Peer Review File

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Reviewer A

The article is relevant. Fluorescence imaging of tumors during surgery is a rapidly developing area of research. New fluorescent agents are being developed. But ICG remains one of the main ones, although its shortcomings are well known.

Reply: Thank you for the Reviewer's constructive and helpful view on the conciseness of the purpose of the research. As the most commonly used near-infrared fluorescent tracer in clinical practice, ICG still has many problems. One of the problems is poor fluorescence imaging performance due to agglomeration. Therefore, we hope to use HSA premixing to improve this situation.

An attempt to improve the accumulation of ICG in the tumor is achieved by combining it with human albumin. The authors did a good job and made several new conclusions. These findings have implications for future research and practice. The article is written very well and logically. There are no fundamental comments.

Reply: Thanks to the reviewer for the recognition here.

Understanding the drawings is hampered by the difficulty of identifying curves represented by different colors. Are there any contradictions between the concentration of ICG and the spectral curves in the figures, is this not entirely clear?

Reply: Thank you for the Reviewer's valuable and kind tips here. There is no contradiction between the concentration and spectral curve of ICG in the figure. In vitro experiments, the ratio of 50:5 showed the worst fluorescence performance, but it was improved in vivo, which benefited from the presence of albumin in vivo. Different molar ratios of ICG-HSA represent different curve colors, Fig.1 and Fig.2 both use the same color to represent the same molar ratios. Although the resolution in the manuscript is low, we have also provided the original images to show the individual curves clearly.

Currently, other fluorophores that are more specific to tumor tissue have been developed; this can be indicated in the Discussion section - this is at the discretion of the authors.

The article is recommended for publication.

Reply: Thank you for the Reviewer's valuable and kind tips here. Another shortcoming of ICG is the lack of specific recognition of tumor tissue. Tumors are imaged only through the EPR effect, which makes it difficult to determine the optimal dose and time for different tumors. There is no doubt that targeted tracers can improve this situation, there are currently many related studies, such as OTL-38, cRGD-ZW800, etc., which have entered the clinical stage.

However, it takes many years to actually advance a new drug to approval for indications. Therefore, the focus of my article is to improve the imaging effect of ICG by changing conditions with the help of currently clinically approved tracers.

Reviewer B

The study by Li et al. investigates the optimal mixing ratio of ICG (indocyanine green) and HSA (human serum albumin) and its effects on the fluorescence characteristics and tumor imaging efficacy of the complex. The data in Table 2 and Figure 7B, however, reveal similar tumor-to-background ratios across different ICG-HSA complex ratios. Notably, the authors did not present a statistical analysis to highlight significant differences between the 4:5 and 50:5 ratio groups. This lack of statistical evidence is a critical issue. Future research should incorporate comprehensive statistical analyses to substantiate the outcomes and conclusions regarding the efficacy of various ICG-HSA ratios in tumor imaging.

Reply: Thank you for the Reviewer's valuable and kind tips here. In vitro experiments, the ratio of 50:5 showed the worst fluorescence performance, but it was improved in vivo, which benefited from the presence of albumin in vivo. We intend to conduct a detailed comparison of the significant differences in imaging between 4:5 and 50:5 ratio groups in future studies.

The authors state that their work is the first to examine the application of a new combined tracer composed of HSA and ICG in tumor imaging. However, Jang et al. have already reported the use of an ICG-HSA complex for targeting glioblastoma. Please refer to the article "Jang HJ, Song MG, Park CR, Youn H, Lee YS, Cheon GJ, Kang KW. Imaging of Indocyanine Green-Human Serum Albumin (ICG-HSA) Complex in Secreted Protein Acidic and Rich in Cysteine (SPARC)-Expressing Glioblastoma. Int J Mol Sci. 2023 Jan 3;24(1):850."

Reply: Thank you for the Reviewer's valuable and kind tips here. I have looked up this article and Jang et al did a really good job. Therefore, we cannot say that we are the first to examine the application of ICG-HSA complexes in tumor imaging. We modified this paper for its first application in breast cancer tumor imaging.

Changes in the text: Change "this work is the first to examine the application of a new combined tracer composed of HSA and ICG in tumor imaging." to "this work is the first to examine the application of a new combined tracer composed of HSA and ICG in breast tumor imaging."

Additionally, the quality of Figure 6 is suboptimal, particularly the image of the mouse, with some photographs appearing excessively dark. It would enhance the clarity of these images if the authors marked the tumors with arrows.

Reply: Thank you for the Reviewer's valuable and kind tips here. I will circle the location of the tumor to improve the quality of the picture.

Changes in the text: Change figure 6 with a new one.

Finally, there is a typographical error in the manuscript: "ICG-HAS" should be corrected to "ICG-HSA" throughout the document to maintain consistency in terminology.
Reply: Thank you for the Reviewer's valuable and kind tips here. We have changed all "ICG-HAS" to "ICG-HSA".

Reviewer C

The authors begin by describing the limitations of the current use of ICG as a single agent for tumour demarcation. They then systematically look at the properties and utility of various ratios of ICG and HSA as a compound agent. These studies are both in vitro and in vivo. They find that a molar ratio of 4:5 ICG:HSA possesses optimal properties and suggest that it be evaluated clinically in breast cancer and other solid tumours.
Reply: Thank you for the Reviewer's constructive and helpful view on the conciseness of the purpose of the research.

Although the background is fairly complete, the authors do not mention the significant amount of clinical work done, particularly in the Netherlands, with indocyanine green complexed with human serum albumin nanocolloids for sentinel node localisation. As in the present work, this involves extemporaneous mixing of two licensed drugs.
Reply: Thank you for the Reviewer's valuable and kind tips here. We mentioned the application of ICG-HSA in lymph node imaging in the introduction. For example, reference 31 is an introduction to this, but there is no detailed introduction. Although the mixing method of the two methods is the same, the injection method is different. We hope that ICG and HSA can be mixed before surgery, and intravenous injection can produce excellent imaging effects for breast cancer.

At the end of the paper the authors discuss some of the limitations of ICG-HSA. I think there should be more emphasis on the practical problem of limited stability of the complex. Another problem which should be mentioned is that ICG is not the optimal fluorescent probe. Much current work is being performed with alternative probes with higher fluorescence output.
Reply: Thank you for the Reviewer's valuable and kind tips here. We used a dynamic particle size scattering instrument to detect that the size of ICG-HSA is stable at around 7nm. At the same time, during clinical use, injection should be started after mixing, so we believe that ICG-HSA will not have stability problems.
Changes in the text: Change "In this study, a new composite ICG-HSA fluorescent tracer for injection and its preparation method were proposed to improve the dispersion and fluorescence

performance of ICG in aqueous solution, and the optimal ICG-HSA mixing ratio (4:5) was identified. In addition, this is the first report of the ICG-HSA complex being applied to breast cancer imaging, and the results confirm that it has significant advantages over traditional ICG tumor imaging, with shortened surgical waiting time, satisfactory TBR, and wider surgical window. Further exploration is needed in the future to see if the complex can aid in the visualization of other types of tumors. Although the complex is not tumor specific, the enhanced tumor uptake due to the presence of HSA also significantly improves the TBR during imaging of the complex, which is promising for future applications in the visualization of a wide range of tumor types. In addition to having characteristics of high biocompatibility and low toxicity, ICG-HSA will have a streamline clinical approval process. Explorations and innovations related to ICG-based NIRFGS technology in tumor imaging have great potential for clinical translation." to "This study proposes a new injectable composite ICG-HSA fluorescent tracer and its preparation method, and confirms that it has significant advantages over traditional ICG tumor imaging, including shortened surgical waiting time, satisfactory TBR, and wider surgical window. However, since this complex is not tumor-specific, it still needs to rely on the EPR effect of the tumor to image the tumor. Therefore, it cannot fundamentally solve the limitations of ICG. For example, the optimal dose and time for different tumors vary widely. With a lot of research on tumor targeted tracers, tumor targeted tracers have gained widespread attention in accurately locating tumor edges, ultra-small tumors, and positive lymph nodes. Compared with tumor-specific targeting tracers, ICG has a wider range of clinical indications. Therefore, the future development trend of near-infrared fluorescence navigation tumor surgery should be ICG combined with tumor-targeting tracers and highly sensitive imaging equipments for imaging of all solid tumors." From line 462 to 474.

MINOR

Page 1, line 3-4, Title. The title is a bit awkward. How about something like: "A human serum albumin-indocyanine green complex offers improved tumor identification in fluorescence-guided surgery"?

Reply: Thank you for the Reviewer's valuable and kind tips here. We have changed this title.

Changes in the text: Change "Effect of a human serum albumin-indocyanine green complex on tumor identification in fluorescence-guided surgery" to "A human serum albumin-indocyanine green complex offers improved tumor identification in fluorescence-guided surgery" From line 3 to line 4.

Page 3, line 90, Introduction. The first sentence is awkward, particularly "have become a high-incidence disease in the world". How about something like: "are an increasingly prominent disease globally"?

Reply: Thank you for the Reviewer's valuable and kind tips here. We have changed this sentence.

Changes in the text: Change “have become a high-incidence disease in the world” to “are an increasingly prominent disease globally” From line 88 to line 89.

Page 8, line 226. The title mentions metabolism, but I don’t think that is actually being studied, only biodistribution.

Reply: Thank you for the Reviewer’s valuable and kind tips here. We measured the fluorescence intensity at different time points to represent the metabolism of ICG-HSA. The specific data are shown in Figure 7A.

The captions for the Tables require further information, such as what the molar ratios represent.

Reply: Thank you for the Reviewer’s valuable and kind tips here.

Changes in the text: Add “50:5, 8:5, 4:5, 1:0 ratio represents molar ratio of ICG:HSA.” in line 679 and 682-683.

TYPOS ETC

Page 6, line 180. Typo – HSA

Page 8, line 216. Typo – nude

Pages 22-28. In most of the Figure captions HSA has been autocorrected to HAS

The references are not formatted entirely consistently, e.g. capitalisation

Reply: Thank you for the Reviewer’s valuable and kind tips here.

Changes in the text: We have changed all errors according Reviewer’s kind tips.