Journal of Controlled Release 206 (2015) 153–160

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Polymeric micelles and nanoemulsions as tumor-targeted drug carriers: Insight through intravital imaging

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ARTICLE INFO

Article history:
Received 8 January 2015
Received in revised form 4 March 2015
Accepted 9 March 2015
Available online 14 March 2015

Keywords:
Drug delivery
Perfluorocarbon
Nanodroplets
Micelles
Paclitaxel
Nanoparticle extravasation
Nanoparticle diffusion
Intravital microscopy

ABSTRACT

Intravital imaging of nanoparticle extravasation and tumor accumulation has revealed, for the first time, detailed features of carrier and drug behavior in circulation and tissue that suggest new directions for optimization of drug nanocarriers. Using intravital fluorescent microscopy, the extent of the extravasation, diffusion in the tissue, internalization by tissue cells, and uptake by the RES system were studied for polymeric micelles, nanoemulsions, and nanoemulsion-encapsulated drug. Discrimination of vascular and tissue compartments in the processes of micelle and nanodroplet extravasation and tissue accumulation was possible. A simple 1-D continuum model was suggested that allowed discriminating between various kinetic regimes of nanocarrier (or released drug) internalization in tumors of various sizes and cell density.

The extravasation and tumor cell internalization occurred much faster for polymeric micelles than for nanoemulsion droplets. Fast micelle internalization resulted in the formation of a perivascular fluorescent coating around blood vessels. A new mechanism of micelle extravasation and internalization was suggested, based on the fast extravasation and internalization rates of copolymer unimers while maintaining micelle/unimer equilibrium in the circulation.

The data suggested that to be therapeutically effective, nanoparticles with high internalization rate should manifest fast diffusion in the tumor tissue in order to avoid generation of concentration gradients that induce drug resistance. However an extra-fast diffusion should be avoided as it may result in the flow of extravasated nanoparticles from the tumor to normal organs, which would compromise targeting efficiency.

The extravasation kinetics were different for nanodroplets and nanodroplet-encapsulated drug F-PTX suggesting a premature release of some fraction of the drug from the carrier.

In conclusion, the development of an "ideal" drug carrier should involve the optimization of both drug retention and carrier diffusion parameters.

1. Introduction

Among various suggested drug carriers, two types, namely liposomes and polymeric micelles, are the most extensively studied and developed. Both are spherical nanoparticles with a core-shell structure. Liposomes have an internal aqueous compartment sequestered by a phospholipid shell, which allows encapsulation of water-soluble drugs in the inner core. In contrast, polymeric micelles are characterized by hydrophobic cores and hydrophilic shells. The hydrophobic micelle cores serve for encapsulation of lipophilic drugs. Micelle formation is thermodynamically driven; micelles are formed by self-assembly of individual molecules (unimers) of amphiphilic block copolymers.

While liposomes entered the market at the end of the last century and have been extensively used in medical practice, only a few polymeric micelles have progressed to clinical trials [1–4] and only one type of micelle has reached the market [3,4] despite more than twenty years of extensive research and development and sometimes excellent preclinical results. Micellar systems have been mostly tested for the delivery of doxorubicin (DOX) [1,2] and paclitaxel (PTX) [3–8]. Micellar encapsulation enhances aqueous drug solubility, prevents drug degradation under the action of body fluids and, most importantly, provides for some extent of tumor targeting. A comprehensive review of micelle application in drug delivery was recently published [9].

In clinical trials, micellar formulations of DOX NK911 and SP 1049C [2] manifested the same spectrum of side effects as free DOX, suggesting a lack of effective tumor targeting. However for Pluronic® micelle encapsulated DOX, this disadvantage has been surpassed by the invaluable benefit of suppressing the development of drug resistance [10–18].

For PTX, no micellar formulation besides NK105 [5] and Genexol PM® [4,6,7,19,20] has progressed to clinical trials due to rapid loss of

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http://dx.doi.org/10.1016/j.jconrel.2015.03.010
0168-3659/

drug from the carrier. Still, despite unfavorable pharmacokinetic parameters [4], the PTX-loaded poly(ethylene oxide)-*co*-poly(D,L-lactide) (PEO_{2000}-*co*-$PDLA_{1750}$) micelles (Genexol®-PM) have shown promising therapeutic results for breast, lung, and pancreatic cancer [3,8,20].

There may be a number of reasons for the conflicting outcomes of preclinical and clinical trials of polymeric micelles. One may be related to the lower permeability of human tumor blood vessels when compared to subcutaneous mouse tumor xenografts. Another might be premature micelle degradation in circulation leading to a premature drug release. As mentioned above, block copolymer micelles are formed by self-assembly of amphiphilic block copolymer molecules in aqueous milieu. The self-assembly occurs when copolymer concentration reaches some critical value called the critical micelle concentration (CMC). Below the CMC, block copolymer molecules exist in solution in the form of individual molecules (unimers). When the copolymer concentration drops below the CMC, micelles dissociate into unimers. Because systemic injections and especially infusion of micellar formulations are associated with a very substantial dilution in the circulatory system, micelles may dissociate into unimers upon injection. To prevent premature micelle dissociation, micellar systems should either have very low CMC (i.e., manifest thermodynamic stability), or have slowly dissociating cores (i.e., manifest kinetic stability).

Unstable micelles may be strengthened by the introduction of strong hydrophobic interactions into micelles cores. This may be achieved, for instance, by the introduction of oil [21] or mixtures of hydrophobic drugs [22]. Recently, one of us (NR) suggested micelle stabilization by the introduction of perfluorocarbon (PFC) compounds [23–28]. Besides stabilization against degradation upon dilution, an important advantage of using perfluorocarbon cores is the opportunity of acoustic droplet vaporization (ADV) [29–31] that instigates drug release from nanodroplets under the action of ultrasound.

With this approach, drug-loaded polymeric micelles serve as starting points for developing drug-loaded nanoemulsions. The stability and drug release properties of the latter depend on the type and structure of the copolymer that forms nanodroplet shells [24,26,28]. Nanoemulsions as well as micelles manifested strong therapeutic effects (dramatically extended life span, suppression of metastases and ascites) in preclinical studies of breast, ovarian, and pancreatic cancer [25–28,32]. However side-by-side comparison of micelles and nanoemulsions as drug carriers and vital information on their stability in circulation, extravasation rates and drug retention in vivo has been lacking in the literature.

In the current study, we used intravital laser fluorescence microscopy and fluorescently labeled drug nanocarriers or fluorescently labeled drug to monitor carrier and drug stability in circulation and extravasation rates upon intravenous injections to pancreatic tumor bearing mice. Two sets of experiments were performed. In the first set, we compared extravasation and interstitial diffusion rates of PEG-PDLA unimers, micelles and PEG-PDLA-stabilized perfluorocarbon nanodroplets. In the second set, extravasation of nanodroplets was compared with that of the nanodroplet encapsulated drug, green-fluorescent paclitaxel (F-PTX).

2. Materials and methods

2.1. Block copolymer

Water soluble, biodegradable block copolymer poly(ethylene oxide)-*co*-poly(D,L-lactide) (PEG-PDLA) with molecular weight of either block of 2000 Da was obtained from Akina, Inc., (West Lafayette, IN, USA).

2.2. Micelle preparation

The empty or PTX-loaded PEG-PDLA micellar solutions were prepared by a solid dispersion technique as described in refs. [26,27].

To prepare fluorescently labeled PEG-PDLA micelles, a 3.76 mol% 1,2-distearoyl-***sn***-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000-N′-carboxyfluorescein] (ammonium salt) (F-PEG-PE) (Avanti Polar Lipid, Alabaster, Alabama, USA) was added to the PEG-PDLA copolymer during micelle preparation. It was shown earlier that F-PEG-PE and PEG-PDLA formed mixed micelles. It was shown by others that F-PEG-PE firmly anchors into micelle cores via its hydrophobic fragment and remains associated with micelles as long as micelles exist [33]. The micelle concentration was found to be 2×10^{-5} M, based on the aggregation number of PEG-PDLA (60 in the electrolyte solution) [34] and the total number of F-PEG-PDLA molecules in the formulation.

2.3. Nanodroplet preparation

Perfluoro-15-crown-5-ether (PFCE) (Oakwoods Products, Inc., West Columbia, SC, USA) was introduced into empty or PTX-loaded micellar solutions and emulsified by sonication on ice (VCX500, Sonics and Materials, Inc., CT, USA) [24].

To ensure the absence of micelles in the formulations used to study nanodroplet extravasation, perfluorocarbon-to-copolymer ratio of 6:1 (3% PFCE/0.5% PEG-PDLA/0.015% F-PEG-PE) was used. The absence of micelles in the formulation was confirmed by the dynamic light scattering measurements. The size of empty or PTX-loaded nanodroplets was in the range of 230 nm to 300 nm. Comparing PFCE volume in an individual particle to the formulation total yields a nanodroplet concentration of 2.8×10^{-8} M.

The fluorescent label concentration for both formulations was 5.4×10^{-5} M. The micelle concentration was close to the probe concentration, with about 3 probe molecules per micelle. In contrast, each nanodroplet contained roughly two thousand probe molecules.

2.4. Drug

Paclitaxel (PTX) was obtained from LC Laboratories (Woburn, MA, USA). Green fluorescence labeled PTX (F-PTX) was obtained from Molecular Probes (Life Technologies, NY; catalog number P22310). Typically, 10% F-PTX was mixed with unlabeled PTX and encapsulated in either PEG-PDLA micelles or PFCE/PEG-PDLA nanodroplets.

Compositions and properties of formulations used are presented in Table S1 of Supplemental materials.

2.5. Subcutaneous PDA MiaPaCa-2 tumor model

Human pancreatic cancer MiaPaCa-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Female nude mice between 6 and 8 weeks of age were utilized (Crl:NU(NCr) Charles River Labs). All experiments were approved by the Institutional Animal Care and Use Committee. Tumors were grown in either the shoulder or thigh region.

2.6. Intravital microscopy

Intravital microscopy (IVM) was performed on live mice using a customized Nikon A1R system [35]. This upright fluorescent imaging system features long-focus air objectives lenses up to 40×, with a 6-color laser illumination system and Nikon NIS Elements 4.0 imaging and analysis software. Two laser wavelengths were used for all experiments: 488 nm (FITC) and 640 nm (DiD). Setup was done via brightfield imaging. All imaging was done with the mice maintained under general anesthesia via isofluorane inhalant. A tail vein catheter was placed in the tail of each mouse prior to placing on the imaging platform. A skin flap was removed to expose the tissue underneath. The wound was flooded with saline and a coverslip was placed on top using minimal pressure. For ultrasound treatment, a physiotherapy device (Metron Accusonic) was used. The transducer head was covered with clear ultrasound gel