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Research paper

pH-Dependent doxorubicin release from terpolymer of starch, polymethacrylic acid and polysorbate 80 nanoparticles for overcoming multi-drug resistance in human breast cancer cells

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ABSTRACT

This work investigated the capability of a new nanoparticulate system, based on terpolymer of starch, polymethacrylic acid and polysorbate 80, to load and release doxorubicin (Dox) as a function of pH and to evaluate the anticancer activity of Dox-loaded nanoparticles (Dox-NPs) to overcome multidrug resistance (MDR) in human breast cancer cells *in vitro*. The Dox-NPs were characterized by Fourier transform infrared spectroscopy (FTIR), isothermal titration calorimetry (ITC), transmission electron microscopy (TEM), and dynamic light scattering (DLS). The cellular uptake and cytotoxicity of the Dox-loaded nanoparticles were investigated using fluorescence microscopy, flow cytometry, and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. The nanoparticles were able to load up to 49.7 ± 0.3% of Dox with a high loading efficiency of 99.9 ± 0.1%, while maintaining good colloidal stability. The nanoparticles released Dox at a higher rate at acidic pH attributable to weaker Dox–polymer molecular interactions evidenced by ITC. The Dox-NPs were taken up by the cancer cells *in vitro* and significantly enhanced the cytotoxicity of Dox against human MDR1 cells with up to a 20-fold decrease in the IC50 values. The results suggest that the new terpolymeric nanoparticles are a promising vehicle for the controlled delivery of Dox for treatment of drug resistant breast cancer.

1. Introduction

Two major limitations of traditional chemotherapy are dose-limiting systemic toxicity and the development of multi-drug resistant (MDR) phenotypes. Traditional chemotherapy is not specific to cancer cells. In most cases, both cancerous and healthy cells are exposed to anticancer drugs. Chemotherapeutic agents such as doxorubicin (Dox) are more toxic toward actively proliferating cells [1]. However, like cancer cells, many normal cells, for example, gastrointestinal tract, hair follicle, and bone marrow cells, are continually proliferating, making them susceptible to the toxicity of chemotherapeutics. Moreover, many anticancer drugs produce reactive oxygen species through redox cycling reactions, which are toxic to both cancerous and normal cells [2].

Another major barrier to effective cancer chemotherapy is drug resistance of cancer cells [3,4]. This drug resistance phenotype can be acquired following failed rounds of chemotherapy, or it can be innate and pre-programmed into the physiome of the cancer cells [3]. Moreover, the resistance phenotype is rarely specific to one drug, but rather manifests itself as a cross-resistance to an array of drugs with different chemical structures [5]. This cross-resistance phenotype is often attributed to cell membrane drug transport proteins, metabolic pathways, and intracellular targets common to cancer chemotherapeutics and is known as MDR [3–6].

Abbreviations: BCRP, breast cancer resistance protein; DAPI, 4′,6-diamidino-2-phenylindole; DDIW, distilled deionized water; DLS, dynamic light scattering; Dox, doxorubicin; Dox-NPs, doxorubicin loaded nanoparticles; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; EE, entrapment efficiency; EPR, enhanced permeation and retention; FA, fluoresceinamine isomer I; FBS, fetal bovine serum; FDA, Federal and Drug Administration; FITC, fluorescein isothiocyanate; FTIR, Fourier transform infrared; IC50, inhibitory concentration for 50% survival; ITC, isothermal titration calorimetry; KPS, potassium persulfate; LC, loading content; LE, loading efficiency; MAA, methacrylic acid; MBA, *N*,*N*′-methylenebisacrylamide; MDR, multi-drug resistance; MRP1, multidrug resistance protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHS, *N*-hydroxysuccinimide; NP, nanoparticle; OD, optical density; PBS, phosphate buffered saline; PdI, polydispersity index; PEG, polyethylene glycol; P-gp, p-glycoprotein; PMAA–PS 80-g-St, poly(methacrylic acid)–polysorbate 80-grafted-starch; PS 80, polysorbate 80; SDS, sodium dodecyl sulfate; STS, sodium thiosulfate; TEM, transmission electron microscopy; WT, wild type.

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Dox, a well known anticancer drug, is a member of the anthracycline ring antibiotics with a broad spectrum of antitumor activity against a variety of human and animal solid tumors [7,8]. The drug, however, has a very narrow therapeutic index and its clinical use is hampered by several undesirable side effects including cardiotoxicity and myelosuppression [7–10]. Another drawback of Dox is that it is a substrate of multiple membrane efflux transporters such as p-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1), and breast cancer resistance protein (BCRP) [11,12]. These transporters prevent intracellular accumulation of many anticancer agents causing a reduction in their cytotoxicity. P-gp mainly prevents active uptake and increases cellular efflux of positively charged amphipathic drugs such as Dox in an ATP-dependent manner. In fact, P-gp over-expression is suggested as one of the main mechanisms of MDR in cancer cells [3,6,11,12].

Encapsulation of Dox in nanoparticles has been found to enhance its cytotoxicity by circumventing membrane transporter-mediated MDR in cancer cells [13–17]. The nanoparticles carry the drug into cancer cells mostly by endocytosis, transporting the drug to the cytoplasm or perinuclear region and releasing the drug inside the cells [15,16,18,19]. Drug molecules are not exposed to the binding sites of trans-membrane efflux pumps, for example, P-gp, and are less likely to be pumped out of the cancer cells.

It has been observed that nanoparticles can accumulate in many solid tumors at much higher concentrations than in normal tissues or organs by a nonspecific targeting process known as the enhanced permeation and retention (EPR) effect [20,21]. The EPR effect is attributed to leaky vasculature and limited lymphatic drainage, typically found in solid tumors. Ideally, the cytotoxic drugs should be released from the nanocarriers within the tumor tissue or inside the tumor cells after uptake by active or passive transport mechanisms. Hence, a nanoparticulate system with minimal drug release in the systemic circulation but accelerated release once in the tumor tissue is highly desirable. One way to achieve this goal is to take advantage of the enhanced acidic environment of tumor tissue and cellular endosomes/lysosomes. It is well documented that tumor extracellular pH is lower than that in normal tissue with average pH values of 6.9 to as low as 5.7 owing to different metabolic pathways in cancer cells [22,23]. To take advantage of this pathophysiological characteristic of the tumor microenvironment, polymeric nanoparticles with pH-dependent drug release properties have been studied by various research groups [24–28]. These systems often change their physical and chemical properties, such as swelling ratio or solubility in response to local pH level leading to an increase in drug release rate at lower pH. Alternatively, drugs have been conjugated to the carrier system through an acid labile bond which promotes accelerated release under mildly acidic conditions [28,29].

Recently, we have developed a new nanoparticulate system based on the terpolymer of poly(methacrylic acid)–polysorbate 80-grafted-starch (PMAA–PS 80-g-St) that exhibits pH-sensitivity due to the presence of PMAA [30]. In this terpolymer system, starch, PS 80, and PMAA (pK_a = 5.6–7) are commonly used ingredients in various pharmaceutical formulations approved by regulatory agencies for use in humans. Starch is a food ingredient degradable by amylase in the body, and hence, this nanoparticle system is expected to possess good biocompatibility and biodegradability properties. Moreover, although not the focus of this study, polysaccharide coatings have recently been considered as an alternative to PEG coatings for giving "stealth" properties to nanoparticles [31]. Certain polysaccharides such as heparin, dextran, and hydroxyethyl starch have been shown to prolong the nanoparticle circulation in blood and minimize their interaction with blood proteins and phagocytotic cells [32–34]. The carboxyl groups in PMAA are expected to provide binding sites for cationic anticancer drugs such as Dox as suggested in previous studies using other carboxyl-containing and negatively charged polymers [15,17].

Polysorbate 80 (PS 80) is a polyethylene sorbitol ester with a molecular weight of 1310 Da. It is widely used as an emulsifier/surfactant/stabilizer in pharmaceutical formulations. The surfactant has a sorbitan ring with ethylene oxide polymers attached at three different hydroxyl positions and contains mixture of fatty acid side chains which are attached through an ester linkage to the ethylene oxide oxygen. The major fatty acid component of the side chain is oleic acid contributing to more than 60% of the total composition of the side chain.

Herein, we investigated the ability of PMAA–PS 80-g-St nanoparticles to efficiently load and release Dox and characterized the molecular interactions of Dox with the polymer particle as a function of pH. We also studied cellular uptake of the nanoparticles and the efficacy of Dox-loaded nanoparticles in overcoming MDR in human breast cancer cells *in vitro*.

2. Materials and methods

2.1. Chemicals

Soluble corn starch (MW = 11,000 g/mol), methacrylic acid (MAA), *N*,*N*′-methylenebisacrylamide (MBA), sodium thiosulfate (STS), potassium persulfate (KPS), polysorbate 80 (PS 80), and sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluoresceinamine isomer I (FA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and all other chemicals, unless otherwise mentioned, were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada). Doxorubicin hydrochloride (Dox) was purchased from Polymed Therapeutics (Houston, TX, USA). Hoechst 33342 and Vybrant™ DiI were purchased from Molecular Probes, Inc. (Eugene, OR, USA). All cell culture plasticware was purchased from Sarstedt (Montreal, QC, Canada). Cell culture medium, α-modified minimal essential medium, and phosphate buffered saline (PBS) were obtained from the Ontario Cancer Institute (Toronto, ON, Canada). Fetal bovine serum (FBS) and trypsin were purchased from Invitrogen, Inc. (Burlington, ON, Canada).

2.2. Cell maintenance

P-gp (MDR1) overexpressing human breast carcinoma cell line MDA-MB435/LCC6/MDR1 and parental cell line MDA-MB435/LCC6/WT were generous gifts from Dr. Robert Clarke (Georgetown University, Washington, DC, USA) [35]. Both cell lines were grown in α-modified minimal essential medium supplemented with 10% FBS (growth medium) at 37 °C in a humidified incubator with 5% CO_2 as monolayers in plastic flasks. When cells reached confluence, they were trypsinized and subcultured at 50-fold dilution. Every 3 months, cultures were renewed by returning to a frozen stock of cells. Cell cultures were routinely checked for absence of mycoplasma.

2.3. Synthesis of PMAA–PS 80-g-St nanoparticles

pH-Sensitive PMAA–PS 80-g-St nanoparticles were synthesized using a novel one-pot dispersion polymerization developed in our laboratory [30]. Briefly, 1.55 g of starch was dissolved in 180 ml of distilled deionized water (DDIW) by heating to 95 °C for 30 min. The solution was cooled down to 70 °C and purged with N_2 for 30 min to remove any dissolved oxygen. Subsequently, 0.2 g of KPS and 0.12 g of KPS dissolved in 5 ml of DDIW were added. After 10 min, 0.75 g of PS 80 and 0.2 g of SDS dissolved in 10 ml of DDIW