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The therapeutic response to multifunctional polymeric nano-conjugates in the targeted cellular and subcellular delivery of doxorubicin

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ABSTRACT

The purpose of this study was to develop polymeric nano-carriers of doxorubicin (DOX) that can increase the therapeutic efficacy of DOX for sensitive and resistant cancers. Towards this goal, two polymeric DOX nano-conjugates were developed, for which the design was based on the use of multi-functionalized poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) micelles decorated with αvβ3 integrin-targeting ligand (i.e. RGD4C) on the micellar surface. In the first formulation, DOX was conjugated to the degradable PEO-*b*-PCL core using the pH-sensitive hydrazone bonds, namely RGD4C-PEO-*b*-P(CL-Hyd-DOX). In the second formulation, DOX was conjugated to the core using the more stable amide bonds, namely RGD4C-PEO-*b*-P(CL-Ami-DOX). The pH-triggered drug release, cellular uptake, intracellular distribution, and cytotoxicity against MDA-435/LCC6^{WT} (a DOX-sensitive cancer cell line) and MDA-435/LCC6^{MDR} (a DOX-resistant clone expressing a high level of P-glycoprotein) were evaluated. Following earlier in vitro results, SCID mice bearing MDA-435/LCC6^{WT} and MDA-435/LCC6^{MDR} tumors were treated with RGD4C-PEO-*b*-P(CL-Hyd-DOX) and RGD4C-PEO-*b*-P(CL-Ami-DOX), respectively. In both formulations, surface decoration with RGD4C significantly increased the cellular uptake of DOX in MDA-435/LCC6^{WT} and MDA-435/LCC6^{MDR} cells. In MDA-435/LCC6^{WT}, the best cytotoxic response was achieved using RGD4C-PEO-*b*-P(CL-Hyd-DOX), that correlated with the highest cellular uptake and preferential nuclear accumulation of DOX. In MDA-435/LCC6^{MDR}, RGD4C-PEO-*b*-P(CL-Ami-DOX) was the most cytotoxic, and this effect correlated with the accumulation of DOX in the mitochondria. Studies using a xenograft mouse model yielded results parallel to those of the *in vitro* studies. Our study showed that RGD4C-decorated PEO-*b*-P(CL-Hyd-DOX) and PEO-*b*-P(CL-Ami-DOX) can effectively improve the therapeutic efficacy of DOX in human MDA-435/LCC6 sensitive and resistant cancer, respectively, pointing to the potential of these polymeric micelles as the custom-designed drug carriers for clinical cancer therapy.

1. Introduction

Conventional chemotherapy is commonly implemented in the management of many cancers with limited clinical success. This is mostly due to the severe side effects of cytotoxic agents and the genetic heterogeneity of tumors, i.e., existence of drug sensitive and resistant phenotypes [1]. Nanotechnology devices can improve the clinical performance of conventional chemotherapy agents by changing the biological disposition, reducing toxicity, enhancing the efficacy and perhaps attenuating the dose of therapy [2–5]. However, design of optimum nano-delivery systems for this purpose often requires careful consideration of the properties of the therapeutic agents and the biology of the disease [6,7]. For the successful implementation of the required functional properties in the nano-delivery system, its structure should be chemically flexible. Polymer based delivery system posses a unique advantage over other nano-carriers in this regard [8–14].

Doxorubicin (DOX) is an effective and widely used chemotherapeutic agent in different cancers. However, its short biological half life, nonspecific distribution leading to intolerable adverse effects and development of drug resistance have limited its success. Several polymer based delivery systems developed for DOX are mostly designed to direct DOX away from sites of drug toxicity (e.g., heart) towards the site of drug action i.e., tumor [13,15–24]. This has mostly resulted in a modest increase in the therapeutic index of DOX usually in DOX sensitive cancers in preclinical and clinical studies. The primary objective of this study was to develop polymer based nano-delivery systems that are not only more efficient in selective delivery of DOX to tumor, but capable of directing DOX to certain sub-cellular targets as means to increase its efficacy in both sensitive and resistant tumor phenotypes.

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Nuclear DNA is the main subcellular site of DOX action for its antitumor activity. Positively charged anthracyclines like DOX with high affinity towards DNA, can efficiently accumulate in nucleus, intercalate DNA, and act as a cytostatic and apoptotic agent against tumor cells. Delivery systems that can prevent DOX distribution in normal cells, but provide selective release of DOX in cancer cells are, therefore, expected to enhance the nuclear disposition and efficacy of DOX in sensitive tumors. However, overexpression of P-glycoprotein (P-*gp*) or other transporters in resistant cells leads to DOX efflux from the cells and/or its sequestration in the cytoplasm, both of which prevent DOX from gaining access to the nucleus [25,26]. In this case, by-pass of P-*gp* and/or controlled delivery of DOX to alternative subcellular sites of DOX action in cancer cells may be pursued to enhance the efficacy of DOX in resistant tumors. In addition to nucleus, DOX also distributes to mitochondria and interferes with major mitochondrial functions leading to cancer cell apoptosis [27]. Recently, strategies aimed at directly interfering with vital mitochondrial functions are proposed to overcome resistance to standard cancer therapy as well as targeting tumor initiating cells [28,29]. In this context, highly lipophilic anthracyclines without primary amine in their structure, demonstrated ability to circumvent multidrug resistance (MDR) by avoiding recognition by P-*gp* and affecting mitochondria functions [30,31].

In the current manuscript, we report on the development of two block copolymer-DOX nano-conjugates for chemotherapy of DOX sensitive and resistant cancers. The delivery systems are based on poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) micelles functionalized on the micellar shell (PEO) as well as the micellar core (PCL). An RGD-containing peptide, i.e., RGD4C, which strongly binds to integrin αvβ3 overexpressed on endothelial and specific cancer cells, was used to modify the micellar surface in both systems for active cancer targeting. Incorporation of DOX to the micellar core was achieved by covalent conjugation to functionalized PCL via two different linkers, i.e., amide bond and hydrazone bond. Evidence is provided that the multifunctional micelles are capable of cancer targeting, receptor-mediated cellular uptake, pH-triggered drug release and controlled sub-cellular delivery *in vitro*, which led to improved therapeutic response to the carried DOX in sensitive and resistant cancers, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals, cell lines and animals

The functional copolymer acetal-poly(ethylene oxide)-*block*-poly(α-carboxyl-ε-caprolactone) (acetal-PEO-*b*-PCCL) was regularly synthesized based on the established protocol in our lab [9,32]. Unless otherwise noted, all the chemicals were obtained from commercial suppliers and used without further purification. 1,3-dicyclohexycarbodiamide (DCC) and N-hydroxy succinimide (NHS) were purchased from Sigma chemicals (St. Louis, MO, USA). Doxorubicin (DOX) was purchased from Hisun Pharmaceutical Co. (Zhejiang, China). RGD4C (KACDCRGDCFCG, MW 1273.9) and cyclic RGDfK (cRGDfK) were purchased from Anaspec (Torrense, CA). All other chemicals were reagent grade. Human cancer cell line MDA-435/LCC6 wide type (MDA-435/LCC6^{WT}) and *mdr1*-transfected phenotype (MDA-435/LCC6^{MDR}), which overexpresses P-*gp* were obtained from Dr. R. Clarke (Georgetown University, Washington, DC). Cells were grown as adherent cultures and maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were used in exponential growth phase, for up to a maximum of 20 *in vitro* passages. The severe combined immunodeficient (SCID) mice were purchased from Jackson Laboratories (Bar Harbor, MI, USA) and were kept in standard housing. All animal experiments were performed in compliance with National Institutes of Health guidelines for animal research under a protocol approved by the Committee on Animal Research at University of Alberta (Edmonton, AB, Canada).

2.2. Synthesis of acetal- and RGD4C-modified poly(ethylene oxide)-block-poly(ester)-DOX conjugates

Conjugation of DOX to the poly(ester) block of block copolymers was accomplished through formation of two different covalent bonds: (i) a pH sensitive hydrazone bound; and (ii) a more stable amide bound. Conjugation of DOX to acetal-PEO-*b*-PCCL through formation of amides bound between the amino group of DOX and the free carboxyl groups on the PCCL chain was achieved using DCC and NHS as the coupling agents according to a previously reported protocol producing acetal-PEO-*b*-P(CL-Ami-DOX) (Scheme 1) [9]. RGD4C-attached PEO-*b*-P(CL-Ami-DOX) was prepared using a previously established method with minor modifications [9]. Briefly, acetal-PEO-*b*-P(CL-Ami-DOX) was assembled to polymeric micelles by solvent evaporation method. The acetal groups on the micelle surface were converted into aldehyde under acidic treatment (pH 2.0 for 2 h). The resulted solution was then neutralized and buffered by sodium phosphate buffer solution (pH = 7.0, ionic strength 0.1 M). RGD4C was added and incubated with aldehyde-PEO-*b*-P(CL-Ami-DOX) micelles at RGD4C:polymer molar ratio of 1:10 at room temperature for 2 h under moderate stirring. Subsequently, $NaBH_3CN$ (10 eq.) was added to the polymer to reduce the Schiff base. The conjugation efficiency of RGD4C to polymeric micelles was assessed by a gradient reverse HPLC method as previously reported. After 90 h of reaction, un-reacted peptide and reducing reagent were removed by extensive dialysis against water.

Conjugation of DOX to acetal-PEO-*b*-PCCL through pH sensitive hydrazone bound was achieved in two steps (Scheme 1). First, Fmoc-protected hydrazine (Fmoc-Hyd) was synthesized as described in literature [33], and then conjugated to the acetal-PEO-*b*-PCCL producing acetal-PEO-*b*-P(CL-Hyd-Fmoc) in the presence of DCC and NHS in THF. Second, the protective Fmoc groups in the copolymer were removed with 20% piperidine DMF for 20 min to obtain acetal-PEO-*b*-P(CL-Hyd). For DOX conjugation, acetal-PEO-*b*-P(CL-Hyd) (100 mg) was dissolved in 40 mL of methanol. 20 mg of DOX-HCL dissolved in 10 mL of methanol was then added with trifluoroacetic acid (TFA) as an acid catalyst. The solution was stirred at room temperature for 48 h while being protected from light till a dark orange solution formed. The resulting solution was purified by Sephadex LH20 (Pharmacia, Uppsala, Sweden) column using methanol as the eluent for separating the acetal-PEO-*b*-P(CL-Hyd-DOX) block copolymer from the unbound free DOX. The applied solution was separated into two fractions, and the first eluted fraction was collected. After evaporation of the methanol, the red wine color product was evaluated by RPLC to confirm the absence of unbound free DOX. The content of conjugated DOX was determined by measuring its absorbance at 485 nm, on the assumption that molar absorptivity of DOX residue bound to the polymer was identical to that of free DOX at 485 nm. DOX content was expressed in mol% with respect to the α-carboxylic-ε-caprolactone residue of acetal-PEO-*b*-PCCL.

For preparation of RGD4C-modified PEO-*b*-P(CL-Hyd-DOX) (RGD4C-PEO-*b*-P(CL-Hyd-DOX)), acetal-PEO-*b*-P(CL-Hyd-Fmoc) micelles were prepared by solvent evaporation method, and RGD4C was conjugated to the micelles at an RGD4C:polymer molar ratio of 1:10 according to the method aforementioned. The resulted RGD4C-attached PEO-*b*-P(CL-Hyd-Fmoc) micelles were then purified by dialysis and freeze dried. DOX was then conjugated to RGD4C-PEO-*b*-P(CL-Hyd-Fmoc) polymer after removal of protective group as described above to prepare RGD4C-PEO-*b*-P(CL-Hyd-DOX). Prepared polymers were characterized for their average molecular weights and polydispersity by 300 MHz ^{1}H NMR (Bruker 300 AM; Billerica MA) and gel permeation chromatography [9].

2.3. Assembly of prepared block copolymer-DOX conjugates and characterization of assembled structures

To prepare DOX-conjugated micelles, the prepared block copolymer, i.e., acetal- or RGD4C-PEO-*b*-P(CL-Ami-DOX) and acetal- or RGD4C-PEO-*b*-P(CL-Hyd-DOX), were dissolved in acetone and added drop-wise to distilled water (1:6, v/v) under moderate stirring. The micellar solution was then left stirring overnight, allowing slow evaporation of acetone. Vacuum was then applied to ensure the removal of residual acetone. The hydrodynamic diameter of the micelles was determined by a dynamic light scattering (DLS) spectrometer (Malvern Zetasizer 3000, UK) at a polymer concentration of 4 mg/mL. The micellar solution was also placed on a clean mica surface and air-dried overnight. The nanosized micelles were visualized by atomic force microscopy (AFM) using a Molecular Force Probe 3D (MFP 3D) from Asylum Research (Santa Barbara, CA) controlled with IGOR PRO software (Wavemetrics, Portland, OR).

2.4. In vitro release of DOX and DOX derivatives from micelles

Dependent on the chemical structure of the conjugate and nature of covalent bonds between DOX and the poly(ester) block, release of either intact DOX or DOX-caprolactone derivatives under acidic or neutral condition (pH 5.0 and 7.4, respectively) is expected. The release study was conducted in Dulbecco's phosphate buffered saline (PBS, pH 7.2) and 0.1 M acetate buffer (pH 5.0) at 37 °C with moderate stirring. Because peptide modification didn't affect drug release significantly [9], the study was conducted using acetal-ended micelles.

For polymeric micelles containing P(CL-Ami-DOX) cores, for which release of DOX-caprolactone derivatives rather than free DOX is expected, micellar solution was incubated in the media. At different time points, 5 mL of incubation medium was withdrawn and extensively dialyzed against water to remove all possible degradation products. The remaining polymer in the dialysis bag was lyophilized, and re-dissolved in $CDCl_3$ for ^{1}H NMR analysis. The percentage of poly(ester) block remained in acetal-PEO-*b*-P(CL-Ami-DOX) at different degradation time points was