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Reversing multidrug resistance by intracellular delivery of Pluronic® P85 unimers

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ABSTRACT

Pluronics have been demonstrated as excellent multidrug resistance (MDR) reversal agent in the form of unimers rather than micelles. However, the effective intracellular delivery of Pluronic® unimers to MDR cancer cells still remains a big challenge. To address this issue, a mixed micellar system based mainly on the pH-sensitive copolymer of poly (L-histidine)-poly (D,L-lactide)-polyethyleneglycol-poly (D,L-lactide)-poly (L-histidine) (PHis-PLA-PEG-PLA-PHis) and Pluronic® F127, some of which was conjugated with folate, was constructed to intracellularly deliver the unimers of Pluronic® P85 to MDR cells. The folate-mediated endosomal pH-sensitive mixed micelles (pH_{endo}SM-P85/f) were prepared by a thin-film hydration method, by which Pluronic® P85 unimers and doxorubicin (DOX) were incoporated into the mixed micelles. The incorporation of Pluronic® P85 unimers was investigated by the surface tension test. The results indicated that the Pluronic® P85 unimers probably first inserted into the binary mixed micelles and then formed a triple-component mixed micelles with Pluronic® F127 and PHis-PLA-PEG-PLA-PHis as the loading content increased. Further analyzed with flow cytometry, confocal laser scanning microscopy (CLSM) and MTT assay, the micelles with inserted Pluronic® P85 unimers demonstrated much more cellular uptake and higher cytotoxicity against MDR cells than the triple-component mixed micelles and plain Pluronic® micelles. The enhanced MDR reversal effect was attributed to the successful intracellular delivery of Pluronic® P85 unimers to the MDR cells, which was confirmed by the subcellular colocalization of Pluronic® P85 unimers with mitochondria, the decreased ATP energy and mitochondrial membrane potential (MP) in the MCF-7/ADR cells. The pH_{endo}SM-P85/f/DOX also demonstrated more dramatic antitumor efficiency and remarkable reduction of ATP energy in the MDR cells in tumors than the control formulations. The intracellular delivery of Pluronic® P85 unimers to the MDR cells based on the targeted and endosomal pH triggerd release mixed micelles has been demonstrated as a promising approach to reverse MDR.

1. Introduction

Nowadays, chemotherapy is still the primary option for cancer therapy. However, the efficacy of chemotherapy treatment has been severely compromised by the development of multidrug resistance (MDR) [1,2]. Even though much effort has been made to overcome the MDR in cancer, limited successes have been achieved with some therapeutic regimens and antineoplastic agents [3–8]. The major reason for that is the complex mechanisms through which the drug resistance exhibited [9,10]. Mechanisms of MDR have been identified to date including reduced drug accumulation due to a superfamily of ATP binding cassette (ABC) proteins, such as the P-glycoprotein and the MDR-associated proteins (MRPs); drug detoxification caused by the glutathione (GSH)/glutathione-S-transferase (GST) system; altered targets involving topoisomerase II; alteration in drug-induced apoptosis owing to the change of Bcl-2 pathway; and the sequestration of drugs within cytoplasmic vesicles. Several independent mechanisms of MDR may act simultaneously and/or in concert, which further increases the difficulty in MDR reversal.

Recently, amphiphilic block copolymers of Pluronics, such as Pluronic® P85, have been identified to be the most promising MDR reversal agent due to their reversal effect on several distinct drug resistance mechanisms. It has been demonstrated that Pluronics

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are able to 1) block drug efflux transporters, such as P-glycoprotein (P-gp) [11], multidrug resistance proteins (MRPs) [12,13], and breast cancer resistance protein (BCRP) [14,15]; 2) incorporate into membranes changing its microviscosity [16]; 3) induce a remarkable reduction of ATP levels in MDR cells [17]; 4) inhibit the glutathione (GSH)/glutathione (GST) detoxification system [18–22]; 5) induce release of cytochrome C and increase of reactive oxygen species (ROS) levels in the cytoplasm [23]; 6) enhance pro-apoptotic signaling and decrease anti-apoptotic defense in MDR cells [24]; 7) abolish drug sequestration in acidic vesicles [25,26]. Remarkably, these reversal effects are most apparent at the copolymer concentrations below their critical micellization concentration CMC [27], suggesting that the Pluronics unimers, i.e. single block copolymer molecules, are responsible for the MDR reversal [28,29]. This was attributed to the hydrophobic PPO chains of the Pluronic® unimers immersed into the biomembrane hydrophobic areas, resulting in the alternations of the membrane structure and decreasing its microviscosity ("membrane fluidization") [30,31]. Many attempts have been made to deliver Pluronics reversal agents to MDR cells using micellar delivery systems due to their ability to self-assemble into micelles [32–34]. For example, Wang and Zhang et al. developed floate-mediated Pluronic® P105 micelles and Pluronic® F127/P123 mixed micelles to overcome MDR, respectively. The authors claimed that the combination of folate-mediated active targeting and Pluronic® MDR reversal effect could enhance the paclitaxel accumulation in both the resistant breast cancer MCF-7/ADR cells and resistant ovarian cancer SKOV-3/PTX cells, leading to enhanced cytotoxicity against MDR cells. However, the major limitations of Pluronic copolymers were the low micellization and solubilization capacity to hydrophobic drugs, as well as the low stability of the self-assembled micelles upon dilution in the blood-stream due to their relatively high CMC values [35]. Therefore, two similar mixed micelles consisting of mPEG-PLA copolymers and Pluronic® L61 were later constructed because of the better micellar stability and higher solubilization to hydrophobic drugs of the mPEG-PLA copolymers than Pluronics [36,37]. Both results showed that the mixed micelles could significantly enhance the cytotoxicity and cellular accumulation of the anticancer drugs, as compared to the plain mPEG-PLA micelles. However, the major concern with the previously developed micellar delivery systems is that whether the Pluronics unimers can be truly transported to the MDR cells. The plain Pluronics micelles were expected to lose the Pluronic MDR reversal agent in the blood circulation due to the poor micellar stability. While the mixed micellar delivery systems based on mPEG-PLA were designed to be stable enough to withstand the severe dilution in the blood circulation in order to carry the Pluronics unimers to the tumor tissue via passive or active targeting [17,38]. However, it is hardly expected that the stabilized micelles were able to effectively release the Pluronics unimers into the cytosol after being taken up into the endosomes or lysosomes of the MDR cells, which created another barrier on the way to the target due to the enhanced sequestration and degradation of MDR cells. The moderate MDR reversal effect of the previously developed micelles was probably attributed to the increased drug accumulation in MDR cells resulting from the active targeting and the micelles bypassing the efflux mediated by MDR-related proteins rather than the Pluronic unimers [39].

The objective of the present research was to evaluate the MDR reversal effect by intracellular delivery of Pluronic® P85 unimers to MDR cells. To achieve this, an endosomal pH-sensitive mixed micellar delivery system with a targeting ligand of folate (pH_{endo}SM-P85/f) based on the pH-sensitive copolymer of PHis-PLA-PEG-PLA-PHis and Pluronic® F127 were constructed (Fig. 1). A small proportional Pluronic® F127 was conjugated with folate to impart the active targeting of the mixed micelles. The relatively longer hydrophilic PEO block (4500 Da) of Pluronic® F127 ensured a prolonged circulation of the micelles and the exposure of the folate ligand at the periphery of the pH_{endo}SM-P85/f for folate receptor-medicated endocytosis [40]. The pH-sensitive copolymer of PHis-PLA-PEG-PLA-PHis was responsible for dissociating the micellar structure in early or late endosomes and subsequently disrupting endosomal membrane due to the protonation of PHis blocks, releasing the Pluronic® P85 unimers into the cytosol for exerting the MDR reversal effect (Fig. 2). In this study, the intracellular delivery of Pluronic® P85 unimers and the MDR reversal effect of pH_{endo}SM-P85/f were evaluated at the cellular and animal levels by performing *in vitro* cytotoxicity, intracellular accumulation, sub-cellular distribution, intracellular ATP level and mitochondrial membrane potential in MCF-7/ADR cells, *in vivo* biodistribution and antitumor effect in MCF-7/ADR tumor-xenografted nude mice.

2. Materials and methods

2.1. Materials

Doxorubicin (DOX) was purchased from Beijing HuaFeng United Technology Co. Ltd. (Beijing, China). Pluronic® F127, Pluronic® P105 and Pluronic® P85 was kindly supplied by BASF Ltd. (Shanghai, China). PEG (M_n: 2000 g/mole), N, N′- Carbonyldiimidazole (CDI), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), fluorescein isothiocyanate (FITC) and Hoechst 33258 were purchased from Sigma (St Louis, MO, USA). RPMI 1640 without folic acid and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersberg, MD, USA). ATP assay kit, mitochondrial membrane potential assay kit with JC-1, MitoTracker Red and Lysotracker Green were purchased from Beyotime® Biotechnology Co. Ltd (Nantong, China). Purified deionized water was prepared by the Milli-Q plus system (Millipore Co., Billerica, MA, USA). All the other reagents and chemicals were of analytical or chromatographic grade and were purchased from Concord Technology (Tianjin, China).

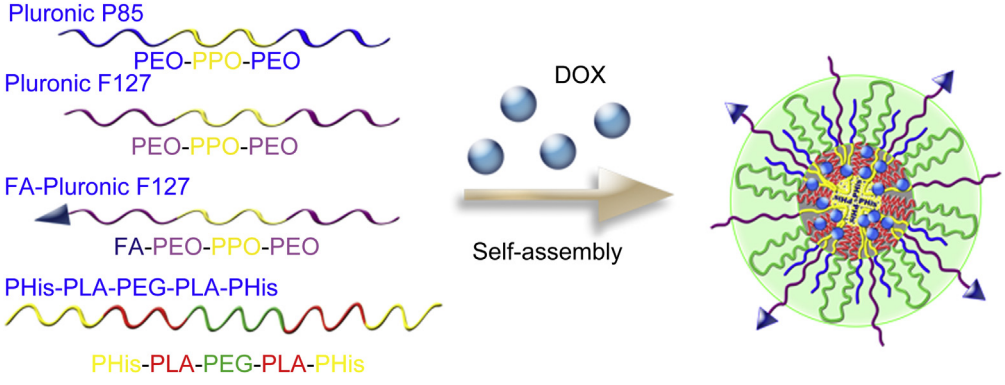

Fig. 1. Schematic diagram of the DOX-loaded folate-mediated endosomal pH-sensitive mixed micellar delivery system (pH_{endo}SM-P85/f/DOX).