Peer Review File

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Reviewer A

The authors provide here a good review of a very important topic. This manuscript primarily reviewed instances where modifications of specific monosaccharides impacted mAbs' characteristics, both in terms of their intrinsic properties and interactions with several cancer targets. Enough studies were cited for each section, but some explanations of the studies were missing support for their claims, and the subtitles were not specific enough to describe the limited scope of the reviews. While we feel positive about the topic and material covered, we feel a comprehensive editing is required to make this manuscript more coherent, comprehensible, and professional. The figures do not have enough descriptions and only seemed loosely relevant to the claims. We encourage the authors to take the following revisions to heart and really bolster the writing and presentation of such important information.

Minor issues

1. Line 88-99 feels more fitting as a part of the introduction because nothing specific was said about glycan profiles or glycoengineering strategies.

Reply 1: Thank you for your comments. We agree with your points. So, as your advise,we move Line 88-99 part into introduction.

Changes in the text:move Line 88-99 as a part of the introduction. (see Page 5, line 85-90)

2. 109-110, if FUT8 KO resulted in higher serum clearance, shouldn't fucose be the variable for differential clearance rate instead of mannose?

Reply 2: That is a really good question, but sorry for the confusing expression. Here we just express high mannosylation influence the the serum clearance. The FUT8KO is just a vector. Actually we did not compare it with cells with FUT8, so this has nothing to do with Fucose. Fucose affects ADCC. In detail, two of the most discussed modifications related to the functionality of antibodies are mannose and fucose. Mannose residues on the N-linked glycan structures of antibodies can increase serum clearance. Mannose residues can be recognized by mannose receptors on the surface of various cells, including liver cells and dendritic cells, leading to rapid endocytosis and degradation of the antibody. Thus, high mannose content can decrease the serum half-life of the antibody. The presence or absence of fucose residues mainly affects the effector functions of antibodies, especially the antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies with low fucose levels bind with higher affinity to FcγRIIIa on immune effector cells, which results in enhanced ADCC. However, fucosylation does not have a significant impact on serum clearance or half-life as mannose does. Instead, it's more about modulating the antibody's interaction with

immune cells. So, when it comes to serum clearance, mannose residues contribute more to this property than fucose residues. Fucose mainly influences antibody interactions with immune effector cells and ADCC activity. To clarify my point here, I will replace the sentence 109-110 as above background of mannose in briefly.
Changes in the text: we have modified our text as advised (see Page 7, line 137-140)

3. 121-123 It would be helpful if the author could support this observation with actual data.
Reply 3: Sorry that is a real case in my GMP inspection experience. However, it is not suitable to publish the actual data here due to the corporate trade secrets and legal provisions. So we will delete this inappropriate sentence here.
Changes in the text: we have deleted this sentence.(see Page 8, line 152)

4. 129-130. Please at least clarify what kind of these "subsequent reactions" are.
Reply 4:sorry for the confusing expression. It has been clarified as " the subsequent reaction of varied modifications seen in complex and hybrid types"
Changes in the text:we have delete this sentence.(see Page 8, line 156-157)

5. 137, citation needed.
Reply 5: sorry we missed this citation in editing. We added the citation in the manuscript.
Changes in the text: we have modified our text as advised (see Page 8, line 165)

6. 138-139, which are these integrated/targeted genes or loci? How are they relevant to the GnT-I locus?
Reply 6: Sorry for my misleading expression. In 138-139 we wanted to express that the GnT-I can be interrupted with the CRISPR/Cas9 tool as well. So we modified the expression to clarify that.
Changes in the text: we have modified our text as advised (see Page 8-9, line 167-168)

7. 142-143 unclear what the statement meant. While fucosylation is prevalent in many mammalian cells, they are by no means necessary for the syntheses of complex type N-glycans based on our previous experiments. Could you please cite this claim?
Reply 7: Thank you for sharing results of your previous experiments. You are totally right that fucosylation is not necessary for the syntheses of complex type N-glycans. We wanted to express that fucose is a prevalent modification in mammalian cells. So, to clarify, we modified the sentence as "Fucose is a prevalent modification of the complex type N-glycans in mammalian cells." Also, we change the order of the first and second sentence in the paragraph. Hope that can help readers to get a better understand.
Changes in the text: we have modified our text as advised (see Page 9, line 170-171)

8. Line 146 and 149 seemed to claim the same thing.

Reply 8： Sorry for that, but Line 146 described the effect of high-level mannose type,

and Line 149 described the effect of ammonia concentration.

9. 190 “for the whole synthesis” of ?
Reply 9: modified as “the two biosynthetic pathways of GDP-fucose”
Changes in the text: we have modified our text as advised (see Page 11, line 215)

10. 192-193, please clarify
Reply 10: this sentence is following the previous sentence to summary Louie et al experiment. To clarification, we modified it as “In that way, Louie et al. achieved the desired ratio of fucosylation without influencing the quality features of final products.”
Changes in the text: we have modified our text as advised (see Page 11, line 226-227)

11. 244 – 245 unclear about the meaning of this sentence
Reply 11: Sorry for my misleading expression. We clarified it as “To overcome the difference in sialylation between the mAbs from human and CHO cells, “humanizing” through genetic and metabolic “glycoengineering” approaches is engaged to improve therapeutics.”
Changes in the text: we have modified our text as advised (see Page14, line 279-282)

12. 273-277. There were no supporting literatures for these claims, and it was not clear which “cell-based” methods the author was referring to.
Reply 12: Sorry for my misleading expression. “cell-based” methods should be “cell-factory” methods. Citations have been added as well.
Changes in the text: we have modified our text as advised (see Page 15, line 318)

13. 280 What kind of research?
Reply 13: Sorry for my misleading expression. We clarified it as “Initially, research on the application of mAbs in cancer therapy focused on Rituximab (Rituxan®), an approved monoclonal antibody for treatment of non-Hodgkin lymphoma and other B-cell related diseases.
Changes in the text: we have modified our text as advised (see Page16, line 322)

14. 293-295 Were you talking about modifying the glycosylation of mAbs to improve its specificity to target sites instead but not “designing targeting sites
Reply 14: Yes. You are totally right. We have modified our text as advised.
Changes in the text: we have modified our text as advised (see Page 16, line 343)

15. Line 298-300 The sentence was worded as if the antibody targeted Asn38 glycosylation somehow. Was that the case?
Reply 15: Sorry for my misleading expression. We clarified it as“ Researchers found that one engineered monoclonal antibody targeting the PD-1 Asn58 glycosylation can blocks the binding between PD-1 and PD-L1/L2 thereby inhibiting tumor growth in vivo trial effectively”
Changes in the text: we have modified our text as advised (see Page 17, line 350-352)

16. Where was Table 1 used in the main text of the manuscript? How was Table 1 relevant to the review?
Reply 16: Sorry for that. That is my mistake in revising and editing the manuscript. we will delete this inappropriate sentence here.
Changes in the text: we have delete this table.

Major issues

17. Please go over the writing carefully。 There are way too many editorial issues that can be resolved with simple proofreading. Some ways of expression are too confusing to be understood clearly. For examples:
a. Line 36-37 “empowers the modern pharmaceutical industry with … and significant challenges.”; Line 28 “US, EU or China”; Line 39 “ in aspects of …); Line 37-38, “a total of … has (should be “have”)”
Changes in the text: we have modified our text as advised.
b. Line 42-43 I think the author meant the drugs are recognized for their “high efficacy, precise targeting, and low side-effect” but not the pipelines?
Reply b: we still believe pipelines is better than drug for here. Because the pipeline means all the drug in market and the candidates under research in biopham corporations.
c. 62-63. Does the author mean to say the glycan structures on glycolipids are as diverse as the types of lipids?
Reply c: The structure of glycolipids is as diverse as the types of lipids moiety.
Changes in the text: we have modified our text as advised (see Page 4, line 66)
d. 63-65 It was unclear what are “them” and there was no supporting evidence or literature cited for the claims.
Reply d: we clarified it as “ Among those types of glycosylation,”
Changes in the text: we have modified our text as advised (see Page 4, line 66)
e. 68 “to improve” not “improving” . Do you mean that “Alternative glycoengineering methods have been developed to improve the attributes of biologics”?
Reply e: Yes, you are right. We have modified our text as advised.
Changes in the text: we have modified our text as advised (see Page 5, line 95)
f. Sentence from 69-70: unclear meaning
Reply f: we clarified it as “With the increasing interest in the biotechnological producing monoclonal antibodies, the application of glycoengineering strategies extend from modify small molecules (like antibotics) to engineer mAbs ”
Changes in the text: we have modified our text as advised (see Page 5, line 97-99)

g. 79 “of glycans”
Reply g: we have modified our text as advise (see Page 5, line 81)
h. 98 the sugars should not be capitalized.
Reply h: All “sugar and glycans in the article has no capital at present.
i. Line 101: I think it would be the modification of glycan but not Golgi
Reply i: we clarified it as “ Mannose can affect mAb folding and stability by avoiding

further modification of the Golgi"
Changes in the text: we have modified our text as advised (see Page 7, line 126)

j. Line 102-104: Different high-mannose N-glycans will have different characteristics but not the numbers.
Reply j: We have changed to "The N-glycans with different numbers of mannose"
k. 106-107 "in in"; 108-109 an awkward sentence
Reply k: The "in in" is not we wrote wrongly , "in vivo means in the body", "play role in" is a set phrase, we have change it to "play a significant role in the *in vivo* therapeutic activity".
we have modified another sentence to "It is reported that the serum clearance of mAbs with high mannose N-glycans is higher than normal ones".
Changes in the text: we have modified our text as advised (see Page 7, line 135-137)

l. Please italicize all Latin phrases or gene names.
Reply l: we have modified our text as advise
There are simply WAY TOO MANY grammatical issues throughout the manuscript, ranging from simple errors to run-on sentences, and I stopped annotating after line 110. Please go over the entire manuscript and fix them as needed. Please also use past tense for past researches unless you are stating well established principles.

18. Some big claims had few or no supportive materials.
a. 73-75 Please cite some past research for this claim
Reply a: sorry we missed this citation in editing. We added the citation in the manuscript.
Changes in the text: we have modified our text as advised (see Page 6, line 102-103)
b. 75-76 Not sure which are those " successful strategies" or "other sources". Also not sure why similar results are expected.
Reply b: Sorry for my misleading expression. We clarified it as" When those successful strategies of glycoengineering in other expression systems apply to mammalian cells, similar results are expected to observe for the alternation of glycosylation."
Changes in the text: we have modified our text as advised (see Page 6, line 103-105)
c. 77 What do you mean by "obtain the issues of aggregation,… or side effects". Could you please specify what natures of monoclonal antibodies or glycosylation may contribute to these issues?
Reply c: Sorry for my misleading expression. We clarified it as"Because of the large molecular weight and complex configuration of monoclonal antibodies, these macromolecules usually inevitable have the issues of aggregation, instability solubility, deficient biological activity, or side effects."
Changes in the text: we have modified our text as advised (see Page 6, line 105-107)
d. 94-96, it seems that the author wanted to say that the N-glycan at Asn297 in the Fc region of an mAb has diverse compositions of monosaccharides and structural complexities, which impact the antibody's therapeutic effect, solubility,… but the wording was too confusing to.

Reply d: Thank you for your question, we have modifiied the article as: "The diversity and complexity of Asn297 in the mAbs Fc region affects the solubility, stability, folding accuracy and biological activity of the mAbs, especially the number of glycosyl, the number of possible bonds, and the structure of the glycan."
Changes in the text: we have modified our text as advised (see Page5, line 90-92)
e. 110-114 citations needed. Is Figure 2 a summary of results from another study? Any figure description that can explain the importance of the structural difference?
Reply e: Good question. The Fig 2 is original. The aim of it is to show readers structures of those three glycans. Followed your advise, we added one more paragraph in introduction to describe and explain the definition of complex type, mannose type, and hybrid type. The Fig 2 was moved to that paragraph as well. By the way, the citation was added for line110-114.
Changes in the text: we have modified our text as advised (see Page4, line 71-83)
f. 203-209, citations please
Reply f: sorry we missed this citation in editing. We added the citation in the manuscript.
Changes in the text: we have modified our text as advised (see Page 12, line237-243)

19. Please double check the relevance of some citations
a. 117-118/124-125 Some materials are not relevant to mannosylation as the section title suggests.
Reply a: Thank you for your comments here. Actually we also hesitate where to put these material, when we writing. For 117-118, we agree with you to move that sentence to sialylation section. But for 124-125, since the decrease in galactosylation and sialylation are due to the rise of mannosylation, we still think it is suitable for this section.
Changes in the text: we have modified our text as advised (see Page14, line289-291)
b. 120-122 There is no comparison here between varied Mn2+ or mannosylation levels. The sentence is not supportive of the preceding claim.
Reply b: Thank you for your good suggestion. We added comparison here to enhance claim.
Changes in the text: we have modified our text as advised (see Page8, line 148-150)
c. 138-139, which are these integrated/targeted genes or loci? How are they relevant to the GnT-I locus?
Reply c: Sorry for my misleading expression. In 138-139 we wanted to express that the GnT-I can be interrupted with the CRISPR/Cas9 tool as well. So we modified the expression to clarify that.
Changes in the text: we have modified our text as advised(see Page 8-9, line 167-168)
d. 169-170. What was the purpose of mentioning the fucose analog here? How did it impact ADCC activity or other fucosylation-related drug properties?
Reply d: That is a really good question. As I mentioned in Q2, the presence or absence of fucose residues mainly affects the effector functions of antibodies, especially the antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies with low fucose levels bind with higher affinity to FcγRIIIa on immune effector cells, which results in enhanced ADCC. Fucose mainly influences antibody interactions with immune effector

cells and ADCC activity. And, here we are talking about antibody-drug conjugate (ADC), not ADCC, we have modified the sentence to be clarified.
Changes in the text: we have modified our text as advised (see Page 10, line 200-202)
e. 171-181 While they were strong pieces of evidence in supporting their respective roles in modulating the IgG's ADCC activity, this part did not seem to fit in the fucosylation section.
Reply e: we added some background between fucosylation and ADCC, these can be easier to understand.

20. The introduction feels a bit too scattered with periphrases and hard to follow. It would be helpful for the readers to grasp the framework of this review if the author can concentrate on explaining one specific claim at a time.
Reply 20: Thank you for this suggestion. That is a really good point. However, there are so many background and definitions need to be introduced before we extend the following topics. To help readers to grasp the framework, we added subtitle in the introduction section. At the same time, a paragraph about the framework of this article was added at the end of the introduction section to help readers to get our train of thought
Changes in the text: we have modified our text as advised (see Page 6, line 115-121)

21. Throughout the manuscript, it was at times hard to distinguish which were the author's conclusions drawn from past research and which were results directly cited from previous literatures. The ambiguity can be risky for publication.
Reply 21: we really thank you for your useful comments here. Yes, we realized this problem here you mentioned and some comments about misleading expression since the same reason now. We have proof read the manuscript throughout again to avoid those misleading and unclear expression. Thank you again.

Reviewer B

Wang et al. present a short review of metabolic glycoengineering strategies for monoclonal antibodies. I found the following significant issues that should be addressed for a new revision of the review:

1. The review title is "metabolic glycoengineering of monoclonal antibodies and its application in cancer therapy." However, an extensive part of the review is focused on the genetic strategies used for glycoengineering antibodies. Review manuscripts based on metabolic approaches are not so abundant in the literature. Thus, it would be more interesting if the authors could give more detailed examples of these metabolic strategies. I also believe that the section "Applications of glycoengineering of monoclonal antibodies in cancer therapy" can be further extended and improved with other examples.
Reply 1: Thank you for your great suggestion. We titled this review as metabolic

glycoengineering because there is other chemoenzymatic glycoengineering method as you mentioned in Q2. We just want to distinguish biological method with chemical method in the title. So, probably “Combinatory glycoengineering” is better than “metabolic glycoengineering” here as the title. We revised the title in the manuscript. Also, as your advice, we extended the sections of “Combinatory glycoengineering” and “Applications of glycoengineering of monoclonal antibodies in cancer therapy” with more examples.

2. Although I understand the review's primary focus are metabolic strategies for glycoengineering antibodies, the authors should briefly mention in the introduction others as chemoenzymatic synthesis as they do with the genetic strategy.
Reply 2: Thank you for your suggestion. You are right that there are many cases of chemoenzymatic glycoengineering of therapeutic antibodies indeed, besides the metabolic strategies for glycoengineering antibodies. As your advice we mentioned chemoenzymatic glycoengineering a little bit in the introduction section. But, just a little bit, because we believe our primary focus are metabolic glycoengineering.
Changes in the text: we have modified our text as advised (see Page 5, line 94)

3. On page 22, there is Table 1 for “comparison of different techniques involved in glycans analysis.” However, I cannot find where the authors refer to this table or write about these techniques in the text.
Reply 3: Sorry for that. That is my mistake in revising and editing the manuscript. we will delete this inappropriate sentence here.
Changes in the text: we have delete this table.

4. The figures are poor and not very informative. A new figure with a representative example of how metabolic strategies used for cancer therapy will be very informative for the reader.
Reply 4: There is a really good suggestion. We added one more figure to be a graph abstract to show how theoretical predictions guide glycoengineering and result in the engineered mAbs used for cancer therapy.
Changes in the text: we added one more figure.(see Figure 6)

Other issues to be address are:

5. Introduce a comma in line 38 between US and EU.
6. The N and O when referring to N-linked, O-glycan and N- acetylglucosamine should be in italic. Please correct this in line 58, line 90, 91, and the rest of the text.
7. Line 69: Substitute monoclonal antibodies by mAbs.
Reply 5,6,7: Thank you for your suggestion, we have modified this kind of problems.
8. Line 71: look group publications to understand the impact in the field.
Reply 8: Sorry for that. That is my mistake in editing the citations. We have revised and double checked the citations again. Thank you.
Changes in the text: we have modified our text as advised (see Page 5, line 100)

9.Line 89: the D of glucuronic acid should be a letter size less than the rest of the text by sugar chemistry nomenclature. Please review this issue in the rest of the manuscript.
Reply 9: Thank you for this suggestion. We have reviewed this issue in the manuscript.
10. In the section glycan profiles and glycoengineering, the authors only mention the sugars that form part of the N-glycans, but I think it would be more informative if in this part the authors briefly explain the general structures of these glycans (e.g definition of complex type, mannose type, and hybrid type) and it would help the reader for the next section of the paper.
Reply 10: Thank you for this suggestion. That is a really good idea. We have added a paragraph to briefly explain the general structures of these glycans. Also, we moved the Fig2 to this section. We hope it would help readers to get a better background and understanding of the next sections.
Changes in the text: we have modified our text as advised (see Page 4, line 69-83)

11. I think a new panel or a figure representing the different high mannose glycoforms (Man5-9GlcNAc2) is needed.
Reply 11: That is a good suggestion since the multiple possibilities of the different high mannose glycoforms. So, we added a new Figure to show that. Also, we added some explanation in the content to help understand.
Changes in the text: we have modified text and added Figure 3 as advised (see Page 7, line 129-134)

12. Line 106: the authors state that high mannose glycoforms are "(usually less than 5% of the total profiles in most mAbs)". The authors should clarify whether this percentage is in human serum or manufactured drugs.
Reply 12: Sorry unclear expression. We have clarify that in text.
Changes in the text: we have modified our text as advised (see Page 7, line135)

13. Line 113: The authors should cite "Kanda's results". They should clarify what Kanda's result means and include references.
Reply 13: sorry we missed this citation in editing. We added the citation in the manuscript.
Changes in the text: we have modified our text as advised (see Page 7, line143-144)

14. In figure 2, the authors represent an afucosylated hybrid type glycan, and in the text the authors mention (line 114) that the hybrid type shows less ADCC than the high mannose. Please confirm if this is correct and include the right reference.
Reply 14: Good question. The aim of Fig2 is to show readers structures of those three glycans. It has no relation with the context herer actually. So, followed your advise, we added one more paragraph in introduction to describe and explain the definition of complex type, mannose type, and hybrid type. The Fig 2 was moved to that paragraph as well.
Changes in the text: we have modified our text as advised (see Page 4, line 71-83)

15. When referring to a cite use the first author last name, followed by et al. For example, in line 117 Costa et al, in line 138 Lee et al, in line 156 Yamane-Ohnuki, in line 159 Mori et al. Please review the rest of the manuscript for this type.
Reply 15: we have modified our text as advised.
16. Line 121: I don't understand "Reviewer has encounter …"
Reply 16: Sorry that is a real case in my GMP inspection experience. However, it is not suitable to publish in a review article. So we will delete this inappropriate sentence here.
Changes in the text: we have deleted this sentence. (see Page 8, line 152)

17. Line 142: rephrase the first sentence because it is difficult to understand what the authors mean.
Reply 17: Got your point. we rephrase it as "When a fucose is added to the chain, the oligosaccharide chain will not be further elongated." Also, we change the order of the first and second sentence in the paragraph. Hope that can help readers to get a better understand.
Changes in the text: we have delete this sentence.(see Page 9, line 170-171)

18. Line 202: correct complement-dependent cytotoxicity by CDC
Reply 18: Thank you for this suggestion. We have corrected it.

19. Line 202-209: please include references for "the ADCC activity is barely influenced by galactosylation"
Reply 19: sorry we missed this citation in editing. We added the citation in the manuscript.
Changes in the text: we have modified our text as advised (see Page 12, line 243)

20. Line 295: authors introduce the PD1 and PD-L1 glycosylation as an antibody target. However, I think this needs to be better explained. The authors write "as mentioned above" but never mention this target in the manuscript.
Reply 20: Agree with your suggestion. We have added more explaination on PD1 and PD-L1 glycosylation to help read.
Changes in the text: we have modified our text as advised (see Page 17, line 344-346)

21. Line 298: block instead of blocks
22. Line 309: substitute decades, by decades.
Reply 21 and 22: Thank you for discovering our mistakes, we have modified our text.