

Photoacoustic Molecular Imaging of Inflammatory Disease

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Abstract---Multiple biomarkers of cardiovascular inflammatory were simultaneously imaged *in vivo* using antibody conjugated gold nanorods (GNRs) injected into a mouse model of vascular injury stimulated by a photochemical reaction of Rose Bengal dye to green light. Mixed solutions of ICAM-1 antibody conjugated GNRs (715 nm) and E-selectin antibody conjugated GNRs (800 nm) were injected to bind to their respective inflammatory markers on the luminal surface of the inferior vena cava of a mouse. Photoacoustic intensity was measured by a commercial ultrasound probe synchronized to a pulsed laser (10-18 mJ/cm²) at 715 nm or 800 nm clearly identified the up-regulation of targeted biomarkers. Histopathology on the harvested tissues confirmed inflammation. The feasibility of simultaneous photoacoustic molecular imaging of inflammation responses in cardiovascular system using a commercial ultrasound system has been demonstrated *in vivo*.

Index Terms---Photoacoustics, Photoacoustic Molecular Imaging, Inflammation, Vascular Injury, Gold Nanorods

I. INTRODUCTION

It has been demonstrated that some inflammatory biomarkers have predictive value for the development of future vascular events among apparently healthy subjects. In particular, prospective epidemiological studies have found increased vascular risk in association with increased basal level of cytokines such as IL-6 and TNF- α ; cell adhesion molecules such as soluble or membrane-bound ICAM-1, P-selectin, and E-selectin; and acute-phase reactants such as CRP and fibrinogen.[1-3] Detection of these specific biomarkers, especially simultaneously, could accurately report the location and degree of inflammation and may provide prognostic information and further characterize systemic diseases such as atherosclerosis. Furthermore, longitudinal monitoring of these transcriptional regulations *in vivo* may lead to a better understanding of the molecular and cellular processes of diseases and might help in accelerating the development and screening of lead compounds. In this study, ICAM-1 and E-selectin were chosen as biomarkers of inflammation and activation of endothelial cells.[3-5] There is

Manuscript received February 1, 2015. This work was supported by the National Research Foundation of Korea (NRF-2014R1A1A2058341, 2012R1A1A2006556) grant funded and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A4A01006895).

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a need for reliable biomarkers that can detect inflamed endothelium *in vivo* and possibly predict risk for vascular disease as these inflammatory markers can predate clinical disease.[6-7]

Photoacoustic molecular imaging (PMI) using target specific contrast agents conjugated with antibody can detect inflammatory biomarkers of interest with significantly enhanced contrast. GNRs conjugated with an antibody have been used to enhance optical absorption (and photoacoustic (PA) signals) in targeted cancer tissue and provide high contrast for non-invasive cancer imaging.[8-12] The feasibility of PMI of inflammation using antibody conjugated GNRs was demonstrated *in vitro*. [13] In the previous *in vitro* single targeting study, endothelial cells were stimulated to over-express ICAM-1, a marker of endothelial cell activation and vascular inflammation. To target stimulated cells, GNRs were conjugated to anti-ICAM-1 antibody which binds to the surface of cells that over-express ICAM-1. The binding of these antibody conjugated GNRs to stimulated endothelial cell monolayers *in vitro* has been confirmed previously using a single-element high frequency transducer centered at 50 MHz and a 700 nm excitation laser. PA images in these studies clearly differentiated targeted inflamed cells from controlled cells and correlated well with fluorescent microscope images.[13] Using a mouse model based on the vascular endothelial injury by a photochemical reaction of Rose Bengal (RB) dye to green light laser, *in vivo* PMI using GNRs as cell-targeted contrast agents was also applied to an inflamed inferior vena cava (IVC) of a mouse. Anti-ICAM-1 conjugated GNRs successfully targeted inflamed endothelial cells and corresponding PA images were obtained *in vivo* using a commercial ultrasound system. PA images clearly locate a targeted inflamed region in the blood vessel and compare well with histology.[14]

In our recent works, simultaneous targeting of ICAM-1 and E-selectin on the inflamed Human umbilical vein endothelial cells (HUVECs) was demonstrated *in vitro* using GNRs of different aspect ratio of 1:3 and 1:3.5.[15] In this paper, *in vivo* multi-target PMI on the inflamed IVC of a mouse using the same GNRs used *in vitro* multi-targeting study is presented.

II. MATERIALS AND METHODS

A. Gold nanorods (GNRs) synthesis and conjugation with antibodies of ICAM-1 and E-selectin

GNRs of aspect ratio (AR) of 1:3 or 1:3.5 were synthesized.[15] Their optical absorption was centered at 715 nm and 800 nm, respectively. The distinct separation in the optical absorption spectrum between GNRs with different ARs enables the multiple targeting on the same site with an insignificant overlap between two different wavelengths.

Synthesized GNRs have been exposed to the surfactant hexadecyltrimethylammonium bromide (CTAB), which forms a bilayer on their surface, which acts as a stabilizer and prevents aggregation. After removing excess CTAB, GNRs settle at the bottom of the tube, and are redispersed in PBS (Phosphate buffered saline, Lonza, Walkersville, MD, USA). A layer of polyacrylic acid (PAA) (Sigma-Aldrich, St. Louis, MO, USA) was absorbed onto the surface of GNRs by adding 100 μ l of 10 mg/ml PAA solution to 1 ml of GNR solution. Excess PAA in solution was removed by brief centrifugation and GNRs were dispersed in 10 ml of PBS followed by the addition of 1.25 ml of 1M N-ethyl-N-(3-dimethylamino propyl)-carbodiimide (EDC) (Sigma-Aldrich, St. Louis, MO, USA) and 1.25 ml of 1M N-hydroxy-succinimide (NHS) (Sigma-Aldrich, St. Louis, MO, USA).[10-14, 16] After 20 min, the reaction mixture was added to 20 μ l of FITC labeled anti-ICAM-1 antibody or PE labeled anti-E-selectin antibody. The EDC/NHS mixture forms an active ester intermediate with the -COOH groups of PAA which undergoes amidation reaction with the -NH₂ group in the FITC labeled anti-ICAM-1 and PE labeled anti-E-selectin antibodies to yield the GNR-antibody conjugates. The reaction mixture was incubated overnight and then unconjugated antibody was removed by centrifugation.

B. Animal Preparation

Under the animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) at University of Pittsburgh, three mice (C57BL/6J, The Jackson Laboratory, Bar Harbor, ME, USA) weighing about 29 to 32g, were used to generate a model of venous inflammation model by photochemical injury.[14, 17-18] The first mice was used for a controlled experiment using blank GNRs, the second was used for the single targeting using anti-ICAM-1 conjugated GNRs (AR1:3), and the third was used for the multiple targeting using mixed anti-ICMA-1 conjugated GNRs (AR1:3) and anti-E-selectin conjugated GNRs (AR1:3.5). All three animals received photothermal injury treatments. Each animal was anesthetized with an inhalation vaporizer system (Drager Vapor 19.1, Drager; isoflurane gas (1.5% to 2%) and oxygen (100%), NV, USA), placed on a plate stage, and illuminated with an incandescent bulb to keep the mouse warm during the surgery and photothermal injury treatments. First, an injection site was prepared using a thin polyethylene tube (PE10, Intramedic™, Sparks, MD, USA), canalizing the exposed femoral vein by dissecting the muscle of the left leg. A small opening was cut into only the femoral vein and a length of PE10 tubing attached to a saline filled 1ml syringe was passed into the vessel and secured with the 6-0 silk looped tie. Second, a midline laparotomy was performed, exteriorizing the small bowel from the body cavity and placing it slightly to the left of the animal, and then IVC was directly approached by blunt dissection. Following a mid-line laparotomy, 0.06 ml of RB solution (10 mg/Kg, Acros organics, NJ, USA) was injected through the prepared injection site to the femoral vein. The exposed IVC was then illuminated with a 1.5 mW Green light Cylindrical Hellow Neon Laser (543.5 nm, Beam diameter 0.81mm, Albuquerque, NM, USA) above the vein for 2 minutes (Figure 1). Exposure of RB to green laser light triggers a photochemical reaction that produces singlet oxygen promoting the formation of other reactive oxygen species that damage the vascular endothelium. All mice were housed and

cared for by the University of Pittsburgh the Division of Laboratory Animal Resources (DLAR) and were free of pathogens.

C. In vivo Photoacoustic Molecular Imaging (PMI)

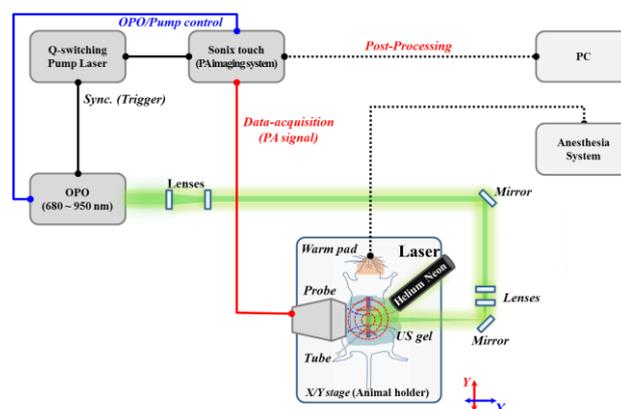


Fig. 1 Experimental set up for *in vivo* photoacoustic molecular imaging. A commercial ultrasound scanner was synchronized to a pulsed laser system. The ultrasound probe and laser light were aligned in (near) parallel. Ultrasound gel in the abdomen provides ultrasound conduction and diffuses incident light

Following the photothermal injury, PMI scans were performed on the animal under anesthesia. A commercial ultrasound scanner (Sonix TOUCH, Ultrasonix, Richmond, BC, Canada) with linear transducer (L14-5, Ultrasonix, Richmond, BC, Canada) centered at 6 MHz was synchronized to the Q-switch of the pulsed laser. A Nd:Yag pulsed Laser (Brilliant, Quantel, France) pumps an optical parametric oscillator (Vibrant HE532I, OpoTek, USA) to generate 5 ns pulses at 10 Hz. (See Figure 1). The laser beam was directed to illuminate from the top of the open abdomen of the mouse (approximately 10-18 mJ/cm² fluence). The wavelength was tuned to either 715 nm or 800 nm to match the peak absorption of the GNRs of AR 1:3 and 1:3.5 respectively. The ultrasound probe was mounted on top of translational stage and held longitudinal to the IVC. The abdomen cavity was filled with ultrasound gel for acoustic coupling. 6-10 cross sectional images of the IVC were obtained by moving the animal holding stage after every set of scans with a step increment of 0.5 mm. The anatomical B-mode images were interleaved with PA imaging with 5 averages at each imaging position. The entire scanning took about 30-50 minutes and these images were used as baseline. Anti-ICAM-1 conjugated GNRs for single targeting or mixed anti-E-selectin and anti-ICAM-1 conjugated GNRs for multiple targeting was slowly injected through the prepared injection site to femoral vein. To reduce the shear force due to blood flow in a relatively large vessel (inferior vena cava) to increase the binding efficiency at the injury site, a semi occlusion of IVC was made by applying mild pressure using a laboratory use cotton ball tip during the injection of GNRs solution. A minimal blood flow was allowed to prevent any undesired blood clot formation and constant flow was evidenced by ultrasound Doppler during the procedure. The concentration of the GNRs solution was 5×10^{12} particles/ml (based on particles entering the conjugation protocol) and the total injected volume was 0.3 ml in all cases. After the completion of the injection of GNRs solution, the blood vessel was remained partially occluded for about 30 minutes to allow

GNRs binding under lower shear force. After removing the cotton ball tip, the same imaging scan sequences were performed as before GNRs injection. The obtained images were compared to the baseline images. At end of the experiment, the excised IVC samples were fixed, paraffin embedded, stained with hematoxylin and eosin (H&E) to be examined using light microscopy.

III. RESULTS AND DISCUSSION

High binding efficiency between prepared GNRs and the biomarkers ICAM-1 and E-selectin was confirmed *in vitro* on inflamed endothelial cells prior to the initiation of *in vivo* experiments using the same protocol in our previous *in vitro* study.¹⁵ The *in vivo* single targeting results on the inflamed IVC of the second mouse are illustrated in Figure 2. The top row presents B-mode and PA images before injection of GNRs and the bottom row presents the B-mode and PA images after injection of GNRs solution. The B-mode image (a) and (d) shows the anatomical view of the IVC inside the abdominal cavity along the long axis of the vessel, including the IVC (yellow arrows; the top and bottom walls of IVC). The darker area above these bright parts of the image represents the abdominal cavity filled with ultrasound gel, which is necessary for acoustic coupling (green arrows). The thick bottom layer of the B-mode (a) and (d) depicts the backbone of the mouse. Note the ultrasound probe is positioned at the top of the image, about 20 mm away from IVC. The PA signal magnitudes before (b) and after (e) injection GNRs (such as GNRs antibody conjugated anti-ICAM-1) were compared. There was about 5dB increase in average PA signal magnitude after injection of GNRs, which corresponds to about 2 folds increase in GNRs binding. The location of the vessel was evidenced by vascular pulsation (including aorta nearby) and ultrasound Doppler images obtained during the course of these experiments. In Figure 2 (c) and (f), PA images were overlaid on top of B-mode images, illustrating the bindings of GNRs to the injury site.

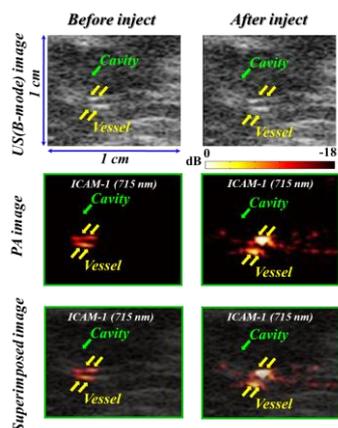


Fig. 2 Single-targeting of the photochemical injury mouse model.

B-mode images before injection (a) and after injection (d) (targeting of antibody conjugated GNRs) are presented. Photoacoustic images before (b) and after (e) GNRs injections are compared. In (c) and (f), PA images are overlaid on top of B-mode image. (green arrows; abdominal cavity, yellow arrows; IVC)

In Figure 3, *in vivo* multi targeting images obtained from the inflamed IVC of the third mouse are presented. The first and second row present B-mode images and corresponding PA images before injecting mixed GNRs conjugated

anti-ICAM-1 (715 nm) and GNRs conjugated anti-E-selectin (800 nm). The third and fourth row present B-mode images and corresponding PA images after injecting GNRs solution. The increase of the overall brightness of PA signal magnitude in the targeted IVC is observed for both ICAM-1 and E-selectin cases (compare (b) to (g) and (d) to (i)). PA images are also overlaid on top of B-mode images before and after injection to localize the source of PA signal. PA signal in the IVC was increased by about 5 dB on average at both wavelengths following the injection of mixed GNRs. Control experiments performed on the first mouse using a mixed solution of un-conjugated GNRs of AR1:3 (715nm) and AR1:3.5 (800nm) displayed insignificant change of PA signal intensity after GNRs injection (data not shown) and confirm insignificant nonspecific bindings.

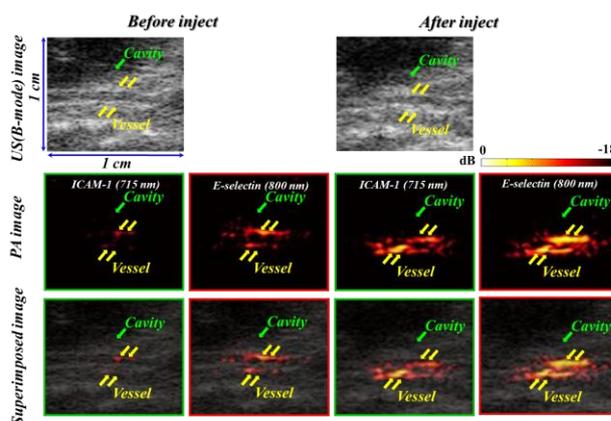


Fig. 3. Multi-targeting of the photochemical injury mouse model.

B-mode images before injection (a) and after injection (f) are presented. Photoacoustic images before (b, 715nm), (d, 800nm) and after (g, 715nm), (i, 800nm) injections are compared. In (c), (e), (h), (j), PA images are overlaid on top of B-mode image. (green arrow; abdominal cavity, yellow arrow; IVC)

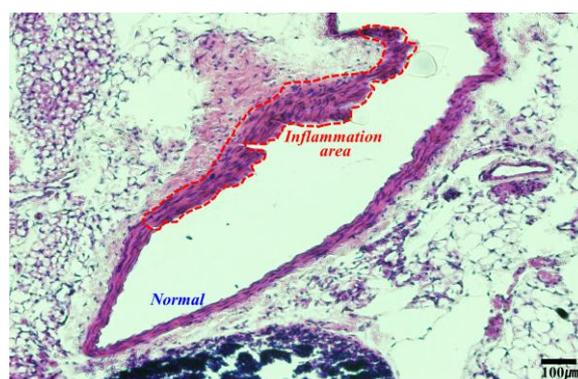


Fig. 4 H&E Histology (Injured endothelium in IVC)

The red arrows locate the injury site with changed morphology and expressed nuclear. The blue arrows indicate uninjured normal site.

Inflamed endothelial cells induced by photochemical injury were confirmed by histological evaluation in Figure 4 on the IVC harvested after the imaging procedure (approximately 4 hours). In Figure 4, we can see a build-up of blood clots (red arrow) in the vessel corresponding to the predicted site of photochemical injury. Also, morphological change to rough and irregular surface of denuded endothelium has been shown to correspond to effect of stimulated vascular. The presence of blood clots has been shown to correspond to high levels of vascular inflammation.[19]

Compared to our previous *in vivo* study for single targeting[14], there are two modifications in this study in animal model preparation and imaging protocol. In our previous single targeting study, GNRs binding was performed on IVC injury by photothermal treatments for about 10 minutes. In the following studies with a larger number of animals, relatively heavy blood clot formation in the lumen was often observed by histopathology. The presence of clotting makes it difficult to differentiate the specific binding of GNRs to the targeted inflammatory biomarkers on the surface of the endothelial cells and the collective specific and nonspecific bindings to blood clots. In other studies, it has been shown that the photothermal injury by green light exposure to RB dye induces significant blood clots depending on the treatment doses.[20] By reducing the photothermal treatment time to 2 minutes, lowered but reasonable endothelial injury was evidenced with significantly decreased clot formation by histopathology, however, further investigation for optimal inflammation conditions without clot formation is needed. In parallel, to maintain the binding efficiency with lowered inflammatory responses under relatively high shear force by blood flow in IVC, a medium occlusion of IVC was made by applying mild pressure on IVC using a laboratory use soft cotton tip, allowing constant blood flow to avoid any natural blood clot formation. No noticeable differences in PA intensity between from the vessel with occlusion and without occlusion were observed. In addition, to avoid any nonspecific binding of GNRs to the aggregating blood clots during the photothermal treatments, GNRs solution was injected after the photothermal injury through a femoral vein. With these modified protocols, single and multi-targeting was successfully performed with a limited number of animals. More studies are planned with an extended number of animals to evaluate GNRs specific bindings to the targeted inflammatory biomarkers under different inflammation and flow conditions.

IV. CONCLUSION

Using antibody conjugated GNRs tuned to different laser light wavelengths, *in vivo* multi-target PMI was performed on an inflamed blood vessel in a mouse. Antibody-conjugated GNRs successfully targeted inflamed endothelium and corresponding PA images were obtained *in vivo* using a commercial ultrasound scanner synchronized to a pulsed laser system. PA images at 715 nm and 800nm clearly locate the inflamed region in the blood vessel targeting ICAM-1 and E-selectin simultaneously, and generally compare well with the presence of inflammation as indicated by histology.

These preliminary results with a limited number of animals demonstrate that, in combination with a commercial ultrasound platform, PMI using antibody conjugated GNRs may detect and possibly monitor multiple inflammatory responses at every stage of the cardiovascular disease progress *in vivo*. This imaging system may be compatible with both small animal and clinical studies.

ACKNOWLEDGMENT

Acknowledge research contributions by people other than the authors. Dr. S. Tripathy who supported error correction of PA system. Dr. A. Carlson who participated in results and discussions. Mis. L.L. Lavery who help animal experiment.

Dr. H.Zhang who provided samples. Dr. A. Agarwal who provided samples. Dr. N.Kotov who provided samples, facilities for synthesis of samples, participated in results and discussions. Dr. K. Kim who provided PA imaging system and facilities, participated in results and discussions. Dr. F.S. Villanueva who provided facilities, participated in results and discussions. The authors would like to thank those doctors for supporting all of these, specially.

This work was supported by the National Research Foundation of Korea (NRF-2014R1A1A2058341, 2012R1A1A2006556) grant funded and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A4A01006895).

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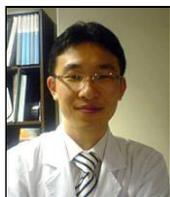
Optical molecular imaging system, and Stereo vision.

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