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NANOMEDICINE

Polymeric micelles for enhanced lymphatic drug delivery to treat metastatic tumors

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ABSTRACT

Polymeric micelles have been proven to be a promising nano-sized system for drug delivery. Understanding its *in vivo* behaviors at the whole body, tissue and cellular levels is critical for translating this drug delivery system into clinical practice. In this study, the 14.5 nm micelles made of polyethylene glycol-phosphatidylethanolamine (PEG-PE) for delivery of doxorubicin and vinorelbine were investigated. Using confocal and two-photon microscopy imaging of live mice or tissue sections, we observed that after systemic administration, the fluorescently labeled PEG-PE micelles encapsulating doxorubicin migrated through blood vessels in entirety into the interstitial tissue, collected by lymphatic vessels, and accumulated in lymph nodes. Importantly, encapsulated drugs such as vinorelbine (Nanovin), preferentially accumulate in lymph nodes when compared to the free drugs. Moreover, the *in vivo* bioluminescent imaging showed that Nanovin significantly reduced lymph node metastasis rate ($P < 0.05$) in 4 T1-luc2 murine breast tumor bearing mice. Finally, we observed that Nanovin enhanced antitumor activity against primary tumors and lung metastases while having low toxicity in various 4 T1 tumor models. This study suggests that PEG-PE micelle is a promising drug delivery system for the treatment of lymphatic metastases, and may also have important applications in other lymphatic system-related diseases.

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1. Introduction

Metastasis affects approximately 90% of cancer patients, yet developing effective therapeutic interventions has proven to be elusive [1,2]. Cancer cells escape from the primary tumor site to secondary organs via two major routes, blood vessels and lymphatic vessels. In fact, the lymphatic dissemination is found to precede hematogenous dissemination in many types of cancers, including melanoma, breast, prostate, colon and lung cancers [3]. Evidence of tumor cells in a lymph node is often the first indicator of cancer spread. Also, lymph node metastasis is correlated with an increased risk of distant metastasis and poor clinical outcome [4]. Therefore, preventing or inhibiting lymph node metastasis is critical for improving the outcome of patients.

Conventional chemotherapeutic drugs fail to enter the lymphatic system effectively due to dose-limiting toxicities or failure to access the lymphatics at adequate concentrations via intravenous (i.v.) injection. Encapsulating chemotherapeutic drugs into nanocarriers may improve drug accumulation in the lymphatic system, if their particle size and administration route are appropriate. Lymphatic uptake requires an optimal size between 10 nm to 100 nm [5], while the nanocarriers need to be small enough to extravasate from blood vessels when administrated intravenously. For this reason, the delivery of drug into regional lymphatic system is mostly facilitated by local injection of colloidal particles, such as liposomes [6–8], emulsion [9] and nanoparticles [10,11]. The polymer-conjugated protein drug SMANCS in lipiodol formulation was found to be delivered to lymph nodes after tumor-feeding artery injection [12–15]. However, there are limited studies regarding lymphatic drug delivery system via conventional intravenous injection. Intravenously injected drug delivery systems have better compliance and suitability for multiple dose administration compared with local injected drug delivery systems.

Among novel drug delivery systems, polymeric micelles are emerging as a promising platform for drug delivery. Polymeric micelles are nanosized supramolecular assemblies of amphiphilic polymers that possess a core-shell type architecture. The hydrophobic core of micelles is a drug reservoir, and the shell is a hydrophilic corona that provides a

Abbreviations: LN, lymph node; Vin, vinorelbine; Dox, doxorubicin; ICG, indocyanine green; Axi, axillary; Ing, inguinal; Mes, mesentery; i.v, intravenous; PEG-PE, distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000].

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protective interface between the core and the external environment [16]. Importantly, alterations in the composition of the constituent polymers influence their characteristics including micelle size and shape, core-drug compatibility, drug loading capacity and release, and stability, enabling manipulation of the encapsulated drug's pharmacokinetic profile, tissue and cellular level distribution [17–19]. The polyethylene glycol-phosphatidylethanolamine (PEG-PE)-based polymeric micelle is one of the most renowned micelle for small molecule anti-cancer drug delivery. Above the critical micelle concentration (CMC), PEG-PE spontaneously self-assembles to form micelles. The flexible PEG serves as a hydrophilic shell, and the PE serves as a hydrophobic core to admit drugs. To achieve specific delivery, a full understanding of the fate of polymeric micelle-based dug formulations is needed. However, knowledge of many points related to micelle fate, such as stability *in vivo* [20], capability of extravasation [21,22], transport within tissue and transport at cellular level [19,23], remains limited.

Previously we have shown that PEG-PE micelles can effectively encapsulate doxorubicin [24]. Micelle-encapsulated doxorubicin showed much better antimetastatic activity than free (unencapsulated) doxorubicin through i.v. injection. However, the mechanism underlying the enhanced antimetastatic activity for this micelle-based drug delivery system remains unclear. We hypothesize that the micelle encapsulation changes the *in vivo* behaviors of chemotherapeutic drugs. Micelle encapsulation may cause more drug accumulation in lymph nodes, which can be sanctuary sites of metastatic tumor cells. To test this hypothesis, we used imaging techniques to investigate the *in vivo* behaviors of PEG-PE micelles alone or with their cargos, and further confirmed the antimetastatic property of this delivery system by encapsulating chemotherapeutic drug vinorelbine.

2. Materials and methods

2.1. Drugs and reagents

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG_{2000}-DSPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000-N′-carboxyfluorescein] (CF-PEG-PE), hydrogenated phos-phatidylcholine (HSPC), cholesterol, and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-n-(lissamine rhodamine B sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids. Doxorubicin hydrochloride and Vinorelbine tartrate were provided by Hisun Pharmaceutical Co. Ltd (Zhejiang, China) and Minsheng Corp (Zhejiang, China), respectively. Indocyanine green (ICG) was from Sigma.

2.2. Animals and cells

Female BALB/c mice or immunodeficient BALB/c nu/nu mice were purchased from the Vital River Laboratory Technology Co. Ltd. (Beijing, China), and treated in compliance with Animal Care and Use Committee of Institute of Biophysics guidelines. The mouse mammary carcinoma cell line 4 T1 and the human melanoma tumor cell line MDA-MB-435S were purchased from American Type Culture Collection (ATCC). The luciferase expressing cell line 4 T1-luc2 was purchased from Caliper Life Science. 4 T1 and 4 T1-luc2 cells were grown in RPMI 1640 medium and MDA-MB-435S cells were grown in L15 medium. All cell lines were cultured in media supplemented with 10% fetal bovine serum (Invitrogen) and maintained at 37 °C in a 5% CO_2, 95% humidity incubator.

2.3. Preparation of micelles and liposomes

The empty PEG-PE micelle stock solution was prepared by dispersing PEG-PE in Milli-Q water at a concentration of 14 mM. To label the micelles, carboxyfluorescein covalently conjugated PEG-PE (CF-PEG-PE) was mixed with unlabeled PEG-PE at 2.5:100 molar ratio to form the CF-labeled micelles. Micelles encapsulating doxorubicin hydrochloride, vinorelbine tartrate, or indocyanine green (ICG) were prepared by a one-step self-assemble method as previously described [25]. Briefly, each drug was dissolved in Milli-Q water, and mix with PEG-PE micelle stock solution at their optimal molar ratios (doxorubicin: PEG-PE = 0.5:1, vinorelbine: PEG-PE = 0.25:1, ICG: PEG-PE = 0.01:1). The mixtures were incubated at 60 °C for 30 min to allow for drug encapsulation. After incubation, the prepared drug-loaded micelles were purified by passage through a 0.22 μm polyethersulfone syringe membrane, and the flow-through part was collected for further study. The encapsulation efficiency of drugs was determined after removing the free drug via ultrafiltration [25]. The drug concentration in micelles was measured by high-performance liquid chromatography (HPLC). The double-labeled drug-loaded micelles were made using a similar procedure by incubating fluorescent drug doxorubicin and CF-labeled micelles.

Labeled liposome was prepared by dissolving HSPC, cholesterol and rhodamine-PE in chloroform at a molar ratio of 55:45:1. Chloroform was removed, and the formed lipid film was hydrated by PBS at 60 °C for 1 h to form the liposomal suspension. This suspension was extruded by the Extruder with 100 nm polycarbonate membrane (Whatman) to become the uniform liposome with a diameter of 100 nm.

2.4. Two photon microscopy (TPM)

To study the extravasation of micelles, mice were i.v. injected with fluorescence labeled micelles or liposomes or free fluorescent dyes as described in the Supplementary methods. Imaging was performed using a multiphoton laser scanning microscope (Olympus' Fluoview FV1000-MPE, Japan). The excitation wavelength was set to 810 nm, and the filter BA495-540 was used for CF labeled PEG-PE fluorescence collection, the filter BA570-625 was used for doxorubicin fluorescence collection. The images around blood vessels in the dorsal skin of live nude mice were continuously collected for 1 min at 1, 2 and 3 h intervals after injection. The fluorescence intensity was quantified using Image J 1.43 m.

2.5. Confocal microscopy and fluorescence molecular tomography

For confocal microscopy studies, female athymic BALB/c *nu*/*nu* mice were injected i.v. with CF-labeled micelles alone or CF-labeled micelles encapsulating doxorubicin, and allowed to circulate for 60 min. To study the lymphatic transportation of micelles, rhodamine-labeled liposomes were i.v. administered 3 min before observation to serve as a blood pool marker. Mice were sacrificed. Skin samples were removed, fixed and scanned by confocal microscope (Fluor View FV500, Olympus) [26]. To study the interstitial tissue and lymph node accumulation, mice were autopsied 60 min after the administration of CF-labeled micelles encapsulating doxorubicin injection. Lymph nodes and small intestines were harvested. Lymph nodes were frozen in OCT and 10-μm sections were cut with a Cryostat microtome (Leica CM3050S). The mesentery was prepared by stretching out a loop of the small intestine on a slide. Specimens were air dried and examined [27].

Full angle fluorescence molecular tomography system was used to evaluate the specific lymph node accumulation of micelle-encapsulated indocyanine green (Nano-ICG). Mice were given an i.v. injection of Nano-ICG (3 mice per group), and were sacrificed at 1 and 3 h after injection. *Ex vivo* spectral fluorescence images from lymph nodes were obtained using laser with excitation/emission: 780 nm/840 nm [28,29].

2.6. Tumor models

In all animal experiments, mice were randomly divided into three groups: the control group, the free vinorelbine treatment group and the micelle-encapsulated vinorelbine (Nanovin) treatment group. Unless otherwise indicated, these groups were treated intravenously with PBS, 5 mg/kg free vinorelbine, or 5 mg/kg Nanovin (based on

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