Manuscript ID: biosensors-1847608

Manuscript Title: **Contrast Agents for Photoacoustic Imaging: A Review Focusing on the Wavelength Range**

Dear Editor,

We greatly appreciate your reviewing our manuscript, and we also thank the reviewers for their valuable comments and questions. We have modified as following the reviewer's comments and made revisions. These revisions have been reflected in the original manuscript and highlighted as yellow: comments from reviewer #1 and blue: comments from reviewer #2 in the manuscript.

Our detailed responses to the reviewers' specific comments are given below, where the reviewers' comments are in italics, and our replies are in regular font.

Sincerely,

The Authors

Reviewer 1:

Han et al reviewed the contrast agents for photoacoustic (PA) imaging technique. Useful scientific information about PA imaging technique ranging from basic physics to application are described. Detail specification of the contrast agents are well listed and organized in terms of wavelength region and material system. The review would provide the deep insight into the selection of optimum imaging system for PA imaging applications, particularly providing the material constructs and wavelength selection. Based on this, we think that this review paper must be published in Biosensors. Few comments for further improvements are as below.

Reply: We appreciate your positive comments.

Comment 1: *Specific advantages of the imaging with PA detection should be described comparing with the fluorescence (FL) detection. In page 2, authors described that PA can achieve both imaging depth (~10–20 mm) and the resolution (~100–500 μm), however, current fluorescence imaging technique such as wavelength-induced frequency filtering (Nature Nanotechnology 17, 643-652 (2022)) can even track the fluorescence signal for depths up to 5.5 ± 0.1 cm.*

Reply: Thank you for the comment. In the point of imaging, the light-in-light-out principle of fluorescence imaging suffers from the two-way diffusion of photons. Therefore, high-resolution fluorescence imaging is only available in shallow depth. Typical fluorescence imaging techniques in deep tissue, including the suggested reference article, demonstrate the detection of the signal, not imaging. In contrast, the light-in-sound-out principle of photoacoustic imaging is beneficial for deep tissue imaging compared to pure optical imaging techniques, because of the one-way diffusion of photons. To clarify this point we have added the following sentences on Page 2–3.

"Compared to the light-in-light-out principle of FLI, which suffers from the low resolution in deep tissue owing to the two-way diffusion of photons, the light-in-sound-out principle of PAI can greatly enhance the imaging depth in deep tissue. Although the FLI can provide high-resolution images in biological tissues, it is highly challenging to achieve such image quality beyond the optical mean transport path (~1 mm). Recently, there have been studies for FL signal detection in deep tissue [61], but they are still limited in image acquisition. In contrast, PAI can achieve images a few centimeters deep with a resolution of several hundred micrometers with the help of acoustic wave propagation, which is less diffusive compared to photons."

[61] V. B. Koman, et al., "A Wavelength-Induced Frequency Filtering Method for Fluorescent Nanosensors In Vivo," *Nature Nanotechnology*, 17, 643-652. (2022)

Comment 2: *It would be fruitful if authors can describe the distinct application characteristics between each material types of contrast agent. Organic dye, organic nanoparticle, and inorganic nanoparticle should have different material stability, versatility, sensing performances, and functionalization availabilities, thus, researchers might need selection condition between the candidates for the PA imaging.*

Reply: We appreciate your suggestion. We have added following sentences in the Conclusion section to guide the selection of materials for contrast enhanced photoacoustic imaging.

"In this review, we introduced various types of PA agents such as organic dye, SPN, low-dimensional nanomaterials, and inorganic nanoparticles. It will be noteworthy to address the pros and cons of each type of PA agent in specific conditions. Organic dye has usually the smallest size providing high biocompatibility in animal studies, which comes from rapid renal clearance. However, they sometimes suffer from irreversible oxidative photobleaching under the high-power laser and it is usually difficult to modify the absorption spectrum of organic dyes over 1000 nm for NIR-II PA imaging agents. SPN also consists of the organic component only, so they have decent biocompatibility for *in vivo* monitoring. SPN is known to possess higher photostability than organic dye-based PA agents. Broad absorption spectra of SPNs would hinder the multiplexed PAI of multiple biotargets in biological systems. Low-dimensional nanomaterial and inorganic nanoparticles have relatively large absorption coefficients and endow higher PA sensitivity in complex media under modest illumination intensity. On the other hand, their poor biocompatibility and toxicity concerns will be the major bottleneck for *in vivo* PA application."

Comment 3: *Not only for dye or nanoparticles, low-dimensional nanomaterials such as CNT or MoS2 have been also widely used as strong PA imaging agents with various micro/nano complex sensor construct (ex. Nature Nanotechnology 3, 557-562 (2008)). It also need to be described.*

Reply: Thank you for the helpful suggestion. We have added the overview of low-dimensional nanomaterials for contrast-enhanced photoacoustic imaging on Page 8.

"Low-dimensional nanomaterials with good biocompatibility and biodegradability have also been applied for contrast-enhanced PAI. De la Zerda et al. developed single-walled carbon nanotubes (CNTs) conjugated with cyclic Arg-Gly-Asp peptides, which showed high affinity to αvβ3 integrin in tumor angiogenesis [92]. They confirmed the PA signal enhancement at 690 nm excitation, after injection of CNTs into U87MG xenograft tumors in mice. Cheng et al. demonstrated contrast-enhanced tumor imaging using tungsten

disulfide (WS_2) nanosheets [93]. They also verified the therapeutic effect through photothermal ablation in 4T1-bearing mice. Chen et al. achieved PA and FL dual-modal imaging using reduced nano-graphene oxide (rNGO) that can deliver ICG molecules with a high loading efficiency of π-π junction structure [94]. The same group also acquired contrast-enhanced PA images using molybdenum disulfide (MoS_2) nanosheets [95]. They achieved 54-fold signal enhancement at 675 nm laser illumination after injection of a single layer MoS_2 into U87 xenograft tumors in mice."

We have also added the corresponding information in Table 2.

[92] A. de la Zerda, et al., "Carbon Nanotubes as Photoacoustic Molecular Imaging Agents in Living Mice," *Nature Nanotechnology*, 3, 557-562. (2008)

[93] L. Cheng, et al., "PEGylated WS_2 Nanosheets as a Multifunctional Theranostic Agent for In Vivo Dual-Modal CT/Photoacoustic Imaging Guided Photothermal Therapy," *Advanced Materials*, 26, 1886-1893. (2014)

[94] J. Chen, et al, "Indocyanine Green Loaded Reduced Graphene Oxide for In Vivo Photoacoustic/Fluorescence Dual-Modality Tumor Imaging," *Nanoscale Research Letters*, 11, 1-11. (2016)

[95] J. Chen, et al., "Single-Layer MoS_2 Nanosheets with Amplified Photoacoustic Effect for Highly Sensitive Photoacoustic Imaging of Orthotopic Brain Tumors," *Advanced Functional Materials*, 26, 8715-8725. (2016)

Comment 4: *Key story of this review is focusing on the wavelength range. Thus, it would be great if authors can provide the comparison of contrast agents between each target NIR window in terms of application fields or imaging performances.*

Reply: We appreciate the reviewer's suggestion. We have added the following paragraph to compare each wavelength window in the terms of imaging application.

"In comparison to visible light, NIR illumination is advantageous for PAI due to low background absorption and high MPE. Especially, NIR-I excitation provides the lowest absorption from biological tissues including water, protein, and lipids. PA imaging performance by NIR-I light can be maximized through the high water loading medium such as artery and vein system. NIR-II light shows higher absorption than NIR-I in the tissues, mostly owing to water absorption, but has the smallest scattering coefficient through many tissue types. PA imaging with NIR-II excitation fits deep tissue imaging in the highly scattering media like brain and bone."

Reviewer 2:

In this review, Kim and coworkers are reporting a concise summary of the Photoacoustic Imaging applications of the contrast agents specifically focusing on the wavelength range. The review seems to be beneficial for the Imaging fields are reasonably well-written. There are few grammatical and textual errors I noticed throughout the manuscript. Therefore, I advise authors to perform a careful revision on this aspect prior to the final publication. There are few comments I would like to add here.

Reply: Thank you for your detailed comments.

Comment 1: *Can authors be specific about the penetration depth of the technique based on the appropriate references?*

Reply: Thank you for the suggestion. The penetration depth of photoacoustic imaging is dependent on two factors: light delivery and acoustic wave detection. The two factors are varied with the configuration of the system. In addition, the imaging depth can be scalable by adjusting the light delivery mechanism according to the imaging target. Therefore, it is difficult to define the penetration depth specifically. We have already stated the range of penetration depth and spatial resolution on Page 2.

"One unique advantage of PAI for achieving adequate images is its scalable spatial resolution and imaging depth according to the application [15]. To achieve high-resolution images (~5–50 μm) in shallow regions (~1 mm), the excitation light must be tightly focused on the specimens, resulting in images of ears [23], brains [24-27], and eyes [28-30] in small animals. In an alternative system, light is moderately focused or even diffused to increase the imaging depth (~10–20 mm) at the expense of the resolution (~100–500 μm), providing whole-body images of small animals *in vivo* [31-38]. Recently, PAI has also been applied in clinical human studies by combining it with clinical US machines to implement real-time imaging platforms [39-44]."

To clarify this variability, we have added the following sentences in the Conclusion section.

"According to the configuration of the PAI system, the penetration depth and spatial resolution of the resulting images are varied [118]."

[15] L. V. Wang, et al., "Photoacoustic Tomography: *In Vivo* Imaging From Organelles to Organs," *Science*, 335, 1458-1462. (2012)

[23] R. Bi, et al., "*In Vivo* Label-Free Functional Photoacoustic Monitoring of Ischemic Reperfusion," *Journal of Biophotonics*, 12, e201800454. (2019)

[24] S. Hu, et al., "Functional Transcranial Brain Imaging by Optical-Resolution Photoacoustic Microscopy," *Journal of Biomedical Optics*, 14, 040503. (2009)

[25] Q. Chen, et al. "Wearable Optical Resolution Photoacoustic Microscopy," *Journal of Biophotonics*, 12, e201900066. (2019)

[26] Y. Liu, et al., "Assessing the Effects of Norepinephrine on Single Cerebral Microvessels using Optical-Resolution Photoacoustic Microscope," *Journal of Biomedical. Optics*, 18, 076007. (2013)

[27] W. Qin, et al., "Large-Field-of-View Optical Resolution Photoacoustic Microscopy," *Optics Express*, 26, 4271-4278. (2018)

[28] W. Liu, et al., "*In Vivo* Corneal Neovascularization Imaging by Optical-Resolution Photoacoustic Microscopy," *Photoacoustics*, 2, 81-86. (2014)

[29] W. Liu, et al., "Photoacoustic Imaging of the Eye: A Mini Review," *Photoacoustics*, 4, 112-123. (2016)

[30] R. H. Silverman, "High-Resolution Photoacoustic Imaging of Ocular Tissues," *Ultrasound in Medicine & Biology*, 36, 733-742. (2010)

[31] K. H. Song, et al., "Deep Reflection-Mode Photoacoustic Imaging of Biological Tissue," *Journal of Biomedical Optics*, 12, 060503. (2007)

[32] K. H. Song, et al., "Noninvasive Photoacoustic Imaging of the Thoracic Cavity and the Kidney in Small and Large Animals," *Medical Physics*, 35, 4524-4529. (2008)

[33] M. Jeon, et al., "Multiplane Spectroscopic Whole-Body Photoacoustic Imaging of Small Animals *In Vivo*," *Medical & Biological Engineering & Computing*, 54, 283-294. (2016)

[34] C. Li, et al., "Real-Time Photoacoustic Tomography of Cortical Hemodynamics in Small Animals," *Journal of Biomedical Optics*, 15, 010509. (2010)

[35] H.-P. Brecht, et al., "Whole-Body Three-Dimensional Optoacoustic Tomography System for Small Animals," *Journal of Biomedical Optics*, 14, 064007. (2009)

[36] J. Xia, et al., "Whole-Body Ring-Shaped Confocal Photoacoustic Computed Tomography of Small Animals *In Vivo*," *Journal of Biomedical Optics*, 17, 050506. (2012)

[37] L. Li, et al., "Single-Impulse Panoramic Photoacoustic Computed Tomography of Small-Animal Whole-Body Dynamics at High Spatiotemporal Resolution," *Nature Biomedical Engineering*, 1, 1-11. (2017)

[38] E.-Y. Park, et al., "Simultaneous Dual-Modal Multispectral Photoacoustic and Ultrasound Macroscopy for Three-Dimensional Whole-Body Imaging of Small Animals," *Photonics*, 8, 13. (2021)

[39] J. Kim, et al., "Multiparametric Photoacoustic Analysis of Human Thyroid Cancers *In Vivo*," *Cancer Research*, 81, 4849-4860. (2021)

[40] B. Park, et al., "3D Wide-Field Multispectral Photoacoustic Imaging of Human Melanomas *In Vivo*: A Pilot Study," *Journal of the European Academy of Dermatology and Venereology*, 35, 669-676. (2020)

[41] G. Diot, et al., "Multispectral Optoacoustic Tomography (MSOT) of Human Breast Cancer," *Clinical Cancer Research*, 23, 6912-6922. (2017)

[42] F. Knieling, et al., "Multispectral Optoacoustic Tomography for Assessment of Crohn's Disease Activity," *New England Journal of Medicine*, 376, 1292-1294. (2017)

[43] W. Roll, et al., "Multispectral Optoacoustic Tomography of Benign and Malignant Thyroid Disorders: A Pilot Study," *Journal of Nuclear Medicine*, 60, 1461-1466. (2019)

[44] A. Becker, et al., "Multispectral Optoacoustic Tomography of the Human Breast: Characterisation of Healthy Tissue and Malignant Lesions using a Hybrid Ultrasound-Optoacoustic Approach," *European Radiology*, 28, 602-609. (2018)

[118] H. Kye, et al., "Whole-Body Photoacoustic Imaging Techniques for Preclinical Small Animal Studies," Sensors, 22, 5130. (2022)

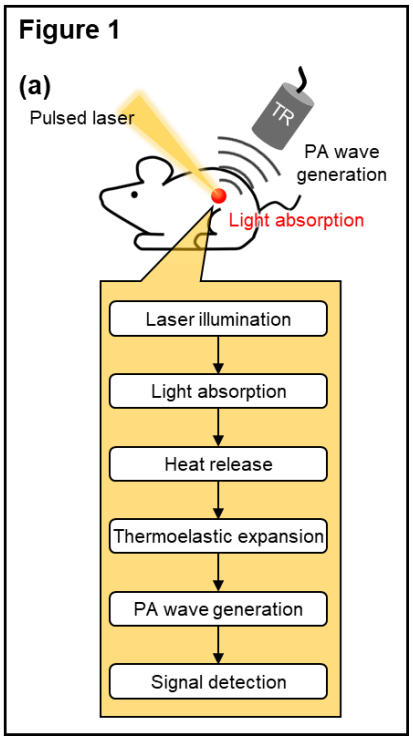
Comment 2: *Can authors provide a more detail summary of chemical structures of the PAI agents reported up to date?*

Reply: We thank the reviewer's suggestion. The summary of the structure for photoacoustic agents is added in the Conclusion section as below.

"A number of organic dyes for low NIR-I PA imaging have been reported including FDA-approved MB, BODIPY, and modified cyanine core structures. However, high NIR-I and NIR-II PA imaging agents have a smaller pool of organic dye structures; for example, a series of naphthalocyanine were reported to have high NIR-I absorption. For the longer absorption spectrum in the NIR-II region, phthalocyanine, fluoroalkyl cyanine, and metal ion-chelated dithiolene dyes have been employed."

Comment 3: *An appropriate diagram with the mechanism of the PA probes will be beneficial to the readers.*

Reply: Thank you for the comment. We have modified Figure 1a to elaborate on the mechanism of photoacoustic signal generation.



Comment 4: *For the section 3.1, is there any other additional PA agents that could be provided here?*

Reply: Thank you for the suggestion. We have added a brief overview of low-dimensional nanomaterials for contrast-enhanced photoacoustic imaging on Page 8. Please see our response to Comment 3 from Reviewer 1.

Comment 5: *Can authors also explain how does the fluorescence quantum yield of the dye would affect the photoacoustic imaging efficiency?*

Reply: Thank you for the suggestion. We have added the following explanation on Page 3.

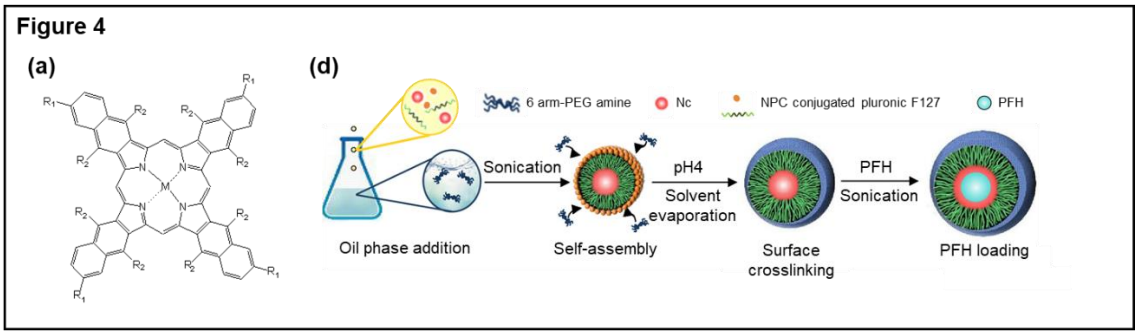
"… σ is the heat conversion efficiency described in Equation (1), …"

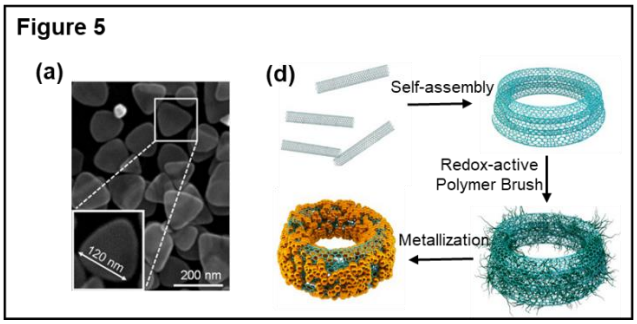
We have also added the following sentences on Page 3.

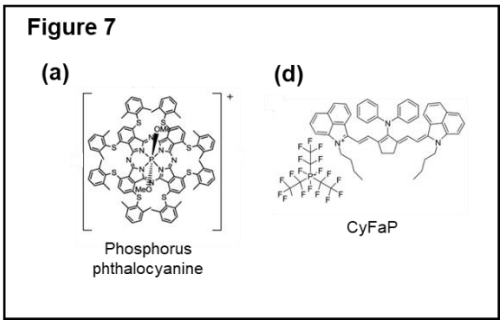
"We can note that the heat conversion efficiency is residual of FL quantum yield as described in Equation (1). Therefore, the lower the FL quantum yield, the higher the PA signals."

Comment 6: *Authors must provide appropriate examples/structures for sections 3.2 and 3.3.*

Reply: Thank you for the helpfule suggestion. We have added figures to show the example structures agents in Figure 4a, 4d, 5a, 5d, 7a, and 7d.







Comment 7: *In the conclusion section, authors can also suggest in brief future directions.*

Reply: Thank you for the valuable suggestion. We have already stated future directions in the conclusion section regarding potential clinical translation.

"For a successful clinical translation, further studies are necessary to evaluate the biocompatibility, biodegradability, toxicity, and solubility of PA agents and achieve FDA approval."

We have added the following sentences to elaborate on the future direction of contrast-enhanced photoacoustic imaging.

"In addition, to enhance the deep tissue imaging capability of PAI, advanced wavelength-tuning of nanoparticles with absorption peaks in the NIR-II region and the development of high-power lasers in the corresponding wavelength are required. Finally, the high photothermal conversion would widen the application area into the theragnostic field."