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Image-based analysis of the size- and time-dependent penetration of polymeric micelles in multicellular tumor spheroids and tumor xenografts

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ABSTRACT

While the heightened tumor accumulation of systemically administered nanomedicines relative to conventional chemotherapeutic agents has been well established, corresponding improvements in therapeutic efficacy have often been incommensurate. This observation may be attributed to the limited exposure of cancer cells to therapy due to the heterogeneous intratumoral distribution and poor interstitial penetration of nanoparticle-based drug delivery systems. In the present work, the spatio-temporal distribution of block copolymer micelles (BCMs) of different sizes was evaluated in multicellular tumor spheroids (MCTS) and tumor xenografts originating from human cervical (HeLa) and colon (HT29) cancer cells using image-based, computational techniques. Micelle penetration was found to depend on nanoparticle size, time as well as tumor and spheroid cell line. Moreover, spheroids demonstrated the capacity to predict relative trends in nanoparticle interstitial transport in tumor xenografts. Overall, techniques are presented for the assessment of nanoparticle distribution in spheroids and xenografts and used to evaluate the influence of micelle size and cell-line specific tissue properties on micelle interstitial penetration.

1. Introduction

Poor penetration of chemotherapeutic agents in solid tumors has been recognized as one of the major challenges limiting the efficacy of macromolecular and nanoparticle-based cancer therapies (Minchinton and Tannock, 2006). Distribution of therapeutic agents within a tumor may be hindered by the heterogeneous architecture of the vascular network, abnormal blood flow, dense nature of the extracellular matrix and interstitial hypertension (Chauhan et al., 2011; Jang et al., 2003; Minchinton and Tannock, 2006). Mitigation of transport barriers may be achieved through the design of optimized drug delivery vehicles using *in vitro* and *in vivo* tumor models that account for these and other critical features of the tumor microenvironment.

Supramolecular assemblies of amphiphilic copolymers known as block copolymer micelles (BCMs) constitute a promising class of nanoparticles for the systemic delivery of chemotherapy (Blanco et al., 2011; Matsumura, 2008; Mikhail and Allen, 2009; Miyata et al., 2011; Vicent et al., 2009; Yokoyama, 2010). In contrast to conventional small molecule surfactants that are commonly used as solubilizing excipients, biocompatible copolymers such as poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG-*b*-PCL) generate micelles with a greater degree of thermodynamic and kinetic stability and readily tunable properties, including their size (Mikhail and Allen, 2010). Stable, long-circulating BCMs can accumulate preferentially within a tumor *via* the enhanced permeability and retention (EPR) effect, resulting in tumor-specific delivery of the encapsulated drug (Iyer et al., 2006; Maeda et al., 2009).

Indeed, the systemic delivery of anti-cancer drugs *via* nanoparticle encapsulation has been shown to increase their tumor accumulation relative to conventional chemotherapeutic agents (Alexis et al., 2010; Miyata et al., 2011; Nishiyama and Kataoka, 2006; Torchilin, 2011). However, seminal research pertaining to the intratumoral fate of macromolecules and nanoparticles suggests that they are heterogeneously distributed and display limited penetration into avascular tumor compartments (Cabral et al., 2011; Dreher et al., 2006; Hoang et al., 2009; Minchinton and Tannock, 2006; Primeau et al., 2005; Tunggal et al., 1999;

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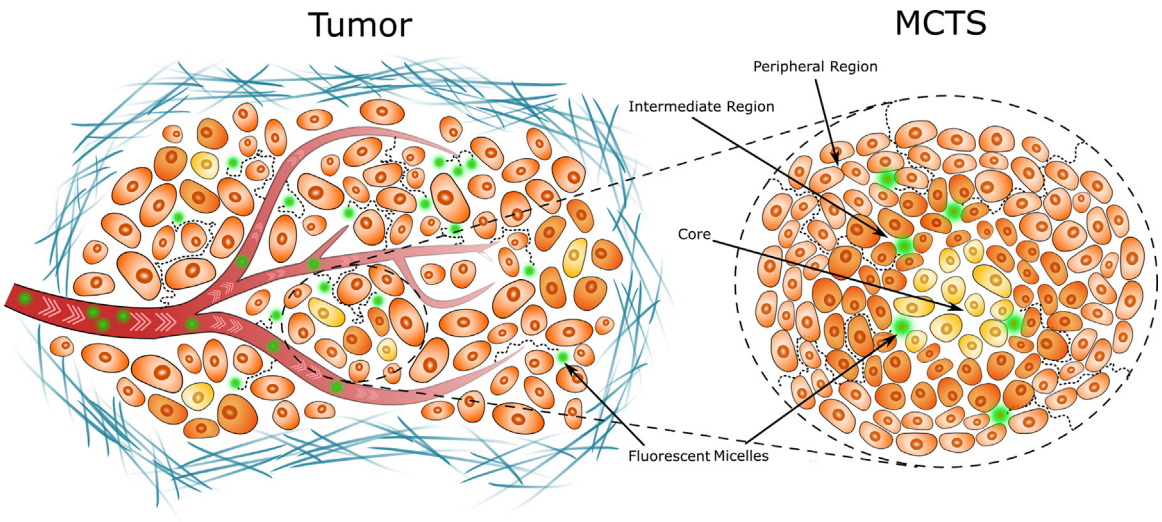

Fig. 1. Schematic representation of tumor and MCTS cross-sections. MCTS are composed of a peripheral region of proliferating cells surrounding an intermediate region of quiescent cells and a nutrient deprived or necrotic core mimicking the radial organization of tissues adjacent to a tumor blood vessel.

Yuan et al., 1994). While evaluating chemotherapeutic nanomedicines in conventional tissue cultures provides insight into therapeutic responses at the cellular level, this approach often fails to accurately predict a formulation's efficacy *in vivo*. This is largely due to the simplified structure of monolayer cultures which does not account for multicellular mechanisms of drug resistance and transport restrictions commonly associated with 3-dimensional (3-D) tumor tissues. In contrast, 3-D cultures such as multicellular tumor spheroids (MCTS) possess an established extracellular matrix as well as pH, oxygen, metabolic and proliferative gradients analogous to the microenvironment in hypovascular and avascular regions of solid tumors (Acker et al., 1987; Nederman et al., 1984; Rotin et al., 1986). MCTS are composed of an outer layer of proliferating cells surrounding a quiescent cell layer and necrotic core, mimicking the radial organization of tissues adjacent to a perfused tumor blood vessel (Fig. 1) (Hirschhaeuser et al., 2010). As such, MCTS may provide a useful platform for studying diffusion-based transport of nanoparticles in a milieu that better reflects the structural and microenvironmental heterogeneity commonly associated with solid tumors.

The primary objectives of the present study were to: (1) develop image-based, computational methods for evaluating the spatial and temporal penetration of nanoparticles in spheroids and corresponding tumor xenografts; (2) apply these methods to evaluate the influence of the size of nanoparticles on their penetration and distribution in tumor tissues; and (3) evaluate the capacity of the spheroid model to predict the interstitial penetration of nanoparticles in tumor xenografts. Ultimately, this study demonstrates important frameworks for the development of novel nanomedicines with enhanced intratumoral penetration.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) of different molecular weights (CH_3O-PEG-OH; $M_n = 5000$, $M_w/M_n = 1.06$; $M_n = 2000$, $M_w/M_n = 1.06$) were obtained from Sigma–Aldrich (Oakville, ON, Canada). ε-Caprolactone and dichloromethane were purchased from Sigma–Aldrich and dried using calcium hydride prior to use. Hydrogen chloride (HCl, 1.0 M in diethyl ether), N,N-dimethylformamide (DMF), diethyl ether, hexane and acetonitrile were also purchased from Sigma–Aldrich and used without further purification. Alexa Fluor® 488 (AF488) carboxylic acid succinimidyl ester was purchased from Molecular Probes (Eugene, OR).

2.2. Synthesis of CH_3O-PEG-b-PCL (PEG-b-PCL) and Alexa Fluor® 488-PEG-b-PCL (AF488-PEG-b-PCL) copolymers

PEG-*b*-PCL copolymers were prepared by metal-free cationic ring opening polymerization of ε-CL with CH_3O-PEG-OH as macroinitiator in the presence of HCl using an established method previously reported (Mikhail and Allen, 2010). For the synthesis of fluorescently labeled copolymer (AF488-PEG-*b*-PCL), NH_2-PEG-OH was used in place of CH_3O-PEG-OH for polymerization. Alexa Fluor® carboxylic acid succinimidyl ester (AF488) was then conjugated to NH_2-PEG-*b*-PCL (2× molar excess with respect to AF488) in DMSO with TEA (30× molar excess with respect to NH_2-PEG-*b*-PCL) for 24 h at room temperature. AF488-PEG-*b*-PCL was purified using a silica gel 100 C8- reverse phase column (Sigma–Aldrich, Oakville, ON, Canada) packed in 50/50 acetonitrile/water and equilibrated with water prior to injection of the reaction mixture. Unbound AF488 was eluted using 3-bed volumes of water followed by elution of the AF488-PEG-*b*-PCL conjugate using 2-bed volumes of a 90/10 solution of acetonitrile/water. The purity of the conjugate was assessed by high performance liquid chromatography (HPLC) using a Styragel HR2 column (effective molecular-weight range of 500–20,000; Waters, MA) connected to a fluorescence detector (Agilent Technologies Inc., CA) with excitation and emission wavelengths of $\lambda_{ex} = 495$ nm and $\lambda_{em} = 519$ nm, respectively. Filtered HPLC grade DMF was used as the mobile phase with a flow rate of 0.5 mL/min.

2.3. Preparation and characterization of BCMs

Micelles were prepared from PEG_{2000}-*b*-PCL_{1000} or PEG_{5000}-*b*-PCL_{5000} copolymers by hydration of thin films. First, copolymers were dissolved in DMF and stirred overnight. For generation of fluorescent BCMs, AF488-conjugated PEG_{2000}-*b*-PCL_{1000} or PEG_{5000}-*b*-PCL_{5000} copolymers dissolved in DMF were added to the unlabeled copolymer in order to achieve an equivalent final AF488 concentration of 100 nM in each BCM sample. DMF was evaporated under N_2 at 30 °C and trace solvent was removed under vacuum. Dry copolymer films were then heated to 60 °C in a water bath prior to the addition of PBS buffer (pH 7.4) at the same temperature with thorough vortexing. Resultant micelle solutions were stirred for