andang miatmoko <andang-m@ff.unair.ac.id>

Regarding your manuscript Submitted to APB

4 messages

apb.tbzmed@gmail.com <apb.tbzmed@gmail.com> Sun, Oct 18, 2020 at 1:21 AM
To: andang-m@ff.unair.ac.id

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Regarding your manuscript Submitted to APB

Dear Dr Andang Miatmoko

We are pleased to accept your paper entitled "Physical Characterization and Biodistribution of Cisplatin Loaded in Surfactant Modified-Hybrid Nanoparticles using Polyethylene Oxide-b-Polymethacrylic Acid" in its current form. You will receive proofs for checking in due course. Your manuscript will be available in "Articles in press" at "http://apb.tbzmed.ac.ir" soon.

The publisher also requests that proofs are checked and returned within 48 hours of receipt. Thanks for your contribution to "Advanced Pharmaceutical Bulletin" and looking forward to receive further submissions from you

Sincerely yours,

Hadi Valizadeh, Pharm. D, Ph.D,

Editor-in-Chief,

Professor of Pharmaceutics,

Faculty of Pharmacy,

Tabriz University of Medical Sciences

Tabriz, Iran

• Email: valizadeh@tbzmed.ac.ir or valizadehh@gmail.com

Tabriz University of Medical Sciences

Andang MIATMOKO <andang-m@ff.unair.ac.id> Tue, Oct 13, 2020 at 6:42 PM
To: apb.tbzmed@gmail.com, valizadeh@tbzmed.ac.ir, valizadehh@gmail.com

Dear Editor,
I have checked the in press manuscript and there is mis typesetting for the equation (2) in Page 4. Please find the attachment.

Many thanks
[Quoted text hidden]
--

Salam,

Andang Miatmoko, PhD., Apt.
Department of Pharmaceutics
Faculty of Pharmacy, Airlangga University
Nanizar Zaman Joenoes Building
Campus C Airlangga University, Mulyorejo, 60115
Surabaya

apb-29003.pdf
813K

Hadi Valizadeh <valizadehh@gmail.com> Wed, Oct 21, 2020 at 7:54 PM
To: Andang MIATMOKO <andang-m@ff.unair.ac.id>

Hi
Thank you. Please correct all mistakes on the manuscript whenever you get the galley proof.
Regards
[Quoted text hidden]

Andang MIATMOKO <andang-m@ff.unair.ac.id> Thu, Oct 15, 2020 at 1:29 PM
To: Hadi Valizadeh <valizadehh@gmail.com>

Dear Editor,
Many thanks. I have checked the manuscript in the galley proof, and i have found some mistakes:
1. mis-typesetting for the equation (2) in Page 4
2. mis-spelling of sodium deoxycholate in page 6.
Please see the attachment.
many thanks

[Quoted text hidden]

apb-29003.pdf
823K

andang miatmoko <andang-m@ff.unair.ac.id>

Regarding your manuscript Submitted to APB

1 message

apb.tbzmed@gmail.com <apb.tbzmed@gmail.com> Tue, Jul 21, 2020 at 11:08 AM
To: andang-m@ff.unair.ac.id

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Regarding your manuscript Submitted to APB

Re: Manuscript ID: apb-29003

Dear Dr Andang Miatmoko

The peer review report on your submission entitled "Physical Characterization and Biodistribution of Cisplatin Loaded in Surfactant Modified-Hybrid Nanoparticles using Polyethylene Oxide-b-Polymethacrylic Acid" is below, which identifies some points to be addressed. Please highlight and underline the corrections and then send the revised manuscript. Consequently, and provided that these points are satisfactorily addressed, we would be happy to consider your manuscript for publication.

By the way, your paper has been identified as requiring English language copy editing. Note that your paper will not be accepted for publication unless this editing has taken place.

Please provide a separate list of responses to the reviewer comments, describing the changes you have made to your manuscript. Please place this in a separate file called 'Responses to review results' and upload this in the "Attach files" step and submit it together with your manuscript. Do not submit this paper as a New Submission. Without a completed "Responses" sheet, your manuscript will not be processed further.

Please submit your revised paper within 2 weeks. After this period it will be treated as a new submission.

http://apb.tbzmed.ac.ir/Login

Sincerely yours,

Dr Fahimeh Zahednezhad,

Assistant editor of Advanced Pharmaceutical Bulletin,

•Email:zahednejadf@tbzmed.ac.ir or zahednezhadf@gmail.com

Prof Hadi Valizadeh, Pharm. D, Ph.D,

Editor-in-Chief,

Professor of Pharmaceutics,

Faculty of Pharmacy,

Tabriz University of Medical Sciences

Tabriz, Iran

• Email: valizadeh@tbzmed.ac.ir or valizadehh@gmail.com

Reviewers' comments:

Reviewer 1: ()
Comments to the Editorial Office:

Dear editor:

The paper entitled "**Physical Characterization and Biodistribution of Cisplatin Loaded in Surfactant Modified-Hybrid Nanoparticles using Polyethylene Oxide-b-Polymethacrylic Acid"** does not have enough quality to be published and the study could be conducted more carefully.

Comments to the Author:

Dear authors:

The present study needs major revision both of the writing and the tests; I think it does not have enough quality to be published. There is some comments that may help you.

1. The English writing needs major revision and there is some mistakes for example:
    - Please change the sentence in line 9: "and encapsulation efficiency was 5-18%"
    - It is better to write "so tumor accumulation may increase" in line 11 and also the conclusion because the tumor accumulation has not been studied.
    - "pharmacokinetic profile" is correct in line 24.
    - "Reduction of …" in line 27
    - …
2. Please explain the detection method of drug in the plasma briefly.
3. Some other test are necessary for formulation characterization for example drug release study or FT-IR and etc.
4. The formulation optimization needs modification because the EE% is too low.
5. The in vivo bio-distribution has not been explained completely.
6. It has been mentioned that the animals were divided into 3 groups but the results show 5 groups.

Reviewer 2: ()
Comments to the Author:

- As methanol is not generally regarded as safe, how did you ensure non-existence of residual methanol in the final product?
- 82 The amount of water should be noted: "the mixture was then dialyzed against water"
- It is highly suggested to draw a schematic figure of the prepared nanoparticle showing the structure for better understanding by the readers about the hybrid conception.
- Please add the HPLC graph, pH of the mobile phase, retention time, and also the calibration curve with R^2 and its equation.
- Was the applied HPLC method, and also the nanoparticle preparation innovative or adopted from a reference? if not please include the reference where necessary.
- Please add Loading capacity percent equation (LC%)
- I suggest to discuss more about the causes of differences in the results obtained from different surfactants, by investigating structural point of view or other possible interactions.
- Please add DOI of the references.

- 141 This is not in accordance with figure 1 data: “The addition of surfactants including sodium deoxycholate, sodium cholate and Tween 80 into hybrid nanoparticles i.e. HNP-SD, HNP-SC, and HNP-TW, respectively, slightly decreased the particle sizes.”
- There were several grammar mistakes, fluency, and vague and literal sentences in the manuscript. A thorough revision has to be performed by an expert. Only some comments are provided below:

line 3 ambiguous: “and the presence of water diffusion-limiting lipid layer”

9 “with encapsulation efficiencies were of”

edit 40 “we modify hybrid nanoparticles loading cisplatin …”, 89 “The hybrid nanoparticles loading cisplatin was obtained”, 101 and 112 “hybrid nanoparticles loading cisplatin”, …

52 Surfactant has been known destabilizing lipid bilayer

101 and 102 “was added with”

104 for 5 minutes.

117 mice were tumor induced

124 “Tumor tissue and kidney were taken care of without any blood, …”

144 were negative ~~charge~~

154 It is linear with the previous study

159 that contained

Reviewer 3: ()
Comments to the Author:

Suggested revisions

1. Line 23- What does the author refers to 'hear losing'? Use appropriate alternate term. Whether authors mean that hair loss then use the term alopecia and if it ers to hearing loss you can use the 'ototoxic'.

2. Line 24- revise sentence

3. The discussion part may be explored in depth.

4. References shall be provided at a suitable place in methods and references shall be in accordance with the journal guidelines.

5. English language editing shall be performed.

Tabriz University of Medical Sciences

Dear Editor,

Many thanks for the email. We really appreciate all comments to improve our manuscript. Below are the answers addressed for the reviewer's comments. We have proofread the manuscript to Colledge & Associates, UK. Please see the attachment.

- **Please highlight the previously added parts in the revised manuscript.**
  Answer:
  Many thanks for the correction. We have highlighted the added parts in the manuscript.

- **Edit reference style as for APB.**
- **Please recheck the English mistakes: "Blood-free tumor and kidney tissue ~~was~~ were taken and weighed".**
  Answer:
  Many thanks for the correction. We have revised the word "was" in line 133 into "were".

- **Add space where necessary. e.g. line 40**
  Answer:
  Many thanks for the correction. We have added space to the necessary part of the manuscript.

- **Please replace a higher quality HPLC figure with minimized solvent picks; otherwise Table 1 and Calibration graph which should be mentioned in-text, is sufficient to be added in the manuscript.**
  Answer:
  Many thanks for the correction. We have added the data of peak area of Cisplatin standard solution in Table 2 and the calibration curve in Figure 2. We have also revised the subsequent figure numbers.
  We have also added some discussion in line 169 as the following:
  "The amount of cisplatin encapsulated in hybrid nanoparticles was determined by using an HPLC method. The peak areas were then plotted against concentration (Table 2) resulted in a good linearity of calibration curve with coefficient of correlation (R^2) of 0.9997 over the cisplatin concentration range of 5-100 μg/mL, as seen in Figure 2."

Reviewer 1:

Comments to the Editorial Office:

Dear editor:

The paper entitled "**Physical Characterization and Biodistribution of Cisplatin Loaded in Surfactant Modified-Hybrid Nanoparticles using Polyethylene Oxide-b-Polymethacrylic**

Acid" does not have enough quality to be published and the study could be conducted more carefully.

Comments to the Author:

Dear authors:

The present study needs major revision both of the writing and the tests; I think it does not have enough quality to be published. There is some comments that may help you.

1. **The English writing needs major revision and there is some mistakes for example:**

   Answer:

   we have proofread the manuscript. Please see the attachment.

   - **Please change the sentence in line 9: "and encapsulation efficiency was 5-18%"**

   Answer:

   Many thanks for the correction. We have revised the sentence.

   Line 9: The addition of cisplatin increased the ζ-potential to slightly positive charges with encapsulation efficiencies of 5-18%.

   - **It is better to write "so tumor accumulation may increase" in line 11 and also the conclusion because the tumor accumulation has not been studied.**

   Answer:

   Many thanks for the comment. In this study, we have evaluated distribution of Cisplatin nanoparticles in plasma, tumor and kidney as it can be seen in Figure 3. The solid tumor tissue itself was excised from the animal, which was transplanted by injecting C-26 cells suspension subcutaneously, after the treatment of samples. Therefore, the result represents the cisplatin accumulation in the tumor tissue.

   - **"pharmacokinetic profile" is correct in line 24.**

   Answer:

   Many thanks for the correction. We have revised the word

   Line 25: has a biphasic pharmacokinetic profile

- **"Reduction of …" in line 27**

Answer:

Many thanks for the correction. We have revised the word

Line 29: may improve the efficacy of cisplatin in addition to reducing its toxicity

2. **Please explain the detection method of drug in the plasma briefly.**

Answer:

Many thanks for the comment. We have added the details of platinum level assay in the method section line 136 as the following:

"Platinum levels were determined by digesting samples with concentrated HNO_3 and heating them for one hour at 70°C, followed by heating at 120°C overnight to obtain dry samples. These were then added to 0.1 N HCl at an appropriate level of dilution. The GF-AAS analysis program involved three steps : (1) a 40-second drying stage at 80-100°C, (2) a 30-second ashing stage at 800°C (3) a 7-second atomization stage at 3000°C, followed by cooling. Absorbances were measured at 265.9 nm with a slit bandwidth of 0.4 nm and the sample volume was 30 µL. The results were expressed as µg Pt/mL plasma and µg Pt/g tissue or organ".

3. **Some other test are necessary for formulation characterization for example drug release study or FT-IR and etc.**

Answer:

Many thanks for the comment. In this study, we focused on the study for evaluation of the efficacy of hybrid nanoparticles in maintaining drug trapped inside the polymer and prevent mature leakage. We have observed in vitro drug release of HNP and HNP-SC in PBS pH 5.5 and pH 7.4. As it can be seen in the Figure A, there was no significant different of cisplatin released from the hybrid nanoparticles (HNP) and sodium cholate-modified (HNP-SC). However, these hybrid nanoparticles released higher cisplatin levels in pH 5.5 than those of pH 7.4.

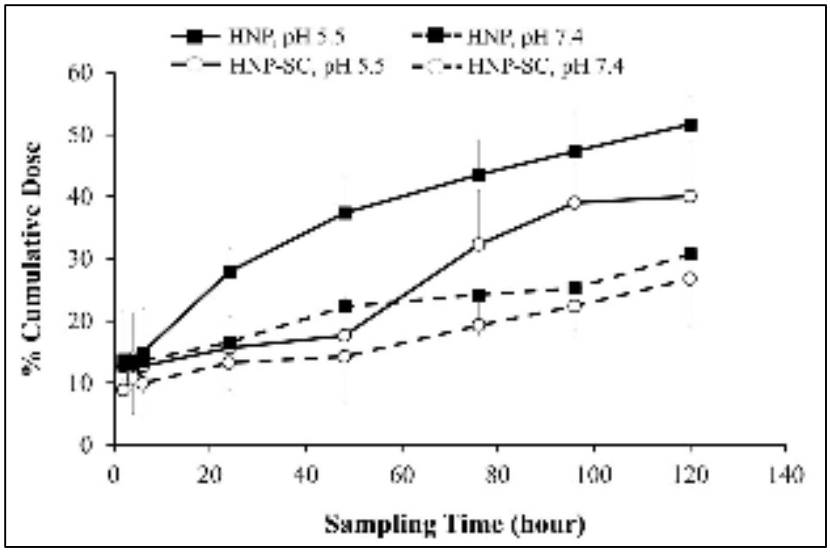

Figure A. The cisplatin release from Hybrid Nanoparticles (HNP) and Sodium Cholate-modified Hybrid Nanoparticles (HNP-SC) in PBS pH 5.5 and 7.4.

For other evaluation such as FTIR, we have not checked it since we would like to observe the *in vivo* efficacy of these HNPs to propose further study.

4. The formulation optimization needs modification because the EE% is too low.

Answer:

Many thanks for the comment. In our preformulation study, the use of polymeric micelles prepared with the PEO-b-PMAA resulted in higher encapsulation efficiency than those of Hybrid Nanoparticles, which was 66%. We also observed that increasing the polymer amount will improve the encapsulation efficiency and reduce the particle size, however, the polymeric micelle was produced in non-homogenous particle size indicated with high polydispersity indexes, and sometimes undetected by particle size analyzer. The formula used in this study had the optimal ratio between the polymer, surfactant, and lipid, but, surely further study importantly required to improve the encapsulation efficiency.

5. The in vivo bio-distribution has not been explained completely.

Answer:

In the manuscript, as it can be seen in line 188-218, we have shown the cisplatin levels found in plasma, tumor and kidney tissue of C-26-induced solid tumor mice at 24

hours after two injections of Cisplatin HNP, Cisplatin-HNP-SD, Cisplatin-HNP-SC, and Cisplatin-HNP-TW. The HNPs provided higher drug concentration in plasma and tumor than free cisplatin. And Cisplatin-HNP-SC had the highest drug concentration in plasma, up to ten times higher than cisplatin solution, and high accumulation in tumor tissue among others. It should be due to prolong drug circulation in plasma, thus increasing tumor drug accumulation, which is known probably due to the enhanced permeation and retention (EPR) effect. On the other hand, the elevation of drug level in the kidney was observed in Cisplatin-HNP-SC generating two-fold higher platinum levels than free Cisplatin, which probably due to release of cisplatin from long circulated Cisplatin-HNP-SC, which has biphasic elimination phase with the late phase within 2-3 days in slow mode, thus accumulating high cisplatin levels in the kidney as the excreting organ.

We have added some discussion in line 207 as the following:

"It is known that these three surfactants have different chemical structures that may affect the lipid layered on the hybrid nanoparticle surfaces. Tween 80 contains non-bulky hydrocarbon chains. On the other hand, sodium deocycholate and sodium cholate have steroid-like structures with differences in the total number of hydroxyl functional groups, which are three and two for sodium cholate and sodium deoxycholate respectively. These structures are bulkier than Tween 80, thus reducing transient hydrophilic hole formation causing rigidity of the lipid layer (47). This may limit water permeability across the lipid layer on the hybrid nanoparticles causing cisplatin to leak out. However, this report states that there were no significant differences in lipid rigidity between sodium cholate and sodium deoxycholate, while in this study sodium cholate produced more stable nanoparticles than sodium dexocyholate – a phenomenon requiring further evaluation."

47. El Zaafarany GM, Awad G a S, Holayel SM, Mortada ND. Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. Int J Pharm. 2010;397(1–2):164–72. DOI: 10.1016/j.ijpharm.2010.06.034

6. **It has been mentioned that the animals were divided into 3 groups but the results show 5 groups.**

   Answer:

   Many thanks for the correction. We have revised the sentence in the method section line 128 as the following: “the mice were divided into five groups of 4-5 subjects”

Reviewer 2:

Comments to the Author:

- **As methanol is not generally regarded as safe, how did you ensure non-existence of residual methanol in the final product?**

  Answer:

  Many thanks for the comment. During the final stage of hybrid nanoparticles preparation, approximately 20 mL of the mixture was dialyzed with 200 mL of water using a regenerated cellulose dialysis membrane (Spectra Por®7) with a molecular weight cut-off (MWCO) of 2,000 and The water was changed a total of eight times on two consecutive days. After Cisplatin binding, we concentrated the Cisplatin-HNPs by centrifuging the samples using centrifugal filter unit with MWCO 30.000, which this steps included the outer media changes with 2 x 5 mL of 5% Dextrose solution.

  Furthermore, we have checked the cytotoxicity of HNPs, without Cisplatin loading on Lewis Lung Cancer (LLC) cells for 48 hours. As seen in Figure 2, there were no cytotoxicities observed for the HNPs’s matrix.

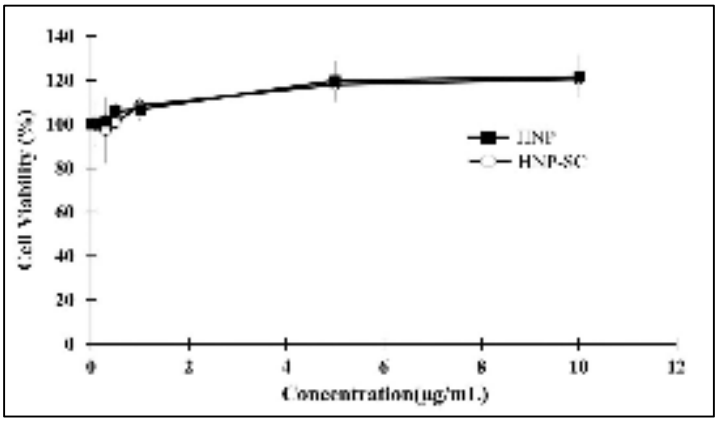

Figure 2. Cell Cytotoxity of Hybrid Nanoparticles without Cisplatin loading on LLC Cells for 48 Hours Incubation

- **82 The amount of water should be noted: "the mixture was then dialyzed against water"**

  Answer:

  Many thanks for the correction. We have revised the sentence into the following:

  Line 86: "In order to remove the organic solvents, approximately 20 mL of the mixture was dialyzed with 200 mL of water using a regenerated cellulose dialysis membrane (Spectra Por®7) with a molecular weight cut-off (MWCO) of 2,000. The water was changed a total of eight times on two consecutive days. "

- **It is highly suggested to draw a schematic figure of the prepared nanoparticle showing the structure for better understanding by the readers about the hybrid conception.**

  Answer:

Many thanks for the comment. We have added the schematic figure of HNP loading Cisplatin as the following:

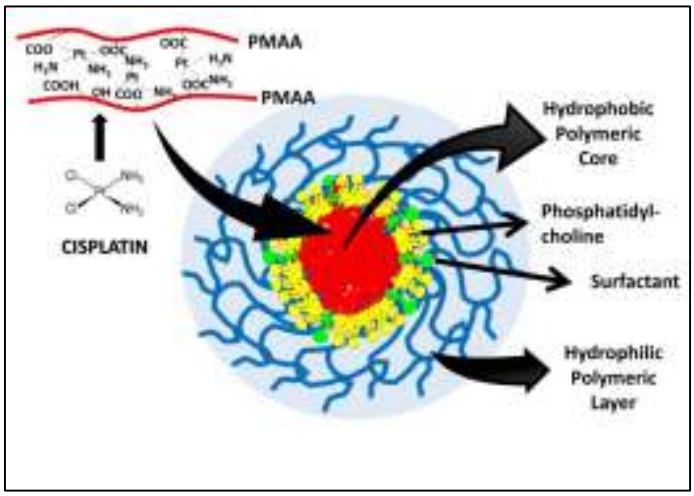

Figure 3. The schematic illustration of Hybrid Nanoparticles loading Cisplatin

- **Please add the HPLC graph, pH of the mobile phase, retention time, and also the calibration curve with R^2 and its equation.**

  Answer:

Many thanks for the comments. The Cisplatin assay was performed by using mobile phase = ethyl acetate:MetOH:milliq water:N,N Dimethyl formamide= 8:40:10:20. The milliQ water itself has pH 5.5, however, we did not measure the pH of the mobile phase. The chromatograms of Cisplatin were presented in Figure

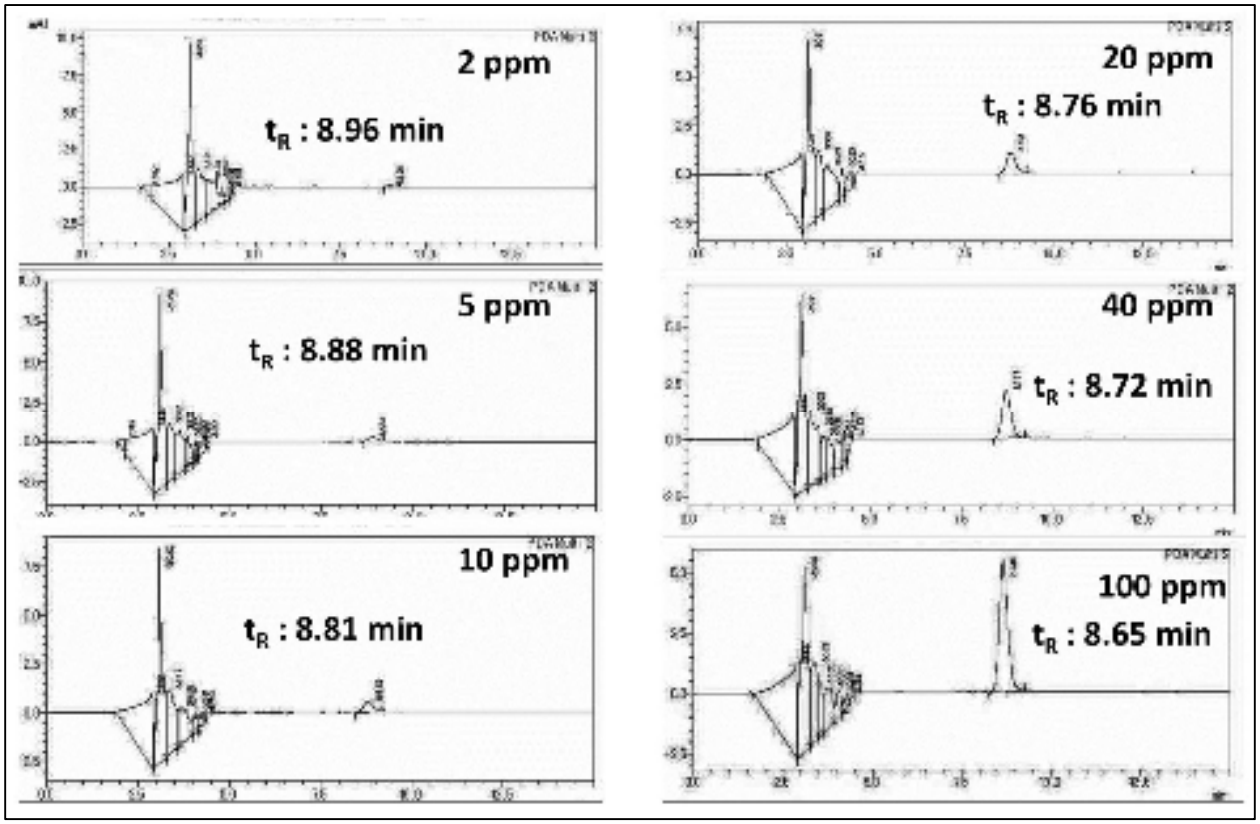

Figure 4. The HPLC chromatograms of Cisplatin at the concentration ranges within 2-100 ppm by using Inertsil AX® anion exchange column with mobile phase consisted of the mixture of ethyl acetate:Methanol:water:N,N Dimethylformamide at a ratio of 8:40:10:20, respectively.

The peak area of cisplatin solution at various concentration was presented in Table 1. The calibration curve and the correlation coefficient was shown in Figure 5.

Table 1. The peak area of standard solution of cisplatin.

Concentration of Cisplatin (ppm)	Peak area

5	4711
10	11933
20	24303
50	57635
100	115688

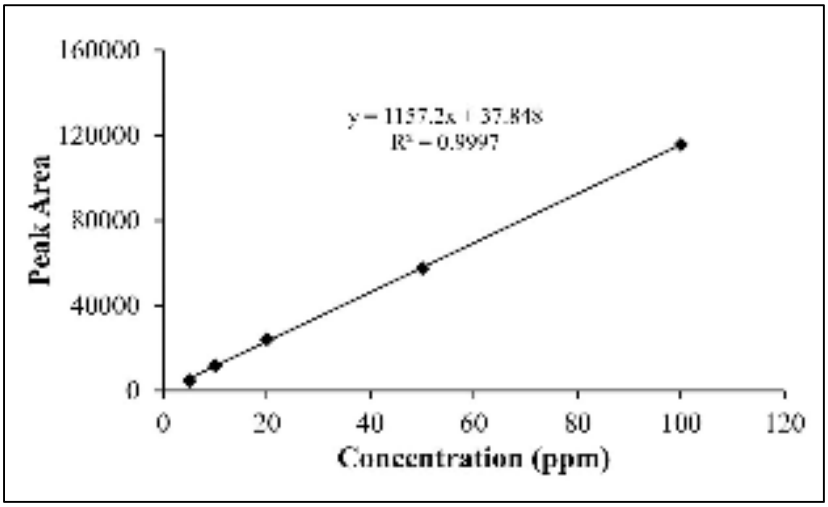

Figure 4. The calibration curve of Cisplatin solution in PBS pH 7.4 at the concentration ranges within 5-100 ppm measured by HPLC using Inertsil AX® anion exchange column with mobile phase consisted of the mixture of ethyl acetate:Methanol:water:N,N Dimethylformamide at a ratio of 8:40:10:20, respectively.

- **Was the applied HPLC method, and also the nanoparticle preparation innovative or adopted from a reference? if not please include the reference where necessary.**

  Answer:

  Many thanks for the comments. The applied HPLC method was a modified adapted method from Supplement II to The Japanese Pharmacopeia XVII edition page 714. We have added the reference to the manuscript as the following:

  Line 111: "The upper aqueous layer containing cisplatin was removed and analyzed by high-performance liquid chromatography (HPLC) method (HPLC Shimadzu, Japan) with an anion exchange column Inertsil AX® (250 mm x 4.6 mm, 5 μm) as the stationary phase at room temperature (40,41)."

40. The Ministry of Health L and W. Supplement II to The Japanese Pharmacopoeia 17th Edition. XVII. Hashida M, Kawasaki N, editors. Tokyo: The Ministry of Health, Labour and Welfare; 2019. 714 p.
41. Miatmoko A, Kawano K, Hattori Y, Maitani Y, Yonemochi E. Evaluation of transfersome and protransfersome for percutaneous delivery of cisplatin in hairless mice. J Pharmaceu Pharmacol. 2015;S(1):1–7.

The preparation method was a modification from a procedure adapted from the literatures for general method for preparing hybrid nanoparticles (38) and the loading cisplatin into polymeric micelle of PEO-b-PMAA (33).

33. Kim JO, Nukolova N V, Oberoi HS, Kabanov A V, Bronich TK. Block ionomer complex micelles with cross-linked cores for drug delivery. Polym Sci Ser A. 2009 Jun;51(6):708–18. DOI: 10.1134/S0965545X09060169
38. Zhang L, Zhang L. Lipid–Polymer hybrid nanoparticles: synthesis, characterization and applications. Nano Life. 2010;1(1&2):163–73. DOI: 10.1142/S179398441000016X

- **Please add Loading capacity percent equation (LC%)**

  Answer:

  Many thanks for the correction. We have added the calculation and equation for loading capacity in line 115 as the following:

  The encapsulation efficiency (EE) and loading capacity (LC) were calculated using equations (1) and (2) respectively (42,43):

"$Entrapment\ Efficiency\ (\%) = \frac{cisplatin\ content\ of\ hybrid\ nanoparticle}{total\ added\ amount\ of cisplatin} \times 100\%$ (1)

$$Loading\ Capacity\ (\%) = \frac{amount\ of\ drug\ encapsulated}{amount\ of\ drug\ encapsulated + total\ amount\ of\ liposomal\ components} \times 100 \quad (2)$$

42. Miatmoko A, Annuryanti F, Sari R, Hendradi E. Dual loading of primaquine and chloroquine into liposome. Eur Pharm J. 2019;66(2):18–25. DOI: 10.2478/afpuc-2019-

0009

43. Qiu L, Jing N, Jin Y. Preparation and in vitro evaluation of liposomal chloroquine diphosphate loaded by a transmembrane pH-gradient method. Int J Pharm. 2008;361(1–2):56–63. DOI: 10.1016/j.ijpharm.2008.05.010

- **I suggest to discuss more about the causes of differences in the results obtained from different surfactants, by investigating structural point of view or other possible interactions.**

  Answer:

  Many thanks for the correction. We have added some discussion in line 207 as the following: "It is known that these three surfactants have different chemical structures that may affect the lipid layered on the hybrid nanoparticle surfaces. Tween 80 contains non-bulky hydrocarbon chains. On the other hand, sodium deocycholate and sodium cholate have steroid-like structures with differences in the total number of hydroxyl functional groups, which are three and two for sodium cholate and sodium deoxycholate respectively. These structures are bulkier than Tween 80, thus reducing transient hydrophilic hole formation causing rigidity of the lipid layer (47). This may limit water permeability across the lipid layer on the hybrid nanoparticles causing cisplatin to leak out. However, this report states that there were no significant differences in lipid rigidity between sodium cholate and sodium deoxycholate, while in this study sodium cholate produced more stable nanoparticles than sodium dexocyholate – a phenomenon requiring further evaluation."

47. El Zaafarany GM, Awad G a S, Holayel SM, Mortada ND. Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. Int J Pharm. 2010;397(1–2):164–72. DOI: 10.1016/j.ijpharm.2010.06.034

- **Please add DOI of the references.**

  Answer:

  Many thanks for the correction. We have added DOIs to the references section; however there are 3 articles that we could not find their DOI.

- **141 This is not in accordance with figure 1 data: "The addition of surfactants including sodium deoxycholate, sodium cholate and Tween 80 into hybrid nanoparticles i.e. HNP-SD, HNP-SC, and HNP-TW, respectively, slightly decreased the particle sizes."**

Answer:

Many thanks for the correction. We have revised the sentence in Line 159 as the following:

"The addition of surfactants including sodium deoxycholate and sodium cholate to the hybrid nanoparticles, i.e. HNP-SD and HNP-SC respectively, produced slightly smaller particle sizes than HNP".

- **There were several grammar mistakes, fluency, and vague and literal sentences in the manuscript. A thorough revision has to be performed by an expert. Only some comments are provided below:**

Answer: we have proofread the manuscript to Colledge & Associates, UK.

line 3 ambiguous: "<u>and the presence of water diffusion-limiting lipid layer"</u>

Answer:

We have revised the sentence into the following:

"and prevent premature drug release because of the presence of a lipid layer"

9 "<u>with encapsulation efficiencies were of"</u>

Answer:

We have revised the sentence into the following:

"with encapsulation efficiencies of 5-18%."

edit 40 <u>"we modify hybrid nanoparticles loading cisplatin …"</u>

Answer:

We have revised the sentence in line 42 into the following:

"hybrid nanoparticle-loading cisplatin was modified"

<u>, 89 "The hybrid nanoparticles loading cisplatin was obtained"</u>,

Answer:

We have revised the sentence in line 94 into the following:
"The hybrid nanoparticle-loaded cisplatin …"

101 and 112 "hybrid nanoparticles loading cisplatin", …

Answer:
We have revised the sentence in line 107 and into the following:
"cisplatin-loaded hybrid nanoparticles"

52 Surfactant has been known destabilizing lipid bilayer

Answer:
We have revised the sentence in line 54 into the following:
"Surfactant has been known to destabilize the lipid bilayer,"

101 and 102 "was added with"

Answer:
We have revised the sentence in Line 107 into the following:
"were added with"

104 for 5 minutes.

Answer:
We have revised the sentence in line 110 into the following:
"for five minutes."

117 mice were tumor induced

Answer:
We have revised the sentence in line 126 into the following:
"the mice were tumor induced"

124 "Tumor tissue and kidney were taken care of without any blood, …"

Answer:
We have revised the sentence in line 133 into the following:

“Blood-free tumor and kidney tissue was taken and weighed.”

144 were negative ~~charge~~

Answer:

We have revised the sentence in line 161 into the following:

“the ζ-potential of all these hybrid nanoparticles was negative and not significantly different.”

154 It is linear with the previous study

Answer:

We have revised the sentence in line 177 into the following:

“These results correlate closely with those of a previous study”

159 that contained

Answer:

We have revised the sentence in line 182 into the following:

“that contained lipid and surfactant were not significantly different”

Reviewer 3:

Comments to the Author:

Suggested revisions

1. Line 23- What does the author refers to 'hear losing'? Use appropriate alternate term. Whether authors mean that hair loss then use the term alopecia and if it ers to hearing loss you can use the 'ototoxic'.

Answer:

Many thanks for the correction. We have revised the word “hear losing” in line 24 into ototoxicity.

2. Line 24- revise sentence

Answer:

Many thanks for the correction. We have revised the sentences in Line 25 into the following:
"It has been reported that cisplatin has a biphasic pharmacokinetic profile with a first half-life of between 25-49 minutes and a second which lasts for 58-73 hours after intravenous injection (11). Moreover, cisplatin also has a high tissue-blood partition ratio in the kidney (16)."

3. The discussion part may be explored in depth.

Many thanks for the correction. We have added some discussion in line 207 as the following:
"It is known that these three surfactants have different chemical structures that may affect the lipid layered on the hybrid nanoparticle surfaces. Tween 80 contains non-bulky hydrocarbon chains. On the other hand, sodium deocycholate and sodium cholate have steroid-like structures with differences in the total number of hydroxyl functional groups, which are three and two for sodium cholate and sodium deoxycholate respectively. These structures are bulkier than Tween 80, thus reducing transient hydrophilic hole formation causing rigidity of the lipid layer (47). This may limit water permeability across the lipid layer on the hybrid nanoparticles causing cisplatin to leak out. However, this report states that there were no significant differences in lipid rigidity between sodium cholate and sodium deoxycholate, while in this study sodium cholate produced more stable nanoparticles than sodium dexocyholate – a phenomenon requiring further evaluation."

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4. References shall be provided at a suitable place in methods and references shall be in accordance with the journal guidelines.

Answer:

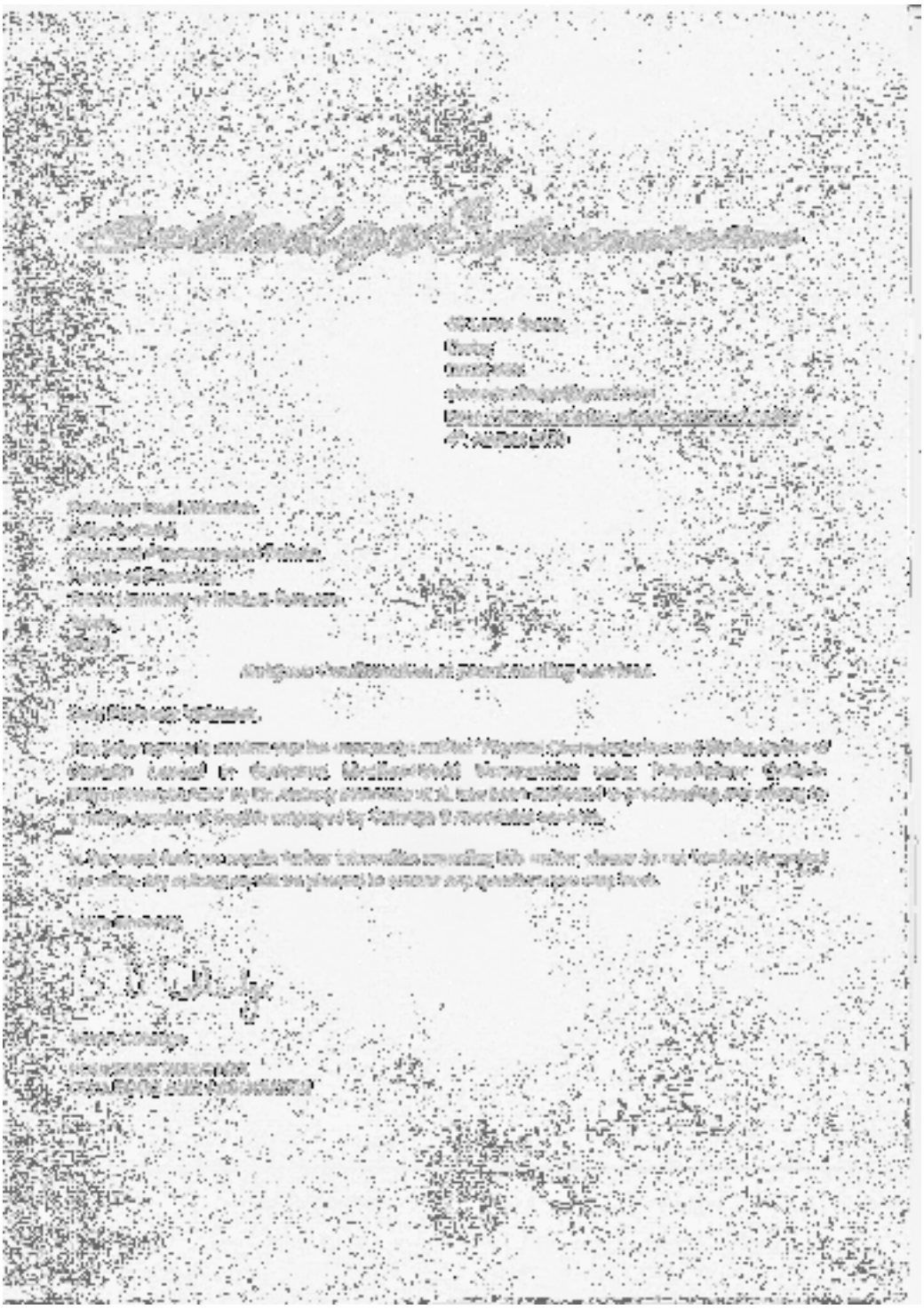
Many thanks for the correction. We have revised the citation and references in the manuscript in accordance with the journal guideline.

5. English language editing shall be performed.

Answer:

Many thanks for the suggestion. We have proofread the manuscript to Colledge & Associates, UK. Please see the attachment.

Certificate of Proofreading



ABSTRACT

Purpose: Conjugating cisplatin into hybrid nanoparticles is intended to enhance tumor accumulation due to drug interaction with polymer and prevent premature drug release because of the presence of a lipid layer. **Methods:** Hybrid nanoparticles composed of polyethylene oxide-b-polymethacrylic acid, egg phosphatidylcholine, and surfactant, i.e. sodium cholate/sodium deoxycholate/Tween 80, were prepared by the injection method. Cisplatin was subsequently loaded by incubating the polymer-drug mixtures at the molar ratio of carboxylate ions of 2:1. **Results:** The results showed that the addition of surfactants produced particle sizes between 33 and 52 nm. The addition of cisplatin increased the ζ-potential to slightly positive charges with encapsulation efficiencies of 5-18%. An *in vivo* biodistribution study of mice identified a cisplatin plasma concentration of sodium cholate-modified hybrid nanoparticles ten times higher than cisplatin solution, thus producing high tumor accumulation. **Conclusion:** Conjugating cisplatin into sodium cholate-modified hybrid nanoparticles improves its accumulation in tumors.

Keywords: cisplatin, egg phosphatidylcholine, hybrid nanoparticles, polyethylene oxide-b-polymethacrylic acid, surfactants

INTRODUCTION

Cisplatin, classified as a platinum complex alkylating-drug, has been widely employed in chemotherapy, either as a single-drug therapy or in combination with other chemotherapeutic agents.[1–4] However, numerous reports exist regarding the severe side effects correlated to dosage and dose intensity of cisplatin therapy including nephrotoxicity,[5,6] distal neuropathy,[7,8] nausea, vomiting, and anorexia,[9–11] ototoxicity,[12,13] and liver toxicity.[14,15]

It has been reported that cisplatin has a biphasic pharmacokinetic profile with a first half-life of between 25-49 minutes and a second which lasts for 58-73 hours after intravenous injection.[11] Moreover, cisplatin also has a high tissue-blood partition ratio in the kidney.[16] Enhancing the circulation lifetime of drugs in the body by drug conjugation into nanoparticles may improve the efficacy of cisplatin in addition to reducing its toxicity. Particulate carriers such as polymeric micelles, liposomes, nanoparticles, and many others have been investigated in order to achieve higher drug accumulation in tumors and reduce distribution to healthy tissues and organs during cisplatin therapy.[17–22]

Hybrid nanoparticles, which combine the properties of lipid vesicles and polymeric micelle, are potentially excellent drug carriers. Generally, hybrid nanoparticles contain diblock polymer and lipid components. The diblock polymer itself possesses two different functional block segments that can generate polymeric micelles. In addition, the lipid layer surrounding the core serves as a highly biocompatible-biomimetic shell and water diffusion constraint.[23,24] This lipid layer may also support and protect the inner layer from premature disintegration caused by excessive dilutions upon systemic administration.[25] A previous study reported that hybrid nanoparticles enhance the stability of cisplatin-polymer conjugation and result in superior *in vitro* stability to polymeric micelle.[26] However, it indicated no improved levels of cisplatin in

tumor tissue. Therefore, hybrid nanoparticle-loading cisplatin was modified by the addition of a surfactant whose presence affects lipid deformability.[27,28]

In this study, a biodegradable block ionomer, polyethylene oxide-b-polymethacrylic acid (PEO-b-PMAA), was used to prepare the hybrid nanoparticles. PEO is a relatively non-toxic water soluble non-ionic homopolymer[29] which demonstrates the ability to maintain the recognition of nanoparticles by the immune system providing longer drug circulation in plasma.[30,31] PMAA is a pH-dependently ionized weak anionic polymer which will conjugate with the platinum ions, together forming the inner core and controlling the drug release specific to the acidic tumor environment via reversible ion exchanges.[32] Given the presence of abundant counterions, the drug-polymer interaction will cease leading to the disintegration of the micelle, thus releasing the encapsulated drug.[33] The addition of lipid such as egg phosphatidylcholine and surfactant, as the hybrid nanoparticle component, is intended to enhance the stability of nanoparticles as well as high tumor drug accumulation. Surfactant has been known to destabilize the lipid bilayer, thereby altering the membrane's permeability and flexibility.[34–37] The use of surfactant may affect the drug released from the carrier resulting in changes to the drug levels in plasma and tumor tissue. Conjugating cisplatin into hybrid nanoparticles was intended to enhance the stabilization of polymer-drug interaction during blood circulation, thus promoting tumor drug accumulation.

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MATERIALS & METHODS

Materials

Cisplatin was purchased from Wako Pure Chemical Industries Co., Ltd. (Tokyo, Japan). Egg phosphatidylcholine (EPC, Coatsome® NC-50) and Tween 80 were products of NOF Inc. (Tokyo, Japan). Sodium Deoxycholate (SD) was acquired from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Sodium cholate (SC) was supplied by Sigma Aldrich (Tokyo, Japan). The diblock polymer used in this experiment was polyethylene oxide-b-polymethacrylic acid (PEO-b-PMAA; M_w of PEO = 7,500; M_n of PMAA= 11,000) was obtained from Polymer Source, Inc. (Canada). Saline was purchased from Otsuka Co. Ltd. (Japan). In order to undertake high-performance liquid chromatography (HPLC) analysis, all solvents were of HPLC analytical grade. For graphite furnace atomic absorption spectrophotometry (GF-AAS) measurements, nitric acid (1.38; analytical grade, Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) was employed as a digestive acid solution, while hydrochloric acid (AAS analytical grade) was used for the sample solvent (Kanto Chemical Co., Inc., Tokyo, Japan). The solvents; ethanol, methanol, acetone, and chloroform; were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents employed in this study were of the highest quality available. Milli-Q water was used in all experiments.

Preparation of cisplatin hybrid nanoparticles

Firstly, the matrix of hybrid nanoparticles was prepared by injection method.[38] The diblock polymer, PEO-b-PMAA, was dissolved in methanol, while EPC, Sodium Deoxycholate, Sodium Cholate and Tween 80 were dissolved in methanol. As seen from the contents of Table 1, appropriate amounts of EPC and each surfactant were mixed by vortexing, with the polymer

solution subsequently being added and agitated until it was homogenous. The solution was quickly injected into the water and left at room temperature. In order to remove the organic solvents, approximately 20 mL of the mixture was dialyzed with 200 mL of water using a regenerated cellulose dialysis membrane (Spectra Por®7) with a molecular weight cut-off (MWCO) of 2,000. The water was changed a total of eight times on two consecutive days.

Cisplatin was incorporated into the hybrid nanoparticles by direct mixing of cisplatin solution in an alkaline condition with a molar ratio of carboxylate ions [COO^-]:[Cisplatin] of 2:1.[33] The carboxylate molar concentration was determined by acid-base titration method. The pH of the mixture was adjusted to pH 9 by means of an ammonia solution and it was then incubated for two days in a shaking water bath at 37°C. The hybrid nanoparticle-loaded cisplatin was obtained by filtering the mixtures with a centrifugal filter unit (Merck Millipore Ltd., Carrigtwohill, Ireland) with an MWCO of 30,000 at 2,500 G for 20 minutes. The filtrate was further used to determine encapsulation efficiency.

Measurement of particle size, ζ-potential, entrapment efficiency, and loading capacity of Cisplatin hybrid nanoparticles.

The average particle size and ζ-potential of cisplatin-loaded hybrid nanoparticles was measured by cumulant method and electrophoretic mobility with a light scattering photometer (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C. The undiluted samples were subsequently measured directly.

The entrapment efficiency was determined by calculating the cisplatin content of the hybrid nanoparticles. As previously reported, the sample extraction was performed using the Bligh and Dyer method.[26,39] Approximately 200 µL of cisplatin-loaded hybrid nanoparticles were

added with chloroform:methanol mixtures (1:1, v/v) and a vortexed well. This mixture was added to 250 µL chloroform followed by vortexing. Approximately 250 µL of 0.1N HCl solution was added to the mixture and mixed thoroughly before being centrifuged at 10,000 rpm for five minutes. The upper aqueous layer containing cisplatin was removed and analyzed by high-performance liquid chromatography (HPLC) method (HPLC Shimadzu, Japan) with an anion exchange column Inertsil AX® (250 mm x 4.6 mm, 5 µm) as the stationary phase at room temperature.[40,41] The mobile phase was composed of ethyl acetate: methanol: Milli-Q water: *N,N*-dimethylformamide (8:40:10:20, v/v) with a flow rate of 1 mL/min. The encapsulation efficiency (EE) and loading capacity (LC) were calculated using equations (1) and (2) respectively:[42,43]

$$Entrapment\ Efficiency\ (\%) = \frac{cisplatin\ content\ of\ hybrid\ nanoparticle}{total\ added\ amount\ of\ cisplatin} \times 100\% \qquad (1)$$

$$Loading\ Capacity\ (\%) = \frac{amount\ of\ drug\ encapsulated}{amount\ of\ drug\ encapsulated + total\ amount\ of\ liposomal\ components} \times 100 \qquad (2)$$

In vivo biodistribution study of hybrid nanoparticles loading cisplatin.

In order to evaluate the drug biodistribution, 6-week old, female CDF1 mice weighing 20-25 grams represented the subjects of this study. They were all purchased from Sankyo Labo (Tokyo, Japan) and treated in accordance with the conditions stipulated by the Guiding Principles for the Care and Use of Laboratory Animals of the Animal Research Committee of Hoshi University. Firstly, the mice were tumor induced by means of xenograft method of C-26 cells, which were transplanted by injecting cell suspension ($1x10^6$ cells) subcutaneously. After the tumor had reached a size of 100 mm^3, the mice were divided into five groups of 4-5 subjects.

The samples were administered intravenously twice via tail vein injection at a dose equivalent to 4 mg cisplatin per kg mice per injection. Forty eight hours after the first injection, a second was administered, twenty-four hours after which the subjects were sacrificed and their blood collected in heparinized tubes. In order to extract the plasma fraction, the blood samples were centrifuged for ten minutes at 9,100 G. Blood-free tumor and kidney tissue were taken and weighed. The tissues and plasma were stored at -20ºC until platinum analysis by graphite furnace atomic absorption spectrophotometry (GF-AAS, Z-8100 Polarized Zeeman, Hitachi, Japan) was performed as previously reported.[26] Platinum levels were determined by digesting samples with concentrated HNO_3 and heating them for one hour at 70ºC, followed by heating at 120ºC overnight to obtain dry samples. These were then added to 0.1 N HCl at an appropriate level of dilution. The GF-AAS analysis program involved three steps: (1) a 40-second drying stage at 80-100ºC, (2) a 30-second ashing stage at 800ºC (3) a 7-second atomization stage at 3000ºC, followed by cooling. Absorbances were measured at 265.9 nm with a slit bandwidth of 0.4 nm and the sample volume was 30 µL. The results were expressed as µg Pt/mL plasma and µg Pt/g tissue or organ.

Statistical analysis.

All data was in three replicates and presented with the mean ± SD. To evaluate the significance of the differences, the data was analyzed by means of a one way ANOVA test followed by a Tukey's posthoc test performed using SPSS Software v.17.0 with a p value <0.05.

RESULTS AND DISCUSSION

Physical characterizations of surfactant-modified cisplatin loading hybrid nanoparticles

In this study, the hybrid nanoparticles were prepared by injection method. The polymer was prepared by, firstly, dissolving it in the organic solvent and precipitating it in aqueous media to form nanoparticles. The surfactants used in this study were of two types; anionic surfactant, i.e. sodium cholate (SC) and sodium deoxycholate (SD), and non-ionic surfactant (Tween 80/TW). As seen from Figure 1A, all hybrid nanoparticles (HNP) had a particle size less than 100 nm. The addition of surfactants including sodium deoxycholate and sodium cholate to the hybrid nanoparticles, i.e. HNP-SD and HNP-SC respectively, produced slightly smaller particle sizes than HNP. Moreover, as shown in Figure 1B, the ζ-potential of all these hybrid nanoparticles was negative and not significantly different.

The drug loading was achieved simply by mixing the cisplatin solution with an aqueous dispersion of the hybrid nanoparticles matrix in alkaline conditions. The results showed that the addition of cisplatin to hybrid nanoparticles did not significantly affect the particle sizes (Figure 1A). On the other hand, this addition neutralized the ζ-potential of all preparations from a negative charge (around -9.4 to -13.8 mV) to a relatively neutral charge of between 1.4 and 4.4 mV, as shown in Figure 1B.

The amount of cisplatin encapsulated in hybrid nanoparticles were determined by using an HPLC method. The peak areas were then plotted against concentration (Table 2) resulted in a good linearity of calibration curve with coefficient of correlation (R^2) of 0.9997 over the cisplatin concentration range of 5-100 µg/mL, as seen in Figure 2. The cisplatin entrapment efficiency of Cisplatin-HNP, Cisplatin-HNP-SD, Cisplatin-HNP-SC, and Cisplatin-HNP-TW were between 5.4-17.8%, with loading capacity were 1.4-7.1%, as shown in Figure 3A-B.

175 Sodium cholate-modified hybrid nanoparticles (Cisplatin-HNP-SC) had the highest drug loading
176 capacity of all the hybrid nanoparticles.

177 These results correlate closely with those of a previous study which reported that SC
178 coexistence with phospholipid can undergo changes in shape and size due to the formation of
179 mixed micelle and mixed bilayer micelle.[27,39] However, in this study, hybrid nanoparticles also
180 contained diblock polymer of PEO-b-PMAA whose presence may also contribute to the final
181 hybrid nanoparticle size. Nevertheless, further investigation is required in order to analyze this
182 phenomenon. After the addition of cisplatin, the particle size of hybrid nanoparticles that
183 contained lipid and surfactant were not significantly different. Although the loading of cisplatin
184 has been reported as affecting the cross-linking state of the polymer on micelle formation,[21]
185 through inter- and intramolecular bonds between carboxylic acid groups of PMAA with active
186 aquatic species of cisplatin,[33] it was probably insufficient to produce denser particles. Once
187 maximum packed particles had been obtained in the pre-conjugation state, resulted from the
188 spontaneous precipitation of polymer-lipid mixtures, the compaction effects of cisplatin binding
189 to polymer would not produce any further size reduction. On the other hand, the cisplatin loading
190 process generated neutralization of ζ-potential of hybrid nanoparticles complexes as presented in
191 Figure 1B.

192

193 ***In vivo* biodistribution study of cisplatin-loading hybrid nanoparticles**

194 An *in vivo* biodistribution study was performed by administering the drugs twice due to a short
195 first-half lifetime.[11] The data represents the drug concentrations at 24 hours after the second
196 injection. As shown in Fig. 3A-B, generally the hybrid nanoparticles, i.e. Cisplatin HNP,
197 Cisplatin-HNP-SD, Cisplatin-HNP-SC, and Cisplatin-HNP-TW, produced higher drug

concentration in plasma and tumors than the cisplatin solution group. Furthermore, it can be clearly seen from these figures that the addition of sodium cholate to hybrid nanoparticles (Cisplatin-HNP-SC) produced the highest drug concentration in plasma, up to ten times higher than that of the cisplatin solution, and high accumulation in tumor tissue among others. This explains how incorporating cisplatin into hybrid nanoparticles (Cisplatin-HNP-SC) successfully prolonged the drug circulation in plasma, thus increasing tumor drug accumulation via the enhanced permeation and retention (EPR) effect.[46] These results may also correlate with the lipid barrier of hybrid nanoparticles for water diffusion and the cisplatin-polymer (PMAA) conjugation states, However, the effect of sodium cholate in prolonging circulation of cisplatin hybrid nanoparticles, though it affects membrane deformability, is still being investigated. It is known that these three surfactants have different chemical structures that may affect the lipid layered on the hybrid nanoparticle surfaces. Tween 80 contains non-bulky hydrocarbon chains. On the other hand, sodium deocycholate and sodium cholate have steroid-like structures with differences in the total number of hydroxyl functional groups, which are three and two for sodium cholate and sodium deoxycholate respectively. These structures are bulkier than Tween 80, thus reducing transient hydrophilic hole formation causing rigidity of the lipid layer.[47] This may limit water permeability across the lipid layer on the hybrid nanoparticles causing cisplatin to leak out. However, this report states that there were no significant differences in lipid rigidity between sodium cholate and sodium deoxycholate, while in this study sodium cholate produced more stable nanoparticles than sodium dexocyholate – a phenomenon requiring further evaluation.

On the other hand, as shown in Figure 3C, elevated drug levels in the kidney were observed in this group generating two-fold higher platinum levels than in the cisplatin solution

221 treatment group. The long circulated Cisplatin-HNP-SC probably releases cisplatin which
222 experiences a biphasic elimination phase with the late phase within 2-3 days,[11] in slow mode,
223 thus leading to the accumulation of high cisplatin levels in the kidney as the excreting organ.
224
225

CONCLUSIONS

The hybrid nanoparticles loading cisplatin were prepared with the addition of surfactants. Sodium Cholate modification had successfully reduced the particle size of the cisplatin hybrid nanoparticles and improved plasma drug circulation as well as the tumor drug accumulation of cisplatin. However, further investigation is required to evaluate the effects of sodium cholate on the biological barrier with the presence of diblock polymer as the core of cisplatin hybrid nanoparticles.

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FINANCIAL DISCLOSURES

None

ETHICAL CONDUCT OF RESEARCH STATEMENT

The animal experimental investigation employed in this study complied with the Guiding Principles for the Care and Use of Laboratory Animals as established by the Animal Research Committee of Hoshi University

CONFLICT OF INTEREST

The author declare no conflicts of interest or financial interests in any product or service mentioned in this article, including grants, employment, gifts, stock holdings, honoraria, consultancies, expert testimony, patents, and royalties.

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391 **Table Legend**

392 **Table 1.** Molar compositions of hybrid nanoparticles prepared with different surfactants.

Formula	Molar Ratio		
	EPC	Surfactant (SD, SC, TW)	PEO-b-PMAA
HNP	50	-	2.8
HNP-SD	50	5.0	2.8
HNP-SC	50	5.0	2.8
HNP-TW	50	5.0	2.8

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Table 2. The peak area of cisplatin standard solution.

Concentration of Cisplatin (ppm)	Peak area
5	4711
10	11933
20	24303
50	57635
100	115688