Photoacoustics 2 (2014) 119–127

Contents lists available at ScienceDirect

Photoacoustics

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

Research Article

Cellulose nanoparticles are a biodegradable photoacoustic contrast agent for use in living mice

Jesse V. Jokerst [a], Dominique Van de Sompel [a], Sarah E. Bohndiek [a,b], Sanjiv S. Gambhir [a,b,*]

[a] Molecular Imaging Program at Stanford (MIPS), Department of Radiology, Stanford University, 318 Campus Drive, Stanford, CA 94305-5427, United States[1]
[b] Bioengineering, Materials Science & Engineering, Bio-X Stanford University, Stanford, CA 94305, United States

ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 4 April 2014
Accepted 16 July 2014
Available online 12 August 2014

Keywords:
Photoacoustic imaging
Optoacoustic
Biodegradation
Cellulose
Nanoparticle
Ovarian cancer

ABSTRACT

Molecular imaging with photoacoustic ultrasound is an emerging field that combines the spatial and temporal resolution of ultrasound with the contrast of optical imaging. However, there are few imaging agents that offer both high signal intensity and biodegradation into small molecules. Here we describe a cellulose-based nanoparticle with peak photoacoustic signal at 700 nm and an *in vitro* limit of detection of 6 pM (0.02 mg/mL). Doses down to 0.35 nM (1.2 mg/mL) were used to image mouse models of ovarian cancer. Most importantly, the nanoparticles were shown to biodegrade in the presence of cellulase both through a glucose assay and electron microscopy.

1. Introduction

Photoacoustic imaging (PAI) has been used extensively in preclinical models of human disease including prostate [1], breast [2], and ovarian cancer [3]. This modality produces contrast by converting nanosecond light pulses into an acoustic signal and offers significant improvements in spatial resolution relative to other optics-based approach [4–6]. Ovarian cancer in particular could eventually benefit from PAI due to the existing widespread use of trans-abdominal or trans-vaginal ultrasound in the screening and management of ovarian cancer [7,8]. PAI can use either endogenous signal from hemoglobin, deoxyhemoglobin, melanin, etc. or an exogenous imaging agent can be applied, which is typical for in molecular imaging experiments.

Many materials produce photoacoustic signal and can be broadly grouped as small molecules and nanoparticles. Both types offer a range of PA signal. Small molecule agents include methylene blue [9] or indocyanine green [10] with very intensephotoacoustic signal. Fluorophore, fluorescent proteins [11], quenchers [12], and activatable hybrid molecules [13,14] are also members of this category. Nanoparticle-based PAI uses gold nanoparticles, gold/silica hybrids [15,16], carbon-based particles [17,18], porphysomes [19], iron oxide nanoparticles, copper sulfide [20], and others [14,21,22]. Other nanoparticles such as poly-lactic-glutamic acid or poly-caprolactone have poor photoacoustic signal. However—on a molar basis—nanoparticles usually out-perform small molecule fluorophore.

While nanoparticles do offer robust and stable photoacoustic signal, they are hampered by poor biodistribution and clearance profiles. Indeed, one of the most common limitations of all nanoparticle imaging agents is non-specific, long term liver and spleen accumulation. While porphysomes [19] and plasmonic nanoclusters containing 5 nm gold particles linked for red-shifted resonances [23,24] may offer renal clearance, their full utility in small animal models remains unclear. Any agent that combines the signal intensity of nanoparticles with the renal clearance of small molecules would have a significant advantage toward clinical translation.

We thus considered a wide variety of naturally occurring, optically active biodegradable materials. Of interest as an alternative to solid metal nanoparticles or carbon nanotubes is cellulose [25–27]. This material has a crystalline structure [28], is readily available from renewable sources, well characterized, and easily manipulated through a variety of chemical processes [29]. Cellulose is a routine component of the human diet and has very

* Corresponding author at: Molecular Imaging Program at Stanford (MIPS), Department of Radiology, Stanford University, 318 Campus Drive, Stanford, CA 94305-5427, United States.
E-mail addresses: jokerst@stanford.edu (J.V. Jokerst), sgambhir@stanford.edu (S.S. Gambhir).
[1] Tel.: +1 650 725 2309; fax: +1 650 724 4948.

http://dx.doi.org/10.1016/j.pacs.2014.07.001

well defined clearance pathways in ruminants and rodents via cellulase. Furthermore, cellulose has been transformed into a variety of crystalline nanoparticle forms for materials science applications [26,30]. These cellulose nanoparticles are formed by treating the biomass with concentrated sulfuric acid for acid hydrolysis to remove disordered or paracrystalline cellulose with highly ordered crystalline areas remaining intact [31].

In this work, we hypothesized that cellulose in crystalline nanoparticle form could be used as a biodegradable PAI agent. We made cellulose nanoparticles (CNPs) from cotton cellulose and performed physical and toxicological characterization. We then did a series of *ex vivo* experiments to quantitate the PA signal produced by the material as well as *in vivo* experiments to understand its utility in a small animal model of human ovarian cancer. Finally, we demonstrate that the imaging agent can biodegrade into simple sugars. To the best of our knowledge, this is the first report of a cellulose-based imaging agent and among the few reported biodegradable photoacoustic imaging agents with important advantages for clinical translation.

2. Materials and methods

2.1. Reagents

The cellulose source was cotton linters from Arnold Grummer Corp. Concentrated sulfuric acid and phosphosphate buffered saline (PBS) was purchased from Fisher. Cellulase from *Aspergillus niger* and glacial acetic acid were purchased from Sigma and Fisher, respectively, and used without further purification. Modified Alamar Blue reagent ("Presto Blue") was acquired from Invitrogen. All water was purified to 18 MΩ and then filtered through 0.2 μm filter.

2.2. CNP synthesis

The CNPs were made with a protocol adapted from the literature [26]. Briefly, 500 mg of cellulose was added to 15 mL of water in an Erlenmeyer flask with magnetic stirring. 28 mL of 18 M sulfuric acid was added and the exothermic reaction was allowed to cool back to room temperature over 3 h. The material was then centrifuged for 10 min at 6000 RPM and the supernatant decanted and the pellet re-suspended with distilled water. The product was then dialyzed with a 3500 molecular weight cutoff membrane (Pierce) for at least 24 h and then adjusted to pH 7 with 0.1 N NaOH. We dried known volumes of CNPs in a 90 °C oven overnight and weighed the resulting powder to calculate the mass concentration.

2.3. Equipment

The CNP size and zeta potential were obtained via dynamic light scattering (DLS) on a Zetasizer-90 instrument from Malvern Instruments (Worcestershire, UK). The measurements were made in 50% PBS/50% water. A Synergy 4 (Biotek) microplate reader was used for cell assays and absorbance measurements. All transmission electron microscopy (TEM) and energy-dispersive x-ray spectroscopy (EDS) was performed with a Tecnai G2 X-Twin (FEI Co.) instrument operating at 200 kV.

A tomographic photoacoustic scanner (Nexus 128; Endra Life Sciences) was used for animal imaging [32,33]. Briefly, the Nexus uses an optical parametric oscillator (OPO) tunable laser and 128 detectors submerged in hemispherical bowl filled with water stabilized at 38 °C. The animal or sample to be imaged is placed in a tray that lies on top of the water in the center of the bowl. This tray contains a central indentation or dimple to immobilize a subcutaneous tumor or *ex vivo* sample for consistent spatial location. Optimization scans used 60 views with 25 replicate pulses. Animal scans rotated the bowl through 120 views (3° each) with 75 pulses per view with 8 min scan times. The incident radiation was selected during scan setup. The fluence is ~4 mJ/cm^2.

For spectral PA studies, a linear array scanner (LAZR; Visualsonics Corp.) was used for planar imaging due to the high throughput nature of this imaging instrument. It was equipped with a 21 MHz-centered transducer as described previously [34,35]. This instrument also uses an OPO laser operating at 20 Hz between 680 and 970 nm. Step sizes are 1 nm with 4–6 ns pulse width. The spot size is 1 mm × 24 mm and the full field-of-view is 14–23 mm wide. Images were acquired at 5 frames per second, and peak energy at the source is 45 ± 5 mJ at 20 Hz.

2.4. Cell culture and animal handling

In vivo imaging and *in vitro* studies used the OV2008 (also known as 2008) cell line. These cells were grown in DMEM supplemented with fetal bovine serum and antibiotics/antimycotics. Toxicity assays used a derivative of the Alamar Blue assay (Presto Blue) [37]. Here, 10,000 cells/well were plated and analyzed in replicate ($n = 8$). Cells were exposed to increasing concentrations of CNPs for 18 h, 24 h after plating. Assay readout used 540 nm excitation and 600 nm emission.

Female nu/nu mice age 6–16 weeks were used for these studies and each data point includes three mice unless otherwise noted. Before handling, animals were anesthetized with 2% isofluorane in oxygen at 1–3 L/min. To create subcutaneous xenograft tumors, we implanted 10^7 cells in 50% growth factor reduced matrigel/50% PBS into the hind limb of a nude mouse. Tumors were imaged when they reached 500 mm^3, typically 1–2 weeks after implantation. The Administrative Panel on Laboratory Animal Care at Stanford University approved all work with animals.

2.5. Biodegradation experiments

These experiments followed established protocols [38]. A solution containing 1.5 mM nicotinamide adenine dinucleotide (NAD), 1.0 mM ATP, 1.0 unit/mL of hexokinase, and 1.0 unit/mL of glucose-6-phosphate dehydrogenase was obtained from Sigma (p/n G3293). CNPs and cellulose standards were brought to 1 mg/mL in 0.05 M acetic acid (pH = 5.0). D-Glucose standards (250–5 μg/mL) were prepared in the same acetic acid solution. Cellulase (5 U/mL) was prepared in cold distilled water. 4 mL of CNPs and controls were added to borosilicate test tubes followed by 1 mL of cellulase or water as a control. The solution was incubated at 37 °C with shaking. Aliquots were periodically removed and the cellulase activity quenched by placing the aliquots in an ice bath. The samples were centrifuged for 12 min at 12,000 RPM to removed unreacted materials. 40 μL aliquots of the supernatant and glucose standards were then placed in triplicate in a 96 well plate; 100 μL of the HK solution was added and allowed to react at room temperature for 15–17 min. Absorbance at 340 nm was measured and used to construct a standard curve and estimate available glucose.

2.6. Data analysis

PA data was reconstructed with a filtered backprojection algorithm proposed by Wang et al. [39]. Amide software (http://sourceforge.net/projects/amide/) was used to create renderings of the images and all images were thresholded to the same value [40]. To quantitate the images, we used MicroView (General Electric Corp.) software. A region of interest (ROI) 15 mm × 15 mm × 15 mm was created around the sample and the mean intensity