Ultra pH-Sensitive Polymeric Nanosystems for Tumor Targeting and chemotherapy

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Introduction

For cell cycle-phase non-specific anticancer drugs such as doxorubicin (DOX), the dose-response curves present that the survival fraction of both wild-type and resistant cancer cells decreases in a sigmoidal pattern as a function of log (dose size). The inflection point of the curves is drug specific and is shifted to a lower concentration with increasing duration of drug exposure. The inflection point for the resistant cells occurs at a higher concentration region and shows more gradual decrease in viability with increasing drug concentration. Overall, the experimental results and theoretical fitting of the results give us a simple implication that high dose exposure for longer duration results in effective kill. Considering the dose-response relationship, it was mentioned that future improvement in carrier design by providing a triggerable mechanism for drug release on reaching the tumor sites could be the most efficacious delivery strategy. Various approaches for externally stimulated triggered release have been attempted. For example, hyperthermic conditions were used for thermosensitive liposomes or polymeric micelles and sonication was used to trigger release from for Pluronic micelles.

In this contribution, ultra pH-sensitive polymeric micelles for tumor targeting and triggered release either by slightly acidic extracellular pH (pH_e) or by early endosomal pH are introduced. This approach may allow localized high-dose therapy which was effective for sensitive cells. Active intracellular translocation of the micellar carriers via specific interactions combined with triggered release in early endosomes and endosomolytic acitivity of destrabilized micelle components has been proven to be effective for treating multidrug resistant tumors.

Experimental

Poly(L-histidine)-*b*-PEG. L-Histidine (His) was derivatized by introducing carbobenzoxy (CBZ) group to α amino group and amino group in imidazole ring of N^{α}-CBZ-L-histidine was protected with dinitrophenyl (DNP) group. N^{α}-CBZ-N^{im}-DNP-L-His was then transformed to N-carboxy anhydride (NCA) form by thionyl chloride. The ring opening polymerization of N^{α}-CBZ-N^{im}-DNP-L-His NCA·HCl in DMF was performed using different molar ratios of the monomer to initiator (isopropylamine or n-hexylamine) (M/I ratio). Monocarboxy-PEG was used to prepare an activated NHS-PEG. The coupling reaction between poly(N^{im}-DNP-L-His) and NHS-PEG (1:1 functional group ratio) was carried out in THF for two days at room temperature.

PolyHis-*b*-PEG micelle. The deprotonated polyHis at high pH is hydrophobic, while PEG is soluble in water at all pH's. This amphiphilicity was responsible for the formation of polymeric micelles. Lowering the solution pH below the pK_b can affect the micellar structure because protonation converts the hydrophobic polyHis to a more hydrophilic block. The polyHis/PEG block copolymer micelles were prepared by the dialysis of polymer solution in DMSO against a pH 8.0 medium. Micelle formation was monitored by fluorometry in the presence of pyrene as a fluorescent probe. The change of total emission intensity *vs.* polymer concentration indicated the formation of micelle or the change from micelle to unimer (dissociated polymer from the micelle).

Mixed micelles of polyHis/PEG and PLLA/PEG. PolyHis-*b*-PEG micelle was relatively unstable and began to gradually disintegrate at pH 7.4. The incorporation of a non-ionizable block copolymer (pLLA/PEG) in the micellar structure of polyHis/PEG improved micelle stability at pH 7.4 and shifted destabilization to lower pH. In particular, the mixed micelles composed of polyHis/PEG (75 wt %) and PLLA/PEG (25 wt %) showed a unimodal size distribution with an average size of 70 nm in diameter at pH 9.0. Other mixed micelles prepared from different contents of PLLA/PEG were ranged 70-100 nm in diameter with unimodal size distributions at pH 9.0. For receptor mediated endocytosis, folic acid was conjugated to the block copolymer prior to micelle fabrication.

Synthesis of pLLA-*b*-PEG-*b*-polyHis-biotin. For the preparation of biotin-NH_2, 1 mmol biotin was preactivated with 1.25 mmol N,N'-dicyclohexylcarbodiimide (DCC) and 1.5 mmol hydroxysuccinimide (NHS) in 30 ml dimethylformamide (DMF) at room temperature for 1 day. The dried biotin-NHS (1 mmol) was aminated with ethylene diamine (10 mmol, in DMF (30 ml, at room temperature for 8 h, in presence of 100 μl pyridine. After being recrystallized from diethyl ether, product was dried *in vacuo* for 2 days. The synthesized polymer was coupled with pLLA (M_n 3K)-*b*-PEG (M_n 2K)-COOH (pLLA-*b*-PEG-COOH) prepared by conventional method to yield block copolymers. The final product was pLLA (M_n 3K)-*b*-PEG (M_n 2K)-*b*-polyHis (M_n 1K)-biotin. Before cell interaction test, the test micelle was labeled with FITC. PolyHis-*b*-PEG (1 mmol) in 30 ml DMSO was conjugated with 0.03 mmol FITC and 10 mg of dilaurated dibutyltin at 60 °C for 3 h. The mixture was cooled down to room temperature, and dialyzed against de-ionized water for 2 days, and then freeze-dried.

Cell interaction. The interaction between breast MCF-7 tumor cells and FITC-labeled test micelle or DOX-loaded FITC-labelled test micelle was observed with the cells (1×10^5 cells/ml) grown on a Lab-Tek^R II chamber slide (Nalge Nunc International, Napevillem, IL). The RPMI 1640 medium (pH 7.4-6.8) containing the above micelle was prepared as described before. The cells were treated with the test micelle (polymer concentration; 5 μg/ml) for 30 min, and washed three times with PBS pH 7.4 solution. The cells were fixed with 1 wt% formaldehyde (Sigma) in PBS for 10 min at room temperature. A coverslip was mounted on a glass microscope slide with a drop of anti-fade mounting media (5 % n-propyl galate, 47.5 % glycerol, Sigma) and 47.5 % Tris-HCl pH 8.4 (Gibco) to reduce fluorescence photo bleaching. The polymer distribution in cell was examined by a confocal microscopy (Leica TCS NT).

For flow cytometry analysis, the cells (1×10^5 cells/ml) were harvested by 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution (Gibco), pelleted by centrifuge (1000 rpm for 3 min) and resuspended in phosphate buffer saline (PBS, pH 7.4-7.0) supplemented with 0.5 % BSA and 20 mM HEPES (Simga). The cells in pH 7.4.-7.0 buffer solution were incubated with the FITC-labelled test micelle (polymer concentration; 5 μg/ml) for 30 min at 37 °C. After interaction, to remove unbound micelle, gently pipetting was done and the cells were rinsed with 0.1 M Dulbecco's phosphate-buffered saline (Gibco) supplemented 0.5 % bovine serum albumin (BSA, Sigma). After centrifugation (1000 rpm for 3 min), cells were pelleted and resuspended in 1 ml of 0.1 M PBS supplemented with BSA and analyzed on a flow cytometer (Becton Dickenson FacScan).

In vivo study. *In vivo* study was performed in 5-6 weeks old female nude mice (BALB/c mice, Charles River Lab. Wilmington, USA). Mice were maintained in autoclaved microisolator cages housed in a positive pressure containment rack and maintained under the guidelines of an approved protocol from University of Utah Institutional Animal Care and Use Committee.

To establish human breast cancer xenografts, a cell suspension (100 μl) containing 5×10^6 MCF-7 cells was injected *s.c.* into the left inguinal mammary line. The parental MCF-7 cell line responds to estrogen with the increased level of progesterone receptors. Therefore, MCF-7 tumor growth was facilitated by feeding one mg estrogen per liter of water to mice. The administration of estrogen in water to mice bearing MCF-7 xenograft was stopped one wk prior to antitumor treatments. Tumor volume was calculated using a formula, tumor volume = 0.52 × length × width × height. The dimension was measured by an electronic digital caliper. After 2-3 wk inoculation (tumor volume 150-200 mm^3) for *s.c.* MCF-7 xenografts, *in vivo* antitumor studies were performed.

Results and Discussion

The polyHis-*b*-PEG in dimethylsulfoxide (DMSO) formed polymeric micelles on dialysis against a borate buffer at pH 8. Dynamic light scattering (DLS) and atomic force microscopy (AFM) showed the micelles were spherical, diameter ~114nm, with a unimodal distribution. The critical micelle concentration (CMC) at pH 8.0 was 2.3 mg/L. The CMC increased markedly on decreasing the pH of the dialysis medium below 7.2. Micelles prepared at pH 8.0 were gradually destabilized below pH 7.4, as evidenced by a slight increase

in light transmittance, an alteration in size distribution, and a decrease in the pyrene fluorescence intensity. It was concluded that the ionization of the polyHis block forming the micelle core determined the pH-dependent CMC and stability [1].

These pH-sensitive micelles were investigated for pH-dependent drug release, folate receptor mediated internalization and cytotoxicity with MCF-7 cells *in vitro*. The polyHis-*b*-PEG micelles showed accelerated DOX release as decreasing pH from 8.0. When the cumulative release for 24 hrs was plotted as a function of pH, the gradual transition in release rate appeared in a pH range from 8.0 to 6.8. To target triggering pH of polymeric micelles to more acidic tumor extracellular pH while improving the micelle stability at pH 7.4, PLLA/PEG block copolymer was blended with polyHis/PEG to form mixed micelles. This blending shifted the triggering pH to a lower value. Depending on the amount of PLLA/PEG, the mixed micelles were destabilized in the pH range of 7.2-6.6 (triggering pH for adriamycin release). When the mixed micelles were conjugated with folic acid for receptor-mediated endocytosis, the *in vitro* results demonstrated that the micelles were more effective in tumor cell kill due to accelerated drug release and folate receptor-mediated tumor uptake [2].

In order to overcome multidrug resistance in solid tumors, DOX loaded pH-sensitive micelles of which surface was decorated with folate (PHSM/f) were evaluated both *in vitro* and *in vivo* experiments. PHSM/f were fabricated from a mixture of two block copolymers of poly(L-histidine) (M_n: 5K)-*b*-PEG (M_n: 2K)-folate (polyHis/PEG-folate) (75 wt %) and poly(L-lactic acid) (M_n: 3K)-*b*-PEG (M_n: 2K)-folate (PLLA/PEG-folate) (25 wt%). The PHSM/f showed more than 90% cytotoxicity of DOX resistant MCF-7 (MCF-7/DOX^R) when cultured with PHSM/f at a concentration of 10 μg/ml DOX. The result was interpreted by a sequential event of active internalization of PHSM/f via folate-receptor mediated endocytosis and ionization of His residues which result in micelle destabilization and probably disturbance of endosomal membranes. This potential mechanism may endow the drug carriers to bypass Pgp efflux pump and sequestration of DOX in acidic intracellular compartments, yielding high cytotyoxicity. Experimental evaluation of tumor regression was carried out in a small animal model bearing *s.c.* MCF-7 or MCF-7/DOX^R xenografts. The tumor (MCF-7/DOX^R) volumes of mice treated with PHSM/f was significantly less than control groups treated with free DOX or similar micelles but without folate (PHSM) as presented in Fig. 1. In the MCF-7/DOX^R xenograft model, the accumulated DOX level of PHSM/f in solid tumors was 20 times higher than free DOX group, and 3 times higher than PHSM group. The results demonstrate that PHSM/f is a viable means for treating drug resistant tumors [3].

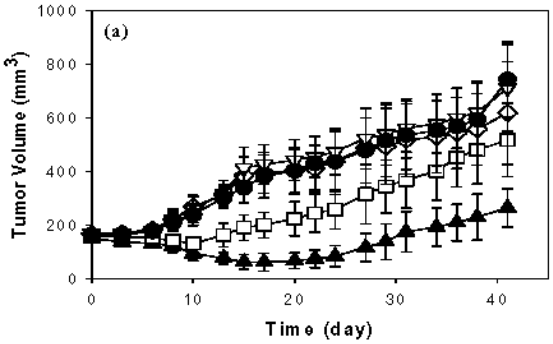

Fig. 1. Tumor growth inhibition of *s.c.* human breast MCF-7/DOX^R carcinoma xenografts in BALB/c nude mice. Mice were injected *i.v.* with 10 mg/kg DOX equivalent dose: DOX loaded pH-sensitive micelle (□), pH-sensitive micelle with folate (▲), pH-insensitive micelle (pLLA-PEG) (▽), pH-insensitive micelle with folate (◇), and free DOX (●). Three *i.v.* injections on day 0, 3, and 6 were made.

A core-shell type micelle was constituted from two block copolymer components of polyHis-*b*-PEG and pLLA-*b*-PEG-*b*-polyHis-biotin. PolyHis was utilized in this work for multifunctionalities for tumor extracellular pH-sensitive actuator for ligand exposure, early endosomal pH-induced micelle destabilization, followed by endosomal membrane disruption. The differentiation of pH for ligand exposure and for destabilization of the micelle was obtained by blending polyHis-*b*-PEG with pLLA-*b*-PEG-*b*-polyHis-biotin, resulting in a mixed micelle system of which core was composed of polyHis block from polyHis-*b*-PEG and pLLA block from pLLA-*b*-PEG-*b*-polyHis-biotin. When the two block copolymers dissolved in dimethyl sulfoxide (DMSO), which is water miscible and a common solvent for all components in the block copolymers, at a concentration of 5.0 mg/ml and exposed to excess amount of pH 9.0 buffer solution through dialysis membrane, solvent exchange occurs. Because of insolubility of polyHis and pLLA in pH 9.0 buffer, the polymers gradually self-assemble into hydrophobic core-hydrophilic shell type micelles as increasing water content in the dialysis tubing. The produced micelle was about 100 nm in average diameter with unimodal distribution and its atomic forced microscopic picture showed a spherical shape. During the dialysis process, it is assumed that short polyHis block in pLLA-*b*-PEG-*b*-polyHis-biotin is located at the interface of hydrophobic pLLA/polyHis core and hydrophilic PEG shell due to high water solubility of PEG and biotin. And the interfacial polyHis causes PEG block bending and biotin buried in the PEG chain forest (shell) derived from polyHis-b-PEG block copolymer. All measurements of pH_e of human and animal solid tumors by either invasive and noninvasive methods showed that more than 80% of all measured values falls below pH 7.2. The tumor pH_e can be further manipulated by glucose administration. This small difference in pH between blood and tumor tissues is used as a signal for biotin exposure. The micelle was stable above pH 7.2 and hide conjugated biotins. As lowering pH below pH 7.2, the degree of ionization of polyHis increases. The interfacial short polyHis became first ionized and at critical degree of ionization its hydrophobic interaction with the core phase weakened. As a result the PEG-*b*-polyHis-biotin portion expanded, exposing biotin out of PEG shell. The pH 7.0 seems the critical pH for this expansion as demonstrated in Fig. 2 [4].

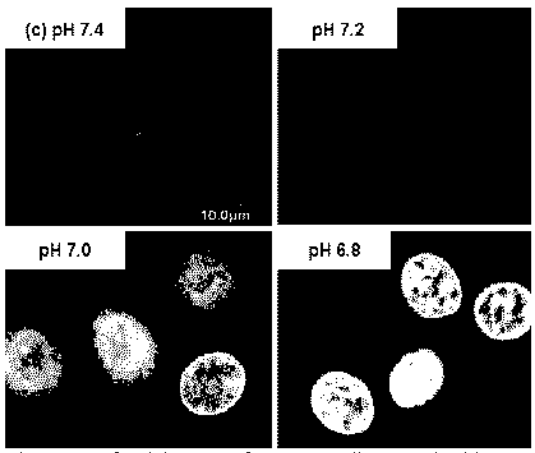

Fig. 2. Confocal images of MCF-7 cells treated with FITC-labeled polyHis-*b*-PEG/pLLA-*b*-PEG-*b*-polyHis-biotin micelles under pH 7.4, 7.2, 7.0, and 6.8 RPMI-1640 buffer medium for 30 min.

Conclusion

Taken together, the ultra pH-sensitive polymeric mixed micelles present four functionalities as decreasing pH: ligand exposure at pH 7.0, micelle destabilization below pH 6.8, enhanced DOX release and endosomal membrane disruption. The first functionality is expected to endow tumor pH specificity to nonspecific ligands and the rest ones may help to treat solid tumors that are hard-to-treat by conventional chemotherapy (resistant tumors).

References

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