

Enzymatically Catalyzed Molecular Aggregation

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors report two compounds that they claim to undergo enzyme triggered aggregation (both in vitro and vivo) leading to effective PDT outcomes, partly due to a "type-I PDT" process they claim to be better than "type II".

This referee finds important design flaws and problems with the implementation of the work.

Here are the major issues:

Problem with Aggregates:

While AIE compounds may be interesting for imaging perhaps mostly in cell culture and mice models (considering limited light penetration) as a therapeutic agent, aggregate structures are ill-defined and especially in high protein milieus, not very stable and difficult to standardize. More to the point, there is really no real justification for the use of aggregates, considering the fact molecular drugs or photosensitizers are better than the aggregates in many aspects, and nothing new is offered or even suggested by these compounds.

Excitation wavelength:

The absorption peak of the monomeric compounds and the aggregates in this work is around 450 nm. This means essentially no penetration in tissues (just single cell width). This is the reason why the authors use a White LED light source, which is a non-descript identification of a light source, but is known that LEDs of this type have a very strong near UV peak. Part of the cell death in cell cultures is clearly due to white LED.

Confusion about the Type-I and Type-II PDT:

In recent articles regarding PDT, it seems like a misreading of PDT processes getting entrenched. PDT is a combination of both of these processes. Most ROS species are interconvertible by various enzymatic processes in vivo. Some articles also push the misconception that Type-I process (which are partly based on the degradation of the photosensitizers) are better, because it is less oxygen dependent; it is not and it is not easy to separate these two processes (I/II).

Enhancement of emission "AND" PDT efficiency.

The authors should also keep in mind that any emission from the aggregates, is a loss in ROS generation efficiency. So, AIE-PDT carries a certain self-contradictory character.

Reviewer #2

(Remarks to the Author)

Tang and colleagues reported a γ -glutamyl transferase (GGT) activatable aggregation-induced emission photosensitizer (AIE-PS) named TBmA-Glu. TBmA-Glu is designed to specifically target and aggregate cancer cells through the catalytic action of tumor-overexpression GGT. Selective tumor cell aggregation not only enhances AIE-PS emission and

photodynamic activity but also induces ferroptosis in cancer cells by depleting GSH and promoting lipid peroxidation. Both in vitro cell assays and in vivo animal models were used to validate the phototoxicity and antitumor effects of TBmA-Glu, providing a comprehensive assessment of its potential as a therapeutic agent. The aggregation strategies in this paper allow a controlled release of the photodynamic effect, which is critical for therapies such as PDT. AIE-PS remains dormant until it reaches the cancerous environment where GGT is present, ensuring minimal impact on healthy cells and maximizing the therapeutic effect on cancer cells. This study highlights the significance of targeting activation of AIE-PSs for targeting and enhanced cancer photodynamic therapy. It is also a sophisticated strategy for targeted delivery and activation of a photodynamic therapeutic agent for disease. I suggest this article be published with minor revisions.

1. The authors claimed that TBmA-Glu could be activated by GGT. Is there evidence to suggest that TBmA-Glu could be effective against other types of cancer that overexpress GGT, or is its application currently limited to the cancer types studied?
2. How photostable is TBmA-Glu under the conditions used for PDT, and does its aggregation state affect its photostability?
3. The author claimed the aggregate size has a great impression on the PDT efficiency of AIE-PSs, What are the typical sizes and shapes of the TBmA-Glu aggregates formed in the presence of GGT? How do these properties affect the emission properties and PDT efficacy?
4. Can aggregated TBmA be expelled from cancer cells via exocytosis, potentially reducing its therapeutic efficacy? Long-term (48 h) cellular imaging results should be provided by the author.
5. The abbreviations, such as DCF, DCFH-DA, HPF, ABDA, CLSM, et al., should be defined at the first time they are used.

Reviewer #3

(Remarks to the Author)

This work presents a novel approach to targeted cancer therapy by leveraging the tumor-overexpressed enzyme γ -Glutamyl Transferase (GGT) to induce aggregation of an aggregation-induced emission photosensitizer (AIE-PS), TBmA-Glu. This innovative strategy not only enhances the photosensitivity of the AIE-PS but also results in the degradation of GGT and the accumulation of lipid peroxides, leading to cancer cell ferroptosis. The study is significant for its potential to advance targeted photodynamic therapy (PDT) and the development of smart therapeutics that exploit enzyme activity for controlled molecular aggregation within cancer cells. The authors have demonstrated a clear understanding of the complex interactions between molecular aggregation and biological environments, and the manuscript is well-structured, presenting a logical flow of information from synthesis and characterization to in vivo efficacy. The results are compelling, showing the selective activation of TBmA-Glu by GGT, its enhanced photodynamic activity, and the subsequent therapeutic effects on cancer cells. The manuscript is well-written and provides a solid foundation for further research in the field of nanomedicine and targeted drug delivery. I recommend publication following minor modifications, my concerns are outlined below:

1. The author could incorporate a concise, visual representation of key discoveries and TBmA-Glu's proposed mechanism of action through a mechanistic cartoon or schematic.
2. The stability of the aggregates of TBmA, especially the photostability of it in physiological conditions should be discussed.
3. The author claimed the lipid peroxides (LPOs) resulting from the photodynamic process of activated AIE-PS induce the ferroptosis of cancer cells, the changes in the level of LPOs in cancer cells after photodynamic therapy should be quantified.
4. Detailed experimental procedures for minimally invasive PDT should be provided.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer 1 responses to the authors' comments is highlighted in red.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors report two compounds that they claim to undergo enzyme triggered aggregation (both in vitro and vivo) leading to effective PDT outcomes, partly due to a "type-I PDT" process they claim to be better than "type II".

This referee finds important design flaws and problems with the implementation of the work.

Response: We thank the reviewer for the constructive comments on our paper. In this manuscript, we actually did not intend to compare the Type I and Type II PDT processes, and the efficiency of type I and type II photosensitizers is not the key point of this work. We just want to present the objective performance of the developed photosensitizers based on their ROS generation capability. As clearly stated in the manuscript: "It was found that TBmA and TBpA produced significantly higher ROS compared to TBmA-Glu and TBpA-Glu, even surpassing the commercial photosensitizer, Rose Bengal (RB). Moreover, TBmA was identified as the most potent photosensitizer among the four compounds. Further analysis revealed

that TBmA and TBpA functioned as strong type I photosensitizers (Fig. 1c and Fig. S25), while TBmA-Glu and TBpA-Glu acted as very weak type II photosensitizers (Fig. 1c and Fig. S26).”

***The authors are accurate in stating that a comparison was not made (see the green text below from the manuscript). However, they claim that oxygen content has a negligible influence on the observed activity under hypoxia, which is linked to Type-I process, based on previous claims. These claims were not supported by “negative controls” with standard PDT photosensitizers, in a fair comparison. 251. “only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d). This suggests that oxygen content has negligible influence on its photodynamic activity. The cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process, which is consistent with the prior findings.”

Here are the major issues:

Problem with Aggregates:

While AIE compounds may be interesting for imaging perhaps mostly in cell culture and mice models (considering limited light penetration) as a therapeutic agent, aggregate structures are ill-defined and especially in high protein milieu, not very stable and difficult to standardize. More to the point, there is really no real justification for the use of aggregates, considering the fact molecular drugs or photosensitizers are better than the aggregates in many aspects, and nothing new is offered or even suggested by these compounds.

Response and revision: Thank you for your thoughtful comments regarding AIE compounds. We appreciate your concerns and would like to address them point by point:

(i) Stability and standardization: we acknowledge that stability is crucial for bio-application. Our recent studies have shown promising results regarding the stability of TBmA aggregates in high-protein environments, specifically: (a) Long-term stability: TBmA aggregates showed no significant degradation when dispersed in FBS for 72 hours (Fig. S28a and S28c).

*** Aggregation is a type of supramolecular association which is perfectly reversible. It is only natural to expect deaggregation in the biological media with so many different gradients of hydrophobicity. FBS is not a good approximation for intracellular medium as its protein content is very low. Of course, a simple pharmacokinetics study would reveal how stable is those aggregates are in vivo.

(ii) (b) Photostability: The aggregates remained stable under continuous light irradiation for 30 minutes (Fig. S28a and S28b). These findings collectively highlight the exceptional stability exhibited by TBmA. These findings demonstrate the exceptional stability of TBmA aggregates in biologically relevant conditions.

Moreover, numerous AIEgens, including small molecules or AIE nanoparticles, have been extensively reported for their long-term monitoring and theranostic applications.^{1, 2, 3} These pieces of evidence underscore the remarkable stability of AIEgens, making them highly promising candidates for theranostic applications.

(iii) Aggregate structure: To address concerns about ill-defined aggregate structures, we extensively investigated the aggregate size of TBmA using Dynamic Light Scattering (DLS) and Transmission Electron Microscope (TEM). The results suggest that the TBmA forms spherical particles with 140 nm in 99% PBS and 150 nm diameter after the GGT catalysis reaction (12 h, Fig. S31). These results indicate that TBmA consistently forms nanoparticles of definite shape and size in aqueous environments, regardless of the specific conditions. Numerous works have been reported to show the definite shape and size, as well as the excellent stability and biocompatibility of the AIE aggregates.^{4, 5, 6}

*** References 4, 5 and 6 were carefully checked. Stability of the aggregates “in vivo” was not studied in these articles.

Retention of fluorescence is not necessarily a sign of stability.

(iv) A comparative analysis of small molecular drugs and AIE materials: Although molecular drugs and traditional photosensitizers have their advantages, AIE compounds offer unique benefits such as enhanced emission upon aggregation, responsiveness to stimuli, and multifunctional potential. Revealing reports increasingly indicate that small molecular photosensitizers, such as CE6, exhibit low solubility and undergo aggregation in solution, resulting in the deactivation of their photosensitizing activity and hindering their bioapplication.^{7, 8, 9, 10} We believe that AIE compounds can serve as complementary agents, rather than substitutes, for small molecule drugs. Recent literature has demonstrated the potential of AIE compounds for combining imaging and therapeutic functions in a single entity opening up new possibilities for personalized medicine and real-time treatment monitoring.^{4, 11, 12, 13}

We believe that AIE compounds, including TBmA-Glu, offer valuable and unique properties that complement existing molecular drugs and photosensitizers. While challenges remain, the growing body of research on AIE materials suggests significant potential for advancing biomedical imaging and therapeutic applications. We appreciate the reviewer's perspective and believe that continued research and development in this field will address current limitations and unlock new possibilities in biomedical science.

*** While AIE compounds seem to provide potentially useful imaging opportunities, their relevance in PDT or other therapeutic schemes remain questionable. A therapeutic agent which would change size on meeting hydrophobic membranes or proteins, which could lead to different properties has to be handled very carefully. It would be advisable to avoid hype terminology such as “personalized medicine and real-time treatment monitoring”.

Changes in the Revised Manuscript:

Moreover, the TBmA aggregates exhibited excellent long-term stability (Fig. S28a and S28c) and photodynamic stability (Fig. S28a and S28b), no significant aggregation or degradation was found after dispersed in FBS (fetal bovine serum) solution for 72 h or light irradiated for 30 min.

Changes in the Supporting Information:

Fig. S28. (a) The average hydrodynamic diameter (Z-average) of TBmA aggregates measured by Dynamic Light Scattering

(DLS). The distribution of TBmA aggregates during 30 min light irradiation and 72 h FBS preservation.

Fig. S31. (a) The average hydrodynamic diameter (Z-average) of TBmA aggregates produced in GGT catalytic reaction measured by DLS. (b-g) Distribution of TBmA aggregates formed at different times of GGT catalytic reaction. (h) The transmission electron microscope (TEM) of the TBmA aggregates formed after the GGT catalytic reaction for 12 h.

References

1. Zuo J, et al. Long-term spatiotemporal and highly specific imaging of the plasma membrane of diverse plant cells using a near-infrared AIE probe. *Angew. Chem. Int. Ed.* 14, 2139-2148 (2023).
2. Wang Z, et al. Long-term fluorescent cellular tracing by the aggregates of aie bioconjugates. *J. Am. Chem. Soc.* 135, 8238-8245 (2013).
3. Li K, et al. Photostable fluorescent organic dots with aggregation-induced emission (AIE dots) for noninvasive long-term cell tracing. *Sci. Rep.* 3, 1150 (2013).
4. Wang J, et al. Nanolab in a cell: Crystallization-induced in situ self-assembly for cancer theranostic amplification. *J. Am. Chem. Soc.* 144, 14388-14395 (2022).
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7. Li Y, et al. Near-infrared light and redox dual-activatable nanosystems for synergistically cascaded cancer phototherapy with reduced skin photosensitization. *Biomaterials* 288, 121700 (2022).
8. Tian S, He J, Lyu D, Li S, Xu Q-H. Aggregation enhanced photoactivity of photosensitizer conjugated metal nanoparticles for multimodal imaging and synergistic phototherapy below skin tolerance threshold. *Nano Today* 45, 101534 (2022).
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12. Liu Z, Wang Q, Zhu Z, Liu M, Zhao X, Zhu W-H. AIE-based nanoaggregate tracker: high-fidelity visualization of lysosomal movement and drug-escaping processes. *Chem. Sci.* 11, 12755-12763 (2020).
13. Yu Y, et al. Cytophilic Fluorescent Bioprobes for Long-Term Cell Tracking. *Adv. Mater.* 23, 3298-3302 (2011).

Excitation wavelength:

The absorption peak of the monomeric compounds and the aggregates in this work is around 450 nm. This means essentially no penetration in tissues (just single cell width). This is the reason why the authors use a White LED light source, which is a non-descript identification of a light source, but is known that LEDs of this type have a very strong near UV peak. Part of the cell death in cell cultures is clearly due to white LED.

Response and revision: We appreciate the reviewer's concern regarding light penetration and the effects of our light source. Our analysis of the white LED light shows predominant peaks at 450 and 570 nm, with no detectable UV peak, which could address the concerns of the reviewers about unintended UV-induced effects (Fig. S27). Furthermore, all anticancer IC₅₀ values of tested compounds were detected using the MTT assays, and no significant effect on cell viability was detected in the control group after exposure to LED irradiation. MTT assays and control experiments demonstrate that the observed cell death is due to TBmA-Glu's photodynamic properties, not the LED light itself.

*** May be it wasn't clear in my earlier statement of concern, I did say near UV, but I was specifically referring to 450 nm peak. There are literature reports of blue (450 nm) light causing cellular damage.

Depth of Penetration in Tissues: While it is true that the penetration depth of light at 450 nm is limited, this wavelength is still within the range where some penetration can occur in biological tissues. The actual penetration depth can be influenced by factors such as tissue type, pigmentation, and the optical properties of the tissue. Furthermore, we employed a minimally invasive approach for PDT to optimize the efficiency of photodynamic therapy and minimize the impact of light penetration.

*** One of the most important issues here is the fact that short wavelength irradiation is required to excite the chromophore, whether it is in organic or aqueous medium. 450 nm is not compatible with PDT. The typical penetration length as 450 nm is less than 1 mm, which is significantly less than needed for an effective "photo"-driven process.

Changes in the Revised Manuscript:

The generation of total ROS generation (2',7'-dichlorodihydrofluorescein, DCF), hydroxyl radical (hydroxyphenyl fluorescein, HPF) and singlet oxygen (9,10-anthracenediyl-bis(methylene)dimalonic Acid, ABDA) by photosensitizers (5 μ M) after white LED light (predominant emission peaks at 450 and 570 nm, Fig. S27) irradiation (20 mW-cm⁻²) for 15 min using the corresponding ROS indicator in PBS/DMSO (v/v = 99:1). DCF, λ_{ex} = 488 nm.

Changes in the Supporting Information:

Fig. S27. The emission wavelength analysis of the LED light.

Confusion about the Type-I and Type-II PDT:

In recent articles regarding PDT, it seems like a misreading of PDT processes getting entrenched. PDT is a combination of both of these processes. Most ROS species are interconvertible by various enzymatic processes in vivo. Some articles also push the misconception that Type-I process (which are partly based on the degradation of the photosensitizers) are better, because it is less oxygen dependent; and it is not easy to separate these two processes (I/II).

Response: We appreciate the reviewer's insightful comments on the Type-I and Type-II PDT processes. We agree that PDT often involves a combination of both processes and that ROS species can undergo interconversion through various enzymatic processes in vivo. Our study focused on characterizing the predominant mechanism of TBmA under specific conditions, not comparing the superiority of Type-I vs Type-II processes. We found that the cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process. And aligning with this finding, we observed the oxygen independence of TBmA's photodynamic activity in the hypoxia condition, which is potentially advantageous in hypoxic tumor environments.

We acknowledge the complexity of PDT processes in biological systems, which may reflect both directly generated species and enzymatic interconversions. However, the ROS we detected in cells are coordinating with the results we detected in vitro, which validates the validity of our conclusion.

*** First of all, no PDT is independent of oxygen (please refer to Baptista, et al., *Photochemistry and Photobiology*, 2017, 93 (4) 912-919.) So, instead of 1 O₂ % hypoxia, if the authors were to switch to 0.5 % O₂ hypoxia, or anoxia, the effectiveness would be much more different.

I am also worried about the fact that the type-I designation is partly based on Figure 4b, there is some inconsistencies between the legend and the plot. Ebselen found in the legend, is not found on the plot, which is a singlet oxygen quencher. Also, Trolox, just like azide (N₃⁻) is a singlet oxygen quencher

Enhancement of emission "AND" PDT efficiency.

The authors should also keep in mind that any emission from the aggregates, is a loss in ROS generation efficiency. So, AIE-PDT carries a certain self-contradictory character.

Response: We appreciate the reviewer's insights regarding the competitive nature of fluorescence and reactive oxygen species (ROS) generation in AIE-PDT systems. While both processes utilize energy from the excited state, our findings on the simultaneous enhancement of aggregate luminescence and photodynamic activity are not contradictory. Here is some reported literature.

(i) Aggregation-induced intermolecular intersystem crossing (AI-ISC): Jiang et al. proposed a new mechanism called aggregation-induced intersystem crossing (AI-ISC) to understand the effect of aggregation on increasing ISC efficiency.^{1, 2} According to the AI-ISC theory, more excitonic couplings cause excited-state energy splitting and overlapping of singlet and triplet in aggregate. The energy splitting and overlapping significantly produce many ISC channels with very small ΔE_{ST} in aggregates, which is available for ISC processes. Therefore, the formation of aggregates can facilitate the production of triplet excitons. In addition to emitting phosphorescent radiation, these triplet excitons can also undergo a non-radiative pathway known as the aggregation-enhanced photodynamic effect to return to their ground state.^{3, 4, 5}

*** Regardless of the mechanism, the total quantum yield of all radiative and non-radiative processes is not going to be larger than 1. So far, I did not come across a quantum yield of ROS formation, or emission quantum yield reported with aggregated structures. However, that should be the first thing to be studied when reporting a novel photosensitizer, but especially so, when both emission and ISC is claimed to be enhanced.

(ii) Restriction of intramolecular motion (RIM): The aggregation of AIE molecules results in a restriction of intramolecular rotations and vibrations, effectively suppressing molecular motions, which is also beneficial for the ISC process.^{6, 7} All the evidence highlights the potential of AIE materials in PDT. The aggregation-induced changes in the molecular environment can optimize both the imaging and therapeutic aspects of the treatment.^{8, 9, 10}

*** Imaging on surface tumors or in mice, perhaps; but not therapeutics. Short wavelength excitation, and their aggregate structure, which would most likely disintegrate as it travels through the body into different sized nanoparticles would limit their potential.

References:

1. Li Q, et al. Time-dependent photodynamic therapy for multiple targets: A highly efficient aie-active photosensitizer for selective bacterial elimination and cancer cell ablation. *Angew. Chem. Int. Ed.* 59, 9470-9477 (2020).
2. Liu Z, et al. Tuning organelle specificity and photodynamic therapy efficiency by molecular function design. *ACS Nano* 13, 11283-11293 (2019).
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9. Lee E, et al. A boronic acid-functionalized phthalocyanine with an aggregation-enhanced photodynamic effect for combating antibiotic-resistant bacteria. *Chem. Sci.* 11, 5735-5739 (2020).

10. Wan Q, et al. Molecular engineering to boost aie-active free radical photogenerators and enable high-performance photodynamic therapy under hypoxia. *Adv. Func. Mater.* 30, 2002057 (2020).

Reviewer #2

(Remarks to the Author)

The authors have addressed all the concerns in the revisions. And the manuscript is ready to be published.

Reviewer #3

(Remarks to the Author)

The paper's focus is the enzymatically catalyzed molecular aggregation for improving the response and PDT treatment. The paper has been revised accordingly, and ready for publication.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Response

General opinion

The present manuscript claims to achieve a better targeting of a proposed photosensitizer (PS) which can be activated by GGT and be excited at short wavelengths.

Also, activated (aggregated structure) may have a better cytotoxic effect, compared to its non-activated form, which is another example of activated-PS

There have been countless photosensitizers which can be targeted one way or another. Many reviews exist about activatable photosensitizers. Some enzymatically, some by hypoxia, by higher H₂O₂ or GSH concentrations, or acidic pH.

The main, may be the only reason why PDT did not develop significantly since 70's is that fact that light, even at the so-called "therapeutic window" does not go through tissues. And of course, there is no real justification for a 450 nm chromophore to be proposed as a novelty. There are very specific, niche cases, where a single cell layer penetration may be useful. But citing these, is missing the point of all PDT-work.

Type-I processes being less oxygen dependent has been proposed without real evidence. The new data provided by the authors is also not a fair comparison (see below).

Thus the manuscript does not bring any novelty to the field. The requirement for aggregation, if anything, complicates the picture very unnecessarily.

Reviewer #1 (Remarks to the Author):

Q1: The authors are accurate in stating that a comparison was not made (see the green text below from the manuscript). However, they claim that oxygen content has a negligible influence on the observed activity under hypoxia, which is linked to Type-I process, based on previous claims. These claims were not supported by "negative controls" with standard PDT photosensitizers, in a fair comparison.

251. "only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d). This suggests that oxygen content has negligible influence on its photodynamic activity. The cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process, which is consistent with the prior findings."

Response and revision: We appreciate the valuable suggestions provided by the reviewer. To enhance the accuracy of our study, we conducted additional experiments using Rose Bengal (RB), a well-established type II photosensitizer¹, as a control. These experiments revealed that RB's photodynamic efficiency decreased significantly under hypoxia conditions (2% O₂) compared to normoxia conditions, while TBmA maintained relatively consistent activity across both environments (Fig. R1).

To discuss this result more accurately, we have revised our manuscript by replacing the statement "This suggests that oxygen content has negligible influence on its photodynamic activity." with "This suggests that TBmA exhibits tolerance towards hypoxic conditions."

Fig. S36. The effects of hypoxia (2% O₂) and normoxia (20% O₂) conditions on the anticancer photodynamic efficiency of Rose Bengal against HepG2 cells.

Revised in manuscript:

Additionally, only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d), while the type-II PS, RB, showed a significant decrease in photodynamic efficiency under hypoxia conditions (2% O₂) compared to normoxia conditions (Fig. S36). This suggests that TBmA exhibits tolerance towards hypoxic conditions.

Reference:

1. Fischer BB, Krieger-Liszky A, Eggen RIL. Oxidative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different molecular responses in *Chlamydomonas reinhardtii*. *Plant Sci.* 168, 747-759 (2005).

response

+++ Light source in Fig S36 was not given, if it is white LED, it is not a fair comparison, because LED emission profile fits TbmA/aggregate better.

Q2: Aggregation is a type of supramolecular association which is perfectly reversible. It is only natural to expect deaggregation in the biological media with so many different gradients of hydrophobicity. FBS is not a good approximation for intracellular medium as its protein content is very low. Of course, a simple pharmacokinetics study would reveal how stable is those aggregates are *in vivo*.

Response: We appreciate the reviewer's insightful comments regarding the nature of supramolecular aggregation and the potential for de-aggregation in biological media. We would like to clarify several key points that address these concerns.

Firstly, it's crucial to emphasize that TBmA-Glu is a water-soluble prodrug. The aggregation process only occurs after the Glu moiety is cleaved by GGT in HepG2 cells. This design ensures that TBmA-Glu remains soluble in the blood, avoiding premature aggregation. Aggregation is triggered specifically in the intracellular environment of GGT-overexpressing tumor cells.

We acknowledge that FBS is not an ideal model for the intracellular environment. To address this issue, we further conducted stability studies using a 30% BSA (Bovine Serum Albumin) solution, which is a better model for the protein-rich intracellular milieu. The intracellular protein concentration typically ranges from 50-400 mg/mL, and our 30% BSA solution (~300 mg/mL) falls within this range. TBmA aggregates showed remarkable stability in this environment, with no significant degradation observed over 72 hours (Fig. R1).

We also agree that pharmacokinetics studies would be valuable. However, our system presents unique challenges for such studies, as the aggregates form intracellularly rather than in circulation. Collecting and analyzing intracellular aggregates from tumor sections poses significant technical difficulties. Our approach using a highly concentrated protein solution provides valuable insights into aggregate stability in a physiologically relevant environment.

Importantly, beyond structural stability, we have observed that the aggregates maintain their photodynamic properties in the 30% BSA solution for 72 h (Fig. R2). This functional stability is crucial for the compound's theranostic applications.

Fig. R1. The long-term stability of TBmA aggregates in 30% BSA solutions.

Fig. R2. The ROS generation capacity of TBmA aggregates after dispersed in 30% BSA solution for 0 h (a) and 72 h (b). The ROS was identified using DCFH as an indicator. (c) The plot of the relative emission intensity (I/I_0) of DC versus the irradiation ($20 \text{ mW} \cdot \text{cm}^{-2}$) time, where I_0 = PL intensity of DCFH in solutions without light irradiation.

Q3: References 4, 5 and 6 were carefully checked. Stability of the aggregates "in vivo" was not studied in these articles. Retention of fluorescence is not necessarily a sign of stability.

Response: We thank the reviewer for the critical feedback. The unique photophysical properties of AIE compounds stem from the restriction of intramolecular motion (RIM) mechanism, where aggregation limits molecular rotations and vibrations, leading to enhanced fluorescence. Therefore, the fluorescence behavior of AIE materials does provide valuable insights into their molecular state and environment.

This interpretation is supported by several factors. First of all, TBmA-Glu is engineered to aggregate specifically in response to GGT activity, which is overexpressed in certain tumor cells. This targeted approach minimizes premature aggregation in circulation. Secondly, the crowded, protein-rich cytoplasmic environment of tumor cells likely provides conditions that favor aggregate stability once formed. Additionally, we observed that the photosensitivity of TBmA was maintained in our 30% BSA studies, suggesting a preservation of the aggregate structure.

Q4: While AIE compounds seem to provide potentially useful imaging opportunities, their relevance in PDT or other therapeutic schemes remain questionable. A therapeutic agent which would change size on meeting hydrophobic membranes or proteins, which could lead to different properties has to be handled very carefully. It would be advisable to

avoid hype terminology such as “personalized medicine and real-time treatment monitoring”.

Response: We appreciate the reviewer’s thoughtful comments regarding the therapeutic relevance of AIE compounds and the importance of careful characterization of their behavior in biological systems.

Regarding the stability and behavior of TBmA, we emphasize that TBmA-Glu is designed as a water-soluble prodrug that only forms aggregates within tumor cells following enzymatic reaction. This targeted approach minimizes potential issues related to premature aggregation or size changes in circulation. Furthermore, we have demonstrated the stability of TBmA aggregates in a 30% BSA solution for 72 hours, providing initial evidence of their potential stability in protein-rich environments.

About “personalized medicine and real-time treatment monitoring.” in the previous response letter: The full sentence is “Recent literature has demonstrated the potential of AIE compounds for combining imaging and therapeutic functions in a single entity opening up new possibilities for personalized medicine and real-time treatment monitoring.” We agree that such terminology should be used judiciously, especially in early-stage research, however, our intention here is to highlight the potential of AIE materials to contribute to these fields in the future, rather than to claim immediate clinical applicability. The unique properties of AIE materials, including their AIE and potential for stimuli-responsive behavior, do offer intriguing possibilities for both imaging and therapeutic applications. However, we agree that rigorous investigation is needed to establish their efficacy and safety for PDT or other therapeutic schemes. Moving forward, we will focus on providing concrete evidence for the specific advantages of AIE compounds in relevant biological contexts, rather than speculating on broad future applications. We believe this approach will better serve the scientific community and responsibly advance the field.

Q5: May be it wasn’t clear in my earlier statement of concern, I did say near UV, but I was specifically referring to 450 nm peak. There are literature reports of blue (450 nm) light causing cellular damage.

Response: We acknowledge that there are indeed literature reports of blue light (450 nm) causing cellular damage. This is an important consideration in photodynamic therapy and other light-based treatments. However, we would like to emphasize that the biological effects of light exposure are highly dependent on both wavelength and dosage.

In our experiments, we carefully controlled the light dosage to minimize potential phototoxicity while maintaining therapeutic efficacy. Under the experimental conditions described in our manuscript, we did not observe any significant effects on cell viability following LED light irradiation (Fig. R3).

To address the reviewer’s concern, we also conducted a blue light irradiation (450 nm, 12 J/cm²) PDT assay. In this experiment, we also found no significant effect on cellular viability. This suggests that at the dosages used in our study, the blue light alone does not cause substantial cellular damage.

However, we agree that the potential for phototoxicity is an important consideration in developing light-based therapies. In future studies, we plan to conduct a more comprehensive dose-response analysis to determine the threshold at which blue light exposure may begin to affect cell viability. We also intend to investigate the potential long-term effects of repeated light exposure and compare the effects of our AIE-based approach with traditional photosensitizers at equivalent light doses.

Fig. R3 The impact of white light and 450 nm light exposure (12 J/cm²) on the cellular viability of HepG2 cells.

Q6: One of the most important issues here is the fact that short wavelength irradiation is required to excite the chromophore, whether it is in organic or aqueous medium. 450 nm is not compatible with PDT. The typical penetration length as 450 nm is less than 1 mm, which is significantly less than needed for an effective “photo”-driven process.

Response: It is correct that the typical penetration depth of 450 nm light is less than 1 mm in tissue, which is indeed less than ideal for treating deep-seated tumors. However, we would like to highlight several important considerations:

First, though direct light penetration is restricted, the effective depth of PDT damage may increase due to light reflection and scattering within tissues. This occurrence can expand the scope of the photodynamic impact beyond the initial penetration depth.

Secondly, several clinical scenarios exist where shallow light penetration is sufficient or even advantageous. For instance, PDT with blue light excitation could be particularly useful for superficial skin cancers and precancerous lesions, intraoperative treatment of residual tumor cells after surgical resection, treatment of early-stage mucosal cancers in inaccessible areas (e.g., oral cavity, bladder), and endoscopic applications for gastrointestinal tumors.

Finally, numerous published studies demonstrate the successful use of 450 nm light and white light (including the blue spectrum) for PDT when the photosensitizers have maximum absorption around 450 nm. 2, 3, 4, 5, 6, 7

Nevertheless, we fully agree that blue light’s limited tissue penetration restricts the broader applicability of our current system for treating deep-seated tumors. Given this limitation, our future research directions include exploring two-photon excitation to achieve deeper tissue penetration, investigating upconversion nanoparticles to convert longer-wavelength light to blue light locally, and developing new AIE photosensitizers with red-shifted absorption for improved tissue penetration. We believe that addressing these challenges will expand the potential applications of our AIE-based PDT system while utilizing its unique properties.

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4. Mei Y, et al. A Novel Photosensitizer Based 450-nm Blue Laser-Mediated Photodynamic Therapy Induces Apoptosis in Colorectal Cancer - in Vitro and in Vivo Study. *Front. Biosci. (Landmark Ed)* 29, 199 (2024).

5. Chen Y, et al. Photoactivatable metal organic framework for synergistic ferroptosis and photodynamic therapy using 450 nm laser. *Chem. Eng. J.* 454, 140438 (2023).
6. Sun P, et al. A water-soluble phosphorescent conjugated polymer brush for tumor-targeted photodynamic therapy. *Polym. Chem.* 8, 5836-5844 (2017).
7. An J, et al. An unexpected strategy to alleviate hypoxia limitation of photodynamic therapy by biotinylation of photosensitizers. *Nat. Commun.* 13, 2225 (2022).

Q7: First of all, no PDT is independent of oxygen (please refer to Baptista, et al., *Photochemistry and Photobiology*, 2017, 93 (4) 912-919.) So, instead of 1 O₂ % hypoxia, if the authors were to switch to 0.5 % O₂ hypoxia, or anoxia, the effectiveness would be much more different.

I am also worried about the fact that the type-I designation is partly based on Figure 4b, there is some inconsistencies between the legend and the plot. Ebselen found in the legend, is not found on the plot, which is a singlet oxygen quencher. Also, Trolox, just like azide (N₃⁻) is a singlet oxygen quencher.

Response and revision: We agree with the reviewer that oxygen plays a pivotal role in the Type I and Type II PDT processes. However, from the PDT mechanism, we know that the type I photosensitizers could directly transfer electrons to the substrate, forming a radical cation or neutral radical. These radicals could immediately react with O₂ or H₂O to generate hydrogen peroxide (H₂O₂), hydroxyl radicals (\cdot OH), or superoxide anions (\cdot O₂⁻) (Fig. R4).^{1, 2}

We have tried but could not finish the antitumor PDT assays in the anaerobic conditions, because the anoxia condition resulted in death of the tumor cells (Fig. R5a). So, we re-evaluated the photodynamic efficiency of TBmA and RB using a deoxidized PBS solution. The results showed that TBmA could also induce the oxidation of DFCH under the anoxia condition (Fig. R5b), while the photodynamic efficiency of RB showed significant degradation. Hence, type-I photosensitizers exhibit relatively higher tolerance towards oxygen concentrations, which implies that, even under low oxygen conditions, they can still engage in substrate reactions through electron transfer.

We are sorry for the mistake in the figure legend in Figure 4b. "Ebselen" has been revised as "Trolox." However, it should be noted that Trolox is not only a 1O₂ scavenger but also a scavenger of peroxy and alkoxy groups.³ The type-I designation is mainly based on the ROS species we detected in vitro (Fig. R5c).

Fig. R4 Scheme of the photochemical reactions for type I and type II PDT.⁹

Fig. R5 (a) Cellular viability of HepG2 cells in normoxia and anoxia conditions. (b) Fluorescence emission changes of DCFH (Dichlorodihydrofluorescein, 10 μ M) in the presence of 5 μ M photosensitizers in DMSO-PBS (v:v = 1:99) after irradiation (20 mW·cm⁻²) for a different time under anoxia conditions. (b) TBmA, (c) Rose Bengal (RB). DCHF, λ_{ex} = 488 nm.

Revised in manuscript:

Trolox: 50 μ M (ROO \cdot scavenger and 1O₂ scavenger); D-mannitol: 50 mM (\cdot OH scavenger); Tiron: 10 mM (\cdot O₂⁻ scavenger); NaN₃: 5 mM (1O₂ scavenger)

References:

1. Zhao X, Liu J, Fan J, Chao H, Peng X. Recent progress in photosensitizers for overcoming the challenges of photodynamic therapy: from molecular design to application. *Chem. Soc. Rev.* 50, 4185-4219 (2021).
2. Fan W, Huang P, Chen X. Overcoming the Achilles' heel of photodynamic therapy. *Chem. Soc. Rev.* 45, 6488-6519 (2016).
3. Lúcio M, Nunes C, Gaspar D, Ferreira H, Lima JLFC, Reis S. Antioxidant Activity of Vitamin E and Trolox: Understanding of the Factors that Govern Lipid Peroxidation Studies In Vitro. *Food Biophys.* 4, 312-320 (2009).

+++ 0.5 or 1 % hypoxia may be better.

Q8: Regardless of the mechanism, the total quantum yield of all radiative and not radiative processes is not going to be larger than 1. So far, I did not come across a quantum yield of ROS formation, or emission quantum yield reported with aggregated structures. However, that should be the first thing to be studied when reporting a novel photosensitizer, but especially so, when both emission and ISC is claimed to be enhanced.

Response: Indeed, the total quantum yield of all radiative and non-radiative processes cannot exceed 1. However, the energy consumption in no radiative processes contains both the energy for ISC processes and the molecular motion as well. Molecular aggregation could induce the restriction of intramolecular motions (RIM) and, as a result, reduce energy loss through non-radiative molecular motion, potentially increasing the energy available for emission and ISC processes. So, the energy efficiency of both emission and ISC can be enhanced in aggregated structure due to RIM.

However, in specific cases, such as the graphene quantum dots reported by Zhang et al., the apparent quantum yield could be larger than 1.¹ This occurs when the energy gaps between Δ EST and Δ ETG (the energy gap between T1 and Ground state) are larger than the formation energy of 1O₂ (22.5 kcal mol⁻¹). In such cases, 1O₂ generation happens through multiple pathways: energy transfer from T1 (ET(1) in Fig. R6), but also the energy transfer from S1 to 3O₂ during the S1-T1 intersystem crossing transition (ET(2) in Figure R6). This multi-pathway mechanism can lead to an overall 1O₂ quantum

yield greater than 1.0, as more than one $1O_2$ molecule can be produced per absorbed photon.²

Fig. R6 Schematic illustration of the $1O_2$ generation mechanisms by conventional PDT agents (left) and GQDs (right).

References

1. Ge J, et al. A graphene quantum dot photodynamic therapy agent with high singlet oxygen generation. *Nat. Commun.* 5, 4596 (2014).
2. Kanner RC, Foote CS. Singlet oxygen production from singlet and triplet states of 9,10-dicyanoanthracene. *J. Am. Chem. Soc.* 114, 678-681 (1992).

+++ Both of these articles while interesting, hardly relevant to PDT considering the absorption peaks of the proposed sensitizers are in blue, and the fact that they are very unique cases. The first one reached to a surprising conclusion without doing any photophysical work. Vibrational (or rotational) relaxation and their control by micro- or molecular environments, by molecular steric hindrance is well known. However, only accurate quantum yield determinations would prove simultaneous increases in emission and singlet oxygen quantum yields. This is not done in Ref 1.

Q9: Imaging on surface tumors or in mice, perhaps; but not therapeutics. Short wavelength excitation, and their aggregate structure, which would most likely disintegrate as it travels through the body into different sized nanoparticles would limit their potential.

Response: As previously discussed, TBmA-Glu is a water-soluble molecule that forms aggregates within tumor cells upon activation by GGT to produce TBmA. Consequently, most of these aggregates are localized in the tumor cells. Furthermore, we have demonstrated the stability of TBmA aggregates for 72 hours in a 30% BSA solution. Additionally, considering that PDT processes were conducted 12 hours after administration of TBmA-Glu, it can be inferred that the TBmA aggregates exhibit sufficient stability to complete the PDT processes.

+++ The problem is that now "activated" aggregates, will not stay forever in tumor cells, as these cells disintegrate.

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Point-by-Point Responses

We are very grateful to the reviewers for their insightful comments, which have significantly enhanced the quality of our paper. Following their valuable feedback and suggestions, we have carefully revised the manuscript. Our responses and revisions are presented in a blue font for easy reference.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors report two compounds that they claim to undergo enzyme triggered aggregation (both in vitro and vivo) leading to effective PDT outcomes, partly due to a "type-I PDT" process they claim to be better than "type II".

This referee finds important design flaws and problems with the implementation of the work.

Response: We thank the reviewer for the constructive comments on our paper. In this manuscript, we actually did not intend to compare the Type I and Type II PDT processes, and the efficiency of type I and type II photosensitizers is not the key point of this work. We just want to present the objective performance of the developed photosensitizers based on their ROS generation capability. As clearly stated in the manuscript: "It was found that TBmA and TBpA produced significantly higher ROS compared to TBmA-Glu and TBpA-Glu, even surpassing the commercial photosensitizer, Rose Bengal (RB). Moreover, TBmA was identified as the most potent photosensitizer among the four compounds. Further analysis revealed that TBmA and TBpA functioned as strong type I photosensitizers (Fig. 1c and Fig. S25), while TBmA-Glu and TBpA-Glu acted as very weak type II photosensitizers (Fig. 1c and Fig. S26)."

Here are the major issues:

Problem with Aggregates:

While AIE compounds may be interesting for imaging perhaps mostly in cell culture and mice models (considering limited light penetration) as a therapeutic agent, aggregate structures are ill-defined and especially in high protein milieus, not very stable and difficult to standardize. More to the point, there is really no real justification for the use of aggregates, considering the fact molecular drugs or photosensitizers are better than the aggregates in many aspects, and nothing new is offered or even suggested by these compounds.

Response and revision: Thank you for your thoughtful comments regarding AIE compounds. We appreciate your concerns and would like to address them point by point:

(i) **Stability and standardization:** we acknowledge that stability is crucial for bio-application. Our recent studies have shown promising results regarding the stability of TBmA aggregates in high-protein environments, specifically: (a) **Long-term stability:** TBmA aggregates showed no significant degradation when dispersed in FBS for 72 hours (Fig. S28a and S28c). (b) **Photostability:** The aggregates remained stable under continuous light irradiation for 30 minutes (Fig. S28a and S28b). These findings collectively highlight the exceptional

stability exhibited by TBmA. These findings demonstrate the exceptional stability of TBmA aggregates in biologically relevant conditions.

Moreover, numerous AIEgens, including small molecules or AIE nanoparticles, have been extensively reported for their long-term monitoring and theranostic applications.^{1, 2, 3} These pieces of evidence underscore the remarkable stability of AIEgens, making them highly promising candidates for theranostic applications.

(ii) **Aggregate structure:** To address concerns about ill-defined aggregate structures, we extensively investigated the aggregate size of TBmA using Dynamic Light Scattering (DLS) and Transmission Electron Microscope (TEM). The results suggest that the TBmA forms spherical particles with 140 nm in 99% PBS and 150 nm diameter after the GGT catalysis reaction (12 h, Fig. S31). These results indicate that TBmA consistently forms nanoparticles of definite shape and size in aqueous environments, regardless of the specific conditions. Numerous works have been reported to show the definite shape and size, as well as the excellent stability and biocompatibility of the AIE aggregates.^{4, 5, 6}

(iii) **A comparative analysis of small molecular drugs and AIE materials:** Although molecular drugs and traditional photosensitizers have their advantages, AIE compounds offer unique benefits such as enhanced emission upon aggregation, responsiveness to stimuli, and multifunctional potential. Revealing reports increasingly indicate that small molecular photosensitizers, such as CE6, exhibit low solubility and undergo aggregation in solution, resulting in the deactivation of their photosensitizing activity and hindering their bioapplication.^{7, 8, 9, 10} We believe that AIE compounds can serve as complementary agents, rather than substitutes, for small molecule drugs. Recent literature has demonstrated the potential of AIE compounds for combining imaging and therapeutic functions in a single entity opening up new possibilities for personalized medicine and real-time treatment monitoring.^{4, 11, 12, 13}

We believe that AIE compounds, including TBmA-Glu, offer valuable and unique properties that complement existing molecular drugs and photosensitizers. While challenges remain, the growing body of research on AIE materials suggests significant potential for advancing biomedical imaging and therapeutic applications. We appreciate the reviewer's perspective and believe that continued research and development in this field will address current limitations and unlock new possibilities in biomedical science.

Changes in the Revised Manuscript:

Moreover, the TBmA aggregates exhibited excellent long-term stability (Fig. S28a and S28c) and photodynamic stability (Fig. S28a and S28b), no significant aggregation or degradation was found after dispersed in FBS (fetal bovine serum) solution for 72 h or light irradiated for 30 min.

Changes in the Supporting Information:

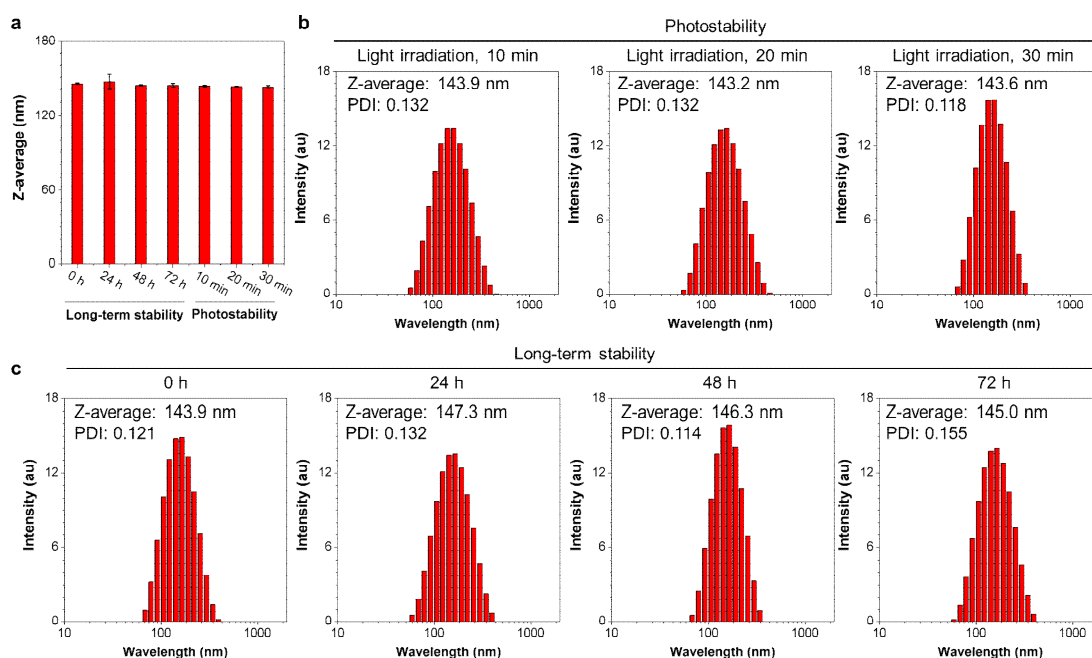


Fig. S28. (a) The average hydrodynamic diameter (*Z*-average) of TBmA aggregates measured by Dynamic Light Scattering (DLS). The distribution of TBmA aggregates during 30 min light irradiation and 72 h FBS preservation.

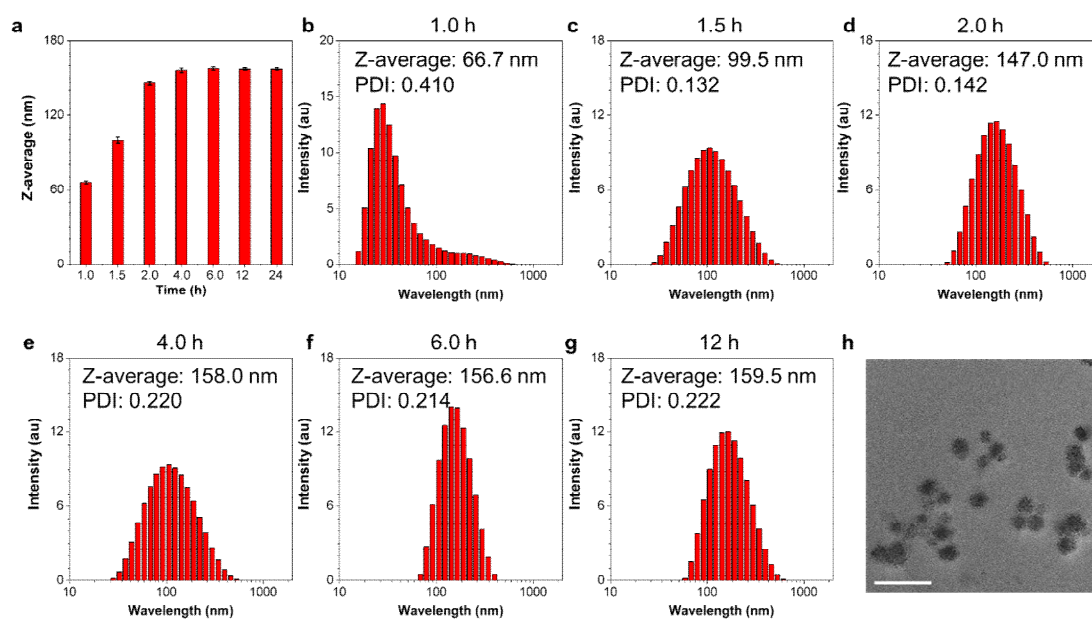


Fig. S31. (a) The average hydrodynamic diameter (*Z*-average) of TBmA aggregates produced in GGT catalytic reaction measured by DLS. (b-g) Distribution of TBmA aggregates formed at different times of GGT catalytic reaction. (h) The transmission electron microscope (TEM) of the TBmA aggregates formed after the GGT catalytic reaction for 12 h.

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1. Zuo J, et al. Long-term spatiotemporal and highly specific imaging of the plasma membrane of diverse plant cells using a near-infrared AIE probe. *Angew. Chem. Int. Ed.* **14**, 2139-2148 (2023).
2. Wang Z, et al. Long-term fluorescent cellular tracing by the aggregates of aie bioconjugates. *J. Am. Chem. Soc.* **135**, 8238-8245 (2013).
3. Li K, et al. Photostable fluorescent organic dots with aggregation-induced emission (AIE dots) for noninvasive long-term cell tracing. *Sci. Rep.* **3**, 1150 (2013).
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7. Li Y, et al. Near-infrared light and redox dual-activatable nanosystems for synergistically cascaded cancer phototherapy with reduced skin photosensitization. *Biomaterials* **288**, 121700 (2022).
8. Tian S, He J, Lyu D, Li S, Xu Q-H. Aggregation enhanced photoactivity of photosensitizer conjugated metal nanoparticles for multimodal imaging and synergistic phototherapy below skin tolerance threshold. *Nano Today* **45**, 101534 (2022).
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10. Li X, et al. Nanostructured Phthalocyanine Assemblies with Protein-Driven Switchable Photoactivities for Biophotonic Imaging and Therapy. *J. Am. Chem. Soc.* **139**, 10880-10886 (2017).
11. Chen C, Zhang X, Gao Z, Feng G, Ding D. Preparation of AIEgen-based near-infrared afterglow luminescence nanoprobes for tumor imaging and image-guided tumor resection. *Nat. Protoc.*, in press (2024).
12. Liu Z, Wang Q, Zhu Z, Liu M, Zhao X, Zhu W-H. AIE-based nanoaggregate tracker: high-fidelity visualization of lysosomal movement and drug-escaping processes. *Chem. Sci.* **11**, 12755-12763 (2020).
13. Yu Y, et al. Cytophilic Fluorescent Bioprobes for Long-Term Cell Tracking. *Adv. Mater.* **23**, 3298-3302 (2011).

Excitation wavelength:

The absorption peak of the monomeric compounds and the aggregates in this work is around

450 nm. This means essentially no penetration in tissues (just single cell width). This is the reason why the authors use a White LED light source, which is a non-descript identification of a light source, but is known that LEDs of this type have a very strong near UV peak. Part of the cell death in cell cultures is clearly due to white LED.

Response and revision: We appreciate the reviewer's concern regarding light penetration and the effects of our light source. Our analysis of the white LED light shows predominant peaks at 450 and 570 nm, with no detectable UV peak, which could address the concerns of the reviewers about unintended UV-induced effects (Fig. S27). Furthermore, all anticancer IC₅₀ values of tested compounds were detected using the MTT assays, and no significant effect on cell viability was detected in the control group after exposure to LED irradiation. MTT assays and control experiments demonstrate that the observed cell death is due to TBmA-Glu's photodynamic properties, not the LED light itself.

Depth of Penetration in Tissues: While it is true that the penetration depth of light at 450 nm is limited, this wavelength is still within the range where some penetration can occur in biological tissues. The actual penetration depth can be influenced by factors such as tissue type, pigmentation, and the optical properties of the tissue. Furthermore, we employed a minimally invasive approach for PDT to optimize the efficiency of photodynamic therapy and minimize the impact of light penetration.

Changes in the Revised Manuscript:

The generation of total ROS generation (2',7'-dichlorodihydrofluorescein, DCF), hydroxyl radical (hydroxyphenyl fluorescein, HPF) and singlet oxygen (9,10-anthracenediyl-bis(methylene)dimalonic Acid, ABDA) by photosensitizers (5 μ M) after white LED light (predominant emission peaks at 450 and 570 nm, Fig. S27) irradiation (20 mW·cm⁻²) for 15 min using the corresponding ROS indicator in PBS/DMSO (v/v = 99:1). DCF, λ_{ex} = 488 nm.

Changes in the Supporting Information:

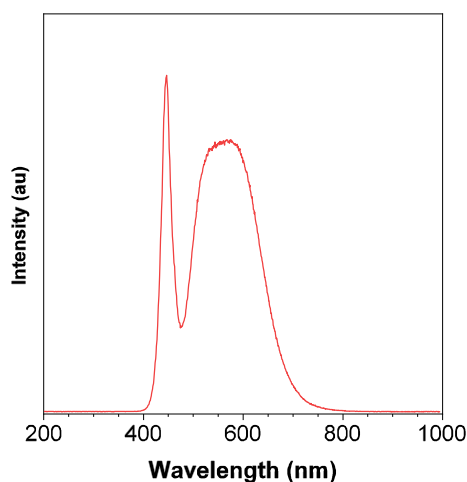


Fig. S27. The emission wavelength analysis of the LED light.

Confusion about the Type-I and Type-II PDT:

In recent articles regarding PDT, it seems like a misreading of PDT processes getting entrenched. PDT is a combination of both of these processes. Most ROS species are interconvertible by various enzymatic processes *in vivo*. Some articles also push the misconception that Type-I process (which are partly based on the degradation of the photosensitizers) are better, because it is less oxygen dependent; and it is not easy to separate these two processes (I/II).

Response: We appreciate the reviewer's insightful comments on the Type-I and Type-II PDT processes. We agree that PDT often involves a combination of both processes and that ROS species can undergo interconversion through various enzymatic processes *in vivo*. Our study focused on characterizing the predominant mechanism of TBmA under specific conditions, not comparing the superiority of Type-I vs Type-II processes. We found that the cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process. And aligning with this finding, we observed the oxygen independence of TBmA's photodynamic activity in the hypoxia condition, which is potentially advantageous in hypoxic tumor environments.

We acknowledge the complexity of PDT processes in biological systems, which may reflect both directly generated species and enzymatic interconversions. However, the ROS we detected in cells are coordinating with the results we detected *in vitro*, which validates the validity of our conclusion.

Enhancement of emission "AND" PDT efficiency.

The authors should also keep in mind that any emission from the aggregates, is a loss in ROS generation efficiency. So, AIE-PDT carries a certain self-contradictory character.

Response: We appreciate the reviewer's insights regarding the competitive nature of fluorescence and reactive oxygen species (ROS) generation in AIE-PDT systems. While both processes utilize energy from the excited state, our findings on the simultaneous enhancement of aggregate luminescence and photodynamic activity are not contradictory. Here is some reported literature.

(i) Aggregation-induced intermolecular intersystem crossing (AI-ISC): Jiang et al. proposed a new mechanism called aggregation-induced intersystem crossing (AI-ISC) to understand the effect of aggregation on increasing ISC efficiency.^{1,2} According to the AI-ISC theory, more excitonic couplings cause excited-state energy splitting and overlapping of singlet and triplet in aggregate. The energy splitting and overlapping significantly produce many ISC channels with very small ΔE_{ST} in aggregates, which is available for ISC processes. Therefore, the formation of aggregates can facilitate the production of triplet excitons. In addition to

emitting phosphorescent radiation, these triplet excitons can also undergo a non-radiative pathway known as the aggregation-enhanced photodynamic effect to return to their ground state.^{3, 4, 5}

(ii) Restriction of intramolecular motion (RIM): The aggregation of AIE molecules results in a restriction of intramolecular rotations and vibrations, effectively suppressing molecular motions, which is also beneficial for the ISC process.^{6, 7}

All the evidence highlights the potential of AIE materials in PDT. The aggregation-induced changes in the molecular environment can optimize both the imaging and therapeutic aspects of the treatment.^{8, 9, 10}

We hope that these clarifications address the reviewer's concerns and reinforce the validity of our findings. We are grateful for the opportunity to discuss these important aspects of AIE-PDT and hope that our response provides a clearer understanding of the complex interplay between fluorescence and photodynamic activity in our study.

References:

1. Li Q, *et al.* Time-dependent photodynamic therapy for multiple targets: A highly efficient aie-active photosensitizer for selective bacterial elimination and cancer cell ablation. *Angew. Chem. Int. Ed.* **59**, 9470-9477 (2020).
2. Liu Z, *et al.* Tuning organelle specificity and photodynamic therapy efficiency by molecular function design. *ACS Nano* **13**, 11283-11293 (2019).
3. Lee E, *et al.* A boronic acid-functionalized phthalocyanine with an aggregation-enhanced photodynamic effect for combating antibiotic-resistant bacteria. *Chem. Sci.* **11**, 5735-5739 (2020).
4. Wan Q, *et al.* Molecular engineering to boost aie-active free radical photogenerators and enable high-performance photodynamic therapy under hypoxia. *Adv. Func. Mater.* **30**, 2002057 (2020).
5. Ji C, Lai L, Li P, Wu Z, Cheng W, Yin M. Organic dye assemblies with aggregation-induced photophysical changes and their bio-applications. *Aggregate* **2**, e39 (2021).
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9. Lee E, *et al.* A boronic acid-functionalized phthalocyanine with an aggregation-enhanced photodynamic effect for combating antibiotic-resistant bacteria. *Chem. Sci.* **11**, 5735-5739

(2020).

10. Wan Q, *et al.* Molecular engineering to boost aie-active free radical photogenerators and enable high-performance photodynamic therapy under hypoxia. *Adv. Func. Mater.* **30**, 2002057 (2020).

Reviewer #2 (Remarks to the Author):

Tang and colleagues reported a γ -glutamyl transferase (GGT) activatable aggregation-induced emission photosensitizer (AIE-PS) named TBmA-Glu. TBmA-Glu is designed to specifically target and aggregate cancer cells through the catalytic action of tumor-overexpression GGT. Selective tumor cell aggregation not only enhances AIE-PS emission and photodynamic activity but also induces ferroptosis in cancer cells by depleting GSH and promoting lipid peroxidation. Both in vitro cell assays and in vivo animal models were used to validate the phototoxicity and antitumor effects of TBmA-Glu, providing a comprehensive assessment of its potential as a therapeutic agent. The aggregation strategies in this paper allow a controlled release of the photodynamic effect, which is critical for therapies such as PDT. AIE-PS remains dormant until it reaches the cancerous environment where GGT is present, ensuring minimal impact on healthy cells and maximizing the therapeutic effect on cancer cells. This study highlights the significance of targeting activation of AIE-PSs for targeting and enhanced cancer photodynamic therapy. It is also a sophisticated strategy for targeted delivery and activation of a photodynamic therapeutic agent for disease. I suggest this article be published with minor revisions.

Response: We would like to express our gratitude for the favorable remarks provided by the reviewer regarding our manuscript.

1. The authors claimed that TBmA-Glu could be activated by GGT. Is there evidence to suggest that TBmA-Glu could be effective against other types of cancer that overexpress GGT, or is its application currently limited to the cancer types studied?

Response and revision: We appreciate the reviewer's suggestion and subsequently conducted further investigations into the anticancer efficacy of TBmA-Glu on additional cancer cell lines overexpressing GGT, including OVCAR-5 cells and 4T1 cells (mouse breast cancer cells), as well as HLF-1 cells with regular GGT expression, using MTT assays. The IC₅₀ values for photodynamic therapy were determined to be $5.13 \pm 0.69 \mu\text{M}$ (OVCAR-5) and $5.28 \pm 1.56 \mu\text{M}$ (4T1, Fig. S35), respectively. Moreover, no significant photocytotoxicity was observed in HLF-1 cells with regular GGT expression, nor any significant dark cytotoxicity. These results demonstrate the broad-spectrum anticancer potential of TBmA-Glu against GGT-overexpressing cancer cells, and the rationale for the broader application of TBmA-Glu lies in the overexpression of GGT in various cancerous conditions. Therefore, the potential

effectiveness of TBmA-Glu could extend to other cancers that exhibit elevated GGT levels.

Changes in the Revised Manuscript:

The broad-spectrum anticancer potential of TBmA-Glu against GGT-overexpressing cancer cells was further demonstrated through subsequent investigations, including OVCAR-5 and murine 4T1 cancer cells (Fig. S35). The IC_{50} values for photodynamic therapy were determined to be $5.13 \pm 0.69 \mu\text{M}$ (OVCAR-5) and $5.28 \pm 1.56 \mu\text{M}$ (4T1), respectively. Moreover, no significant photocytotoxicity was observed in HLF-1 cells with regular GGT expression, nor any significant dark cytotoxicity. Therefore, the potential effectiveness of TBmA-Glu could extend to other cancers that exhibit elevated GGT levels.

Changes in the Supporting Information:

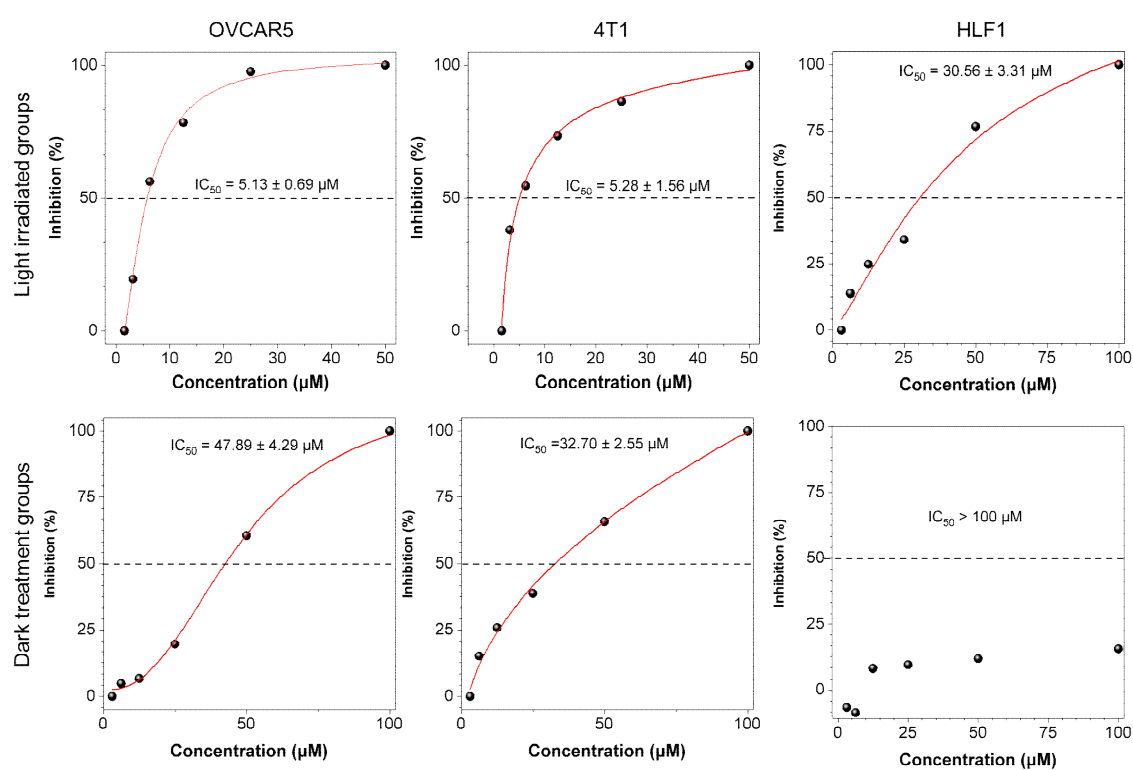


Fig. S35. The anticancer activity (IC_{50} , μM) of TBmA-Glu against GGT overexpressing OVCAR5 and 4T1, and GGT normally expressing HLF1 cells.

2. How photostable is TBmA-Glu under the conditions used for PDT, and does its aggregation state affect its photostability?

Response and revision: We appreciate the reviewer's question regarding the photostability of TBmA-Glu. To address this, we conducted comprehensive stability studies on TBmA-Glu and its active form, TBmA, produced in cancer cells. We extensively investigated the long-term stability and photostability of TBmA aggregates in FBS (Fig. S28c). The results demonstrate

that no significant degradation or aggregation was observed after TBmA aggregates were dispersed in PBS for 72 h or subjected to continuous light irradiation for 30 minutes. Then, we monitored the photostability of TBmA-Glu in HepG2 cells during light irradiation (Fig. S28b). The results show no significant decrease in fluorescence intensity during the light exposure period (Fig. S34). All these findings, along with the stability studies in PBS, collectively demonstrate the high photostability of TBmA-Glu and its active form TBmA under PDT conditions, both in solution and in the cellular environment.

Changes in the Revised Manuscript:

Moreover, the TBmA aggregates exhibited excellent long-term stability (Fig. S28a and S28c) and photodynamic stability (Fig. S28a and S28b), no significant aggregation or degradation was found after dispersed in FBS solution for 72 h or light irradiated for 30 min.

Additionally, TBmA-Glu showed remarkable specific accumulation in HepG2 cells compared to TBmA (Fig. 3b and Fig. S33c), along with exceptional photostability within living cells (Fig. S34a), as evidenced by the absence of significant bleaching even after continuous light irradiation (Fig. S34b).

Changes in the Supporting Information:

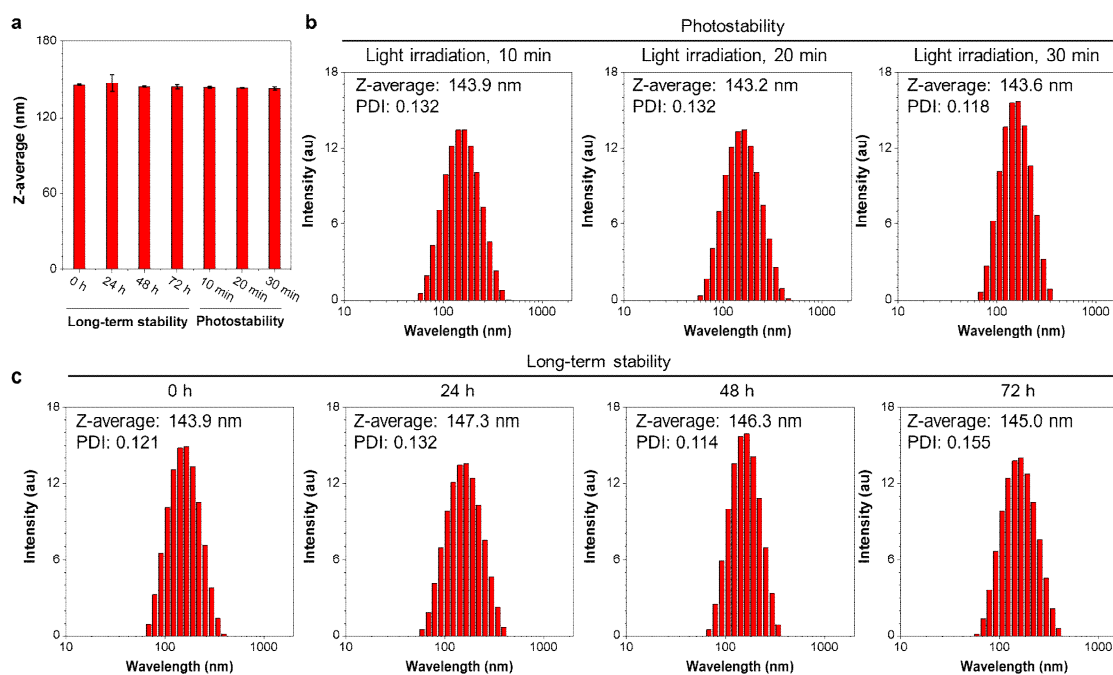


Fig. S28. (a) The average hydrodynamic diameter (*Z*-average) of TBmA aggregates measured by Dynamic Light Scattering (DLS). The distribution of TBmA aggregates during 30 min light irradiation (b) and 72h FBS preservation (c).

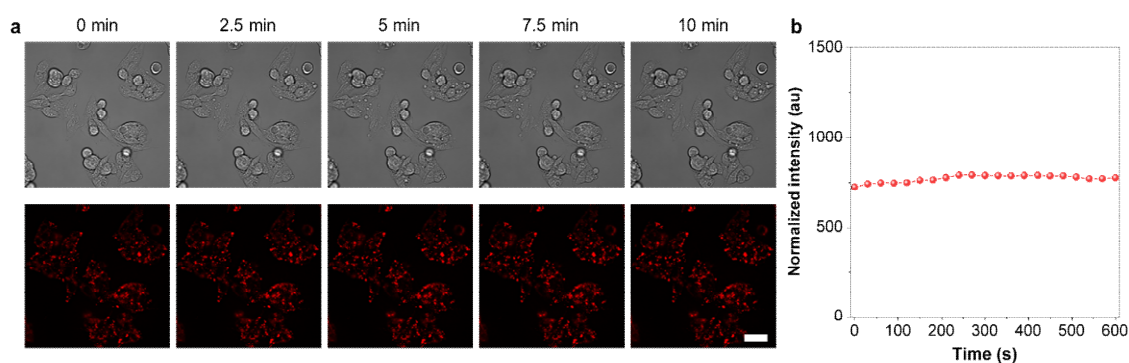


Fig. S34. The HepG2 cells were imaged after incubation with TBmA-Glu (5 μM) for 12 hours. Subsequently, the cells were exposed to a 465 nm laser for 10 minutes, and images were captured every minute. $\lambda_{\text{ex}} = 465 \text{ nm}$; $\lambda_{\text{em}} = 700 \pm 20 \text{ nm}$, Scale bar, 20 μm.

3. The author claimed the aggregate size has a great impression on the PDT efficiency of AIE-PSs, What are the typical sizes and shapes of the TBmA-Glu aggregates formed in the presence of GGT? How do these properties affect the emission properties and PDT efficacy?

Response and revision: We monitored the aggregate sizes of TBmA produced from the GGT-catalyzed reaction using DLS (Fig. S31). The sizes of TBmA aggregates increase from 66.7 nm (1 h, Fig. S31b) to 158.0 nm (4 h, Fig. S31e). Subsequently, no further changes in size were observed as the incubation time was extended. The morphology of the aggregate in 12 h was further analyzed using the transmission electron microscope (TEM, Fig. S34h). The aggregate size measured by TEM is about 100 nm, which is in accord with the size detected by DLS. The stable aggregates produced by GGT catalysis were found to be slightly larger than those detected in 99% PBS (156.8 nm vs 139.5 nm).

Additionally, we evaluated the total ROS generation properties of stable TBmA aggregates generated through a GGT-catalyzed reaction (12 h) using DCFH (Fig. S32). Upon 15 minutes of white LED light exposure ($20 \text{ mW} \cdot \text{cm}^{-2}$), GGT-catalyzed TBmA aggregates exhibited an approximately 164-fold (Fig. S32c) increase in fluorescent intensity. While this enhancement was slightly lower than the aggregates formed in 99% PBS (188-fold), it was significantly higher than Rose Bengal (67.0-fold). These findings are consistent with previous observations that smaller aggregate size tends to enhance photodynamic efficiency. Our results demonstrate that TBmA aggregates generated by GGT possess potent photodynamic activity.

Changes in the Revised Manuscript:

Furthermore, we investigated the morphology of TBmA aggregates formed at the endpoint GGT catalytic reaction (12 h) and its ROS generation capability by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). The DLS results revealed a gradual increase in the size of aggregates to approximately 158.0 nm over the initial 4-hour period.

Subsequently, a steady state size was attained after 4 hours of incubation, with no further changes observed even when extending the incubation time to 12 hours (Fig. S31). The TEM analysis revealed that GGT facilitated the formation of spherical TBmA aggregates, exhibiting an average diameter of approximately 100 nm (Fig. S31h). Additionally, evaluation of the total ROS generation properties of stable TBmA aggregates showed that it induced an approximately 164-fold increase in the intensity of DCFH after 15 min light irradiation (Fig. S32). While this enhancement was slightly lower than the aggregates formed in 99% PBS (188-fold), it was significantly higher than Rose Bengal (67.0-fold). These findings are consistent with previous observations that smaller aggregate size tends to enhance photodynamic efficiency. Our results demonstrate that TBmA aggregates generated by GGT possess potent photodynamic activity.

Changes in the Supporting Information:

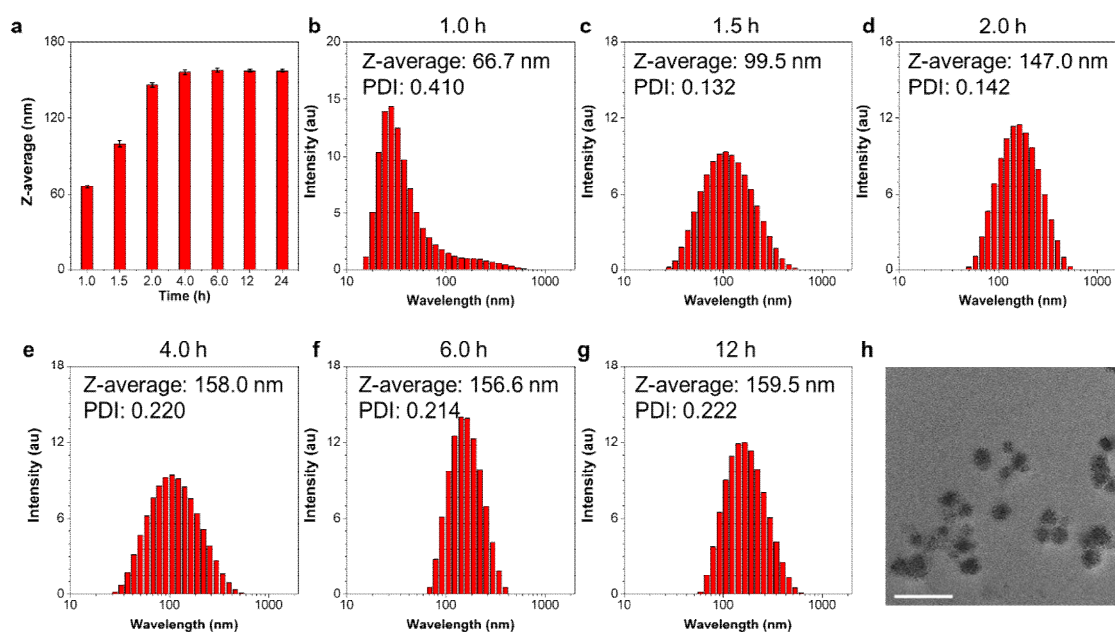


Fig. S31. (a) The average hydrodynamic diameter (Z-average) of TBmA aggregates produced in GGT catalytic reaction measured by DLS. (b-g) Distribution of TBmA aggregates formed at different times of GGT catalytic reaction. (h) The transmission electron microscope (TEM) of the TBmA aggregates formed after the GGT catalytic reaction for 12 h.

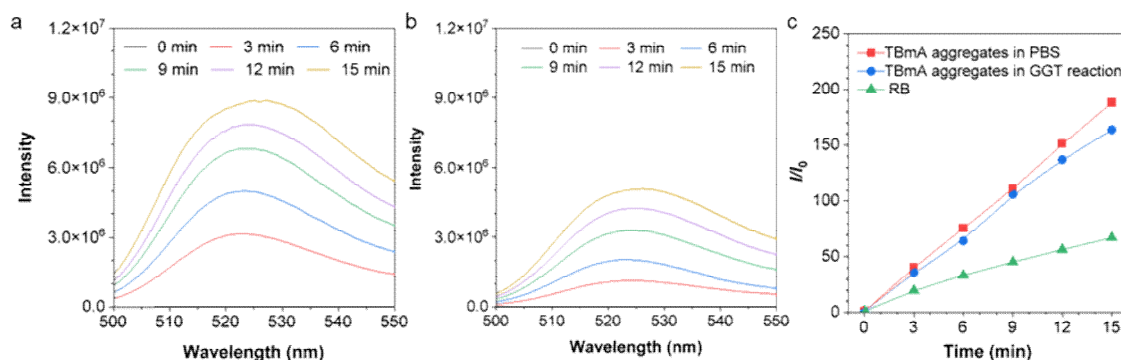


Fig. S32. Fluorescence emission changes of DCFH (10 μM) in the presence of 5 μM photosensitizers in DMSO-PBS ($v:v= 1/99$) after irradiation ($20 \text{ mW} \cdot \text{cm}^{-2}$) for different time. (a) TBmA aggregates in PBS, (b) TBmA aggregates produced in GGT catalytic reaction, DCFH, $\lambda_{\text{ex}} = 488 \text{ nm}$. (c) Plot of the relative emission intensity (I/I_0) of DCF (10 μM) in presence of TBmA (5 μM), TBmA aggregates produced in GGT reaction (5 μM) or Rose Bengal (RB, 5 μM) versus the irradiation ($20 \text{ mW} \cdot \text{cm}^{-2}$) time, where $I_0 = \text{PL intensity of DCFH in solutions with different water fraction } (f_w) \text{ without light irradiation. } \lambda_{\text{ex}} = 488 \text{ nm}$.

4. Can aggregated TBmA be expelled from cancer cells via exocytosis, potentially reducing its therapeutic efficacy? Long-term (48 h) cellular imaging results should be provided by the author.

Response and revision: We conducted confocal imaging to investigate the intracellular retention of TBmA-Glu. Compared with the short treatment groups, no significant decrease in the imaging fluorescent intensity was detected after 48 h of incubation (Fig. S33a).

Changes in the Revised Manuscript:

The results demonstrated that TBmA-Glu has excellent selectivity towards HepG2 cells compared to LO2 cells (Fig. S33a and S33b) and long-term retention ability in HepG2 cells (Fig. S33a).

Changes in the Supporting Information:

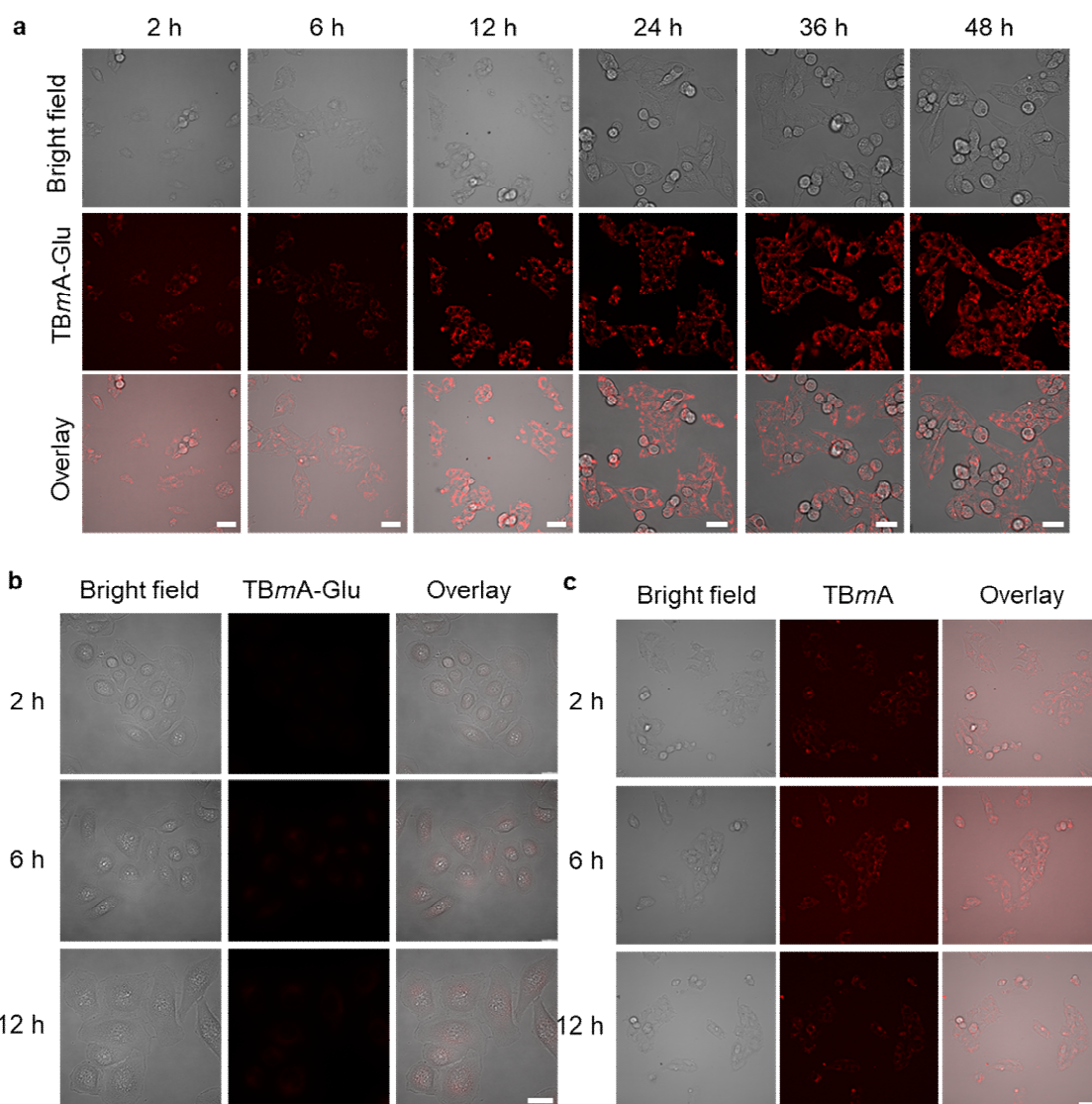


Fig. S33. The time-dependent uptake process of TBmA-Glu (a) / TBmA (b) in HepG2 cells. (c) The time-dependent uptake process of TBmA-Glu in LO2 cells. All the cells were incubated with 5 μM TBmA-Glu / TBmA and imaged at the indicated time. $\lambda_{\text{ex}} = 465 \text{ nm}$; $\lambda_{\text{em}} = 700 \pm 20 \text{ nm}$, Scale bar, 20 μm .

5. The abbreviations, such as DCF, DCFH-DA, HPF, ABDA, CLSM, et al., should be defined the first time they are used.

Response and revision: we have ensured that all the abbreviations are defined when they first appear in the revised manuscript.

Changes in the Revised Manuscript:

“(c) The generation of total ROS generation (2',7'-dichlorodihydrofluorescein, DCF), hydroxyl radical (hydroxyphenyl fluorescein, HPF) and singlet oxygen (9,10-anthracenediyl-bis(methylene)dimalonic Acid, ABDA) by photosensitizers (5 μM) after white LED light

(predominant emission peaks at 450 and 570 nm, Fig. S27) irradiation ($20 \text{ mW}\cdot\text{cm}^{-2}$) for 15 min using the corresponding ROS indicator in PBS/DMSO (v/v = 99:1).”

“**Fig. 3** (a) Confocal laser scanning microscope (CLSM) images of co-incubated cancer (HepG2; luciferase-transfected) and normal (LO2) cells after treatment with TBmA-Glu ($5 \mu\text{M}$, 12 h).“

Reviewer #3 (Remarks to the Author):

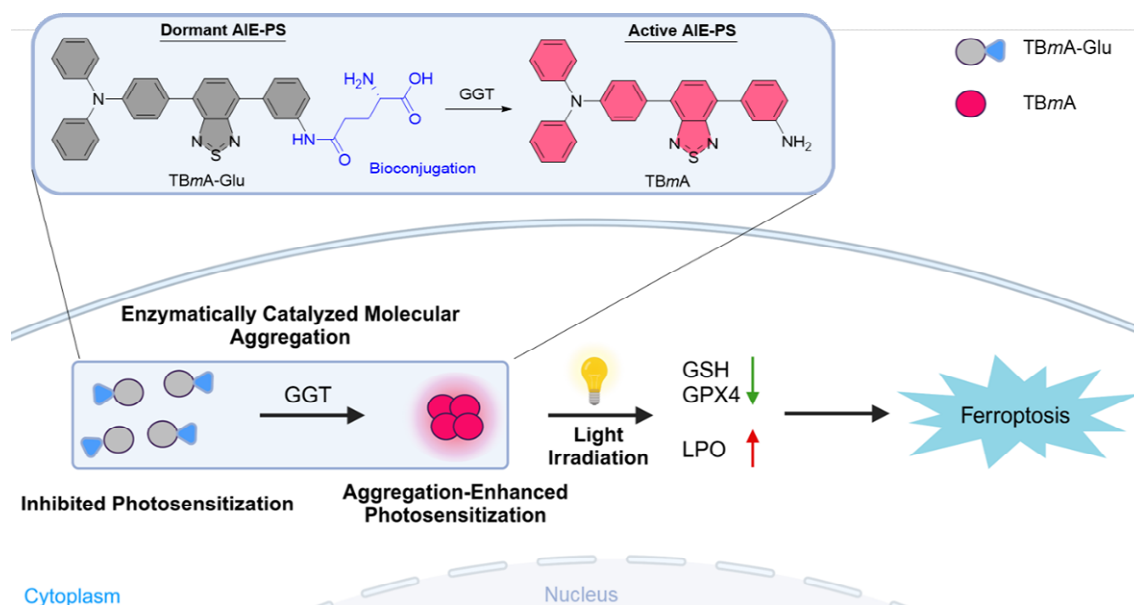
This work present a novel approach to targeted cancer therapy by leveraging the tumor-overexpressed enzyme γ -Glutamyl Transferase (GGT) to induce aggregation of an aggregation-induced emission photosensitizer (AIE-PS), TBmA-Glu. This innovative strategy not only enhances the photosensitivity of the AIE-PS but also results in the degradation of GGT and the accumulation of lipid peroxides, leading to cancer cell ferroptosis. The study is significant for its potential to advance targeted photodynamic therapy (PDT) and the development of smart therapeutics that exploit enzyme activity for controlled molecular aggregation within cancer cells. The authors have demonstrated a clear understanding of the complex interactions between molecular aggregation and biological environments, and the manuscript is well-structured, presenting a logical flow of information from synthesis and characterization to in vivo efficacy. The results are compelling, showing the selective activation of TBmA-Glu by GGT, its enhanced photodynamic activity, and the subsequent therapeutic effects on cancer cells. The manuscript is well-written and provides a solid foundation for further research in the field of nanomedicine and targeted drug delivery. I recommend publication following minor modifications, my concerns are outlined below:

Response: Firstly, we appreciate the reviewer's positive comments on our manuscript.

1. The author could incorporate a concise, visual representation of key discoveries and TBmA-Glu's proposed mechanism of action through a mechanistic cartoon or schematic.

Response and revision: As suggested by the reviewer, we supplemented a schematic diagram of the mechanism of TBmA-Glu's anticancer activities (**Scheme 1**).

Changes in the Revised Manuscript:



Scheme 1: Schematic illumination of the aggregation-enhanced photodynamic therapeutic mechanism mediated by TBmA-Glu.

2. The stability of the aggregates of TBmA, especially the photostability of it in physiological conditions should be discussed.

Response and revision: The long-term stability and photostability of the TBmA aggregates were investigated through DLS. The results suggested no significant degradation was observed when TBmA aggregates were dispersed in FBS for 72 hours or subjected to continuous light irradiation for 30 minutes (Fig. S28). TBmA-Glu also exhibited well anti-bleaching properties in living cells (Fig. S34).

Changes in the Revised Manuscript:

Moreover, the TBmA aggregates exhibited excellent long-term stability (Fig. S28a and S28c) and photodynamic stability (Fig. S28a and S28b), no significant aggregation or degradation was found after dispersed in FBS solution for 72 h or light irradiated for 30 min.

Additionally, TBmA-Glu showed remarkable specific accumulation in HepG2 cells compared to TBmA (Fig. 3b and Fig. S33c), along with exceptional photostability within living cells (Fig. S34a), as evidenced by the absence of significant bleaching even after continuous light irradiation (Fig. S34b).

Changes in the Supporting Information:

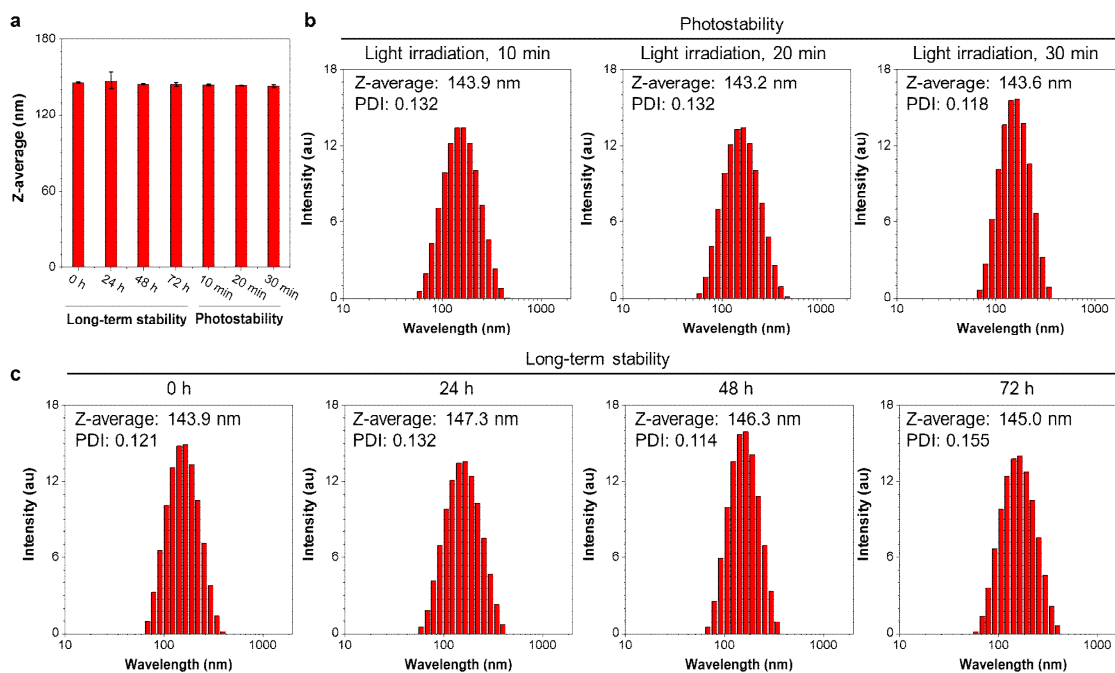


Fig. S28. (a) The average hydrodynamic diameter (*Z*-average) of TBmA aggregates measured by Dynamic Light Scattering (DLS). The distribution of TBmA aggregates during 30 min light irradiation and 72h FBS preservation.

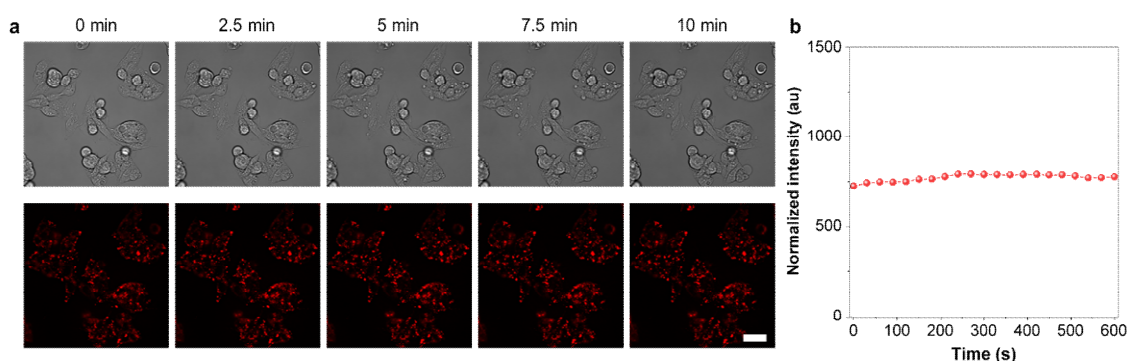


Fig. S34. The HepG2 cells were imaged after incubation with TBmA-Glu (5 μ M) for 12 hours. Subsequently, the cells were exposed to a 465 nm laser for 10 minutes, and images were captured every minute. $\lambda_{ex} = 465$ nm; $\lambda_{em} = 700 \pm 20$ nm, Scale bar, 20 μ m.

3. The author acclaimed the lipid peroxides (LPOs) resulting from the photodynamic process of activated AIE-PS induce the ferroptosis of cancer cells, the changes in the level of LPOs in cancer cells after photodynamic therapy should be quantified.

Response and revision: The amounts of oxidative products of lipids in HepG2 cells after the photodynamic therapy are quantified using the Lipid Peroxidation MDA (malondialdehyde) Assay Kit. It can be seen from the results that, compared with the control group and dark-treatment groups, TBmA-Glu induced a significant accumulation of the LPOs in HepG2 cells

after light irradiation treatment (Fig. S39), which corresponds with the previous ROS species and lipids oxidation in our results.

Changes in the Revised Manuscript:

The peroxidation products of DOPE and the lipid peroxidation products, malondialdehyde (MDA), were also detected in the DOPE/TBmA mixture (Fig. S38) and the cells after light irradiation (Fig. S39).

Measurement of MDA levels. HepG2 cells were cultured in 6 cm dishes until they reached approximately 80% confluency, followed by treatment with TBmA-Glu (2 μ M) for 12 h. The cells were then harvested, counted, and lysed with RAPI lysis buffer at 4 °C for 15 min. MDA levels in each group were determined using the Lipid Peroxidation MDA Assay Kit (Beyotime, China), according to the manufacturer's instructions.

Changes in the Supporting Information:

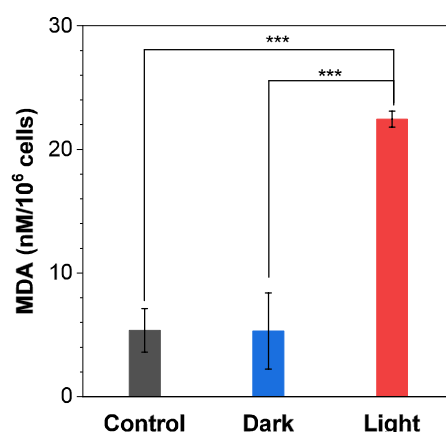


Fig. S39. Malondialdehyde (MDA) levels in HepG2 cells after treated with the TBmA-Glu (2 μ M) for 12 h. Then, the cells were irradiated with a white laser array (12 J \cdot cm⁻²) and the MDA levels were detected using a Lipid Peroxidation MDA Assay Kit. Data expressed as average \pm standard error, n = 3. Statistical significance: P values, ***P < 0.001, calculated with the Student's T-test.

4. Detailed experimental procedures for minimally invasive PDT should be provided.

Response and revision: The experimental procedure for minimally invasive PDT assay has been incorporated into the "In vivo antitumor assay" section in the "Methods" of the manuscript.

Changes in the Revised Manuscript:

For the minimally invasive photodynamic therapy, the mice were initially anesthetized using an isoflurane inhalant anesthesia apparatus. The TBmA fluorescence was utilized to precisely

locate the tumor section in the liver, and a small incision (2 mm) was meticulously made with a scalpel at the localized region. Subsequently, the laser probe was carefully inserted into the incision to execute the photodynamic therapy. Finally, the wound was sutured and disinfected.

Finally, we would like to reiterate our sincere gratitude to all the reviewers and the editorial office for their invaluable suggestions and diligent efforts, which have significantly enhanced the quality of this manuscript.

Point-by-point Response

We express our sincere gratitude to the reviewers for their insightful comments, which have significantly enhanced the scholarly quality of our paper. In response to their valuable feedback and suggestions, we have carefully revised the manuscript. Our responses and revisions are presented in a distinguishable blue font for convenient reference.

Reviewer #1 (Remarks to the Author):

Q1: The authors are accurate in stating that a comparison was not made (see the green text below from the manuscript). However, they claim that oxygen content has a negligible influence on the observed activity under hypoxia, which is linked to Type-I process, based on previous claims. These claims were not supported by “negative controls” with standard PDT photosensitizers, in a fair comparison.

251. “only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d). This suggests that oxygen content has negligible influence on its photodynamic activity. The cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process, which is consistent with the prior findings.”

Response and revision: We appreciate the valuable suggestions provided by the reviewer. To enhance the accuracy of our study, we conducted additional experiments using Rose Bengal (RB), a well-established type II photosensitizer¹, as a control. These experiments revealed that RB's photodynamic efficiency decreased significantly under hypoxia conditions (2% O₂) compared to normoxia conditions, while TBmA maintained relatively consistent activity across both environments (**Fig. R1**).

To discuss this result more accurately, we have revised our manuscript by replacing the statement “This suggests that oxygen content has negligible influence on its photodynamic activity.” with “*This suggests that TBmA exhibits tolerance towards hypoxic conditions.*”

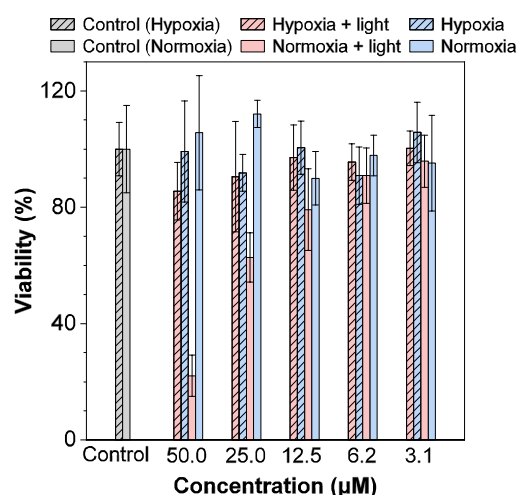


Fig. S36. The effects of hypoxia (2% O₂) and normoxia (20% O₂) conditions on the anticancer photodynamic efficiency of Rose Bengal against HepG2 cells.

Revised in manuscript:

Additionally, only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d), while the type-II PS, RB, showed a significant decrease in photodynamic efficiency under hypoxia conditions (2% O₂) compared to normoxia conditions (Fig. S36). This suggests that TBmA exhibits tolerance towards hypoxic conditions.

Reference:

1. Fischer BB, Krieger-Liszky A, Eggen RIL. Oxidative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different molecular responses in *Chlamydomonas reinhardtii*. *Plant Sci.* **168**, 747-759 (2005).

Q2: Aggregation is a type of supramolecular association which is perfectly reversible. It is only natural to expect deaggregation in the biological media with so many different gradients of hydrophobicity. FBS is not a good approximation for intracellular medium as its protein content is very low. Of course, a simple pharmacokinetics study would reveal how stable those aggregates are in vivo.

Response: We appreciate the reviewer's insightful comments regarding the nature of supramolecular aggregation and the potential for de-aggregation in biological media. We would like to clarify several key points that address these concerns.

Firstly, it's crucial to emphasize that TBmA-Glu is a water-soluble prodrug. The aggregation process only occurs after the Glu moiety is cleaved by GGT in HepG2 cells. This design ensures that TBmA-Glu remains soluble in the blood, avoiding premature aggregation. Aggregation is triggered specifically in the intracellular environment of GGT-overexpressing tumor cells.

We acknowledge that FBS is not an ideal model for the intracellular environment. To address this issue, we further conducted stability studies using a 30% BSA (Bovine Serum Albumin) solution, which is a better model for the protein-rich intracellular

milieu. The intracellular protein concentration typically ranges from 50-400 mg/mL, and our 30% BSA solution (~300 mg/mL) falls within this range. TBmA aggregates showed remarkable stability in this environment, with no significant degradation observed over 72 hours (**Fig. R1**).

We also agree that pharmacokinetics studies would be valuable. However, our system presents unique challenges for such studies, as the aggregates form intracellularly rather than in circulation. Collecting and analyzing intracellular aggregates from tumor sections poses significant technical difficulties. Our approach using a highly concentrated protein solution provides valuable insights into aggregate stability in a physiologically relevant environment.

Importantly, beyond structural stability, we have observed that the aggregates maintain their photodynamic properties in the 30% BSA solution for 72 h (**Fig. R2**). This functional stability is crucial for the compound's theranostic applications.

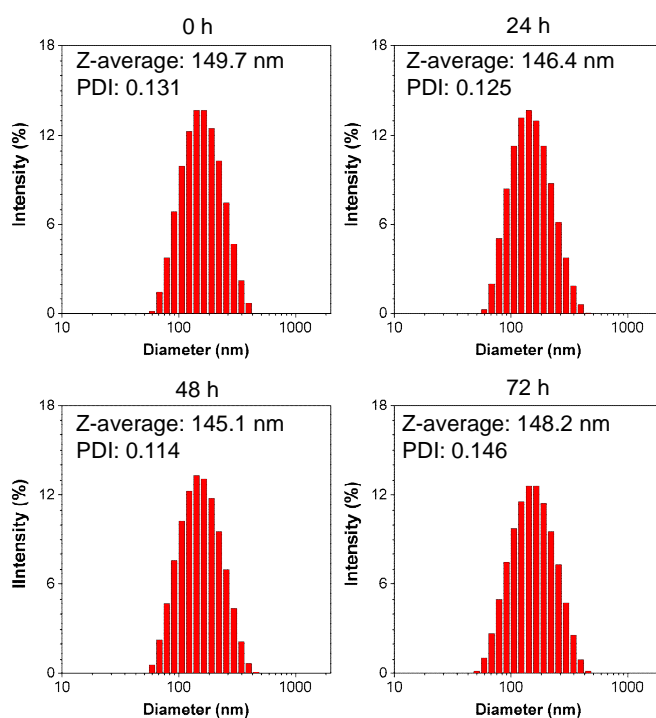


Fig. R1. The long-term stability of TBmA aggregates in 30% BSA solutions.

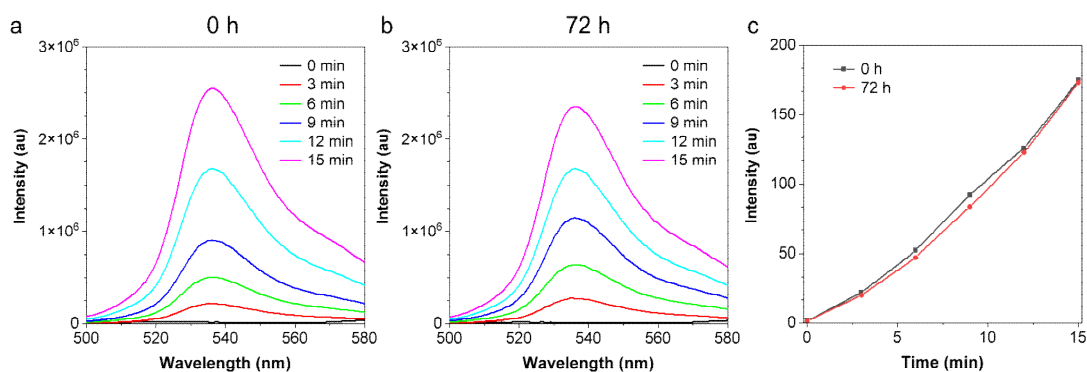


Fig. R2. The ROS generation capacity of TBmA aggregates after dispersed in 30% BSA solution for 0 h (a) and 72 h (b). The ROS was identified using DCFH as an indicator. (c) The plot of the relative emission intensity (I/I_0) of DC versus the irradiation ($20 \text{ mW} \cdot \text{cm}^{-2}$) time, where $I_0 = \text{PL intensity of DCFH in solutions without light irradiation}$.

Q3: References 4, 5 and 6 were carefully checked. Stability of the aggregates “in vivo” was not studied in these articles. Retention of fluorescence is not necessarily a sign of stability.

Response: We thank the reviewer for the critical feedback. The unique photophysical properties of AIE compounds stem from the restriction of intramolecular motion (RIM) mechanism, where aggregation limits molecular rotations and vibrations, leading to enhanced fluorescence. Therefore, the fluorescence behavior of AIE materials does provide valuable insights into their molecular state and environment.

This interpretation is supported by several factors. First of all, TBmA-Glu is engineered to aggregate specifically in response to GGT activity, which is overexpressed in certain tumor cells. This targeted approach minimizes premature aggregation in circulation. Secondly, the crowded, protein-rich cytoplasmic environment of tumor cells likely provides conditions that favor aggregate stability once formed. Additionally, we observed that the photosensitivity of TBmA was maintained in our 30% BSA studies, suggesting a preservation of the aggregate structure.

Q4: While AIE compounds seem to provide potentially useful imaging opportunities, their relevance in PDT or other therapeutic schemes remain questionable. A therapeutic agent which would change size on meeting hydrophobic membranes or proteins, which could lead to different properties has to be handled very carefully. It would be advisable to avoid hype terminology such as “personalized medicine and real-time treatment monitoring”.

Response: We appreciate the reviewer's thoughtful comments regarding the therapeutic relevance of AIE compounds and the importance of careful characterization of their behavior in biological systems.

Regarding the stability and behavior of TBmA, we emphasize that TBmA-Glu is designed as a water-soluble prodrug that only forms aggregates within tumor cells following enzymatic reaction. This targeted approach minimizes potential issues related

to premature aggregation or size changes in circulation. Furthermore, we have demonstrated the stability of TBmA aggregates in a 30% BSA solution for 72 hours, providing initial evidence of their potential stability in protein-rich environments.

About "personalized medicine and real-time treatment monitoring." in the previous response letter: The full sentence is "Recent literature has demonstrated the potential of AIE compounds for combining imaging and therapeutic functions in a single entity opening up new possibilities for personalized medicine and real-time treatment monitoring." We agree that such terminology should be used judiciously, especially in early-stage research, however, our intention here is to highlight the potential of AIE materials to contribute to these fields in the future, rather than to claim immediate clinical applicability.

The unique properties of AIE materials, including their AIE and potential for stimuli-responsive behavior, do offer intriguing possibilities for both imaging and therapeutic applications. However, we agree that rigorous investigation is needed to establish their efficacy and safety for PDT or other therapeutic schemes. Moving forward, we will focus on providing concrete evidence for the specific advantages of AIE compounds in relevant biological contexts, rather than speculating on broad future applications. We believe this approach will better serve the scientific community and responsibly advance the field.

Q5: May be it wasn't clear in my earlier statement of concern, I did say near UV, but I was specifically referring to 450 nm peak. There are literature reports of blue (450 nm) light causing cellular damage.

Response: We acknowledge that there are indeed literature reports of blue light (450 nm) causing cellular damage. This is an important consideration in photodynamic therapy and other light-based treatments. However, we would like to emphasize that the biological effects of light exposure are highly dependent on both wavelength and dosage.

In our experiments, we carefully controlled the light dosage to minimize potential phototoxicity while maintaining therapeutic efficacy. Under the experimental conditions described in our manuscript, we did not observe any significant effects on cell viability following LED light irradiation (**Fig. R3**).

To address the reviewer's concern, we also conducted a blue light irradiation (450 nm, 12 J/cm²) PDT assay. In this experiment, we also found no significant effect on cellular viability. This suggests that at the dosages used in our study, the blue light alone does not cause substantial cellular damage.

However, we agree that the potential for phototoxicity is an important consideration in developing light-based therapies. In future studies, we plan to conduct a more comprehensive dose-response analysis to determine the threshold at which blue light exposure may begin to affect cell viability. We also intend to investigate the potential

long-term effects of repeated light exposure and compare the effects of our AIE-based approach with traditional photosensitizers at equivalent light doses.

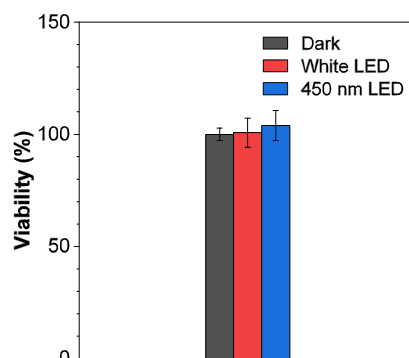


Fig. R3 The impact of white light and 450 nm light exposure (12 J/cm^2) on the cellular viability of HepG2 cells.

Q6: One of the most important issues here is the fact that short wavelength irradiation is required to excite the chromophore, whether it is in organic or aqueous medium. 450 nm is not compatible with PDT. The typical penetration length as 450 nm is less than 1 mm, which is significantly less than needed for an effective “photo”-driven process.

Response: It is correct that the typical penetration depth of 450 nm light is less than 1 mm in tissue, which is indeed less than ideal for treating deep-seated tumors. However, we would like to highlight several important considerations:

First, though direct light penetration is restricted, the effective depth of PDT damage may increase due to light reflection and scattering within tissues. This occurrence can expand the scope of the photodynamic impact beyond the initial penetration depth.

Secondly, several clinical scenarios exist where shallow light penetration is sufficient or even advantageous. For instance, PDT with blue light excitation could be particularly useful for superficial skin cancers and precancerous lesions, intraoperative treatment of residual tumor cells after surgical resection, treatment of early-stage mucosal cancers in inaccessible areas (e.g., oral cavity, bladder), and endoscopic applications for gastrointestinal tumors.

Finally, numerous published studies demonstrate the successful use of 450 nm light and white light (including the blue spectrum) for PDT when the photosensitizers have maximum absorption around 450 nm. ^{2, 3, 4, 5, 6, 7}

Nevertheless, we fully agree that blue light's limited tissue penetration restricts the broader applicability of our current system for treating deep-seated tumors. Given this limitation, our future research directions include exploring two-photon excitation to achieve deeper tissue penetration, investigating upconversion nanoparticles to convert longer-wavelength light to blue light locally, and developing new AIE photosensitizers with red-shifted absorption for improved tissue penetration. We believe that addressing

these challenges will expand the potential applications of our AIE-based PDT system while utilizing its unique properties.

References:

2. Fan L, *et al.* A Bioactive Photosensitizer for Hypoxia-Tolerant Molecular Targeting-Photo-Immunotherapy of Malignant Tumor. *Adv. Funct. Mater.* **34**, 2313755 (2023).
3. Li X, *et al.* A novel 450-nm laser-mediated sinoporphyrin sodium-based photodynamic therapy induces autophagic cell death in gastric cancer through regulation of the ROS/PI3K/Akt/mTOR signaling pathway. *BMC Med.* **20**, 475 (2022).
4. Mei Y, *et al.* A Novel Photosensitizer Based 450-nm Blue Laser-Mediated Photodynamic Therapy Induces Apoptosis in Colorectal Cancer - in Vitro and in Vivo Study. *Front. Biosci. (Landmark Ed)* **29**, 199 (2024).
5. Chen Y, *et al.* Photoactivatable metal organic framework for synergistic ferroptosis and photodynamic therapy using 450 nm laser. *Chem. Eng. J.* **454**, 140438 (2023).
6. Sun P, *et al.* A water-soluble phosphorescent conjugated polymer brush for tumor-targeted photodynamic therapy. *Polym. Chem.* **8**, 5836-5844 (2017).
7. An J, *et al.* An unexpected strategy to alleviate hypoxia limitation of photodynamic therapy by biotinylation of photosensitizers. *Nat. Commun.* **13**, 2225 (2022).

Q7: First of all, no PDT is independent of oxygen (please refer to Baptista, et al., *Photochemistry and Photobiology*, 2017, 93 (4) 912-919.) So, instead of 1 O₂ % hypoxia, if the authors were to switch to 0.5 % O₂ hypoxia, or anoxia, the effectiveness would be much more different.

I am also worried about the fact that the type-I designation is partly based on Figure 4b, there is some inconsistencies between the legend and the plot. Ebselen found in the legend, is not found on the plot, which is a singlet oxygen quencher. Also, Trolox, just like azide (N₃⁻) is a singlet oxygen quencher.

Response and revision: We agree with the reviewer that oxygen plays a pivotal role in the Type I and Type II PDT processes. However, from the PDT mechanism, we know that the type I photosensitizers could directly transfer electrons to the substrate, forming a radical cation or neutral radical. These radicals could immediately react with O₂ or H₂O to generate hydrogen peroxide (H₂O₂), hydroxyl radicals (\cdot OH), or superoxide anions (\cdot O₂⁻) (**Fig. R4**).^{1, 2}

We have tried but could not finish the antitumor PDT assays in the anaerobic conditions, because the anoxia condition resulted in death of the tumor cells (**Fig. R5a**). So, we re-evaluated the photodynamic efficiency of TBmA and RB using a deoxidized PBS solution. The results showed that TBmA could also induce the oxidation of DFCH under the anoxia condition (**Fig. R5b**), while the photodynamic efficiency of RB showed significant degradation. Hence, type-I photosensitizers exhibit relatively higher tolerance towards oxygen concentrations, which implies that, even under low oxygen conditions, they can still engage in substrate reactions through electron transfer.

We are sorry for the mistake in the figure legend in Figure 4b. “Ebselen” has been revised as “Trolox.” However, it should be noted that Trolox is not only a $^1\text{O}_2$ scavenger but also a scavenger of peroxy and alkoxy groups.³ The type-I designation is mainly based on the ROS species we detected *in vitro* (Fig. R5c).

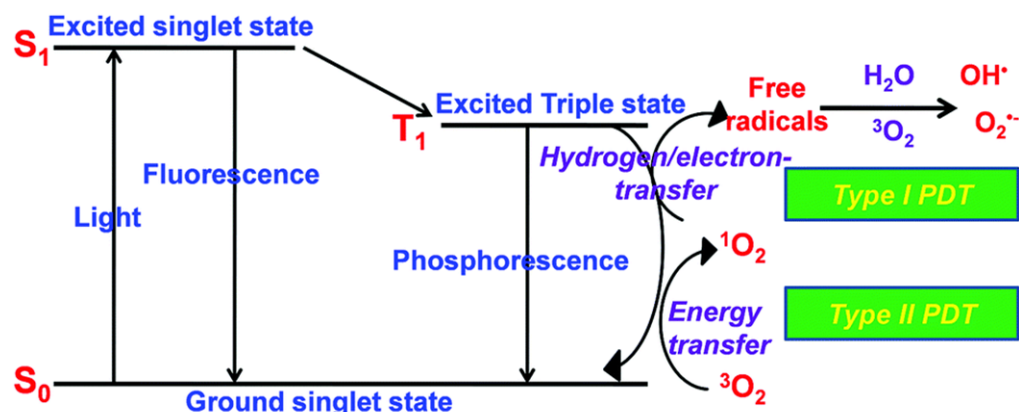


Fig. R4 Scheme of the photochemical reactions for type I and type II PDT.⁹

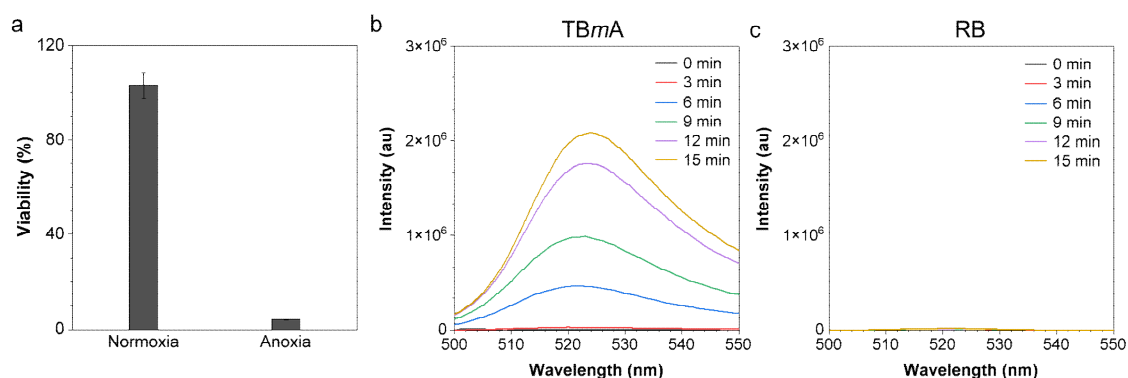


Fig. R5 (a) Cellular viability of HepG2 cells in normoxia and anoxia conditions. (b) Fluorescence emission changes of DCFH (Dichlorodihydrofluorescein, $10\ \mu\text{M}$) in the presence of $5\ \mu\text{M}$ photosensitizers in DMSO-PBS ($v:v = 1:99$) after irradiation ($20\ \text{mW}\cdot\text{cm}^{-2}$) for a different time under anoxia conditions. (b) TBmA, (c) Rose Bengal (RB). DCFH, $\lambda_{\text{ex}} = 488\ \text{nm}$.

Revised in manuscript:

Trolox: $50\ \mu\text{M}$ ($\text{ROO}\cdot$ scavenger and $^1\text{O}_2$ scavenger); D-mannitol: $50\ \text{mM}$ ($\cdot\text{OH}$ scavenger); Tiron: $10\ \text{mM}$ ($\cdot\text{O}_2^-$ scavenger); NaN_3 : $5\ \text{mM}$ ($^1\text{O}_2$ scavenger)

References:

1. Zhao X, Liu J, Fan J, Chao H, Peng X. Recent progress in photosensitizers for overcoming the challenges of photodynamic therapy: from molecular design to application. *Chem. Soc. Rev.* **50**, 4185-4219 (2021).
2. Fan W, Huang P, Chen X. Overcoming the Achilles' heel of photodynamic therapy. *Chem. Soc. Rev.* **45**, 6488-6519 (2016).

3. Lúcio M, Nunes C, Gaspar D, Ferreira H, Lima JLFC, Reis S. Antioxidant Activity of Vitamin E and Trolox: Understanding of the Factors that Govern Lipid Peroxidation Studies In Vitro. *Food Biophys.* **4**, 312-320 (2009).

Q8: Regardless of the mechanism, the total quantum yield of all radiative and not radiative processes is not going to be larger than 1. So far, I did not come across a quantum yield of ROS formation, or emission quantum yield reported with aggregated structures. However, that should be the first thing to be studied when reporting a novel photosensitizer, but especially so, when both emission and ISC is claimed to be enhanced.

Response: Indeed, the total quantum yield of all radiative and non-radiative processes cannot exceed 1. However, the energy consumption in no radiative processes contains both the energy for ISC processes and the molecular motion as well. Molecular aggregation could induce the restriction of intramolecular motions (RIM) and, as a result, reduce energy loss through non-radiative molecular motion, potentially increasing the energy available for emission and ISC processes. So, the energy efficiency of both emission and ISC can be enhanced in aggregated structure due to RIM.

However, in specific cases, such as the graphene quantum dots reported by Zhang et al., the apparent quantum yield could be larger than 1.¹ This occurs when the energy gaps between ΔE_{ST} and ΔE_{TG} (the energy gap between T_1 and Ground state) are larger than the formation energy of 1O_2 (22.5 kcal mol⁻¹). In such cases, 1O_2 generation happens through multiple pathways: energy transfer from T_1 (ET(1) in **Fig. R6**), but also the energy transfer from S_1 to 3O_2 during the S_1 - T_1 intersystem crossing transition (ET(2) in Figure R6). This multi-pathway mechanism can lead to an overall 1O_2 quantum yield greater than 1.0, as more than one 1O_2 molecule can be produced per absorbed photon.²

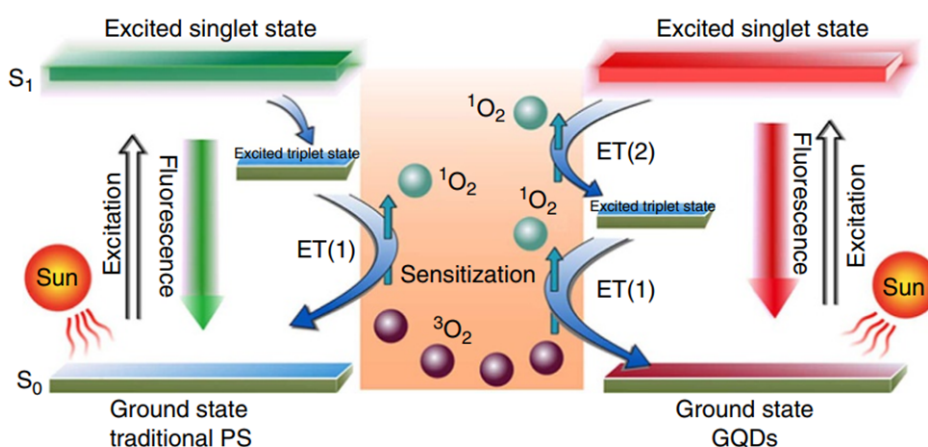


Fig. R6 Schematic illustration of the 1O_2 generation mechanisms by conventional PDT agents (left) and GQDs (right).

References

1. Ge J, *et al.* A graphene quantum dot photodynamic therapy agent with high singlet oxygen generation. *Nat. Commun.* **5**, 4596 (2014).
2. Kanner RC, Foote CS. Singlet oxygen production from singlet and triplet states of 9,10-dicyanoanthracene. *J. Am. Chem. Soc.* **114**, 678-681 (1992).

Q9: Imaging on surface tumors or in mice, perhaps; but not therapeutics. Short wavelength excitation, and their aggregate structure, which would most likely disintegrate as it travels through the body into different sized nanoparticles would limit their potential.

Response: As previously discussed, TBmA-Glu is a water-soluble molecule that forms aggregates within tumor cells upon activation by GGT to produce TBmA. Consequently, most of these aggregates are localized in the tumor cells. Furthermore, we have demonstrated the stability of TBmA aggregates for 72 hours in a 30% BSA solution. Additionally, considering that PDT processes were conducted 12 hours after administration of TBmA-Glu, it can be inferred that the TBmA aggregates exhibit sufficient stability to complete the PDT processes.

Reviewer #2 (Remarks to the Author):

Q1: The authors have addressed all the concerns in the revisions. And the manuscript is ready to be published.

Response: We would like to express our sincere gratitude to the reviewer again for their invaluable suggestions, which have significantly enhanced the comprehensiveness and rigor of this paper.

Reviewer #3 (Remarks to the Author):

Q1: The paper's focus is the enzymatically catalyzed molecular aggregation for improving the response and PDT treatment. The paper has been revised accordingly, and ready for publication.

Response: We would like to thank the reviewer for their invaluable suggestions, which have significantly improved the comprehensiveness and rigor of this paper.

Point-by-point Response

We express our sincere gratitude to the reviewers, which have significantly enhanced the scholarly quality of our paper. In response to your valuable feedback and suggestions, we have carefully revised the manuscript. Our responses and revisions are presented in a distinguishable blue font for convenient reference.

Reviewer #1 (Remarks to the Author)

General opinion

The present manuscript claims to achieve a better targeting of a proposed photosensitizer (PS) which can be activated by GGT and be excited at short wavelengths.

Also, activated (aggregated structure) may have a better cytotoxic effect, compared to its non-activated form, which is another example of activated-PS

There have been countless photosensitizers which can be targeted one way or another. Many reviews exist about activatable photosensitizers. Some enzymatically, some by hypoxia, by higher H₂O₂ or GSH concentrations, or acidic pH.

The main, may be the only reason why PDT did not develop significantly since 70's is that fact that light, even at the so-called "therapeutic window" does not go through tissues. And of course, there is no real justification for a 450 nm chromophore to be proposed as a novelty. There are very specific, niche cases, where a single cell layer penetration may be useful. But citing these, is missing the point of all PDT-work.

Type-I processes being less oxygen dependent has been proposed without real evidence. The new data provided by the authors is also not a fair comparison (see below).

Thus the manuscript does not bring any novelty to the field. The requirement for aggregation, if anything, complicates the picture very unnecessarily.

Response: We would like to thank the reviewer for the insightful comments, which have significantly enhanced the quality of our paper.

Reviewer #1 (Remarks to the Author):

Q1: Light source in Fig S36 was not given, if it is white LED, it is not a fair comparison, because LED emission profile fits TbmA/aggregate better.

Response: Rose Bengal (RB) has its maximum absorption at 558 nm. We can see from the emission spectrum of the LED light (Fig. S28) that the LED has a broad maximum emission at 550-600 nm. It means that LED emission also fits the absorption of RB.

Q2: 0.5 or 1 % hypoxia may be better.

Response and revision: In previous experiments, we have proved that TBmA has more potent PDT efficiency than Rose Bengal (RB) even under anoxia conditions. So, we think the 0.5 or 1 % hypoxia would not lead to the different PDT efficiency of TBmA and RB. Furthermore, to address your concerns about the hypoxia PDT efficiency, we decided to remove the discussion of the hypoxia photosensitization of AIE photosensitizers in our manuscript.

Q3: Both of these articles while interesting, hardly relevant to PDT considering the absorption peaks of the proposed sensitizers are in blue, and the fact that they are very unique cases. The first one reached to a surprising conclusion without doing any photophysical work. Vibrational (or rotational) relaxation and their control by micro- or molecular environments, by molecular steric hindrance is well known. However, only accurate quantum yield determinations would prove simultaneous increases in emission and singlet oxygen quantum yields. This is not done in Ref 1.

Response and revision: I would like to clarify that our discussion here is indeed focused on the quantum yield during the photodynamic therapy (PDT) process. It is important for you to know that, regardless of the light source used to excite the photosensitizer, all photodynamic processes share the same underlying photophysical mechanisms.

In the first reference, the authors demonstrated the quantum yield of graphene quantum dots (GQDs) using electron paramagnetic resonance (EPR) assays, which is a critical aspect of validating the multistate sensitization (MSS) mechanism. The authors also focused on the fluorescence intensity at 680 nm and the singlet oxygen ($^1\text{O}_2$) quantum yield of GQDs in solutions with varying oxygen concentrations. All the results are in agreement with the proposed MSS mechanism, which suggests that an overall $^1\text{O}_2$ quantum yield greater than 1.0 can be achieved under specific conditions. However, we fully concur with your assessment that more work is necessary before any definitive conclusions.

Q4: The problem is that now “activated” aggregates, will not stay forever in tumor cells, as these cells disintegrate.

Response and revision: It is important to point out that we do not want the aggregates to stay in cells forever. What we need to make sure is that the "activated" aggregates work properly during treatment. The stability and photodynamic efficiency assays have proved that the activated aggregates could meet the therapeutic requirement in the study.

Reviewer 1 responses to the authors' comments is highlighted in red.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors report two compounds that they claim to undergo enzyme triggered aggregation (both in vitro and vivo) leading to effective PDT outcomes, partly due to a "type-I PDT" process they claim to be better than "type II".

This referee finds important design flaws and problems with the implementation of the work.

Response: We thank the reviewer for the constructive comments on our paper. In this manuscript, we actually did not intend to compare the Type I and Type II PDT processes, and the efficiency of type I and type II photosensitizers is not the key point of this work. We just want to present the objective performance of the developed photosensitizers based on their ROS generation capability. As clearly stated in the manuscript: "It was found that TBmA and TBpA produced significantly higher ROS compared to TBmA-Glu and TBpA-Glu, even surpassing the commercial photosensitizer, Rose Bengal (RB). Moreover, TBmA was identified as the most potent photosensitizer among the four compounds. Further analysis revealed that TBmA and TBpA functioned as strong type I photosensitizers (Fig. 1c and Fig. S25), while TBmA-Glu and TBpA-Glu acted as very weak type II photosensitizers (Fig. 1c and Fig. S26)."

***The authors are accurate in stating that a comparison was not made (see the green text below from the manuscript). However, they claim that oxygen content has a negligible influence on the observed activity under hypoxia, which is linked to Type-I process, based on previous claims. These claims were not supported by "negative controls" with standard PDT photosensitizers, in a fair comparison.

251. "only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d). This suggests that oxygen content has negligible influence on its photodynamic activity. The cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process, which is consistent with the prior findings."

Here are the major issues:

Problem with Aggregates:

While AIE compounds may be interesting for imaging perhaps mostly in cell culture and mice models (considering limited light penetration) as a therapeutic agent, aggregate structures are ill-defined and especially in high protein milieus, not very stable and difficult to standardize. More to the point, there is really no real justification for the use of aggregates, considering the fact molecular drugs or photosensitizers are better than the aggregates in many aspects, and nothing new is offered or even suggested by these compounds.

Response and revision: Thank you for your thoughtful comments regarding AIE compounds. We appreciate your concerns and would like to address them point by point:

(i) **Stability and standardization:** we acknowledge that stability is crucial for bio-application. Our recent studies have shown promising results regarding the stability of TBmA aggregates in high-protein environments, specifically: **(a) Long-term stability:** TBmA aggregates showed no significant degradation when dispersed in FBS for 72 hours (Fig. S28a and S28c).

*** Aggregation is a type of supramolecular association which is perfectly reversible. It is only natural to expect deaggregation in the biological media with so many different gradients of hydrophobicity. FBS is not a good approximation for intracellular medium as its protein content is very low. Of course, a simple pharmacokinetics study would reveal how stable is those aggregates are in vivo.

(ii) **(b) Photostability:** The aggregates remained stable under continuous light irradiation for 30 minutes (Fig. S28a and S28b). These findings collectively highlight the exceptional stability exhibited by TBmA. These findings demonstrate the exceptional stability of TBmA aggregates in biologically relevant conditions.

Moreover, numerous AIEgens, including small molecules or AIE nanoparticles, have been extensively reported for their long-term monitoring and theranostic applications.^{1, 2, 3} These pieces of evidence underscore the remarkable stability of AIEgens, making them highly promising candidates for theranostic applications.

(iii) **Aggregate structure:** To address concerns about ill-defined aggregate structures, we extensively investigated the aggregate size of TBmA using Dynamic Light Scattering (DLS) and Transmission Electron Microscope (TEM). The results suggest that the TBmA forms spherical particles with 140 nm in 99% PBS and 150 nm diameter after the GGT catalysis reaction (12 h, Fig. S31). These results indicate that TBmA consistently forms nanoparticles of definite shape and size in aqueous environments, regardless of the specific conditions. Numerous works have been reported to show the definite shape and size, as well as the excellent stability and biocompatibility of the AIE aggregates.^{4, 5, 6}

*** References 4, 5 and 6 were carefully checked. Stability of the aggregates “in vivo” was not studied in these articles. Retention of fluorescence is not necessarily a sign of stability.

(iv) **A comparative analysis of small molecular drugs and AIE materials:** Although molecular drugs and traditional photosensitizers have their advantages, AIE compounds offer unique benefits such as enhanced emission upon aggregation, responsiveness to stimuli, and multifunctional potential. Revealing reports increasingly indicate that small molecular photosensitizers, such as CE6, exhibit low solubility and undergo aggregation in solution, resulting in the deactivation of their photosensitizing activity and hindering their bioapplication.^{7, 8, 9, 10} We believe that AIE compounds can serve as complementary agents,

rather than substitutes, for small molecule drugs. Recent literature has demonstrated the potential of AIE compounds for combining imaging and therapeutic functions in a single entity opening up new possibilities for personalized medicine and real-time treatment monitoring.^{4, 11, 12, 13}

We believe that AIE compounds, including TBmA-Glu, offer valuable and unique properties that complement existing molecular drugs and photosensitizers. While challenges remain, the growing body of research on AIE materials suggests significant potential for advancing biomedical imaging and therapeutic applications. We appreciate the reviewer's perspective and believe that continued research and development in this field will address current limitations and unlock new possibilities in biomedical science.

*** While AIE compounds seem to provide potentially useful imaging opportunities, their relevance in PDT or other therapeutic schemes remain questionable. A therapeutic agent which would change size on meeting hydrophobic membranes or proteins, which could lead to different properties has to be handled very carefully. It would be advisable to avoid hype terminology such as “personalized medicine and real-time treatment monitoring”.

Changes in the Revised Manuscript:

Moreover, the TBmA aggregates exhibited excellent long-term stability (Fig. S28a and S28c) and photodynamic stability (Fig. S28a and S28b), no significant aggregation or degradation was found after dispersed in FBS (fetal bovine serum) solution for 72 h or light irradiated for 30 min.

Changes in the Supporting Information:

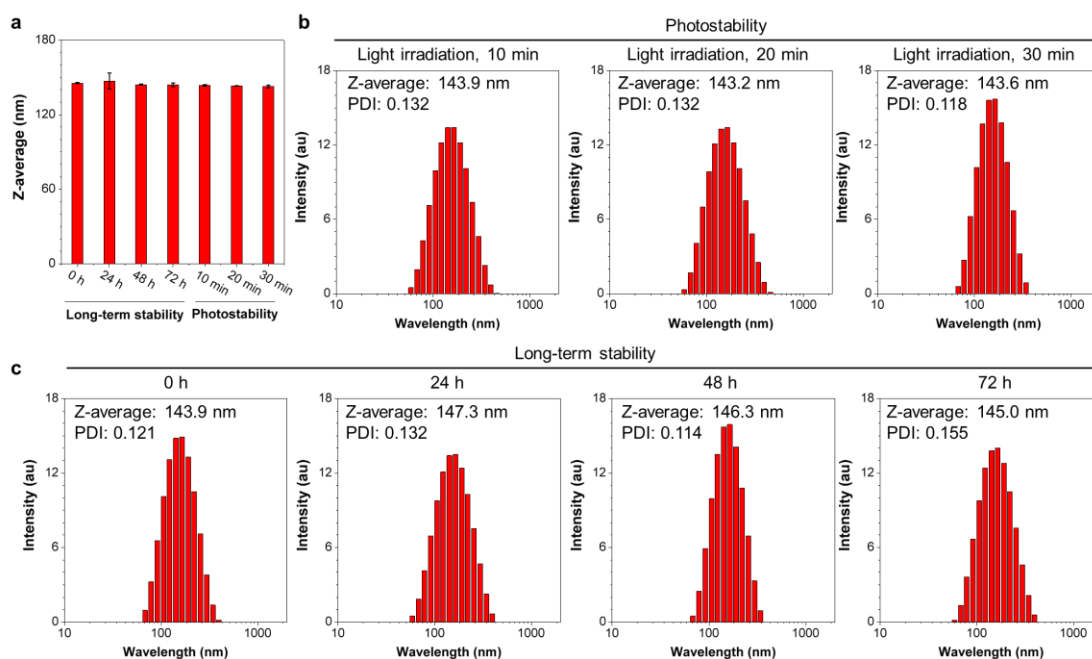


Fig. S28. (a) The average hydrodynamic diameter (*Z*-average) of TBmA aggregates measured

by Dynamic Light Scattering (DLS). The distribution of TBmA aggregates during 30 min light irradiation and 72 h FBS preservation.

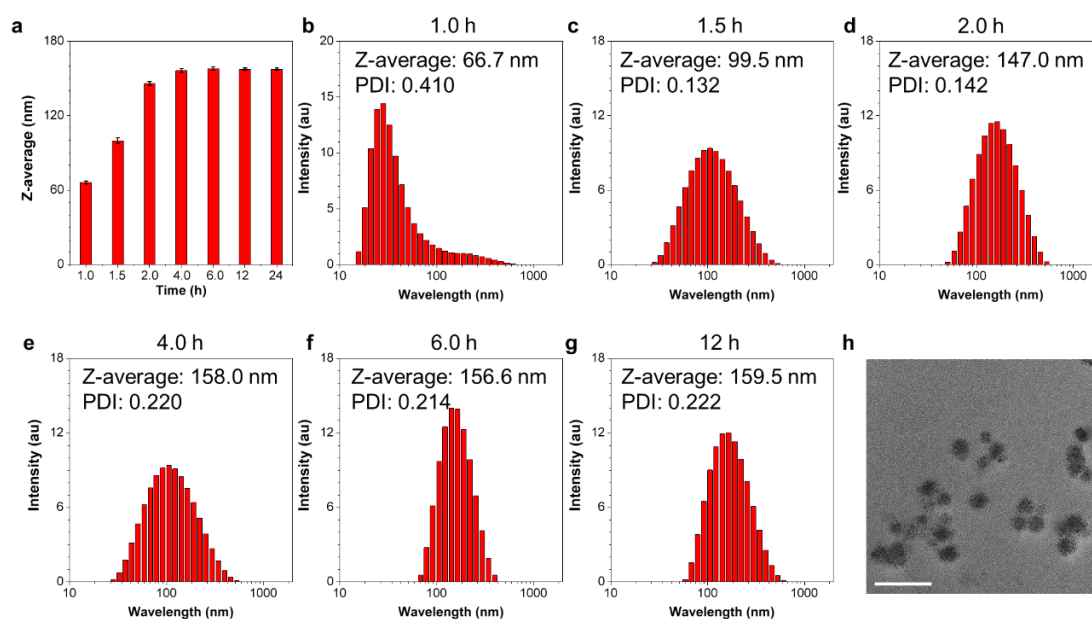


Fig. S31. (a) The average hydrodynamic diameter (*Z*-average) of TBmA aggregates produced in GGT catalytic reaction measured by DLS. (b-g) Distribution of TBmA aggregates formed at different times of GGT catalytic reaction. (h) The transmission electron microscope (TEM) of the TBmA aggregates formed after the GGT catalytic reaction for 12 h.

References

1. Zuo J, et al. Long-term spatiotemporal and highly specific imaging of the plasma membrane of diverse plant cells using a near-infrared AIE probe. *Angew. Chem. Int. Ed.* **14**, 2139-2148 (2023).
2. Wang Z, et al. Long-term fluorescent cellular tracing by the aggregates of aie bioconjugates. *J. Am. Chem. Soc.* **135**, 8238-8245 (2013).
3. Li K, et al. Photostable fluorescent organic dots with aggregation-induced emission (AIE dots) for noninvasive long-term cell tracing. *Sci. Rep.* **3**, 1150 (2013).
4. Wang J, et al. Nanolab in a cell: Crystallization-induced in situ self-assembly for cancer theranostic amplification. *J. Am. Chem. Soc.* **144**, 14388-14395 (2022).
5. Li Y, et al. Trojan Horse-Like Nano-AIE Aggregates Based on Homologous Targeting Strategy and Their Photodynamic Therapy in Anticancer Application. *Adv. Sci.* **8**, 2102561 (2021).
6. Yan Z, et al. Preparation of ultrasmall AIE nanoparticles with tunable molecular packing via freeze assembly. *Nano Lett.* **23**, 1030-1035 (2023).
7. Li Y, et al. Near-infrared light and redox dual-activatable nanosystems for synergistically

- cascaded cancer phototherapy with reduced skin photosensitization. *Biomaterials* **288**, 121700 (2022).
8. Tian S, He J, Lyu D, Li S, Xu Q-H. Aggregation enhanced photoactivity of photosensitizer conjugated metal nanoparticles for multimodal imaging and synergistic phototherapy below skin tolerance threshold. *Nano Today* **45**, 101534 (2022).
 9. Wang H, Xue K-F, Yang Y, Hu H, Xu J-F, Zhang X. In Situ Hypoxia-Induced Supramolecular Perylene Diimide Radical Anions in Tumors for Photothermal Therapy with Improved Specificity. *J. Am. Chem. Soc.* **144**, 2360-2367 (2022).
 10. Li X, et al. Nanostructured Phthalocyanine Assemblies with Protein-Driven Switchable Photoactivities for Biophotonic Imaging and Therapy. *J. Am. Chem. Soc.* **139**, 10880-10886 (2017).
 11. Chen C, Zhang X, Gao Z, Feng G, Ding D. Preparation of AIEgen-based near-infrared afterglow luminescence nanoprobe for tumor imaging and image-guided tumor resection. *Nat. Protoc.*, in press (2024).
 12. Liu Z, Wang Q, Zhu Z, Liu M, Zhao X, Zhu W-H. AIE-based nanoaggregate tracker: high-fidelity visualization of lysosomal movement and drug-escaping processes. *Chem. Sci.* **11**, 12755-12763 (2020).
 13. Yu Y, et al. Cytophilic Fluorescent Bioprobes for Long-Term Cell Tracking. *Adv. Mater.* **23**, 3298-3302 (2011).

Excitation wavelength:

The absorption peak of the monomeric compounds and the aggregates in this work is around 450 nm. This means essentially no penetration in tissues (just single cell width). This is the reason why the authors use a White LED light source, which is a non-descript identification of a light source, but is known that LEDs of this type have a very strong near UV peak. Part of the cell death in cell cultures is clearly due to white LED.

Response and revision: We appreciate the reviewer's concern regarding light penetration and the effects of our light source. Our analysis of the white LED light shows predominant peaks at 450 and 570 nm, with no detectable UV peak, which could address the concerns of the reviewers about unintended UV-induced effects (Fig. S27). Furthermore, all anticancer IC₅₀ values of tested compounds were detected using the MTT assays, and no significant effect on cell viability was detected in the control group after exposure to LED irradiation. MTT assays and control experiments demonstrate that the observed cell death is due to TBmA-Glu's photodynamic properties, not the LED light itself.

*** May be it wasn't clear in my earlier statement of concern, I did say near UV, but I was specifically referring to 450 nm peak. There are literature reports of blue (450 nm) light

causing cellular damage.

Depth of Penetration in Tissues: While it is true that the penetration depth of light at 450 nm is limited, this wavelength is still within the range where some penetration can occur in biological tissues. The actual penetration depth can be influenced by factors such as tissue type, pigmentation, and the optical properties of the tissue. Furthermore, we employed a minimally invasive approach for PDT to optimize the efficiency of photodynamic therapy and minimize the impact of light penetration.

*** One of the most important issues here is the fact that short wavelength irradiation is required to excite the chromophore, whether it is in organic or aqueous medium. 450 nm is not compatible with PDT. The typical penetration length at 450 nm is less than 1 mm, which is significantly less than needed for an effective “photo”-driven process.

Changes in the Revised Manuscript:

The generation of total ROS generation (2',7'-dichlorodihydrofluorescein, DCF), hydroxyl radical (hydroxyphenyl fluorescein, HPF) and singlet oxygen (9,10-anthracenediyl-bis(methylene)dimalonic Acid, ABDA) by photosensitizers (5 μM) after white LED light (predominant emission peaks at 450 and 570 nm, Fig. S27) irradiation (20 $\text{mW}\cdot\text{cm}^{-2}$) for 15 min using the corresponding ROS indicator in PBS/DMSO (v/v = 99:1). DCF, $\lambda_{\text{ex}} = 488 \text{ nm}$.

Changes in the Supporting Information:

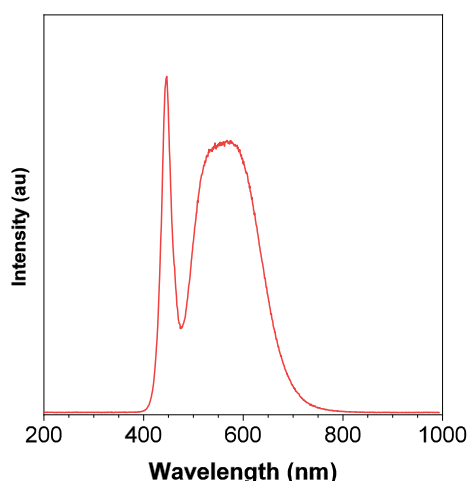


Fig. S27. The emission wavelength analysis of the LED light.

Confusion about the Type-I and Type-II PDT:

In recent articles regarding PDT, it seems like a misreading of PDT processes getting entrenched. PDT is a combination of both of these processes. Most ROS species are interconvertible by various enzymatic processes *in vivo*. Some articles also push the misconception that Type-I process (which are partly based on the degradation of the photosensitizers) are better, because it is less oxygen dependent; and it is not easy to separate these two processes (I/II).

Response: We appreciate the reviewer's insightful comments on the Type-I and Type-II PDT processes. We agree that PDT often involves a combination of both processes and that ROS species can undergo interconversion through various enzymatic processes *in vivo*. Our study focused on characterizing the predominant mechanism of TBmA under specific conditions, not comparing the superiority of Type-I vs Type-II processes. We found that the cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process. And aligning with this finding, we observed the oxygen independence of TBmA's photodynamic activity in the hypoxia condition, which is potentially advantageous in hypoxic tumor environments.

We acknowledge the complexity of PDT processes in biological systems, which may reflect both directly generated species and enzymatic interconversions. However, the ROS we detected in cells are coordinating with the results we detected *in vitro*, which validates the validity of our conclusion.

*** First of all, no PDT is independent of oxygen (please refer to Baptista, et al., *Photochemistry and Photobiology*, 2017, 93 (4) 912-919.) So, instead of 1 O₂ % hypoxia, if the authors were to switch to 0.5 % O₂ hypoxia, or anoxia, the effectiveness would be much more different.

I am also worried about the fact that the type-I designation is partly based on Figure 4b, there is some inconsistencies between the legend and the plot. Ebselen found in the legend, is not found on the plot, which is a singlet oxygen quencher. Also, Trolox, just like azide (N₃-) is a singlet oxygen quencher

Enhancement of emission "AND" PDT efficiency.

The authors should also keep in mind that any emission from the aggregates, is a loss in ROS generation efficiency. So, AIE-PDT carries a certain self-contradictory character.

Response: We appreciate the reviewer's insights regarding the competitive nature of fluorescence and reactive oxygen species (ROS) generation in AIE-PDT systems. While both processes utilize energy from the excited state, our findings on the simultaneous enhancement of aggregate luminescence and photodynamic activity are not contradictory. Here is some reported literature.

(i) **Aggregation-induced intermolecular intersystem crossing (AI-ISC):** Jiang et al. proposed a new mechanism called aggregation-induced intersystem crossing (AI-ISC) to

understand the effect of aggregation on increasing ISC efficiency.^{1,2} According to the AI-ISC theory, more excitonic couplings cause excited-state energy splitting and overlapping of singlet and triplet in aggregate. The energy splitting and overlapping significantly produce many ISC channels with very small ΔE_{ST} in aggregates, which is available for ISC processes. Therefore, the formation of aggregates can facilitate the production of triplet excitons. In addition to emitting phosphorescent radiation, these triplet excitons can also undergo a non-radiative pathway known as the aggregation-enhanced photodynamic effect to return to their ground state.^{3,4,5}

*** Regardless of the mechanism, the total quantum yield of all radiative and not radiative processes is not going to be larger than 1. So far, I did not come across a quantum yield of ROS formation, or emission quantum yield reported with aggregated structures. However, that should be the first thing to be studied when reporting a novel photosensitizer, but especially so, when both emission and ISC is claimed to be enhanced.

(ii) Restriction of intramolecular motion (RIM): The aggregation of AIE molecules results in a restriction of intramolecular rotations and vibrations, effectively suppressing molecular motions, which is also beneficial for the ISC process.^{6,7}

All the evidence highlights the potential of AIE materials in PDT. The aggregation-induced changes in the molecular environment can optimize both the imaging and therapeutic aspects of the treatment.^{8,9,10}

***Imaging on surface tumors or in mice, perhaps; but not therapeutics. Short wavelength excitation, and their aggregate structure, which would most likely disintegrate as it travels through the body into different sized nanoparticles would limit their potential.

References:

1. Li Q, *et al.* Time-dependent photodynamic therapy for multiple targets: A highly efficient aie-active photosensitizer for selective bacterial elimination and cancer cell ablation. *Angew. Chem. Int. Ed.* **59**, 9470-9477 (2020).
2. Liu Z, *et al.* Tuning organelle specificity and photodynamic therapy efficiency by molecular function design. *ACS Nano* **13**, 11283-11293 (2019).
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4. Wan Q, *et al.* Molecular engineering to boost aie-active free radical photogenerators and enable high-performance photodynamic therapy under hypoxia. *Adv. Func. Mater.* **30**, 2002057 (2020).

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6. Kwok RTK, Leung CWT, Lam JWY, Tang BZ. Biosensing by luminogens with aggregation-induced emission characteristics. *Chem. Soc. Rev.* **44**, 4228-4238 (2015).
7. Li Q, *et al.* Time-dependent photodynamic therapy for multiple targets: A highly efficient aie-active photosensitizer for selective bacterial elimination and cancer cell ablation. *Angew. Chem. Int. Ed.* **59**, 9470-9477 (2020).
8. Liu Z, *et al.* Tuning organelle specificity and photodynamic therapy efficiency by molecular function design. *ACS Nano* **13**, 11283-11293 (2019).
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Response

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The present manuscript claims to achieve a better targeting of a proposed photosensitizer (PS) which can be activated by GGT and be excited at short wavelengths.

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The main, may be the only reason why PDT did not develop significantly since 70's is that fact that light, even at the so-called "therapeutic window" does not go through tissues. And of course, there is no real justification for a 450 nm chromophore to be proposed as a novelty. There are very specific, niche cases, where a single cell layer penetration may be useful. But citing these, is missing the point of all PDT-work.

Type-I processes being less oxygen dependent has been proposed without real evidence. The new data provided by the authors is also not a fair comparison (see below).

Thus the manuscript does not bring any novelty to the field. The requirement for aggregation, if anything, complicates the picture very unnecessarily.

Reviewer #1 (Remarks to the Author):

Q1: The authors are accurate in stating that a comparison was not made (see the green text below from the manuscript). However, they claim that oxygen content has a negligible influence on the observed activity under hypoxia, which is linked to Type-I process, based on previous claims. These claims were not supported by "negative controls" with standard PDT photosensitizers, in a fair comparison.

251. "only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d). This suggests that oxygen content has negligible influence on its photodynamic activity. The cleaved TBmA-Glu (TBmA) primarily exerts its anticancer effects through the type I PDT process, which

is consistent with the prior findings.”

Response and revision: We appreciate the valuable suggestions provided by the reviewer. To enhance the accuracy of our study, we conducted additional experiments using Rose Bengal (RB), a well-established type II photosensitizer¹, as a control. These experiments revealed that RB's photodynamic efficiency decreased significantly under hypoxia conditions (2% O₂) compared to normoxia conditions, while TBmA maintained relatively consistent activity across both environments (Fig. R1).

To discuss this result more accurately, we have revised our manuscript by replacing the statement “This suggests that oxygen content has negligible influence on its photodynamic activity.” with “*This suggests that TBmA exhibits tolerance towards hypoxic conditions.*”

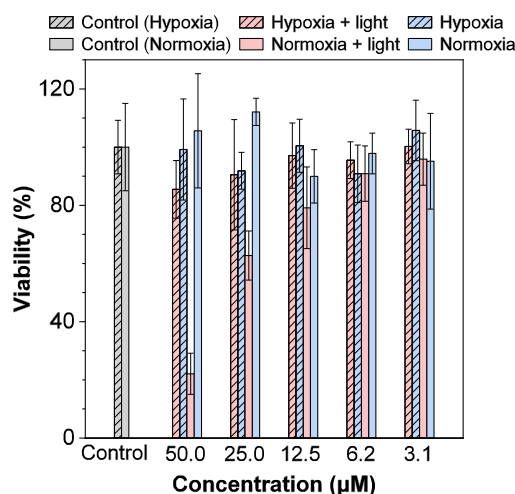


Fig. S36. The effects of hypoxia (2% O₂) and normoxia (20% O₂) conditions on the anticancer photodynamic efficiency of Rose Bengal against HepG2 cells.

Revised in manuscript:

Additionally, only marginal variation was observed in the phototoxicity of TBmA-Glu between normoxic and hypoxic conditions (Fig. 3d), while the type-II PS, RB, showed a significant decrease in photodynamic efficiency under hypoxia conditions (2% O₂) compared to normoxia conditions (Fig. S36). This suggests that TBmA exhibits tolerance towards hypoxic conditions.

Reference:

1. Fischer BB, Krieger-Liszky A, Eggen RIL. Oxidative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different molecular responses in *Chlamydomonas reinhardtii*. *Plant Sci.* **168**, 747-759 (2005).

response

+++ Light source in Fig S36 was not given, if it is white LED, it is not a fair comparison, because LED emission profile fits TbmA/aggregate better.

Q2: Aggregation is a type of supramolecular association which is perfectly reversible. It is only natural to expect deaggregation in the biological media with so many different gradients of hydrophobicity. FBS is not a good approximation for intracellular medium as its protein content is very low. Of course, a simple pharmacokinetics study would reveal how stable those aggregates are in vivo.

Response: We appreciate the reviewer's insightful comments regarding the nature of supramolecular aggregation and the potential for de-aggregation in biological media. We would like to clarify several key points that address these concerns.

Firstly, it's crucial to emphasize that TB*m*A-Glu is a water-soluble prodrug. The aggregation process only occurs after the Glu moiety is cleaved by GGT in HepG2 cells. This design ensures that TB*m*A-Glu remains soluble in the blood, avoiding premature aggregation. Aggregation is triggered specifically in the intracellular environment of GGT-overexpressing tumor cells.

We acknowledge that FBS is not an ideal model for the intracellular environment. To address this issue, we further conducted stability studies using a 30% BSA (Bovine Serum Albumin) solution, which is a better model for the protein-rich intracellular milieu. The intracellular protein concentration typically ranges from 50-400 mg/mL, and our 30% BSA solution (~300 mg/mL) falls within this range. TB*m*A aggregates showed remarkable stability in this environment, with no significant degradation observed over 72 hours (**Fig. R1**).

We also agree that pharmacokinetics studies would be valuable. However, our system presents unique challenges for such studies, as the aggregates form intracellularly rather than in circulation. Collecting and analyzing intracellular aggregates from tumor sections poses significant technical difficulties. Our approach using a highly concentrated protein solution provides valuable insights into aggregate stability in a physiologically relevant environment.

Importantly, beyond structural stability, we have observed that the aggregates maintain their photodynamic properties in the 30% BSA solution for 72 h (**Fig. R2**). This functional stability is crucial for the compound's theranostic applications.

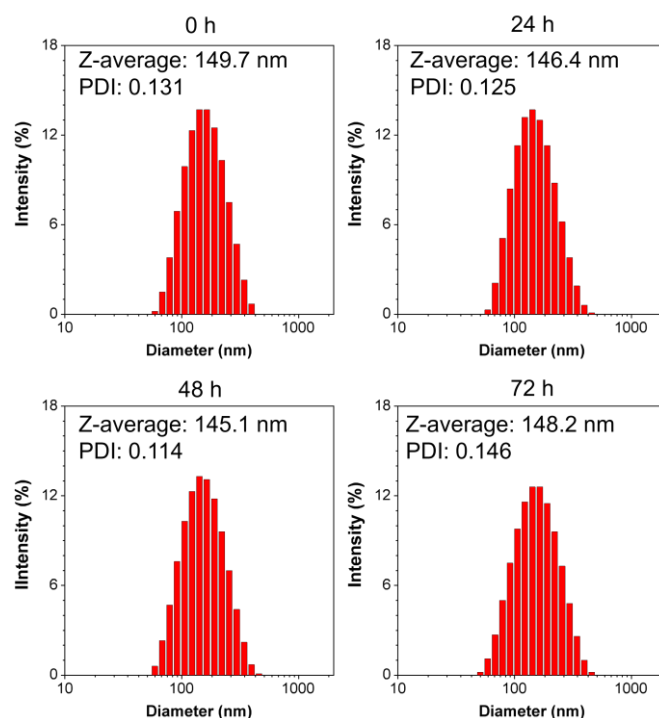


Fig. R1. The long-term stability of TBmA aggregates in 30% BSA solutions.

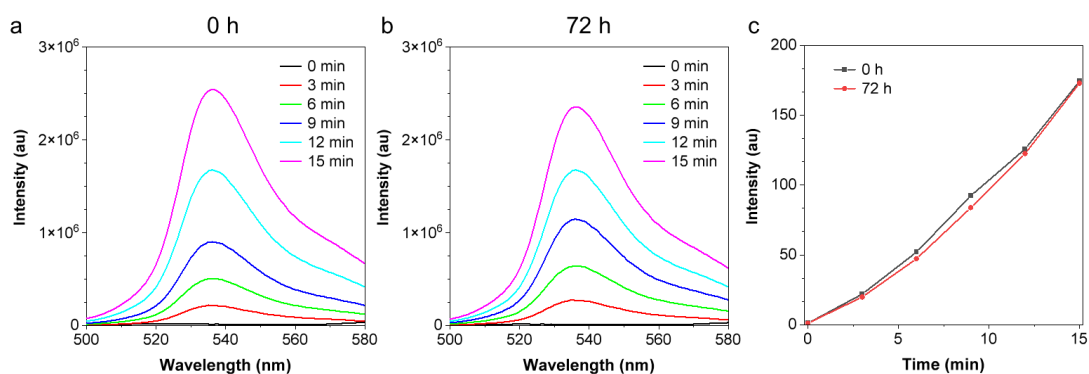


Fig. R2. The ROS generation capacity of TBmA aggregates after dispersed in 30% BSA solution for 0 h (a) and 72 h (b). The ROS was identified using DCFH as an indicator. (c) The plot of the relative emission intensity (I/I_0) of DC versus the irradiation ($20 \text{ mW} \cdot \text{cm}^{-2}$) time, where $I_0 = \text{PL intensity of DCFH in solutions without light irradiation}$.

Q3: References 4, 5 and 6 were carefully checked. Stability of the aggregates “in vivo” was not studied in these articles. Retention of fluorescence is not necessarily a sign of stability.

Response: We thank the reviewer for the critical feedback. The unique photophysical properties of AIE compounds stem from the restriction of intramolecular motion (RIM) mechanism, where aggregation limits molecular rotations and vibrations, leading to enhanced fluorescence. Therefore, the fluorescence behavior of AIE materials does provide valuable insights into their molecular state and environment.

This interpretation is supported by several factors. First of all, TBmA-Glu is

engineered to aggregate specifically in response to GGT activity, which is overexpressed in certain tumor cells. This targeted approach minimizes premature aggregation in circulation. Secondly, the crowded, protein-rich cytoplasmic environment of tumor cells likely provides conditions that favor aggregate stability once formed. Additionally, we observed that the photosensitivity of TBmA was maintained in our 30% BSA studies, suggesting a preservation of the aggregate structure.

Q4: While AIE compounds seem to provide potentially useful imaging opportunities, their relevance in PDT or other therapeutic schemes remain questionable. A therapeutic agent which would change size on meeting hydrophobic membranes or proteins, which could lead to different properties has to be handled very carefully. It would be advisable to avoid hype terminology such as “personalized medicine and real-time treatment monitoring”.

Response: We appreciate the reviewer's thoughtful comments regarding the therapeutic relevance of AIE compounds and the importance of careful characterization of their behavior in biological systems.

Regarding the stability and behavior of TBmA, we emphasize that TBmA-Glu is designed as a water-soluble prodrug that only forms aggregates within tumor cells following enzymatic reaction. This targeted approach minimizes potential issues related to premature aggregation or size changes in circulation. Furthermore, we have demonstrated the stability of TBmA aggregates in a 30% BSA solution for 72 hours, providing initial evidence of their potential stability in protein-rich environments.

About "personalized medicine and real-time treatment monitoring." in the previous response letter: The full sentence is “Recent literature has demonstrated the potential of AIE compounds for combining imaging and therapeutic functions in a single entity opening up new possibilities for personalized medicine and real-time treatment monitoring.” We agree that such terminology should be used judiciously, especially in early-stage research, however, our intention here is to highlight the potential of AIE materials to contribute to these fields in the future, rather than to claim immediate clinical applicability.

The unique properties of AIE materials, including their AIE and potential for stimuli-responsive behavior, do offer intriguing possibilities for both imaging and therapeutic applications. However, we agree that rigorous investigation is needed to establish their efficacy and safety for PDT or other therapeutic schemes. Moving forward, we will focus on providing concrete evidence for the specific advantages of AIE compounds in relevant biological contexts, rather than speculating on broad future applications. We believe this approach will better serve the scientific community and responsibly advance the field.

Q5: May be it wasn't clear in my earlier statement of concern, I did say near UV,

but I was specifically referring to 450 nm peak. There are literature reports of blue (450 nm) light causing cellular damage.

Response: We acknowledge that there are indeed literature reports of blue light (450 nm) causing cellular damage. This is an important consideration in photodynamic therapy and other light-based treatments. However, we would like to emphasize that the biological effects of light exposure are highly dependent on both wavelength and dosage.

In our experiments, we carefully controlled the light dosage to minimize potential phototoxicity while maintaining therapeutic efficacy. Under the experimental conditions described in our manuscript, we did not observe any significant effects on cell viability following LED light irradiation (**Fig. R3**).

To address the reviewer's concern, we also conducted a blue light irradiation (450 nm, 12 J/cm²) PDT assay. In this experiment, we also found no significant effect on cellular viability. This suggests that at the dosages used in our study, the blue light alone does not cause substantial cellular damage.

However, we agree that the potential for phototoxicity is an important consideration in developing light-based therapies. In future studies, we plan to conduct a more comprehensive dose-response analysis to determine the threshold at which blue light exposure may begin to affect cell viability. We also intend to investigate the potential long-term effects of repeated light exposure and compare the effects of our AIE-based approach with traditional photosensitizers at equivalent light doses.

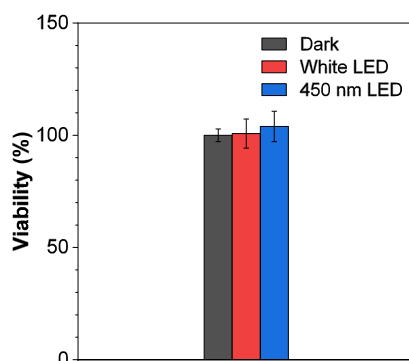


Fig. R3 The impact of white light and 450 nm blue light exposure (12 J/cm²) on the cellular viability of HepG2 cells.

Q6: One of the most important issues here is the fact that short wavelength irradiation is required to excite the chromophore, whether it is in organic or aqueous medium. 450 nm is not compatible with PDT. The typical penetration length as 450 nm is less than 1 mm, which is significantly less than needed for an effective “photo”-driven process.

Response: It is correct that the typical penetration depth of 450 nm light is less than 1 mm in tissue, which is indeed less than ideal for treating deep-seated tumors.

However, we would like to highlight several important considerations:

First, though direct light penetration is restricted, the effective depth of PDT damage may increase due to light reflection and scattering within tissues. This occurrence can expand the scope of the photodynamic impact beyond the initial penetration depth.

Secondly, several clinical scenarios exist where shallow light penetration is sufficient or even advantageous. For instance, PDT with blue light excitation could be particularly useful for superficial skin cancers and precancerous lesions, intraoperative treatment of residual tumor cells after surgical resection, treatment of early-stage mucosal cancers in inaccessible areas (e.g., oral cavity, bladder), and endoscopic applications for gastrointestinal tumors.

Finally, numerous published studies demonstrate the successful use of 450 nm light and white light (including the blue spectrum) for PDT when the photosensitizers have maximum absorption around 450 nm.^{2, 3, 4, 5, 6, 7}

Nevertheless, we fully agree that blue light's limited tissue penetration restricts the broader applicability of our current system for treating deep-seated tumors. Given this limitation, our future research directions include exploring two-photon excitation to achieve deeper tissue penetration, investigating upconversion nanoparticles to convert longer-wavelength light to blue light locally, and developing new AIE photosensitizers with red-shifted absorption for improved tissue penetration. We believe that addressing these challenges will expand the potential applications of our AIE-based PDT system while utilizing its unique properties.

References:

2. Fan L, *et al.* A Bioactive Photosensitizer for Hypoxia-Tolerant Molecular Targeting-Photo-Immunotherapy of Malignant Tumor. *Adv. Funct. Mater.* **34**, 2313755 (2023).
3. Li X, *et al.* A novel 450-nm laser-mediated sinoporphyrin sodium-based photodynamic therapy induces autophagic cell death in gastric cancer through regulation of the ROS/PI3K/Akt/mTOR signaling pathway. *BMC Med.* **20**, 475 (2022).
4. Mei Y, *et al.* A Novel Photosensitizer Based 450-nm Blue Laser-Mediated Photodynamic Therapy Induces Apoptosis in Colorectal Cancer - in Vitro and in Vivo Study. *Front. Biosci. (Landmark Ed)* **29**, 199 (2024).
5. Chen Y, *et al.* Photoactivatable metal organic framework for synergistic ferroptosis and photodynamic therapy using 450 nm laser. *Chem. Eng. J.* **454**, 140438 (2023).
6. Sun P, *et al.* A water-soluble phosphorescent conjugated polymer brush for tumor-targeted photodynamic therapy. *Polym. Chem.* **8**, 5836-5844 (2017).
7. An J, *et al.* An unexpected strategy to alleviate hypoxia limitation of photodynamic therapy by biotinylation of photosensitizers. *Nat. Commun.* **13**, 2225 (2022).

Q7: First of all, no PDT is independent of oxygen (please refer to Baptista, et al., Photochemistry and Photobiology, 2017, 93 (4) 912-919.) So, instead of 1 O₂ % hypoxia, if the authors were to switch to 0.5 % O₂ hypoxia, or anoxia, the effectiveness would be much more different.

I am also worried about the fact that the type-I designation is partly based on Figure 4b, there is some inconsistencies between the legend and the plot. Ebselen found in the legend, is not found on the plot, which is a singlet oxygen quencher. Also, Trolox, just like azide (N₃⁻) is a singlet oxygen quencher.

Response and revision: We agree with the reviewer that oxygen plays a pivotal role in the Type I and Type II PDT processes. However, from the PDT mechanism, we know that the type I photosensitizers could directly transfer electrons to the substrate, forming a radical cation or neutral radical. These radicals could immediately react with O₂ or H₂O to generate hydrogen peroxide (H₂O₂), hydroxyl radicals (\cdot OH), or superoxide anions (\cdot O₂⁻) (**Fig. R4**).^{1,2}

We have tried but could not finish the antitumor PDT assays in the anaerobic conditions, because the anoxia condition resulted in death of the tumor cells (**Fig. R5a**). So, we re-evaluated the photodynamic efficiency of TBmA and RB using a deoxygenated PBS solution. The results showed that TBmA could also induce the oxidation of DFCH under the anoxia condition (**Fig. R5b**), while the photodynamic efficiency of RB showed significant degradation. Hence, type-I photosensitizers exhibit relatively higher tolerance towards oxygen concentrations, which implies that, even under low oxygen conditions, they can still engage in substrate reactions through electron transfer.

We are sorry for the mistake in the figure legend in Figure 4b. “Ebselen” has been revised as “Trolox.” However, it should be noted that Trolox is not only a ¹O₂ scavenger but also a scavenger of peroxy and alkoxy groups.³ The type-I designation is mainly based on the ROS species we detected *in vitro* (**Fig. R5c**).

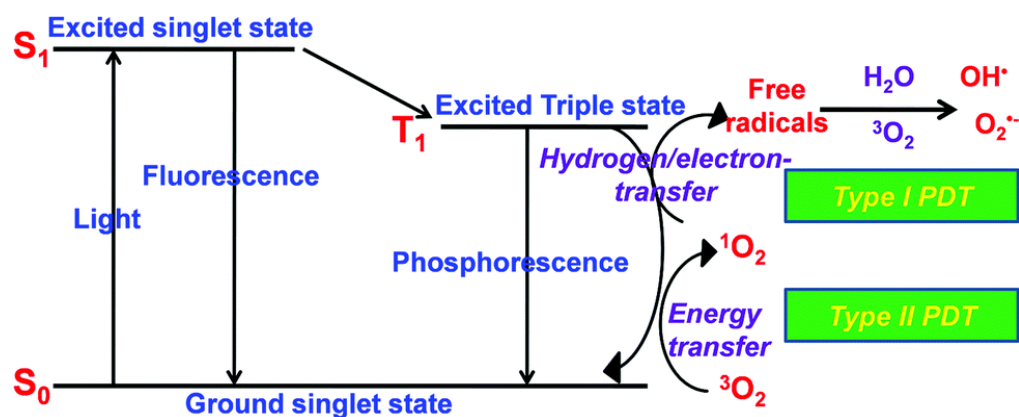


Fig. R4 Scheme of the photochemical reactions for type I and type II PDT.⁹

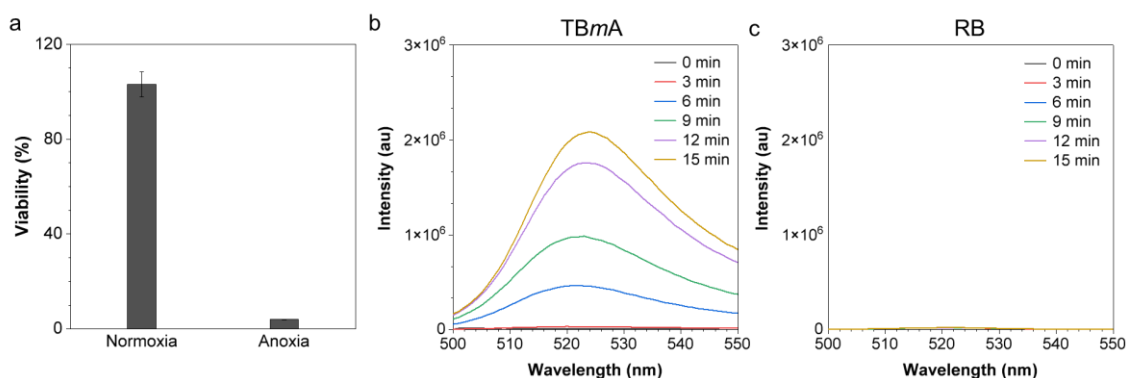


Fig. R5 (a) Cellular viability of HepG2 cells in normoxia and anoxia conditions. (b) Fluorescence emission changes of DCFH (Dichlorodihydrofluorescein, 10 μM) in the presence of 5 μM photosensitizers in DMSO-PBS ($v:v = 1:99$) after irradiation (20 $\text{mW}\cdot\text{cm}^{-2}$) for a different time under anoxia conditions. (b) TBmA, (c) Rose Bengal (RB). DCHF, $\lambda_{\text{ex}} = 488 \text{ nm}$.

Revised in manuscript:

Trolox: 50 μM ($\text{ROO}\cdot$ scavenger and $^1\text{O}_2$ scavenger); D-mannitol: 50 mM ($\cdot\text{OH}$ scavenger); Tiron: 10 mM ($\cdot\text{O}_2^-$ scavenger); NaN_3 : 5 mM ($^1\text{O}_2$ scavenger)

References:

1. Zhao X, Liu J, Fan J, Chao H, Peng X. Recent progress in photosensitizers for overcoming the challenges of photodynamic therapy: from molecular design to application. *Chem. Soc. Rev.* **50**, 4185-4219 (2021).
2. Fan W, Huang P, Chen X. Overcoming the Achilles' heel of photodynamic therapy. *Chem. Soc. Rev.* **45**, 6488-6519 (2016).
3. Lúcio M, Nunes C, Gaspar D, Ferreira H, Lima JLFC, Reis S. Antioxidant Activity of Vitamin E and Trolox: Understanding of the Factors that Govern Lipid Peroxidation Studies In Vitro. *Food Biophys.* **4**, 312-320 (2009).

+++ 0.5 or 1 % hypoxia may be better.

Q8: Regardless of the mechanism, the total quantum yield of all radiative and not radiative processes is not going to be larger than 1. So far, I did not come across a quantum yield of ROS formation, or emission quantum yield reported with aggregated structures. However, that should be the first thing to be studied when reporting a novel photosensitizer, but especially so, when both emission and ISC is claimed to be enhanced.

Response: Indeed, the total quantum yield of all radiative and non-radiative processes cannot exceed 1. However, the energy consumption in no radiative processes contains both the energy for ISC processes and the molecular motion as well. Molecular aggregation could induce the restriction of intramolecular motions (RIM) and, as a result, reduce energy loss through non-radiative molecular motion, potentially

increasing the energy available for emission and ISC processes. So, the energy efficiency of both emission and ISC can be enhanced in aggregated structure due to RIM.

However, in specific cases, such as the graphene quantum dots reported by Zhang et al., the apparent quantum yield could be larger than 1.¹ This occurs when the energy gaps between ΔE_{ST} and ΔE_{TG} (the energy gap between T_1 and Ground state) are larger than the formation energy of 1O_2 (22.5 kcal mol⁻¹). In such cases, 1O_2 generation happens through multiple pathways: energy transfer from T_1 (ET(1) in Fig. R6), but also the energy transfer from S_1 to 3O_2 during the S_1 - T_1 intersystem crossing transition (ET(2) in Figure R6). This multi-pathway mechanism can lead to an overall 1O_2 quantum yield greater than 1.0, as more than one 1O_2 molecule can be produced per absorbed photon.²

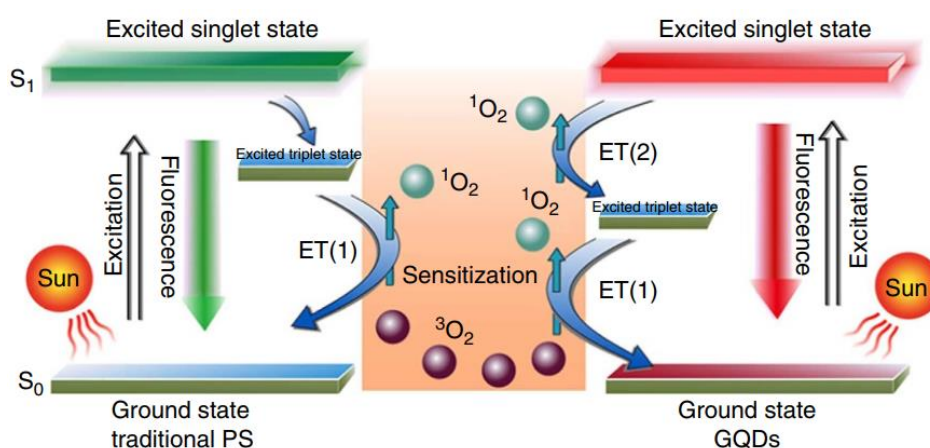


Fig. R6 Schematic illustration of the 1O_2 generation mechanisms by conventional PDT agents (left) and GQDs (right).

References

1. Ge J, *et al.* A graphene quantum dot photodynamic therapy agent with high singlet oxygen generation. *Nat. Commun.* **5**, 4596 (2014).
2. Kanner RC, Foote CS. Singlet oxygen production from singlet and triplet states of 9,10-dicyanoanthracene. *J. Am. Chem. Soc.* **114**, 678-681 (1992).

+++ Both of these articles while interesting, hardly relevant to PDT considering the absorption peaks of the proposed sensitizers are in blue, and the fact that they are very unique cases. The first one reached to a suprising conclusion without doing any photophysical work. Vibrational (or rotational) relaxation and their control by micro- or molecular environments, by molecular steric hinderence is well known. However, only accurate quantum yield determinations would prove simulataneous increases in emission and singlet oxygen quantum yields. This is not done in Ref 1.

Q9: Imaging on surface tumors or in mice, perhaps; but not therapeutics. Short

wavelength excitation, and their aggregate structure, which would most likely disintegrate as it travels through the body into different sized nanoparticles would limit their potential.

Response: As previously discussed, TBmA-Glu is a water-soluble molecule that forms aggregates within tumor cells upon activation by GGT to produce TBmA. Consequently, most of these aggregates are localized in the tumor cells. Furthermore, we have demonstrated the stability of TBmA aggregates for 72 hours in a 30% BSA solution. Additionally, considering that PDT processes were conducted 12 hours after administration of TBmA-Glu, it can be inferred that the TBmA aggregates exhibit sufficient stability to complete the PDT processes.

+++ The problem is that now “activated” aggregates, will not stay forever in tumor cells, as these cells disintegrate.