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Biostable Poly(ethylene oxide)-*b*-poly(methacrylic acid) Micelles for pH-triggered Release of Doxorubicin

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ABSTRACT – pH-sensitive cross-linked polymeric micelles were synthesized by using block ionomer complexes of poly(ethylene oxide)-b-poly(methacrylic acid) (PEO-b-PMA) with calcium ions as micellar templates. An anticancer drug, doxorubicin (DOX) was conjugated on the cross-linked ionic cores of micelles via acid-labile hydrozone bonds. The resulting DOX-conjugated, pH-sensitive micelles are stable at physiological conditions, whereas the release of DOX was significantly increased at the acidic pH. Such micelles were internalized to lysosomes, and acidic pH in lysosomes triggers the release of DOX upon internalization in MCF-7 breast cancer cells. The released DOX entered the cell nucleus and eventually killed cancer cells. Therefore, these data demonstrate that the pH-sensitive micelles could be a promising nanocarrier for delivery of anticancer drug, DOX.

Key words – Doxorubicin, Block ionomer, Calcium, Cross-linked, Polymeric micelles

Self-assembled polymeric micelles formed by amphilphilic block copolymers have been utilized as potential drug delivery vehicles for anti-cancer drugs (Allen et al., 1999; Cohy, 2005; Croy et al., 2006; Duncan, 2003; Kabanov et al., 2009; Lavasanifara et al., 2002; Riess, 2003; Rösler, 2001). They are nanoparticles prepared by self-assembly of block copolymers consisted of two different blocks, which are hydrophilic and hydrophobic blocks. Advantages of polymeric micelles for tumor targeting include core-shell type architecture with nanoscale size (10 to 100 nm in diameter), which prolong circulation time of polymeric micelles in the body. Hydrophilic nonionic shell such as poly(ethylene oxide) allows for long-circulation of nanocarriers by reducing non-specific uptake and clearance by the macrophages of the reticuloendothelial system (RES). Interestingly, such polymeric nanocarriers have been shown to accumulate in tumors and improve anti-tumor activity of anti-cancer drugs (Alakhov et al., 1999; Yokoyama et al., 1999; Lavasanifara et al., 2002; Duncan, 2003; Davis et al., 2008), by the enhanced permeability and retention effect (EPR effect) (Maeda, 2001).

Nanofabrication of the polymeric micelles has been extensively advanced through employment of double hydrophilic diblock copolymers, one of which is hydrophilic ionic block and the other is hydrophilic nonionic block. These copolymers spontaneously form nanoparticles called "*block ionomer complexes* (BIC)" or "*polyion complex micelles* (PIC)", through electrostatic interaction with oppositely charged molecules. However, the stability of polymeric micelles can be significantly affected by environmental factors such as pH, ionic strength, dilution and shear forces after administration in the blood stream, which may lead to loss of their ability to deliver chemotherapeutic drugs to target tissues.

In order to address this problem, nano-sized polymeric micelles based on a cross-linked network of amphiphilic block copolymers have been developed for delivery of anti-cancer drugs (Bronich et al., 2005; Bontha et al., 2006; Kim et al., 2009; Kim et al., 2010). It contains several key features such as cross-linked ionic core, a hydrophilic PEO shell and nanoscale size. Due to cross-linked ionic cores of micelles, these micelles exhibited distinctive properties as drug delivery carriers, such as their high loading capacity, high stability, and responses to environmental stimuli, such as ionic strength, pH, and temperature (Bontha et al., 2006; Kim et al., 2009). Furthermore, the entry of the cross-linked micelles is inhibited in tight junctions in normal epithelial cells but permitted in cancer cells that do not form tight junctions, via caveolae-mediated endocytosis (Sahay et al., 2010). These favorable characteristics of cross-linked micelles can lead to develop-

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ment of drug delivery systems with reduced side effects and higher efficacy in cancer chemotherapy.

In the present study, cross-linked micelles were prepared by condensation reaction of PEO-*b*-PMA with metal ions (Ca^{2+}) and following chemical cross-linking of the polyion chains in the ionic cores. Doxorubicin (DOX), a potent anticancer drug, was conjugated to micelles via acidic pH-sensitive hydrazone linkers. The release profiles, in vitro cellular uptake and cytotoxicity of the resulting DOX-conjugated micelles via pH-sensitive linker (CLM-DOX) were examined to evaluate their potentials in cancer treatment.

Experimental

Materials

PEO-*b*-PMA diblock copolymer (M_w/M_n = 1.45) was purchased from Polymer Source Inc., Canada. The block lengths were 170 and 180 repeating units for PEO and PMA, respectively. Doxorubicin hydrochloride (DOX) is a kind gift from Dong-A Pharm. Co. (Suwon, South Korea). Calcium chloride, 1,2-ethylenediamine (ED), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), triethylamine (TEA), ethylenediaminetetraacetic acid (EDTA), methanol and dimethylformamide were obtained from Sigma-Aldrich (St Louis, MO). Lysotracker™ (green) was purchased from Invitrogen Inc (Carlsbad, CA). MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Research Products International (Prospect, IL). All other chemicals were of reagent grade and used without further purification.

Synthesis of CLM-DOX

The CLM-DOX was prepared by the method previously published (Kim et al., 2009; Sahay et al, 2010). In brief, DOX was conjugated to PEO-*b*-PMA copolymer through an acid-labile hydrazone linkage. DOX-conjugated PEO-*b*-PMA/Ca^{2+} complexes were prepared by mixing an aqueous solution of corresponding of PEO-*b*-PMA with a solution of $CaCl_2$ at a molar ratio of $[Ca^{2+}]/[COO^-]$=1.3, followed by addition of ED as bifunctional cross-linkers in the presence of EDC. After completion of the reaction for 1 day, EDTA (1.5 molar equivalent) was added followed by dialysis to remove metal ions and byproducts of the cross-linking reaction.

Physicochemical characterizations of CLM-DOX

Effective hydrodynamic diameter (D_{eff}) and ζ-potential of CLM-DOX were determined using ZetaPlus Analyzer with multi-angle sizing option (Brookhaven Instrument Co.). All measurements were performed in automatic mode, at 25°C. Software provided by the manufacturer was used to calculate the particle size, polydispersity indices and ζ-potential of CLM-DOX. The values were calculated from the measurements performed at least in triplicate. The ^{1}H NMR spectra for the CLM-DOX were acquired by ^{1}H NMR (Varian 500 MHz spectrometer, D_2O 25°C.

In vitro release studies

The release studies were conducted in phosphate buffered saline (PBS, pH 7.4) and acetate buffered saline (ABS, pH 5.5) by dialysis method (Membrane with 3,500 Da cut-off). DOX was sampled at selected time intervals. UV-Vis spectroscopy and HPLC (Agilent 1200 HPLC system) techniques were used to determine the concentration of DOX present in the dialysate. The concentration of DOX released from CLM-DOX was expressed as a percentage of the total DOX available and plotted as a function of time.

Confocal microscopy on live cell

Cellular uptake of CLM-DOX was characterized by live cell confocal imaging using Carl Zeiss LSM 510 Meta confocal microscope (Peabody, MA). MCF-7 human breast cancer cells (1×10^6) were plated in live cell chambers (Fischer Scientific, Waltham, MA) and after two days (37°C, 5% CO_2) were exposed to CLM-DOX for 60 min, followed by incubation with Lysotracker Red® for 10 min. Finally, cells were washed and kept in complete media for confocal imaging.

In vitro cytotoxicity studies

Cytotoxicity of CLM-DOX was assessed in MCF-7 cells by a standard MTT assay as described previously (Bontha et al., 2006). Cells were seeded in a 96-well microtiter plates with 5,000 cells per well and allowed to adhere for 24 h prior to the assay. Cells were exposed to various concentrations of free DOX and CLM-DOX for 24 h at 37°C, followed by washing with PBS, and maintaining in DMEM medium with 10% FBS for additional 72 h. Cell viability was determined using MTT assay method. Based on the results of the test, the IC_{50} values (the concentration which kill 50% of cells) were calculated by using GraphPad Prism Software (GraphPad Software, San Diego California, USA).

Results and Discussion

Synthesis and characterization of CLM-DOX

Doxorubicin (DOX) is an active anthracyline anticancer drug. It is highly effective against a wide range of cancers, but

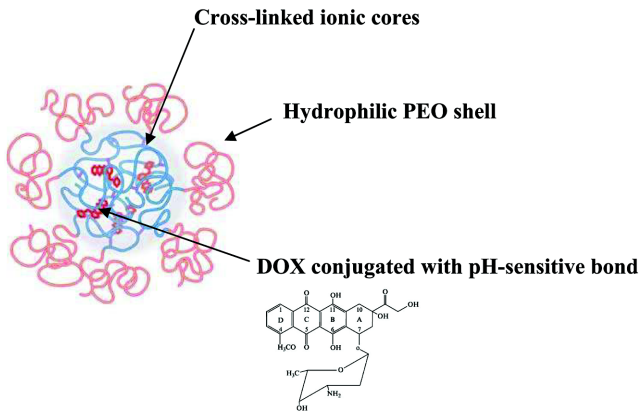

Figure 1. DOX-conjugated Cross-linked polymeric micelles CLM-DOX.

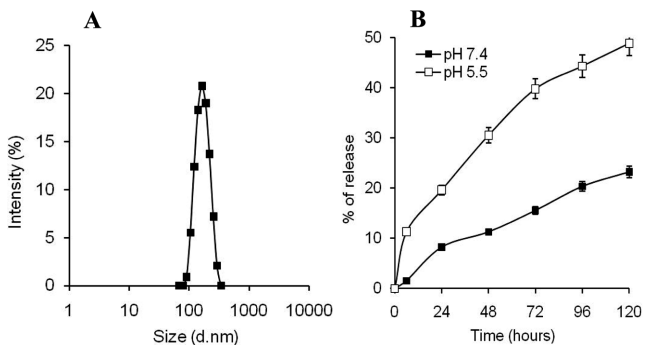

Figure 2. (A) Size distribution of CLM-DOX. (B) In vitro release profiles of DOX from CLM-DOX with a hydrazone linkage at pH 7.4 and pH 5.5.

severe side effects of DOX such as cardiotoxicity and multi-drug resistance could be problems for patient treatment. To enhance its therapeutic efficacy, reduce adverse side effects and abrogate the emergence of drug resistance, Cross-linked polymeric micelles as nanocarriers for DOX were prepared by using block ionomer complex (BIC) of PEO-*b*-PMA copolymer and calcium ions as templates (Bronich et al., 2005; Kim et al., 2009; Kim et al., 2010). DOX was introduced into the micellar core via a pH-sensitive linker. The resulting polymer micelles with cross-linked ionic cores are a special type of nanomaterials formed by double hydrophilic block copolymers containing ionic and nonionic water-soluble blocks ("*block ionomer*"). As shown in Figure 1, CLM-DOX are soft, hydrogel-like structures that have cross-linked PMA polyanion cores and nonionic PEO shell. At physiological pH, the particle size and zeta potential of CLM-DOX were ca.150 nm in diameter and ca. -18 mV, while at pH 5.0 the effective diameter and negative charge of CLM-DOX decreased to ca. 110 nm and -7 mV, due to protonation of carboxylic groups in the cores. In addition, the particle size distribution of CLM-DOX was practically uniform size distribution (0.084 of polydispersity index by DLS) (Figure 2A). It is important to note that relatively low degrees of DOX was conjugated (1 DOX per ca. 60-90 carboxyl groups) conjugated so that the size, charge, and swelling behavior of cross-linked micelles was not affected by the presence of DOX. The detail physico-chemical properties of cross-linked micelles were reported in our previous publications (Kim et al., 2009; Kim et al., 2010; Sahay et al., 2010).

pH-responsive release of DOX from CLM-DOX

The release of DOX from the micelles is essential for exhibiting the drug activities in physiological conditions. Hence, the DOX release profiles of CLM-DOX micelles were evaluated by equilibrium dialysis at 37°C at pH 5.5 (ABS) or pH 7.4 (PBS), which reflect intracellular or plasma conditions, respectively. As shown in Figure 2B, these systems displayed very slow release profiles of DOX under pH 7.4. In contrast, CLM-DOX exhibit rapid release profile of DOX at pH 5.5. For instance, only ca. 23% of drug from CLM-DOX was released at pH 7.4 for 5 days, while ca. 50% of drug released at pH 5.5 during the same period. The observed pH-dependent release of DOX may be due to the acid-labile nature of the hydrazone linkage between DOX and PEO-*b*-PMA backbone. The drug release may be also related to the structural characteristics of the cross-linked ionic cores. Since the micelles have cross-linked ionic cores, some parts of the released DOX molecules could be retained within the cross-linked core region through physical entrapment as well as electrostatic interaction.

The accelerated release of DOX in acidic pH condition is highly desirable characteristic for targeted cancer therapy in the body, due to a lower pH in the interstitial space of solid tumors relative to the normal tissues. The chemotherapeutic drug will be more efficiently delivered to the tumor by preventing the premature drug release. Importantly, since cross-linked micelles were internalized into cancer cells by endocytic pathway and transported into intracellular lysosomal compartments, where the pH values are acidic (Kim et al., 2010; Sahay et al., 2010), the release of drug from these nanocarries can be potentially triggered. Therefore, these results indicate that DOX release can be facilitated in the intracellular acidic condition by the cleavage of acid-labile linkages in the cores of the micelles. Such delivery systems can minimize its release in systemic circulation but effectively release their payload at the disease site.

Intracellular delivery of CLM-DOX

There are multiple endocytic pathways for internalization of nanomaterials in the cells. Endocytosis can be broadly classified as clathrin-dependent and clathrin independent endocy-

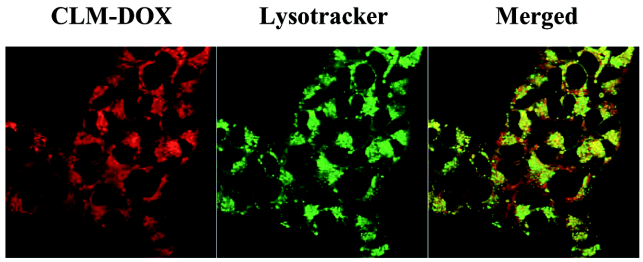

Figure 3. Cellular uptake of CLM-DOX in MCF-7 cells. MCF-7 cells were exposed for 60 min, at 37°C to CLM-DOX (Red) and stained with Lysotracker Green® (Green) for 10 min.

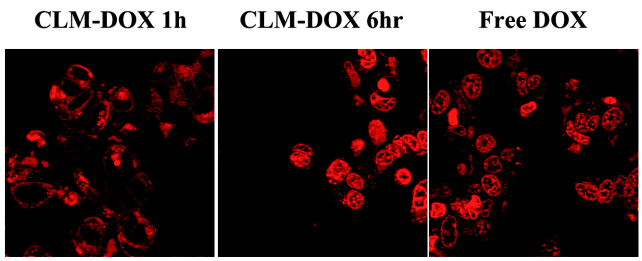

Figure 4. Cellular uptake of CLM-DOX in MCF-7 cells treated with free DOX, CLM-DOX after incubation for 1 hr and 6 hr.

tosis. Clathrin independent endocytosis includes caveolae-mediated endocytosis, clathrin- and caveolae-independent pathways and macropinocytosis (Sahay et al., 2010). The mechanisms of cellular entry and final sub-cellular distribution of nanomaterials could affect the performance of the drug. Particularly, understanding of cellular entry mechanism of pH-sensitive-micelles is very important to design efficient drug carriers.

Our previous study demonstrated that the cellular entry of the cross-linked micelles is limited in normal epithelial cells which form tight junctions, but the cross-linked micelles preferentially entered cancer cells that do not form tight junctions (Sahay et al., 2010). In particular, these micelles are selectively transported to the lysosomes via caveolae-mediated endocytosis. Based on these interesting founding, we utilized this property for lysosomes-specific delivery of DOX in MCF-7 breast cancer cells. For this purpose pH-sensitive, DOX-conjugated micelles (CLM-DOX) were synthesized and evaluated whether the drug can be released in intracellular acidic environment. Consistent with the previous report by us, the red fluorescence of CLM-DOX show a significant co-localization with a lysosomal marker, Lysotrackter Green® in MCF-7 cells after 1h incubation (Figure 3). The vesicle-like structures of CLM-DOX were observed in the lysosomal compartments. We further incubated CLM-DOX for additional 6h in the MCF-7 cells, to evaluate whether acid-labile linkages of CLM-DOX can be cleavable in the intracellular compartments.

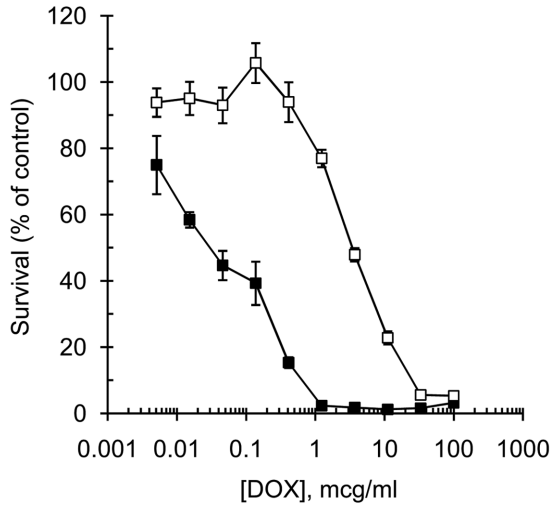

Figure 5. *In vitro* cytotoxic effect of CLM-DOX in MCF-7 cells. (□) free DOX, (■) CLM-DOX after incubation for 24 hr at 37°C (n=8).

Additional incubation of CLM-DOX for 6h in the MCF-7 cells led to the release and accumulation of DOX from micelles in the nucleus of the MCF-7 cells (Figure 4). Since DOX was conjugated into the micelle core via a pH-sensitive hydrazone linker, it suggests that acidic pH of the lysosomes triggers the release of DOX upon internalization in MCF-7 cells.

In vitro cytotoxicity

The cytotoxicity of CLM-DOX was evaluated in comparison with that of free DOX by using conventional MTT assay in MCF-7 breast cancer cells. After treatment, the cell viability declined progressively with increase of the concentration of CLM-DOX. IC_{50} values for free DOX and CLM-DOX were 0.58 ±0.1 μg/mL and 13.9±1.2 μg/mL, respectively. Evidently, CLM-DOX displayed nearly 24-fold lower cytotoxic activity than free DOX, which is consistent with the sustained release of DOX (Figure 5). The reduced *in vitro* cytotoxicity of CLM-DOX can be also explained by incubation time of CLM-DOX. In this study, incubation time of CLM-DOX with MCF-7 cells might not be sufficient for the complete cleavage of pH-sensitive hydrazone linkages to release DOX from CLM-DOX, as shown in the release profile (Figure 2B). Despite the lower cytotoxicity of CLM-DOX, substantial benefits can be expected in such nanocarriers in vivo that might alter pharmacokinetics of drug and accumulate drug in tumor tissue via EPR mechanism. Taken together, these data indicate that these nanocarriers trigger the release of drug and cytotoxic activity of anti-cancer in pH-dependent manner in the acidic environment of lysosomes.

Conclusion

This study demonstrated the possibility of pH-sensitive cross-linked micelles for delivery of DOX using a pH-sensitive linker. Such pH-sensitive DOX-loaded micelles can trigger drug in lysosomal compartments and exhibit selective toxicity to the cancer cells. Therefore, the polymer micelles with cross-linked ionic cores are promising nanocarriers that may lead to development of drug delivery systems with reduced side effects and higher efficacy in cancer chemotherapy.

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