nature portfolio

Peer Review File

A Stable and Biocompatible SWIR Nanoribbon for Dual-channel in vivo Imaging

Corresponding Author: Professor Youjun Yang

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

Editorial Note: Reviewer 1 was asked to look over comments from Reviewer 3 in the third round of review

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The development of high-performance shortwave infrared (SWIR) dyes is important and challenging research endeavor. Any research progress along this line of research will benefit multiple fields. In particular, ultra-stable and biocompatible SWIR absorbing/emitting materials are attracting ever-increasing interests in bioimaging and biomedical applications. Thus, robust strategies to develop ultra-stable and biocompatible SWIR dyes are highly soughtafter and beneficial to the field.
Indeed, SWIR active materials can be accessed via supramolecular dye aggregation. Unfortunately, development of dye aggregation remains a serendipitous endeavor. In this work, based on their recently developed bisbenzannulated silicon-rhodamine dye framework, Yang et al. introduces a novel Crystal-Aided Aggregate Synthesis (CAASH) approach for the systematic development of J-aggregates. The adoption of this approach facilitated the creation of a distinctive, water-soluble SWIR JV-aggregate, incorporating a bisbenzannulated silicon rhodamine scaffold (ESi5). The SWIR-aggregates produced demonstrate remarkable stability against a range of factors, including organic solvents, varying pH conditions, sonication, photodegradation, thiols, and endogenous oxidative species. Notably, the aggregates can be further refined through heating or annealing procedures to minimize aggregation irregularities. Moreover, the biocompatibility of these aggregates underscores their immense potential for applications in in vivo imaging and beyond, opening avenues for innovative biomedical research and diagnostic tools.
Overall, I find the work very interesting, tackling a long-standing challenge of rational design of dye aggregates. The scholar presentation of this manuscript is of high quality. I feel that this work could be a very important contribution to the field, and strongly recommend acceptance of this manuscript after the following minor revisions to further improve the quality of this manuscript.

1. Have the authors investigated the influence of low-temperature preparation on the formation and properties of ESi5-S aggregates?
2. Fig.3, TEM photography, is it done immediately after sample preparation, or after storing for a few days? Because these nanofibers usually require time to grow.
3. Fig. S30, the labels for Oxidant and Thiol are reversed.
4. “An aliquot of ESi5-S agg solution (500 μM in PBS, 100 μL) was injected into a BALB/c mouse through the tail vein. The first set of images was acquired 10 min post injection (Figure 5C).” “Figure 5C” should be “Figure 5E”.
5. Abstract: “The aggregates are biocompatible and has tremendous potential in in vivo imaging and more.” “Has” should be “have”.
6.In supporting material, page S26, the content of Scheme S2 was missing.

Reviewer #2

(Remarks to the Author)
In this work, Yao et al. report a method to construct SWIR aggregates. These JV-aggregates with a bisbenzannulated silicon rhodamine scaffold exhibit high stability and could be applied in dual-channel in vivo bioimaging. Although these results were interesting, there are some important concerns to be better studied.
1) Silicon rhodamine dyes have the intrinsic good stability. The design and synthesis of this amphiphilic bisbenzannulated silicon rhodamine scaffold (ESi5) were pretty similar to their previous work, such as J. Am. Chem. Soc. 2022, 144, 14351−14362; Angew. Chem. 2017, 129, 3025–3029. After reading the introduction, I cannot easily get the key point of the

innovation in chemistry to construct excellent SWIR J-aggregates. Compared to previous NIR-II J-aggregation strategies (Adv. Mater. 2023,2304848), I'm not convinced that the reported results contain the sort of original chemical insights to develop NIR-II J-aggregates.
2) Typically, J-aggregates are noticed by accident. The key challenge in the development of SWIR J-aggregates is a lack of clear reference and design guidelines. The authors claimed that they report a rational approach, termed Crystal-Aided Aggregate SyntHesis (CAASH), to develop J-aggregates. However, it's hard to understand the advancement of Crystal-Aided. Based on the structure of ESi5, different types of aggregation are probably caused by electronic and steric effects. I suggest the authors should focus on the study of structure-property relationships.
3) Figure 1 should be re-organized to clearly convey information.
4) Besides slipping angle, the slipping distance and stacking distance should be clearly presented and discussed.
5) It's not easy to understand the V-type intra-chain packing. It seems like intermolecular packing in Figure 1B.
6) These SWIR aggregates exhibit two red-shifted and one blue-shifted sharp peaks. It's better to add more discussions about blue-shifted sharp peaks.
7) These SWIR aggregates suffer from low quantum yields. I suggest to figure out a solution to increase brightness via structure modification.
8) Many figures in the supporting information were not discussed in the main text. ESi5-S and ESi5-C1(-C5) aggregated instantaneously upon the addition its MeOH stock into H2O (Figure S14). Figure S14 was not correctly cited.
9) What are the advantages of V-aggregation?
10) How does the charge of pendent groups affect the aggregation behaviors?
11) ESi5-S agg was essentially non-cytotoxic when its concentration was smaller than 10 μM, since the cells viability was higher than 85% after a 24 hr incubation with HeLa cells (Figure S34). 10 μM was obviously lower than the dose used in mice. The in vivo toxicity of ESi5-S agg should be tested.
12) It's better to examine the aggregation behavior of ESi5-S agg with 10% FBS or in serum.
13) In 180 min, the monomer was metabolized from the liver to the intestine (Figure 5J). Figure 5J should be Figure 5F.
14) The authors should discuss the reason why ESi5-S agg could bind to the skeletal system in the main text.

Reviewer #3

(Remarks to the Author)
In the manuscript "A rational approach to ultra-stable and biocompatible SWIR nanoribbion for dual-channel in vivo imaging" Yang and coworkers report the J-aggregate of their previously reported ESi5 dye. They frame the manuscript as a new method to predict/engineer J-aggregation: CAASH. The J-aggregate characterization within the manuscript is well-done and thorough. The J-aggregates are then applied for in vivo imaging using multiplexing to image both the monomer and the aggregate. Overall, this reviewer has concerns over the generalizability of the CAASH method and the toxicity/usefulness of the probes for in vivo imaging which prevent this manuscript from being at the level necessary for Nature Communications.

The CAASH protocol requires having a crystal structure of the fluorophore and being able to model in substituents with the assumption that the packing does not change when amphiphilic character is added. It is hard to do step 1 for most fluorophores and this is likely the more reliable step of the two. It is very impressive that this system worked for ESi5 and the crystal packing and structures obtained are beautiful. These structures really help in the analysis of the H and J coupling in the aggregates. However, this method should not be put forward as generalizable.

After characterization, the J-aggregates are used from two color imaging where the bluer channel allows for the monomer to be imaged and the red channel is the aggregate. This is a clever way to see the dissociation of the aggregate in vivo. The aggregate has surprising stability in vivo. The authors state that the blood vessels cannot be observed in either channel citing aggregate stability– this comment is true for the monomer but shouldn't aggregate emission be observed in the vessels upon injection? What does the 0 min timepoint look like (Figure S36 indicates there should be a 0 min but is labeled as 10 min in the figure). Intensity bars, exposure times and all imaging metrics should be included in Figure 5 and S36. There should be at least one biological replicate of the imaging experiments shown.

The concentration of probe i.v. injected is 500 uM with 4% DMSO. The J-aggregate starts to show toxicity in cell culture at 10 uM (50x less than injected!). The hemolysis test is also not performed as these concentrations. Finally, DMSO should not be injected intravenously. These are significant concerns about the stated utility of the probe.

Other comments/concerns:

What does the J-aggregate look like in the presence of FBS?

The conclusion states that the aggregates are superradiant but this analysis was performed/discussed

Relative fluorescence quantum yields should be performed with available standards and references should be given for their quantum yield values. The value for IR-1061 is incorrect and over estimates the quantum yield of the aggregates.

The camera used for imaging is not stated.

If the mice are shaved before imaging is not stated.

Figure S29 should report the photobleaching rates. There is a typo in Figure S29 legend.

In Figure S30 the thiol and oxidant labels are reversed.

The procedure corresponding to Figure S40 should be in the SI.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This revised manuscript well addressed all my previous concerns/comments. The authors provided a TEM imaging of the aggregation process at different time points to highlight the entire process from particulate, to a mixture of particulate and short ribbon, and eventually to a network of long ribbon. These are very informative and high quality results.
I also enjoyed reading the responses of the authors to the other two reviewers. In particular, the authors showcased the proof-of-concept application of the use of this aggregate for photoacoustic mouse imaging. The figure G is very impressive. This is the first time that deep-buried bones could be imaged with such elaborate details. This experiment indeed greatly expanded the potential of this aggregate for biological applications.
I have a suggestion regarding photoacoustic imaging. Considering the narrow-bandwidth of the aggregate absorption peak, it becomes immediately obviously that the authors may further develop system with slightly different absorption maxima. All these aggregates with different absorption maxima would allow multiplexing in the NIR spectral region. This would address the current bottleneck for multiplexing with monomeric NIR fluorophores. Their absorption bands are usually very broad, exhibit extensive overlapping and therefore leads to serious cross-talk between different channels. I found that the authors did not mentions its potentials in multiplexing in the response and in the manuscript. Of course, for this to be feasible, the authors would need to figure out how to let different aggregate target different tissues in vivo. This is not obvious, but still practical. Addition of this discussion in the manuscript help the audience better appreciate the significance of this aggregate. With these additional data and discussion, this reviewer feel that this quality of this manuscript has been further greatly improved.
As I previously indicated, development of well-defined dye aggregates, especially with favorable properties including strong absorption/emission in NIR-II region, remains a serendipitous endeavor. The authors proposed an elegant way to give a distinctive, water-soluble SWIR JV-aggregate. More importantly, the authors proposed a rational approach (CAASH) toward judicious design of rarely encountered V-type aggregates. I believe that this work would open up a new and dynamic research direction of dye aggregation, and highly recommend the acceptance of this manuscript for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
I agree the dye ESi5 is an awesome NIR fluorophore. However, as the authors claimed, if two zwitterionic chains were installed, a blue-shifted spectrum of H-aggregation was observed. With two cationic ammonium chains, the dye molecules did not aggregate. Only the two chains contain carboxylate or sulfate groups, the dyes aggregate into the nanoribbon (J-aggregation). The strategy of crystal-aided aggregate synthesis (CAASH) did not provide a general design guideline for the construction of highly ordered J-aggregates.

Reviewer #3

(Remarks to the Author)
The authors have responded to the reviewer comments but there are still concerns regarding this manuscript that prevent publication in Nature Communications.

1) The rational design/CAASH strategy is not a generalizable approach to J-aggregate formation. The authors agree with this in the reviewer response

“We totally agree with the reviewer for the generalizability of any given method with respect to the development of an ordered dye aggregate. It is exactly for this reason that beautiful aggregates are so rare and developing novel ordered dye aggregates are challenging.
The whole field would agree that development of ordered dye aggregate remains largely serendipitous. What we wish is to introduce a layer of rationality over the development of ordered dye aggregate. The crystal structures of a dye is the only experimental grasp that a chemist can have. It is fortunate that we succeeded in this piece of work and developed a beautiful double-layer nano-ribbon exhibiting SWIR absorption/emission. But, we absolutely agree with the reviewer that our success does not necessarily mean that this method is surely generally applicable to other class of dye molecules.”

Furthermore in other points of the reviewer response they state that they indeed made a bunch of different variants for this approach to be successful.

“The success growth of the crystal is not a guarantee that a certain aggregate would be obtained. Tremendous laboratory dedications of the graduate students tinkering the structural nuances of ESi5should not be taken by granted. For example, when two zwitterionic chains are installed, a blue-shifted spectra was obtained upon presumably an H-aggregate. With two cationic ammonium chains, the dye molecules did not aggregate until a fairly high concentration. Only with two chains with a carboxylate or sulfate, the dyes aggregates into the anticipated nano-ribbon. The aforementioned structure-propertystudy is displayed in Figure 2 of the manuscript.”

Yet they have only made two minor changes to their manuscript.

Abstract: “Herein, we propose a crystal-aided aggregate synthesis (CAASH) approach to introduce a layer of rationality for the development of J-aggregate.”
Conclusion: “In conclusion, we proposed an approach, i.e., crystal-aided aggregate synthesis (CAASH), to make J-aggregate development more of a rational and less of a serendipitous endeavor.”

The title and main figures putting forward CAASH as a strategy for J-aggregate formation still remain, when even the authors agree this isn’t an approach that is going to be broadly beneficial.

2) The description of superradiance in the main text is incorrect. The supperradiance values should be calculated.

3) The response to determining quantum yields in the SWIR region is unsatisfactory. SWIR detectors have advanced significantly and multiple papers have been published on accurately determining the quantum yield of IR-26 which is accepted as a standard for relative quantum yield measurements in the field.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all the comments properly. I recommend the publication of the manuscript in Nat. Comm. in the present form.

Comments and the revision suggestions of the Reviewer #1:

The development of high-performance shortwave infrared (SWIR) dyes is important and challenging research endeavor. Any research progress along this line of research will benefit multiple fields. In particular, ultra-stable and biocompatible SWIR absorbing/emitting materials are attracting ever-increasing interests in bioimaging and biomedical applications. Thus, robust strategies to develop ultra-stable and biocompatible SWIR dyes are highly soughtafter and beneficial to the field.

Indeed, SWIR active materials can be accessed via supramolecular dye aggregation. Unfortunately, development of dye aggregation remains a serendipitous endeavor. In this work, based on their recently developed bisbenzannulated silicon-rhodamine dye framework, Yang et al. introduces a novel Crystal-Aided Aggregate Synthesis (CAASH) approach for the systematic development of J-aggregates. The adoption of this approach facilitated the creation of a distinctive, water-soluble SWIR JV-aggregate, incorporating a bisbenzannulated silicon rhodamine scaffold (ESi5). The SWIR-aggregates produced demonstrate remarkable stability against a range of factors, including organic solvents, varying pH conditions, sonication, photodegradation, thiols, and endogenous oxidative species. Notably, the aggregates can be further refined through heating or annealing procedures to minimize aggregation irregularities. Moreover, the biocompatibility of these aggregates underscores their immense potential for applications in in vivo imaging and beyond, opening avenues for innovative biomedical research and diagnostic tools.

1. Have the authors investigated the influence of low-temperature preparation on the formation and properties of ESi5-S aggregates?

   Reply:

   We tested the influence of low temperature on the aggregation outcome via UV-Vis absorption spectroscopy, which reliably reflects the aggregation pattern. We pipetted an aliquot of **ESi5-S agg** (2 μL in MeOH) into a solution of PBS (pH = 7.4, 10 μM, 2 mL, containing 5% MeOH) at 10 °C. The mixture was vigorously stirred for 30 s before the absorption spectrum of the resulted solution was acquired. Over the next course of ca. 15 mins, the temperature of the solution gradually warmed to the room temperature and the absorption spectrum of the solution was acquired frequently. The absorption spectra at different temperature completed overlaid with each other, suggesting that the aggregation pattern of **ESi5-S agg** in this tested temperature range remain unchanged. The following graph is now incorporated in the SI as Figure S37. Temperature lower than 10 °C was not tested due to condensation of the cuvette.

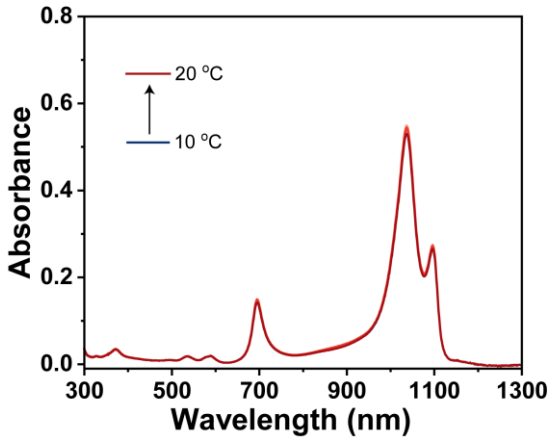

Figure S37: The UV-Vis absorption spectra of a solution of **ESi5-S agg** (5 μM in PBS, containing 5% methanol) at different temperature (10 °C to 20 °C).

2. Fig.3, TEM photography, is it done immediately after sample preparation, or after storing for a few days? Because these nanofibers usually require time to grow.

   Reply:

We thank the reviewer for this thoughtful question. We followed the suggestion of the reviewer and acquired the TEM images of the aggregate solution at different delay upon mixing the **ESi5-S agg** and the H_2O.

The TEM sample for imaging was prepared essentially immediately upon mixing. We pipetted 5 μL of the **ESi5-S agg** solution (0.2 mM in H_2O, containing 4% MeOH), dispensed it onto the a 400-mesh formvar supported copper grids coated with ultra-thin carbon film, and waited 10 minutes before the excess liquid was absorbed with filter paper. Then the TEM image of the sample was acquired to produce the image at 0 h. Similarly, the TEM images at 6 h and 24 h were acquired after the solution was allowed to sit unperturbed for 6 h and 24 h respectively before the solution was transferred onto the support grid.

"*The morphology of the **ESi5-S agg** was different with respect to the sitting time of the solution. Without sitting time, the aggregate adopted the form of small particulate with a diameter of 7.7 ± 0.6 nm (Figure S29A). In 6 h, the particulate could still be seen while the aggregate predominantly existed in form of short nano-ribbon with an average width of 39.6 ± 1.6 nm, and an average length of 0.44 μm(Figure S29B). In 24 h, the short nano-ribbon further grew into a network. Some ribbon exhibited an increased width of up to 116.2 ± 2.2 nm. (Figure 3A, Figure S29C)" "Despite the morphological change, it was interesting to note that the absorption spectra of the bulk aggregate solution remained unchanged. This indicated that the molecular packing of the monomer in the particulate at 0 h, short nano-ribbon at 6 h, and long nano-ribbon network at 24 h remained unchanged.*"

The results of this experiment provided an understanding of the growth of the nano-ribbon and we thank the reviewer for his/her input. The italicized section is incorporated in the manuscript and the Figure S29 is updated with the following images.

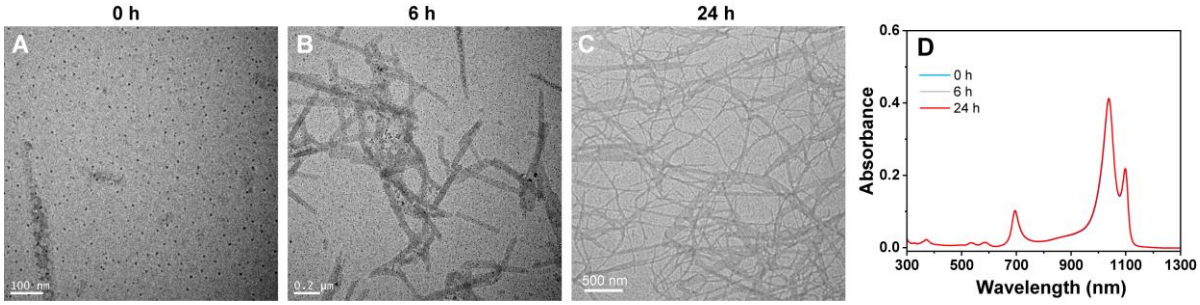

Figure S29. (A, B, C) The TEM images and (D) absorbance spectrum of **ESi5-S agg** (200 μM in H_2O, containing 4% MeOH) self-assembled for 0 h, 6 h and 24 h, respectively.

3. Fig. S30, the labels for Oxidant and Thiol are reversed.

Reply:

The error is now corrected.

4. "An aliquot of ESi5-S agg solution (500 μM in PBS, 100 μL) was injected into a BALB/c mouse through the tail vein. The first set of images was acquired 10 min post injection (Figure 5C)." "Figure 5C" should be "Figure 5E".

Reply:

The error is now corrected.

5. Abstract: "The aggregates are biocompatible and has tremendous potential in in vivo imaging and more." "Has" should be "have".

Reply:

The error is now corrected.

6. In supporting material, page S26, the content of Scheme S2 was missing.

Reply:

The error is now corrected.

Comments and revision suggestions of the reviewer 2:

In this work, Yao et al. report a method to construct SWIR aggregates. These JV-aggregates with a bisbenzannulated silicon rhodamine scaffold exhibit high stability and could be applied in dual-channel in vivo bioimaging. Although these results were interesting, there are some important concerns to be better studied.

1. Silicon rhodamine dyes have the intrinsic good stability. The design and synthesis of this amphiphilic bisbenzannulated silicon rhodamine scaffold (ESi5) were pretty similar to their previous work, such as J. Am. Chem. Soc. 2022, 144, 14351−14362; Angew. Chem. 2017, 129, 3025−3029.

Reply:

We thank the reviewer for the interests in our work. We are a synthetic dye chemistry group focusing on rational development of novel xanthenoid dyes absorbing/emitting beyond 800 nm.

The **ECX** dyes reported in *Angew Chem* **2017** and the **ESi5** scaffold in *J. Am. Chem. Soc.* **2022** differs structurally from each other in the following aspects. First, the central rigidifying moiety of the **ECX**, *i.e*., a xanthene unit, is replaced by the dimethyl or diphenyl substituted silicon atom. The silicon atom is larger in atomic radius and therefore reduces the vibrational normal modes. **ESi5** dyes become brighter. The silicon atom also bears an empty d orbital, which results in a desirable spectral red-shift of *ca.* 40 nm. Second, the julolidine unit of the **ECX** is replaced by a chloropropyl substituted tetrahydroquinoline unit. This is critical for further functionalization of the **ESi5** scaffold.

These structural changes are based on rational design to further improve the photophysical or stability properties.

2. After reading the introduction, I cannot easily get the key point of the innovation in chemistry to construct excellent SWIR J-aggregates. Compared to previous NIR-II J-aggregation strategies (Adv. Mater. 2023,2304848), I'm not convinced that the reported results contain the sort of original chemical insights to develop NIR-II J-aggregates.

Reply:

Development of ordered dye aggregates could be considered the holy grail of the field of supramolecular dye chemistry. They are the cornerstones of natural wonders of photosynthetic machineries. It could be considered a great lifetime triumph of a dye chemist if he/she can develop a signature dye aggregate with an ordered molecular packing. There are generally two approaches to access ordered molecular packing of dyes for red-shifted spectral properties.

The first and less challenging approach is the self-assembly or precipitation of hydrophobic dye scaffolds in aqueous medium driven by the hydrophobicity. The most notable examples of the assembly is the J-aggregation of pseudocyanine dyes discovered by Jelley and Scheibe in 1930s. The most notable examples of precipitation is the AIE dyes discovered by Benzhong Tang in 2011. Essentially all examples in the *Adv. Mater.* **2023**, 2304848 fall in this category.

The aforementioned examples are undoubtedly beautiful progresses of the field of supramolecular dye aggregation. However, there lacks of a judicious control over how and why the dye monomers aggregate. In other words, the first approach is the serendipitous approach.

As chemists, we always wish to further have a layer of control over whether and how the dye monomer will aggregate. It is extremely challenging, as unanimously acknowledged by all three reviewers, *e.g.*, "*dye aggregation remains a serendipitous endeavor*" by the reviewer 1, "*Typically, J-aggregates are noticed by accident.*" by the reviewer 2, and "*However, this method should not be put forward as generalizable.*" by the reviewer 3. We tackled this challenge and had some success with **ESi5**.

The second and the more challenging approach is the planned or judicious supramolecular dye

aggregation. By saying “planned” or “judicious”, we are not trying to deny or exclude the requirement of that element of serendipity in our research. We simply wish to have an additional layer of control over the dye aggregation. The most notable example of this approach is the beautiful assembly of **C8S3** into the double-walled nanotube, whose structure was eventually unambiguously solved by UCLA scientists (*Nanoscale*, **2023,** 15, 3841). The most important structural feature of **C8S3** is that it is an amphiphilic molecule, with two hydrophobic alkyl chains with eight carbon atoms at one end of the molecules and two hydrophilic chains with three carbon atoms at the other end.

Our work was inspired by the **C8S3** and progressed exactly the way we had reported in this manuscript. We obtained two crystal structures of **ESi5**. Analysis of the packing of **ESi5** dyes in these two crystals revealed two different intrachain packing styles, *i.e.*, J-type and a V-type. Both types of packing would yield a spectral red-shifts. Therefore, we wondered, if we could trim the excess layers of molecules until there are only two layers of dyes. Then, we can pull up the two chloropropyl chains and install two water soluble groups. Would this modification stabilize the two-layer packing into a robust two-layer nano-ribbon, which could persist in aqueous milieu? It turned out that this idea achieved a great success. For this reason, we wish to report both the planning or judicious design, *i.e.*, Crystal-Aided Aggregate SyntHesis (CAASH), and the actual outcome of this design, *i.e.*, the double-layer nano-ribbon of **ESi5-S agg** exhibiting beautiful SWIR spectral properties.

With this, we hope that we have made clear the challenge of our research and the originality of our approach to address this challenge.

3. Typically, J-aggregates are noticed by accident. The key challenge in the development of SWIR J-aggregates is a lack of clear reference and design guidelines. The authors claimed that they report a rational approach, termed Crystal-Aided Aggregate SyntHesis (CAASH), to develop J-aggregates. However, it’s hard to understand the advancement of Crystal-Aided. Based on the structure of ESi5, different types of aggregation are probably caused by electronic and steric effects. I suggest the authors should focus on the study of structure-property relationships.

Reply:

The growth of the crystal(/s) helps the research in two aspects. First, the success growth of the crystal is a strong indication that the dye monomers can potentially stack with each through all non-covalent interactions. Second, the way they pack, *i.e.*, J-/H-/V-/X-packing, offers a reliable indication of the potential spectral outcome upon aggregation.

The success growth of the crystal is not a guarantee that a certain aggregate would be obtained. Tremendous laboratory dedications of the graduate students tinkering the structural nuances of **ESi5** should not be taken by granted. For example, when two zwitterionic chains are installed, a blue-shifted spectra was obtained upon presumably an H-aggregate. With two cationic ammonium chains, the dye molecules did not aggregate until a fairly high concentration. Only with two chains with a carboxylate or sulfate, the dyes aggregates into the anticipated nano-ribbon. The aforementioned structure-property study is displayed in Figure 2 of the manuscript.

4. Figure 1 should be re-organized to clearly convey information.

Reply:

We have revised the Figure 1 accordingly so that it is easier for the audience to understand the design rationale.

5. Besides slipping angle, the slipping distance and stacking distance should be clearly presented and discussed.

Reply:

The following figures are incorporated in the SI to highlight the slip angle and distance.

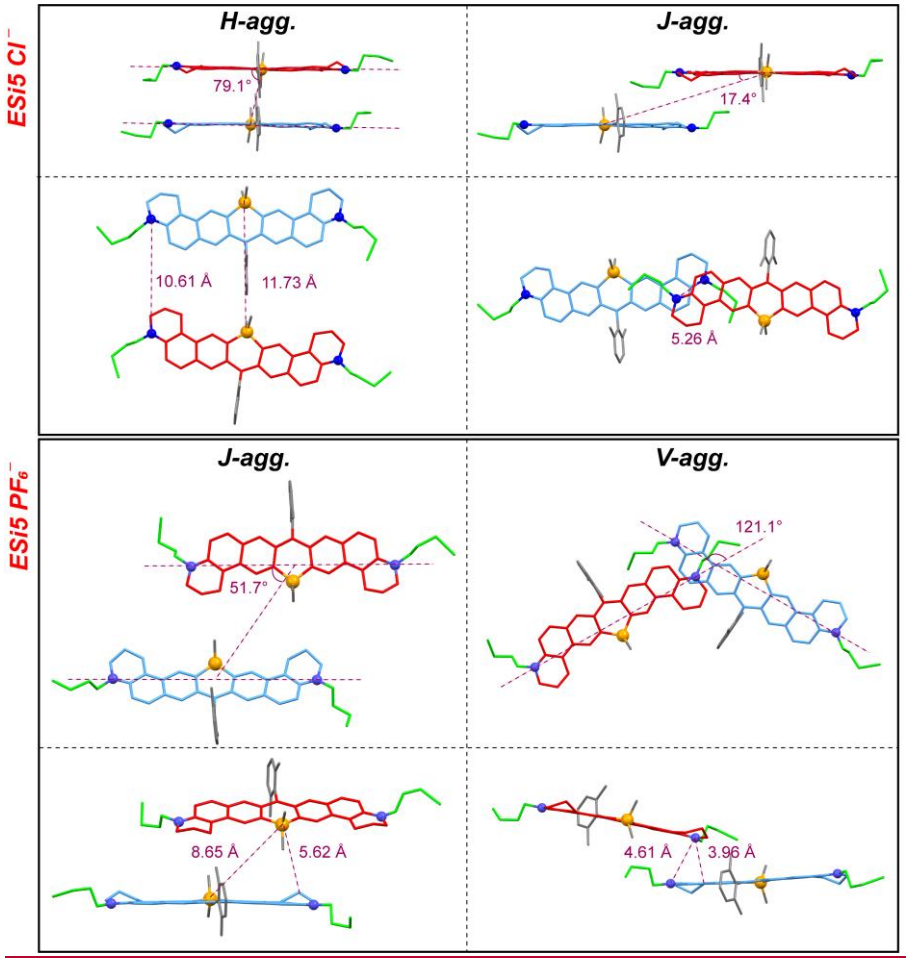

Figure S2. The detailed packing mode of **ESi5•Cl**$^-$ (H-aggregate and J-aggregate) and **ESi5•PF$_6$**$^-$ (J-aggregate and V-aggregate). Including slipping angle and interatomic distance.

6. It's not easy to understand the V-type intra-chain packing. It seems like intermolecular packing in Figure 1B.

Reply:

Packing of the dye molecules is intermolecular interaction. The molecules first pack in a linear fashion into a chain. The relative orientation of the two molecules varies. In this work, the packing of **ESi5** with Cl^- is J-type, in which the two dye molecules are parallel to each other and the slipping angle between the two molecules are 17.4º, which is smaller than 54º. The packing of **ESi5** with PF_6^- is a rare V-type packing, in which the transition dipole moments of the two dyes exhibits an angle of 121.1º. The chains of J-type or V-type packing of **ESi5** further align with each in a parallel fashion and grow into a layer. Layers further stack over each other into the crystal.

7. These SWIR aggregates exhibit two red-shifted and one blue-shifted sharp peaks. It's better to add more discussions about blue-shifted sharp peaks.

Reply:

We thank the reviewer for the thoughtful suggestion. The text is now revised accordingly.

"*As for **ESi5**, a single red (or blue)-shifted peak would suggest a J- (or H-) aggregate, while a V-aggregate would result in a red-shifted peak along with a blue-shifted peak via energy-splitting. The relative intensities of the two peaks depends on the angle in between. Suppose the angle (α) remains at ca. 120º, the oscillation strengths of the J-band (f_J) is expected to be roughly three times that of the H-band (f_H), according to $f_J/f_H = tg^2(\alpha/2)$.*"

8. These SWIR aggregates suffer from low quantum yields. I suggest to figure out a solution to increase

brightness via structure modification.

Reply:

Generously speaking, the dyes or aggregate absorbing in the shorter wavelength exhibit a larger fluorescence quantum yield. When the absorption maximum shifts to the longer-wavelength region, the fluorescence quantum yield decreases as mandated by the energy gap law, *i.e.*, a smaller bandgap promotes the rate constant of non-radiative internal conversion. As the absorption maximum goes beyond 1000 nm, any finite fluorescence quantum yield in aqueous medium is actually a success. To my knowledge, the SWIR materials typically require nano-encapsulation for them to be fluorescent in aqueous medium. Therefore, the fact that **ESi5-S agg** is fluorescent in aqueous medium makes it an outstanding SWIR bioimaging material.

There still lacks a viable strategy to judiciously improve the fluorescence quantum yield of aggregate. Some recent work by Caram, Justin R. might have offered some insight over this topic. Prof. Caram, Justin R. stated in his publication that——"*The relative slip between adjacent monomers as well as length of the monomer determines the relative contributions of short- and long-range couplings, which in turn, result in the distinct excitonic band structures of H-, I-, and J-aggregates. This leads to an internally consistent description of the distinct photophysical behaviors—enhanced or suppressed quantum yields relative to their monomers, differences in superradiance and red or blue shifts with temperature.*" Yet, how to chemically implement this proposed strategy remains unaddressed.

9. Many figures in the supporting information were not discussed in the main text. ESi5-S and ESi5-C1(-C5) aggregated instantaneously upon the addition its MeOH stock into H2O (Figure S14). Figure S14 was not correctly cited.

Reply:

The following paragraph is now in the manuscript.

*"The solutions of **ESi5-C1(-C5)** aggregates in PBS with 5% MeOH were heated to 90 ^{o}C and then cooled to room temperature (Figure S15). The effect was very obvious. Three major bands at ca. 1098 nm, ca. 1038 nm, and ca. 696 nm became sharper in shape. The two spectrally unresolved red-bands were now totally resolved. The two red-bands slightly red-shifted by ca. 8 nm, and the blue-band slightly blue-shifted by ca. 12 nm. These were indications of the reduction of aggregate disorder. **ESi5-S agg** was also stable toward sonication and pH change (Figure S34-S35)".*

10. What are the advantages of V-aggregation?

Reply:

The V-type aggregation is a distinct packing style from the more commonly encountered J-type or H-type. The J-type aggregation results in a red-shifted absorption band compared to the monomer as the result of an excitonic coupling, the H-type aggregates a blue-shift absorption band. The V-type packing yields both a blue-shifted and a red-shifted peak, via Davydov splitting. And notably, the relative peak intensities could be adjusted by the relative angle between the two interacting dyes and therefore spectrally more versatile than J/H-type packing.

The V-type aggregates are rarely encountered and actively sought-after for photophysical mechanisctic studies and they are more versatile for cutting-edge technologies, including bioimaging, energy harvesting, and potentially electro-optic materials.

11. How does the charge of pendent groups affect the aggregation behaviors?

Reply:

We have carried out a systematic structure-function relationship study over the influence of the pendent water-soluble groups over the aggregation behavior.

When two zwitterionic chains are installed, a blue-shifted spectra was obtained upon presumably

an H-aggregate. With two cationic ammonium chains, the dye molecules did not aggregate until a fairly high concentration. Only with two chains with a carboxylate or sulfate, the dyes aggregates into the anticipated nano-ribbon.

12. ESi5-S agg was essentially non-cytotoxic when its concentration was smaller than 10 μM, since the cells viability was higher than 85% after a 24 hr incubation with HeLa cells (Figure S34). 10 μM was obviously lower than the dose used in mice. The in vivo toxicity of ESi5-S agg should be tested.

Reply:

We apologize for the confusion. We wrote "*An aliquot of **ESi5-S agg** solution (500 μM in PBS, 100 μL) was injected into mouse*". The injected **ESi5-S agg** would be diluted in the circulating blood, the volume of which of a BALB/c mouse (~ 20 g) is typically about 1.5 mL. Therefore, the final blood concentration of **ESi5-S agg** was estimated to be ca. 30 μM, which is biocompatible both in *in vitro* and *in vivo* toxicity studies. The manuscript is revised accordingly.

"*An aliquot of **ESi5-S agg** solution was injected into a BALB/c mouse through the tail vein to render a final vasculature concentration of 2.5 mg/kg, equivalent to a biocompatible blood concentration of ca. 30 μM.*"

SWIR dyes are generally lipophilic and require DMSO as a co-solvent for delievery. DMSO is routinely used in cell or small animal. In cells, typically the final DMSO concentration in culture is suggested not to exceed 1% in in vitro studies. *In vivo* experiments, it has been established that up to 20% DMSO could be used as a co-solvent in oral or *i.v.* injection (*Int. J. Pharm.*, **2007**, *341*, 1-19).

To further prove the biosecurity of **ESi5-S agg,** we carried out the suggested in vivo toxicity study. S ix-week BALB/c female mouse (n=6) was used as an animal model and randomly divided into three groups. An aliquot of PBS (10 mM, pH=7.4, 0.1 mL) and **ESi5-S agg** (2.5 mg/kg, 5.0 mg/kg) solution was injected through the tail vein, respectively. The mice were raised for a period of 14 days, during which period the body weight and vitality of the mouse is recorded. The mice showed no significant abnormalities and the body weight of all three groups increased steadily.

In a second study, the mouse is sacrificed 24 hours post *i.v.* injection of **ESi5-S agg** (2.5 mg/kg) and the major organs are harvested for H&E histology analysis for acute toxicity features. No obvious organ damage or lesion was observed for **ESi5-S agg** injected mice.

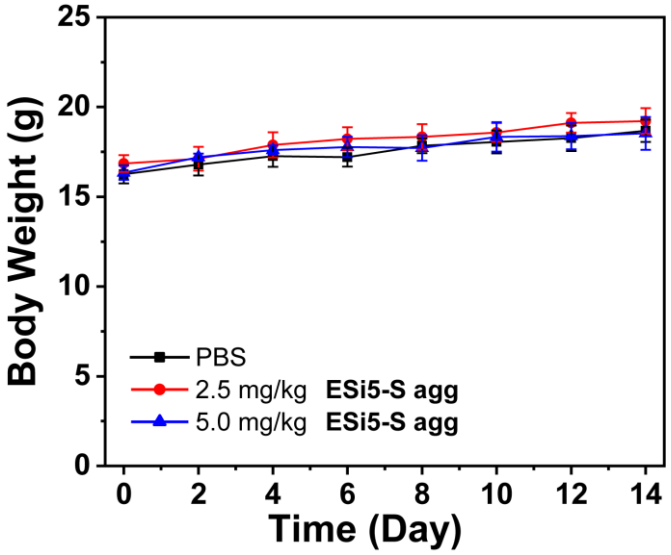

Figure S45. The change of the body weight of BALB/c mice after intravenous injected with PBS (100 μL), 2.5 mg/kg **ESi5-S agg** and 5.0 mg/kg **ESi5-S agg** (n=6).

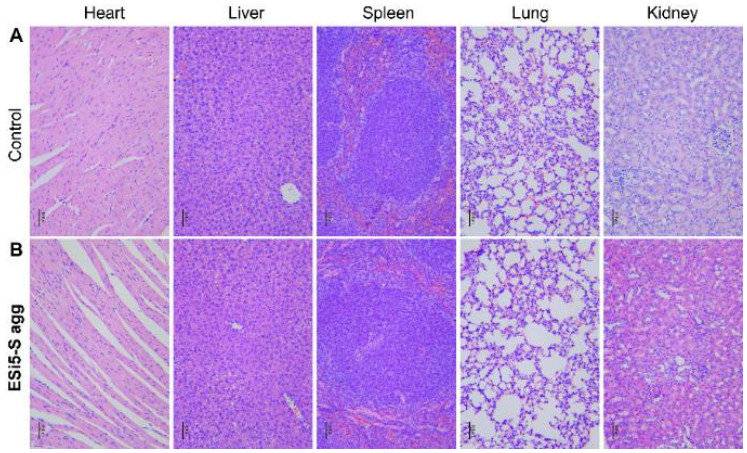

Figure S46. H&E images of major organs (including heart, liver, spleen, lung and kidney) collected from (A) BALB/c mice treated with PBS (100 μL) and (B) BALB/c mice at 24 h post-injection of **ESi5-S agg** (2.5 mg/kg). No obvious organ damage or lesion was observed for **ESi5-S agg** injected mice. Scale bar: 100 μm.

13. It's better to examine the aggregation behavior of ESi5-S agg with 10% FBS or in serum.

Reply:

We tested the aggregation of **ESi5-S agg** in 10% FBS. The absorption spectrum of the solution was acquired and the signature bands of the JV-aggregate at 1038 nm, 1098 nm and 696 nm were observed. Compared to the absorption in PBS, the monomer peak at 868 nm was slightly more pronounced, suggesting the presence of protein in FBS is slightly unfavorable for aggregation, but does not inhibit the aggregation, and does not change the aggregation pattern as well.

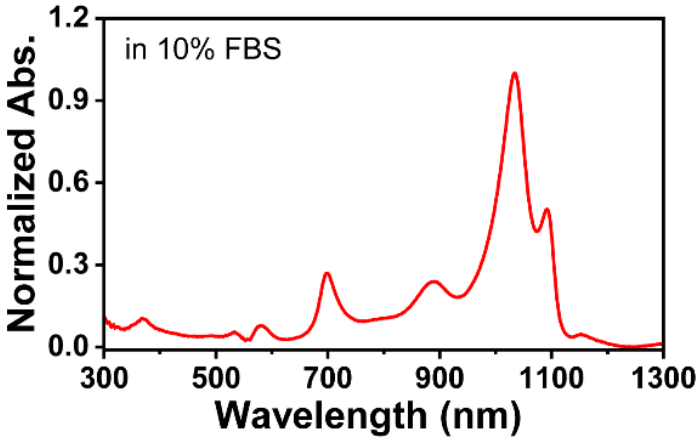

Figure S22. The absorption spectrum of **ESi5-S agg** in PBS (5 μM, containing 10% FBS).

14. In 180 min, the monomer was metabolized from the liver to the intestine (Figure 5J). Figure 5J should be Figure 5F.

Reply:

The error is now corrected.

15. The authors should discuss the reason why ESi5-S agg could bind to the skeletal system in the main text.

Reply:

The following paragraph is now in the manuscript.

"*Its affinity toward bone was likely due to its polyanionic nature. This bone-affinity was also in agreement with the fact that many other polyanionic materials exhibits bone-targetting capability by interaction with the hydroxyapatite (Figure S47)*."

Comments and revision suggestions of the reviewer 3:

In the manuscript "A rational approach to ultra-stable and biocompatible SWIR nanoribbion for dual-channel in vivo imaging" Yang and coworkers report the J-aggregate of their previously reported ESi5 dye. They frame the manuscript as a new method to predict/engineer J-aggregation: CAASH. The J-aggregate characterization within the manuscript is well-done and thorough. The J-aggregates are then applied for in vivo imaging using multiplexing to image both the monomer and the aggregate.

1. The CAASH protocol requires having a crystal structure of the fluorophore and being able to model in substituents with the assumption that the packing does not change when amphiphilic character is added. It is hard to do step 1 for most fluorophores and this is likely the more reliable step of the two. It is very impressive that this system worked for ESi5 and the crystal packing and structures obtained are beautiful. These structures really help in the analysis of the H and J coupling in the aggregates. However, this method should not be put forward as generalizable.

   Reply:

   We totally agree with the reviewer for the generalizability of any given method with respect to the development of an ordered dye aggregate. It is exactly for this reason that beautiful aggregates are so rare and developing novel ordered dye aggregates are challenging.

   The whole field would agree that development of ordered dye aggregate remains largely serendipitous. What we wish is to introduce a layer of rationality over the development of ordered dye aggregate. The crystal structures of a dye is the only experimental grasp that a chemist can have. It is fortunate that we succeeded in this piece of work and developed a beautiful double-layer nano-ribbon exhibiting SWIR absorption/emission. But, we absolutely agree with the reviewer that our success does not necessarily mean that this method is surely generally applicable to other class of dye molecules.

   For this reason, we have carefully revised the manuscript to remove any unsuitable over-emphasis on the generalizability of this method. We thank the reviewer for the insightful suggestion and believe that the scholarly presentation of the manuscript is greatly improved this way.

   Abstract: "*Herein, we propose a crystal-aided aggregate synthesis (CAASH) approach to* ***introduce a layer of rationality*** *for the development of J-aggregate.*"

   Conclusion: "*In conclusion, we proposed an approach, i.e., crystal-aided aggregate synthesis (CAASH), to make J-aggregate development* ***more of a rational and less of a serendipitous endeavor.***"

2. After characterization, the J-aggregates are used from two color imaging where the bluer channel allows for the monomer to be imaged and the red channel is the aggregate. This is a clever way to see the dissociation of the aggregate in vivo. The aggregate has surprising stability in vivo. The authors state that the blood vessels cannot be observed in either channel citing aggregate stability– this comment is true for the monomer but shouldn't aggregate emission be observed in the vessels upon injection? What does the 0 min timepoint look like (Figure S36 indicates there should be a 0 min but is labeled as 10 min in the figure). Intensity bars, exposure times and all imaging metrics should be included in Figure 5 and S36. There should be at least one biological replicate of the imaging experiments shown.

   Reply:

   We thank the reviewer for this thoughtful question. We believe that the fluorescence signal of the aggregate should indeed be observable in the vasculature upon injection. Only, we could not possibly carry out such an experiment.

   The *i.v.* injection of **ESi5-S agg** needs to be carried out in the laboratory animal room. Then, the animal has to be transferred to the equipment room and then be anesthetized before imaged. This process took us ca. 10 minutes. We apologize for not being able to provide an imaging at time zero.

   The intensity bars, exposure times and other imaging parameters are included in both Figure 5

and S36 as suggested.

The imaging was repeated for multiple times. We now included a batch of different set of *in vivo* imaging in the SI as Figure S42

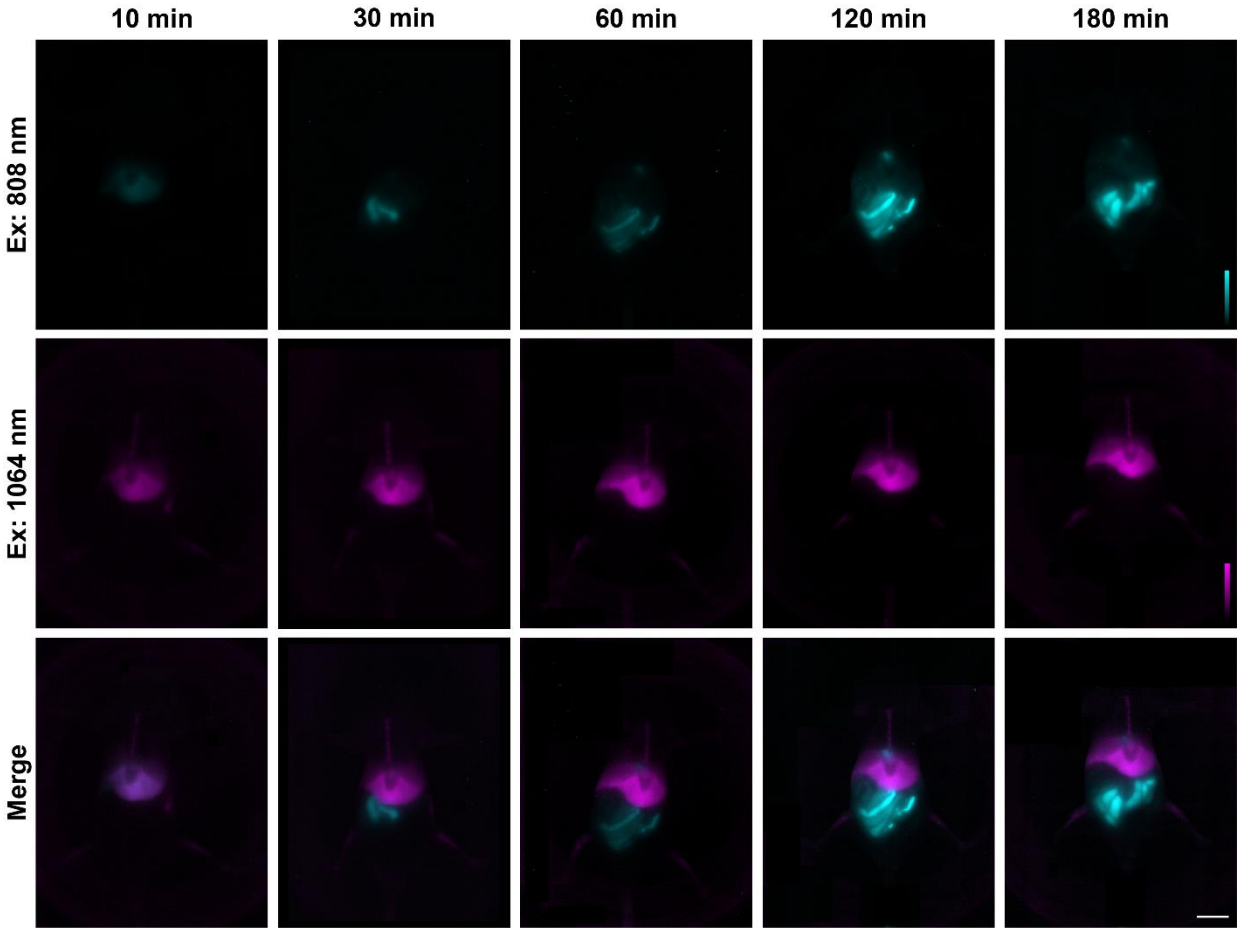

Figure S42. The repeat dual-channel fluorescence images of BALB/c mice in supine position acquired 0 min, 30 min, 60 min, 120 min, or 180min post *i.v.* injection of **ESi5-S agg**. Scale bar: 10 mm. 808 nm channel: λ_{ex} = 808 nm (60 mW•cm^{-2}), long pass filter: 1200 nm, exposure time: 100 ms. 1064 nm channel: λ_{ex} = 1064 nm (80 mW•cm^{-2}), long pass filter: 1200+1350 nm, exposure time: 500 ms

3. The concentration of probe i.v. injected is 500 uM with 4% DMSO. The J-aggregate starts to show toxicity in cell culture at 10 uM (50x less than injected!). The hemolysis test is also not performed as these concentrations. Finally, DMSO should not be injected intravenously. These are significant concerns about the stated utility of the probe.

Reply:

We apologize for the confusion. We wrote "*An aliquot of **ESi5-S agg** solution (500 μM in PBS, 100 μL) was injected into mouse*". The injected **ESi5-S agg** would be diluted in the circulating blood, the volume of which of a BALB/c mouse (~ 20 g) is typically about 1.5 mL. Therefore, the final blood concentration of **ESi5-S agg** was estimated to be ca. 30 μM, which is biocompatible both in *in vitro* and *in vivo* toxicity studies. The manuscript is revised accordingly.

"*An aliquot of **ESi5-S agg** solution was injected into a BALB/c mouse through the tail vein to render a final vasculature concentration of 2.5 mg/kg, equivalent to a biocompatible blood concentration of ca. 30 μM*."

SWIR dyes are generally lipophilic and require DMSO as a co-solvent for delievery. DMSO is routinely used in cell or small animal. In cells, typically the final DMSO concentration in culture is suggested not to exceed 1% in in vitro studies. *In vivo* experiments, it has been established that up to 20% DMSO could be used as a co-solvent in oral or *i.v.* injection (*Int. J. Pharm.*, **2007**, *341*, 1-19).

To further prove the biosecurity of **ESi5-S agg,** we carried out the suggested in vivo toxicity study. S ix-week BALB/c female mouse (n=6) was used as an animal model and randomly divided into three

groups. An aliquot of PBS (10 mM, pH=7.4, 0.1 mL) and **ESi5-S agg** (2.5 mg/kg, 5.0 mg/kg) solution was injected through the tail vein, respectively. The mice were raised for a period of 14 days, during which period the body weight and vitality of the mouse is recorded. The mice showed no significant abnormalities and the body weight of all three groups increased steadily.

In a second study, the mouse is sacrificed 24 hours post *i.v.* injection of **ESi5-S agg** (2.5 mg/kg) and the major organs are harvested for H&E histology analysis for acute toxicity features. No obvious organ damage or lesion was observed for **ESi5-S agg** injected mice.

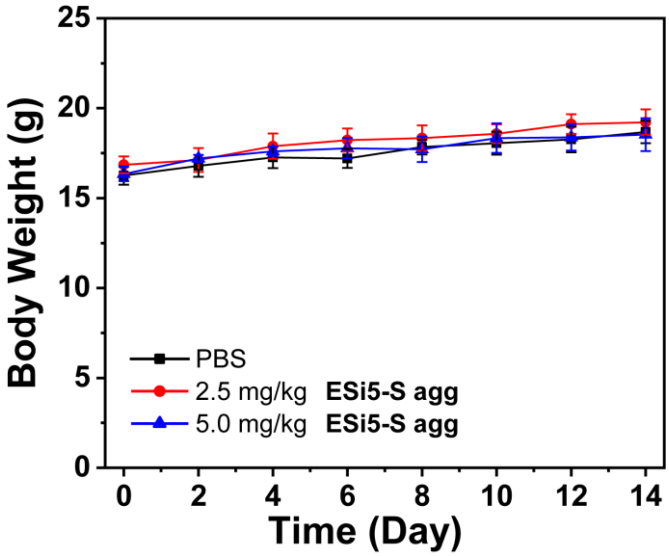

Figure S45. The change of the body weight of BALB/c mice after intravenous injected with PBS (100 μL), 2.5 mg/kg **ESi5-S agg** and 5.0 mg/kg **ESi5-S agg** (n=6).

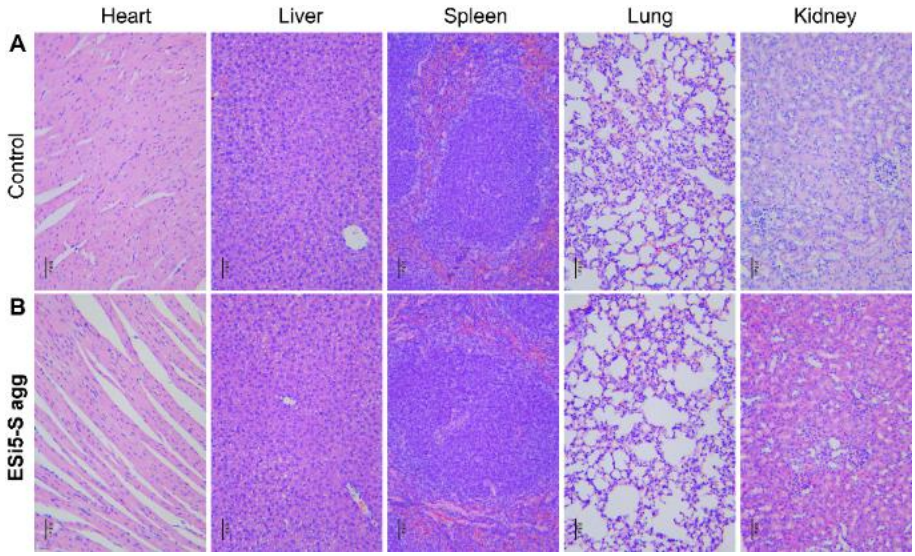

Figure S46. H&E images of major organs (including heart, liver, spleen, lung and kidney) collected from (A) BALB/c mice treated with PBS (100 μL) and (B) BALB/c mice at 24 h post-injection of **ESi5-S agg** (2.5 mg/kg). No obvious organ damage or lesion was observed for **ESi5-S agg** injected mice. Scale bar: 100 μm.

4. What does the J-aggregate look like in the presence of FBS?

Reply:

We tested the aggregation of **ESi5-S agg** in 10% FBS. The absorption spectrum of the solution was acquired and the signature bands of the JV-aggregate at 1038 nm, 1098 nm and 696 nm were observed. Compared to the absorption in PBS, the monomer peak at 868 nm was slightly more pronounced, suggesting the presence of protein in FBS is slightly unfavorable for aggregation, but does not inhibit the aggregation, and does not change the aggregation pattern as well.

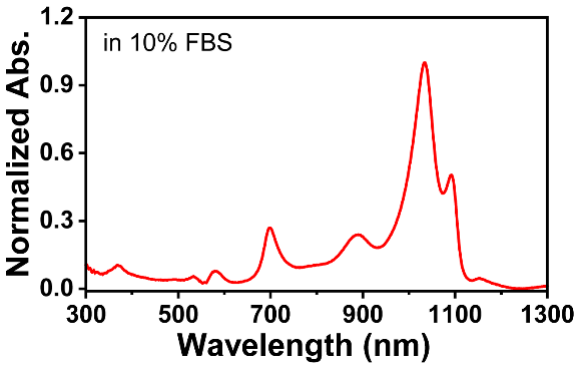

Figure S22. The absorption spectrum of **ESi5-S agg** in PBS (5 μM, containing 10% FBS).

5. The conclusion states that the aggregates are superradiant but this analysis was performed/discussed.

Reply:

We thank the reviewer for the excellent suggestion. Superradience is the accelerated deactivation of the excited via excitonic coupling and the hallmark is a greatly reduced fluorescence lifetime with an extremely sharp emission band. The fluorescence lifetime of the aggregate was measured to be 58 ps, which is greatly reduced compared to the monomer. The resonant fluorescence emission was very sharp. We incorporated a short discussion regarding the potential existence of superradience with the **ESi5-S agg**.

*"Superradiance is a behavior of aggregated dyes and its hallmarks are reduced fluorescence quantum yield with an extremely sharp resonant emission band. Both were observed with **ESi5-S agg** and suggested that **ESi5-S agg** also likely exhibited superradiance."*

6. Relative fluorescence quantum yields should be performed with available standards and references should be given for their quantum yield values. The value for IR-1061 is incorrect and over estimates the quantum yield of the aggregates.

Reply:

This is a very insightful suggestion. SWIR dyes are at its early days. There still lacks a standard method to reliably measure the fluorescence quantum yields. For the dyes absorbing/emitting in the visible range. There exist many standard reference dyes to choose. For dyes absorbing/emitting in the range of 800-1000 nm, both HORIBA and Hamamatsu offer instruments to measure their absolute fluorescence quantum yields. However, for dyes beyond 1000 nm, there are no bright and stable dyes for reference and there are also no commercial instruments for absolution fluorescence quantum yield.

Hongjie Dai in his **2013** Angew paper reported that the fluorescence quantum yield of nano-encapsulated IR1061 was 1.8% with IR-26 as the reference (*Angew. Chem. Int. Ed.*, **2013**, *52*, 13002 –13006). In this paper, Dai also cited that **IR-1061** exhibited a fluorescence quantum of 1.7±0.5% in CH_2Cl_2 (Quaela, et al. *Chem. Phys. Lett.*, **2003**, *373*, 372–378). Many groups later adopted the value of 1.7% when using **IR-1061** as the reference for fluorescence quantum yield measurements. In 2019, Tobila *et al.* wrote a manuscript to alert the field that the fluorescence quantum yield of **IR-1061** has been overestimated and provided a new value of 0.59% (*Anal. Chem.*, **2020**, *92*, 607−611).

Following the reviewer's suggestion, we recalculated the quantum yield of our aggregate using this revised value. We also wish to note that we are very careful with the practice of the relative fluorescence quantum yield measurements. We kept the concentration very low to avoid aggregation-induced quenching. We used the anhydrous CH_2Cl_2 to avoid the potential nucleophilic addition of H_2O to IR1061. Also, we kept the room dark to avoid photobleaching of IR1061 upon sitting.

Besides this modification to the fluorescence quantum yield revision, the following sentence is incorporated in the SI to notify the reader.

"*Due to the fact that there still lacks a stable and bright dye absorbing in SWIR region with robustly calibrated fluorescence quantum yield, this quantum yield should be considered as an*

estimate."

7. The camera used for imaging is not stated.

Reply:

The SI is now updated with the camera information as "*The in vivo fluorescence imaging was carried out with MARS (Artemis Intelligent Imaging) equipped with an InGaAs camera (NIRvana, Teledyne Princeton Instruments)*"

8. If the mice are shaved before imaging is not stated.

Reply:

The SI is now updated with the information as "All mice were shaved before fluorescence imaging." and "All mice were shaved before photoacoustic imaging."

9. Figure S29 should report the photobleaching rates. There is a typo in Figure S29 legend.

Reply:

The error is now corrected.

10. In Figure S30 the thiol and oxidant labels are reversed.

Reply:

The error is now corrected.

11. The procedure corresponding to Figure S40 should be in the SI.

Reply:

The procedure is now updated in SI as ***"In vitro Calcium-Binding Experiments***" in page S6.

12. The reviewer has concern over the potential of aggregation for bioimaging.

Reply:

We carried out an additional proof-of-concept photoacoustic imaging application to further showcase the potential of this aggregate for various modality of bioimaging.

The following two italicized paragraphs and the figures are incorporated in the revised manuscript.

"*An additional proof-of-concept photoacoustic imaging application was carried out to further showcase the potential of this series of aggregates with **ESi5-S agg** as an example for different modality of bioimaging. PA signal is mainly related to the photothermal conversion and subsequent thermal-to-acoustic transformation of a light-absorbing material. The temperature increase profiles of the **ESi5-S agg** solution in PBS correlated with both the aggregate concentrations and laser power density (Figure 6A-B). Fitting of the photothermal conversion profile yielded a photothermal conversion efficiency of 58.1% (Figure 6C). Further, the thermal stability of the **ESi5-S** agg was evaluated by repeated heating/cooling cycles in the range of 25 ^{o}C to 46.6 ^{o}C (Figure 6D). After five cycles, no sign of fatigue was observed, while IR1061 did not survive two around of heating/cooling cycles under the same conditions. The photoacoustic effect of **ESi5-S agg** was further tested at various concentrations (0-50 μM) (Figure 6E). A dose-dependence was observed between the photoacoustic intensity and the **ESi5-S agg** concentration. Notably, the photoacoustic signal intensity was 3.5 times higher than that of **IR-1061** (50 μM in PBS).*

*The feasibility of **ESi5-S agg** for in vivo photoacoustic imaging was carried out with BALB/c mouse. First, we acquired the background photoacoustic imaging with mouse without injecting **ESi5-S agg**. The presence of weak signals from the vasculature-rich area/organs was due to a weak absorption of hemoglobin at this SWIR region (Figure 6F). Then, a solution of **ESi5b-S agg** (2.5 mg/kg) in PBS was injected into shaved BALB/c mouse through tail vein. Due to its intense absorption at 1064 nm, a good contrast could be obtained over the signal from the hemoglobin. In 180 min, the*

photoacoustic experiment was carried out with a 1064 nm excitation and a three-dimention photoacoustic tomography was reconstructed (Figure 6G). The photoacoustic signal was concentrated the liver and the bone (including sternum, costae and lumbar), in agreement with the fluorescence imaging results. For lumbar, specifically, vertical and horizontal analysis (ROI 1 and ROI 2) along the lumbar vertebra showed eight and three peaks of photoacoustic intensity respectively, and the FWHMs were high enough to discriminate different vertebra including posterior articular protrusion (Figure 6H-I). From the above experimental results, it can be concluded that the ESi5-S exhibit an excellent PA imaging performance."

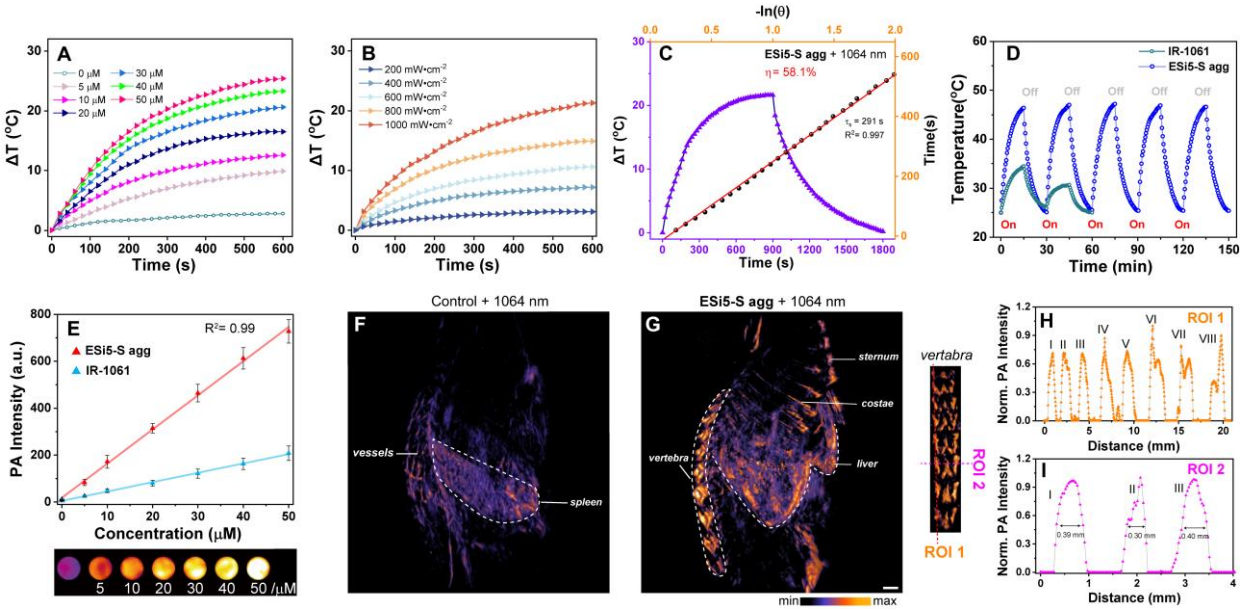

Figure 6. The *in vivo* photoacoustic imaging of BALB/c mouse using **ESi5-S agg**. (**A**, **B**) The concentration and laser intensity dependent photothermal property of **ESi5-S agg** in PBS. (**C**) The calculation of the photothermal conversion efficiency of **ESi5-S agg** (30 μM) under continuous 1064 nm laser (1000 mW•cm^{-2}) irradiation. (**D**) Photothermal stability of **ESi5-S agg** (30 μM) in PBS and **IR-1061** (30 μM) in PBS under 1064 nm laser (1000 mW•cm^{-2}) irradiation. (**E**) The photoacoustic intensity of the **ESi5-S agg** and **IR-1061** in PBS excited by 1064 nm laser. (**F**, **G**) Photoacoustic imaging of BALB/c mice acquired (**F**) without **ESi5-S agg** and (**G**) 180 min post-injection of **ESi5-S agg** (2.5 mg/kg in PBS). (**H**, **I**) Line intensities of the highlighted ROIs of the images of vertebra.

Comments and the revision suggestions of the Reviewer #1:

I have a suggestion regarding photoacoustic imaging. Considering the narrow-bandwidth of the aggregate absorption peak, it becomes immediately obviously that the authors may further develop system with slightly different absorption maxima. All these aggregates with different absorption maxima would allow multiplexing in the NIR spectral region. This would address the current bottleneck for multiplexing with monomeric NIR fluorophores. Their absorption bands are usually very broad, exhibit extensive overlapping and therefore leads to serious cross-talk between different channels. I found that the authors did not mentions its potentials in multiplexing in the response and in the manuscript. Of course, for this to be feasible, the authors would need to figure out how to let different aggregate target different tissues in vivo. This is not obvious, but still practical. Addition of this discussion in the manuscript help the audience better appreciate the significance of this aggregate.

Reply:

We appreciate for the insightful inputs of the reviewer 1 and revised the discussion accordingly.

"*Multiplexed imaging with minimal cross-talks between different channels is a challenge for the near infrared region due to the broad absorption bands of the NIR dyes. Sletten et al. noted that the aggregates with sharp absorption bands are superior candidates for multiplexing in this spectral region. Therefore, developing aggregates that can pair with* ***ESi5-S agg*** *for multiplexed imaging is a viable direction for future research.*"

Comments and the common revision suggestion of the Reviewer #2 and #3 regarding the generality of this approach:

1. **The reviewer 2:** The strategy of crystal-aided aggregate synthesis (CAASH) did not provide a general design guideline for the construction of highly ordered J-aggregates.
2. **The reviewer 3:** The rational design/CAASH strategy is not a generalizable approach to J-aggregate formation. The title and main figures putting forward CAASH as a strategy for J-aggregate formation still remain, when even the authors agree this isn't

an approach that is going to be broadly beneficial.

Reply:

While I might have over-emphasized the rationality in this work. The reviewer 2 and 3 definitely underestimated our rationality. To find a proper balance, not to completely overlook the power of rational design and yet to acknowledge the role of serendipity in this work, we further revised the relevant discussion as follows.

First, the title is now revised to "*An Ultra-stable, Biocompatible SWIR Nanoribbon for Dual-channel in vivo Imaging*".

Second, the first sentence of the paragraph 3 of the introduction now reads "*Herein, we report an ultra-stable and biocompatible SWIR aggregate via Crystal-Aided Aggregate SyntHesis (CAASH).*"

Third, unnecessary use of "*rational*" is avoided throughout the text.

Other comments and the revision suggestions of the Reviewer #3:

3. The description of superradiance in the main text is incorrect. The superradiance values should be calculated.

Reply:

We thank the reviewer for pointing out this. We feel that the superradiance is not critical for this work and we avoided the use of "*superradiance*" in the text.

4. The response to determining quantum yields in the SWIR region is unsatisfactory. SWIR detectors have advanced significantly and multiple papers have been published on accurately determining the quantum yield of IR-26 which is accepted as a standard for relative quantum yield measurements in the field.

Reply:

We thank the reviewer for this suggestion.

The fluorescence quantum yield of **ESi5** JV-aggregates in PBS (λ_{em} >1000 nm) were re-calculated with **IR26** (ϕ = 0.5% in 1,2-dichloroethane) as an reference, with excitation at 980 nm.

Alexander L. Antaris, Hao Chen, Kai Cheng, Yao Sun, Guosong Hong, Chunrong Qu, Shuo Diao, Zixin Deng, Xianming Hu, Bo Zhang, Xiaodong Zhang, Omar K. Yaghi, Zita R. Alamparambil, Xuechuan Hong, Zhen Cheng & Hongjie Dai. A small-molecule dye for NIR-II imaging. *Nat. Mater.*, **2016**, 15, 235 – 242.