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Ultrasound-sensitive nanoparticle aggregates for targeted drug delivery

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ABSTRACT

Here we describe injectable, ultrasound (US)-responsive, nanoparticle aggregates (NPAs) that disintegrate into slow-release, nanoscale, drug delivery systems, which can be targeted to selective sites by applying low-energy US locally. We show that, unlike microbubble based drug carriers which may suffer from stability problems, the properties of mechanical activated NPAs, composed of polymer nanoparticles, can be tuned by properly adjusting the polymer molecular weight, the size of the nanoparticle precursors as well as the percentage of excipient utilized to hold the NPA together. We then apply this concept to practice by fabricating NPAs composed of nanoparticles loaded with Doxorubicin (Dox) and tested their ability to treat tumors via ultrasound activation. Mouse studies demonstrated significantly increased efficiency of tumor targeting of the US-activated NPAs compared to PLGA nanoparticle controls (with or without US applied) or intact NPAs. Importantly, when the Dox-loaded NPAs were injected and exposed to US energy locally, this increased ability to concentrate nanoparticles at the tumor site resulted in a significantly greater reduction in tumor volume compared to tumors treated with a 20-fold higher dose of the free drug.

1. Introduction

Effective concentrations of cancer chemotherapy agents often cannot be administered due to dose-limiting side effects or to active impediments to delivery of high drug doses at tumor sites [1]. Nanotechnology-based drug delivery systems have been designed to combat these limitations by increasing achievable drug concentrations locally, primarily due to their size-dependent ability to passively accumulate in tumors via the enhanced permeability and retention (EPR) effect [2,3]. Once these particles accumulate in the tumor, the poorly developed lymphatic system in the tumor microenvironment retains them and allows particles to deliver their drug payload [4,5]. Indeed, several chemotherapeutic agents loaded in nanoparticles have been approved by the FDA since 1999 mainly based on their improved side effect profile, including Abraxane (an albumin particle-based formulation), Mariqbo, Doxil and DaunoXome (liposomal formulations) [6,7]. However, these nanoparticulate drug formulations still have to overcome high interstitial pressures to penetrate solid tumors [1], and while these nanotherapies can reduce side effects and improve outcomes in animals [4,5] as well as humans [6,7], there is still a significant need to improve their effectiveness for treatment of cancers [8].

US-triggered drug release from liposomes [9], lipid nanobubbles [10] or even PLGA nanoparticles [11] has been investigated with US being applied after the particles passively homed to the tumor site via the EPR effect. Indeed, US also has been explored to remotely

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induce release of drug payloads utilizing microbubbles [12] (which are used clinically as US contrast agents) injected intravenously [13]. However, the effectiveness of microbubbles as drug carriers is significantly limited by their low loading capacity, which is driven by their surface area and shell thickness. To remedy this shortfall, researchers have functionalized the surface of microbubbles with liposomes or other nanoparticles loaded with drugs [14] adding complexity and stability challenges. An even greater limitation of microbubbles, however, is that US exposure only triggers a short-lived burst of drug release within the vasculature near the targeted site as micron-sized drug-release carriers cannot extravasate into tumor tissue due to their size. The triggered rupture of these microbubbles near the endothelial wall can also provoke endothelial damage or hemorrhage [15,16]. In the present study, we therefore set out to develop a new approach that would leverage low energy US to induce deployment and concentration of nanoscale, controlled drug release polymers that could then extravasate at tumor sites.

Here we describe the development of US-responsive poly(lactic-*co*-glycolic acid) (PLGA) nanoparticle aggregates (NPAs) loaded with doxorubicin (Dox) that when injected in the bloodstream are selectively induced to disassemble and deploy multiple, drug-loaded, sustained release nanoparticles at the tumor sites by applying low-energy US radiation in these regions (Fig. 1A). To develop this capability, we leveraged a recently developed spray drying method that creates NPAs from many component 180 nm PLGA nanoparticles, which can be activated by abnormally high fluid shear stresses that occur at sites of vascular occlusion when injected intravenously; when coated with the clot-busting drug, tissue plasminogen activator (tPA), these NPAs were shown to significantly increase animal survival in a mouse pulmonary embolism model even when injected at $1/100^{th}$ the dose required to produce similar effects using free drug [17,18]. In the current study, Dox was encapsulated within the PLGA nanoparticles to create nanoscale drug delivery vehicles before they were assembled to form microscale (~4 μm diameter) NPAs, which were then injected intravenously via the retro-orbital route and induced to disassemble into individual nanoparticles at tumor sites by applying low-energy US radiation locally. We also characterized the Dox-NPAs, evaluated their therapeutic effectiveness with US stimulation, and demonstrated their superiority relative to intact Dox-NPAs, isolated Dox-nanoparticles or free Dox in terms of therapeutic efficacy and survival in a mouse model.

2. Materials and methods

2.1. Procedure for spray drying nanoparticles into NPAs

Doxorubicin PLGA nanoparticles from Phosphorex, Inc. (50:50, 17 kDa, 182.2 ± 3.5 nm, Dox:PLGA w:w ratio 1.4:100) or coumarin PLGA nanoparticles from Phosphorex, Inc. (50:50, 17 kDa, 188.0 ± 6.4 nm, coumarin:PLGA w:w ratio 1:100) were resuspended in water (10 mg/mL) and supplemented with 2 mg/mL L-leucine (Spectrum Chemicals & Laboratory Products, CA). The suspension was spray dried into nanoparticle aggregates (Dox-NPAs) with an ethanol to water ratio of 3:2 injected at a rate of (30:20) mL/min (85–87 °C) using a Mobile Minor spray dryer (Niro, Inc.; Columbia, MD) [19]. The gas flow was set at 25 g/min and nozzle pressure was kept between 33 and 35 psi. This procedure leads to production of NPAs that average 4.5 μm in diameter, with only a small fraction (2–4%) of residual nanoparticles.

2.2. Procedure for US-activation of NPAs

Following spray drying, suspensions of NPAs (1 mg/mL in serum free RPMI, 3 mL) underwent ultrasound stimulation for 3 min using Sonicator 740 from Mettler Electronics Corp. (intensity: 2.2 Watt/cm^2; 5 cm^2 applicator; pulse 20%) and an acoustic gel. The power density (2.2 W/cm^2) is presented as the spatial average temporal peak (SATP); the acoustic pressure represents the peak-to-peak value.

2.3. Physicochemical characterization of nanoparticles and NPAs

Size distribution of nanoparticles and microaggregates were analyzed using the Nano ZS and Mastersizer Malvern Instruments. For DLS measurements, we used a 10 μg/mL suspension of nanoparticles prepared in PBS. We also confirmed that the size distribution remains unchanged when cell culture medium (i.e. RPMI without FBS and with penicillin/streptomycin) is used to resuspend the nanoparticles (data not shown). Data were recorded at 25 °C.

Laser diffraction measurements were performed at room temperature using 3 mg of NPAs resuspended in 100 mL of distilled water (30 μg/mL). To analyze the stability of NPAs in plasma, blood from healthy donors was centrifuged at 290*g* for 10min to pellet red blood cells and the upper layer of platelet rich plasma (PRP) was collected. The PRP was then centrifuged at 1000*g* for 10 min to pellet platelets, and the platelet poor plasma was collected to assess the effect of blood proteins on NPA stability, with a focus on potential aggregation. NPAs were incubated in the platelet-depleted plasma for 20min before being assessed by laser diffraction.

Scanning Electron micrographs were obtained using a Zeiss Supra 55VP microscope operating at 4.5 keV while Transmission Electron Microscopy was done utilizing a JEOL JEM-1400 TEM operating at 80 keV. Doxorubicin loading in the particles was assessed by analyzing UV–visible absorbance at λ_{max} 482 nm after dissolving particles in a 2:1 mixture of 1 N HCl:DMSO.

2.4. Internalization of coumarin-loaded nanoparticles, NPAs and US-activated NPAs in 4T1 cells

4T1 breast cancer cells were seeded in 6-well plates (10,000 cells/cm^2) in RPMI media supplemented with 10% FBS and 1% Penicillin-Streptomycin. After 24 h, cells were incubated for 2 h with coumarin-loaded nanoparticles, coumarin-loaded NPAs and US-activated coumarin-loaded NPAs at a particle concentration equivalent to 0.05, 0.5 and 5 μM of doxorubicin in serum free medium (1mL/well). Cells were then washed twice with PBS, stained with CellMask™ Deep Red Plasma Membrane Stain (Life Technologies) and DAPI (Life Technologies) according to manufacturer protocols. As coumarin is naturally fluorescent, the coumarin-loaded nanoparticles and NPAs appear yellow when visualized by fluorescence microscopy. Cells were then washed with complete medium and imaged by confocal microscopy, or trypsinized and collected in complete RPMI before internalization evaluation was performed by flow cytometry.

2.5. Cell viability assay (MTS)

4T1 cells were seeded in 96 well plates (4000 cells/well) in RPMI supplemented with 10% FBS and 1% Penicillin-Streptomycin. After 24 h, cells were incubated for 2 h with multiple doses of doxorubicin, doxorubicin-loaded nanoparticles, doxorubicin-loaded NPAs or US-activated doxorubicin-loaded NPAs in serum free media (100 μL/well). The cells were subsequently rinsed twice with PBS and fresh RPMI was added. The MTS assays (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) were performed at 24, 48 and 72 h.