International Journal of Pharmaceutics 496 (2015) 903–911

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The targeted delivery of doxorubicin with transferrin-conjugated block copolypeptide vesicles

Brian S. Lee, Allison T. Yip, Alison V. Thach, April R. Rodriguez, Timothy J. Deming, Daniel T. Kamei*

Department of Bioengineering, University of California, Los Angeles, 420 Westwood Plaza, 5121 Engineering V, Los Angeles, CA 90095, United States

ARTICLE INFO

Article history:
Received 18 May 2015
Received in revised form 10 September 2015
Accepted 5 October 2015
Available online 9 October 2015

Chemical compounds studied in this article:
N-hydroxysuccinimide (PubChem CID: 80170)
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (PubChem CID: 15908)
Tetrahydrofuran (PubChem CID: 8028)
Ammonium sulfate (PubChem CID: 6097028)
Dimethyl sulfoxide (PubChem CID: 679)

Keywords:
Vesicle
Block copolypeptide
Drug delivery
Drug release
Doxorubicin
Mathematical modeling

ABSTRACT

We previously investigated the intracellular trafficking properties of our novel poly(L-glutamate)$_{60}$-*b*-poly(L-leucine)$_{20}$ ($E_{60}L_{20}$) vesicles (EL vesicles) conjugated to transferrin (Tf). In this study, we expand upon our previous work by investigating the drug encapsulation, release, and efficacy properties of our novel EL vesicles for the first time. After polyethylene glycol (PEG) was conjugated to the vesicles for steric stability, doxorubicin (DOX) was successfully encapsulated in the vesicles using a modified pH-ammonium sulfate gradient method. Tf was subsequently conjugated to the vesicles to provide active targeting to cancer cells and a mode of internalization into the cells. These Tf-conjugated, DOX-loaded, PEGylated EL (Tf-DPEL) vesicles exhibited colloidal stability and were within the allowable size range for passive and active targeting. A mathematical model was then derived to predict drug release from the Tf-DPEL vesicles by considering diffusive and convective mass transfer of DOX. Our mathematical model reasonably predicted our experimentally measured release profile with no fitted parameters, suggesting that the model could be used in the future to manipulate drug carrier properties to alter drug release profiles. Finally, an *in vitro* cytotoxicity assay was used to demonstrate that the Tf-DPEL vesicles exhibited enhanced drug carrier efficacy in comparison to its non-targeted counterpart.

1. Introduction

Doxorubicin (DOX) is one of the most widely used small molecule drugs for the treatment of several cancers, such as breast and lung cancer (Keizer et al., 1990). However, the major limitation of the naked delivery of DOX is its nonspecificity, often resulting in undesirable toxicity to healthy organs and tissues (Imordino et al., 2006). Therefore, research has been performed in hopes of targeting the delivery of drugs towards only cancer cells by encapsulating the drug within nano-sized particles. Nano-sized drug delivery vehicles are advantageous since they can protect the drug from degradation during its circulation in the body, release the drug in a controlled manner, and provide passive targeting to the tumor tissue through the enhanced permeability and retention (EPR) effect (Greish, 2010; Sahoo et al., 2008).

Liposomes have shown great promise as nano-sized drug delivery vehicles. One of the most well-known liposome drug systems in the market is DOXIL®, which is a formulation of doxorubicin encapsulated within PEGylated liposomes. DOXIL® is currently FDA approved for treating Kaposi's sarcoma and recurrent ovarian cancer, and is under clinical trials for the treatment of multiple myeloma, breast cancer, and high-grade glioma (Imordino et al., 2006).

In addition to liposome drug systems, many researchers have been investigating new types of building blocks for developing more effective drug delivery vesicles. An emerging class of drug

Abbreviations: $E_{60}L_{20}$, poly(L-glutamate)$_{60}$-*b*-poly(L-leucine)$_{20}$ block copolypeptide; EL, vesicles: self-assembled vesicular structures from $E_{60}L_{20}$ block copolypeptides; Tf-DPEL, vesicles: transferrin-conjugated, DOX-loaded, PEGylated EL vesicles.

* Corresponding author at: 420 Westwood Plaza, 5121J Engineering V, P.O. Box 951600, University of California, Los Angeles, CA 90095-1600, United States.
E-mail address: kamei@seas.ucla.edu (D.T. Kamei).

http://dx.doi.org/10.1016/j.ijpharm.2015.10.028
0378-5173/

delivery vehicles is the block copolypeptide vesicle since it has properties that makes it promising as an effective carrier for therapeutics. The advantages of block copolypeptides include synthetic control of chain lengths, incorporation of secondary structure, ability to be functionalized, and potential to be biocompatible (Carlsen and Lecommandoux, 2009). Our group previously investigated a novel block copolypeptide vesicle construct, the poly(L-glutamate)$_{60}$-*b*-poly(L-leucine)$_{20}$ ($E_{60}L_{20}$). These polypeptides self-assembled into vesicles that could be controlled in size, encapsulate hydrophilic molecules, and exhibit very low cytotoxicity towards cells (Choe et al., 2013). However, the main limitation of the $E_{60}L_{20}$ vesicles (EL vesicles) as a potential drug carrier was their inability to efficiently enter cancer cells due to the electrostatic repulsions between the negatively-charged surface and the net negatively-charged cell membrane, thus preventing interactions for cellular uptake. To overcome this limitation, transferrin (Tf) was previously conjugated onto the surfaces of the EL vesicles as Tf is a well-known targeting ligand for cancer and its intracellular trafficking properties have also been well studied (Aisen and Listowsky, 1980; Karin and Mintz, 1981; Mayle et al., 2012). Fluorescence and endocytosis inhibitor studies demonstrated that the Tf-EL vesicles exhibited enhanced cellular uptake into cancer cells, primarily through clathrin-mediated endocytosis (Choe et al., 2013). Since the Tf-EL vesicles were able to effectively enter cancer cells, it was hypothesized that this would translate into enhanced therapeutic efficacy if small molecule drugs were encapsulated within the EL vesicles since many chemotherapeutics, such as DOX, have intracellular targets (Tacar et al., 2012).

This study is the first investigation of the drug delivery capabilities of the EL vesicles. After conjugating polyethylene glycol (PEG) to the EL vesicles to form PEGylated EL vesicles, DOX was successfully encapsulated into the vesicles using a modified pH-ammonium sulfate gradient. Tf was then conjugated to the vesicles to create a targeted drug delivery system: the Tf-conjugated, DOX-loaded, PEGylated EL (Tf-DPEL) vesicle. The size and stability of the Tf-DPEL vesicles were monitored using dynamic light scattering, and the drug loading ratio was evaluated after the formation of the Tf-DPEL vesicles. Zeta potential measurements were additionally taken to evaluate the stability of the vesicles throughout the conjugation process and the resulting vesicles were imaged using transmission electron microscopy (TEM). Moreover, a mathematical model was derived to predict the drug release properties of the Tf-DPEL vesicles. In our mathematical model, we considered the transient diffusion of DOX across the vesicle bilayer as described by the Conservation of Species equation. Mass balance and convective mass transfer equations modeled drug release from the vesicle surface to the bulk solution. The resulting system of differential equations was solved numerically using finite difference equations and the method of lines. *In vitro* release studies were performed to compare with the drug release properties predicted by the mathematical model. *In vitro* cytotoxicity studies also demonstrated that the Tf-DPEL vesicles exhibited an improved therapeutic effect compared to the non-targeted DPEL vesicles.

2. Materials and methods

2.1. Materials

The Bradford reagent was obtained from Bio-Rad (Hercules, California). Dialysis bags (MWCO = 8000 Da) were obtained from Spectrum Laboratories (Rancho Dominguez, California). The 1000, 400, and 200 nm polycarbonate membranes were purchased from Whatman Nuclepore (Florham Park, New Jersey). The Avanti Mini-Extruder was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama). Zeba desalt spin columns (MWCO = 8000 Da), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Pierce (Rockford, Illinois). The methoxy-poly(ethylene glycol)$_{5000}$-amine (mPEG) and orthopyridyl disulfide-poly(ethylene glycol)$_{5000}$-amine (biPEG) molecules that were conjugated onto the vesicles were purchased from Nanocs (New York, New York). Both of these molecules have 5000 MW PEG, where mPEG is amine functionalized on one end, while biPEG is amine functionalized on one end and functionalized with an orthopyridyl disulfide (OPSS) group on the other end. Spin concentrators (MWCO = 10,000 Da) were purchased from Millipore (Billerica, Massachussetts). UltraPure Sterile Water was purchased from Rockland Immunochemicals (Limerick, Pennsylvania). The prostate cancer cell line PC3 was obtained from the American Type Culture Collection (Manassas, Virginia). Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin-streptomycin (P/S), sodium pyruvate (NaPyr), phosphate-buffered saline (PBS), and 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was obtained from Hyclone (Waltham, Massachusetts). The CellTiter 96® AQueous Non-radioactive Cell Proliferation Assay (MTS assay) was purchased from Promega (Madison, Wisconsin). All other reagents, such as apo-transferrin (apo-Tf), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and Sepharose CL-4B cross linked beads, were purchased from Sigma–Aldrich (St. Louis, Missouri) unless otherwise noted.

2.2. Synthesis of the $E_{60}L_{20}$ block copolypeptide

The $E_{60}L_{20}$ block copolypeptide was synthesized using the transition metal-mediated α-amino acid *N*-carboxyanhydride (NCA) polymerization technique, as previously described (Deming, 1997; Holowka et al., 2005).

2.3. Processing the EL vesicles

A solution of 0.5% w/v polypeptide in tetrahydrofuran (THF) was first prepared. This solution was sonicated for 30 min, followed by a 30 min interval of inactivity, and then another 30 min of sonication to ensure dissolution of the polypeptide. Subsequently, filtered water was added dropwise to the solution while vortexing such that the final suspension was a 2:1 volume ratio of THF to water. This resulted in a vesicle concentration of 0.333% w/v. In order to remove the remaining THF, the resulting suspension was dialyzed (MWCO = 8000 Da) against filtered water overnight with water bath changes every hour for the first 3 h. After dialysis, the final EL vesicle concentration was diluted to 0.2% w/v with filtered water.

2.4. Extrusion of EL vesicles

To prepare the processed vesicles for subsequent drug loading procedures, the appropriate amount of a 50 μM ammonium sulfate solution was added such that the final suspension had an ammonium sulfate concentration of 5 μM. The vesicles were then serially extruded through 1000, 400, and 200 nm Whatman nuclepore polycarbonate membranes using the Avanti Mini-Extruder. The size and polydispersity index (PdI) were measured using the Malvern Zetasizer Nano ZS model ZEN3600 (Malvern Instruments Inc., Westborough, Massachusetts). The Bradford assay was then performed using the Coomassie Blue Reagent to quantify the final concentration of vesicles by using the post-dialyzed vesicles as the standard. 450 μL of the extruded EL vesicles in an aqueous solution of 5 μM ammonium sulfate were then measured using dynamic light scattering (DLS) to determine the sample size and PdI. A viscosity of 0.8872 cP and a dispersant