

Integrated Theranostic Nanoplatfrom Empowers Precision Cancer Care via Radionuclide-Labeled NIR-II Aggregation-Induced Emission Luminogens

Corresponding Author: Professor Wanhai Xu

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study describes a therapeutic nanoplatfrom that integrates radiolabeled NIR-II AIEgens for precision cancer therapy. The authors demonstrate an approach combining targeted drug delivery, PET/CT imaging, fluorescence navigation, and multimodal therapy that addresses major limitations of conventional cancer diagnosis and treatment. By using folic acid and DOTA-modified AIEgens followed by ^{68}Ga radiolabeling, a tumor-targeting nanoprobe (^{68}Ga -2TT-oC6B-4COOH NPs) was generated with excellent PET/CT imaging capabilities, allowing clear tumor demarcation to be achieved within 0.5 h of injection, as reported by the one of the fastest time-targeted diagnostics for nanomaterials. In addition, the AIE-based platform showed strong photothermal and ROS-generating effects, avoiding tumor recurrence. This well-written paper provides a clear description of the experiments and methods, and the proposed AIEgens radiolabeling strategy is of significant innovative value. The topic of radiolabeled-AIE, Multimodal diagnostic and therapeutic nanoplatfroms will be of high interest to the readers of Nature Communication. Therefore, I recommend publishing the manuscript with minor revisions.

1. Different tumor microenvironments may significantly affect the permeability and retention effects of nanoparticles, and the therapeutic effects in other cells (e.g., cervical cancer HeLa cells, bladder cancer T24 cells, etc.) need to be verified.
2. The toxicity of ^{68}Ga -2TT-oC6B-4COOH NPs towards normal cells should be added as control.
3. The fluorescence pictures of ^{68}Ga -2TT-oC6B-4COOH versus ICG at the tumor site over time in Figs. 5d and 5e alone could not visually differentiate the window time for optimal surgical resection, and it is necessary to supplement the quantitative analysis of fluorescence intensity of ^{68}Ga -2TT-oC6B-4COOH versus ICG at the tumor site over time in Figs. 5d and 5e.
4. In addition, Figs. 5f and 5g should also be supplemented with ^{68}Ga -2TT-oC6B-4COOH versus ICG to guide the quantitative analysis of fluorescence intensity in various organs after the tumor.
5. Experimental conditions need to be detailed and some spelling mistakes needs to be corrected.
6. Some experimental protocol (Preparation of ^{68}Ga -2TT-oC6B-4COOH NPs, In vivo PET/CT imaging) is confusing to readers, need to be present properly.
7. The statistical methods should be added in the caption (Fig. 6d). Besides, the p value analysis should be performed in Fig. 7c, Fig. 7e and Figs. 8a-j.
8. The discussion section can be elaborated by citing more relevant examples of nanomaterials designed for PET/CT imaging.

Reviewer #2

(Remarks to the Author)

- What are the noteworthy results?

This manuscript describes the synthesis and proof-of-concept studies of a first-in class folate-targeted multifunctional theranostic nanoplatfrom for folate-expressing tumors (^{68}Ga -PET, fluorescence imaging (FLI), aggregation-induced emission-active photosensitizer for photodynamic therapy (PDT) and photothermal therapy (PTT)). However, it is difficult to gauge how noteworthy these data are since this could be viewed as an extension of prior work published by Tang (one of the corresponding authors of the current study). This describes the modification of 2TT-oC6B-4COOH NPs (PMID:

33889836 + 4COOH) + DOTA chelate + folate molecules for folate receptor alpha (FR- α). Also, since DOTA is such a universal chelator of radiometals, there is potential for radiotherapy (for e.g., with ^{177}Lu or ^{225}Ac).

• Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

This work has the potential to be of significance if this one of the first reports of a truly “all-in-one” tumor-antigen specific biocompatible nanoplatfrom that offers PET staging (although ^{68}Ga ’s short physical half-life may be a barrier for optimal clinical utilization), fluorescent-guided surgical resection, coupled with a conditionally activated post-operative photothermal therapeutic that is well-suited for management of residual microscopic disease.

A major strength of the work is the highly rigorous evaluation of the performance of the multimodal phototherapy, especially the ROS characterization including mechanistic confirmation with electron spin resonance. These data provide important benchmarks for optimization and comparison with other multifunctional theranostic nanoplatfroms.

Unclear what the significance is of the observed laser stability and reporter photothermal conversion efficiency of ~40% (is this high/low/typical compared with other photothermal agents?).

• Does the work support the conclusions and claims, or is additional evidence needed?

A weakness in the in vivo studies is the lack of non-targeted NPs (i.e., identical formulation but without folate molecules). It would be desirable to see the tumor targeting profile of this control to demonstrate the gain in tumor targeting and residence time conferred by the folate targeting. Furthermore, it is unclear if folate acid here is an informative control. In vivo blocking radiotracer studies with folate would support the claim of active targeting of folate receptor alpha. Also, it would be desirable to see extended radiotracer biodistribution data beyond 2 h to evaluate tumor clearance kinetics. For example, if ^{68}Ga was swapped with ^{111}In , then the pharmacokinetics and biodistribution over the course of days could be studied to support the claim (As shown in Fig. 5d, through intravenous administration of ^{68}Ga -2TT-oC6B-4COOH NPs, the tumor site demonstrated a progressive increase in fluorescence intensity over 24 h, followed by a gradual decline). We typically don’t see progressive tumor increase unless the drug has slow clearance from blood.

• Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision? None noted.

• Is the methodology sound? Does the work meet the expected standards in your field?

The ^{68}Ga radiochemistry methodology and associated quality control is lacking. It is also unclear what the folate receptor alpha expression is in the U87 cells/xenograft model. A weakness is that only a single cell line/animal model was studied.

• Is there enough detail provided in the methods for the work to be reproduced?

As mentioned above, the ^{68}Ga radiochemistry would be very difficult to reproduce based on the provided methods. Also, it is preferable to see the ^{68}Ga tracer characteristics in the main text. For example, along with administered activity of 20 μCi , what was the mass/moles of drug (specific activity?). Tracer specific activity can have a tremendous effect on tumor targeting. There is also a discrepancy between the administered activity in the main text (20 μCi) versus the methods (200 μCi).

Reviewer #3

(Remarks to the Author)

In this manuscript, Zhang et al. reports a theranostic nanoplatfrom for precision cancer care by integrating a radionuclide with NIR-II aggregation induced emission (AIE) luminogens. A comprehensive set of experiments (from synthesis to in vivo imaging and cancer therapy) has been conducted. However, many experimental details are missing, making it difficult to fully understand and evaluate the research. Many critical questions need to be addressed in this manuscript.

1. The multimodality theranostic imaging involving NIR dye integrated with PET modality have been previously reported. The authors should review the relevant literature in the Introduction section (e.g., Mol Imaging Biol. 2023 Jun 21;25(4):727–734) and clearly highlight how the current work differs from existing studies.

2. The authors have previously developed and published NIR-II theranostic systems based on AIE luminogen. (ACS Nano 2018, 12, 11, 11282–11293; J. Am. Chem. Soc. 2022, 144, 33, 15391–15402) The design rationale of NIR-II luminogen for study should be discussed, particularly in comparison to their earlier work.

3. In supplementary Fig.23, The TEM image of 2TT-oC6B-4COOH NPs clearly showed a mixture of large and small nanoparticles. The average TEM size and size distribution histogram of the nanoparticles should be provided in the manuscript. There will be a concern on broad size distribution because large nanoparticles have different pharmacokinetic behaviors compared to that of small nanoparticles. The broad size distribution may affect the biodistribution in vivo.

4. In the manuscript, it is stated that “Additionally, infrared thermal imaging confirmed that 2TT-oC6B-4COOH NPs exhibit a favorable photothermal effect (Fig. 2h).”

The authors should provide a clearer description or definition of what is meaning of favorable photothermal effects from Fig. 2h.

5. In the manuscript, it is stated that “Cellular uptake and blocking experiments (Fig. 4c) revealed that both ^{68}Ga -2TT-oC6B-4COOH NPs and ^{68}Ga -folic acid exhibited significantly higher uptake rates in GBM cells ($36.31 \pm 1.07\%$ and $34.61 \pm 2.96\%$, respectively) compared to the control group ($4.70 \pm 1.14\%$), confirming the targeted intracellular delivery capability of ^{68}Ga -2TT-oC6B-4COOH NPs.”

The methods for labeling folic acid with ^{68}Ga should be clearly described. Was a chelator used? If so, what was the conjugation strategy? This information is critical for interpreting and repeat the results shown in Fig. 4.

6. In the manuscript, it is stated that “Comparative analysis of half-maximal inhibitory concentrations demonstrated that 68Ga-folic acid and 68Ga-labeled 2TT-oC6B-4COOH NPs gave comparable binding affinities for folic acid receptors (5.23 nM vs. 2.81 nM, respectively) (Fig. 4d).”

Details of the inhibitory assay described in Fig. 4d are missing and should be included. Furthermore, clarification is needed: does the reported binding affinity of 2.81 nM correspond to the concentration of 68Ga-labeled NPs? How many folic acid molecules are present per nanoparticle?

7. In the preparation of 68Ga-2TT-oC6B-4COOH NPs, the manuscript state “which was then directly mixed with 2TT-oC6B-4COOH NPs in 0.1 M NaHCO₃ (at specified pH) for radiolabeling”

How are the nanoparticles purified from the free Ga-68 after radiolabeling? Was radiochemical purity assessed, and by what method? These are essential information for PET/CT imaging.

8. The control experiments of 2TT-oC6B-4COOH NPs without folic acid on the nanoparticle are missing. The control of 2TT-oC6B-4COOH NPs without folic acid should be included in the study of targeting effect of 68Ga-2TT-oC6B-4COOH NPs such as Figure 4, Figure 5, Figure 6, Figure 7, and Figure 8.

9. In the study of in vivo PET/CT imaging for diagnosis & NIR-II FLI navigation, it is stated that “The primary excretion was through the kidneys and bladder, leading to low background activity and a favorable tumor-to-background ratio.” It is observed that the nanoparticles are majorly clearance by kidney and bladder. Given the fact that the hydrodynamic size of nanoparticles are relatively big (~100 nm), the mechanism behind this unusual clearance profile should be explored and discussed.

10. More experimental details need to be provided for Figure 5. Was the data shown in Fig. 5c obtained via in vivo PET imaging or from ex vivo gamma counting following sacrifice? This should be clearly stated.

11. There is a discrepancy between Fig. 5b and Fig. 5d. Fig. 5b shows peak tumor uptake at 0.5 h with a subsequent decline, while Fig. 5d shows an increase in uptake up to 24 h. The authors should explain this inconsistency.

12. The statement “with minimal fluorescence observed in reticuloendothelial system (RES) organs, including the liver, spleen, and lungs.” Should be revised.

The lungs are generally not considered part of the reticuloendothelial system.

13. A comparative discussion of the biodistribution of 68Ga-2TT-oC6B-4COOH NPs versus other nanoparticles with similar size reported in the literature should be included.

14. In the section “Postoperative Tumor Recurrence Detection”

Since it is designed to monitor the tumor recurrence. It is not clear why laser is used in groups III and V. Where is laser applied to? Tumor? However, the tumor is supposed to have been excised.

15. The manuscript states “Group IV exhibited moderate tumor growth inhibition.” (Figure 7d and 7e).

Since the 68Ga-2TT-oC6B-4COOH NPs are biocompatible, why do they show inhibition of tumor. There is no therapeutic component in the nanoparticles. In addition, histological study in Figure 7g does not show any differences for group IV compared to group I-III.

16. In Figure 8, the biosafety experiment compares PBS+laser and NPs+laser groups. However, the laser application method is unclear. If healthy mice (no tumors?) were injected, where was the laser applied? Please provide details on the laser treatment procedure.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I think that the authors have addressed my concerns well. The quality of the manuscript has been improved. I thus recommend the acceptance of the manuscript by the journal as is.

Reviewer #3

(Remarks to the Author)

The authors have revised the manuscript in response to the reviewers' comments. Additional experimental details have been included, and several confusing sections have been clarified. However, some key issues remain insufficiently addressed, and new questions have arisen from the authors' responses. My main concerns are outlined below.

3. In supplementary Fig. 23, The TEM image of 2TT-oC6B-4COOH NPs clearly showed a mixture of large and small

nanoparticles. The average TEM size and size distribution histogram of the nanoparticles should be provided in the manuscript. There will be a concern on broad size distribution because large nanoparticles have different pharmacokinetic behaviors compared to that of small nanoparticles. The broad size distribution may affect the biodistribution in vivo.

Response: We thank the reviewer for their constructive suggestions. We have replaced the TEM image in Fig. 2d and supplemented it with the average nanoparticle size from TEM measurements and a size distribution histogram. Both Fig. 5d and Supplementary Fig. 23 demonstrate the relatively uniform size of the nanoparticles, and this addition has significantly enhanced the quality of the manuscript.

Revisions in the main body of the text: while transmission electron microscopy (TEM) and the size distribution histogram confirmed their relatively uniform spherical morphology (Fig. 2d, Supplementary Fig. 23).

Comments: From the new zoomed-out TEM image (Figure 2d), a mixture of large and small particles can be observed, which is similar to the previous TEM image in the original Supplementary Figure 23. In the nanoparticle size distribution histogram of DOTA-FA-BBTD-4C NPs (new Supplementary Figure 23), two distinct peaks are evident. Therefore, the size distribution should not be considered “uniform,” as claimed by the authors. This issue is important because the coexistence of large and small nanoparticles can complicate biodistribution and clearance.

8. The control experiments of 2TT-oC6B-4COOH NPs without folic acid on the nanoparticle are missing. The control of 2TT-oC6B-4COOH NPs without folic acid should be included in the study of targeting effect of 68Ga-2TT-oC6B-4COOH NPs such as Figure 4, Figure 5, Figure 6, Figure 7, and Figure 8.

Response: We thank the reviewer for this constructive suggestion. We have added control experiments without folic acid on the [68Ga]DOTA-BBTD-4C NPs in Figs. 4-8. This additional experiment significantly enhances the scientific rigor and data integrity of the paper.

In Fig. 4, we included two control groups (VI and VII) of non-targeted NPs (i.e., identical formulation but without folate molecules).

Comments: In the representative PET/CT images of [68Ga]DOTA-BBTD-4C NPs (without FA targeting ligands) in tumor-bearing mice (new Fig. 5 d), high uptake is observed in the kidney and bladder. However, since the particle size is assumed to be around 100 nm as [68Ga]DOTA-FA-BBTD-4C NPs (with FA), This represents an abnormal clearance pattern for nanoparticles of this size. The experiment is very important as it can provide insights into the clearance mechanism of [68Ga]DOTA-FA-BBTD-4C NPs. We will discuss in Question 9.

9. In the study of in vivo PET/CT imaging for diagnosis & NIR-II FLI navigation, it is stated that “The primary excretion was through the kidneys and bladder, leading to low background activity and a favorable tumor-to-background ratio.” It is observed that the nanoparticles are majorly clearance by kidney and bladder. Given the fact that the hydrodynamic size of nanoparticles are relatively big (~100 nm), the mechanism behind this unusual clearance profile should be explored and discussed.

Response: We are grateful for the reviewers' valuable comments. The folate receptor (FR) is highly overexpressed in various human cancers, but exhibits low expression in normal tissues. However, the kidney is a major exception. Renal proximal tubule cells highly express FR- α , which facilitates the reabsorption of folate from urine. When folate-based imaging agents (such as radionuclide-labeled compounds) are administered (Nano Today, 2021, 37, 101173), the tracer accumulates in the kidneys (Mol. Pharmaceutics 2024, 21, 5681-5689). Additionally, the kidneys act as a major site for drug uptake and retention, leading to subsequent drug accumulation. In response, we have performed a comparative analysis between the biodistribution profile of [68Ga]DOTA-FA-BBTD-4C NPs and that of other similarly-sized nanoparticles to provide clearer interpretation of the in vivo behavior.

As reported in Literature Mol. Pharmaceutics 2024, 21, 5681-5689, the biodistribution results are consistent with our experimental findings.

Furthermore, we compared the results of this study with those of other reported fluorescent probes, all of which have a size distribution of approximately 100 nm, and the results demonstrated excellent agreement.

In summary, we propose that the clearance mechanism via the bladder and kidneys is as follows: Renal proximal tubule cells highly express FR- α , which mediates the reabsorption of folate from urine. Due to the folate-modified surface layer of the material used in this study, the tracer specifically binds to FR- α , resulting in significant accumulation in the kidneys. Furthermore, as the primary filtration and excretory organs, the kidneys allow partially unreabsorbed tracer to enter the urine via glomerular filtration and ultimately reach the bladder. Therefore, the drug is primarily cleared through the renal and bladder pathways.

Comments: The proposed clearance mechanism is not convincing. Further questions need to be addressed:

a). Figure 5d shows that the nanoparticles without FA also undergo significant excretion through the kidney and bladder. This suggests that FA may not be the major factor driving renal clearance.

b). Although FA could potentially bind to the kidney, the large particle size should limit nanoparticle excretion into the bladder.

c). In the paper cited by the authors (Mol. Pharmaceutics 2024, 21, 5681–5689), the goal was to reduce kidney uptake by increasing nanoparticle size. Their conclusion was that larger particles show reduced renal clearance. Moreover, most reagents listed in their table are small-molecule compounds. It is reasonable that such compounds exhibit high kidney

clearance because of their small size.

d). In other cited references (Adv. Funct. Mater. 2022, 32, 2206346; J. Control. Release 2021, 330, 1–14), some kidney uptake is observed, but the majority of nanoparticles are taken up by the liver and spleen, with no obvious clearance into the bladder. This biodistribution pattern is quite different from this work, where dominant uptake is seen in the kidney and bladder.

e). Even at 0.5 h, this study shows very strong kidney uptake and rapid clearance through the bladder, with minimal liver and spleen uptake over time. This pattern closely resembles the behavior of small molecules. The authors might need to consider testing the stability of their nanoparticles.

Additionally, the authors mention “reticuloendothelial system (RES) organs (liver and kidneys) and lung tissue.” In fact, RES generally refers to the liver and spleen, not the kidney. The authors should double-check this concept.

11. There is a discrepancy between Fig. 5b and Fig. 5d. Fig. 5b shows peak tumor uptake at 0.5 h with a subsequent decline, while Fig. 5d shows an increase in uptake up to 24 h. The authors should explain this inconsistency.

Response: We thank the reviewer for this constructive suggestion. The PET imaging results in Fig. 5b demonstrate that the ⁶⁸Ga-labeled nanoparticles reached peak tumor uptake within 0.5 h post-injection. This observation clearly indicates the nanoparticles' rapid tumor accumulation through folate receptor-mediated active targeting. PET/CT imaging technology exhibits three key advantages: (1) completely non-invasive detection, (2) exceptional tissue penetration capability, and (3) sub-centimeter high spatial resolution, making it particularly valuable for early diagnosis of deep-seated tumors. Notably, the PET signal intensity decreased over time, primarily due to three factors: First, the inherent physical properties of ⁶⁸Ga radionuclide (half-life of only 68 min) caused signal decay. Second, efficient renal clearance of free ⁶⁸Ga took place. These factors collectively contributed to the temporal changes in PET signals at tumor sites. Furthermore, through systematic comparison with published ⁶⁸Ga-PET studies in this field, we found that the dynamic changes in tumor signals observed by PET imaging in our study are highly consistent with the pharmacokinetic characteristics of ⁶⁸Ga-labeled nanoprobe reported in the literature (e.g., J. Nucl. Med. 2022, 63, 862-868; J. Nucl. Med. 2023, 64, 1449-1455; J. Nucl. Med. 2024, 65, 394-401).

Fig. 5d demonstrates a dynamic fluorescence signal trend characterized by initial enhancement followed by gradual attenuation over time. This phenomenon primarily stems from the inherent physical limitations of fluorescence imaging technology: firstly, the restricted tissue penetration depth compromises imaging efficacy in deep tissues; secondly, during deep-tissue imaging, significantly intensified photon scattering effects lead to diminished spatial resolution and signal-to-noise ratio, consequently impairing the sensitivity and accuracy of real-time dynamic monitoring at tumor sites. Notably, through systematic comparative analysis with published ⁶⁸Ga-PET/FLI imaging studies in this field, the tumor fluorescence signal dynamics observed in our experiments show remarkable consistency with the fluorescent behavior of nanoprobe reported in the literature (e.g., Mol. Pharmaceutics. 2023, 20, 6262-6271).

To enable readers to more clearly observe the dynamic changes in fluorescence intensity at the tumor site, we have supplemented the following content in both the main text and supplementary information.

Comments: The authors state that the PET/CT data are consistent with other Ga-68-labeled nanoprobe in the literature. While this is possible, the key question remains: why are there differences between Fig. 5b (5c) and Fig. 5d in this work? These figures use the same probe but different imaging modalities.

The authors attribute the decrease in tumor uptake in Fig. 5c to the decay of Ga-68. Although Ga-68 does decay, PET/CT imaging should be calibrated, and internal standards are typically used. The quantitative analysis must account for the decay of Ga-68.

The authors also suggest that release of DOTA– Ga-68 may be another factor. This explanation is more plausible, but if correct, it raises concerns about the reliability of the entire study. It could also explain why the nanoparticles, despite their ~100 nm size, show unusually high renal clearance.

The authors acknowledge the limitations of FLI, which is valid. However, they should be aware that the study is supposed to be done in the same mice, at different times. The differing conclusions drawn from Fig. 5b and Fig. 5d remain unresolved.

12. The statement “with minimal fluorescence observed in reticuloendothelial system (RES) organs, including the liver, spleen, and lungs.” Should be revised.

The lungs are generally not considered part of the reticuloendothelial system.

Response: We thank the reviewer for this constructive suggestion. We have already revised this sentence in the article. This has significantly enhanced the quality of the article.

Revisions in the main body of the text: fluorescent signals were observed in reticuloendothelial system (RES) organs (liver and kidneys) and lung tissue.

Comments: The authors exclude the lungs from the RES but classify the kidney as part of it. However, the kidney is generally not considered an RES organ.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

Based on the authors' responses and revisions, several major concerns regarding the manuscript remain not fully addressed. In the manuscript, the biodistribution data shows very high renal clearance and much lower liver and spleen uptakes (Figure 5b and 5c). As the nanoparticles are approximately 100 nm (TEM and DLS), they typically cannot undergo renal clearance. The authors attribute the observed renal clearance to targeting FA on the surface of nanoparticles to folate receptor alpha over expressed in kidney. However, the control experiment shows that nanoparticles without FA modification also exhibit almost the same renal clearance (Figure 5d, signal from bladder) although the kidney signal is lower. Below are comments on some of authors' responses.

9.

a). Figure 5b and 5d are PET/CT images of [⁶⁸Ga]DOTA-FA-BBDT-4C NPS (with folate modification) and [⁶⁸Ga]DOTA-BBDT-4C NPS (without folate modification), respectively. As shown in the circled area, the NPs with FA show high uptake in both bladder and kidney, whereas the NPs without FA exhibit significant renal clearance in bladder (as evidenced by the strong bladder signal in Figure 5d). Although the NPs with FA have higher kidney uptake, the bladder uptake is nearly identical to that of the NPs without FA.

The elevated kidney signal observed for the NPs with FA may result from FA binding to folate receptor alpha in the kidney. FA binding could lead to accumulation in the kidney before eventual excretion into the bladder. However, since both NPs (with FA and without FA) show similar bladder clearance (eventual clearance), the data suggest that renal clearance is not primarily modulated by FA conjugation, contrary to the authors' claim. How do the authors explain these findings?

b). If the authors truly believe that these about 100 nm nanoparticles can be completely cleared through the renal system, they are encouraged to collect urine samples and confirm the presence of intact nanoparticles (approximately 100 nm) using TEM. This suggested experiment would help validate the unusual clearance behavior.

c). It is worth to mention that the authors cited the reference (Mol. Pharmaceutics 2024, 21, 5681–5689) in the first revision response. The intention was to demonstrate a similar renal clearance phenomenon in the cited reference. Regardless of the degree of comparability between the two studies, it is important to emphasize the key conclusion from the reference: although many factors influence renal clearance, it is widely accepted that small-sized molecules are cleared through the renal system, whereas larger molecules/particles are not. This principle is fundamental in designing and controlling pharmacokinetics.

e). To more convincingly demonstrate nanoparticle stability, the authors are advised to conduct stability studies in different buffers and at various pH values.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The authors have responded to my questions; I do not have further comments.

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REVISION: “Integrated Theranostic Nanoplatfrom Empowers Precision Cancer Care via Radionuclide-Labeled NIR-II Aggregation-Induced Emission Luminogens”

List of changes in the revised manuscript and supporting information:

- (1) We have carefully provided key information of the work references in the revised manuscript.
- (2) We have provided some personal opinions in the conclusion of revised manuscript.
- (3) We have checked all Figures and supplement more details of the corresponding concepts and strategies in the revised manuscript.
- (4) We have systematically refined and standardized the molecular naming conventions and experimental group terminology in the revised manuscript.
- (5) We have systematically evaluated the tumor imaging and therapeutic effects of nanoparticles in different tumor cells in the revised manuscript.
- (6) We have supplemented the quantitative analysis of fluorescence intensity over time for [⁶⁸Ga]DOTA-FA-BBTD-4C NPs and ICG at tumor sites of Fig. 5d and 5e in the revised manuscript.
- (7) We have added control experiments without folic acid on the [⁶⁸Ga]DOTA-BBTD-4C NPs in Figs. 4-8. in the revised manuscript.
- (8) We have carefully revised English grammar, spelling, and sentence structure in the revised manuscript.
- (9) All authors' details and affiliations have been verified.
- (10) All the revisions in the revised manuscript were highlighted in red.

Questions and point-by-point responses are as follows:

Reviewer #1 (Remarks to the Author):

This study describes a therapeutic nanoplatform that integrates radiolabeled NIR-II AIEgens for precision cancer therapy. The authors demonstrate an approach combining targeted drug delivery, PET/CT imaging, fluorescence navigation, and multimodal therapy that addresses major limitations of conventional cancer diagnosis and treatment. By using folic acid and DOTA-modified AIEgens followed by ^{68}Ga radiolabeling, a tumor-targeting nanoprobe (^{68}Ga -2TT-oC6B-4COOH NPs) was generated with excellent PET/CT imaging capabilities, allowing clear tumor demarcation to be achieved within 0.5 h of injection, as reported by the one of the fastest time-targeted diagnostics for nanomaterials. In addition, the AIE-based platform showed strong photothermal and ROS-generating effects, avoiding tumor recurrence. This well-written paper provides a clear description of the experiments and methods, and the proposed AIEgens radiolabeling strategy is of significant innovative value. The topic of radiolabeled-AIE, Multimodal diagnostic and therapeutic nanoplatforms will be of high interest to the readers of Nature Communication. Therefore, I recommend publishing the manuscript with minor revisions.

To address the reviewers' comments regarding the supplementation of experimental components and to ensure precise and standardized nomenclature of experimental materials, we have systematically refined and standardized the molecular naming conventions and experimental group terminology throughout the manuscript. Additionally, the detailed composition of each material has been provided in the Supporting Information.

As demonstrated in the following:

1. "Folic acid" has been uniformly replaced with "[^{68}Ga]DOTA-FA NPs" in the text.
2. "2TT-oC6B-4COOH" has been uniformly replaced with "BBTD-4C " in the text.
3. "2TT-oC6B-4COOH NPs" has been uniformly replaced with "DOTA-FA-BBTD-4C NPs" in the text.
4. " ^{68}Ga -2TT-oC6B-4COOH NPs" has been uniformly replaced with "[^{68}Ga]DOTA-FA-BBTD-4C NPs" in the text.
5. The non-targeted nanoparticles (i.e., nanoparticles with identical composition but without folate molecules) are designated as "[^{68}Ga]DOTA-BBTD-4C NPs" in the text.

Revisions in the main body of the text: The chemical compositions of materials for in vitro and in vivo experiments are provided in **Supplementary Table 1**.

Revisions in the Supporting Information:

Supplementary Table 1. The composition of each nanoparticle.

Name	Folic acid targeting	Chelating agent	AI Egen	Radionuclide
DOTA-FA NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	/	/
DOTA-FA-BBTD-4C NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	/
[⁶⁸ Ga]DOTA-FA NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	/	⁶⁸ Ga
[⁶⁸ Ga]DOTA-BBTD-4C NPs	/	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	⁶⁸ Ga
[⁶⁸ Ga]DOTA-FA-BBTD-4C NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	⁶⁸ Ga

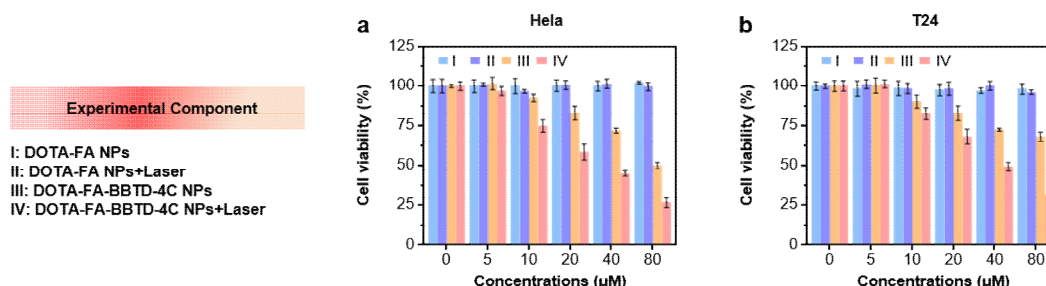
1. Different tumor microenvironments may significantly affect the permeability and retention effects of nanoparticles, and the therapeutic effects in other cells (e.g., cervical cancer HeLa cells, bladder cancer T24 cells, etc.) need to be verified.

Response: We thank the reviewer for this constructive suggestion. We have systematically evaluated the therapeutic effects of nanoparticles at varying concentrations under laser irradiation in both cervical cancer (HeLa) and bladder cancer (T24) cell lines, with detailed results presented in the main text and supporting information.

Revisions in the main body of the text:

Under 660 nm (0.8 W) laser irradiation, the experimental results showed that nanoparticles with the same composition but lacking folic acid molecules (DOTA-BBTD-4C NPs) did not exhibit significant inhibitory effects on U87 cells at low concentrations (< 20 μ M). In contrast, nanoparticles with the same composition but containing folic acid molecules (DOTA-FA-BBTD-4C NPs) demonstrated marked growth inhibition against GBM cells (U87), human cervical cancer cells (HeLa), and human bladder cancer cells (T24) (Fig. 4e, Supplementary Fig. 32), further confirming the tumor cell-targeting property of folic acid.

Revisions in the supporting information:



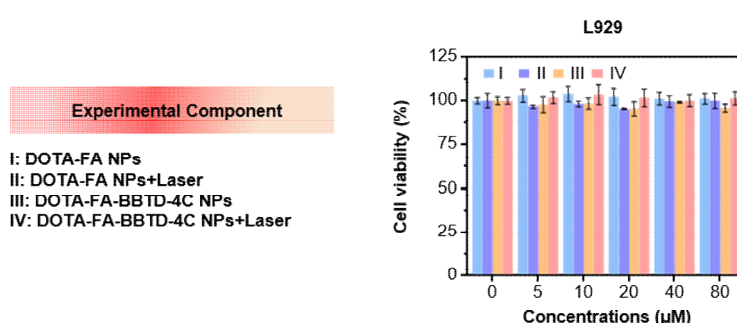
Supplementary Fig. 32 Cell viability of **a** HeLa and **b** T24 cells after laser treatment with different concentrations of DOTA-FA-BBTD-4C NPs.

2. The toxicity of ^{68}Ga -2TT-oC6B-4COOH NPs towards normal cells should be added as control.

Response: We thank the reviewer for this constructive suggestion. We have incorporated the cytotoxicity data of DOTA-FA-BBTD-4C NPs toward normal cells as controls in both the main text and supporting information.

Revisions in the main body of the text: Notably, even at relatively high concentrations, the nanoparticles maintained high cell viability when interacting with normal cells, demonstrating favorable dose-dependent safety (Supplementary Fig. 33).

Revisions in the supporting information:



Supplementary Fig. 33 Cell viability of NCTC clone 929 (L929) after laser treatment with different concentrations of DOTA-FA-BBTD-4C NPs.

3. The fluorescence pictures of ^{68}Ga -2TT-oC6B-4COOH versus ICG at the tumor site over time in Figs. 5d and 5e alone could not visually differentiate the window time for optimal surgical resection, and it is necessary to supplement the quantitative analysis of fluorescence intensity of ^{68}Ga -2TT-oC6B-4COOH versus ICG at the tumor site over time in Figs. 5d and 5e.

Response: We thank the reviewer for this constructive suggestion. We have supplemented the quantitative analysis of fluorescence intensity over time for ^{68}Ga]DOTA-FA-BBTD-4C NPs and ICG at tumor sites in Fig. 5d and 5e, and incorporated these data into the main text.

Revisions in the main body of the text:

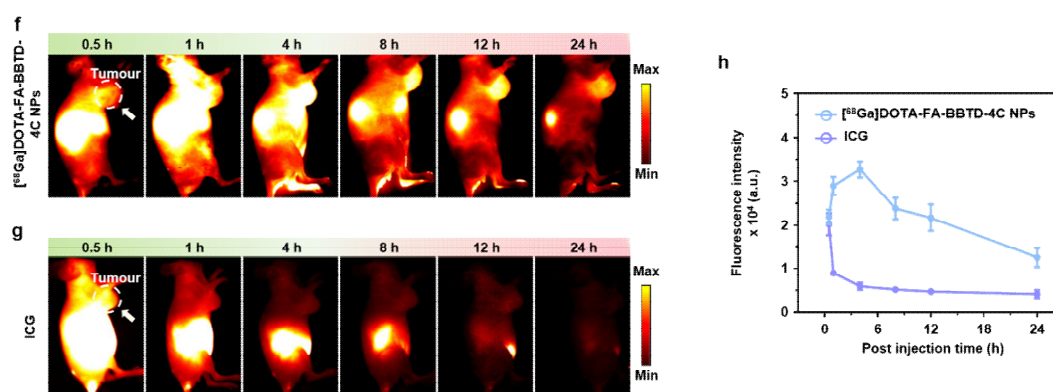
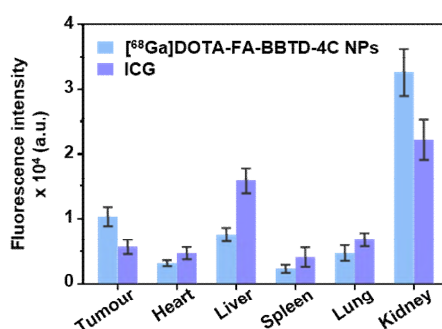


Fig. 5 h Quantitative analysis of time-dependent fluorescence intensity of [^{68}Ga]DOTA-FA-BBTD-4C NPs and ICG at tumor sites.

4. In addition, Figs. 5f and 5g should also be supplemented with ^{68}Ga -2TT-oC6B-4COOH versus ICG to guide the quantitative analysis of fluorescence intensity in various organs after the tumor.

Response: We thank the reviewer for this constructive suggestion. We have supplemented the quantitative analysis of fluorescence intensity in various organs following tumor resection between [^{68}Ga]DOTA-FA-BBTD-4C NPs and ICG, and incorporated these data into the main text.

Revisions in the supporting information:



Supplementary Fig. 35 Quantitative analysis of fluorescence intensity in various organs following tumor resection for [^{68}Ga]DOTA-FA-BBTD-4C NPs vs. ICG.

5. Experimental conditions need to be detailed and some spelling mistakes needs to be corrected.

Response: We thank the reviewer for this constructive suggestion. We have supplemented the detailed experimental procedures in the main text and conducted thorough proofreading of the entire manuscript to ensure linguistic accuracy.

6. Some experimental protocol (Preparation of ^{68}Ga -2TT-oC6B-4COOH NPs, In vivo PET/CT imaging) is confusing to readers, need to be present properly.

Response: We thank the reviewer for this constructive suggestion. To enhance experimental reproducibility and facilitate reader comprehension, we have systematically optimized and refined the preparation of [^{68}Ga]DOTA-FA-BBTD-4C NPs and the procedures for in vivo PET/CT imaging experiments.

Revisions in the main body of the text:

Radiolabelling of [^{68}Ga]DOTA-FA-BBTD-4C NPs

$^{68}\text{GaCl}_3$ (2.5 mL, 0.05 M HCl, 25mCi) was eluted from the $^{68}\text{Ge}/^{68}\text{Ga}$ generator and mixed with 50 μL of 0.25 M NaOAc containing 1 mg/mL of DOTA-FA-BBTD-4C NPs to achieve a pH of 4.0. The mixture was incubated at 100 $^{\circ}\text{C}$ for 10 min and purified by centrifugation with ultrafiltration device.

In vivo PET/CT imaging

To construct U87 xenograft mice, a density of 5×10^6 U87 cells resuspended in Matrigel were subcutaneously injected into the right hind of BALB/c nude mice (6-8 weeks, 16-18 g). When the average tumor volume reached around 200 mm³, the U87 xenograft mice were intravenously injected with [⁶⁸Ga]DOTA-FA-BBTD-4C NPs (200 μCi in 150 μL PBS) for in vivo PET/CT imaging at different time points with a Super Nova® PET/CT system. In blocking experiments, mice bearing U87 xenografts were co-injected with [⁶⁸Ga]DOTA-FA-BBTD-4C NPs (200 μCi in 150 μL PBS) and DOTA-FA-BBTD-4C NPs (20 mg/kg) as a competitor.

Biodistribution

U87 xenograft mice were intravenously injected with [⁶⁸Ga]DOTA-FA-BBTD-4C NPs (200 μCi in 150 μL PBS) and sacrificed at 0.5, 1, 2, 4 h post-injection, of which the main organs and tumors were collected, weighed, and measured for radioactivity using a γ-counter.

7. The statistical methods should be added in the caption (Fig. 6d). Besides, the p value analysis should be performed in Fig. 7c, Fig. 7e and Figs. 8a-j.

Response: We thank the reviewer for this constructive suggestion. We have carefully added the statistical methods in the caption of Fig. 6d and Fig. 7e. Besides, the p value analysis was performed in Fig. 7c, Fig. 7e and Figs. 8a-j. This contributes substantially to the quality of the article.

8. The discussion section can be elaborated by citing more relevant examples of nanomaterials designed for PET/CT imaging.

Response: We thank the reviewer for this constructive suggestion. To provide readers with a more comprehensive understanding of the research progress in PET/CT imaging, we have listed relevant examples of nanomaterials used in PET/CT imaging and elaborated on them in detail in the introduction section.

Revisions in the main body of the text:

Detrille, A. et al.⁵⁸ demonstrated in a non-human primate model of COVID-19 that a single administration of [⁸⁹Zr]COVA1-27-DFO - a radiolabeled human monoclonal antibody targeting a conserved epitope on the SARS-CoV-2 spike protein - permitted longitudinal viral tracking through PET/CT imaging. Three months post-infection, convalescent animals maintained detectable [⁸⁹Zr]COVA1-27-DFO PET signals in both pulmonary and cerebral tissues. Furthermore, this imaging modality successfully identified viral presence in multiple organ systems (including the respiratory tract and kidneys) throughout the acute phase of infection. Additionally, Lee, H. J. et al.⁵⁹ radiolabeled PEGylated FUD (PEG-FUD) with ⁶⁴Cu and monitored its spatiotemporal biodistribution in bleomycin-induced murine models of lung injury and fibrosis using PET/CT imaging. The study demonstrated significant [⁶⁴Cu]Cu-PEG-FUD uptake at both 3 and 11 d post-

bleomycin administration, suggesting this radiolabeled PEG-FUD has potential as an imaging probe for assessing disease activity in pulmonary fibrotic disorders.

References:

58. Detrille, A., et al. Whole-body visualization of SARS-CoV-2 biodistribution in vivo by immunoPET imaging in non-human primates. *Nat. Commun.* **16**, 2816 (2025).
59. Lee, H. J., et al. [⁶⁴Cu]Cu-PEG-FUD peptide for noninvasive and sensitive detection of murine pulmonary fibrosis. *Sci. Adv.* **10**, eadj1444 (2024).

Reviewer #2 (Remarks to the Author):

To address the reviewers' comments regarding the supplementation of experimental components and to ensure precise and standardized nomenclature of experimental materials, we have systematically refined and standardized the molecular naming conventions and experimental group terminology throughout the manuscript. Additionally, the detailed composition of each material has been provided in the Supporting Information.

As demonstrated in the following:

1. **"Folic acid"** has been uniformly replaced with **"[⁶⁸Ga]DOTA-FA NPs"** in the text.
2. **"2TT-oC6B-4COOH"** has been uniformly replaced with **"BBTD-4C "** in the text.
3. **"2TT-oC6B-4COOH NPs"** has been uniformly replaced with **"DOTA-FA-BBTD-4C NPs"** in the text.
4. **"⁶⁸Ga-2TT-oC6B-4COOH NPs"** has been uniformly replaced with **"[⁶⁸Ga]DOTA-FA-BBTD-4C NPs"** in the text.
5. The non-targeted nanoparticles (i.e., nanoparticles with identical composition but without folate molecules) are designated as **"[⁶⁸Ga]DOTA-BBTD-4C NPs"** in the text.

Revisions in the main body of the text: The chemical compositions of materials for in vitro and in vivo experiments are provided in Supplementary Table 1.

Revisions in the Supporting Information:

Supplementary Table 1. The composition of each nanoparticle.

Name	Folic acid targeting	Chelating agent	AI Egen	Radionuclide
DOTA-FA NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	/	/
DOTA-FA-BBTD-4C NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	/
[⁶⁸ Ga]DOTA-FA NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	/	⁶⁸ Ga
[⁶⁸ Ga]DOTA-BBTD-4C NPs	/	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	⁶⁸ Ga
[⁶⁸ Ga]DOTA-FA-BBTD-4C NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	⁶⁸ Ga

- What are the noteworthy results?

This manuscript describes the synthesis and proof-of-concept studies of a first-in class folate-targeted multifunctional theranostic nanoplatform for folate-expressing tumors (⁶⁸Ga-PET, fluorescence imaging (FLI), aggregation-induced emission-active photosensitizer for photodynamic therapy (PDT) and photothermal therapy (PTT)). However, it is difficult to gauge how noteworthy these data are since this could be viewed as an extension of prior work published by Tang (one of the corresponding authors of the current study). This describes the modification of 2TT-oC6B-4COOH NPs (PMID: 33889836 + 4COOH) + DOTA

chelate + folate molecules for folate receptor alpha (FR- α). Also, since DOTA is such a universal chelator of radiometals, there is potential for radiotherapy (for e.g., with ^{177}Lu or ^{225}Ac).

Response: We thank the reviewer for this constructive suggestion. Previous work by Tang (a co-corresponding author of this study), including *Nat. Nanotechnol.* **2024**, *19*, 834-845; *J. Am. Chem. Soc.* **2024**, *146*, 35462-35477; *Angew. Chem. Int. Ed.* **2025**, *64*, e202413219, etc., while focusing on cancer theranostics, had not yet established a fully integrated diagnostic-therapeutic system. This study presents an innovative integration of PET/CT's high sensitivity with the exceptional spatiotemporal resolution of AIEgen-based NIR-II fluorescence imaging. The developed dual-modal imaging platform demonstrates remarkable synergistic effects, achieving both enhanced detection sensitivity for comprehensive visualization of primary and metastatic tumors and effective resolution of two critical technical limitations - the inherent sensitivity constraints of NIR-II imaging and the temporal restrictions imposed by ^{68}Ga -PET's short half-life. A key advancement lies in the establishment of a fully integrated theranostic system that seamlessly bridges precise diagnostic imaging with targeted therapeutic intervention. The system employs the organic multidentate ligand DOTA as a chelating agent, which not only effectively coordinates diagnostic radionuclides (such as ^{68}Ga and ^{64}Cu) for precise molecular imaging, but also stably binds therapeutic isotopes (including ^{177}Lu and ^{225}Ac) for targeted radiotherapy. This design demonstrates remarkable versatility and broad applicability, exhibiting outstanding multifunctionality and promising clinical translation potential.

The Noteworthy results of this work can be summarized as follows:

1. This platform represents the first realization of a tripartite "radioactive diagnosis-fluorescence-guided surgery-targeted therapy" integrated system for precision oncology. The all-in-one therapeutic paradigm demonstrates the following transformative advantages: (1) Innovative application of multimodal image fusion technology effectively resolves the "information gap" challenge caused by spatiotemporal heterogeneity in conventional staged treatments; (2) Establishment of a "diagnosis-to-targeted therapy" closed-loop system enables progressive precision intervention, achieving significant reduction in tumor recurrence rates. The established diagnostic-therapeutic paradigm not only represents the state-of-the-art in current comprehensive cancer treatment, but more importantly, provides a transferable theoretical framework and technical system for precision oncology management.

2. Leveraging a folate receptor-mediated targeted delivery system, this nanoplatform enables specific drug enrichment at tumor sites. This reduced nonspecific toxicity and ensured precise drug delivery.

3. For diagnostic purposes, it innovatively combines the high sensitivity of PET-CT imaging with the superior spatiotemporal resolution of NIR-II fluorescence imaging, not only facilitating highly sensitive whole-body visualization of primary and metastatic tumors but also overcoming the clinical limitations posed by the short half-life of ^{68}Ga -PET isotopes through complementary advantages of the dual-modal imaging. This complementary approach demonstrates significant potential for optimizing radiotracers with short half-lives, addressing a key limitation in current nuclear medicine applications.

4. The nano-platform establishes a hierarchical precision therapy paradigm where preoperative high-resolution imaging enables precise tumor localization, intraoperative real-time NIR-II fluorescence guidance

ensures accurate tumor resection, and postoperative AIEgen-mediated combinatorial phototherapy (activated by laser irradiation) induces localized hyperthermia and ROS generation at the surgical site to effectively trigger tumor cell apoptosis through cell cycle disruption, thereby eliminating residual microlesions and preventing tumor recurrence.

5. This study conducted a systematic and in-depth investigation into the performance of the multimodal phototherapy system and the working mechanism of AIEgens. For mechanistic exploration, we comprehensively characterized the ROS generation process through electron spin resonance (ESR) spectroscopy and other techniques, while providing profound insights into the luminescence mechanism of AIEgens based on the restricted intramolecular motion (RIM) effect. These comprehensive experimental data not only provide crucial guidance for the structural optimization of AIEgens, but also establish a reliable evaluation benchmark for comparing the performance of various multifunctional therapeutic nanoplateforms.

6. We have constructed an integrated theranostic nano-platform utilizing radioactive NIR-II AIEgen, offering innovative insights and implementation frameworks for applying aggregate science in biomedicine. This advancement significantly broadens the scope of aggregate science while pioneering a new technical approach for precise cancer diagnosis and treatment.

• Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

This work has the potential to be of significance if this one of the first reports of a truly “all-in-one” tumor-antigen specific biocompatible nanoplateform that offers PET staging (although ^{68}Ga 's short physical half-life may be a barrier for optimal clinical utilization), fluorescent-guided surgical resection, coupled with a conditionally activated post-operative photothermal therapeutic that is well-suited for management of residual microscopic disease.

Response: We thank the reviewer for this constructive suggestion. The AIEgen-based theranostic systems for tumor (e.g., *Angew. Chem. Int. Ed.* **2022**, 61, e202202614; *Aggregate*. **2024**, 5, e458; *Adv. Sci.* **2024**, 11, 2302713; *Angew. Chem. Int. Ed.* **2025**, 64, e202419759 etc.) still face multiple technical bottlenecks in clinical translation. In terms of diagnostic modalities, current technologies exhibit significant limitations: fluorescence imaging suffers from restricted penetration depth due to tissue scattering effects, while photoacoustic imaging demonstrates substantially reduced signal-to-noise ratio owing to strong ultrasound attenuation by bone tissues and acoustic impedance mismatch at air-tissue interfaces. Therapeutically, single-modality photothermal therapy (PTT) carries risks of collateral tissue damage caused by heat diffusion, and photodynamic therapy (PDT) shows limited efficacy in hypoxic tumor microenvironments - both modalities fail to achieve complete ablation of deep-seated tumors. Most critically, current delivery systems relying on passive EPR targeting demonstrate generally low tumor accumulation efficiency and lack microenvironment-responsive targeting mechanisms, significantly hindering the implementation of spatiotemporally precise treatment.

This study on the radio-AIEgen-based theranostic nanoplatfrom was designed with reference to the work reported in *Chem. Asian J.* **2023**, *18*, e202300189. However, for the first time, we innovatively integrated a trimodal synergistic therapy system combining "radio-diagnosis/fluorescence-guided surgery/targeted therapy" for precision oncology, representing a completely original design concept. Experimental results demonstrate that this nanoplatfrom not only possesses significant scientific research value but also exhibits remarkable clinical translation potential. Compared with this previous study, our current work demonstrates several distinct differences:

1. The novel NIR-II AIEgen designed and synthesized in this study demonstrates outstanding comprehensive performance. This molecular system exhibits significantly enhanced reactive oxygen species generation efficiency coupled with excellent photothermal conversion capability, enabling synergistic optimization of theranostic functions. Such multi-mechanism cooperative effects effectively overcome the limitations inherent in single-modality therapeutic approaches and markedly reduce tumor cell treatment resistance (e.g., *Adv. Funct. Mater.* **2024**, *34*, 2401627, *Nat. Commun.* **2019**, *10*, 2206).

2. Although the EPR effect has demonstrated favorable tumor-selective accumulation in preclinical animal models, its performance in human clinical trials has fallen far short of expectations (e.g., *J Control Release.* **2016**, *28*, 108-121). To address this translational challenge, our study has innovatively developed a targeted nanodelivery system based on the amphiphilic material DSPE-PEG₂₀₀₀-FA. This system achieves receptor-mediated active targeting through specific molecular recognition between surface-conjugated folate ligands and overexpressed folate receptor α on tumor cells. The approach not only significantly enhances drug accumulation efficiency in tumor tissues but also ensures efficient cellular uptake by promoting receptor-mediated endocytosis. More importantly, this precision delivery strategy effectively reduces nonspecific toxicity to normal tissues, providing a viable solution for translating nanomedicines from basic research to clinical applications.

3. This study adopted a graded treatment strategy, which involved surgical resection for tumor debulking followed by combined PTT and PDT for synergistic treatment. This sequential therapeutic approach fully leverages the advantages of different treatment modalities: surgical resection rapidly eliminates the primary tumor burden, while the subsequent PTT/PDT synergistic therapy overcomes the limitations of monotherapy through the combined effects of photothermal conversion and ROS generation, effectively eradicating residual lesions and preventing tumor recurrence. This strategy achieves precise and multimodal comprehensive therapy.

4. This study presents a systematic and in-depth investigation of the multimodal phototherapy system's performance and the working mechanism of AIEgens. For mechanistic exploration, we employed ESR spectroscopy coupled with complementary analytical techniques to comprehensively characterize the ROS generation process. Furthermore, we elucidated the luminescence mechanism of AIEgens through detailed analysis of the restricted intramolecular motion (RIM) effect. The comprehensive experimental dataset obtained not only provides fundamental insights for AIEgens' structural optimization but also establishes a robust evaluation framework for comparative assessment of multifunctional therapeutic nanoplatforms.

5. In the experimental investigation, this study systematically elucidated the photophysical properties of AIEgen materials using multi-scale characterization techniques, complemented by theoretical calculations to delineate the underlying molecular mechanisms. Building upon this theoretical foundation, we developed a groundbreaking NIR-II AIEgen-based theranostic nanoplatform through comprehensive in vitro cellular studies and in vivo animal models. This platform represents the first realization of a tripartite "radioactive diagnosis-fluorescence-guided surgery-targeted therapy" integrated system for precision oncology. The all-in-one therapeutic paradigm demonstrates the following transformative advantages: (1) Innovative application of multimodal image fusion technology effectively resolves the "information gap" challenge caused by spatiotemporal heterogeneity in conventional staged treatments; (2) Establishment of a "diagnosis-to-therapy" closed-loop system enables progressive precision intervention, achieving significant reduction in tumor recurrence rates. The established diagnostic-therapeutic paradigm not only represents the state-of-the-art in current comprehensive cancer treatment, but more importantly, provides a transferable theoretical framework and technical system for precision oncology management.

6. The development of AIEgen-based nanoplatforms for radiotherapy has pioneered groundbreaking research directions in the application of aggregation science in biomedical fields. This innovative platform successfully establishes a novel theranostic paradigm through the ingenious integration of radiation physics and molecular imaging principles. Particularly noteworthy is that the platform not only effectively addresses the temporal limitations of short-half-life isotopes (e.g., ^{68}Ga -PET) in clinical applications, but also provides innovative solutions for optimizing clinical utilization of short-lived radiotracers through fluorescence-radiation dual-modal tracking strategy. This technological breakthrough is anticipated to overcome critical bottlenecks in current nuclear medicine diagnostics and therapeutics, while simultaneously laying important theoretical foundations and technical support for the advanced application of aggregation science in precision medicine.

To better highlight the novelty and advantages of our work compared to previous studies, we have added the following content in the main text:

Current research on combining photosensitizers with radionuclide labeling has primarily focused on PET/FLI dual-modality imaging diagnostics.⁶⁰ However, conventional photosensitizers inherently suffer from aggregation-caused quenching (ACQ) effects.^{61,62} While some studies have attempted to integrate diagnostic and therapeutic functions, their treatment strategies remain relatively simplistic, predominantly limited to photosensitizer-based single-modality photothermal therapy,⁶³ which fails to prevent tumor recurrence. More critically, conventional drug delivery systems relying solely on the passive enhanced permeability and retention (EPR) effect encounter fundamental limitations, including suboptimal tumor-specific accumulation and absence of intelligent microenvironment-responsive targeting capabilities,⁶⁴ which profoundly restricts the clinical translation of spatiotemporal precision therapeutics.

Herein, we present the first-ever construction of an integrated "radio-diagnosis/fluorescence-guided surgery/targeted therapy" trinity synergistic therapeutic system, offering a groundbreaking solution for cancer treatment. As shown in Fig. 1, BBTD-4C was first encapsulated in functionalized amphiphilic

molecules DSPE-PEG₂₀₀₀-FA and DSPE-PEG₂₀₀₀-DOTA, successfully preparing water-soluble nanoparticles (DOTA-FA-BBTD-4C NPs) with excellent solubility.

The platform achieves: (1) highly sensitive whole-body visualization of both primary and metastatic tumors, with the complementary advantages of bimodal imaging overcoming the clinical limitation of ⁶⁸Ga-PET isotopes' short half-life—demonstrating remarkable potential for optimizing short-lived radiotracers and addressing a critical bottleneck in current nuclear medicine applications; (2) the first-ever integration of the "radio-diagnosis/fluorescence-guided surgery/targeted therapy" triad in precision oncology; (3) innovative application of multimodal image fusion technology that effectively resolves the "information gap" challenge caused by spatiotemporal heterogeneity in conventional staged treatments; and (4) establishment of a closed-loop "diagnosis-to-treatment" system enabling progressive precision intervention that significantly reduces tumor recurrence rates. This established diagnostic-therapeutic paradigm not only represents the state-of-the-art in comprehensive cancer care but, more importantly, provides a transferable theoretical framework and technical system for precision tumor management.

References:

60. Lwin, T. M., et al. Multimodality PET and near-infrared fluorescence intraoperative imaging of CEA-positive colorectal cancer. *Mol. Imaging. Biol.* **25**, 727-734 (2023).

61. Zhang, G. L., et al. Multifunctional AIEgen Visualizing a 3D Scaffold for Skull Repair and Highly Effective Anti-inflammatory Activity. *ACS Appl. Mater. Interfaces*. **17**, 36109-36120 (2025).

62. Su, H., et al. Near-Infrared II AIE Luminogens with Mitochondria-Targeting Characteristics for Combinational Phototherapies of Breast Tumors Through Synergistic Cell Apoptosis and Pyroptosis. *Adv. Funct. Mater.* **35**, 2414976 (2025).

63. Yu, K., et al. Radiolabeled AIE Probes as Dual-modality Imaging Agents for PET/NIR-II Fluorescence-Guided Photothermal Therapy. *Chem-Asian J.* **18**, e202300189 (2023).

64. Goos, J., et al. Delivery of polymeric nanostars for molecular imaging and endoradiotherapy through the enhanced permeability and retention (EPR) effect. *Theranostics*, **10**, 567-584 (2020).

• A major strength of the work is the highly rigorous evaluation of the performance of the multimodal phototherapy, especially the ROS characterization including mechanistic confirmation with electron spin resonance. These data provide important benchmarks for optimization and comparison with other multifunctional theranostic nanoplatfroms. Unclear what the significance is of the observed laser stability and reporter photothermal conversion efficiency of ~40% (is this high/low/typical compared with other photothermal agents?).

Response: We thank the reviewer for this constructive suggestion. Conventional single-modality phototherapies have inherent limitations: PTT causes collateral tissue damage due to thermal diffusion, while PDT exhibits restricted therapeutic efficacy in hypoxic tumor microenvironments. To address these challenges, we engineered AIEgen molecules through precise singlet-triplet energy level modulation, enabling multiplexed energy conversion pathways. Upon photoexcitation, AIEgens undergo $S_0 \rightarrow S_n \rightarrow S_1$ transitions followed by intersystem crossing (ISC) to generate long-lived T_1 states for ROS production. Concurrently, the S_1 state yields both fluorescence via radiative decay and hyperthermia through

nonradiative transition, achieving imaging-guided PDT/PTT synergistic therapy. AIEgen demonstrates excellent photostability in laser irradiation tests (Supplementary Fig. 25), maintaining highly stable photothermal conversion performance over multiple on-off cycles (Fig. 2i). This characteristic not only effectively suppresses material degradation but also significantly improves material utilization efficiency, thereby reducing operational costs in practical applications.

Compared with reported photothermal agents in the literature (e.g., *Nat. Commun.* **2024**, *15*, 6362; *Adv. Funct. Mater.* **2024**, *34*, 2310237; *J. Am. Chem. Soc.* **2025**, *147*, 2010-2020), this AIEgen represents a typical class of photothermal materials. However, its photothermal conversion efficiency is higher than that of most previously reported photothermal agents. While its photothermal efficiency not surpass the highest recorded values, its distinctive merit resides in the precisely engineered singlet-triplet energy level system that facilitates multiplexed energy conversion: the absorbed optical energy can be simultaneously channeled into reactive oxygen species (ROS) generation, fluorescence emission, and photothermal conversion, thereby achieving an integrated theranostic platform with multifunctional capabilities.

To help readers better understand the photothermal capability of this material, we have supplemented the following content in the main text:

This material demonstrates a photothermal conversion efficiency significantly surpassing those of conventional photothermal agents (e.g., $\approx 9.5\%$ and 8.4% for cryptocyanine dyes vs. $\approx 3.1\%$ for ICG).⁷²

References:

72. Jung, H. S., et al. A Mitochondria-Targeted Cryptocyanine-Based Photothermogenic Photosensitizer. *J. Am. Chem. Soc.* **139**, 9972-9978 (2017).

• Does the work support the conclusions and claims, or is additional evidence needed?

A weakness in the in vivo studies is the lack of non-targeted NPs (i.e., identical formulation but without folate molecules). It would be desirable to see the tumor targeting profile of this control to demonstrate the gain in tumor targeting and residence time conferred by the folate targeting. Furthermore, it is unclear if folate acid here is an informative control. In vivo blocking radiotracer studies with folate would support the claim of active targeting of folate receptor alpha. Also, it would be desirable to see extended radiotracer biodistribution data beyond 2 h to evaluate tumor clearance kinetics. For example, if ⁶⁸Ga was swapped with ¹¹¹In, then the pharmacokinetics and biodistribution over the course of days could be studied to support the claim (As shown in Fig. 5d, through intravenous administration of ⁶⁸Ga-2TT-oC6B-4COOH NPs, the tumor site demonstrated a progressive increase in fluorescence intensity over 24 h, followed by a gradual decline). We typically don't see progressive tumor increase unless the drug has slow clearance from blood.

Response: We thank the reviewer for this constructive suggestion.

1. We have included control data for non-targeted NPs (i.e., identical formulation but without folate molecules) in Figs. 4-7. These comparative experiments clearly demonstrate that folate targeting

modification significantly enhances tumor-specific accumulation and prolongs the retention time of the nanoparticles.

In Fig. 4, we included two control groups (VI and VII) of non-targeted NPs (i.e., identical formulation but without folate molecules).

Revisions in the main body of the text:

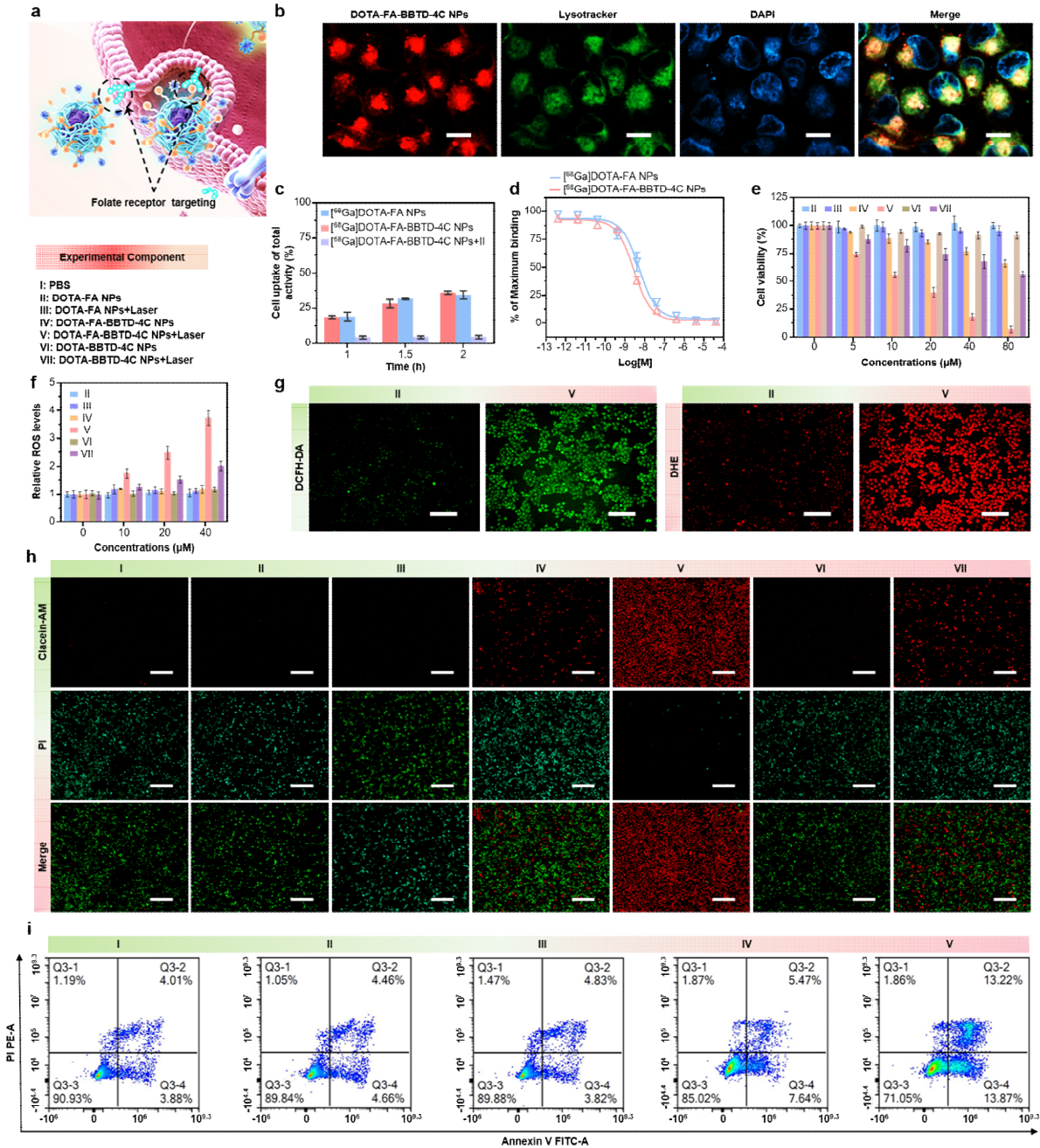


Fig. 4 Comprehensive evaluation of cellular uptake, cytotoxicity and phototherapeutic efficacy in vitro.

In contrast, nanoparticles with the same formulation but without folate modification failed to demonstrate significant tumor-targeting capability (Fig. 5d).

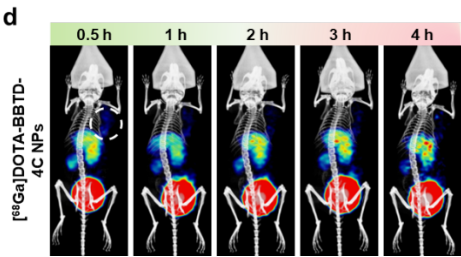


Fig. 5 d Representative PET/CT images of $[^{68}\text{Ga}]\text{DOTA-BBTD-4C NPs}$ in tumor-bearing mice.

In Fig. 6, we included two control groups (**VI** and **VII**) of non-targeted NPs (i.e., identical formulation but without folate molecules).

Revisions in the main body of the text:

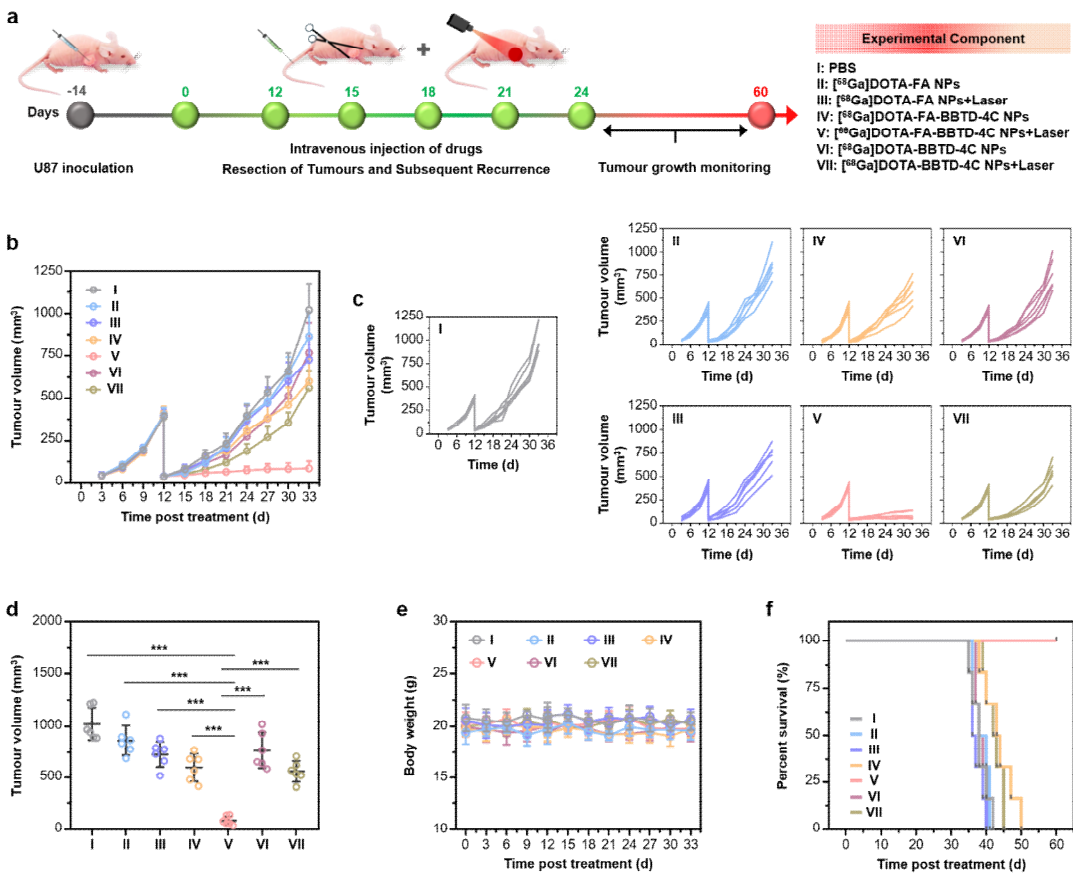


Fig. 6 Longitudinal monitoring of postoperative tumor recurrence.

In Fig. 7, we included two control groups (VI and VII) of non-targeted NPs (i.e., identical formulation but without folate molecules).

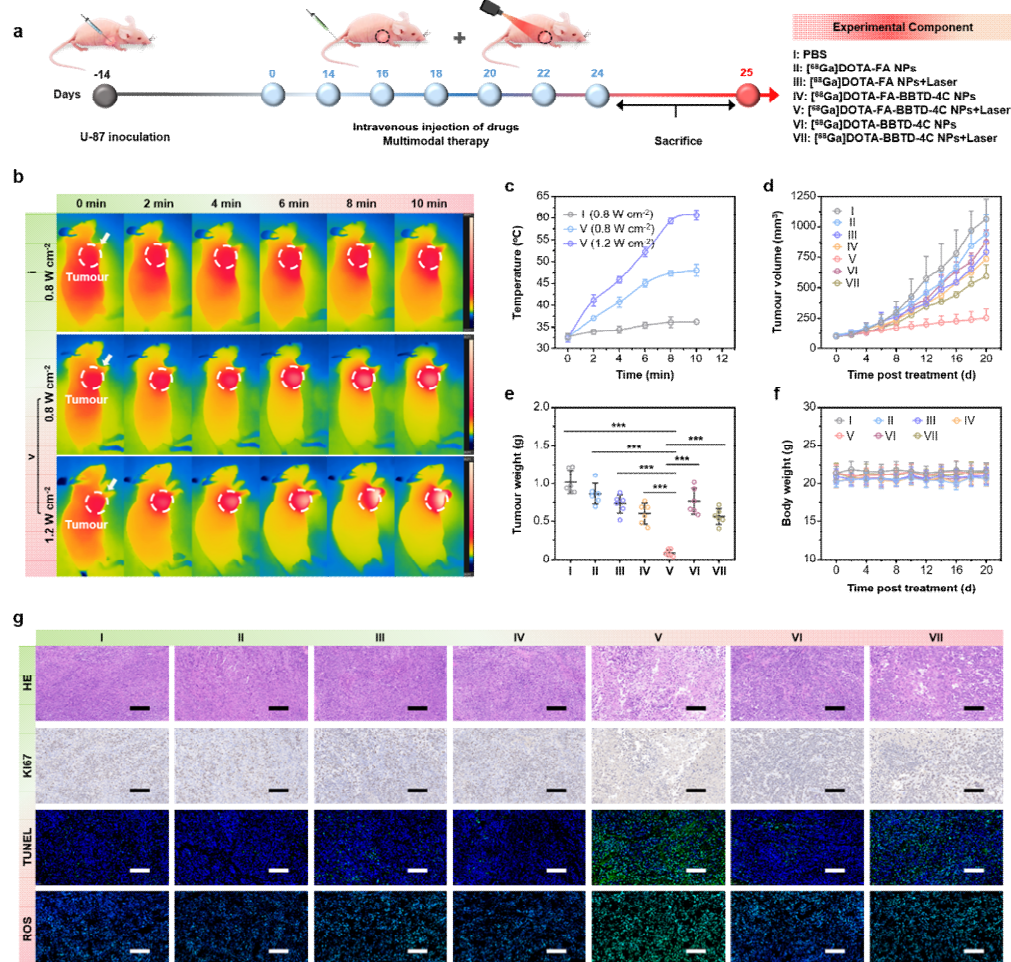


Fig. 7 Mechanistic investigation of combined photodynamic/photothermal therapy (PDT/PTT) synergistic effects.

In Fig. 8, we included control groups ([⁶⁸Ga]DOTA-BBTD-4C NPs + Laser) of non-targeted NPs (i.e., identical formulation but without folate molecules).

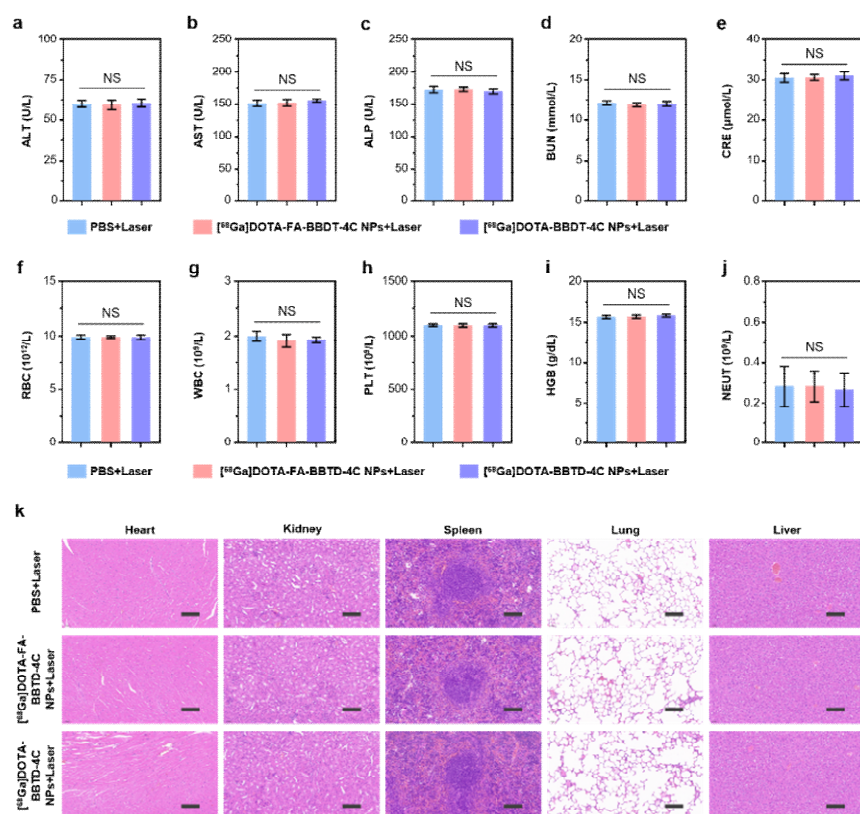


Fig. 8 Comprehensive biosafety profiling of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NPs: toxicology assessment.

These data confirm that the formulation without folic acid molecules (i.e., identical formulation but without folate molecules). exhibits significantly lower tumor-targeting ability and tumor retention capacity compared to the folate-mediated formulation.

2. Sorry for the confusion, the folate used in the manuscript was $[^{68}\text{Ga}]\text{DOTA-FA}$ NPs consist of DSPE-PEG₂₀₀₀-FA and DSPE-PEG₂₀₀₀-DOTA. Besides, to thoroughly validate the reference value of folate as an effective control, we conducted additional folate-mediated in vivo blocking experiments, which further confirmed the active targeting mechanism of folate receptor α through radiotracer studies.

Revisions in the main body of the text: Subsequently, to validate the active targeting mechanism mediated by FR- α in vivo, we performed competitive folate inhibition assays (Fig. 5e). The results demonstrated that administration of exogenous folate significantly attenuated the targeting capacity of the nanoparticles, providing direct evidence for FR α -mediated active targeting.

Revisions in the supporting information of the text:

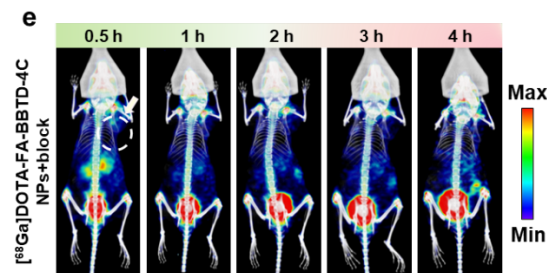


Fig. 5 e Representative PET/CT images of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NPs + block in tumor-bearing mice from the folate competition inhibition group.

3. Under your suggestion, we added 4-h biodistribution data of the radiotracer to more clearly evaluate tumor kinetics.

Revisions in the main body of the text:

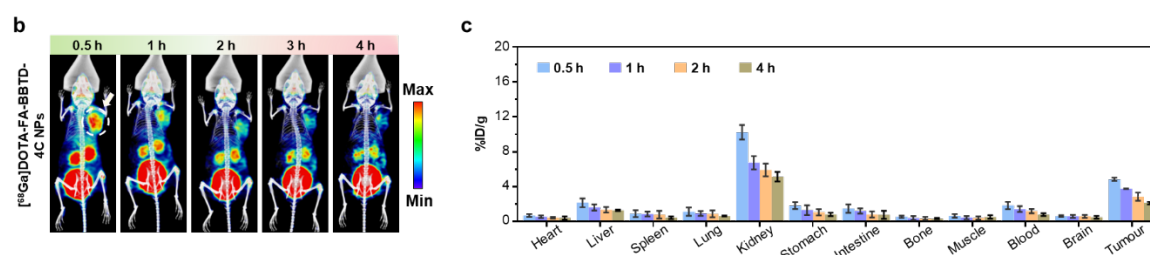


Fig 5. c Dynamic time-activity curves of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NPs in heart, liver, spleen, kidney, stomach, intestine, bone, muscle, blood, brain, and tumor of tumor-bearing mice.

The results show that the intensity of the radiotracer biodistribution gradually decreases over time, which is consistent with previously reported literature (*J. Nucl. Med.*, **2024**, 65, 394-401 and *J. Nucl. Med.*, **2022**, 63, 862-868).

[editorial note: third party material redacted]

References (*J. Nucl. Med.*, **2024**, 65, 394-401 and *J. Nucl. Med.*, **2022**, 63, 862-868.)

The design concept of this study is to combine radionuclide imaging with fluorescence bimodal imaging to achieve relay imaging, thereby reducing the radiation impact of persistent radionuclides on the organism. Although radionuclides possess excellent tissue penetration capability, their prolonged retention in the body can cause significant safety concerns. You suggested that a comparative study using ^{68}Ga and ^{111}In would help to more systematically assess the pharmacokinetic and biodistribution profiles of the nanoparticles over several days, thereby supporting our assertion (as indicated in Fig. 5d, where fluorescence intensity at the tumor site increased progressively over 24 h after intravenous injection of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NPs, followed by a gradual decline). However, as our laboratory currently does not have the permit to use the radionuclide ^{111}In (half-life: 2.8 d), we regret that we are unable to perform long-term observations to more comprehensively reflect its in vivo distribution patterns. To further enhance our research capabilities, we

plan to obtain certification for additional radionuclides in future studies. In order to better substantiate the abovementioned assertion, we have incorporated a comparative analysis of the fluorescence intensity at the tumor site between $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ and ICG in Fig. 5 of the main text. This includes quantitative tracking of fluorescence intensity over time, as well as a comparison between $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ and ICG to facilitate quantitative evaluation of fluorescence in each organ following tumor resection. Changes in fluorescence intensity at the tumor site over a 24-h period can be directly observed in Fig. 5h, while the distribution of fluorescence intensity across various organs is clearly shown in Fig. 5k. These additional data provide strong support for our conclusion.

Revisions in the main body of the text:

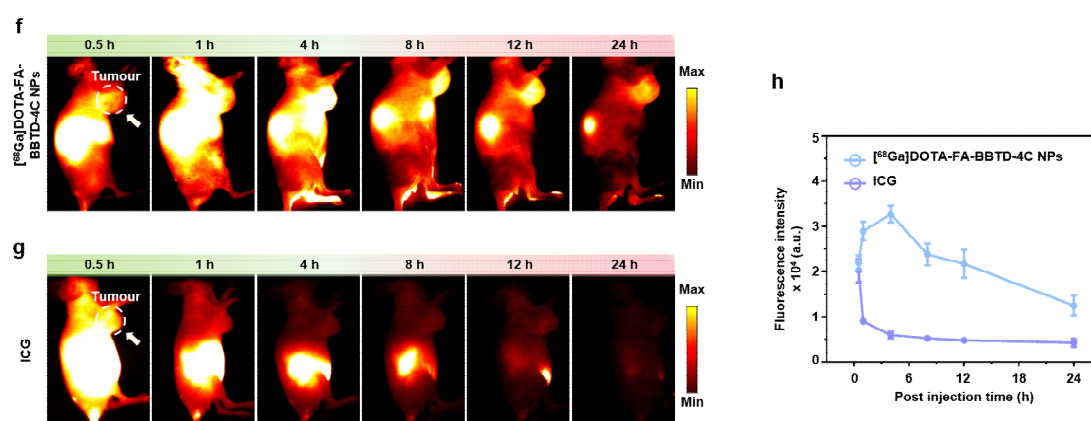
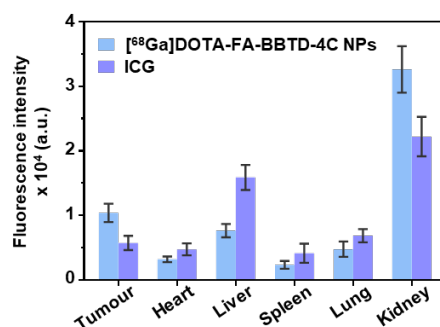


Fig. 5 h Quantitative analysis of time-dependent fluorescence intensity of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ and ICG at tumor sites.



Supplementary Fig. 35 Quantitative analysis of fluorescence intensity in various organs following tumor resection for $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ vs. ICG.

• Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision? None noted.

Response: We sincerely appreciate the reviewers' constructive comments and suggestions. We have carefully reviewed the entire manuscript, with particular attention to key aspects including data analysis, results interpretation, and conclusion derivation. We have ensured the professionalism of language

expression and the reproducibility of the research process, thereby providing readers with more rigorous and scientifically sound research outcomes.

- Is the methodology sound? Does the work meet the expected standards in your field?

The ^{68}Ga radiochemistry methodology and associated quality control is lacking. It is also unclear what the folate receptor alpha expression is in the U87 cells/xenograft model. A weakness is that only a single cell line/animal model was studied.

Response: We thank the reviewer for this constructive suggestion.

1. The ^{68}Ga radiochemistry methodology and associated quality control was added in the revised manuscript.

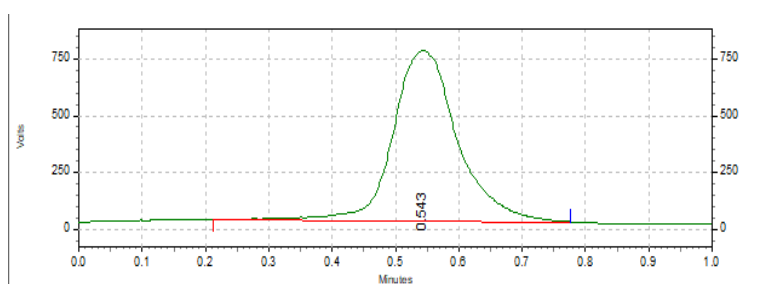
Revisions in the main body of the text:

Radiolabelling of [^{68}Ga]DOTA-FA-BBTD-4C NPs

$^{68}\text{GaCl}_3$ (2.5 mL, 0.05 M HCl, 25mCi) was eluted from the $^{68}\text{Ge}/^{68}\text{Ga}$ generator and mixed with 50 μL of 0.25 M NaOAc containing 1 mg/mL of DOTA-FA-BBTD-4C NPs to achieve a pH of 4.0. The mixture was incubated at 100 $^{\circ}\text{C}$ for 10 min and purified by centrifugation with ultrafiltration device.

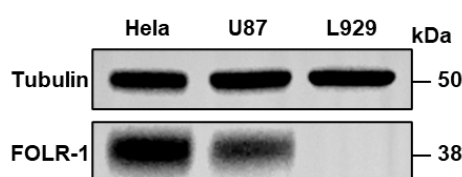
Subsequently, a novel radiolabeled probe for PET/CT imaging was successfully obtained by reacting DOTA-FA-BBTD-4C NPs with $^{68}\text{GaCl}_3$ (eluted from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator), and its radiochemical purity (More than 99%) was confirmed by thin-layer chromatography analysis (Supplementary Fig. 30, Supplementary Fig. 31).

Revisions in the supporting information:



Supplementary Fig. 31 Thin-Layer Chromatography (TLC) analysis of [^{68}Ga]DOTA-FA-BBTD-4C NPs.

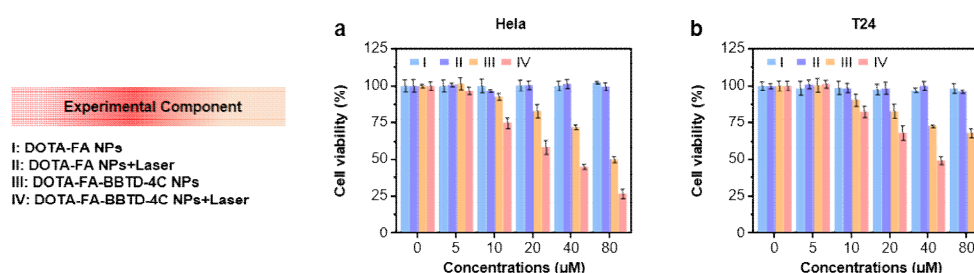
2. The folate receptor alpha expression in the U87, HeLa and L929 cells was investigated by WB. As a result, the folate receptor alpha was proved to be high expressed in U87 and HeLa cells.



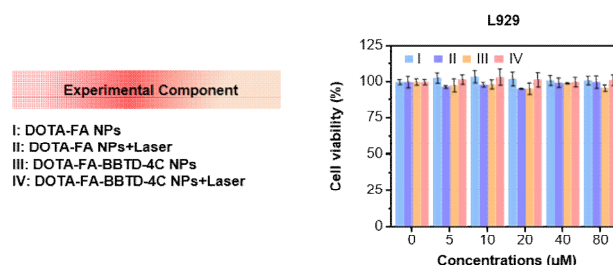
Western blot analysis of Folate Receptor alpha in U87, HeLa and L929 cells.

3. This study further employed a multidimensional experimental approach to systematically evaluate the antitumor efficacy of the nano-system. At the cellular level, we conducted in vitro experiments using bladder cancer T24 cells, cervical cancer HeLa cells, and L929 fibroblast cells as controls, which confirmed the broad-spectrum inhibitory effect of this system on tumors of different origins. In the in vivo evaluation, we established a HeLa cell-derived cervical cancer mouse model and utilized small-animal PET/CT imaging to dynamically monitor the targeting efficiency and biodistribution of the nanoplatform, thereby providing comprehensive experimental evidence to support its clinical translation.

Revisions in the supporting information:

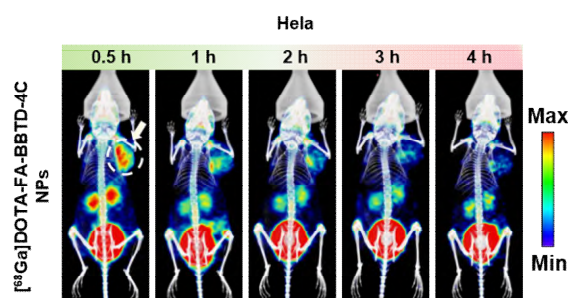


Supplementary Fig. 32 Cell viability of **a** HeLa and **b** T24 cells after laser treatment with different concentrations of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NPs.



Supplementary Fig. 33 Cell viability of NCTC clone 929 (L929) after laser treatment with different concentrations of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NPs.

Revisions in the main body of the text: After intravenous injection of 200 μCi in U87 and HeLa tumor models, distinct tumor visibility was observed within 0.5 h (Fig. 5b, Supplementary Fig. 34).



Supplementary Fig. 34 Representative PET/CT images of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs in Hela tumor-bearing mice.

- Is there enough detail provided in the methods for the work to be reproduced?

As mentioned above, the ⁶⁸Ga radiochemistry would be very difficult to reproduce based on the provided methods. Also, it is preferable to see the ⁶⁸Ga tracer characteristics in the main text. For example, along with administered activity of 20 uCi, what was the mass/moles of drug (specific activity?). Tracer specific activity can have a tremendous effect on tumor targeting. There is also a discrepancy between the administered activity in the main text (20 uCi) versus the methods (200 uCi).

Response: We thank the reviewer for this constructive suggestion and sorry for our mistakes. The administered activity of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs in the main text should be 200 μCi consisting with that in methods (200 μCi). Besides, we have enhanced the experimental methodology and provided additional detailed descriptions to ensure the reproducibility of the research work. The concentration of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs used for in vivo PET/CT imaging was 0.02 mg/mL in 150 μL (200 μCi).

Revisions in the main body of the text:

Radiolabelling of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs

⁶⁸GaCl₃ (2.5 mL, 0.05 M HCl, 25mCi) was eluted from the ⁶⁸Ge/⁶⁸Ga generator and mixed with 50 μL of 0.25 M NaOAc containing 1 mg/mL of DOTA-FA-BBTD-4C NPs to achieve a pH of 4.0. The mixture was incubated at 100 °C for 10 min and purified by centrifugation with ultrafiltration device.

Reviewer #3 (Remarks to the Author):

In this manuscript, Zhang et al. reports a theranostic nanoplatform for precision cancer care by integrating a radionuclide with NIR-II aggregation induced emission (AIE) luminogens. A comprehensive set of experiments (from synthesis to in vivo imaging and cancer therapy) has been conducted. However, many experimental details are missing, making it difficult to fully understand and evaluate the research. Many critical questions need to be addressed in this manuscript.

To address the reviewers' comments regarding the supplementation of experimental components and to ensure precise and standardized nomenclature of experimental materials, we have systematically refined and standardized the molecular naming conventions and experimental group terminology throughout the manuscript. Additionally, the detailed composition of each material has been provided in the Supporting Information.

As demonstrated in the following:

1. "Folic acid" has been uniformly replaced with "[⁶⁸Ga]DOTA-FA NPs" in the text.
2. "2TT-oC6B-4COOH" has been uniformly replaced with "BBTD-4C " in the text.
3. "2TT-oC6B-4COOH NPs" has been uniformly replaced with "DOTA-FA-BBTD-4C NPs" in the text.
4. "⁶⁸Ga-2TT-oC6B-4COOH NPs" has been uniformly replaced with "[⁶⁸Ga]DOTA-FA-BBTD-4C NPs" in the text.
5. The non-targeted nanoparticles (i.e., nanoparticles with identical composition but without folate molecules) are designated as "[⁶⁸Ga]DOTA-BBTD-4C NPs" in the text.

Revisions in the main body of the text: The chemical compositions of materials for in vitro and in vivo experiments are provided in Supplementary Table 1.

Revisions in the Supporting Information:

Supplementary Table 1. The composition of each nanoparticle.

Name	Folic acid targeting	Chelating agent	AIEgen	Radionuclide
DOTA-FA NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	/	/
DOTA-FA-BBTD-4C NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	/
[⁶⁸ Ga]DOTA-FA NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	/	⁶⁸ Ga
[⁶⁸ Ga]DOTA-BBTD-4C NPs	/	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	⁶⁸ Ga
[⁶⁸ Ga]DOTA-FA-BBTD-4C NPs	DSPE-PEG ₂₀₀₀ -FA	DSPE-PEG ₂₀₀₀ -DOTA	BBTD-4C	⁶⁸ Ga

1. The multimodality theranostic imaging involving NIR dye integrated with PET modality have been previously reported. The authors should review the relevant literature in the Introduction section (e.g., Mol

Imaging Biol. 2023 Jun 21; 25(4): 727–734) and clearly highlight how the current work differs from existing studies.

Response: We sincerely appreciate the reviewer's insightful suggestion, which has helped improve the quality of our manuscript.

To better highlight the novelty and advantages of our work compared to previous studies, we have added the following content in the main text:

Current research on combining photosensitizers with radionuclide labeling has primarily focused on PET/FLI dual-modality imaging diagnostics.⁶⁰ However, conventional photosensitizers inherently suffer from aggregation-caused quenching (ACQ) effects.^{61,62} While some studies have attempted to integrate diagnostic and therapeutic functions, their treatment strategies remain relatively simplistic, predominantly limited to photosensitizer-based single-modality therapy,⁶³ which fails to prevent tumor recurrence. More critically, conventional drug delivery systems relying solely on the passive enhanced permeability and retention (EPR) effect encounter fundamental limitations, including suboptimal tumor-specific accumulation and absence of intelligent microenvironment-responsive targeting capabilities,⁶⁴ which profoundly restricts the clinical translation of spatiotemporal precision therapeutics.

Herein, we present the first-ever construction of an integrated "radio-diagnosis/fluorescence-guided surgery/targeted therapy" trinity synergistic therapeutic system, offering a groundbreaking solution for cancer treatment. As shown in Fig. 1, BBTD-4C was first encapsulated in functionalized amphiphilic molecules DSPE-PEG₂₀₀₀-FA and DSPE-PEG₂₀₀₀-DOTA, successfully preparing water-soluble nanoparticles (DOTA-FA-BBTD-4C NPs) with excellent solubility.

The platform achieves: (1) highly sensitive whole-body visualization of both primary and metastatic tumors, with the complementary advantages of bimodal imaging overcoming the clinical limitation of ⁶⁸Ga-PET isotopes' short half-life—demonstrating remarkable potential for optimizing short-lived radiotracers and addressing a critical bottleneck in current nuclear medicine applications; (2) the first-ever integration of the "radio-diagnosis/fluorescence-guided surgery/targeted therapy" triad in precision oncology; (3) innovative application of multimodal image fusion technology that effectively resolves the "information gap" challenge caused by spatiotemporal heterogeneity in conventional staged treatments; and (4) establishment of a closed-loop "diagnosis-to-treatment" system enabling progressive precision intervention that significantly reduces tumor recurrence rates. This established diagnostic-therapeutic paradigm not only represents the state-of-the-art in comprehensive cancer care but, more importantly, provides a transferable theoretical framework and technical system for precision tumor management.

References:

60. Lwin, T. M., et al. Multimodality PET and near-infrared fluorescence intraoperative imaging of CEA-positive colorectal cancer. *Mol. Imaging. Biol.* **25**, 727-734 (2023).

61. Zhang, G. L., et al. Multifunctional AIEgen Visualizing a 3D Scaffold for Skull Repair and Highly Effective Anti-inflammatory Activity. *ACS Appl. Mater. Interfaces.* **17**, 36109-36120 (2025).

62. Su, H., et al. Near-Infrared II AIE Luminogens with Mitochondria-Targeting Characteristics for Combinational Phototherapies of Breast Tumors Through Synergistic Cell Apoptosis and Pyroptosis. *Adv. Funct. Mater.* **35**, 2414976 (2025).

63. Yu, K., et al. Radiolabeled AIE Probes as Dual-modality Imaging Agents for PET/NIR-II Fluorescence-Guided Photothermal Therapy. *Chem-Asian J.* **18**, e202300189 (2023).

64. Goos, J., et al. Delivery of polymeric nanostars for molecular imaging and endoradiotherapy through the enhanced permeability and retention (EPR) effect. *Theranostics*, **10**, 567-584 (2020).

2. The authors have previously developed and published NIR-II theranostic systems based on AIE luminogen. (ACS Nano 2018, 12, 11, 11282–11293; J. Am. Chem. Soc. 2022, 144, 33, 15391–15402) The design rationale of NIR-II luminogen for study should be discussed, particularly in comparison to their earlier work.

Response: We appreciate the reviewer's constructive suggestion. To better highlight the design principles of the NIR-II luminophores compared to previously published works (ACS Nano **2018**, 12, 11, 11282-11293; J. Am. Chem. Soc. **2022**, 144, 15391-15402), we have added the following content in the main text:

Revisions in the main body of the text:

Through precise molecular engineering, we constructed the BBTD-4C compound featuring a “Donor- π -Acceptor- π -Donor” structural framework. This molecular system employs triphenylamine-propionate as a strong electron donor and benzothiazole as an electron acceptor, bridged by a sterically hindered hexylthiophene π -conjugated spacer, endowing it with remarkable advantages over previously reported analogs.⁶⁵⁻⁶⁹ Propionate, a short-chain fatty acid (SCFA),⁷⁰ is a natural metabolite produced by gut microbial fermentation and is beneficial to human health.⁷¹ Thus, the incorporation of the propionyl group confers unique biocompatibility and metabolic adaptability. Furthermore, the hydrophilic propionyl group serves a dual function: it not only effectively counterbalances the hydrophobicity of the TPA backbone, significantly enhancing water solubility, but also successfully suppresses ACQ through steric hindrance. This biocompatibility-driven molecular design not only addresses the key limitations of conventional photosensitizers but also pioneers a novel strategy for developing low-toxicity, high-efficiency therapeutic agents.

References:

65. Alifu, N., et al. Single-Molecular Near-Infrared-II Theranostic Systems: Ultrastable Aggregation-Induced Emission Nanoparticles for Long-Term Tracing and Efficient Photothermal Therapy. *ACS Nano*. **12**, 11282-11293 (2018).

66. Shen, H., et al. Rational Design of NIR-II AIEgens with Ultrahigh Quantum Yields for Photo- and Chemiluminescence Imaging. *J. Am. Chem. Soc.* **144**, 15391–15402 (2022).

67. Chen, Y., et al. Engineering a Near-Infrared Spiro-Based Aggregation-Induced Emission Luminogen for DNazyme-Sensitized Photothermal Therapy with High Efficiency and Accuracy. *J. Am. Chem. Soc.* **146**, 35462–35477 (2024).

68. Yang, X., et al. Biomimetic Aggregation-Induced Emission Nanodots with Hitchhiking Function for T Cell-Mediated Cancer Targeting and NIR-II Fluorescence-Guided Mild-Temperature Photothermal Therapy. *Adv. Funct. Mater.* **32**, 2206346 (2022).

69. Qi, J., et al. Highly stable and bright AIE dots for NIR-II deciphering of living rats. *Nano Today*. **34**, 100893 (2020)

70. Anna, M. et al. Experimental Capacity of Human Fecal Microbiota to Degrade Fiber and Produce Short-Chain Fatty Acids Is Associated with Diet Quality and Anthropometric Parameters. *J. Nutr.* **153**, 2827-2841 (2023).

71. Débora, A. C., et al. Impact of functional flours from pineapple by-products on human intestinal microbiota. *J. Funct. Foods*. **67**, 103830 (2020).

3. In supplementary Fig. 23, The TEM image of 2TT-*o*C6B-4COOH NPs clearly showed a mixture of large and small nanoparticles. The average TEM size and size distribution histogram of the nanoparticles should be provided in the manuscript. There will be a concern on broad size distribution because large nanoparticles have different pharmacokinetic behaviors compared to that of small nanoparticles. The broad size distribution may affect the biodistribution in vivo.

Response: We thank the reviewer for their constructive suggestions. We have replaced the TEM image in Fig. 2d and supplemented it with the average nanoparticle size from TEM measurements and a size distribution histogram. Both Fig. 5d and Supplementary Fig. 23 demonstrate the relatively uniform size of the nanoparticles, and this addition has significantly enhanced the quality of the manuscript.

Revisions in the main body of the text: while transmission electron microscopy (TEM) and the size distribution histogram confirmed their relatively uniform spherical morphology (Fig. 2d, Supplementary Fig. 23).

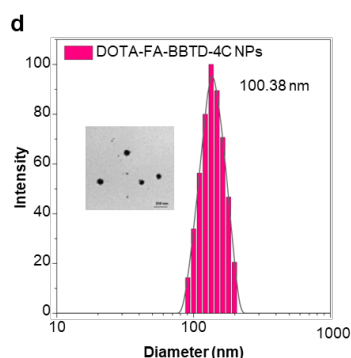
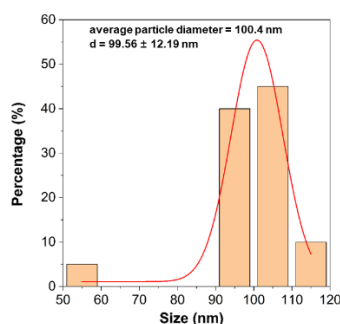


Fig. 2 d Size distribution and TEM image of DOTA-FA-BBTD-4C NPs. Scale bar: 300 nm.

Revisions in the supporting information:



Supplementary Fig. 23 The particle size distribution histogram of DOTA-FA-BBTD-4C NPs.

4. In the manuscript, it is stated that “Additionally, infrared thermal imaging confirmed that 2TT-oC6B-4COOH NPs exhibit a favorable photothermal effect (Fig. 2h).”

The authors should provide a clearer description or definition of what is meaning of favorable photothermal effects from Fig. 2h.

Response: We sincerely appreciate the reviewers' constructive comments. To provide readers with a more comprehensive understanding of the material's photothermal properties, we have incorporated in the main text a clearer description of the favorable photothermal effects illustrated in Fig. 2h.

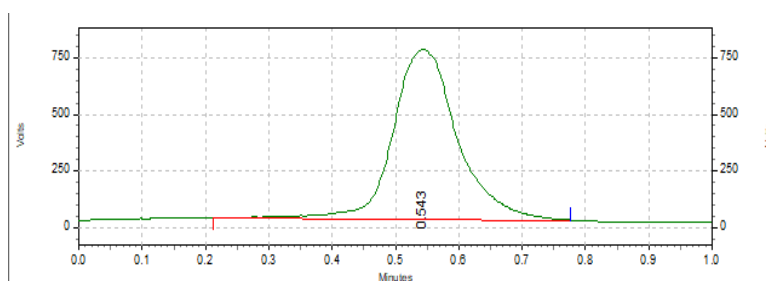
Revisions in the main body of the text: Additionally, further infrared thermography confirmed that the photothermal effect in the central region of DOTA-FA-BBTD-4C NPs nanoparticles was significantly enhanced with increasing concentration under 660 nm laser irradiation (0.8 W/cm²) (Fig. 2h).

5. In the manuscript, it is stated that “Cellular uptake and blocking experiments (Fig. 4c) revealed that both ⁶⁸Ga-2TT-oC6B-4COOH NPs and ⁶⁸Ga-folic acid exhibited significantly higher uptake rates in GBM cells (36.31 ± 1.07% and 34.61 ± 2.96%, respectively) compared to the control group (4.70 ± 1.14%), confirming the targeted intracellular delivery capability of ⁶⁸Ga-2TT-oC6B-4COOH NPs.”

The methods for labeling folic acid with ⁶⁸Ga should be clearly described. Was a chelator used? If so, what was the conjugation strategy? This information is critical for interpreting and repeat the results shown in Fig. 4.

Response: We sincerely appreciate the reviewers' constructive comments and sorry for the confusion. "Folic acid" has been uniformly replaced with "[⁶⁸Ga]DOTA-FA NPs" in the revised manuscript since [⁶⁸Ga]DOTA-FA NPs was consist of DSPE-PEG₂₀₀₀-FA and DSPE-PEG₂₀₀₀-DOTA. The methods for labeling folic acid with ⁶⁸Ga should was added in the revised manuscript.

Revisions in the supporting information:



Supplementary Fig. 31 Thin-Layer Chromatography (TLC) analysis of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs.

Revisions in the main body of the text:

Radiolabelling of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs

⁶⁸GaCl₃ (2.5 mL, 0.05 M HCl, 25mCi) was eluted from the ⁶⁸Ge/⁶⁸Ga generator and mixed with 50 μL of 0.25 M NaOAc containing 1 mg/mL of DOTA-FA-BBTD-4C NPs to achieve a pH of 4.0. The mixture was incubated at 100 °C for 10 min and purified by centrifugation with ultrafiltration device.

6. In the manuscript, it is stated that “Comparative analysis of half-maximal inhibitory concentrations demonstrated that ⁶⁸Ga-folic acid and ⁶⁸Ga-labeled 2TT-oC6B-4COOH NPs gave comparable binding affinities for folic acid receptors (5.23 nM vs. 2.81 nM, respectively) (Fig. 4d).”

Details of the inhibitory assay described in Fig. 4d are missing and should be included. Furthermore, clarification is needed: does the reported binding affinity of 2.81 nM correspond to the concentration of ⁶⁸Ga-labeled NPs? How many folic acid molecules are present per nanoparticle?

Response: We sincerely appreciate the reviewers' constructive comments and sorry for the confusion. The details of the inhibitory assay were added in the revised manuscript. The reported binding affinity of 2.81 nM was corresponded to the concentration of AIE molecular (DOTA-FA-BBTD-4C).

However, it is hard to determine how many folic acid molecules are present per nanoparticle. As we know, DOTA-FA-BBTD-4C NPs was prepared by adding 1 mL of a THF solution containing 5 mg of DSPE-PEG₂₀₀₀-FA, 5 mg of DSPE-PEG₂₀₀₀-DOTA, and 1.3 mg of BBTD-4C molecules to a 50 mL centrifuge tube containing 10 mL of ultra-pure water. Therefore, the mass ratio of DSPE-PEG₂₀₀₀-FA to DSPE-PEG₂₀₀₀-DOTA to BBTD-4C is 3.85:3.85:1. The folate molecules involved in this study are not prodrugs but rather constitute a drug system constructed through a loading process. The system features an external hydrophilic layer and an internal hydrophobic core: the outer layer is composed of DSPE-PEG-FA and DSPE-PEG-DOTA, while the core encapsulates an AIEgen luminescent molecule. A review of existing literature on AIEgen and DSPE-PEG composite systems reveals that no studies have reported methods to quantify the surface content of DSPE-PEG-FA or other DSPE-PEG components (eg., *Adv. Funct. Mater.* **2023**, 33, 2300342, *Angew. Chem. Int. Ed.* **2025**, 64, e202417865, *Adv. Mater.* **2024**, 36, 2410142, *Adv. Mater.* **2025**, 37, 2413164, *Sci. Adv.* **2025**, 11, 10.1126/sciadv.adp7126 and so on). Although this question is not the primary focus of our research, we also believe that investigating surface density of DSPE-PEG-FA of nanoparticles is a very important and promising research direction. Accordingly, we further examined relevant literature and found that researchers such as Wolfgang J. Parak and Warren Chan are at the forefront of studies in this field.

Guided by your valuable suggestions, we endeavored to calculate the number of folic acid molecules per AIEgen nanoparticle, as detailed below:

$M_{\text{BBTD-4C}}=1300 \text{ g/mol}$, $m_{\text{BBTD-4C}}=1.3 \text{ mg}$. $M_{\text{DSPE-PEG2000-FA}}=3230 \text{ g/mol}$, $m_{\text{DSPE-PEG2000-FA}}=5 \text{ mg}$. $M_{\text{DSPE-PEG2000-DOTA}}=3176 \text{ g/mol}$, $m_{\text{DSPE-PEG2000-DOTA}}=5 \text{ mg}$.

$n_{\text{BBTD-4C}}=m_{\text{BBTD-4C}}/M_{\text{BBTD-4C}}=1 \times 10^{-6} \text{ mol}$, $n_{\text{DSPE-PEG2000-FA}}=m_{\text{DSPE-PEG2000-FA}}/M_{\text{DSPE-PEG2000-FA}}=1.5 \times 10^{-6} \text{ mol}$, $n_{\text{DSPE-PEG2000-DOTA}}=m_{\text{DSPE-PEG2000-DOTA}}/M_{\text{DSPE-PEG2000-DOTA}}=1.5 \times 10^{-6} \text{ mol}$

$n_{\text{BBTD-4C}}:n_{\text{DSPE-PEG2000-FA}}:n_{\text{DSPE-PEG2000-DOTA}}=N_{\text{A}} \cdot A_{\text{BBTD-4C}}:N_{\text{A}} \cdot A_{\text{DSPE-PEG2000-FA}}:N_{\text{A}} \cdot A_{\text{DSPE-PEG2000-DOTA}}=1:1.5:1.5$

Therefore, we can calculate that there are approximately 1.5 folic acid molecules and 1.5 DOTA molecules per BBTD-4C molecule.

Revisions in the main body of the text:

In vitro competitive binding assays

The cell uptake assays were performed by incubating cells with [⁶⁸Ga]DOTA-FA-BBTD-4C NPs, [⁶⁸Ga]DOTA-FA NPs (37 kBq/well) or [⁶⁸Ga]DOTA-FA-BBTD-4C NPs + unlabeled precursors (DOTA-FA-BBTD-4C NPs) for 60 min. For the competitive cell-binding assay, a gradient concentration (5.6×10^{-5} to 5.6×10^{-15} M) of DOTA-FA-BBTD-4C NPs or DOTA-FA NPs was added to the U87 cells and incubated with [⁶⁸Ga]DOTA-FA NPs for 60 min. Cells were washed twice with 500 μ L of ice-cold PBS containing 0.2% BSA and solubilized with 1 M sodium hydroxide solution. Cell lysates were collected for radioactivity detection by a γ -counter (2480 Wizard2, PerkinElmer, USA). The 50% inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism.

7. In the preparation of ⁶⁸Ga-2TT-oC6B-4COOH NPs, the manuscript state “which was then directly mixed with 2TT-oC6B-4COOH NPs in 0.1 M NaHCO₃ (at specified pH) for radiolabeling”

How are the nanoparticles purified from the free Ga-68 after radiolabeling? Was radiochemical purity assessed, and by what method? These are essential information for PET/CT imaging.

Response: We thank the reviewer for this constructive suggestion and sorry for our mistakes. We have rewritten the experimental methodology of radiolabelling to ensure the reproducibility of this research work.

The nanoparticles were purified by centrifugation with ultrafiltration device to remove the free ⁶⁸Ga after radiolabeling.

The radiochemical purity of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs was assessed by thin layer chromatography (TLC).

Revisions in the main body of the text:

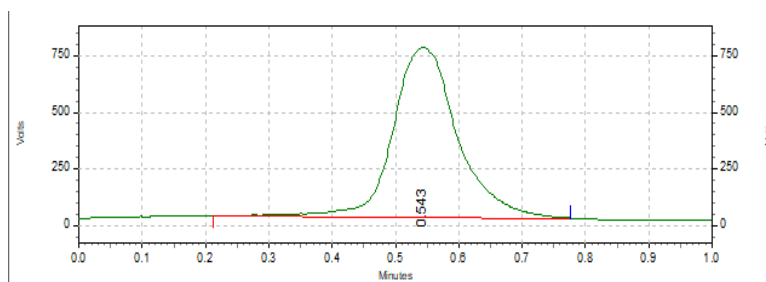
Radiolabelling of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs

⁶⁸GaCl₃ (2.5 mL, 0.05 M HCl, 25mCi) was eluted from the ⁶⁸Ge/⁶⁸Ga generator and mixed with 50 μ L of 0.25 M NaOAc containing 1 mg/mL of DOTA-FA-BBTD-4C NPs to achieve a pH of 4.0. The mixture was incubated at 100 °C for 10 min and purified by centrifugation with ultrafiltration device.

Revisions in the main body of the text:

Subsequently, a novel radiolabeled probe for PET/CT imaging was successfully obtained by reacting DOTA-FA-BBTD-4C NPs with ⁶⁸GaCl₃ (eluted from a ⁶⁸Ge/⁶⁸Ga generator), and its radiochemical purity (More than 99%) was confirmed by thin-layer chromatography (TLC) analysis (Supplementary Fig. 30, Supplementary Fig. 31).

Revisions in the supporting information:



Supplementary Fig. 31 Thin-Layer Chromatography (TLC) analysis of [^{68}Ga]DOTA-FA-BBTD-4C NPs.

8. The control experiments of 2TT-oC6B-4COOH NPs without folic acid on the nanoparticle are missing. The control of 2TT-oC6B-4COOH NPs without folic acid should be included in the study of targeting effect of ^{68}Ga -2TT-oC6B-4COOH NPs such as Figure 4, Figure 5, Figure 6, Figure 7, and Figure 8.

Response: We thank the reviewer for this constructive suggestion. We have added control experiments without folic acid on the [^{68}Ga]DOTA-BBTD-4C NPs in Figs. 4-8. This additional experiment significantly enhances the scientific rigor and data integrity of the paper.

In Fig. 4, we included two control groups (**VI** and **VII**) of non-targeted NPs (i.e., identical formulation but without folate molecules).

Revisions in the main body of the text:

The Calcein-AM/PI staining results also revealed that at a concentration of $80\ \mu\text{M}$, only a small number of apoptotic cells were observed in the group treated with DOTA-FA-BBTD-4C NPs without laser irradiation (Fig. 4h). In contrast, the group treated with DOTA-FA-BBTD-4C NPs under irradiation ($660\ \text{nm}$, $0.8\ \text{W}/\text{cm}^2$) exhibited significant killing effects against GBM cells, with a much higher level of apoptosis than that in the non-targeted DOTA-BBTD-4C NPs group. Furthermore, no signs of apoptosis were observed in the laser irradiation group (DOTA-FA NPs), indicating that the laser irradiation parameters were safe at this power level.

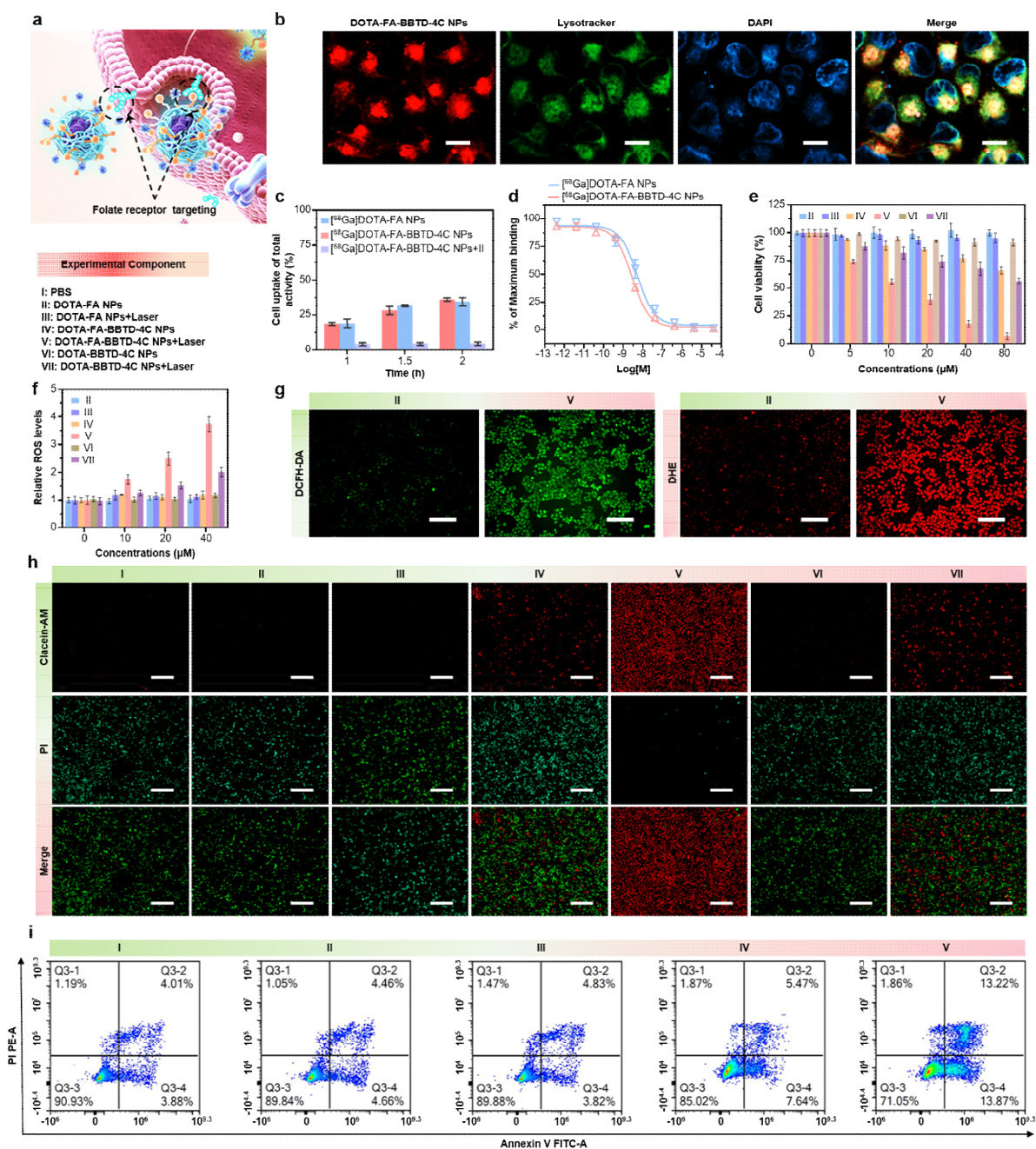


Fig. 4 Comprehensive evaluation of cellular uptake, cytotoxicity and phototherapeutic efficacy in vitro.

Revisions in the main body of the text: In contrast, nanoparticles with the same formulation but without folate modification failed to demonstrate significant tumor-targeting capability (Fig. 5d).

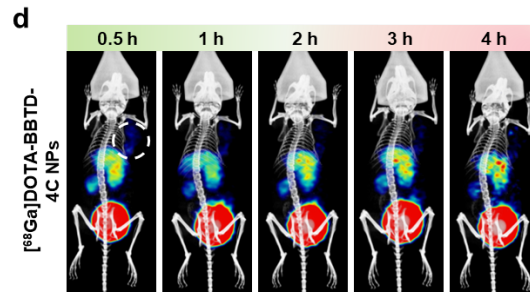


Fig. 5 d Representative PET/CT images of [^{68}Ga]DOTA-BBTD-4C NPs in tumor-bearing mice.

Revisions in the main body of the text: To investigate tumor recurrence dynamics following surgical intervention, we established a subcutaneous resection model in immunocompetent mice (n=6 each) using the following experimental protocol: Group I (PBS control), Group II ([^{68}Ga]DOTA-FA NPs), Group III ([^{68}Ga]DOTA-FA NPs + Laser), Group IV ([^{68}Ga]DOTA-FA-BBTD-4C NPs), Group V ([^{68}Ga]DOTA-FA-BBTD-4C NPs + Laser), Group VI ([^{68}Ga]DOTA-BBTD-4C NPs) and Group VII ([^{68}Ga]DOTA-BBTD-4C NPs + Laser) (Fig. 6a). This experimental design enabled comparison between conventional surgical resection and fluorescence-guided tumor removal in terms of postoperative recurrence rates. The experimental results demonstrated that the control groups exhibited similar tumor recurrence rates and growth patterns (Groups I and II). In the fluorescence-guided folic acid-modified targeting group (Group IV), tumor recurrence was partially suppressed but moderate recurrence was still observed, while the non-folic acid-modified group (Group VI) showed significant recurrence. These findings confirm that relying solely on fluorescence-guided technology cannot completely eliminate postoperative recurrence risk (Fig. 6b, c).

To address this limitation, we developed a combined therapeutic strategy involving immediate laser phototherapy after precise fluorescence-guided tumor resection. As shown in Fig. 6d, Group III, which received laser treatment alone, still exhibited significant tumor recurrence, indicating that laser ablation alone has limited efficacy. The non-folic acid-modified Group VII showed only slight tumor suppression. In contrast, Group V, treated with [^{68}Ga]DOTA-FA-BBTD-4C NPs combined with laser irradiation, demonstrated not only a significant reduction in tumor volume but also complete inhibition of tumor recurrence. This synergistic strategy of "precision surgical resection combined with dual photodynamic/photothermal targeted therapy" enables more efficient elimination of residual tumor cells and significantly improves tumor eradication efficacy.

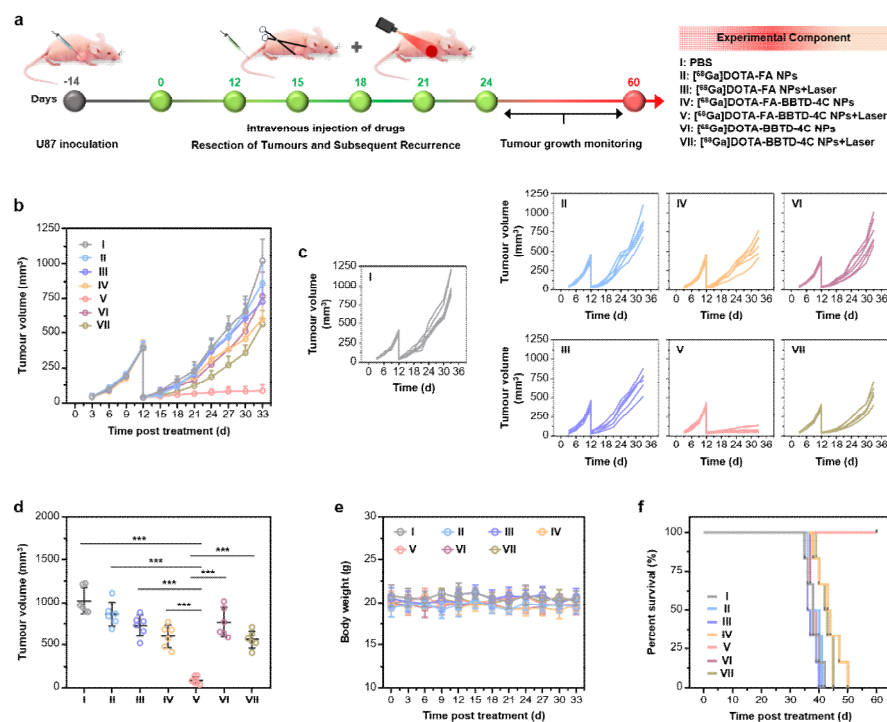


Fig. 6 Longitudinal monitoring of postoperative tumor recurrence.

Group I (PBS control), Group II ([^{68}Ga]DOTA-FA NPs), Group III ([^{68}Ga]DOTA-FA NPs + Laser), Group IV ([^{68}Ga]DOTA-FA-BBTD-4C NPs), Group V ([^{68}Ga]DOTA-FA-BBTD-4C NPs + Laser), Group VI ([^{68}Ga]DOTA-BBTD-4C NPs) and Group VII ([^{68}Ga]DOTA-BBTD-4C NPs + Laser)

The control groups (Groups I-IV and VI) showed continuous tumor growth without significant therapeutic response. In contrast, the non-targeted Group VII with combined laser irradiation exhibited slight tumor suppression. Group V demonstrated significant tumor volume reduction (Fig. 7d, e), confirming the synergistic effect of combined photodynamic and photothermal targeted therapy.

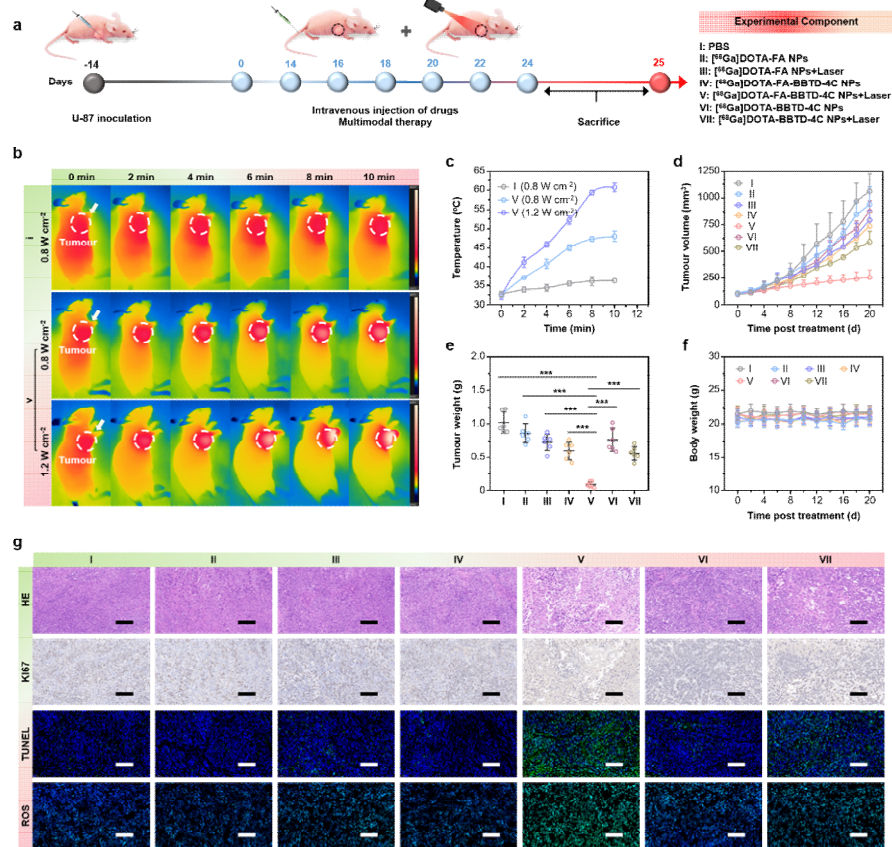


Fig. 7 Mechanistic Study on the Synergistic Effects of Targeted Photodynamic/Photothermal Combined Therapy (PDT/PTT).

The biosafety of NPs is a critical factor for clinical applications. Therefore, we conducted a comprehensive assessment of systemic toxicity in U87 xenograft mice following $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C}$ NP administration combined with laser therapy using multiple evaluation approaches. Drug administration was performed via tail vein injection, followed by precisely controlled laser irradiation of the axillary tumor target area using an adjustable-parameter precision laser system. Upon completion of the treatment protocol, subjects were euthanized and major organs (including liver and kidneys) were harvested from U87 xenograft mice for comprehensive serum biochemistry and complete blood count analyses.

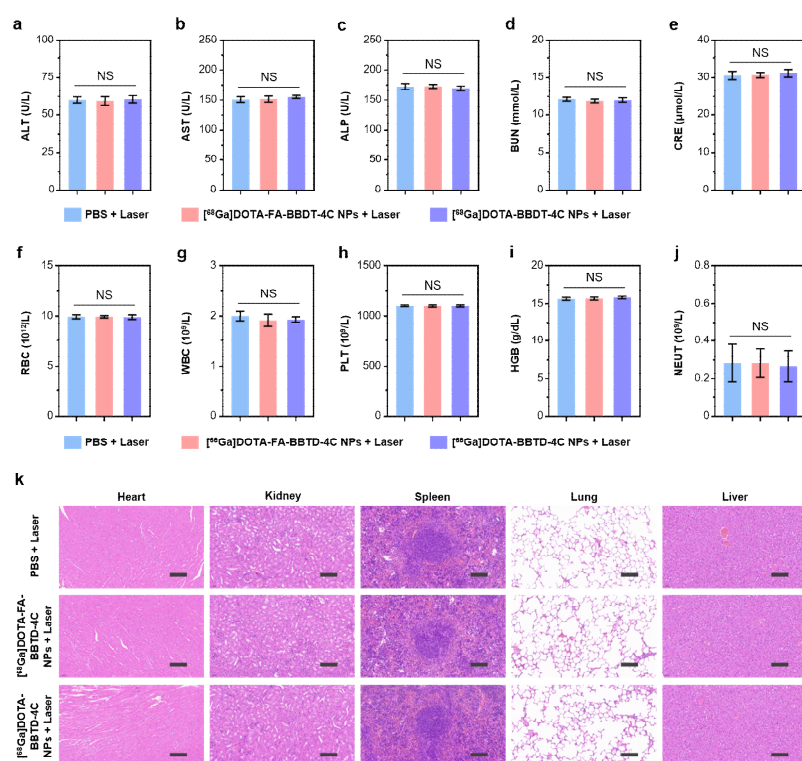


Fig. 8 Comprehensive biosafety profiling of ^{68}Ga -BBTD-4C NPs: toxicology assessment.

9. In the study of in vivo PET/CT imaging for diagnosis & NIR-II FLI navigation, it is stated that “The primary excretion was through the kidneys and bladder, leading to low background activity and a favorable tumor-to-background ratio.”

It is observed that the nanoparticles are majorly clearance by kidney and bladder. Given the fact that the hydrodynamic size of nanoparticles are relatively big (~100 nm), the mechanism behind this unusual clearance profile should be explored and discussed.

Response: We are grateful for the reviewers' valuable comments. The folate receptor (FR) is highly overexpressed in various human cancers, but exhibits low expression in normal tissues. However, the kidney is a major exception. Renal proximal tubule cells highly express FR- α , which facilitates the reabsorption of folate from urine. When folate-based imaging agents (such as radionuclide-labeled compounds) are administered (*Nano Today*, **2021**, 37, 101173), the tracer accumulates in the kidneys (*Mol. Pharmaceutics* **2024**, 21, 5681-5689). Additionally, the kidneys act as a major site for drug uptake and retention, leading to subsequent drug accumulation. In response, we have performed a comparative analysis between the biodistribution profile of [^{68}Ga]DOTA-FA-BBTD-4C NPs and that of other similarly-sized nanoparticles to provide clearer interpretation of the in vivo behavior.

As reported in Literature *Mol. Pharmaceutics* **2024**, 21, 5681-5689, the biodistribution results are consistent with our experimental findings.

[editorial note: third party material redacted]

Furthermore, we compared the results of this study with those of other reported fluorescent probes, all of which have a size distribution of approximately 100 nm, and the results demonstrated excellent agreement.

[editorial note: third party material redacted]

References (*Adv. Funct. Mater.* **2022**, *32*, 2206346, *J. Control. Release.* **2021**, *330*, 1-14).

In summary, we propose that the clearance mechanism via the bladder and kidneys is as follows: Renal proximal tubule cells highly express FR- α , which mediates the reabsorption of folate from urine. Due to the folate-modified surface layer of the material used in this study, the tracer specifically binds to FR- α , resulting in significant accumulation in the kidneys. Furthermore, as the primary filtration and excretory organs, the kidneys allow partially unreabsorbed tracer to enter the urine via glomerular filtration and ultimately reach the bladder. Therefore, the drug is primarily cleared through the renal and bladder pathways.

Revisions in the main body of the text: Renal proximal tubule cells highly express FR- α , which mediates the reabsorption of folate from urine. Due to the folate-modified surface layer of the material used in this study, the tracer specifically binds to FR- α , resulting in significant accumulation in the kidneys. Furthermore, as the primary filtration and excretory organs, the kidneys allow partially unreabsorbed tracer to enter the urine via glomerular filtration and ultimately reach the bladder. Therefore, the drug is primarily cleared through the renal and bladder pathways. This efficient urinary clearance mechanism helps reduce systemic background activity, thereby achieving superior tumor-to-background contrast. These biological distribution characteristics are consistent with those observed by other ^{68}Ga -labeled PET imaging agents.^{63,73}

In vivo fluorescence imaging at 12 h post-injection of [^{68}Ga]DOTA-FA-BBTD-4C NPs (Fig. 5i) demonstrated predominant signal accumulation in tumor tissue. Given the probe's particle size distribution (~100 nm), which is comparable to similar NPs, fluorescent signals were observed in reticuloendothelial system (RES) organs (liver and kidneys) and lung tissue. The organ accumulation characteristics and clearance pathways were consistent with those of other similarly-sized nanoparticles.^{74, 75}

References:

73. Xue, X. et al. PET/NIR Fluorescence Bimodal Imaging for Targeted Tumor Detection. *Mol. Pharmaceutics* 2023, *20*, 12, 6262–6271

74. Yang, X. et. al. Biomimetic Aggregation-Induced Emission Nanodots with Hitchhiking Function for T Cell-Mediated Cancer Targeting and NIR-II Fluorescence-Guided Mild-Temperature Photothermal Therapy. *Adv. Funct. Mater.* **32**, 2206346 (2022).

75. Park, Y. I. et. al. pH-sensitive multi-drug liposomes targeting folate receptor β for efficient treatment of non-small cell lung cancer. *J. Control. Release.* **330**, 1-14 (2021).

10. More experimental details need to be provided for Figure 5. Was the data shown in Fig. 5c obtained via in vivo PET imaging or from ex vivo gamma counting following sacrifice? This should be clearly stated.

Response: We sincerely appreciate the reviewers' constructive comments and sorry for the confusion. We have carefully provided more experimental details for Fig. 5 in the revised manuscript. In fact, the data shown in Fig. 5c was obtained from ex vivo gamma counting following sacrifice.

Revisions in the main body of the text:

Biodistribution

U87 xenograft mice were intravenously injected with [⁶⁸Ga]DOTA-FA-BBTD-4C NPs (200 μCi in 150 μL PBS) and sacrificed at 0.5, 1, 2, 4 h post-injection, of which the main organs and tumors were collected, weighed, and measured for radioactivity using a γ-counter.

11. There is a discrepancy between Fig. 5b and Fig. 5d. Fig. 5b shows peak tumor uptake at 0.5 h with a subsequent decline, while Fig. 5d shows an increase in uptake up to 24 h. The authors should explain this inconsistency.

Response: We thank the reviewer for this constructive suggestion. The PET imaging results in Fig. 5b demonstrate that the ⁶⁸Ga-labeled nanoparticles reached peak tumor uptake within 0.5 h post-injection. This observation clearly indicates the nanoparticles' rapid tumor accumulation through folate receptor-mediated active targeting. PET/CT imaging technology exhibits three key advantages: (1) completely non-invasive detection, (2) exceptional tissue penetration capability, and (3) sub-centimeter high spatial resolution, making it particularly valuable for early diagnosis of deep-seated tumors. Notably, the PET signal intensity decreased over time, primarily due to three factors: First, the inherent physical properties of ⁶⁸Ga radionuclide (half-life of only 68 min) caused signal decay. Second, efficient renal clearance of free ⁶⁸Ga took place. These factors collectively contributed to the temporal changes in PET signals at tumor sites. Furthermore, through systematic comparison with published ⁶⁸Ga-PET studies in this field, we found that the dynamic changes in tumor signals observed by PET imaging in our study are highly consistent with the pharmacokinetic characteristics of ⁶⁸Ga-labeled nanoprobe reported in the literature (e.g., *J. Nucl. Med.* **2022**, 63, 862-868; *J. Nucl. Med.* **2023**, 64, 1449-1455; *J. Nucl. Med.* **2024**, 65, 394-401).

Fig. 5d demonstrates a dynamic fluorescence signal trend characterized by initial enhancement followed by gradual attenuation over time. This phenomenon primarily stems from the inherent physical limitations of fluorescence imaging technology: firstly, the restricted tissue penetration depth compromises imaging efficacy in deep tissues; secondly, during deep-tissue imaging, significantly intensified photon scattering effects lead to diminished spatial resolution and signal-to-noise ratio, consequently impairing the sensitivity and accuracy of real-time dynamic monitoring at tumor sites. Notably, through systematic comparative analysis with published ⁶⁸Ga-PET/FLI imaging studies in this field, the tumor fluorescence signal dynamics observed in our experiments show remarkable consistency with the fluorescent behavior of nanoprobe reported in the literature (e.g., *Mol. Pharmaceutics.* **2023**, 20, 6262-6271).

[editorial note: third party material redacted]

To enable readers to more clearly observe the dynamic changes in fluorescence intensity at the tumor site, we have supplemented the following content in both the main text and supplementary information.

Revisions in the main body of the text:

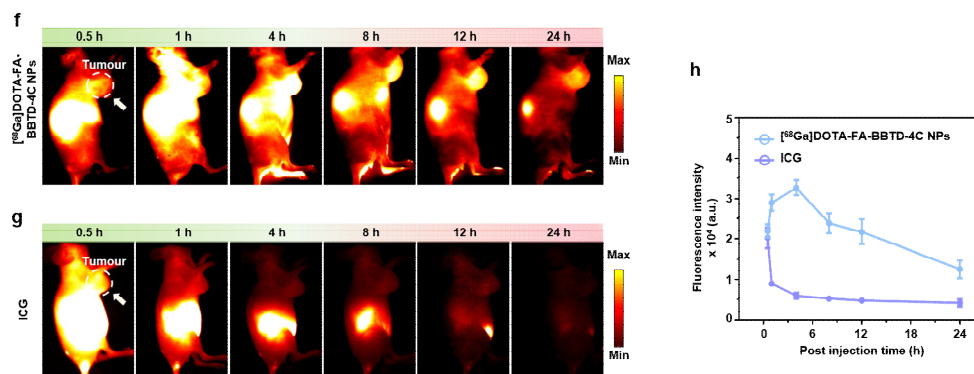
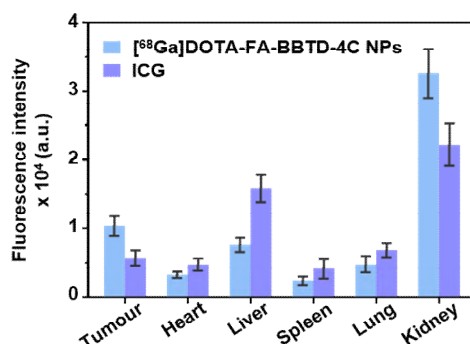


Fig. 5 h Quantitative analysis of time-dependent fluorescence intensity of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ and ICG at tumor sites.



Supplementary Fig. 35 Quantitative analysis of fluorescence intensity in various organs following tumor resection for $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ vs. ICG.

12. The statement “with minimal fluorescence observed in reticuloendothelial system (RES) organs, including the liver, spleen, and lungs.” Should be revised.

The lungs are generally not considered part of the reticuloendothelial system.

Response: We thank the reviewer for this constructive suggestion. We have already revised this sentence in the article. This has significantly enhanced the quality of the article.

Revisions in the main body of the text: fluorescent signals were observed in reticuloendothelial system (RES) organs (liver and kidneys) and lung tissue.

13. A comparative discussion of the biodistribution of ^{68}Ga -2TT-oC6B-4COOH NPs versus other nanoparticles with similar size reported in the literature should be included.

Response: We are grateful for the reviewers' valuable comments. The folate receptor (FR) is highly overexpressed in various human cancers, but exhibits low expression in normal tissues. However, the kidney is a major exception. Renal proximal tubule cells highly express FR- α , which facilitates the reabsorption of folate from urine. When folate-based imaging agents (such as radionuclide-labeled compounds) are administered (*Nano Today*, **2021**, 37, 101173), the tracer accumulates in the kidneys (*Mol. Pharmaceutics* **2024**, 21, 5681-5689). Additionally, the kidneys act as a major site for drug uptake and retention, leading to subsequent drug accumulation. In response, we have performed a comparative analysis between the biodistribution profile of [^{68}Ga]DOTA-FA-BBTD-4C NPs and that of other similarly-sized nanoparticles to provide clearer interpretation of the in vivo behavior.

As reported in Literature *Mol. Pharmaceutics* **2024**, 21, 5681-5689, the biodistribution results are consistent with our experimental findings.

[editorial note: third party material redacted]

References (*Mol. Pharmaceutics* **2024**, 21, 5681-5689)

Furthermore, we compared the results of this study with those of other reported fluorescent probes, all of which have a size distribution of approximately 100 nm, and the results demonstrated excellent agreement.

[editorial note: third party material redacted]

References (*Adv. Funct. Mater.* **2022**, 32, 2206346, *J. Control. Release.* **2021**, 330, 1-14).

In summary, we propose that the clearance mechanism via the bladder and kidneys is as follows: Renal proximal tubule cells highly express FR- α , which mediates the reabsorption of folate from urine. Due to the folate-modified surface layer of the material used in this study, the tracer specifically binds to FR- α , resulting in significant accumulation in the kidneys. Furthermore, as the primary filtration and excretory organs, the kidneys allow partially unreabsorbed tracer to enter the urine via glomerular filtration and ultimately reach the bladder. Therefore, the drug is primarily cleared through the renal and bladder pathways.

Revisions in the main body of the text: Renal proximal tubule cells highly express FR- α , which mediates the reabsorption of folate from urine. Due to the folate-modified surface layer of the material used in this study, the tracer specifically binds to FR- α , resulting in significant accumulation in the kidneys. Furthermore, as the primary filtration and excretory organs, the kidneys allow partially unreabsorbed tracer to enter the urine via glomerular filtration and ultimately reach the bladder. Therefore, the drug is primarily cleared through the renal and bladder pathways. This efficient urinary clearance mechanism helps reduce systemic

background activity, thereby achieving superior tumor-to-background contrast. These biological distribution characteristics are consistent with those observed by other ^{68}Ga -labeled PET imaging agents.^{63,73}

In vitro fluorescence imaging at 12 h post-injection of [^{68}Ga]DOTA-FA-BBTD-4C NPs (Fig. 5i) demonstrated predominant signal accumulation in tumor tissue. Given the probe's particle size distribution (~100 nm), which is comparable to similar NPs, fluorescent signals were observed in reticuloendothelial system (RES) organs (liver and kidneys) and lung tissue. The organ accumulation characteristics and clearance pathways were consistent with those of other similarly-sized nanoparticles.^{74, 75}

References:

73. Xue, X. et al. PET/NIR Fluorescence Bimodal Imaging for Targeted Tumor Detection. *Mol. Pharmaceutics* 2023, 20, 12, 6262–6271

74. Yang, X. et. al. Biomimetic Aggregation-Induced Emission Nanodots with Hitchhiking Function for T Cell-Mediated Cancer Targeting and NIR-II Fluorescence-Guided Mild-Temperature Photothermal Therapy. *Adv. Funct. Mater.* **32**, 2206346 (2022).

75. Park, Y. I. et. al. pH-sensitive multi-drug liposomes targeting folate receptor β for efficient treatment of non-small cell lung cancer. *J. Control. Release.* **330**, 1-14 (2021).

14. In the section “Postoperative Tumor Recurrence Detection” Since it is designed to monitor the tumor recurrence. It is not clear why laser is used in groups III and V. Where is laser applied to? Tumor? However, the tumor is supposed to have been excised.

Response: We sincerely appreciate the reviewers' valuable comments. Through careful examination, we identified inconsistencies between the original subsection content and its title. To better align the content with the heading and improve readability, we have revised "**Postoperative Tumor Recurrence Detection**" to "**Surgical Resection & Adjuvant Therapy**," thereby enhancing the structural rigor and logical coherence of this section. As shown in Figs. 6b-d, the conventional treatment group I (PBS control) and group II ([^{68}Ga]DOTA-FA NPs) both exhibited significant tumor recurrence characteristics. While the single fluorescence-guided surgery Group IV ([^{68}Ga]DOTA-FA-BBTD-4C NPs) partially suppressed tumor recurrence, moderate recurrence was still observed, indicating that relying solely on fluorescence-guided technology cannot completely eliminate the risk of postoperative recurrence. To address this limitation, we introduced a combined therapeutic strategy involving immediate laser phototherapy on the surgical margin area following precise fluorescence-guided tumor resection. Notably, control group III ([^{68}Ga]DOTA-FA NPs + Laser) still exhibited significant tumor recurrence, demonstrating the ineffectiveness of the current laser power setting for tumor ablation. In contrast, experimental data from group V ([^{68}Ga]DOTA-FA-BBTD-4C NPs + Laser) confirmed that this synergistic "surgical resection combined with photodynamic/photothermal dual therapy" approach more effectively eliminated residual tumor cells, significantly enhancing tumor eradication efficacy.

To ensure the author's clear comprehension of this chapter, we have incorporated the **following additions into the main text:**

The experimental results demonstrated that the control groups exhibited similar tumor recurrence rates and growth patterns (Groups I and II). In the fluorescence-guided folic acid-modified targeting group (Group IV), tumor recurrence was partially suppressed but moderate recurrence was still observed, while the non-folic acid-modified group (Group VI) showed significant recurrence. These findings confirm that relying solely on fluorescence-guided technology cannot completely eliminate postoperative recurrence risk (Fig. 6b, c).

To address this limitation, we developed a combined therapeutic strategy involving immediate laser phototherapy after precise fluorescence-guided tumor resection. As shown in Fig. 6d, Group III, which received laser treatment alone, still exhibited significant tumor recurrence, indicating that laser ablation alone has limited efficacy. The non-folic acid-modified Group VII showed only slight tumor suppression. In contrast, Group V, treated with [^{68}Ga]DOTA-FA-BBTD-4C NPs combined with laser irradiation, demonstrated not only a significant reduction in tumor volume but also complete inhibition of tumor recurrence. This synergistic strategy of “precision surgical resection combined with dual photodynamic/photothermal targeted therapy” enables more efficient elimination of residual tumor cells and significantly improves tumor eradication efficacy.

Furthermore, we have revised the experimental flowchart (Fig. 6a) in the main text to ensure better comprehension and a smoother reading experience.

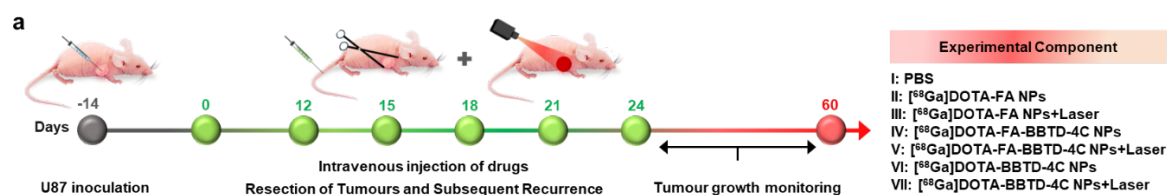


Fig. 6a NIR-II FLI-guided tumor resection in the experimental and control groups.

15. The manuscript states “Group IV exhibited moderate tumor growth inhibition.” (Figure 7d and 7e).

Since the ^{68}Ga -2TT-oC6B-4COOH NPs are biocompatible, why do they show inhibition of tumor. There is no therapeutic component in the nanoparticles. In addition, histological study in Figure 7g does not show any differences for group IV compared to group I-III.

Response: We would like to express our sincere gratitude for your rigorous review and valuable suggestions regarding the linguistic expressions in our manuscript. Through re-analysis of the tumor inhibition efficacy data presented in Figs. 7d and 7e for groups I, II, and III, along with systematic comparison with group IV, no significant differences were observed. By carefully examining the histopathological sections shown in Figure 7g, we have come to recognize that the original description of "moderate inhibitory effect" did not accurately reflect the essential characteristics of the experimental data. We apologize for any linguistic inaccuracies in our expression. Based on this understanding, we have implemented comprehensive and meticulous revisions in the relevant sections of the manuscript. These corrections have not only enabled

more precise presentation of our research findings but have also significantly enhanced the academic rigor and reliability of the conclusions throughout the paper.

Revisions in the main body of the text: The control groups (Groups I-IV and VI) showed continuous tumor growth without significant therapeutic response. In contrast, the non-targeted Group VII with combined laser irradiation exhibited slight tumor suppression. The tumor volume in the targeted Group V showed a significant reduction (Fig. 7d, e), confirming the synergistic effect of combined targeted photodynamic and photothermal therapy.

16. In Figure 8, the biosafety experiment compares PBS+laser and NPs+laser groups. However, the laser application method is unclear. If healthy mice (no tumors?) were injected, where was the laser applied? Please provide details on the laser treatment procedure.

Response: We sincerely appreciate the reviewers' constructive comments and sorry for the confusion. We recognize that the erroneous description of using healthy mice in our original manuscript has caused significant confusion among readers. We have modified the “healthy mice” to “U87 xenograft mice” to ensure the authenticity and completeness of the data. In addition, we have refined the detailed laser treatment protocol to ensure the completeness and reproducibility of the experiments.

Revisions in the main body of the text: The biosafety of NPs is a critical factor for clinical applications. Therefore, we conducted a comprehensive assessment of systemic toxicity in U87 xenograft mice following [⁶⁸Ga]DOTA-FA-BBTD-4C NPs administration combined with laser therapy using multiple evaluation approaches. Drug administration was performed via tail vein injection, followed by precisely controlled laser irradiation of the axillary tumor target area using an adjustable-parameter precision laser system. Upon completion of the treatment protocol, subjects were euthanized and major organs were harvested from U87 xenograft mice for comprehensive serum biochemistry and complete blood count analyses.

In vivo toxicology evaluation of IAS system

A suspension of U87 cells (5×10^6 cells) in 100 μ L Matrigel was subcutaneously inoculated into the right flank of 6-8-week-old BALB/c nude mice (16-18 g). When the average tumor volume reached 100 mm³, the tumor-bearing mice were randomly divided into three groups (N=6 per group) for intravenous treatment: PBS + Laser, [⁶⁸Ga]DOTA-FA-BBTD-4C NPs + Laser, and [⁶⁸Ga]DOTA-BBTD-4C NPs + Laser. At the end of the treatment period, major organs including liver and kidney of U87 xenograft mice were excised for histological analysis by Hematoxylin and Eosin (H&E) staining, which was conducted by Servicebio Technology Co., Ltd. (Wuhan, China). Meanwhile, the mice were sacrificed for blood chemistry and blood routine analyses performed by Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

REVISION: “Integrated Theranostic Nanoplatfrom Empowers Precision Cancer Care via Radionuclide-Labeled NIR-II Aggregation-Induced Emission Luminogens”

List of changes in the revised manuscript and supporting information:

- (1) We have carefully provided key information of the work references in the revised manuscript.
- (2) We have provided some personal opinions in the conclusion of revised manuscript.
- (3) We have checked all Figures and supplement more details of the corresponding concepts and strategies in the revised manuscript.
- (4) We have carefully revised English grammar, spelling, and sentence structure in the revised manuscript.
- (5) All authors' details and affiliations have been verified.
- (6) All the revisions in the revised manuscript were highlighted in red.

Questions and point-by-point responses are as follows:

Reviewer #3 (Remarks to the Author):

3. Comments: From the new zoomed-out TEM image (Figure 2d), a mixture of large and small particles can be observed, which is similar to the previous TEM image in the original Supplementary Figure 23. In the nanoparticle size distribution histogram of DOTA-FA-BBTD-4C NPs (new Supplementary Figure 23), two distinct peaks are evident. Therefore, the size distribution should not be considered “uniform,” as claimed by the authors. This issue is important because the coexistence of large and small nanoparticles can complicate biodistribution and clearance.

Response: We sincerely appreciate your valuable feedback. Regarding your observation that the zoomed-out TEM image (Fig. 2d) shows a mixture of large and small particles, which is similar to the previous TEM image in the original Supplementary Fig. 23, and that the nanoparticle size distribution histogram of DOTA-FA-BBTD-4C NPs (new Supplementary Fig. 23) exhibits two distinct peaks, you raised the concern that this coexistence of different-sized nanoparticles might complicate the biodistribution and clearance. We would like to provide the following explanation.

PEG-coated AIE nanoparticles represent a strategy distinct from the direct self-assembly of AIE molecules. They are typically prepared via nanoprecipitation, a process that co-precipitates hydrophobic AIE molecules and amphiphilic PEG polymers from a good solvent into a poor solvent (e.g., water). In this process, the hydrophobic AIE molecules aggregate to form a core, minimizing aqueous contact, while the hydrophilic PEG chains extend into the water, forming a hydrated shell that stabilizes the nanoparticle. The size of these nanoparticles is not solely determined by the AIE molecules themselves but is predominantly influenced by the molecular weight of PEG and the kinetics of the preparation process. Consequently, an inherent and relatively broad size distribution is often observed. In contrast, the self-assembly of AIE molecules does not rely on auxiliary polymers like PEG. The AIE molecules themselves, under specific solvent conditions (e.g., a mixture of good and poor solvents), spontaneously and orderly arrange into structurally defined aggregates driven by intrinsic intermolecular interactions. As this process is governed by molecular structure and thermodynamic equilibrium, the resulting assemblies typically exhibit a more uniform size distribution. The observed particle size distribution arises from this main factors:

1) The commercially sourced PEG itself has an inherent chain length distribution, where longer chains may form a thicker shell that differentially restricts core growth, contributing to size variation.

2) The nanoprecipitation process is inherently heterogeneous—local THF concentration and mixing efficiency vary across regions, leading to inconsistent supersaturation levels. Near the injection point, lower supersaturation favors fewer but larger particles, while farther regions with higher supersaturation promote instantaneous nucleation of numerous small particles, systematically resulting in a polydisperse population.

3) The observation of a single large particle peak in DLS results (Fig. 2d), alongside the presence of small particles in TEM images, is not contradictory. This precisely indicates that while a population of small particles exists in the sample system, the large particles are dominant in number and overwhelmingly dominate the scattering intensity.

4) Although the nanoparticle size distribution histogram of DOTA-FA-BBTD-4C NPs (new Supplementary Fig. 23), which is based on TEM images, shows two distinct peaks, this outcome is consistent with the DLS data. Both methods clearly show the absolute dominance of the larger particles (~100 nm).

5) By integrating the findings from our previous publications and reviewing the literature on PEG-coated nanoparticles, we have observed that the presence of some smaller particles is a common feature in TEM images. References: *Angew. Chem. Int. Ed.* **2025**, 64, e202417865, *Adv. Sci.* **2025**, e07956, *Adv. Mater.* **2025**, 37, 2411133, *Adv. Mater.* **2024**, 36, 2309748, et al.

[editorial note: third party material redacted]

Figure S26. TEM image of 4TT-PBPT NPs. (*Angew. Chem. Int. Ed.* **2025**, 64, e202417865)

[editorial note: third party material redacted]

Figure 2 d) transmission electron microscopy image, e) hydrodynamic diameter distribution. (*Adv. Sci.* **2025**, e07956)

[editorial note: third party material redacted]

Figure 4 Dynamic light scattering (DLS) of G) BTS-2F Lip, H) BTS-2Cl Lip, I) TTS-2F Lip, and J) TTS-2Cl Lip in aqueous solution, inset: representative TEM image of BTS-2F Lip (G, scale bar: 300 nm), BTS-2Cl Lip (H, scale bar: 300 nm), TTS-2F Lip (I, scale bar: 200 nm) and TTS-2Cl Lip (J, scale bar: 200 nm). (*Adv. Mater.* **2025**, 37, 2411133)

[editorial note: third party material redacted]

Figure 2. A) Size distributions and TEM images of BETT-1 and BETT-2 NPs. (*Adv. Mater.* **2024**, 36, 2309748)

6) In addition, we have supplemented the TEM images and the average particle size distribution diagram. Based on the comprehensive analysis of **Fig.2d** and **Supplementary Fig. 23**, as well as references from the literature, we believe this sufficiently demonstrates the size characteristics of the material.

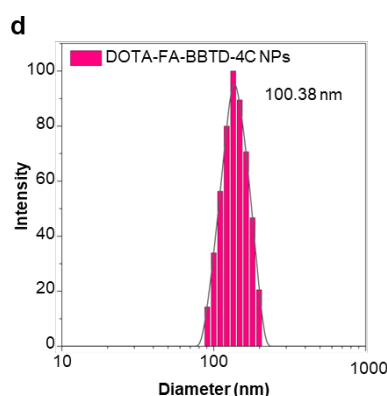
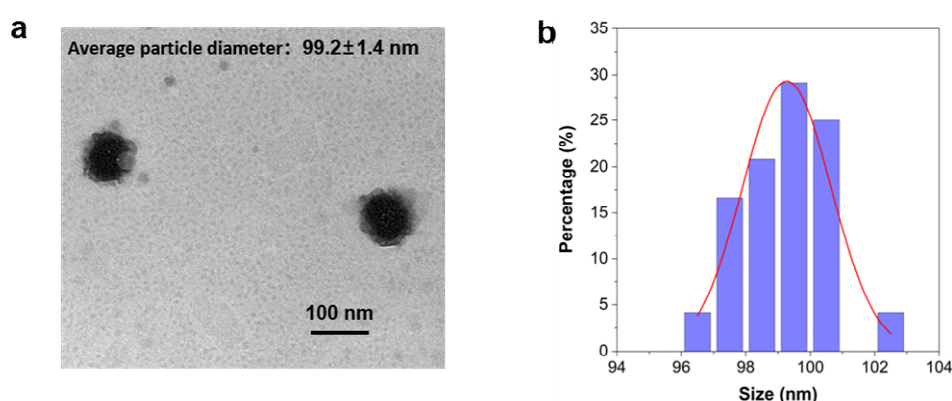


Fig. 2 d Size distribution of DOTA-FA-BBTD-4C NPs.



Supplementary Fig. 23 a The TEM of DOTA-FA-BBTD-4C NPs and **b** the particle size distribution histogram of DOTA-FA-BBTD-4C NPs.

8. Comments: In the representative PET/CT images of [^{68}Ga]DOTA-BBTD-4C NPs (without FA targeting ligands) in tumor-bearing mice (new Fig. 5 d), high uptake is observed in the kidney and bladder. However, since the particle size is assumed to be around 100 nm as [^{68}Ga]DOTA-FA-BBTD-4C NPs (with FA), This represents an abnormal clearance pattern for nanoparticles of this size. The experiment is very important as it can provide insights into the clearance mechanism of [^{68}Ga]DOTA-FA-BBTD-4C NPs. We will discuss in Question 9.

Response: We sincerely value and appreciate your insightful feedback. The specific mechanisms of biological clearance will be discussed and addressed in detail in Question 9.

9. Comments: The proposed clearance mechanism is not convincing. Further questions need to be addressed:

a). Figure 5d shows that the nanoparticles without FA also undergo significant excretion through the kidney and bladder. This suggests that FA may not be the major factor driving renal clearance.

Response: We sincerely value and appreciate your insightful feedback.

a) In the PET/CT images of Fig. 5d ([⁶⁸Ga]DOTA-BBDT-4C NPS, without folate modification) and Fig. 5b ([⁶⁸Ga]DOTA-FA-BBDT-4C NPS, with folate modification), radioactive signals can be observed in the kidney and bladder regions of the mice. However, within the same time frame, the renal region of mice injected with the folate-modified tracer (Fig. 5b) exhibited significantly higher radionuclide uptake compared to the non-folate-modified group.

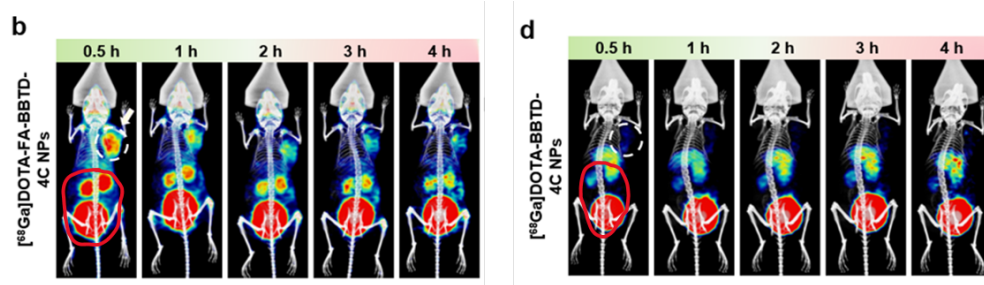


Fig. 5b and 5d (As clearly observed in the red-marked regions of the figure, the folate-modified nanoparticles exhibit significantly stronger radiolabeling signals than the unmodified nanoparticles.)

Folate receptor α (Folate Receptor α , FR α) is a high-affinity, glycosylphosphatidylinositol (GPI)-anchored receptor expressed in normal tissues such as pulmonary cells and renal tubules (References: *Nutrients*. **2019**, 11(10), 2353). It has also been reported that folate receptor is a protein present in various tissues of the body, including the kidneys, lungs, intestines, and central nervous system (References: *Int. J. Mol. Sci.* **2024**, 25, 1046). These findings collectively demonstrate that folate modification is pivotal for directing the targeted accumulation of NPs in the kidney, particularly the renal tubules. The congruent radionuclide and fluorescence signals in Fig. 5 provide direct evidence for this renal targeting. Furthermore, experimental data indicate that the nanoparticles (NPs) are primarily cleared through the renal and hepatic pathways. The mechanisms underlying biological clearance will be described in detail in a subsequent section (Question 11).

[editorial note: third party material redacted]

(References: *Nutrients*. **2019**, 11(10), 2353)

[editorial note: third party material redacted]

(References: *Int. J. Mol. Sci.* **2024**, 25, 1046)

b). Although FA could potentially bind to the kidney, the large particle size should limit nanoparticle excretion into the bladder.

Response: The biodistribution experiments were repeatedly conducted with consistent results. Despite the nanoparticle size significantly exceeding the conventional renal filtration threshold, their in vivo distribution profiles showed high reproducibility—a finding of considerable interest.

Extensive literature research revealed that the clearance mechanism aligns with the pathway established in a previous study (References: *Sci. Adv.* **2024**, *10*, eadp8719). This mechanism thereby provides a comprehensive theoretical framework for interpreting the biodistribution patterns observed in our work.

As shown in Figs. 2B and 2D of the literature, the nanoparticles have a size of 459 nm, considerably larger than the threshold for renal metabolism/clearance.

[editorial note: third party material redacted]

Fig. 2 (B) hydrodynamic diameters of cy7.5AA and cy7.5AA@cdP in PBS determined by dLS analysis. (D) Representative teM image of cy7.5AA@cdP. (References: *Sci. Adv.* **2024**, *10*, eadp8719)

Following subsequent tail vein injection, the biodistribution and clearance of the material in the kidneys and liver can be clearly observed in the images (Fig. 2I-K). Observations indicate a shift in the clearance pathway from hepatic to renal elimination. Moreover, the author also conducted a comparison with ICG, which is primarily metabolized in the liver and to a lesser extent in the kidneys. This aligns with the results presented in our Figs. 5f-j (Our work).

[editorial note: third party material redacted]

Fig. 2 (I) Fluorescence images of live mice post- intravenous administration of various supramolecular probes (100 μ M, 100 μ l) at different intervals. Red and yellow arrows indicate the kidneys and liver on the dorsal and ventral sides, respectively, while yellow circles indicate the bladder. (J and K) Post- injection fluorescence intensities over time in the kidneys (J) and liver (K) of mice administered with different supramolecular probes. cy7.5AA@cdP, having the highest K_a , displayed the brightest kidney fluorescence. conversely, weaker kidney signals and enhanced liver signals were noted for complexes with diminished binding strengths. the data are the means $\pm$ Sd. n=3 independent experiments. (References: *Sci. Adv.* **2024**, *10*, eadp8719)

Renal clearance occurs via three primary pathways: glomerular filtration, tubular secretion, and tubular reabsorption (Fig. 4A). Since the supramolecular probe Cy7.5AA@CDP exceeds the size threshold for glomerular filtration, it is hypothesized to be cleared primarily through tubular mechanisms. To investigate this, Wu et al. performed a perturbation experiment using cimetidine or probenecid to inhibit tubular secretion and reabsorption, respectively (Figs. 4B-E). The results showed that both inhibitors caused significant probe retention in the kidneys and a marked reduction in bladder signal. In contrast, control mice exhibited efficient renal clearance and a strong, rapid bladder signal. This confirms the probe's reliance on tubular pathways for clearance. Subsequent

fluorescence imaging of kidney sections revealed widespread distribution of the probe in the renal cortex and medulla (Fig. 4F). High-resolution images of the cortex specifically localized the probe to the proximal tubules, providing direct evidence that the probe is cleared in vivo predominantly via the tubular secretion pathway.

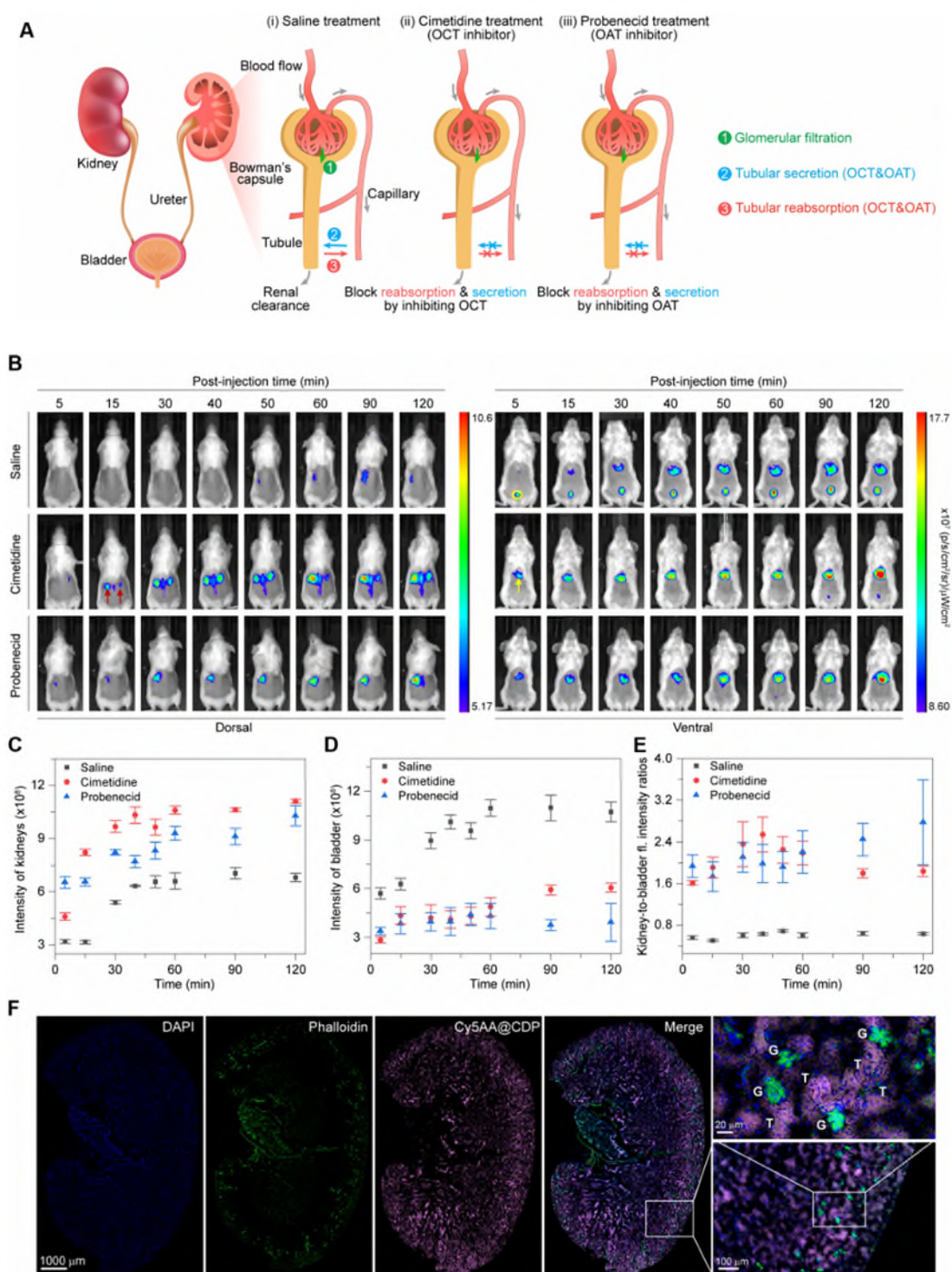


Fig. 4. Renal clearance mechanism of the supramolecular probes. (A) Schematic illustration of the renal clearance mechanisms, including glomerular filtration, tubular secretion, and tubular

reabsorption. (B) Representative fluorescence images of live mice at diverse intervals after intraperitoneal administration of either saline, organic cation transporter (Oct) cimetidine (150 mg/kg), or organic anion transporter (OAt) probenecid (150 mg/kg), followed by an intravenous injection of cy7.5AA@cdP (100 μ M, 100 μ l). Red and yellow arrows highlight the locations of the kidneys and liver, dorsally and ventrally, respectively, while the yellow circle indicates the bladder on the ventral side. (C and D) time- dependent fluorescence intensities measured from the kidneys (c) and bladder (d) after administering cy7.5AA@cdP to mice that were pretreated with either saline, cimetidine, or probenecid. (E) time- dependent ratios of kidney- to- bladder fluorescence intensity post- injection of cy7.5AA@cdP in mice pretreated with saline, cimetidine, or probenecid. (F) Fluorescence microscopy images of entire kidney sections from mice post- intravenous injection of cy5AA@cdP. Fluorescent Alexa Fluor 568 phalloidin was used to highlight the abundant filamentous actin present in both glomeruli and the brush border. dAPi, 4',6- diamidino- 2- phenylindole; G, glomerulus; t, tubules. intense fluorescence attributed to cy5AA@cdP was primarily detected in the proximal tubule. this observation suggests that the supramolecular probe primarily follows a renal secretion mechanism for in vivo renal clearance. the data are the means $\pm$ Sd. n=3 independent mice.

Therefore, it can be concluded that even relatively large nanoparticles can undergo biological clearance through the renal tubules.

c). In the paper cited by the authors (*Mol. Pharmaceutics* 2024, 21, 5681–5689), the goal was to reduce kidney uptake by increasing nanoparticle size. Their conclusion was that larger particles show reduced renal clearance. Moreover, most reagents listed in their table are small-molecule compounds. It is reasonable that such compounds exhibit high kidney clearance because of their small size.

Response: The core premise and data of this paper (*Mol. Pharmaceutics*. 2024, 21, 5681–5689) demonstrate that introducing a phenyl group (increasing molecular hydrophobicity) reduces renal clearance, as evidenced by decreased kidney uptake. While a comparison with their previous work confirms that this structural modification, resulting in a larger molecular structure, significantly reduces renal accumulation and retention, it is crucial to note fundamental differences from our PEG-coated nanoparticle system. Firstly, the discussed study involves a small molecule coordination complex, not a nanoformulation with a defined nanoparticle size, which is central to our work. Secondly, the preparation strategies are distinctly different. Therefore, while the general principle of modulating physicochemical properties to influence renal clearance is noteworthy, the findings cannot be directly equated with our research and should be treated as a reference only.

d). In other cited references (Adv. Funct. Mater. 2022, 32, 2206346; J. Control. Release 2021, 330, 1–14), some kidney uptake is observed, but the majority of nanoparticles are taken up by the liver and spleen, with no obvious clearance into the bladder. This biodistribution pattern is quite different from this work, where dominant uptake is seen in the kidney and bladder.

Response: In a published study (Adv. Funct. Mater. 2022, 32, 2206346), the authors noted that "DC@BPBBT dots and BPBBT dots are spherical with average sizes of 147.2 nm and 136 nm, respectively," indicating that the synthesized nanoparticles are relatively large. Furthermore, ex vivo biodistribution results revealed significant fluorescence signals in the liver, spleen, and kidneys, a phenomenon consistent with our own experimental observations. However, the cited study did not provide an in-depth analysis of the bladder signal. As we are the first to construct such a multifunctional theranostic system, our interpretations are necessarily based on comparisons with similar previous works, while acknowledging that our system is not entirely equivalent to those reported previously.

[editorial note: third party material redacted]

References: Adv. Funct. Mater. 2022, 32, 2206346

e). Even at 0.5 h, this study shows very strong kidney uptake and rapid clearance through the bladder, with minimal liver and spleen uptake over time. This pattern closely resembles the behavior of small molecules. The authors might need to consider testing the stability of their nanoparticles.

Additionally, the authors mention “reticuloendothelial system (RES) organs (liver and kidneys) and lung tissue.” In fact, RES generally refers to the liver and spleen, not the kidney. The authors should double-check this concept.

Response: As we have confirmed in Fig. 2e, the size dynamics and polydispersity index of the nanoparticles show no significant changes over 7 days. Furthermore, the results in Supplementary Fig. 25 demonstrate that the nanoparticles maintain structural stability even under continuous laser irradiation, collectively evidencing their excellent stability.

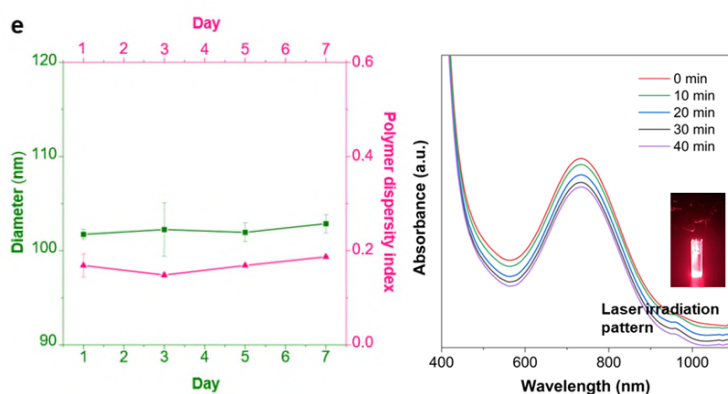


Fig. 2e and Supplementary Fig. 25 (Our work)

The Reticuloendothelial System (RES) is a concept introduced in the early 20th century to describe cells dedicated to phagocytosing and clearing foreign substances, senescent cells, and microbes from the blood. The term "Reticuloendothelial System (RES)" is a concept dating back to the early 20th century, describing cells specialized in phagocytosing and clearing foreign substances, senescent cells, and microbes from the blood. As this concept is no longer up-to-date, we thank the reviewer for the reminder. Based on current understanding and for the sake of scientific accuracy, the relevant text in the manuscript has been revised accordingly.

Revisions in the main body of the text: The probe observed fluorescence signals in liver, kidney, and lung tissues.

in vitro imaging of organs collected 4 h after ICG administration revealed substantial signal accumulation in the liver and kidneys organs.

11. Comments: The authors state that the PET/CT data are consistent with other Ga-68-labeled nanoprobes in the literature. While this is possible, the key question remains: why are there differences between Fig. 5b (5c) and Fig. 5d in this work? These figures use the same probe but different imaging modalities.

The authors attribute the decrease in tumor uptake in Fig. 5c to the decay of Ga-68. Although Ga-68 does decay, PET/CT imaging should be calibrated, and internal standards are typically used. The quantitative analysis must account for the decay of Ga-68.

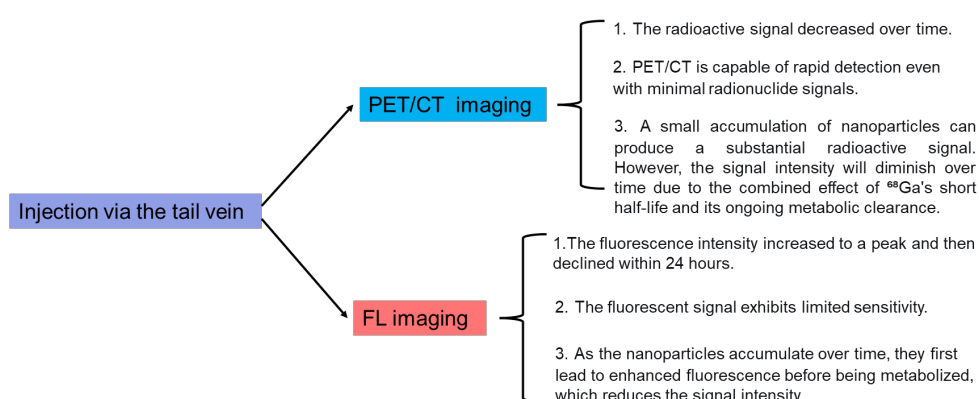
The authors also suggest that release of DOTA–Ga-68 may be another factor. This explanation is more plausible, but if correct, it raises concerns about the reliability of the entire study. It could also explain why the nanoparticles, despite their ~100 nm size, show unusually high renal clearance.

The authors acknowledge the limitations of FLI, which is valid. However, they should be aware that the study is supposed to be done in the same mice, at different times. The differing conclusions drawn from Fig. 5b and Fig. 5d remain unresolved.

Response: First, I want to assure you of the complete authenticity of our findings. Regarding the issues you raised about the signal discrepancy between PET/CT and fluorescence imaging for the same nanoparticles and their seemingly anomalous biodistribution, we highly appreciate your valuable input and provide herein a comprehensive mechanistic explanation.

All PET/CT and fluorescence (FL) imaging studies were performed on a single mouse. As shown in Fig. 5b, the radioactive signal from the tumor site in PET/CT images was detectable at 0.5 h, with the intensity peaking at this earliest time point and subsequently declining over time. In contrast, the fluorescence signal at the tumor site (Fig. 5d) exhibited a different pharmacokinetic

profile, increasing initially before eventually decreasing. The folate-modified nanoparticles effectively targeted the tumor. PET/CT imaging, with its superior tissue penetration, enabled immediate and sensitive tumor detection, even with a low radiotracer dose—a level of sensitivity far surpassing that of fluorescence imaging. Over time, the nanoparticles underwent a dynamic process of accumulation, retention, and clearance at the tumor site. The high sensitivity of PET/CT allowed for rapid tumor visualization even during the initial accumulation phase. Concurrently, the fluorescence signal initially increased as more nanoparticles accumulated. However, the signal profile was shaped by the relatively short half-life of ^{68}Ga (leading to a decaying radioactive signal) and the subsequent metabolic clearance of the nanoparticles, which collectively resulted in the observed rise and fall of the fluorescence intensity. The experiment was conducted through numerous repetitions, which verified its reproducibility and reliability.



Brief description diagram

We thank the reviewer for highlighting this point (regarding the half-life of ^{68}Ga). In fact, its short half-life was a deliberate choice in our experimental design, precisely to address the central premise of our study: mitigating the long-term radiological exposure associated with prolonged isotope retention in the human body, for which fluorescence imaging offers a potential solution. To ensure the accuracy and reliability of our PET/CT quantitative data, all imaging results were meticulously calibrated for radioactive decay, using the injected dose as an internal standard.

Folate receptor α (FR α) is a high-affinity, glycosylphosphatidylinositol (GPI)-anchored cell membrane receptor. Studies have shown that it is constitutively expressed in normal tissues such as pulmonary cells and renal tubules. Furthermore, folate receptors have been reported to be widely distributed as proteins in various human tissues, including the kidneys, lungs, intestines, and central nervous system. Recent work by Wu et al. further indicates that even large-sized nanoparticles can be metabolized via the proximal tubule pathway. Collectively, this evidence suggests that folate modification may play a key role in directing the targeted accumulation of nanoparticles in the kidneys, particularly in the renal tubules. Based on the above understanding, we propose that although the nanoparticles used in this study are relatively large, their surface folate modification may further enhance active targeting to renal tubular epithelial cells, thereby promoting metabolism

and clearance primarily via the renal tubular pathway. This proposed metabolic mechanism is clearly illustrated in the accompanying "Biodistribution and Clearance Mechanism Schematic."

Revisions in the main body of the text: Renal clearance is primarily mediated through three distinct pathways: glomerular filtration, tubular secretion, and tubular reabsorption⁷⁴. Given that the size of our nanomaterial exceeds the glomerular filtration threshold⁷⁵, we further examined its biological clearance mechanisms. Work by Wu et al⁷⁶. has shown that even substantially sized nanoparticles can be processed via the proximal tubule pathway⁷⁷. Furthermore, FR α —a high-affinity, GPI-anchored membrane receptor—is constitutively expressed in normal tissues including the lung and renal tubules. Functionalization of the nanoparticles with folate enhanced their specific accumulation in proximal tubular epithelium, thereby facilitating renal clearance through an active tubular mechanism (Supplementary Fig. 36).

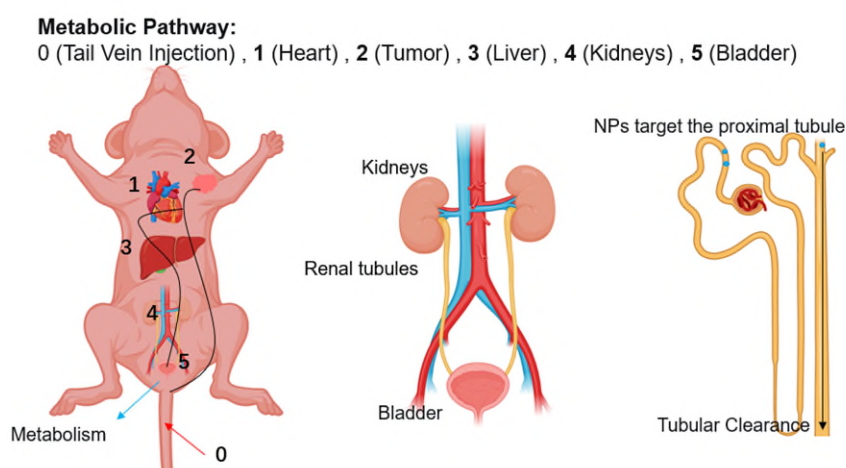
74. Huang, J., et al. Renal- clearable molecular semiconductor for second near- infrared fluorescence imaging of kidney dysfunction. *Angew. Chem. Int. Ed.* **58**, 15120-15127 (2019).

75. Du, B., et al. Glomerular barrier behaves as an atomically precise bandpass filter in a sub-nanometre regime. *Nat. Nanotechnol.* **12**, 1096-1102 (2017).

76. Wu, D., et al. Multivalent supramolecular fluorescent probes for accurate disease imaging. *Sci. Adv.* **10**, eadp8719 (2024).

77. Du, B., et al. transport and interactions of nanoparticles in the kidneys. *Nat. Rev. Mater.* **3**, 358-374 (2018).

Revisions in the main body of the Supporting information:



Supplementary Fig. 36 Mechanisms of Biological Clearance. Created in BioRender. Guoling, Z. (2026) <https://BioRender.com/9ni604q>

12. Comments: The authors exclude the lungs from the RES but classify the kidney as part of it. However, the kidney is generally not considered an RES organ.

Response: We sincerely appreciate your valuable feedback. The Reticuloendothelial System (RES) is a concept introduced in the early 20th century to describe cells dedicated to phagocytosing and clearing foreign substances, senescent cells, and microbes from the blood. The term "Reticuloendothelial System (RES)" is a concept dating back to the early 20th century, describing cells specialized in phagocytosing and clearing foreign substances, senescent cells, and microbes from the blood. As this concept is no longer up-to-date, we thank the reviewer for the reminder. Based on current understanding and for the sake of scientific accuracy, the relevant text in the manuscript has been revised accordingly.

Revisions in the main body of the text: The probe observed fluorescence signals in liver, kidney, and lung tissues.

in vitro imaging of organs collected 4 h after ICG administration revealed substantial signal accumulation in the liver and kidneys organs.

Questions and point-by-point responses are as follows:

Reviewer #3 (Remarks to the Author):

Based on the authors' responses and revisions, several major concerns regarding the manuscript remain not fully addressed. In the manuscript, the biodistribution data shows very high renal clearance and much lower liver and spleen uptakes (Figure 5b and 5c). As the nanoparticles are approximately 100 nm (TEM and DLS), they typically cannot undergo renal clearance. The authors attribute the observed renal clearance to targeting FA on the surface of nanoparticles to folate receptor alpha over expressed in kidney. However, the control experiment shows that nanoparticles without FA modification also exhibit almost the same renal clearance (Figure 5d, signal from bladder) although the kidney signal is lower. Below are comments on some of authors' responses.

Response: We sincerely appreciate your insightful comments, which have significantly contributed to the improvement of this work. The nanoparticles involved in this study are relatively large in size (~100 nm). Under normal physiological conditions, these particles are generally unable to undergo direct glomerular filtration (with a pore size of approximately 5-10 nm) followed by rapid metabolic clearance via the kidney-bladder pathway. This observation presents a certain discrepancy with the classical renal excretion theory. To ensure the scientific rigor and accuracy of our experimental results, we conducted a systematic validation of this phenomenon. We re-synthesized new batches of nanoparticles and independently repeated the PET/CT imaging experiments to comprehensively track the metabolic trajectory of the nanoparticles in vivo. Additional experimental validation and a schematic diagram of the biological clearance mechanism have been incorporated into the manuscript to clarify the biological clearance mechanism.

9.

a). Figure 5b and 5d are PET/CT images of [⁶⁸Ga]DOTA-FA-BBDT-4C NPS (with folate modification) and [⁶⁸Ga]DOTA-BBDT-4C NPS (without folate modification), respectively. As shown in the circled area, the NPs with FA show high uptake in both bladder and kidney, whereas the NPs without FA exhibit significant renal clearance in bladder (as evidenced by the strong bladder signal in Figure 5d). Although the NPs with FA have higher kidney uptake, the bladder uptake is nearly identical to that of the NPs without FA.

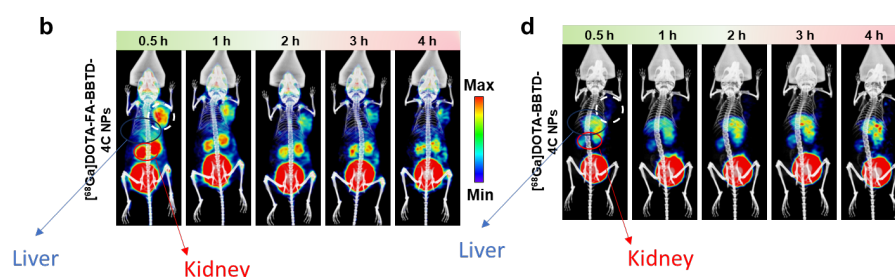
The elevated kidney signal observed for the NPs with FA may result from FA binding to folate receptor alpha in the kidney. FA binding could lead to accumulation in the kidney before eventual excretion into the bladder. However, since both NPs (with FA and without FA) show similar bladder clearance (eventual clearance), the data suggest that renal clearance is not primarily modulated by FA conjugation, contrary to the authors' claim. How do the authors explain these findings?

Response: We sincerely thank you for your valuable comments. As shown in Figs 5b and 5d from the previous experiment, both the unmodified and folate-modified substances were encapsulated using polyethylene glycol (PEG), with low molecular weight PEG used in all cases. Notably, both the folate-

modified and unmodified substances were metabolized through the liver and kidneys (as indicated in the figures), but the folate-modified substance exhibited a stronger binding affinity for kidney tissue.

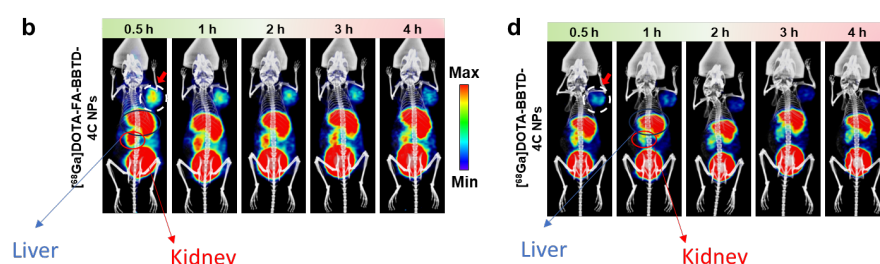
The reasons are as follows:

- (1) Literature has demonstrated that surface modification of nanoparticles with PEG significantly reduces serum protein adsorption, thereby decreasing their uptake by organs of the reticuloendothelial system, such as the liver and spleen (Ref. *Nat. Rev. Mater.* **3**, 358-374 (2018)).
- (2) The modified molecules exhibit a propensity for renal accumulation. This is likely attributable to PEGylation-induced alterations in molecular charge or size, thereby influencing the processes of glomerular filtration and tubular reabsorption (Ref. *Amino Acids.* **56**, 44 (2024)).
- (3) Publications indicated that it is possible for some large NPs, even ones sized 100–200 nm (Ref. *Nat. Commun.* **9**, 4551, (2018); *Hypertension.* **71**, 87–94 (2018)), to escape degradation and then bypass the GFB.



Figs 5b and 5d of the previous experiment

In response to the reviewer's comment regarding the clearance pathway, and taking into full consideration the non-traditional nature of this metabolic route, we re-synthesized the nanoparticles and repeated the PET/CT imaging experiments to further enhance the accuracy, scientific rigor, and reproducibility of the study. The results of the repeated experiments were largely consistent with our previous observations: regardless of whether the nanoparticles were modified with folic acid, they exhibited significant accumulation in the liver and kidneys, and radioactive signals were also detected in the bladder region (Figs 5b and 5d of the new experiment). Subsequently, we conducted a quantitative analysis of the intensity in the kidney region for different materials. The results indicated that the folate-modified material exhibits a stronger binding affinity for kidney tissue (Newly added Supplementary Fig. 34).



Figs 5b and 5d of the new experiment

This phenomenon suggests that although these particles are relatively large in size, typically exceeding the conventional size threshold for renal filtration, their renal clearance may still be achieved through multiple mechanisms. These mechanisms may act synergistically to facilitate the clearance and excretion of large-sized particles from the body. Full experimental details and the biological clearance mechanisms are described in the main text and the supporting Information.

To more clearly elucidate the clearance mechanism, we added the following content to the main text: In the U87 model, the maximum tumor retention reached $4.94 \pm 1.06\%$ ID/g at 0.5 h, remaining relatively stable at 1 h ($3.86 \pm 0.67\%$ ID/g) and 2 h ($3.31 \pm 0.31\%$ ID/g) (Fig. 5c). In contrast, nanoparticles with the same formulation but without folate modification did not exhibit significant tumor-targeting ability and were mainly cleared through both renal and hepatic pathways (Fig. 5d). Quantitative analysis of mouse kidney tissue further clarified whether folate modification enhances renal targeting and promotes urinary excretion via the bladder (Supplementary Fig. 35). The results indicated that the folate-modified material exhibits a stronger binding affinity for kidney tissue. This biodistribution pattern is consistent with that reported for other ^{68}Ga -labeled PET imaging agents.^{63,73}

Renal clearance is primarily mediated by glomerular filtration, tubular secretion, and tubular reabsorption.⁷⁴ Although the nanomaterials used in this study exceed the typical size threshold for glomerular filtration,⁷⁵ they still exhibit pronounced renal clearance. Previous studies have shown that certain nanomaterials above this size cutoff can be actively cleared via proximal tubule-mediated pathways.^{76,77} Additionally, PEGylation may enhance renal tropism by altering the hydrodynamic radius or surface charge of the nanoparticles, thereby shifting the balance between glomerular filtration and tubular reabsorption.⁷⁸ The folate moieties on the nanoparticle surface may specifically bind to the high-affinity receptor FR α —constitutively expressed on renal tubular epithelial cells—thereby facilitating efficient uptake and accumulation of the nanoparticles in these cells.⁷⁹ Supporting evidence comes from transmission electron microscopy analysis of urine samples from treated mice, which revealed the presence of nanoparticle fragments (Supplementary Fig. 37), and stability assessments under varying pH conditions (Supplementary Fig. 38) suggest that the physically adsorbed PEG layer on the nanoparticle surface may dissociate upon entry into the circulation, driven by interactions with blood components and the complex pH environment in vivo. Based on the above findings, we propose that a synergistic clearance mechanism occurs in vivo: the nanoparticles initially accumulate in the liver and undergo metabolic degradation; the resulting fragments subsequently enter the bloodstream and, by virtue of folate-mediated targeting affinity for renal tubules, are cleared via glomerular filtration and ultimately excreted into the urine through the bladder (Supplementary Fig. 39). This clearance process is likely co-regulated by physicochemical properties and active biological transport mechanisms.

References:

78. Zhang, S., et al. PEGylation renders carnosine resistant to hydrolysis by serum carnosinase and increases renal carnosine levels. *Amino Acids* **56**, 44 (2024).
79. Larenkov, A., et al. Pharmacokinetic Properties of ^{68}Ga -labelled Folic Acid Conjugates: Improvement Using HEHE Tag. *Molecules* **25**, 2712 (2020).

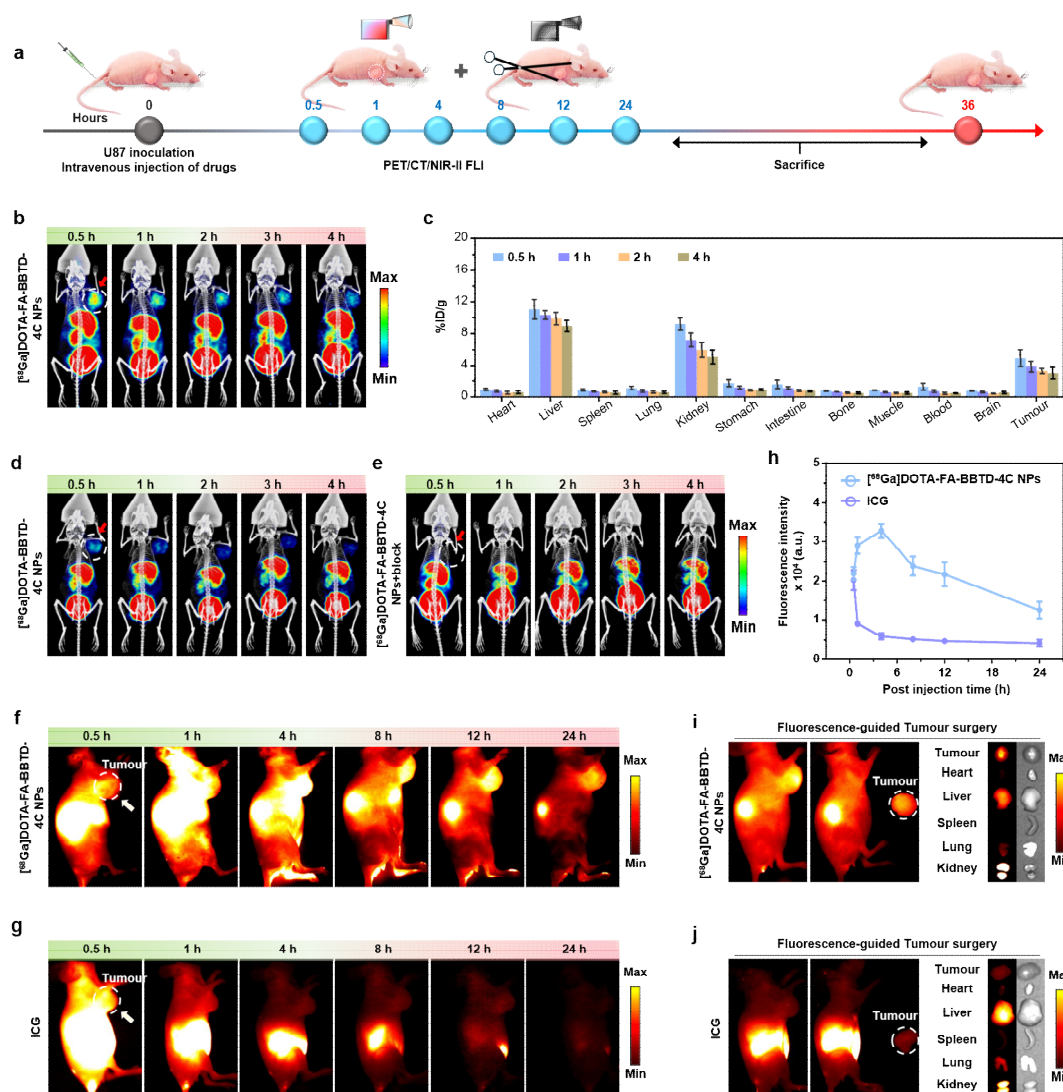
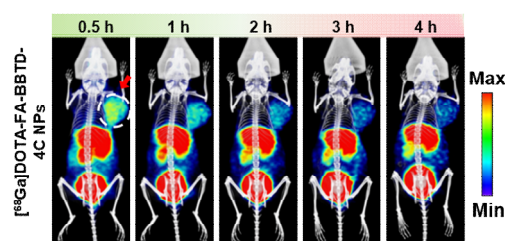


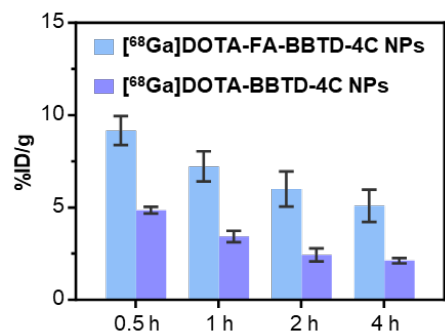
Fig. 5 **b** Representative PET/CT images of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ in tumor-bearing mice. **c** Dynamic time-activity curves of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ in heart, liver, spleen, kidney, stomach, intestine, bone, muscle, blood, brain, and tumor of tumor-bearing mice. **d** Representative PET/CT images of $[^{68}\text{Ga}]\text{DOTA-BBTD-4C NPs}$ in tumor-bearing mice. **e** Representative PET/CT images of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs} + \text{block}$ in tumor-bearing mice.

The following content is added to the Supporting Information:

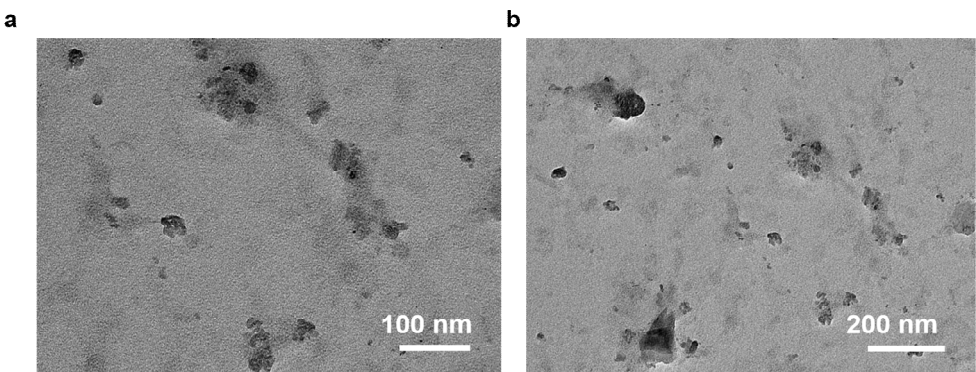


Supplementary Fig. 34 Representative PET/CT images of $[^{68}\text{Ga}]\text{DOTA-FA-BBTD-4C NPs}$ in Hela

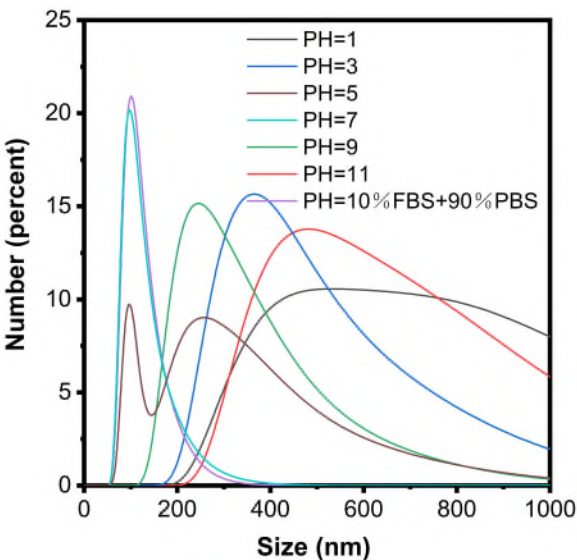
tumor-bearing mice.



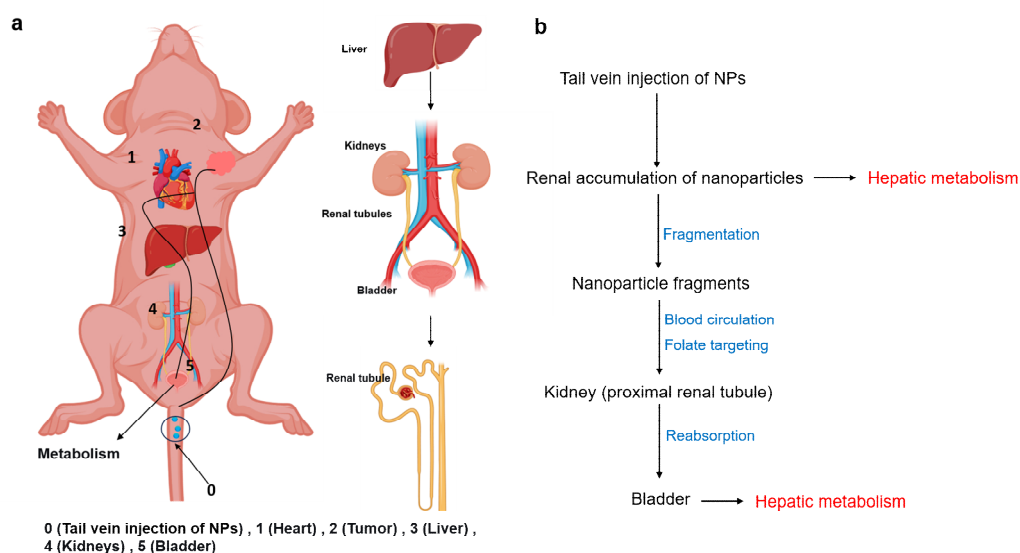
Supplementary Fig. 35 Quantitative analysis of different materials intensity in mouse kidney tissue.



Supplementary Fig. 37 Transmission electron microscopy images of urine collected from mice treated with **a** [⁶⁸Ga]DOTA-BBTD-4C NPs and **b** [⁶⁸Ga]DOTA-FA-BBTD-4C NPs.



Supplementary Fig. 38 Particle size of [⁶⁸Ga]DOTA-FA-BBTD-4C NPs under different pH conditions.

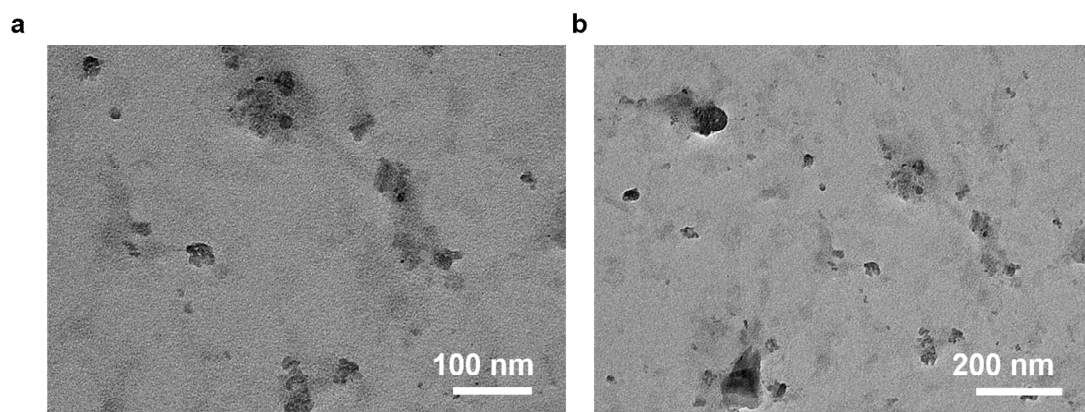


Supplementary Fig. 39 **a** Schematic diagram of the biological clearance mechanism. **b** Flowchart of the biological clearance mechanism. Created in BioRender. Guoling, Z. (2026) <https://BioRender.com/9ni604q>

b). If the authors truly believe that these about 100 nm nanoparticles can be completely cleared through the renal system, they are encouraged to collect urine samples and confirm the presence of intact nanoparticles (approximately 100 nm) using TEM. This suggested experiment would help validate the unusual clearance behavior.

Response: We sincerely thank you for your valuable comments. Based on your suggestions and in combination with conventional biological clearance mechanisms, we resynthesized the nanoparticles and repeated the experiments to ensure accuracy and reproducibility. Urine samples were collected from the experimental mice and examined using transmission electron microscopy (TEM). The results showed that no intact nanoparticles were observed in the urine samples; however, nanoparticle fragments and other substances were detected. This experimental finding differs from the literature we initially referenced regarding the renal metabolism of large particles. On this basis, we propose that the biological clearance mechanism is as follows: the nanoparticles initially accumulate in the liver and undergo metabolic degradation; the resulting fragments subsequently enter the bloodstream and, by virtue of folate-mediated targeting affinity for renal tubules, are cleared via glomerular filtration and ultimately excreted into the urine through the bladder. This clearance process is likely co-regulated by the physicochemical properties of the nanoparticles and active biological transport mechanisms. Our experimental results support the view that this biological clearance pathway is consistent with the conventional understanding that large particles are difficult to clear through renal metabolism.

The following content is added to the Supporting Information:



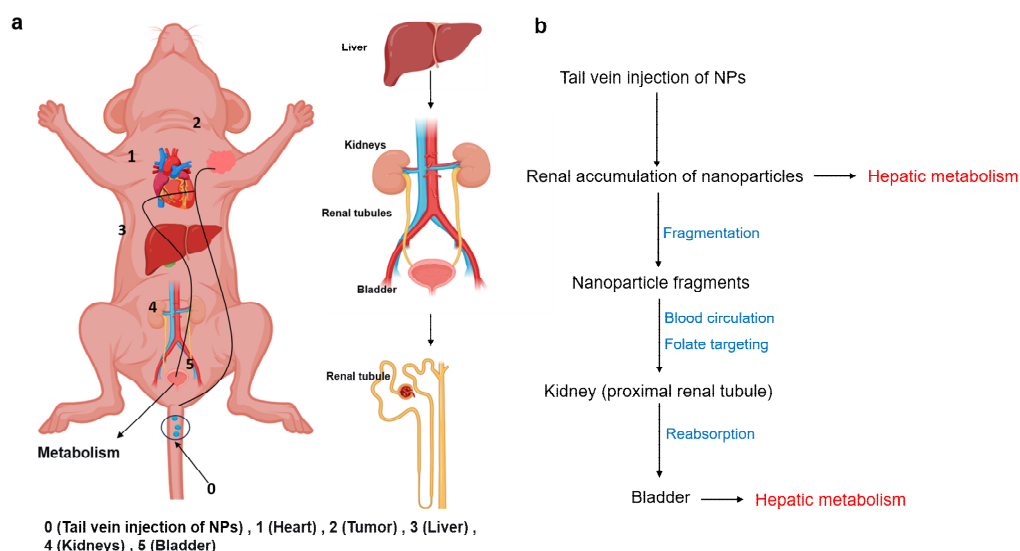
Supplementary Fig. 37 Transmission electron microscopy images of urine collected from mice treated with **a** [^{68}Ga]DOTA-BBDT-4C NPs and **b** [^{68}Ga]DOTA-FA-BBDT-4C NPs.

c). It is worth to mention that the authors cited the reference (Mol. Pharmaceutics 2024, 21, 5681–5689) in the first revision response. The intention was to demonstrate a similar renal clearance phenomenon in the cited reference. Regardless of the degree of comparability between the two studies, it is important to emphasize the key conclusion from the reference: although many factors influence renal clearance, it is widely accepted that small-sized molecules are cleared through the renal system, whereas larger molecules/particles are not. This principle is fundamental in designing and controlling pharmacokinetics.

Response: We sincerely thank you for your valuable comments. As you pointed out, small molecules are typically cleared primarily via the renal pathway, whereas large molecules or particles are difficult to eliminate through this route—a view that initially aligned with the general consensus in the field. However, some studies have reported that larger nanoparticles can also be cleared through the renal pathway, and current research is actively exploring the potential mechanisms underlying the renal metabolism of large particles. This has led to some uncertainty regarding the biological clearance mechanism of the nanoparticles in our experiments.

In response to the question of the biological clearance mechanism, and following your expert advice, we resynthesized the nanoparticles and repeated the PET/CT experiments. The results showed that the nanoparticles used in this study (100 nm) undergo dual metabolism via the liver and kidneys. Combined with TEM analysis of mouse urine samples (which confirmed the non-integrity of the nanoparticles) and assessments of nanoparticle stability under varying pH conditions, we propose that a complex biological clearance mechanism occurs *in vivo*: the nanoparticles first enter the liver, where they are degraded and fragmented; due to the folate modification on the nanoparticle surface, which enhances targeting affinity for renal tubules, the fragmented nanoparticles subsequently enter the bloodstream, target the kidneys, and are ultimately excreted into the urine via the bladder. We believe that this mechanism provides valuable insights for the design and control of the pharmacokinetics of folate-modified large nanoparticles. We have also included a complete schematic diagram of the biological clearance mechanism in the article to help readers better understand this clearance process.

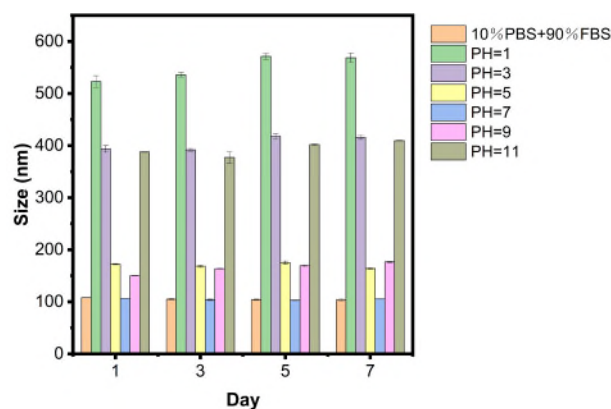
The following content is added to the Supporting Information:



Supplementary Fig. 39 **a** Schematic diagram of the biological clearance mechanism. **b** Flowchart of the biological. Created in BioRender. Guoling, Z. (2026) <https://BioRender.com/9ni604q>

e). To more convincingly demonstrate nanoparticle stability, the authors are advised to conduct stability studies in different buffers and at various pH values.

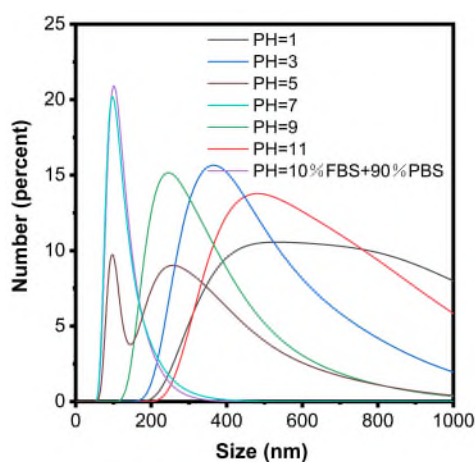
Response: We sincerely thank you for your valuable comments. In Figure 2e, we have already investigated the stability of this material in PBS. Building on this, we first explored the stability of the nanomaterial under different conditions, and then extended the observation period to further assess its stability. Its stability was evaluated in a simulated blood environment (10% FBS+90% PBS) and in aqueous solutions with different pH conditions (pH = 1, 3, 5, 7, 9, 11) over a duration of 7 days. The combined results indicated that in pure PBS, pure water, or 10% FBS+90% PBS, no significant changes in particle size were observed within 7 days. However, under pH conditions of 1, 3, 5, 9, and 11, the stability of the nanoparticles changed significantly. We hypothesize that this phenomenon is due to the disruption of the PEG modification structure on the particle surface under extreme acidic or alkaline conditions. This phenomenon is likely attributable to the instability of the PEG layer modified on the nanoparticle surface under acidic or alkaline conditions. Acidic or alkaline conditions may catalyze the cleavage of bonds linking PEG to the particle surface, leading to the shedding or conformational changes of the PEG chains. This causes the nanoparticles to lose colloidal stability and exposes their hydrophobic core, thereby triggering subsequent aggregation.



Particle sizes of [^{68}Ga]DOTA-FA-BBDT-4C NPs under different storage durations and pH conditions.

we added the following content to the main text: Supporting evidence comes from transmission electron microscopy analysis of urine samples from treated mice, which revealed the presence of nanoparticle fragments (Supplementary Fig. 37), and stability assessments under varying pH conditions (Supplementary Fig. 38) suggest that the physically adsorbed PEG layer on the nanoparticle surface may dissociate upon entry into the circulation, driven by interactions with blood components and the complex pH environment in vivo.

The following content is added to the Supporting Information:



Supplementary Fig. 38 Particle size of [^{68}Ga]DOTA-FA-BBDT-4C NPs under different pH conditions.