SUPPLEMENTARY MATERIALS

A Nanoscale Tool for Photoacoustic-based Measurements of Clotting Time and Therapeutic Drug Monitoring of Heparin

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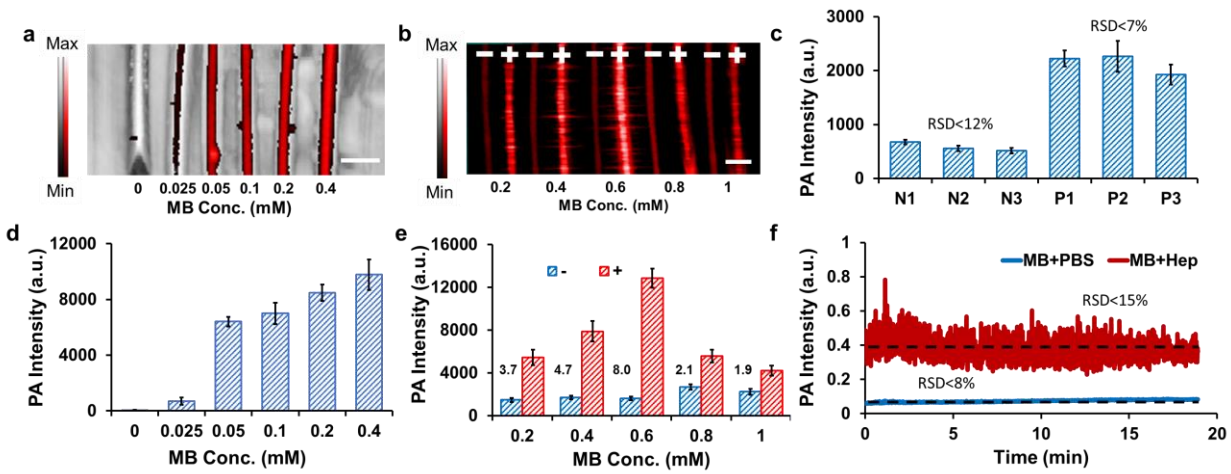

Figure S1. Stability and reproducibility of methylene blue/heparin complex. (**a**) Ultrasound (black and white) and photoacoustic (red) image of plastic tubing filled with increasing concentrations of methylene blue. Photoacoustic signal of sample was quantified in (**d**). (**b**) Increasing concentrations of methylene blue were added to PBS and heparin solution (6.4 U/mL) to identify the methylene blue (MB) concentration for optimal contrast. In panels **b** and **e**, negative (-) and positive (+) signal correspond to methylene blue only and methylene blue with heparin, respectively. The data was quantified in (**e**), and the overlaid numbers are the contrast of photoacoustic signal between negative and positive. Panel **c** shows the signal reproducibility of three methylene blue only samples (0 U/mL heparin; N1-N3) and three methylene blue/heparin samples in PBS (6.4 U/mL heparin; P1-P3). Panel **f** shows signal stability after imaging the samples continuously for 19 minutes.

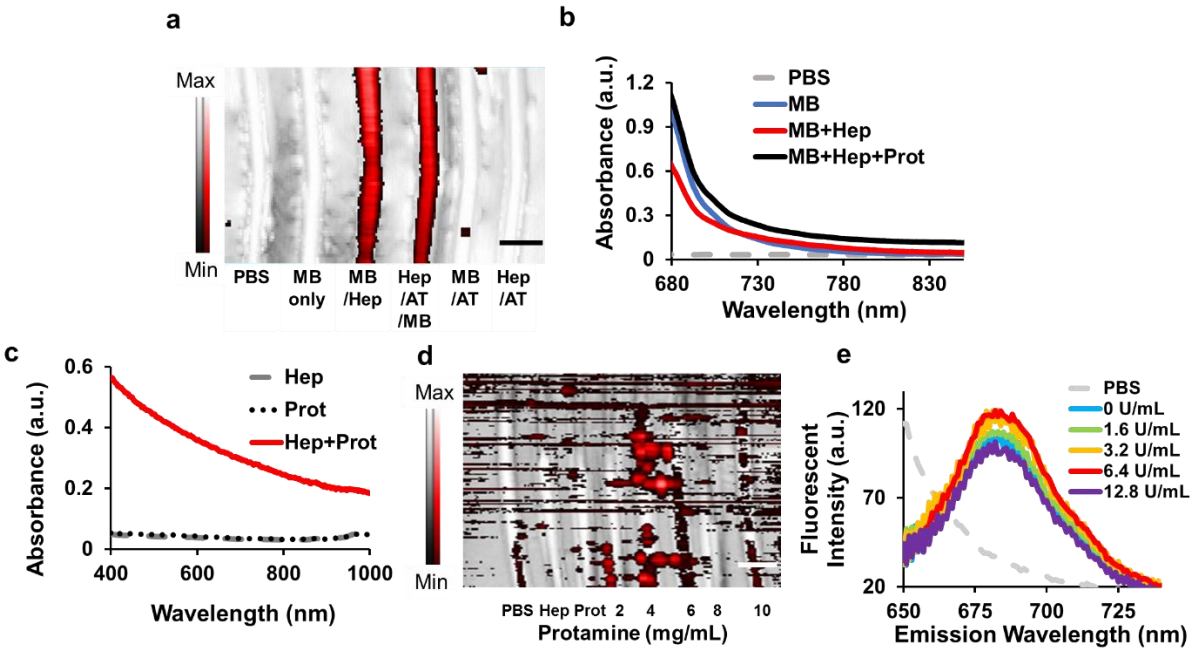

Figure S2. Mechanism of the binding between methylene and heparin. (**a**) Overlaid ultrasound and photoacoustic images show that both methylene blue/heparin complex and heparin treated with antithrombin followed by methylene blue have similar photoacoustic signal, which confirms the utility of this detection approach in the presence of antithrombin. Methylene blue with only antithrombin or heparin with only antithrombin have no signal. (**b**) Absorbance of methylene blue in the presence of heparin and protamine shows slightly elevated absorbance for the MB+Hep sample versus MB only above ~720 nm. (**c**) Absorbance of heparin, protamine and heparin/protamine complex shows that the heparin/protamine solution became turbid and had reduced transmission due to scattering, however these species do not produce photoacoustic signal because there is no chromophore (**d**) despite the absorbance in (**c**)**.** Note that in panel (**d**), the images were collected at a very high gain resulting in significant background. This was to illustrate the lack of signal. Panel (**e**) shows the effect of increasing heparin concentration on methylene blue fluorescence. No increase in fluorescence or quenching is seen. PBS: phosphate buffered saline; MB: methylene blue; Hep: Heparin; AT: antithrombin.

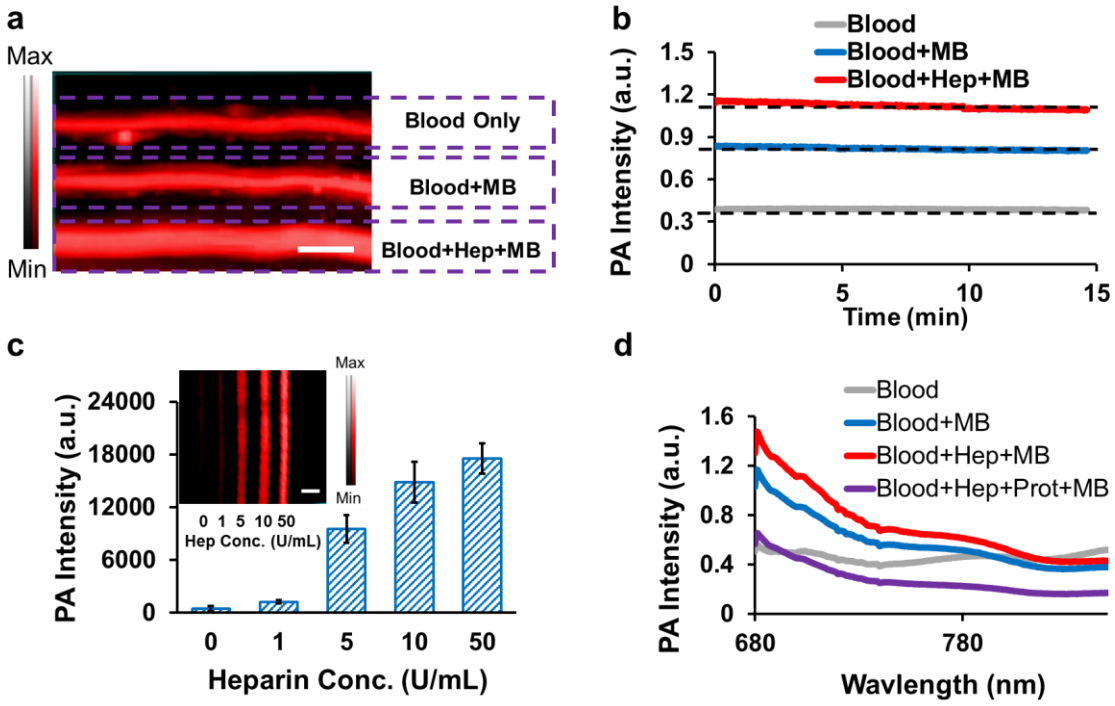

Figure S3. Utility of photoacoustic imaging in whole blood. Panel (**a**) contains whole blood, blood with 0.8 mM methylene blue, and blood with methylene blue and 5 U/mL heparin. The data show that endogenous glycosaminoglycans have little impact on signal creation relative to heparin. Panel (**b**) highlights the signal stability of these same blood samples with continuous imaging (<5%). Panel (**c**) shows the photoacoustic image of plastic tubing containing human blood with 0.8 mM methylene blue, and increasing concentrations of heparin from 0 to 50 U/mL with 0.8 mM methylene blue. Note that the gain is reduced here to that the background signal is near zero. (**d**) Photoacoustic spectra of human blood as well as blood sequentially treated with methylene blue, heparin, and protamine. Scale bars in a and c are 3 mm.

Video S1: Real-time reversibility test in blood. Photoacoustic signal changes of blood/methylene blue mixture as a function of time when heparin (at 27 seconds) and protamine (at 68 seconds) were added. The photoacoustic signal increased 3.6-fold 31 seconds after the injection of 0.1 mL of 50 U/mL heparin (working concentration of 3 U/mL) to 1.7 mL of blood/methylene blue complex (methylene blue concentration of 0.8 mM) (Fig. 3d). It took 32 seconds for the signal to decrease back to baseline after injection of 0.1 mL of 0.5 mg/mL protamine.

Material and Methods

Reagents.

Methylene blue (98%) and human antithrombin III were purchased from Fisher. Heparin (sodium injection at 5,000 and 10,000 United States Pharmacopeia (USP) U/mL) was purchased from SAGENT pharmaceuticals. Protamine sulfate salt from salmon (grade I-A) was purchased from Sigma. UltraPure™ agarose was purchased from Invitrogen. Tetraethylorthosilicate (TEOS, >= 90.0%), ammonium hydroxide solution (28.0-30.0% NH_3 basis), and (3-mercaptopropyl)trimethoxy saline (MPTMS, 95%) were purchased from Sigma-Aldrich. Toluene was purchased from ACROS, and 200 proof pure ethanol was purchased from Koptec. PBS tablets were purchased from Fisher Scientific. Laboratory polyethylene tubing (OD: 1.27 mm, ID: 0.85 mm) was purchased from Harvard apparatus. Vacutainer coagulation tubes with 3.2% citrate solution or 75 USP sodium heparin were purchased from Greiner bio-one.

PBS Sample Preparation

One PBS tablet was dissolved per 200 mL deionized water for a 1X solution. Methylene blue was always prepared fresh by dissolving reagent-grade powder in PBS and filtering through 0.22 µm filter; 10 µL of the heparin solutions (0 – 496 U/mL in PBS) was added in 90 µL 0.6 mM methylene blue. The 10 µL of 8 mg/mL protamine was added to 6.4 U/mL to reverse methylene blue and heparin binding for absorbance measurements. To demonstrate photoacoustic reversibility, 10 µL of 1, 2, 3, 4, 5 mg/mL protamine was added in to 100 µL methylene blue/heparin solution containing 5 U heparin. An aliquot of the methylene blue/heparin solution was used for photoacoustic imaging. LMWH solutions were prepared in several dilutions (0, 0.2, 0.4, 0.8, and 1.6 mg/mL) and 20 µL solutions were added into 180 µL 0.4 mM methylene blue.

Human Blood Sample Preparation

Whole human blood was collected in citrate tubes from a healthy donor according to institutional guidelines. Samples (90 µL) were treated with 10 µL heparin (0 – 500 U/mL) followed by 100 µL methylene blue (1.6 mM) and imaged within 4 hours. To determine the correlation between photoacoustic intensity and blood clotting time, 50 µL of heparin at 0, 0.25, 0.5, 1, 2, and 4 U/mL was added to 450 µL sodium citrate blood, respectively. This sample was repeated three times for error analysis. Citrated platelet-poor plasma was prepared by centrifuging 400 µL citrated blood at 2000 rcf for 15 minutes at 22 ^{o}C twice. The plasma was stored at -80 ^{o}C until aPTT analysis. A separate whole blood aliquot from this same sample was treated with methylene blue and imaged. To neutralize heparin, 0 (PBS), 40, or 80 µg of protamine sulfate was added to the blood collected in a sodium heparin vacutainer. For LWMH studies, concentrations from 0 to 0.16 mg/mL were used and analyzed with aPTT analysis as well as imaging. The blood and buffer samples were placed polyethylene tubing (~ 2 cm long); the ends of the tubing were sealed with heat. These were placed in a 1% agarose phantom for imaging or imaged with a customized phantom.

aPTT assay. Clotting times were determined in duplicate with an ST4 semi-automated mechanical coagulation instrument (Diagnostica Stago, NJ). Here, 30 µl of the aPTT reagent (Diagnostica Stago, NJ) was added to 30 µl of citrated plasma and incubated at 37°C for 5 min. This was followed by 30 µl of 25 mM $CaCl_2$ to initiate clotting. The time was measured in seconds required for blood clot.

Instrumentation. Absorbance measurements used a SpectraMax M5 spectrophotometer. The hydrodynamic radius and zeta potential were measured using dynamic light scattering (DLS) on a Zetasizer-90 instrument from Malvern Instruments (Worcestershire, UK) using 50% PBS in water as the diluent. Scanning electron microscopy (SEM) was performed with a XL30 ultrahigh resolution SEM (FEI Co.).

PA images were scanned using a Vevo 2100 commercial instrument (Visualsonics) equipped with a 21 MHz-centered transducer (LZ250) described previously[1]. The system uses a flashlamp pumped Q-switched Nd:YAG laser with optical parameteric oscillator and second harmonic generator operating at 20 Hz between 680 and 970 nm with a 1 nm step size and a pulse of 4 to 6 ns. The peak energy is 45±5 mJ at 20 Hz at the source. The full field-of-view is 14-23 mm wide. The acquisition rate is 5 frames per second. The samples were aligned under the transducer at a depth of ~ 10 mm. The laser energy was calibrated and optimized using the build-in energy power meter and software before measurements. Typically, we used 100% laser energy with 10-40 dB gain and 21 MHz frequency. 3D scans were performed to image all part of the tubing at 680-710 nm excitation; photoacoustic spectra were collected from 680 to 850 nm.

SSNP@Agarose Hybrid.

Silica nanoparticles were made by mixing 50 mL ethanol, 5 mL Millipore water, and 2.2 mL ammonium hydroxide in a water bath at 30 ^{o}C for 5 minutes with stirring at 300 rpm[2].The stirring speed was increased to 1000 rpm for adding 4.2 mL TEOS in the mixture. Then, the stirring speed was reduced to 500 rpm, and the reaction continued for 2 hours. The product was washed with ethanol and Millipore water twice and dehydrated. To modulate the charge, 200 mg of the SSNPs were incubated with 0.2 mL MPTMS in 40 mL toluene with stirring at 300 rpm in either 110 ^{o}C silicone oil bath for 4 hours or at room temperature overnight. The nanoparticles were washed with toluene 3 times followed by pure ethanol for another 3 times. The nanoparticles were dissolved in 50% PBS after 5 minutes of sonication for zeta potential measurements to confirm the surface modification. The modified nanoparticles were incubated with 3.0 – 4.0 mM methylene blue in Millipore water for overnight. The methylene loaded thiol-modified SSNPs (SSNP-SH) were washed 4 times with Millipore water. The supernatants were saved for absorbance measurement to determine the amount of methylene blue loaded on nanoparticles. The amount of free methylene blue was determined by measuring the absorbance of incubation supernatant.

The amount of MB on silica nanoparticle was calculated by measuring the absorbance of the initial methylene blue solution and the supernatant after each wash. The amount of MB on the silica nanoparticle was back calculated based on Beer's law where the molar extinction coefficients were referred to the data published by Oregon Medical Laser Center (http://omlc.org/spectra/mb/mb-water.html). The size of the silica nanoparticle (>500 nanoparticles) was measured via TEM, and the mass of each particle was calculated based on the silica density. The silica nanoparticles were weighted before incubation and therefore the number of nanoparticle could be determined. The amount of MB on each nanoparticle was determined by dividing the total mole of methylene blue coated on the nanoparticles by the nanoparticle number. Then 2% hot agar was added to the nanoparticles with sonication in hot water for 10 minutes. The SSNP/agar were poured in polyethylene molds simulating an 18 gauge intravenous catheter and cooled for demolding.

Real-time reversibility test in blood

The real-time reversibility test was performed using a customized chamber that allows us to inject heparin and protamine in methylene blue/fresh human blood mixture under water as an ultrasound-coupling medium. The chamber was made by a 1.5 mL microcentrifuge tube with three holes drilled through the cap. The holes in the cap served as ports through which tubing could be secured. One port delivered 50 U/mL heparin, and the other delivered 0.5 mg/mL protamine. The third port served as a vent. The chamber was filled with 0.85 mL 1.6 mM methylene blue and 0.85 mL fresh human blood. The chamber was immersed in water and aligned 11 mm below the transducer and imaged. Then, 0.1 mL heparin and 0.1 mL protamine was injected in the chamber 30 and 68 seconds after the imaging started, respectively. The photoacoustic signal was recorded for more than 120 seconds; three data points were excluded as artifacts due to dust in the sample.

Sample Measurements

Tubing filled with sample were organized in parallel and placed 1 cm beneath the transducer. The 2D gain was optimized so that the background noise was negligible. The 3D scans were performed, and the image was processed as a maximum intensity projection. Spectral measurements were performed by measuring the cross-section of the sample tubing with persistence set to 10. A ROI was drawn on each sample area, and the photoacoustic intensity versus excitation wavelength were analyzed. For stability measurements, the sample tubing was continually exposed for about 10 minutes and recorded at 5 Hz.

Data Analysis and Statistical Treatment.

Photoacoustic images were exported as TIFF files and analyzed via ImageJ 1.49v[3]. Images were changed to 8-bit images and analyzed with region of interest (ROI) analysis using either the mean or integrated density function in ImageJ. Eight groups of data were collected using the same ROI for each tubing. Average and standard deviation were calculated using Microsoft excel functions “AVERAGE” and “STDEV”. The p values were calculated using 8 ROIs and Student’s t-test. Pearson’s r values were calculated with GraphPad PRISM. The detection limits were calculated at 3 standard deviations above the mean of the background signal.

REFERENCES

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