Journal of Controlled Release 187 (2014) 39–49

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Ultrasound-enhanced drug delivery in prostate cancer xenografts by nanoparticles stabilizing microbubbles

Siv Eggen [a,*], Stein-Martin Fagerland [a], Ýrr Mørch [b], Rune Hansen [c,f], Kishia Søvik [a], Sigrid Berg [c], Håkon Furu [a], Audun Dybvik Bøhn [a], Magnus B. Lilledahl [a], Anders Angelsen [d,e], Bjørn Angelsen [f], Catharina de Lange Davies [a]

[a] Department of Physics, Norwegian University of Science and Technology, Trondheim, Norway
[b] SINTEF Materials and Chemistry, Dept. of Polymer Particles and Surface Chemistry, Trondheim, Norway
[c] SINTEF Technology and Society, Dept. of Medical Technology, Trondheim, Norway
[d] Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway
[e] Department of Urological Surgery, St. Olavs Hospital, Trondheim University Hospital, Norway
[f] Department of Circulation and Diagnostic Imaging, Norwegian University of Science and Technology, Trondheim, Norway

ARTICLE INFO

Article history:
Received 24 February 2014
Accepted 9 May 2014
Available online 20 May 2014

Keywords:
Ultrasound
Drug delivery
Solid tumor
Nanoparticles
Microbubbles
Microdistribution

ABSTRACT

The delivery of nanoparticles to solid tumors is often ineffective due to the lack of specificity towards tumor tissue, limited transportation of the nanoparticles across the vascular wall and poor penetration through the extracellular matrix of the tumor. Ultrasound is a promising tool that can potentially improve several of the transportation steps, and the interaction between sound waves and microbubbles generates biological effects that can be beneficial for the successful delivery of nanocarriers and their contents. In this study, a novel platform consisting of nanoparticle-stabilized microbubbles has been investigated for its potential for ultrasound-enhanced delivery to tumor xenografts. Confocal laser scanning microscopy was used to study the supply of nanoparticles from the vasculature and to evaluate the effect of different ultrasound parameters at a microscopic level. The results demonstrated that although the delivery is heterogeneous within tumors, there is a significant improvement in the delivery and the microscopic distribution of both nanoparticles and a released model drug when the nanoparticles are combined with microbubbles and ultrasound. The mechanisms that underlie the improved delivery are discussed.

1. Introduction

Nanotechnology has brought about a new era of engineering multifunctional nanoparticles (NPs) for improved cancer diagnosis and therapy, incorporating both contrast agents for imaging and drugs for therapy. The rationale for using NPs for drug delivery is to improve the pharmacokinetics of cytotoxic drugs by aiming for the specific accumulation in tumor tissue and reducing the toxic effects towards healthy tissue. Circulating NPs may passively accumulate in solid tumors due to fenestrations in the tumor capillaries and a lack of functional lymphatics, a phenomenon that is known as the enhanced permeability and retention (EPR) effect [1,2]. Several different types of nanocarriers have been developed for delivering their payload to tumor cells including liposomes [3], polymeric micelles [4], virus particles [5], conjugates of polymer-drug [6] or lipid-drug [7] and polymeric NPs [8]. Despite the extensive research in this field and the number of particle formulations being developed, only a few pharmacological products have received FDA approval and are ready for use in the clinic [9].

A common feature among nanocarrier systems is that the particles encounter several barriers on their journey to the disease site. To obtain sufficient tumor accumulation, the NPs need to remain in the circulation for a sufficiently long time. This implies that the particles need to escape phagocytosis by the mononuclear phagocyte system (MPS). A common approach to avoid this is to coat the surface of the NPs with poly(ethylene glycol) (PEG) [10,11]. Nonetheless, to reach the cancer cells, the particles need to leave the circulation and enter the extracellular matrix (ECM) of the tumor. This is possible due to the EPR effect, although the hyperpermeability is heterogeneous [12,13]. Furthermore, the high interstitial fluid pressure (IFP) in a tumor [1,14,15] reduces convection-mediated transport of NPs across the capillary wall and throughout the ECM, and hence only a small population of cancer cells located close to blood vessels is exposed to the particles and their cargo [16,17].

Several strategies to improve the therapeutic effect, by improving the delivery and accumulation of NPs to cancer tissue, are being explored. The use of therapeutic ultrasound (US) has received great interest during the past two decades. US is non-invasive and can be focused on an area of interest in the body without affecting the surrounding tissue. US has been shown to enhance the delivery of NPs [18–20], drugs encapsulated in the shell of microbubbles (MBs) [21] and drugs co-

* Corresponding author. Tel.: +47 73593465.
E-mail address: siv.eggen@ntnu.no (S. Eggen).

http://dx.doi.org/10.1016/j.jconrel.2014.05.020
0168-3659/

injected with MBs [22]. Furthermore, in a clinical study of patients with pancreatic tumors, combining US and the co-injection of gemcitabine and MBs revealed promising results [23].

The effects of therapeutic US can roughly be divided into thermal and non-thermal effects. The induction of hyperthermia has been reported to increase blood flow and enhance the permeability of the tumor vasculature [24,25] and to release the contents of thermosensitive nanocarriers. Non-thermal effects, also known as mechanical effects, include acoustic radiation forces and acoustic cavitation. The acoustic radiation force is the transfer of momentum from the propagating US beam to the tissue in the sonicated field, which may induce the translation of objects in the tissue in the same direction as the US beam [26]. Acoustic cavitation is the formation, growth, oscillation and collapse of gas bubbles under the influence of US waves. Stable cavitation is characterized by repetitive, non-collapse oscillations of gas bubbles, which can generate local streaming, shear stresses on nearby objects and increase the permeability of blood vessels [27]. Inertial cavitation occurs when bubble growth during the rarefactional phase of an US wave becomes large enough to cause the bubble to collapse violently during the compression phase of the wave, potentially inducing jet streams, shock waves and temperature elevations [28].

Gas bubbles with a size of 1–10 μm can be administered intravenously to improve the backscattered signal from blood in US diagnostic imaging. In addition, artificially introduced MBs act as cavitation nuclei and will significantly reduce the threshold for obtaining *in vivo* cavitation. In this study, we have combined US and a novel particle platform consisting of MBs stabilized by polymeric NPs to improve the delivery of a hydrophobic model drug to tumor xenografts growing in mice. The polymeric NPs consist of poly(butylcyanoacrylate) (PBCA), which is biodegradable and biocompatible and can be functionalized with PEG and conjugated with bioactive molecules for targeting [29]. Furthermore, the size of both the NPs and the MBs can be tailored, and different drugs and contrast agents can be encapsulated in the NPs [30]. The integration of NPs surrounding a gas bubble makes this a promising agent both for US contrast enhanced imaging and for US-mediated drug delivery (Fig. 1). The NP synthesis involves only a one-step miniemulsion polymerization followed by high-speed mixing of NPs and air to produce the MBs. In the present study, the hydrophobic fluorescent dye Nile red was chosen due to its unique spectral properties, emitting light at wavelengths that depend on the hydrophobicity of the molecule binding to Nile red [31]. Confocal laser scanning microscopy (CLSM) was used to investigate the effects of different US exposures on the tumor uptake of NPs on a microscopical level, estimating both the extravasation and penetration into the ECM. Optical imaging using a whole animal imaging system was used to investigate the clearance of the NPs from the circulation.

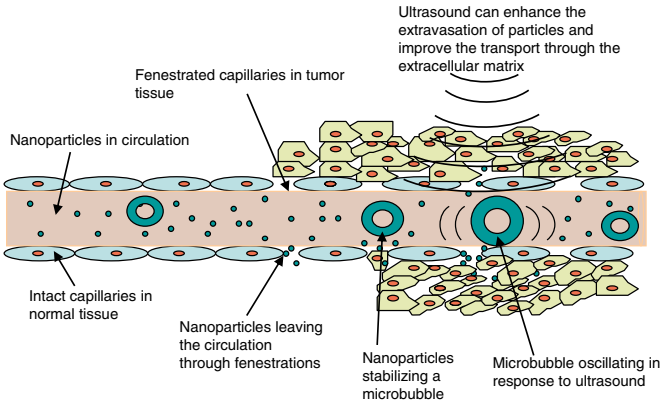

Fig. 1. Illustration of a tumor capillary with circulating nanoparticles and microbubbles stabilized by nanoparticles, exposed to focused ultrasound.

2. Materials and methods

2.1. Synthesis and characterization of nanoparticles and microbubbles

Miniemulsion polymerization was used to prepare PEGylated NPs of the polymer PBCA. Oil-in-water emulsions were prepared by emulsifying (Digital Sonifier®, Branson Ultrasonics, USA) a monomer phase, consisting of butyl-2-cyanoacrylate (BCA; Henkel Loctite, Ireland), a co-stabilizer (Miglyol 810N®, 2 wt.%, Cremer, Germany) and the fluorescent dye Nile red (0.4 wt.%,) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR; 0.1 wt.%) in an acidic aqueous medium (0.1 M HCl) containing sodium dodecyl sulfate (SDS) as a surfactant. The initiation of the anionic polymerization was performed by adding Jeffamine M-1000® (Huntsman Corporation, Belgium) to the emulsion, resulting in PEGylated NPs [30]. Excess PEG and SDS were removed by extensive dialysis against distilled water using dialysis membranes with a MWCO of 12–14,000. The size and the zeta potential of the NPs were measured by dynamic light scattering in a Zetasizer Nano ZS (Malvern, UK).

Stable air MBs were prepared by self-assembly of the NPs (1 wt.%) at the air–water interface by the addition of bovine serum albumin (BSA) and vigorous stirring using an ultra-turrax (IKA Werke, Germany) as described by Mørch et al. [30]. The average MB diameter could be controlled by varying the stirring speed. The size distribution and concentration of MBs was determined using both a Coulter Counter (Beckman Multisizer 3) and light microscopy. The shelf stability of MBs was studied by light microscopy imaging. The behavior of the MBs during US was investigated using a flow mimicking phantom under different transmit frequencies and 10 transmit oscillations, using a conventional pulse inversion technique.

2.2. Cells and animals

Human PC3 prostate adenocarcinoma cells (American Type Culture Collection, USA) were cultured in Dulbecco's Modified Eagle Medium (Life Technologies Corporation, USA) with 10% fetal bovine serum at 37 °C and 5% CO_2.

Balb/c nude mice (Taconic, Denmark) were purchased at 8 weeks of age and housed in groups of 5 in individually ventilated cages (Tecniplast) at temperatures between 19 and 22 °C and a relative humidity of 50–60%. The housing conditions were free of specific pathogens according to the recommendations set by the Federation of European Laboratory Animal Science Associations [32], and the mice had free access to food and sterile water. All experimental procedures with the animals were conducted in compliance with protocols approved by the Norwegian National Animal Research Authorities.

2.3. Tumor models

The human prostate adenocarcinoma PC3 cells were grown as xenografts in athymic mice either subcutaneously or in dorsal window chambers. Before the inoculation of subcutaneous xenografts, the animals were anesthetized with 2% isoflurane, and a suspension containing 3×10^6 cells was slowly injected subcutaneously on the lateral aspect of one hind leg. The tumors were allowed to grow until they had reached a diameter of 5–10 mm.

Before implantation of dorsal skin-fold window chambers, the mice were anesthetized with 0.15 ml of a solution of fentanyl and fluanisone (Hypnorm® vet, VetaPharma Ltd, UK), midazolam (5 mg/ml; Roche Norge AS, Norway) and sterile water (1:1:2). Two symmetrical plastic frames were attached to an extended dorsal skin fold and a window was made and sealed with a cover slip as previously described [33]. For analgesia, the mice were given a subcutaneous injection of 0.05 mg meloxicam (Boehringer Ingelheim GmbH, Germany) prior to the surgery as well as on the two subsequent days. On the day following the surgery, the cover slip was removed, and a bolus of approximately