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

nature portfolio

Peer Review File

Heat-guided drug delivery via thermally induced crosslinking of polymeric micelles

Corresponding Author: Professor Kenjiro Hanaoka

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, a thermoresponsive polymeric micelle was fabricated from poly(N-isopropylacrylamide) with DBCO/azide functionalities for a heat-triggered cancer nanomedicine. The obtained results demonstrated that the micelle was successfully prepared to form aggregates above LCST. Thus, this study is valuable for publication. However, there are some controversial issues prior to publication, as below.

1. The polymer and micelle compositions should also be briefly described in the main manuscript for better understanding because they should be critical for the thermoresponsive functionalities of polymeric micelles.
2. Serum proteins may affect the aggregation behavior of non-Az/DBCO-functionalized micelles. Thus, the aggregation behavior of non-functionalized TRM in serum-containing media should also be examined to clarify the impact of Az/DBCO functionalities in biological milieu.
3. The aggregation behavior of micelles should be affected by the micelle concentration. Thus, the critical aggregation concentration of micelles should be further determined to estimate the least concentration of injected micelles into the body.

Reviewer #2

(Remarks to the Author)

In this manuscript, the authors reported a heat-guided drug delivery system based on the irreversible aggregation of polymeric micelles. The authors carried out thermosensitive micelle formulations and demonstrated convincing anti-tumor effects in a tumor-bearing mouse model. Some minor concerns could be addressed to improve the manuscript.

Q1: It appears that some molecular weights are mislabeled in the Mn (NMR) column of Table S1.

Q1: The authors introduced one DBCO group at the end of P(NIPAAm-co-AAm)-b-PBMA, while the azide group was multiple randomly modified on the side chain of P(GA-co-AAm)-b-PBMA. It implies that one Az-TRM micelles can react with several DBCO-TRM micelles. However, the aggregation study showed that DBCO-TRM and Az-TRM were aggregated at a ratio of 1:1. It might be caused because the starting ratio of the two micelles is 1:1. More evidence is needed to clarify.

Q2: The authors noted that Az-TRM (long) was partly aggregated with DBCO-TRM compared with Az-TRM at 37oC, with increased aggregation upon heating. Since both DBCO-TRM and Az-TRM (long) expose their reactive group on the surface of micelles. It is unclear why 100% aggregation is not achieved at 37oC. The authors should provide an explanation for this finding.

Q3: Considering that aggregation of micelles might influence DOX release behavior, an in vitro DOX release study is recommended.

Q4: The authors mentioned that at 48 h after injection of DBCO-TRM@DOX and DiD-loaded Az-TRM, tumor tissue was collected and homogenized, and the fluorescence of DiD in the tissue lysate was measured using a fluorometer. It is recommended to measure the concentration of DOX in the tumor lysate for clarity.
Additionally, the authors noted that infarction of surface blood vessels was observed on the heated tumor (body surface side) in the mice 48 h post-administration of DBCO-TRM@DOX and Az-TRM. It would be better to compare the micelle amounts in the surface blood vessels to those inside the tumor. Meanwhile, the micelles or drug may penetrate the tumor after 48 h of injection. The authors need to monitor the accumulation of micelles in the tumor over a longer period.

Q5: Given that aggregation of micelles can induce embolization, the authors should address the degradation of micelle aggregates within the blood vessels.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Chemistry initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
This article describes a drug delivery strategy that uses heat-induced aggregation of polymeric micelles to achieve targeted and long-lasting drug delivery. The key aspects include the micelles with thermo-responsive shells that aggregate upon mild heating; crosslinking between the micelles to form irreversible aggregates and efficient tumor accumulation and retention of the drug-loaded aggregates. I recommend publish after minor revision.

1. Why were the LCST values of the micelles tuned to be slightly higher than physiological temperature? The rationale behind of this design could be included in the manuscript.
2. The aggregation of the micelles at 42/47°C was completely reversed upon cooling to 32°C. Is this reversibility an important characteristic for the proposed application?
3. Could the author describe on the measurement sizes measurement of the DBCO-TRM and Az-TRM micelles below and above the LCST?
4. How could the homogeneity and reproducibility of the micelle formulations be ensured to minimize the possibility of batch-to-batch variation ?
5. It has been mentioned that the size of the aggregates after heating at 42°C increased with increasing serum concentration. Is that an indication of the aggregates binding to the serum protein ?
6. After the intravenously administration to tumor-bearing model mice, the subcutaneous tumor was reported to be heated at 42°C. How could the temperature of the whole tumor region be ensured during the heating and how to determine the selected heating duration of 30 min?
7. Could the release profile of the anti-cancer drug be addressed as it would also be helpful for the estimation of the release efficiency in the tumor.
8. Would the in vitro toxicity work also be included as well ?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript has been sufficiently revised based on the reviewer's comments.

Reviewer #2

(Remarks to the Author)
Recommendation: Publish after minor revisions noted.

Comment: It seems that some molecular weights are still incorrectly labeled in the Mn (NMR) column of Table S1. The authors stated that the molecular weights of P(Az-co-AAm)-b-PBMA were determined using 1H NMR (entries 11, 13, and 15 in Table S1). However, these values appear to be mislabeled in entries 10, 12, and 14.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Chemistry initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Recommendation: Publish after minor revisions noted.
Comments:

Q1: It seems that some molecular weights are still incorrectly labeled in the Mn (NMR) column of Table S1. The authors stated that the molecular weights of P(Az-co-AAm)-b-PBMA were determined using 1H NMR (entries 11, 13, and 15 in Table S1). However, these values appear to be mislabeled in entries 10, 12, and 14.

Q2: The additional experiments and responses provided satisfactorily address my other concerns.

Reviewer #4

(Remarks to the Author)
The authors have addressed all questions made by the reviewers, supporting their findings with sufficient convincing data in easily understandable manner. The manuscript is now more complete and acceptance is recommended after correcting the following typos:

1. The sentence “Dextran, a model macromolecule, was not incorporated into the irreversible aggregates of DBCO-TRM and AZ-TRM (Figure S13).” appears strangely in the paragraph. The author may consider adding a transition like “To examine the possibility of trapping serum component into the aggregates, FITC-labelled dextrans were mixed and heated with DBCO-TRM and Az-TRM, where the ratio of fluorescent intensity suggested that neglectable amount of dextran …. (Figure S13).”

2. The format of the title “CONCLUSIONS” should be bolded to maintain consistency.

Response to Reviewer #1

Comment (1)

"1. The polymer and micelle compositions should also be briefly described in the main manuscript for better understanding because they should be critical for the thermoresponsive functionalities of polymeric micelles."

Thank you for the suggestion. The requested information is included in Tables S1 and S2, and we added the following sentence in the left column on page 2.

"The copolymerization ratio of the polymers and the mixing ratio of polymers in the micelles are shown in Tables S1 and S2."

Comment (2)

"2. Serum proteins may affect the aggregation behavior of non-Az/DBCO-functionalized micelles. Thus, the aggregation behavior of non-functionalized TRM in serum-containing media should also be examined to clarify the impact of Az/DBCO functionalities in biological milieu."

We newly examined the effect of serum on the aggregation of DBCO-TRM and TRM (non-azide control) and added the results as Figure S15 in the supporting information. The following sentence was also added in the right column on page 3.

"Further, no aggregation was observed in a mixture of DBCO-TRM and TRM (non-azide control) after heating at 42°C (Figure S15)."

Comment (3)

"3. The aggregation behavior of micelles should be affected by the micelle concentration. Thus, the critical aggregation concentration of micelles should be further determined to estimate the least concentration of injected micelles into the body."

Thank you for the suggestion. We determined the critical micelle concentrations (CMCs) of the micelles using pyrene as a fluorescent probe, and the CMCs of DBCO-TRM, Az-TRM and TRM have been added in Table S2, together with the experimental method in the supporting information.

In the in vivo experiments, we injected both DBCO-TRM (CMC: 4.7 μg/mL) and Az-TRM (CMC: 3.0 μg/mL) into mice at 120 mg/kg each (i.e., 2.4 mg per 20 g mouse). If the blood volume is as assumed to be 10% of body weight (i.e., 2 mL blood per 20 g mouse), the expected micelle concentration in the blood is 1.2 mg/mL, which is much higher than the CMCs.

Response to Reviewer #2

Comment (1)

"Q1: It appears that some molecular weights are mislabeled in the Mn (NMR) column of Table S1."

Thank you for the comment. The amphiphilic diblock polymers were synthesized by two-step polymerization as shown in Schemes S1 and S2. The hydrophilic domain was firstly polymerized, then the resulting polymer was used as a macro-chain transfer agent for the second polymerization to add a hydrophobic domain. For the thermo-responsive polymer, P(NIPAAm-*co*-AAm)-*b*-PBMA, the increase of molecular weight upon polymerization of the second block was not correctly reflected in the GPC measurements, so in this case, the molecular weights were also analyzed by means of NMR measurements (entries 2, 4, 7 and 9 in Table S1).

On the other hand, the molecular weights of azide-containing polymers, P(Az-*co*-AAm)-*b*-PBMA, were increased by unexpectedly large amounts after the second polymerization (entries 11, 13 and 15 in Table S1). Since the ratio of the monomer to the chain transfer agent was 50 eq. in the second step, which is lower than that in the first step (60–200 eq.), we considered that the molecular weights of the first block might not have been correctly measured by GPC, and so the molecular weights of the first block were analyzed by NMR.

We corrected the labels and the text in Table S1 to explain this more clearly.

Comment (2)

"Q1: The authors introduced one DBCO group at the end of P(NIPAAm-co-AAm)-b-PBMA, while the azide group was multiple randomly modified on the side chain of P(GA-co-AAm)-b-PBMA. It implies that one Az-TRM micelles can react with several DBCO-TRM micelles. However, the aggregation study showed that DBCO-TRM and Az-TRM were aggregated at a ratio of 1:1. It might be caused because the starting ratio of the two micelles is 1:1. More evidence is needed to clarify."

We agree with the referee's comment. In addition to Az-TRM, DBCO-TRM can react with several micelles, because DBCO-TRM was prepared from polymer mixture consisting of 30% DBCO-introduced polymer. Therefore, we newly examined the effect of the starting ratio of DBCO-TRM and Az-TRM on the aggregation. As expected, the aggregation ratio depended on the starting ratio of DBCO-TRM and Az-TRM. We added the following text in the left column on page 3, together with Table S4.

"and the aggregation ratio depended on the mixing ratio of micelles (Table S4),"

Comment (3)

"Q2: The authors noted that Az-TRM (long) was partly aggregated with DBCO-TRM compared with Az-TRM at 37oC, with increased aggregation upon heating. Since both DBCO-TRM and Az-TRM (long) expose their reactive group on the surface of micelles. It is unclear why 100% aggregation is not achieved at 37oC. The authors should provide an explanation for this finding."

Thank you for the comment. When we examined the role of the phase transition in the micelle aggregation (Figure 3d), the efficiency of aggregation of DBCO-TRM(high) (LCST: 52°C) with Az-TRM at 47°C was much lower than that of DBCO-TRM. This suggests that hydrophobic aggregation above the LCST drives the micellar crosslinking. Neither DBCO-TRM (LCST: 39.6°C) nor Az-TRM (long) (LCST: 39.8°C) exhibits phase transition at 37°C, and this is presumably the reason for the low efficiency of aggregation at 37°C. We added the following sentence in the left column on page 3.

"This result also supports the idea that hydrophobic interactions above the LCST promote the micellar crosslinking."

Comment (4)

"Q3: Considering that aggregation of micelles might influence DOX release behavior, an in vitro DOX release study is recommended."

As suggested, we newly performed an experiment to assess in vitro Dox release, using a dialysis method. To evaluate the influence of the micellar crosslinking on the release behavior, a mixture of DBCO-TRM@Dox and Az-TRM was pre-heated and then transferred into a dialysis tube. As shown in Figure S18, the release rate of Dox showed some decrease as a result of pre-heating, but sufficient Dox release was observed even after the crosslinking. We have added the following sentences in the left column on page 4, together with Figure S18 and the experimental method in the supporting information.

"Further, although the release rate of Dox showed some decrease by pre-heating in the *in vitro* assay, sufficient release of Dox from the micelles was observed even after the crosslinking (Figure S18)."

Comment (5)

"Q4: The authors mentioned that at 48 h after injection of DBCO-TRM@DOX and DiD-loaded Az-TRM, tumor tissue was collected and homogenized, and the fluorescence of DiD in the tissue lysate was measured using a fluorometer. It is recommended to measure the concentration of DOX in the tumor lysate for clarity."

Thank you for the suggestion. We added the result of the measurement of Dox in the tumor lysate by fluorometry (Figure S20). When DBCO-TRM@Dox and DiD-loaded Az-TRM were coadministered, the accumulation of Dox in the tumor was significantly increased by heating, and this enhancement was retained to some extent even at 48 h postinjection. We have added the following sentence in the left column on page 4.

"A similar result was obtained for Dox accumulation (Figure S20)."

Comment (6)

"Additionally, the authors noted that infarction of surface blood vessels was observed on the heated tumor (body surface side) in the mice 48 h post-administration of DBCO-TRM@DOX and Az-TRM. It would be better to compare the micelle amounts in the surface blood vessels to those inside the tumor. Meanwhile, the micelles or drug may penetrate the tumor after 48 h of injection. The authors need to monitor the accumulation of micelles in the tumor over a longer period."

The aim of the present experiments is to provide a proof-of-concept of our heat-guided drug delivery system, and we think that the precise behavior of the micelles accumulated in tumor tissues is beyond the scope of the present paper, though we agree it will be important for clinical translation of this system.

We believe the data already provided are sufficient to establish the efficacy of the system in our experimental model. Specifically, the treatment of DBCO-TRM (without Dox) and Az-TRM with heating had no marked therapeutic effect (Figure 4e) and produced no infarction of blood vessels (Figure S22), suggesting that Dox was essential for the therapeutic effect, and this is supported by the observation of infarcted blood vessels in the group treated with DBCO-TRM@DOX and Az-TRM with heating. We consider that there are two possible mechanisms for the effect of Dox released from the micelles: (1) Dox penetrates the tumor and directly damages the tumor cells, or (2) the cytotoxic action of Dox on endothelial cells within the tumor causes the occlusion of blood vessels. We will examine these possibilities in future studies. We have added the following sentences in the conclusion section (left column on page 5).

"Further studies are needed to understand the precise mechanism of the therapeutic effect, for example, whether Dox delivered to the tumor directly damages the tumor cells, or whether it causes the occlusion of the blood vessels feeding the tumors."

Comment (7)

"Q5: Given that aggregation of micelles can induce embolization, the authors should address the degradation of micelle aggregates within the blood vessels."

The backbone of the polymers (C-C bonds) is not biodegradable, so we consider that the disappearance of the micelle aggregates may be mainly due to the gradual release of polymer from micelle into the bloodstream. We added the following comment in the left column on page 5.

"The backbone of the polymers (C-C bonds) is not biodegradable, so we consider that the disappearance of the micelle aggregates may be mainly caused by the gradual release of polymer from the micelles."

Response to Reviewer #4

Comment (1)

"1. Why were the LCST values of the micelles tuned to be slightly higher than physiological temperature? The rationale behind of this design could be included in the manuscript."

We aimed to control the pharmacokinetics of the micelles by external heating. Therefore, we designed micelles that do not respond to physiological temperature, i.e. the LCSTs were tuned to be above the physiological temperature. For clarity, we have added the following sentences in the left column on page 2.

"To enable control of the pharmacokinetics of the micelles by external heating,"

Comment (2)

"2. The aggregation of the micelles at 42/47°C was completely reversed upon cooling to 32°C. Is this reversibility an important characteristic for the proposed application?"

The reversibility in response to temperature of the individual micelles (DBCO-TRM and Az-TRM) is derived from the properties of the polymeric micelles with NIPAAm-based shells, as previously reported (refs. 24-26).

To clarify the text, we added the words ", when examined individually," in the left column on page 2 as follows.

"As expected, when examined individually, DBCO-TRM (119 nm in mean diameter) and Az-TRM (114 nm) formed micellar aggregates with apparent sizes of 400 nm at 42°C and more than 1 μm at 47°C (Figure 2c), …"

To achieve persistent drug targeting even after cessation of heating, we aimed to construct micelles that respond irreversibly to heating. As can be seen in Fig. 3(a), we successfully showed that the aggregation of a mixture of the two micelles upon heating was not reversible. Overall, our results demonstrate that the irreversible crosslinking of micelles is both necessary and effective for targeted delivery to heated tissue.

Comment (3)

"3. Could the author describe on the measurement sizes measurement of the DBCO-TRM and Az-TRM micelles below and above the LCST?"

Thank you for the suggestion. We measured the sizes of DBCO-TRM and Az-TRM micelles below and above the LCST, and added these data in the left column on page 2.

Comment (4)

"4. How could the homogeneity and reproducibility of the micelle formulations be ensured to minimize the possibility of batch-to-batch variation ?"

To ensure reproducibility, we employed the following procedure every time micelles were prepared. The hydrodynamic diameters and their distribution were measured, and we confirmed that the mean diameter and PDI were essentially the same as the values shown in Table S2. Then, the thermally induced crosslinking of DBCO-TRM and Az-TRM was evaluated to ensure the efficiency of crosslinking remained similar to our previous results. To explain this, we added the following sentences to the experimental method in the supporting information.

"To check the homogeneity and reproducibility of the prepared micelles, the hydrodynamic diameters and their distribution were measured, and the efficiency of crosslinking of DBCO-TRM and Az-TRM was evaluated prior to use in the following experiments."

Comment (5)

"5. It has been mentioned that the size of the aggregates after heating at 42°C increased with increasing serum concentration. Is that an indication of the aggregates binding to the serum protein ?"

Thank you for raising this point. To investigate this further, we newly examined the effect of serum on the aggregation of DBCO-TRM and TRM (non-azide control), because we first wanted to examine whether or not the crosslinking reaction is necessary for the increasing size of aggregates in serum. In the presence of serum, the increase in micellar size upon heating at 42°C was transient and was completely reversed after cooling at 32°C, just as was the case without serum (Figure S14). Interestingly, even when TRM was used instead of Az-TRM, the presence of serum increased the size of the aggregates to almost the same extent during heating at 42°C (Figure S12 and S14).

We next considered the possibility that serum components were incorporated into the aggregates during heating and crosslinking occurred in this state, resulting in the formation of larger aggregates. So, we newly analyzed the micellar aggregates formed in the presence of fluorescently labelled dextran. Dextran, a model macromolecule, was chosen instead of marker proteins because of concerns about protein denaturation by the organic solvents used to dissolve the micellar aggregates. FITC-labelled dextrans of different molecular weights (4, 70 and 500 kDa) were mixed at the same concentration with DBCO-TRM and Az-TRM and heated at 42°C, and we found that very little dextran was trapped in the aggregates (Figure S13).

Based on these results, we consider that the aggregation of the micelles due to the phase transition above the LCST is enhanced in the presence of serum due to hydrophobic interaction. However, further study will be needed to establish the mechanism in detail, and we think this is beyond the scope of the present paper. We have added the following sentences in the right column on page 3.

"Dextran, a model macromolecule, was not incorporated into the irreversible aggregates of DBCO-TRM and Az-TRM (Figure S13). Furthermore, serum similarly increased the size of aggregates during heating when TRM (non-azide control) was used instead of Az-TRM (Figure S14). These results suggest that micellar aggregation above the LCST may be enhanced by hydrophobic interaction between the aggregates and serum proteins."

Comment (6)

"6. After the intravenously administration to tumor-bearing model mice, the subcutaneous tumor was reported to be heated at 42°C. How could the temperature of the whole tumor region be ensured during the heating and how to determine the selected heating duration of 30 min?"

Thank you for the comment. For local heating, an electric hand warmer with constant temperature control set at 42°C was placed over the tumor site for 30 min, which we considered to be a sufficiently long time for this experiment, based on the in vitro aggregation assay results. We did not measure the temperature inside the tumor, but at least the surface of the subcutaneous tumor in contact with the warmer was heated to 42°C. We have added a photograph of the heating process to the supporting information (Figure S19).

Comment (7)

"7. Could the release profile of the anti-cancer drug be addressed as it would also be helpful for the estimation of the release efficiency in the tumor."

We newly examined the in vitro Dox release by means of a dialysis method. Please refer to our response to Comment (4) of Reviewer 2.

Comment (8)

"8. Would the in vitro toxicity work also be included as well ?"

Thank you for the comment. DBCO-TRM@Dox and Az-TRM rapidly formed aggregates with an apparent particle size of more than 1 μm on heating at 42°C (Figure S14). This size is much larger than the diameter of endosomes that are typically involved in the cellular uptake of nanoparticles, which is 85–150 nm in clathrin- and caveolae-dependent endocytosis (*Cold Spring Harb Perspect Biol* 2014, 6, a016725, *Annu Rev Cell Dev Biol* 2018, 34, 111–136). So, we think that the micelles may aggregate outside cells in heated tissues and the aggregates cannot be internalized into cells. Therefore, the toxicity of the aggregates should be derived from Dox. Since Dox is taken up by cells through passive diffusion (*Int. J. Pharm.* 2001, 222, 169–182), the intracellular uptake of Dox in our study probably occurred via release from the micellar aggregates into the tumor tissues. As shown in Figure S18, Dox was released slowly from the aggregates over several days, and the cytotoxicity is expected to parallel this in vitro release profile.

Others

We added details of the sample sizes (n), statistical parameters and the statistical tests to the legends of Figure 3, 4 and S21.

Response to Reviewer #2 and Reviewer #3

Comment

"1. It seems that some molecular weights are still incorrectly labeled in the Mn (NMR) column of Table S1. The authors stated that the molecular weights of P(Az-co-AAm)-b-PBMA were determined using 1H NMR (entries 11, 13, and 15 in Table S1). However, these values appear to be mislabeled in entries 10, 12, and 14."

Thank you for the comment. The molecular weights of P(GA-co-AAm) (entries 10, 12, and 14) were analyzed by ^{1}H NMR and the labels in Table S1 are correct. For azide-containing polymers, the ratio of the monomer to the chain transfer agent ([monomer] / [CTA]) in the second step was 50 eq., which is lower than that in the first step (60–200 eq.). However, the molecular weights were increased by very large amounts after the second step. We considered that the molecular weights of the first block might not have been correctly measured by GPC, so we determined the molecular weights of the first block, P(GA-co-AAm), were also analyzed by ^{1}H NMR. We added the following sentence in the footnote of Table S1 to explain this clearly.

"Since the ratio of the monomer to the chain transfer agent in the second step was lower than that in the first step, we considered that the molecular weights of the first block might not have been correctly measured by GPC."

Response to Reviewer #4

Comment (1)

"1. The sentence "Dextran, a model macromolecule, was not incorporated into the irreversible aggregates of DBCO-TRM and AZ-TRM (Figure S13)." appears strangely in the paragraph. The author may consider adding a transition like "To examine the possibility of trapping serum component intothe aggregates, FITC-labelled dextrans were mixed and heated with DBCO-TRM and Az-TRM, where the ratio of fluorescent intensity suggested that neglectable amount of dextran …. (Figure S13).""

Thank you for the helpful comment. As the reviewer suggested, we corrected the sentence on page 7 as follows.

"To examine the possibility of trapping serum component into the aggregates, FITC-labelled dextrans were mixed and heated with DBCO-TRM and Az-TRM, where the ratio of fluorescence intensity suggested that neglectable amounts of dextrans were incorporated (Figure S13)."

Comment (2)

"2. The format of the title "CONCLUSIONS" should be bolded to maintain consistency."

Thank you for the comment. We corrected it.