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Toward in vivo detection of hydrogen peroxide with ultrasound molecular imaging

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ABSTRACT

We present a new class of ultrasound molecular imaging agents that extend upon the design of micromotors that are designed to move through fluids by catalyzing hydrogen peroxide (H_2O_2) and propelling forward by escaping oxygen microbubbles. Micromotor converters require 62 mM of H_2O_2 to move – 1000-fold higher than is expected in vivo. Here, we aim to prove that ultrasound can detect the expelled microbubbles, to determine the minimum H_2O_2 concentration needed for microbubble detection, explore alternate designs to detect the H_2O_2 produced by activated neutrophils and perform preliminary in vivo testing. Oxygen microbubbles were detected by ultrasound at 2.5 mM H_2O_2. Best results were achieved with a 400–500 nm spherical design with alternating surface coatings of catalase and PSS over a silica core. The lowest detection limit of 10–100 μM was achieved when assays were done in plasma. Using this design, we detected the H_2O_2 produced by freshly isolated PMA-activated neutrophils allowing their distinction from naïve neutrophils. Finally, we were also able to show that direct injection of these nanospheres into an abscess in vivo enhanced ultrasound signal only when they contained catalase, and only when injected into an abscess, likely because of the elevated levels of H_2O_2 produced by inflammatory mediators.

1. Introduction

Hydrogen peroxide (H_2O_2) is a toxic byproduct of many physiologic reactions. It plays an important role in inflammation [1], cancer [2], diabetes, aging, cardiovascular disease, and intercellular signaling [3,4], with stimulated neutrophils producing a steady concentration of up to 65 μM in ex vivo assays [5]. As a strong oxidizer prone to formation of free radicals, it can cause considerable damage of inflamed or infected tissues [6]. As such, there has been broad interest in a robust strategy for imaging H_2O_2 in vivo not only to recognize tissues with elevated levels [7,8], but also to gain insights into a wide variety of H_2O_2 associated diseases.

Ultrasound is the most commonly used clinical cross-sectional imaging technique worldwide. It displays images at real-time speeds by detecting echoes reflected from interfaces separating tissues with different acoustic impedances – the greater the difference in impedance the stronger the echo. Microbubble-based ultrasound contrast agents are approved for clinical use in most countries including the US. Unlike other clinical modalities requiring micromolar (MRI) to millimolar (CT) levels of contrast agent, ultrasound can detect a single microbubble [9], because it can interrogate tissues with specialized pulses to elicit and then recognize the specific non-linear oscillations of the 1–5 μm microbubbles to eliminate most background signals (often referred to as non-linear or contrast mode).

Recent advances in self-propelled chemically-powered catalytic micromotors that move through fluids at velocities as high as 10 mm/s [10,11] have made these devices promising tools to address many biomedical challenges [12,13]. Micromotor converters (MMCs) catalyze the breakdown of H_2O_2 as fuel to propel forward by expelling released oxygen as microbubbles. Under the microscope and with sufficient fuel (>62 mM H_2O_2), MMCs move as projectiles followed by trails of micron-sized microbubbles [10,11].

We report here a new class of ultrasound contrast agents that can catalyze endogenous H_2O_2 to release oxygen microbubbles based on MMC technology. The aims of this study were: 1) demonstrate that ultrasound can detect the expelled oxygen microbubbles; 2) determine if microbubble detection by ultrasound

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is more efficient than microscopy; 3) determine whether optimally designed particles could allow ultrasound to detect in vivo levels of H_2O_2; 4) determine whether this approach could distinguish activated from naïve neutrophils and 5) perform preliminary testing to see whether the optimal particles could detect H_2O_2 produced in vivo in an abscess model of inflammation.

2. Methods

2.1. Manufacture of platinum-PEDOT micromotors

Micromotors were synthesized as previously described [11]. Briefly, the tubular micromotors were prepared using a common template directed electrodeposition protocol. A Cyclopore polycarbonate membrane, containing 2 μm diameter conical-shaped micropores (Catalog No 7060-2511; Whatman, Maidstone, U. K.), was employed as the template. A 75 nm gold film was first sputtered on one side of the porous membrane to serve as working electrode using the Denton Discovery 18. The sputter was performed at room temperature under vacuum of 5×10^{-6} Torr, DC power 200 W and flow Ar to 3.1 mT. Rotation speed is 65. Sputter time 90 s. A Pt wire and an Ag/AgCl with 3 M KCl were ultrasounded as counter and reference electrodes, respectively. The membrane was then assembled in a plating cell with an aluminum foil serving as a contact. Poly(3,4-ethylenedioxythiophene) (PEDOT) microtubes were electropolymerized at +0.80 V using a charge of 0.06 C from a plating solution containing 15 mM EDOT, 7.5 mM KNO_3 and 100 mM sodium dodecyl sulfate (SDS); subsequently, the inner Pt tube was deposited galvanostatically at −2 mA for 1800 s from a commercial platinum plating solution (Platinum RTP; Technic Inc, Anaheim, CA). The sputtered gold layer was completely removed by hand polishing with ~3 μm alumina slurry. The membrane was then dissolved in methylene chloride for 10 min to completely release the microtubes. The latter were collected by centrifugation at 6000 rpm for 3 min and washed repeatedly with methylene chloride, followed by ethanol and ultrapure water (18.2 MΩ cm), three times of each, with a 3 min centrifugation following each wash. All microtubes were stored in ultrapure water at room temperature when not in use.

2.2. Manufacture of catalase micromotors

Catalase lined micromotors were synthesized as per [11]. Briefly PEDOT microtubes were electropolymerized at +0.80 V for a charge of 0.06 C from a plating solution containing 15 mM EDOT, 7.5 mM KNO_3 and 100 mM sodium dodecyl sulfate (SDS); subsequently, the inner gold layer is plated at −0.9 V for 1 C from a commercial gold plating solution (Orotemp 24 RTU RACK; Technic Inc.). The inner Au layer from the bilayer microtubes was functionalized with a mixed MUA/MCH monolayer. A solution of 2.5 mM MUA and 7.5 mM MCH was prepared in ethanol. The microtubes were incubated in the solution overnight. After rinsing the tubes with water for 5 min, they were transferred to an Eppendorf vial containing a 200 μl PBS buffer (pH 5.5) solution with the coupling agents 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxylsulfosuccinimide (Sulfo-NHS) at 0.4 M and 0.1 M respectively, and the enzyme catalase (2 mg mL^{-1}). This incubation was carried out 7 h at 37°C and thereafter rinsed with PBS with a pH of 7.4 and SDS 0.05 wt % for 15 min at each step. Finally the micromotors were washed repeatedly by centrifugation at 6000 rpm for 3 min with water for three times to remove extra catalase in solution before testing.

2.3. Manufacture of layer by layer catalase-coated nanosphere converters (NSCs)

1 μl of negatively charged silicon particles (0.51 μm, SS03N, Sigma) was washed twice by centrifugation for 3 min at 8000 rpm, first with B&W&B and subsequently with DI water. Layers of catalase/PSS were added to the particles by alternate incubation in 50 μl 1 mg/mL catalase solution (C3155-50, Sigma Aldrich) diluted daily into 0.05 M PBS, pH 5.0 and a PSS/saline solution (1 mg/mL PSS diluted into 1 mL 0.05 M PBS, pH 5.0, containing 29 mg NaCl). Between incubations, particles were washed with 100 μl DI water by centrifuging at 8000 rpm for 3 min. These steps were repeated as layers desired. Particles were resuspended in either PBS (pH 7, Gibco) or HBSS (Gibco) prior to experiments.

2.4. Determination of NSC catalase activity

Enzyme activity was determined spectrophotometrically based on the decrease in absorbance of hydrogen peroxide at $\lambda = 240$ nm, according to an adapted method from the Sigma Enzymatic Assay of Catalase (Sigma, protocol EC 1.11.1.6). Briefly, 100 μL of particle solution was washed by centrifugation at 8000 rpm for 3 min and resuspended in 27 μl of PBS pH 7.0 before being added to 773 μl of 11 mM H_2O_2 solution prepared in 50 mM PBS. After 10 s shaking the solution was placed in the 800 μl spectrophotometer cuvette and the decrease in absorbance at 240 nm with time was recorded immediately at 20 °C for 2 min. One unit of catalase is defined as decomposing 1 μmol of H_2O_2 per minute at pH 7.0 and 20 °C.

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) images were obtained with a Phillips XL30 ESEM instrument, using an acceleration potential of 20 kV. Optical images are captured by an inverted optical microscope (Nikon Instrument Inc. Ti-S/L100), coupled with a 20× objective, a Hamamatsu digital camera C11440 using the NIS-Elements AR 3.2 software.

2.6. Determination of NSC concentration

NSCs were diluted 100-fold into PBS, and injected onto a hemocytometer. The number of particles in a 100 μm^3 was counted manually under light microscopy.

2.7. Ultrasound phantom imaging and quantification

NSCs at the indicated concentration were placed into a transfer pipette modified to contain a port that could be pinned to the back of a water bath. 3 mL phosphate buffered saline (PBS, Gibco) +0.04 M sodium hydrate cholate (NaCH, Sigma) was added to the NSCs through the port, and samples were allowed to sit for approximately 5 min. Under ultrasound operating in contrast mode (GE LogiqE9, 6–15 MHz linear transducer, MI < 0.20, 14 frames per second), the concentration of hydrogen peroxide was increased by factors of ten (eg. 8 μM, 80 μM, 800 μM…) delivered in low volume (3 μL or 30 μL). NSCs were tested side by side with control nanospheres of the same geometry without catalase. Detection limits were obtained on the fly by two independent observers blinded to the identity of the tubes. The detection limit was defined as the first point at which characteristic rising bubbles were observed and was recorded at the time of the experiment. All experiments were performed in triplicate.

Stacks of images were analyzed using Image J. An ovoid region of interest was drawn encapsulating the largest area of the tube possible while excluding obvious imaging artifact from the sides of the tube when present. This region was averaged both pre (3–10 frames) and post (5–20 frames) administration of H_2O_2. Total intensity was defined as the average of the pre frames subtracted from the average of the post frames.

2.8. Neutrophil purification and activation

Two methods were used to isolate neutrophils, each giving similar results.

2.8.1. Ammonium chloride preparation (modified from Ref. [14])

30 mL of rabbit blood was drawn into a heparinized syringe and spun down at 3000 rcf × 15 m. The plasma was removed and frozen. The hematocrit and the buffy coat were incubated in isotonic ammonium chloride buffer (15:1 by volume, 8.32 g/L NH_4Cl, 0.84 g/L $NaHCO_3$) for 15–20 min. The samples were then spun at 300 rcf for 15 min, then rinsed twice with HBSS for 10 min. Cells were counted using a hemocytometer.

2.8.2. Dextran sedimentation preparation (modified from Ref. [14])

30 mL of rabbit blood was drawn and added to an equal amount of 3% solution of dextran-500 diluted into normal saline. This was allowed to incubate at room temperature until the hematocrit had settled (approx 20 m). Plasma was then removed and centrifuged at 250× *g*. To reduce the number of red cells present, neutrophils were bathed in 20 mL of ice cold 0.2% saline for exactly 20 s. 1.6% saline was then added and the cells were spun for 250 rcf × 10 min. This was repeated once. We did not perform a Ficoll gradient due to time considerations, and by Wright stain these preparations were estimated to be about 50% pure, consistent with the literature [14].

Neutrophil activation was accomplished by addition of PMA at 15 ng/mL for 1 h.

2.9. Determination of H_2O_2 content

H_2O_2 concentration was assayed using Amplex Red reagent (Life Sciences) using a protocol supplied by the manufacturer. Samples were diluted by 50× prior to use, and calibration samples were spiked with non-activated neutrophils from the same experiment to control for the spectral properties of lysed red blood cells inevitably present in the mixture.

2.10. Neutrophil imaging and quantification

Two experimenters (EO and BY) were blinded to the identity of the nanospheres (NSCs or control lacking catalase) and the neutrophil preparations. 25 μL (1.3×10^7) or 250 μL (1.3×10^8) nanospheres were placed into each tube along with 800 μL of saline. Neutrophils were added to a concentration of 1×10^6 cells per mL (typically ~100–200 μL). Images were assessed for bubble formation at the time of the experiment by both experimenters, and preliminary results were documented while still blinded.

Stacks of images were processed using Image J. For quantification, an ovoid region of interest was drawn. Region intensity was averaged for up to 20 frames prior to injection. Depending on the experiment, 80–200 frames were skipped to avoid artifactually introduced microbubbles, and then up to 30 frames were

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