have been reported to overcome the MDR. The non-specific damage of normal cells around solid tumor limited their clinical applications [30–34]. Recently, intensive studies indicated that custom tailored drug carriers with encapsulated drug combination showed the potential to treat ABC transporter-mediated MDR [35,36]. Liposomal vehicle-based delivery systems [37,38] are expected as one of the most promising platform for clinical cancer treatment owing to their merits on longer blood circulation time, physiological stability, tumor targetability and low cytotoxicity. However, drug release from both conventional and “stealth” liposomes is a slow process,

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which leads to the low intracellular drug concentration resulting in consequent drug insensitivity [39–41]. To increase the effective intracellular drug content, we attempt to co-assemble pH-responsive molecules with liposomes that will simultaneously disrupt the lipid membrane and burst release the loaded drugs upon external stimuli. Previously, we described a co-assembly constructed by a pH-responsive molecule (i.e., malachite green carbinol base (MG)) and liposome for highly efficient doxorubicin (DOX) release in tumor cells. MG insertion in the bilayer was capable of triggering efficient drug release and the increase intracellular drug concentration [42].

Herein, we demonstrate a liposomal cocktail containing MG, DOX and VER to circumvent P-gp mediated drug resistance in breast cancer treatment. The MG is capable of simultaneously converting from the neutral formation to positive charged MG carbocation (MG^+) in acidic pH condition [43,44], which consequently disturbs the charge balance of liposomal cocktail and lead to the dynamic disordering of membrane and rapid release of the payloads (Scheme 1). The liposomal cocktail with Her-2 antibody (**LMDHV**) as a targeting ligand can be internalized by breast cancer cells *via* Her-2 antibody receptor-mediated endocytosis pathway and accumulated in the lysosomes to enhance the spatial enrichment of drugs. Subsequently, the liposomal cocktail in the acidic environment of lysosomes burst and quickly released drugs (Scheme 2). The increased intracellular accumulation of drugs in DOX-resistance breast cancer cells (MCF-7/DOX) was attributed to P-gp pump suppressed by VER for evading DOX efflux and pH-responsive intracellular release of DOX from the break-up liposomal cocktail. Near-infrared dye labeled liposomal cocktail was subjected to optical imaging to evaluate the biodistribution *in vivo* and the tumor accumulation. Subsequently, anti-tumor ability of **LMDHV** on xenograft model bearing MDR tumor was also investigated and the results indicated the enhanced therapeutic efficacy and minimal side effects. We expect our responsive drug encapsulated liposomal cocktail to greatly improve efficacy of reverse drug resistance in the near future.

2. Materials and methods

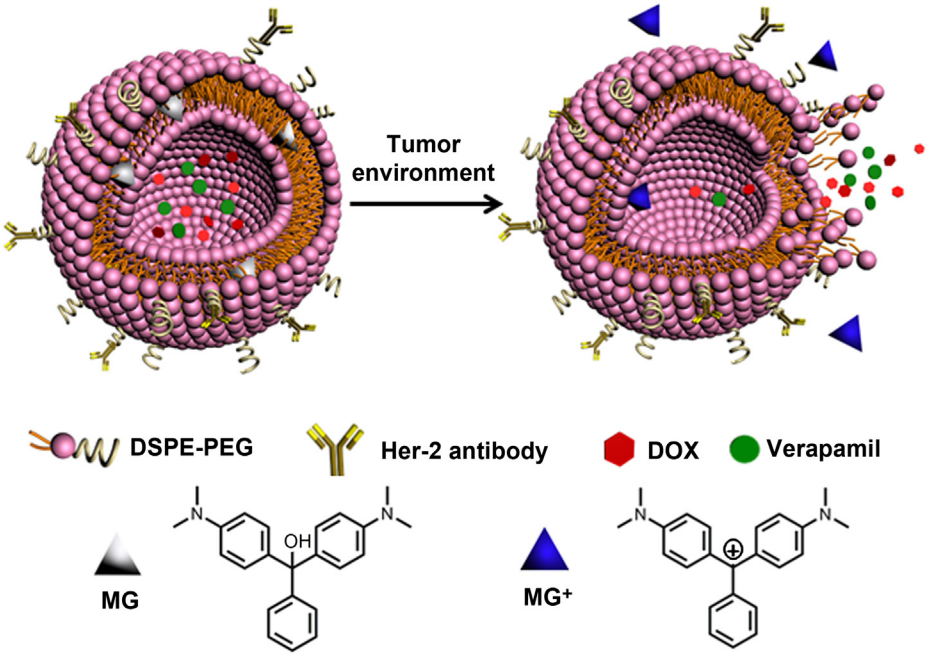
2.1. Materials

L-α-Phosphatidylcholine(PC),1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP),cholesterol(CH),1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N[amino(polyethyleneglycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids. 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[NHS(polyethyleneglycol)$_{2000}$] (DSPE-PEG-NHS) was purchased from Nanocs. Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, phosphate-buffered saline (PBS), fetal bovine serum (FBS), trypsin were obtained from HyClone/Thermo fisher (Beijing, China). MCF-7/DOX and MCF-7 cell line were purchased from Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Doxorubicin hydrochloride (DOX), Hochest 33342, Verapamil hydrochlorid (VER), Her-2 antibody and Malachite green carbinol base (MG) were purchased from Sigma–Aldrich. Cell counting kit assay (CCK-8) was obtained from Beyotime institute of biotechnology (Shanghai, China). 96-well coning culture plates were purchased from Coning Company. BALB/c nude mice (Female, 16–18 g) were purchased from Department of Laboratory Animal Science, Peking University Health Science Center, and all animal experiments were conducted by its center. A squaraine (SQ) fluorophore with near-infrared emission was obtained from Prof. Würthner. All the other solvents used in this research were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd.

2.2. Preparation and characterization of liposomes

2.2.1. Preparation of liposomes

Liposomes were prepared by a thin lipid film hydration method followed by extrusion. Briefly, PC: CH: DOTAP: DSPE-PEG: DSPE-PEG-NHS = 62: 30: 5.5: 2: 0.5 (mol ratio) were dissolved in a chloroform/methanol mixture (4: 1) in a round-bottom flask. To engineer pH-responsive liposomes (**MG⊂L**), MG (0 or 4.5 mol%) dissolved in methanol was added to the lipid mixture before formation of the lipid film. After evaporation of the organic solvents, the lipid film was hydrated with acetate buffer (pH 5.0). In this case, MG has been already carbocationized as an MG^+ form. Following hydration, small unilamillar liposomes were obtained by extrusion through 200 nm and 100 nm polycarbonate membranes filters 5 times. DOX and VER solution with the same concentration (5 mg mL^{-1}) were added to the co-assembly and the external phase of the liposomes was replaced with PBS (pH 8.0). The co-assembly was incubated at 50 °C for 2 h to allow drugs loading. Then the MG^+ returned to hydrophobic form (MG) and embedded into the bilayer of liposomes. Obtained drug-loaded pH-responsive liposomes were dialyzed in PBS (pH 7.4) using a Slide-A-Lyzer dialysis cassette (MWCO 20 kDa) for 12 h at RT to remove excess drugs. Her-2 antibody (1 mg mL^{-1}) was added to the liposomes (**LMDHV**) with gentle stirring for 12 h. Size and surface charge of liposomes were measured by using Zetasizer Nano ZS (Malvern Instruments Ltd).



Scheme 1. Illustration of a co-assembled liposomal cocktail as a spatio-temporal favorable drug delivery system. The neutral malachite green carbinol base (MG) was transformed to its carbocationic form in the low pH values, which directly resulted in the rapid and complete disordering of co-assembly.