Journal of Controlled Release 183 (2014) 114–123

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Phosphatidylserine-targeted bimodal liposomal nanoparticles for *in vivo* imaging of breast cancer in mice

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ARTICLE INFO

Article history:
Received 14 February 2014
Accepted 24 March 2014
Available online 1 April 2014

Keywords:
Phosphatidylserine (PS)
Bimodal liposomal nanoprobe
Tumor vasculature
Magnetic resonance imaging (MRI)
Near-infrared optical imaging
Breast cancer

ABSTRACT

Phosphatidylserine (PS) that is normally constrained to the inner plasma membrane becomes exposed on the surface of endothelial cells (ECs) in tumor vasculature. In the present study, we report the development of a novel tumor vasculature-targeted liposomal nanoprobe by conjugating a human monoclonal antibody, PGN635 that specifically targets PS to polyethylene glycol-coated liposomes. MR contrast, superparamagnetic iron oxide nanoparticles (SPIO) were packed into the core of liposomes, while near-infrared dye, DiR was incorporated into the lipophilic bilayer. The liposomal nanoprobe PGN-L-IO/DiR was fully characterized, and its binding specificity and subsequent internalization into PS-exposed vascular ECs was confirmed by *in vitro* MRI and histological staining. *In vivo* longitudinal MRI and optical imaging were performed after i.v. injection of the liposomal nanoprobes into mice bearing breast MDA-MB231 tumors. At 9.4 T, T_2-weighted MRI detected drastic reduction on signal intensity and T_2 values of tumors at 24 h. Ionizing radiation significantly increased PS exposure on tumor vascular ECs, resulting in a further MRI signal loss of tumors. Concurrent with MRI, optical imaging revealed a clear tumor contrast at 24 h. Intriguingly, PGN-L-IO/DiR exhibited distinct pharmacokinetics and biodistribution with significantly reduced accumulations in liver or spleen. Localization of PGN-L-IO/DiR to tumor was antigen specific, since a control probe of irrelevant specificity showed minimal accumulation in the tumors. Our studies indicate that PS-targeted liposomes may provide a useful platform for tumor-targeted delivery of imaging contrast agents or potentially anti-cancer drugs for cancer theranostics.

1. Introduction

Solid tumors are well known for their ability to grow in the harsh microenvironment characterized by hypoxia, acidosis and oxidative stress. All these factors contribute significantly to genetic and epigenetic instability in tumor cells as well as tumor stromal cells. Studies by Thorpe's lab have demonstrated that the oxidative stress within tumor microenvironment causes redistribution of phosphatidylserine (PS), the most abundant anionic phospholipid of the cell membrane, from the inner to the outer membrane leaflet of tumor endothelial cells (ECs; Schemes in Fig. 1a) [1,2]. These cells are actually found to be viable and not subject to apoptotic process. PS exposure on tumor ECs is inducible and reversible [3,4]. The cells can resume growth and reestablish phospholipid asymmetry, which is distinct from the irreversible process occurring in cell death [4,5]. Examinations of a large panel of tumor types including prostate, breast, lung and brain tumors grown in mice and rats have exhibited that PS exposure on tumor ECs is universal, despite the extent of exposure varying between the tumor types [6–10]. In normal mammalian cells, even in those highly angiogenic ovarian blood vessels during ovulation, PS is asymmetrically distributed across the plasma membrane with essentially all the PS localized in the cell's inner membrane. Thus, PS exposed on tumor but not normal vascular endothelial cells creates a highly specific biomarker for tumor vasculature. A fully human monoclonal PS-targeting antibody, PGN635 has recently been developed. PGN635 binds to PS complexed with the PS-binding protein, β2-glycoprotein I (β2GP1) with a higher specificity and affinity to PS (kD ≈ 10^{-10} M), as compared to annexin V [10–12].

We have previously utilized PGN635 to develop a PS-targeted near infrared fluorescence probe to facilitate *in vivo* optical imaging of glioma in mouse models [10]. Successful visualization of glioma grown in mouse brain provides the proof of principle that PS-targeted imaging probes may be useful for sensitive tumor detection. MRI is the most commonly used imaging modality in clinical oncology, with high spatial resolution and excellent soft tissue contrast. MRI has recently been recommended by the American Cancer Society for breast cancer screening as an adjunct to mammography [13]. However, with the traditional contrast agent, gadolinium, MRI of breast cancer is still encountering the challenges of low sensitivity and specificity. Thus, development of

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http://dx.doi.org/10.1016/j.jconrel.2014.03.043
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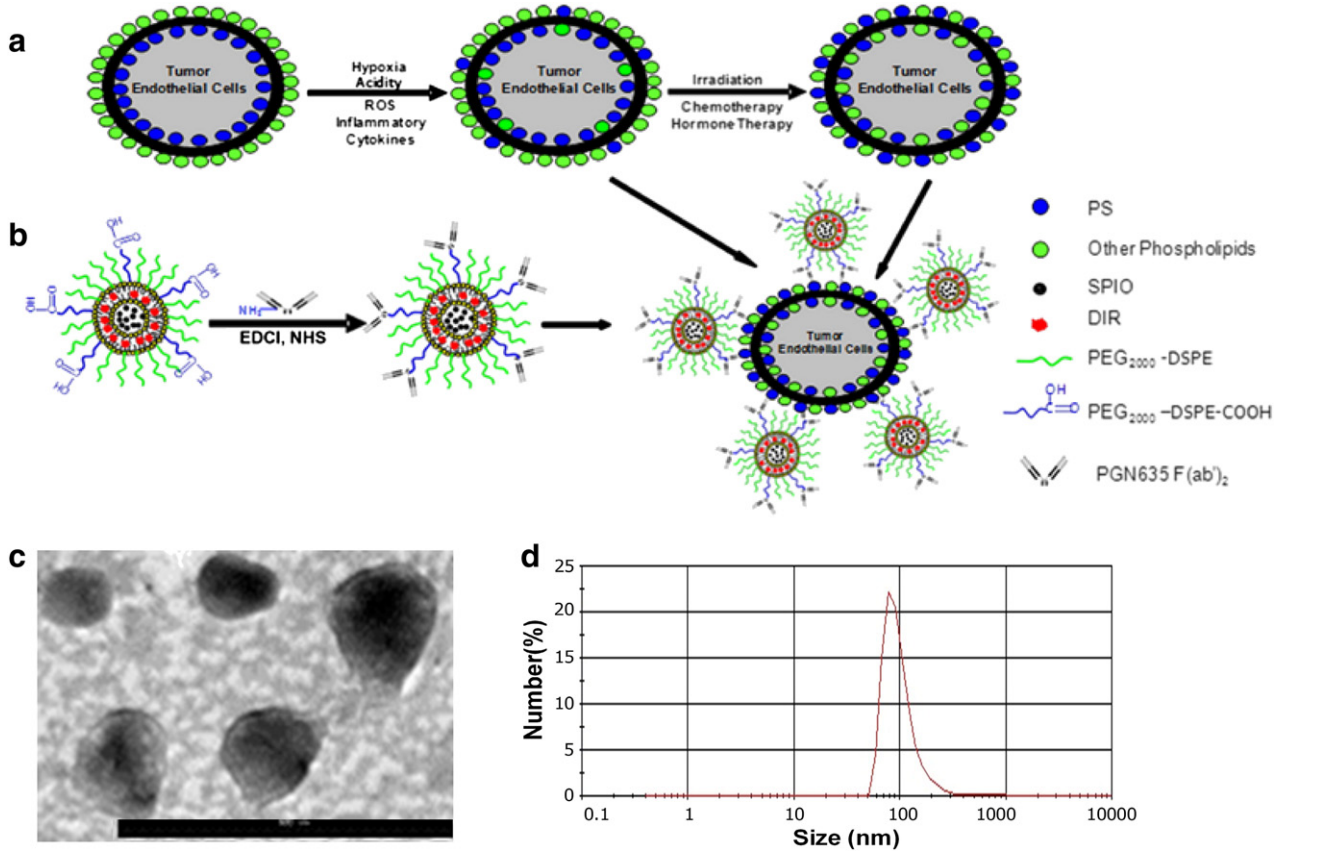

Fig. 1. PS exposure on tumor vascular endothelium targeted specifically by bimodal liposomal nanoprobes. Schematic illustrations: a. PS exposure specifically on tumor vascular endothelial cells, and b. Preparation of liposome loaded with MRI and optical bimodal contrast and functionalized with PGN635F(ab′)$_2$ that bind to exposed PS. c. A representative transmission electron microscopic (TEM) image (bar = 500 nm) of PGN-L-IO/DiR enclosing SPIO in their cores. d. A size distribution curve obtained by dynamic light scattering (DLS) analysis indicates an average hydrodynamic size of PGN-L-IO/DiR = 111 nm.

breast cancer-targeted molecular imaging probes may help to improve the sensitive and specific detection of breast cancer at an early stage.

Many efforts have been made to improve delivery of chemotherapeutics or imaging agents to tumors. Liposome-based nanocarriers have been widely applied for such molecular transport because of their capacity of a large payload and the protective bilayers shielding the enclosed molecules from interaction with the contents of the blood stream [14,15]. By coating the liposomal surface with PEG-polymers, the circulation time of liposomes can be significantly prolonged and thus increased efficacy can be achieved [16,17]. Liposomes are also known for their preference for tumor accumulation due to the enhanced permeation and retention (EPR) effect [18]. However, the selectivity based upon the EPR effect has been demonstrated to vary significantly both intra- and inter-tumorally due to the heterogeneous nature of tumors [19,20]. Thus, functionalization of liposomes with tumor-specific targeting moieties will be essential to achieve enhanced tumor selectivity [20–22].

In this study, we utilized liposomes as nanocarriers to develop PS-targeted dual MRI/optical imaging nanoprobes, PGN-L-IO/DiR. MR contrast, superparamagnetic iron oxide nanoparticles (SPIO, 10 nm in diameter) were entrapped inside the aqueous cores, while near infrared dye, DiR (absorption/emission wavelength: 748/780 nm) was incorporated into the bilayers of liposomes. We then conjugated the F(ab′)$_2$ fragments of PGN635 to the carboxyl groups on the distal terminus of PEG chains that are coated on the surface of liposomes (Schemes in Fig. 1b). PGN-L-IO/DiR nanoprobes were fully characterized and their binding specificity to exposed PS was studied *in vitro* by MRI and histological staining. *In vivo* dual MRI/optical imaging was performed to longitudinally monitor changes in tumor contrast after i.v. injection of PGN-L-IO/DiR into mice bearing human breast MDA-MB231 tumors. Ionizing radiation that is known to induce oxidative stress was used to increase PS exposure on vascular ECs and tumor cells. MDA-MB231 tumors after irradiation were imaged to reveal enhanced tumor contrast due to increased PS binding of PGN-L-IO/DiR. Distribution of PGN-L-IO/DiR nanoprobes in normal organs was also evaluated by *in vivo* MRI and *ex vivo* optical imaging. Superior tumor uptakes and significantly reduced liver and spleen accumulations compared to the control liposomal probes further indicate the highly specific tumor targeting of PGN-L-IO/DiR.

2. Material and methods

2.1. Preparation and characterization of PS-targeted liposomes loaded with MRI and optical imaging contrast

The procedure used for preparation of liposomes has been described previously [23]. In brief, egg phosphatidylcholine (EPC) (Sigma-Aldrich Corporation, MO), cholesterol (Sigma-Aldrich Corporation, MO), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$] (PEG$_{2000}$–DSPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)$_{2000}$] (COOH-PEG$_{2000}$-DSPE) (Avanti Polar Lipids, Alabaster, AL) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine Iodide (DiR, Perkin-Elmer, Waltham, MA) were dissolved in chloroform (53:45:4:1:0.8 μmol/μmol) in a pear-shaped flask. The lipid film was prepared by removing the chloroform using a rotary vacuum evaporator. The rough DiR plus SPIO liposomes were produced by hydration of the film with PBS containing SPIO (10 nm, Ocean Nanotech, AR) with sonication in water bath for 5 min, followed by sonication using a probe-type sonicator (Omni International Inc., Kennesaw, GA) for 5 min. The sample was centrifuged at 1000 *g* for