**Peer Review File**

**Manuscript Title:** Glucose-sensitive insulin with attenuation of hypoglycaemia

## Reviewer Comments & Author Rebuttals

**Reviewer Reports on the Initial Version:**

Referee #1 (Remarks to the Author):

A. Summary of the key results

A glucose-switchable insulin variant is described that gains useful dynamic range and appropriate KD values for application in vivo through the attachment of a synthetic lectin-like module at the truncated C-terminus and an appended competing internal D-glucoside at the N-terminus of the B chain. The convincing in vitro measurements translate to highly promising responsiveness (and 'glycaemic protection') in in vivo models. These studies take advantage of an elegant partitioning of mechanism through the use of L-glucose as a ligand that is proposed to be essentially identical in its engagement with the insulin conjugate yet lacks metabolic participation.

All-in-all I was excited by it and recommend publication. I make some suggestions for revision below.

B. Originality and significance: if not novel, please include reference

The switching of twelvefold in insulin receptor affinity occurs across a concentration range that is relevant and this tuning into a useful physiological range is exciting and represents a key starting point towards a long-standing goal (as the authors note).

The two parts of the model and design given here that I particularly like are:
1) the switch mechanism: it exploits internal competition to moderate affinity and control selectivity in a manner that is noncovalent and so a) likely subject to fewer competitive ligand effects and b) may possess potentially faster kinetics. In this way, as the authors note, it partially resembles prior attempts to use lectins yet by here using a smaller (previously known) synthetic lectin-like moiety. This is an elegant design and one that I think is not only effective but also captures the imagination. I think it's fair to say that although others have explored switch designs (as they cite), thus far the dynamic ranges observed have not yet been applicable.
2) the size of the construct: whilst clearly not a small molecule, this conjugate is still placed within the realms of realistic synthesis. Insulin can now be regarded as an accessible peptide and the pragmatic conjugation chemistry that is used here could be scalable (although of course the designs here are ones for a prototype).

I also admire the key design element of switching, which as the authors note others have explored too – not using an exhaustible depot but instead to create a reversible affinity within principle (noting insulin clearance) could allow pragmatic use.

C. Data & methodology: validity of approach, quality of data, quality of presentation

I found the in vitro competition assays described in figure 3 to be particularly convincing.

Insulin is now a routinely tractable molecule and these conjugates can be characterised at a greater level. The assembly of these is essentially synthetic chemistry and yet the details of corresponding characterization are somewhat light. Synthetic method is given for most of the small molecule chemistry but further details could be added (including further corresponding characterisation data).

The characterisation of the conjugates themselves is also somewhat light. The raw data should be given for e.g. the analysis of the intact conjugate masses as well as the tryptic digests. These digests are sensible analyses employing well-trodden techniques in insulin science but simply quoting the major peaks in a mass spectrum there is a slightly old-fashioned way of presenting – all raw data should be given. The characterisation of this through typical MSMS methods would be appropriate potentially (at the very least MS1 spectra should be given). On this point, I may have missed it, but I couldn't get a sense of the yield for the conjugation chemistry. This is pertinent in the context of understanding purity and reproducibility for an interesting conjugate.

The native MS data is given simply as a non-logarithmic plot in figure 1C. Alternative logarithmic plotting and presentation of corresponding (raw) MS data to allow the reader to understand the basis of this measurement (and potentially its limitations) would be appropriate.

The raw data (gels etc) for all assays should be included (e.g. phosphorylation assays).

In places, 'data not shown' is used as a statement. In the current era, where there is no limit on supplementary data, this seems inappropriate and this should be supplied.

Minor point: Yields could be usefully added to ED figure 4.

D. Appropriate use of statistics and treatment of uncertainties

Valid.

E. Conclusions: robustness, validity, reliability

More characterization details of the conjugates would be useful (see above).

Whilst an advantage, the origin of the five-fold shift in KD observed in the synthetic lectin when placed in the conjugate that lacks glucoside (NNC2215a) is not entirely clear to me – why might this be? Is it that internal competition from insulin (side-chains as ligands) is mitigating potency? Here of course this is somewhat welcome in modulating the dynamic range but the origins are intriguing and the comment that "suitable switch dynamics have been achieved" is a slightly throwaway line.

The statement that native mass spectrometry gives rise to realistic KDs, is one that I agree with in principle but is of course case-specific. Other biophysical experiments would be welcome. Isothermal calorimetry (applied only to the synthetic lectin moiety?) or other modes of biophysical measurement could be applied directly to these conjugates – it would be interesting to know if this was attempted? The constants gathered by nMS appear consistent (and in essence tally with what is seen in the physiological measurements) but further characterisation and detail of these binding constants would be valuable.

F. Suggested improvements: experiments, data for possible revision

The use of L-glucose as a comparable ligand for the 'achiral' synthetic lectin is a nice approach and allowed a powerful dissection of the physiological mechanism from the other downstream/physiological effects of D-glucose. That said an implication is made that the achirality of the macrocycle equates to a lack of 'matching/mismatching' effects in the final conjugate. Whilst it is possible (and even likely) that there is a partitioning of the binding mode to only this moiety away from that of the peptide itself, one can also imagine mechanistic scenarios with this is not the case, especially given the internal role of the (chiral) D-glucoside as the driver of (competitive or not?) inhibition that modulates the observed KD. Experiments should be considered where matched/mismatched pairs are explored in not only the nature of the ligand but also the nature of the conjugate (NNC2215 analogues bearing L glucoside perhaps?). This is also a space where further biophysical measurements could reveal the validity of this assumption. This seems important given the role that the 'L-glucose trick' plays in showing effectiveness of the switch.

I found the structural model somewhat simplistic and whilst dealt with under traditional methods (using relatively short molecular dynamics for optimisation), the full details of how this was performed were somewhat lacking. It wasn't fully clear to me how various poses were established prior to the MD. Was the sugar placed within the synthetic lectin prior to docking? It would be useful to provide further details of the MD (the paper is light in this respect also). Again, I may have missed it but was MD run on the open conformation in glucose-bound yet modified form?

It would of course be absolutely wonderful to have some form of empirical structural data and this system seems ripe for various different techniques. This is in some respects non-trivial (receptor etc) – cryoEM or NMR would give an insight. The use of NMR in particular could also give good insight into the relative dynamics/equilibria of the switchable insulin itself (13C-labelled glucoside in conjugate?). Given the proposal of a switch, which I am reasonably convinced by, it would be good to see some further chemical evidence for switching (buried site 1 glucoside, conformational change) taking place. The structural study of insulin alone provides a useful background for such experiments, which seem worthwhile.

Further biophysical dissection for mechanism (see also above).

More characterization details of the conjugates would be useful (see above).

Minor points:
There are some errors in the naming of the corresponding glucosides (e.g. there are two glycol units).
A small point – why was that particular sulfonate active ester chosen for N-terminal modification?

G. References: appropriate credit to previous work?

Yes – succinct but well-judged in a long-standing field.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions

The paper is clearly written but in some places is somewhat light on technical detail (see above). As a minor stylistic point, perhaps aspects of the narrative overexplain well understood ideas (such as the 'moving' of curves in a dose response).

Referee #2 (Remarks to the Author):

This manuscript provides synthesis of NNC2215 and its in vitro and in vivo characterizations to demonstrate its glucose sensitivity. This research signifies the first genuine demonstration of a self-regulated glucose-sensitive insulin using a molecular switch. Earlier endeavors fell short due to an absence of a potent glucose binder. However, the macrocycle employed in this research successfully overcomes the aforementioned challenge. The Novo Nordisk team performed a suite of robust, convincing experiments, demonstrating the glucose sensitivity of NNC2215. This work culminates numerous prior research breakthroughs and marks a crucial milestone in the field.

I found great pleasure in reading this manuscript, which propels a paradigm shift in the field of insulin engineering. The research provides abundant and definitive evidence to establish the glucose sensitivity of NNC2215, a discovery that immediately piques the interest of all researchers in the diabetes research field.

I wish to bring forth the following queries for the authors' consideration:

1. Reference 34 cites additional examples of insulin derivatives reported in a patent, including one with a glucoside on A1, a design similar to that reported in reference 14. This derivative appears as a suitable candidate for comparison. Was there a specific reason for its exclusion?
2. What factors contribute to the 5-fold glucose affinity disparity between NNC2215a and the free macrocycle?

3. In the in vitro experiments, the authors mention the addition of 1.5% albumin. Can data be provided for the same experiments without the inclusion of albumin? Alternatively, can the authors share data concerning any significant affinity/interaction between NNC2215 and albumin? Moreover, could the authors elaborate on the potential of NNC2215 binding to glycated proteins such as hemoglobin or albumin?

4. Repeating Figure 3d with NNC2215a would be a great comparison.

5. In the in vivo section, I am intrigued by the choice of comparator insulin (human insulin or insulin degludec). Line 181 reads: “Insulin degludec was used as a comparator in the pig study due to similar pharmacokinetic properties following intravenous administration”. Given that this was an IV study, the depot effect (i.e., multi-hexameric structures from insulin degludec) should be minimal. I presume that the pharmacokinetics of insulin degludec is largely dominated by its albumin affinity. Does this suggest that NNC2215 shares similar albumin-binding properties? Furthermore, why was human insulin used in the rat experiments, despite IV dosing?

6. While I concur that the three in vivo studies offer persuasive evidence of NNC2215's glucose sensitivity, I do not see any animals receiving subcutaneous doses. This is the route predominantly adopted by individuals requiring external insulin. It would be enlightening to conduct a comparative study that involves the subcutaneous injection of either NNC2215 or insulin degludec.
Appropriate citations on prior examples were provided. This manuscript was written with great clarity.

Referee #3 (Remarks to the Author):

The study by Hoeg-Jensen et al. describes the engineering of a glucose-sensitive form of insulin. They detail the chemistry used to generate the compound NNC2215, and show that its binding to insulin receptors is influenced by glucose in a concentration-dependent manner. Importantly, NNC2215 shows promising glucose responsiveness in both rat and pig in vivo models.

Insulin was first used in a patient with diabetes in 1922, and successful modification of the molecule was achieved only 14 years later with the development of long-acting insulin. Attempts to produce glucose-stimulated insulin was initiated over 40 years ago. Reducing the risk of hypoglycemia in people requiring insulin therapy is important so the work of Hoeg-Jensen et al, if successful, has potential clinical applications.

A major concern is that much of the work was performed at glucose concentrations that are not physiologically relevant. The reference intervals in healthy adults for random plasma glucose concentrations are 3.9-7.8 mmol/L, while fasting concentrations are 4.1-5.5 (up to 6.1 in some countries) mmol/L. A random plasma glucose concentration >11.1 is considered to be diabetes. The goal of insulin therapy in persons with diabetes is to maintain a range of 3.9 to 10 mmol/L. Analyses at glucose concentrations of 0 have no biological relevance. The authors' claim (p 12) of "..hypoglycemic range, ie 0 to 5.." is erroneous as glucose concentrations ≥4 mmol/L are normoglycemia. Similarly, the claim (p 12) "....over the physiological glucose concentration range of 0 to 20 mM.." needs to be corrected. A plasma glucose of 20 mM is clearly pathological. The text needs to be modified extensively throughout the manuscript to reflect real physiological and hypoglycemic glucose concentrations.

Specific comments:

1. The abstract is misleading as the IR affinity increase of 12-fold is not biologically relevant.
2. Fig. 1c. The rationale for evaluating non-physiological glucose concentrations, particularly 0-2 mM, is unclear and distorts the analysis. This applies throughout the manuscript.
3. The specificity of NNC2215 for the insulin receptor should be evaluated. Does it bind the IGF1 receptor?
4. P. 6. The threshold for hypoglycemia is 3.9 mM. When plasma glucose concentration falls below 3 mM, urgent therapy needs to be instituted. 2.1 mM is extremely low and does not "...matches the hypoglycemic range..."
5. Fig. 3. Statistical analysis should be done where feasible here – and other figures. In panel e, analysis should be performed at physiologically relevant glucose concentrations.
6. The authors need to adequately address how NNC2215 influences insulin signaling in cells. At a minimum, they need to show the data that the compound stimulates IR pY and activates Akt and Erk. While plate-based assays can be used, representative Western blots should be shown.
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7. Fig. 4. What was the concentration of NNC2215 and how was it selected? Panel a – show control without NNC2215. What is the basis for the claim that the increase in D-glucose was a stress

response to L-glucose infusion? Why did NNC2215 not induce a similar response? What is the asterisk in panel b?
Statistics are important for the claims (p 10) regarding comparisons of NNC2215 with insulin degludec eg, “less pronounced”, “smaller”.In 4c, are the differences (about 4.5 vs about 3) statistically significant?
8. How is NNC2215 cleared from the blood?
9. Methods. What is the purity of the insulin receptor? What was the age of the pigs and the “normal” blood glucose concentration? How were blood samples handled prior to glucose measurement and what method was used for measurement? Do any of the antibodies used in the insulin assays cross-react with endogenous pig insulin? The data validating their “novel model ..... acutely diabetic pig” should be shown in the Supplement.
10. The legend to Extended data Fig 6 needs additional information to allow data interpretation.
11. There are no data to support the claim (line 302) that NNC2215 would reduce glucose excursions after a meal. The IVGTT does not mimic food intake. Although not identical to eating, IP GTT is a better – and widely used – approach.

Minor comments
1. “Blood sugar” should be replaced by “blood glucose”.
2. Fig. 1a. I suggest the authors label the A and B chains of insulin for clarity for non-expert readers.
3. Please clarify “glucometer” (line 197). In the Methods it is stated that the YSI analyser was used to measure glucose. “Glucometer” is the brand name for an early glucose meter that is not currently available.

**Author Rebuttals to Initial Comments:**

**Referee 1 Comments**

**A. Summary of the key results**

**A glucose-switchable insulin variant is described that gains useful dynamic range and appropriate KD values for application in vivo through the attachment of a synthetic lectin-like module at the truncated C-terminus and an appended competing internal D-glucoside at the N-terminus of the B chain. The convincing in vitro measurements translate to highly promising responsiveness (and 'glycaemic protection') in in vivo models. These studies take advantage of an elegant partitioning of mechanism through the use of L-glucose as a ligand that is proposed to be essentially identical in its engagement with the insulin conjugate yet lacks metabolic participation.**

**All-in-all I was excited by it and recommend publication. I make some suggestions for revision below.**

RESPONSE: Thank you very much for your thorough assessment and review of our manuscript, including the very positive feedback and the constructive suggestions for improvement.

**B. Originality and significance: if not novel, please include reference**

**The switching of twelvefold in insulin receptor affinity occurs across a concentration range that is relevant and this tuning into a useful physiological range is exciting and represents a key starting point towards a long-standing goal (as the authors note).**

**The two parts of the model and design given here that I particularly like are:**

**1) the switch mechanism: it exploits internal competition to moderate affinity and control selectivity in a manner that is noncovalent and so a) likely subject to fewer competitive ligand effects and b) may possess potentially faster kinetics. In this way, as the authors note, it partially resembles prior attempts to use lectins yet by here using a smaller (previously known) synthetic lectin-like moiety. This is an elegant design and one that I think is not only effective but also captures the imagination.**

**I think it's fair to say that although others have explored switch designs (as they cite), thus far the dynamic ranges observed have not yet been applicable.**

**2) the size of the construct: whilst clearly not a small molecule, this conjugate is still placed within the realms of realistic synthesis. Insulin can now be regarded as an accessible peptide and the pragmatic conjugation chemistry that is used here could be scalable (although of course the designs here are ones for a prototype).**

**I also admire the key design element of switching, which as the authors note others have explored too – not using an exhaustible depot but instead to create a reversible affinity within principle (noting insulin clearance) could allow pragmatic use.**

RESPONSE: Thank you very much for the positive feedback.

**C. Data & methodology: validity of approach, quality of data, quality of presentation**

**1. I found the in vitro competition assays described in figure 3 to be particularly convincing.**

**Insulin is now a routinely tractable molecule and these conjugates can be characterised at a greater level. The assembly of these is essentially synthetic chemistry and yet the details of corresponding characterization are somewhat light. Synthetic method is given for most of the small molecule chemistry but further details could be added (including further corresponding characterisation data).**

**The characterisation of the conjugates themselves is also somewhat light. The raw data should be given for e.g. the analysis of the intact conjugate masses as well as the tryptic digests. These digests are sensible analyses employing well-trodden techniques in insulin science but simply quoting the major peaks in a mass spectrum there is a slightly old-fashioned way of presenting – all raw data should be given. The characterisation of this through typical MSMS methods would be appropriate potentially (at the very least MS1 spectra should be given). On this point, I may have missed it, but I couldn't get a sense of the yield for the conjugation chemistry. This is pertinent in the context of understanding purity and reproducibility for an interesting conjugate.**

RESPONSE: Thank you very much for the constructive suggestion to add more details describing the characterisation of chemistry underlying this molecule. $^{13}C$ NMR values have now been added to the characterisation data for the building blocks in the Supplementary information - Methods. Furthermore, LCMS spectra for insulin conjugates and the tryptic digests have now been included in the Supplementary information – LCMS spectra. Yields for the conjugation chemistry were already provided in Section 4 of the Supplementary information - Methods (38%, 26%, 62% and

60%, respectively). We consider these yields to be decent for a prototype molecule not optimized for large scale production.

**2. The native MS data is given simply as a non-logarithmic plot in figure 1C. Alternative logarithmic plotting and presentation of corresponding (raw) MS data to allow the reader to understand the basis of this measurement (and potentially its limitations) would be appropriate.**
RESPONSE: We have now provided the raw native MS data in Source data fig. 2c (originally fig. 1c). We do not consider logarithmic plotting of these data to add any benefit as compared to plotting on a normal scale.

**3. The raw data (gels etc) for all assays should be included (e.g. phosphorylation assays).**
RESPONSE: As also requested by the Editor, we have now included an Excel file as supplementary information that contains the individual data underlying the tables and figures in the manuscript. The phosphorylation assays were conducted using ELISA and thus no gels were used in any of the studies described here.

**4. In places, 'data not shown' is used as a statement. In the current era, where there is no limit on supplementary data, this seems inappropriate and this should be supplied.**
RESPONSE: This is duly noted, and we have now included the data. Regarding the initial peak in D-glucose upon L-glucose or vehicle injection seen in Fig. 4a, we have now provided data showing that this is also seen in a similar L-glucose protocol testing the non-glucose-sensitive insulin degludec (new Extended data fig. 2). Regarding the statement in the Methods section on the hypoglycaemia study in pigs that suppression of insulin secretion was verified by measuring C-peptide levels, the C-peptide concentration is now shown in Extended data fig. 3c.

**5. Minor point: Yields could be usefully added to ED figure 4.**
RESPONSE: Thank you for this suggestion. Yields have now been added to Extended data fig. 7 (originally Extended data figs. 3 and 4).

**D. Appropriate use of statistics and treatment of uncertainties**
**Valid.**
RESPONSE: Thank you very much for confirming.

**E. Conclusions: robustness, validity, reliability**
**1. More characterization details of the conjugates would be useful (see above).**
RESPONSE: Please refer to the response to Point C1 above.

**2. Whilst an advantage, the origin of the five-fold shift in KD observed in the synthetic lectin when placed in the conjugate that lacks glucoside (NNC2215a) is not entirely clear to me – why might this be? Is it that internal competition from insulin (side-chains as ligands) is mitigating potency? Here of course this is somewhat welcome in modulating the dynamic range but the origins are intriguing and the comment that "suitable switch dynamics have been achieved" is a slightly throwaway line.**
RESPONSE: The proximity of insulin to the macrocycle apparently changes the glucose affinity, which is not surprising. Similarly, when proteins are conjugated with other building blocks, their affinity to whatever binding partner can change relative to the affinity of the free building block. We have now discussed this matter in the Results section 'Glucose binding data for free macrocycle and NNC2215' (Page 6, Lines 120-124).

**3. The statement that native mass spectrometry gives rise to realistic KDs, is one that I agree with in principle but is of course case-specific. Other biophysical experiments would be welcome. Isothermal calorimetry (applied only to the synthetic lectin moiety?) or other modes of biophysical measurement could be applied directly to these conjugates – it would be interesting to know if this was attempted? The constants gathered by nMS appear consistent (and in essence tally with what is seen in the physiological measurements) but further characterisation and detail of these binding constants would be valuable.**
RESPONSE: Thank you very much for these suggestions. Calorimetric readout will, however, likely be perturbed by other molecular interactions around NNC2215 than the glucose binding alone. The trace aldehyde of glucose can be expected to react with the amino groups of insulin (Schiff base formation) at trace level, and such reactions would likely perturb the ITC data. Also, insulin

oligomer equilibrations could perturb the readout. We have not tried ITC for the given purpose, but we thought that native MS would be better suited for the purpose since native MS will only show values for NNC2215 with or without glucose bound, and thus a titration curve with no influence from other interactions around insulin.

**F. Suggested improvements: experiments, data for possible revision**

**1. The use of L-glucose as a comparable ligand for the 'achiral' synthetic lectin is a nice approach and allowed a powerful dissection of the physiological mechanism from the other downstream/physiological effects of D-glucose. That said an implication is made that the achirality of the macrocycle equates to a lack of 'matching/mismatching' effects in the final conjugate. Whilst it is possible (and even likely) that there is a partitioning of the binding mode to only this moiety away from that of the peptide itself, one can also imagine mechanistic scenarios with this is not the case, especially given the internal role of the (chiral) D-glucoside as the driver of (competitive or not?) inhibition that modulates the observed KD. Experiments should be considered where matched/mismatched pairs are explored in not only the nature of the ligand but also the nature of the conjugate (NNC2215 analogues bearing L glucoside perhaps?). This is also a space where further biophysical measurements could reveal the validity of this assumption. This seems important given the role that the 'L-glucose trick' plays in showing effectiveness of the switch.**

RESPONSE: Thank you for the suggestions. In Table 1, we have now added data on the effect of adding L-glucose in the insulin receptor binding assay in order to compare the effect of these stereoisomers of glucose. The response to 20 mM L-glucose is 7.6-fold compared to 0 mM L-glucose, which is lower than the 12.5-fold response to 20 mM D-glucose. Therefore, when L-glucose was used, the lipogenesis assay and the *in vivo* responses were likely underestimated compared to the D-glucose responses. We have now included this point in the Results section '*In vitro* biology' (Page 9, Lines 192-196).

**2. I found the structural model somewhat simplistic and whilst dealt with under traditional methods (using relatively short molecular dynamics for optimisation), the full details of how this was performed were somewhat lacking. It wasn't fully clear to me how various poses were established prior to the MD. Was the sugar placed within the synthetic lectin prior to docking? It would be useful to provide further details of the MD (the paper is light in this respect also). Again, I may have missed it but was MD run on the open conformation in glucose-bound yet modified form?**

RESPONSE: The main purpose of the structural modelling was to illustrate how the modifications applied to the insulin molecule in NNC2215 do not impede its native interactions with the insulin receptor in an open conformation (based on the active conformation in PDB entry 6PXV). In contrast, a model of NNC2215 in an inactive conformation would allow both closure of the switch and steric hindrance with the receptor, as shown in Fig. 2d. The model of the macrocycle in complex with glucose (and the derived model with the glucoside bound) was adapted from the model obtained in Ref. 16 (Tromans, RA et al. Nat Chem 2019;11:52-6, https://doi.org/10.1038/s41557-018-0155-z), where the glucose molecule was placed in the center of the macrocycle, followed by Monte Carlo Molecular Mechanics (MCMM) simulation with OPLS2005 force field in Maestro Version 10.4.018, as described in the Methods. The MD simulation was performed only for the closed conformation to prove that such a model is stable at least on the nanosecond timescale. As requested by this referee, we have now added more details on the modelling and MD simulation in the Results section '3D structural model of NNC2215 interaction with the insulin receptor' (Page 7, Lines 135-144) and in the Methods section '3D modelling studies' (Pages 21-22, Lines 482-506).

**3. It would of course be absolutely wonderful to have some form of empirical structural data and this system seems ripe for various different techniques. This is in some respects non-trivial (receptor etc) – cryoEM or NMR would give an insight. The use of NMR in particular could also give good insight into the relative dynamics/equilibria of the switchable insulin itself (13C-labelled glucoside in conjugate?). Given the proposal of a switch, which I am reasonably convinced by, it would be good to see some further chemical evidence for switching (buried site 1 glucoside, conformational change) taking place. The structural study of insulin alone provides a useful background for such experiments, which seem worthwhile.**

RESPONSE: Thank you very much for the suggestion to analyse the 3D structure of the NNC2215 + insulin receptor complex. However, we agree that such work would indeed be non-trivial, and

thus we have not embarked on the task. Accordingly, we feel that such work would be outside the scope of the current manuscript.

**4. Further biophysical dissection for mechanism (see also above).**
RESPONSE: Please refer to the response to Point E3 above.

**5. More characterization details of the conjugates would be useful (see above).**
RESPONSE: Please refer to the response to Point C1 above.

**Minor points:**
**6. There are some errors in the naming of the corresponding glucosides (e.g. there are two glycol units).**
RESPONSE: In fact, there are not two glycol units in the final glucoside building block. Rather, the glycoside is conjugated to insulin via an ethylene glycol-acetyl linker.

**7. A small point – why was that particular sulfonate active ester chosen for N-terminal modification?**
RESPONSE: Reason was that the given sulfonate phenolic ester gives B1 acylation as the main product. Please see Ref. 18 [cited in the Results section 'Chemistry' (Page 5, Line 101)]. We consider that the current description in the Results section 'Chemistry' covers this point.

**G. References: appropriate credit to previous work?**
**Yes – succinct but well-judged in a long-standing field.**
RESPONSE: Thank you very much for the positive assessment.

**H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions**
**The paper is clearly written but in some places is somewhat light on technical detail (see above). As a minor stylistic point, perhaps aspects of the narrative overexplain well understood ideas (such as the 'moving' of curves in a dose response).**
RESPONSE: Regarding additional technical details, we have now included these in several places based on the input from this referee. Please refer to Points C1-C5 and F1-F2. Furthermore, in the Results section '*In vitro* biology' (Pages 7-8, Lines 147-166), we have reduced the level of explanation.

**Referee 2 Comments**

**This manuscript provides synthesis of NNC2215 and its in vitro and in vivo characterizations to demonstrate its glucose sensitivity. This research signifies the first genuine demonstration of a self-regulated glucose-sensitive insulin using a molecular switch. Earlier endeavors fell short due to an absence of a potent glucose binder. However, the macrocycle employed in this research successfully overcomes the aforementioned challenge. The Novo Nordisk team performed a suite of robust, convincing experiments, demonstrating the glucose sensitivity of NNC2215. This work culminates numerous prior research breakthroughs and marks a crucial milestone in the field.**

**I found great pleasure in reading this manuscript, which propels a paradigm shift in the field of insulin engineering. The research provides abundant and definitive evidence to establish the glucose sensitivity of NNC2215, a discovery that immediately piques the interest of all researchers in the diabetes research field.**

RESPONSE: Thank you very much for your thorough assessment and review of our manuscript, including the very positive feedback and constructive suggestions for improvement.

**I wish to bring forth the following queries for the authors' consideration:**

**1. Reference 34 cites additional examples of insulin derivatives reported in a patent, including one with a glucoside on A1, a design similar to that reported in reference 14. This derivative appears as a suitable candidate for comparison. Was there a specific reason for its exclusion?**

RESPONSE: The insulin A1 derivative in Ref. 38 (example 25) has very low receptor affinity, as A1 derivates typically have. In fact, the receptor affinity of this compound was so low that we could not measure reliable receptor affinity and could not obtain a reliable glucose factor +/- glucose. Also, this compound is quite different from NNC2215 since it has dendrimers on the macrocycle (NNC2215 does not), and the macrocycle is attached to insulin A1 via the pillars, not via the roof as in NNC2215. Overall, we feel the differences between the compound in Ref. 38 and NNC2215 are too numerous to bring the A1 derivative into the discussion in the current manuscript.

**2. What factors contribute to the 5-fold glucose affinity disparity between NNC2215a and the free macrocycle?**

RESPONSE: The proximity of insulin to the macrocycle apparently changes the glucose affinity, which is not surprising. Similarly, when proteins are conjugated with other building blocks, their affinity to whatever binding partner can change relative to the affinity of the free building block. We have now discussed this matter in the Results section 'Glucose binding data for free macrocycle and NNC2215' (Page 6, Lines 120-124).

**3. In the in vitro experiments, the authors mention the addition of 1.5% albumin. Can data be provided for the same experiments without the inclusion of albumin? Alternatively, can the authors share data concerning any significant affinity/interaction between NNC2215 and albumin? Moreover, could the authors elaborate on the potential of NNC2215 binding to glycated proteins such as hemoglobin or albumin?**

RESPONSE: Assessment of the glucose responsiveness *in vitro* was conducted in the insulin receptor binding assay in the presence of 1.5% human serum albumin since this is closer to the physiological conditions (4-5% human serum albumin) compared to complete absence of albumin. Human serum albumin at 1.5% was chosen because that is the highest concentration that can be tolerated in the receptor binding assay. The glucose responsiveness was, however, also assessed in the absence of albumin, where the glucose response decreased from 12.5-fold to 6.8-fold. I.e. albumin binding adds to some extent to the glucose responsiveness. This information has now been added to the Results section '*In vitro* biology' (Page 7-8, Lines 158-163) and in a new Extended data fig. 1a.

The potential ability of NNC2215 to bind to other glycosylated proteins is an interesting question. The glucose specificity of the macrocycle was studied by Tromans et al. (Ref. 16), but it cannot be ruled out that there may be some binding to glycosylated proteins. However, when looking at the insulin receptor, which is a glycosylated protein and was semi-purified using a glycosylation binding column with wheat germ agglutinin (WGA), if there was binding of NNC2215 to the glycans in the insulin receptor, the control compound NNC2215a, with only the macrocycle, should show glucose sensitivity in the binding assay, which it did not. This suggests that NNC2215 does not bind to any major extent to glycosylated proteins. We have now added this point to the Discussion, 1st paragraph (Page 14, Lines 314-321).

**4. Repeating Figure 3d with NNC2215a would be a great comparison.**
RESPONSE: The glucose responsiveness of NNC2215a with 20 mM D-glucose in the presence of 1.5% human serum albumin was assessed. The glucose response of NNC2215a was 2.4-fold suggesting a weak albumin binding of the macrocycle. This information has now been added to the Results section '*In vitro* biology' (Page 8, Lines 163-166) and in a new Extended data fig. 1b.

**5. In the in vivo section, I am intrigued by the choice of comparator insulin (human insulin or insulin degludec). Line 181 reads: "Insulin degludec was used as a comparator in the pig study due to similar pharmacokinetic properties following intravenous administration". Given that this was an IV study, the depot effect (i.e., multi-hexameric structures from insulin degludec) should be minimal. I presume that the pharmacokinetics of insulin degludec is largely dominated by its albumin affinity. Does this suggest that NNC2215 shares similar albumin-binding properties? Furthermore, why was human insulin used in the rat experiments, despite IV dosing?**
RESPONSE: It is correct that insulin degludec after i.v. administration is not influenced by multihexamer formation in the subcutis and that the pharmacokinetic properties after absorption depend on the albumin binding properties of insulin degludec but also on its affinity to the insulin receptor. It is also correct that NNC2215 to some extent binds to albumin (refer to our reponses to this Referee's Points 3 and 4). After i.v. dosing, their half-lives are similar as shown in Extended data table 1. For the purposes of the pig experiment, we wanted to compare NNC2215 to a relevant insulin molecule with similar kinetics in the extravascular space to illustrate the glucose-dependent nature of NNC2215 *in vivo* as purely as possible, which is why we chose i.v. infusion to steady state and insulin degludec as comparator.
Human insulin was used as comparator in the STZ-rat study for two reasons. Firstly, human insulin is the comparator insulin we have used in almost all our glucose clamp studies in rats characterising insulin analogs intravenously. Secondly, as explained in the manuscript, the aim of this study was to quantify the effect of "activated" NNC2215 during the GTT in terms of human insulin equivalents, i.e. to investigate activation of NNC2215 during a meal-like glucose spike.

**6. While I concur that the three in vivo studies offer persuasive evidence of NNC2215's glucose sensitivity, I do not see any animals receiving subcutaneous doses. This is the route predominantly adopted by individuals requiring external insulin. It would be enlightening to conduct a comparative study that involves the subcutaneous injection of either NNC2215 or insulin degludec.**
RESPONSE: We agree that s.c. dosing is the most relevant route of administration and including s.c. dosing would represent a natural next step. In this manuscript, we sought to investigate the glucose sensitive nature of NNC2215 as cleanly as possible, and we felt that using i.v. administration was the best way to achieve that goal. With s.c. administration, any differences in absorption properties between NNC2215 and a comparator could complicate the interpretation of any glucose dependence.

**Appropriate citations on prior examples were provided. This manuscript was written with great clarity.**
RESPONSE: Thank you very much for the positive assessment.

**Referee 3 Comments**

**The study by Hoeg-Jensen et al. describes the engineering of a glucose-sensitive form of insulin. They detail the chemistry used to generate the compound NNC2215, and show that its binding to insulin receptors is influenced by glucose in a concentration-dependent manner. Importantly, NNC2215 shows promising glucose responsiveness in both rat and pig in vivo models.**

**Insulin was first used in a patient with diabetes in 1922, and successful modification of the molecule was achieved only 14 years later with the development of long-acting insulin. Attempts to produce glucose-stimulated insulin was initiated over 40 years ago. Reducing the risk of hypoglycemia in people requiring insulin therapy is important so the work of Hoeg-Jensen et al, if successful, has potential clinical applications.**

RESPONSE: Thank you very much for your thorough assessment and review of our manuscript, including the positive feedback and constructive suggestions for improvement.

**A major concern is that much of the work was performed at glucose concentrations that are not physiologically relevant. The reference intervals in healthy adults for random plasma glucose concentrations are 3.9-7.8 mmol/L, while fasting concentrations are 4.1-5.5 (up to 6.1 in some countries) mmol/L. A random plasma glucose concentration >11.1 is considered to be diabetes. The goal of insulin therapy in persons with diabetes is to maintain a range of 3.9 to 10 mmol/L. Analyses at glucose concentrations of 0 have no biological relevance. The authors' claim (p 12) of "..hypoglycemic range, ie 0 to 5.." is erroneous as glucose concentrations ≥4 mmol/L are normoglycemia. Similarly, the claim (p 12) "....over the physiological glucose concentration range of 0 to 20 mM.." needs to be corrected. A plasma glucose of 20 mM is clearly pathological. The text needs to be modified extensively throughout the manuscript to reflect real physiological and hypoglycemic glucose concentrations.**

RESPONSE: We acknowledge the confusion in the interpretation of our use of the term "physiological" which in this context of a glucose-sensitive insulin refers to the glucose levels that can be observed in the population being treated with insulin, namely approximately 2-20 mM. As you very well point out, this is not physiological but rather pathophysiological at the extreme low and high glucose concentrations. However, with the perspective that previous switch-based glucose-sensitive insulin derivatives needed 50 mM fructose or other non-physiological conditions for triggering, we have come to refer to the glucose levels we tested (0-20 mM) in our *in vitro* studies as being "physiological" since it is possible to observe these glucose concentrations *in vivo*, and in particular, in humans. However, we must admit this is an exaggeration as 0 mM glucose is not realistic in humans. Therefore, we have now refrained from referring to the glucose concentrations as being physiological and have mentioned the specific values. When discussing the human condition, we refer to these levels as being relevant in people with diabetes. This has been changed throughout the manuscript. We thank you for bringing this to our attention.

**Specific comments:**

**1. The abstract is misleading as the IR affinity increase of 12-fold is not biologically relevant.**

RESPONSE: Thank you for pointing out that 0 mM glucose is not physiologically relevant. We aimed to include the glucose levels for a healthy individual and for an individual with diabetes: 3 mM, 5 mM, 10 mM and 20 mM D-glucose. The 0 mM glucose was included as a scientific data point to reveal the full response of the NN2215 molecule and to measure the activity when in the absolute closed state. The 20 mM glucose was selected as the highest tested as it is a pathologically high glucose concentration that is not uncommon in people living with poorly controlled diabetes. Therefore, to reflect the condition in people with diabetes, in Table 1 the fold response between 3 mM and 20 mM D-glucose is included (3.2-fold). We have now also added this value in the Abstract (Page 2, Line 31) and Introduction (Page 5, Line 86).

**2. Fig. 1c. The rationale for evaluating non-physiological glucose concentrations, particularly 0-2 mM, is unclear and distorts the analysis. This applies throughout the manuscript.**

RESPONSE: Please refer to the response to Point 1 above.

**3. The specificity of NNC2215 for the insulin receptor should be evaluated. Does it bind the IGF1 receptor?**
RESPONSE: Thank you for pointing out this important aspect of designing a new insulin molecule. The specificity towards the insulin receptor compared to the IGF1 receptor is very important to avoid any increased mitogenicity. The IGF1 receptor binding was measured in the presence and absence of 20 mM D-glucose. Relative to human insulin, NNC2215 had approximately 10% IGF1 receptor affinity compared to its insulin receptor affinity. Thus, NNC2215 has a higher specificity towards the insulin receptor versus the IGF1 receptor as compared to human insulin. These results have now been added in the Results section '*In vitro* biology' (Page 8, Lines 168-174) and in a new Extended data fig. 1c,d, with the corresponding methodology added in the Methods section 'hIR-A and IGF-1R affinity measurements' (Pages 22-23, Lines 510-534).

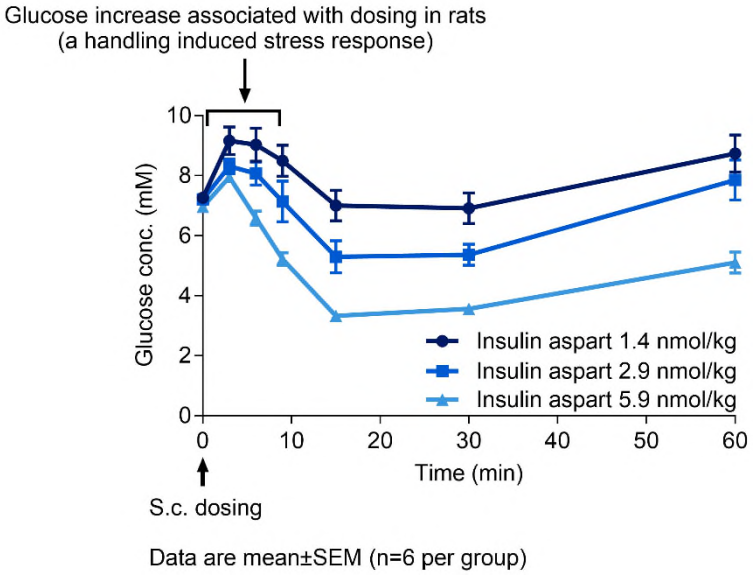
**4. P. 6. The threshold for hypoglycemia is 3.9 mM. When plasma glucose concentration falls below 3 mM, urgent therapy needs to be instituted. 2.1 mM is extremely low and does not "...matches the hypoglycemic range..."**
RESPONSE: Thank you for bringing this to our attention. The sentence has now been corrected and refer to this value as being considered "severe hypoglycaemia" [Results section 'Glucose binding data for free macrocycle and NNC2215' (Page 6, Line 119)].

**5. Fig. 3. Statistical analysis should be done where feasible here – and other figures. In panel e, analysis should be performed at physiologically relevant glucose concentrations.**
RESPONSE: Regarding the choice of glucose concentrations tested in Fig. 3e, please refer to the response to Point 1 above. Furthermore, the lipogenesis experiments were performed in the presence of 3 mM or 20 mM L-glucose which reflect the extreme situations in diabetes, hyperglycaemia (20 mM) and hypoglycaemia (3 mM), in order to examine the difference in the activity of NNC2215 under these conditions. No statistical analyses were performed as these studies were designed simply to obtain an idea as to whether glucose has a qualitative effect on NNC2215 activity which does not occur for a conventional insulin analogue (insulin degludec) or human insulin.

**6. The authors need to adequately address how NNC2215 influences insulin signaling in cells. At a minimum, they need to show the data that the compound stimulates IR pY and activates Akt and Erk. While plate-based assays can be used, representative Western blots should be shown.**
RESPONSE: A description of the data showing that NNC2215 activates Akt and ERK is included in the Results section '*In vitro* biology' (Page 8, Lines 176-182). We have now also included representative curves of IR, Akt and ERK phosphorylation induced by increasing concentrations of NNC2215 versus human insulin in a new Extended data fig. 1e-g. Western blots were not performed for these measurements.

**7. Fig. 4. What was the concentration of NNC2215 and how was it selected? Panel a – show control without NNC2215. What is the basis for the claim that the increase in D-glucose was a stress response to L-glucose infusion? Why did NNC2215 not induce a similar response? What is the asterisk in panel b?**
**Statistics are important for the claims (p 10) regarding comparisons of NNC2215 with insulin degludec eg, "less pronounced", "smaller".In 4c, are the differences (about 4.5 vs about 3) statistically significant?**
RESPONSE: For the rat study in Fig. 4a and b, the dose used was 4.5 nmol/kg [see the Methods section 'L-glucose rat model' (Page 25, Line 569)] and the concentration of the formulations used for i.v. dosing was 4.5 nmol/mL. For the pig study in Fig. 4c and d, the infusion rates and how they were chosen are provided in the Methods section 'Hypoglycaemia study in pigs' (Page 26, Lines 594-602) and the concentration of NNC2215 in the formulation infused i.v. was 3 µM. In plasma, the concentration depended on the dose administered, in steady state ranging from a minimum of about 500 pM in an animal at the lowest dose to a maximum of about 1200 pM in an animal at the highest dose.
We have now included results on a similar L-glucose protocol testing the non-glucose-sensitive insulin degludec as a control (new Extended data fig. 2).
We commonly see initial short lived (<15 min) increases in D-glucose concentrations following dosing of rats, even when dosing with insulin. Please refer to the example in the figure below here, where insulin aspart was dosed s.c. to rats. An increase in D-glucose probably also happened after NNC2215 and insulin degludec dosing, but the signal was not picked up due to the short-lived

nature of the response in relation to the sampling scheme (no samples were drawn between dosing of NNC2215/degludec and L-glucose administration 30 min later).
The asterisk in Fig. 4b indicates that the plasma concentrations of NNC2215 at time point 60 min is significantly lower ($p<0.05$; Students t-test) in the 2 g/kg L-glucose group vs. the 0 g/kg L-glucose group, suggesting faster clearance of NNC2215 when triggered with high levels of L-glucose. Fig. 4b and legend have been updated to clarify.
We agree with the reviewer that statistics are important. The statistical analysis related to Fig. 4c is explained in the legend to Extended data fig. 3a,b. All the animals studied are included in that analysis. In Fig. 4c, two dose groups that showed similar plasma glucose concentrations prior to stopping the glucose infusion are shown for illustrative purposes. We think this way of showing the data is more intuitively understood than the full analysis in Extended data fig. 3a,b. Based on the analysis of all pigs documenting glucose dependence, it is reasonable to select two groups with equi-efficacious doses of NNC2215 and insulin degludec (same starting plasma glucose) for illustration. However, we do not think it is optimal or necessary to make a separate statistical analysis of such selected groups as this could be construed as "cherry picking". A t-test comparing the drop in glucose between the two groups does show a significant difference in glucose drop between NNC2215 and insulin degludec (-4.6±0.9 vs –6.1±0.9 mM, mean±SD, $p<0.01$) as does a t-test comparing the absolute plasma glucose concentration at 7.5 hours between NNC2215 and insulin degludec (4.6±1.1 vs 2.9±0.8 mM, mean±SD, $p<0.005$). We have not added these numbers to the manuscript but can do so if requested.




**8. How is NNC2215 cleared from the blood?**
RESPONSE: Insulin is generally believed to be mainly cleared by the insulin receptor (Sonne O. Physiol Rev 1988;68:1129-96, https://doi.org/10.1152/physrev.1988.68.4.1129) and to a small extent through renal clearance and degradation. We have not specifically studied how NNC2215 is cleared *in vivo*. However, we have no reason to believe it is cleared differently from other insulin molecules, but this cannot be ruled out.

**9. Methods. What is the purity of the insulin receptor? What was the age of the pigs and the "normal" blood glucose concentration? How were blood samples handled prior to glucose measurement and what method was used for measurement? Do any of the antibodies used in the insulin assays cross-react with endogenous pig insulin? The data validating their "novel model ….. acutely diabetic pig" should be shown in the Supplement.**
RESPONSE: The insulin receptor is semipurified by a wheat germ agglutinin (WGA) purification and the preparation therefore contains many different glycosylated proteins. The assay is made specific by binding the insulin receptor to the scintillation beads with the highly specific antibody 83-7 from Prof. K. Siddle (Biochem J 1986;235:199-208, https://doi.org/10.1042/bj2350199). The binding studies are performed with displacement with $^{125}$I-insulin, which has a high selectivity to the insulin receptor and binds in the pM range in this assay. The assay has been used in the characterisation of insulin analogues from Novo Nordisk in several previous studies (e.g. Glendorf T et al. PLoS One 2012;7:e29198, https://doi.org/10.1371/journal.pone.0029198; Hansen BF et

al. PLoS One 2012;7:e34274, https://doi.org/10.1371/journal.pone.0034274; Nishimura E et al. BMJ Open Diabetes Res Care 2021;9:e002301, https://drc.bmj.com/content/9/1/e002301).
The requested information on age of the pigs, normal blood glucose concentration, blood sample handling and glucose measurement have been added in several of the subsections throughout the Methods section '*In vivo* studies' (Pages 25-29).
A description of how cross-reactivity with endogenous insulin was prevented has now been added in the Methods section 'Quantification of insulin and C-peptide concentrations in plasma samples' (Page 28, Lines 646-649).
Thank you for the interest in our model. C-peptide concentrations have now been added in Extended data fig. 3c, showing that despite the relative hyperglycemia during the experiment, C-peptide was similarly suppressed in NNC2215 and insulin degludec treated animals.

**10. The legend to Extended data Fig 6 needs additional information to allow data interpretation.**
RESPONSE: Description of the method used (nonlinear least squares regression analysis) has been added to the legend of Extended data fig. 3a,b (original Extended data fig. 6).

**11. There are no data to support the claim (line 302) that NNC2215 would reduce glucose excursions after a meal. The IVGTT does not mimic food intake. Although not identical to eating, IP GTT is a better – and widely used – approach.**
RESPONSE: Thank you for pointing this out. With our studies we sought to investigate the glucose-dependent nature of NNC2215 for which reason we used i.v. glucose administration. We deliberately did not use oral or IP administration to avoid possible confounding incretin effects and difficulties interpreting non-steady state conditions. We completely agree that oral glucose or mixed meal administration would be a logical next step in the evaluation. Regarding the wording in the original line 302, we have further softened the sentence according to the request from this referee (Page 15, Lines 346-348).

**Minor comments**
**1. "Blood sugar" should be replaced by "blood glucose".**
RESPONSE: Duly noted and done (in the Introduction, Page 3, Line 40). Thank you for pointing out this colloquialism to us.

**2. Fig. 1a. I suggest the authors label the A and B chains of insulin for clarity for non-expert readers.**
RESPONSE: This figure (Fig. 2a in the revised version) has now been updated as requested. Furthermore, we have made the same addition to Extended data fig. 8.

**3. Please clarify "glucometer" (line 197). In the Methods it is stated that the YSI analyser was used to measure glucose. "Glucometer" is the brand name for an early glucose meter that is not currently available.**
RESPONSE: We thank the reviewer for pointing this out. We have changed the wording in the last sentence of the Results section 'L-glucose study in rats' (Page 10, Lines 228-230).

**Reviewer Reports on the First Revision:**

Referee #1 (Remarks to the Author):

Thank you for the revised manuscript. I still consider this work to be exciting and of great promise. I would not wish to be an unnecessary block on work that I do hope is eventually published but there seem some (few) important outstanding issues. Whilst some aspects have been addressed, several have not and I find myself very much torn. I am afraid that in the absence of appropriate data or experiment, certain conclusions cannot be supported.

I will address the points that remain in rough order:

• Further details of synthesis and characterization: Whilst the addition of 13C NMR data is appreciated, this was unassigned and these sections still remain light. The authors will likely be aware of typical methods and characterization standards [see for example http://pubs.acs.org/paragonplus/submission/joceah/joceah_ccc.xls]. There is a duty upon synthetic chemists [also within the Nature guidelines] to provide sufficient data and methods that allow reproduction of synthesis and demonstration of purity. It wasn't clear from the revised methods that any other details had been added. Typical methods for purity demonstration include mp, chromatographic data, provision of NMR spectra. New compounds can require HRMS or microanalysis and structural assignments (e.g. of the 13C NMR resonances) should be complete to allow confidence in the suggested structure; this is not present. I am sorry to press this point but in the additional absence of detailed conjugate characterization [see below] there is a (small) danger that intermediates are impure / not as identified, leading to the possibility of incorrect conjugates and consequent misinterpretation of results. I would simply like to be clearer on this aspect for my own judgement, as I am sure readers would wish to be also.

• Yields and data for conjugates: I note the yields for NNC2215 in the methods and the inclusion now of chromatograms and MS1 for that conjugate – thank you. However, NNC2215a, a key control, still does not seem to have a yield / effiiciency and has not been characterized to the same level as NNC2215 (digests etc). This is perhaps pertinent given the discussion on altered binding (see below and also raised by Referee 2). Although the authors do not find this surprising, two referees did and this might arise instead from incomplete / incorrect construction. The authors may still wish to consider MSMS methods to better validate conjugate identity and, indeed, sites – such methods are now quite routine.

• Native MS data: A request was made for the raw MS data. I realise that I should perhaps have been more specific. Rather than the data points interpreted from the MS, the raw mass spectral data (i.e. the 'native' mass spectra of intact conjugate bound to glucose at varying concentrations) of the native complexes would be important in understanding what remains the central biophysical data supporting the proposed mechanism [see also below].
It is also typical for logarithmic plots to allow proper judgment of what is effectively a dose-response curve at lower concentrations [in regions with higher gradient]. The Source Data will now make it possible for readers to plot the data at lower glucose concentrations in this normal manner should they wish – thank you – but it remains unclear to me exactly how the quoted KDs were estimated

(presumably through non-linear regression?) and what the error, R2 etc is on these? Sorry to press on this but given the dependence on this biophysical data [I note the answer to E3] to the proposed molecular mechanism, I think this will be important.
Minor point: I infer also from the Source Data that n = 3(?) for this plot – I believe all data points should be in the plot. I note too that the authors have incorrectly ticked N/A in the Editorial Policy Checklist – surely this plot [and others] are applicable?

• Structures and further biophysics to support the proposed mechanism: One of the key considerations asked of a referee is whether the data given supports the conclusions drawn. Here, an apparently central thrust of the proposed function is the molecular mechanistic basis of the 'switch'. At one level, only the incomplete native-MS data provides support [see above]. I realise that structures or [at the very least] detailed computation is an additional requirement but as it stands, the MD work is somewhat basic [now seen by the authors as a model – see below] and limited. The authors suggest that empirical structural work or further biophysics are out of scope. Given that this is a potentially profound and exciting design model, for which some further detail is perhaps needed, all I can do is reiterate my suggestions. The suggestion of, at least, more detailed MD work is not that onerous. That said, I would still welcome other experiments.
As things stand, the proposed mechanism is not yet supported. The suggestion now made by the authors that this is simply a model or representation suggests that this borders on speculation and so, again, does not allow the conclusions on mechanism to be fully drawn. I will leave the relative importance of this mechanism to the Editor as a subjective judgement but, for me, this was a key element of novelty identified in my assessment of this work and that seemed to be a central claim of the paper.

• Nomenclature [minor]: I realise that my comment was too obtuse – apologies – I shall be more specific. The naming of the glycosides on page 3,4 of the Supplementary Methods is incorrect. Glycosides do not need the use of "1-beta" - a glycoside is necessarily at C-1. This is specifically a [(2-hydroxy)ethoxy]ethyl glucopyranoside. The O-acetyl positions should be specified etc. On page 4, I realise that the hydroxymethyl of the glycol unit has been oxidised to a carboxyl – therefore the name is wrong (and also missing a glycol unit): carboxyethyl glucoside would have an anomeric substituent of just –CH2CH2COOH. This compound bears –CH2CH2OCH2COOH as a substituent i.e. is a [(carboxy)methoxy]ethyl glucopyranoside etc. See http://publications.iupac.org/pac/1996/pdf/6810x1919.pdf for further details.

Additional points:
I have sought to confine my response only to my prior comments but there is a small arising issue, from the forms, on data availability. The authors suggest that data is only available 'on reasonable request' – I think this is no longer encouraged practice and, given the requests on data noted above, perhaps all the less appropriate.

Referee #2 (Remarks to the Author):

I am pleased to see that most of my previous comments and suggestions were appropriately addressed by the authors. The only remaining suggestion is comment #6, which discussed why only IV administration of NNC2215 was done in the study instead of the SC administration used by millions of people who require external insulin. I agree that IV administration can show the cleanest effect of glucose-sensitive nature. I would like to request an additional experiment that compares NNC2215 and a comparator insulin using SC route for the following reasons:

1. While demonstrating glucose-sensitive properties is an important goal, the ultimate motivation is still to administer such drug candidates to treat diabetes, which will be through the SC route. This manuscript presents the first example of glucose-sensitive insulin without the need for any external materials or matrices. A preclinical animal model in the SC route can serve as an important benchmark for all future studies in this field.
2. Even if the SC study demonstrates a less clear-cut result than the current IV study, it is still an extremely valuable result to the field, and any discussion to rationalize such a result can inform future improvements and developments.
3. The PK profile of SC-administered NNC2215 can be informative.

Referee #3 (Remarks to the Author):

This is a revised and improved manuscript. Glucose-stimulated insulin suitable for treating patients would be useful clinically and this work is of interest to diabetes researchers. The authors have adequately addressed most of my prior concerns. A few items require attention.

1. The text (line 30) “12.5-fold when glucose.....from 0 to 20 mM and” should be removed from the Abstract.
2. P. 6, line 130. The hypoglycemic range in humans is not 0-5 mM. The goal of therapy in individuals with diabetes is to minimise time at glucose 3.0-3.9. The text needs to be corrected for the non-expert reader.
3. Extended data Fig. 1. The data for insulin at 0 mM glucose appear to have been omitted.
4. P. 12. I did not see is the reference interval (“normal range”) for blood glucose in the pigs they studied. Is the “physiological range”3-15 mM as stated?
5. Fig. 4a. The authors should show control without NNC2215, as previously requested.
6. Extended data Fig. 3. The data are not convincing. The enormous SDs cast doubt on the interpretation. Note that the colours used make it very difficult to distinguish the two conditions. What is the reason for the very poor replicates? What are the accuracy and imprecision (analytical variation) of the assay?

**Author Rebuttals to First Revision:**

**Referee 1 Comments**

**Thank you for the revised manuscript. I still consider this work to be exciting and of great promise. I would not wish to be an unnecessary block on work that I do hope is eventually published but there seem some (few) important outstanding issues. Whilst some aspects have been addressed, several have not and I find myself very much torn. I am afraid that in the absence of appropriate data or experiment, certain conclusions cannot be supported.**

RESPONSE: Thank you very much for your assessment of our revised version of the manuscript and the overall positive evaluation. We have now further improved the manuscript based on your input. Below, we provide point by point responses to your specific comments.

**I will address the points that remain in rough order:**

**Further details of synthesis and characterization: Whilst the addition of 13C NMR data is appreciated, this was unassigned and these sections still remain light.**

RESPONSE: Both 1H and 13C NMR data have now been assigned for the final building blocks as used for the insulin conjugations, namely NMR assignments for the alkyne carbonate (Extended data fig. 7a), the glucoside active ester (Extended data fig. 7b) and the macrocycle azide (Extended data fig. 8i). All carbons of the NMR-assigned structures have now been equipped with numbers (on the structures in Extended data figs. 7a,b and 8i), and the corresponding numbered assignments have been added to the synthetic descriptions in the Supplementary information - Methods. Especially for the macrocycle azide, the NMR assignment job was a major undertaking, requiring COSY, HSQC, HMBC and DEPT NMR techniques. Since the assignments of the final building blocks are in agreement with the drawn structures, we do not consider it necessary to NMR-assign all the eight intermediates towards the final building blocks and we hope this referee agrees to that.

**The authors will likely be aware of typical methods and characterization standards [see for example http://pubs.acs.org/paragonplus/submission/joceah/joceah_ccc.xls]. There is a duty upon synthetic chemists [also within the Nature guidelines] to provide sufficient data and methods that allow reproduction of synthesis and demonstration of purity. It wasn't clear from the revised methods that any other details had been added. Typical methods for purity demonstration include mp, chromatographic data, provision of NMR spectra. New compounds can require HRMS or microanalysis and structural assignments (e.g. of the 13C NMR resonances) should be complete to allow confidence in the suggested structure; this is not present. I am sorry to press this point but in the additional absence of detailed conjugate characterization [see below] there is a (small) danger that intermediates are impure / not as identified, leading to the possibility of incorrect conjugates and consequent misinterpretation of results. I would simply like to be clearer on this aspect for my own judgement, as I am sure readers would wish to be also.**

RESPONSE: Prints of all 1H and 13C NMR spectra have now been gathered and included in the Supplementary information - NMR, LCMS, HRMS and native MS spectra (including the COSY, HSQC, HMBC and DEPT spectra for NMR assignments). HRMS data have now been recorded for the building blocks and added to the synthetic descriptions in the Supplementary information - Methods. LCMS and HRMS analysis of the insulin conjugates NNC2215 and NNC2215a were already included in the prior submission.

**Yields and data for conjugates: I note the yields for NNC2215 in the methods and the inclusion now of chromatograms and MS1 for that conjugate – thank you. However, NNC2215a, a key control, still does not seem to have a yield / effiiciency and has not been characterized to the same level as NNC2215 (digests etc). This is perhaps pertinent given the discussion on altered binding (see below and also raised by Referee 2). Although the authors do not find this surprising, two referees did and this might arise instead from incomplete / incorrect construction. The authors may still wish to consider MSMS methods to better validate conjugate identity and, indeed, sites – such methods are now quite routine.**

RESPONSE: Tryptic LCMS analysis has now been performed for the control compound NNC2215a (see LCMS spectra in Supplementary information - NMR, LCMS, HRMS and native MS spectra), showing that the macrocycle is conjugated to the B29 fragment of insulin, as now described in the Supplementary information – Methods, Section 5.

**Native MS data: A request was made for the raw MS data. I realise that I should perhaps have been more specific. Rather than the data points interpreted from the MS, the raw mass spectral data (i.e. the 'native' mass spectra of intact conjugate bound to glucose at varying concentrations) of the native complexes would be important in understanding what remains the central biophysical data supporting the proposed mechanism [see also below].**
RESPONSE: The 72 raw native MS spectra for determining the glucose affinities of the insulin conjugates studies have now been included in the Supplementary information – NMR, LCMS, HRMS and native MS spectra (now referred to in the Fig. 2c legend).

**It is also typical for logarithmic plots to allow proper judgment of what is effectively a dose-response curve at lower concentrations [in regions with higher gradient]. The Source Data will now make it possible for readers to plot the data at lower glucose concentrations in this normal manner should they wish – thank you – but it remains unclear to me exactly how the quoted KDs were estimated (presumably through non-linear regression?) and what the error, R2 etc is on these? Sorry to press on this but given the dependence on this biophysical data [I note the answer to E3] to the proposed molecular mechanism, I think this will be important.**
**Minor point: I infer also from the Source Data that n = 3(?) for this plot – I believe all data points should be in the plot. I note too that the authors have incorrectly ticked N/A in the Editorial Policy Checklist – surely this plot [and others] are applicable?**
RESPONSE: The Kd's were determined by non-linear regression and it is correct that n=3. We have now included the individual values as black squares/circles on this plot (and updated the Editorial Policy Checklist). Furthermore, we have added the $R^2$ values in the legend to Fig. 2c and specified that these are from non-linear regression.

**Structures and further biophysics to support the proposed mechanism: One of the key considerations asked of a referee is whether the data given supports the conclusions drawn. Here, an apparently central thrust of the proposed function is the molecular mechanistic basis of the 'switch'. At one level, only the incomplete native-MS data provides support [see above]. I realise that structures or [at the very least] detailed computation is an additional requirement but as it stands, the MD work is somewhat basic [now seen by the authors as a model – see below] and limited. The authors suggest that empirical structural work or further biophysics are out of scope. Given that this is a potentially profound and exciting design model, for which some further detail is perhaps needed, all I can do is reiterate my suggestions. The suggestion of, at least, more detailed MD work is not that onerous. That said, I would still welcome other experiments.**
**As things stand, the proposed mechanism is not yet supported. The suggestion now made by the authors that this is simply a model or representation suggests that this borders on speculation and so, again, does not allow the conclusions on mechanism to be fully drawn. I will leave the relative importance of this mechanism to the Editor as a subjective judgement but, for me, this was a key element of novelty identified in my assessment of this work and that seemed to be a central claim of the paper.**
RESPONSE: We thank the referee for raising this important point. We agree that the molecular mechanistic basis of the switch is not fully characterized by our experimental and computational methods. However, we believe that our work provides sufficient evidence to support the existence and relevance of the switch, as well as to propose a plausible model for its operation. We also argue that the suggested MD work is not as straightforward or informative as the referee implies, and that it would require a significant amount of time and resources that are beyond the scope of this study. We explain our reasoning below.
First, we would like to clarify that our MD work is not intended to be a detailed computation of the switch mechanism, but rather a simple model that illustrates the key features of the switch, such as the conformational change of the insulin-macrocycle complex, the interaction with the receptor, and the effect of the macrocycle on such interaction. We do not claim that our MD work is conclusive or comprehensive, but rather that it is consistent with our experimental data and provides a useful framework for its rationalization.
Second, we would like to address the referee's suggestion of more detailed MD work. The referee did not specify what kind of simulation he/she is suggesting, but we assume that he/she is referring to longer or more advanced simulations that could capture the full switch dynamics. We do not think that such simulations would be feasible or informative for the following reasons: We can run long simulation of a closed conformation of the insulin+macrocycle alone and see if during the simulation (1-2 microseconds) it opens up, but we do not expect this to happen given the high

affinity of the macrocycle for the insulin core (we already ran 100 nsec simulations and observed no opening). We can run long simulation of an open conformation bound to the receptor and see how stable it is, but we do not think this will provide any new insight into the switch mechanism. Indeed, we do not think it is feasible to observe the switch closing during a simulation of 1-2 microsec. The probability of such an event is extremely low, and it would require much longer simulations or enhanced sampling techniques to observe it.
More advanced simulations, such as free energy calculations, transition path sampling, or metadynamics, usually require months of dedicated work from a postdoc, as well as specialized expertise and software. We do not think they are necessary to support our main findings and conclusions.
In summary, we respectfully consider that the referee's suggestion of more detailed MD work is not feasible or informative for the purpose of this study, which was mainly intended as a model to rationalize and depict the clear experimental evidence of the proposed switch mechanism. We hope that the referee will find our response satisfactory.

**Nomenclature [minor]: I realise that my comment was too obtuse – apologies – I shall be more specific. The naming of the glycosides on page 3,4 of the Supplementary Methods is incorrect. Glycosides do not need the use of "1-beta" - a glycoside is necessarily at C-1. This is specifically a [(2-hydroxy)ethoxy]ethyl glucopyranoside. The O-acetyl positions should be specified etc. On page 4, I realise that the hydroxymethyl of the glycol unit has been oxidised to a carboxyl – therefore the name is wrong (and also missing a glycol unit): carboxyethyl glucoside would have an anomeric substituent of just –CH2CH2COOH. This compound bears –CH2CH2OCH2COOH as a substituent i.e. is a [(carboxy)methoxy]ethyl glucopyranoside etc. See http://publications.iupac.org/pac/1996/pdf/6810x1919.pdf for further details.**
RESPONSE: We thank the reviewer for noting the error in the glucoside nomenclature. It has now been corrected in the synthetic descriptions in Supplementary information – Methods and in the title and legend of Extended data fig. 7.

**Additional points:**
**I have sought to confine my response only to my prior comments but there is a small arising issue, from the forms, on data availability. The authors suggest that data is only available 'on reasonable request' – I think this is no longer encouraged practice and, given the requests on data noted above, perhaps all the less appropriate.**
RESPONSE: Thanks a lot for noting this. We agree that with the inclusion of additional data to the manuscript, the need for further data requests is expected to be limited. Still, in order to keep the possibility for further data requests open, we have now revised the data availability statement to 'Additional data files may be shared upon request.'

**Referee 2 Comments**

**I am pleased to see that most of my previous comments and suggestions were appropriately addressed by the authors. The only remaining suggestion is comment #6, which discussed why only IV administration of NNC2215 was done in the study instead of the SC administration used by millions of people who require external insulin. I agree that IV administration can show the cleanest effect of glucose-sensitive nature. I would like to request an additional experiment that compares NNC2215 and a comparator insulin using SC route for the following reasons:**

**1. While demonstrating glucose-sensitive properties is an important goal, the ultimate motivation is still to administer such drug candidates to treat diabetes, which will be through the SC route. This manuscript presents the first example of glucose-sensitive insulin without the need for any external materials or matrices. A preclinical animal model in the SC route can serve as an important benchmark for all future studies in this field.**

**2. Even if the SC study demonstrates a less clear-cut result than the current IV study, it is still an extremely valuable result to the field, and any discussion to rationalize such a result can inform future improvements and developments.**

**3. The PK profile of SC-administered NNC2215 can be informative.**

RESPONSE: Thank you very much for your assessment of our revised version of the manuscript and the overall positive evaluation. In a new Extended data fig. 4, we have now included pharmacokinetic and plasma glucose results following subcutaneous administration as well as intravenous bolus administration of NNC2215 in normal LYD pigs. Although this study did not include a direct comparator, we have historical subcutaneous pharmacokinetic data in this pig model for both insulin detemir and insulin degludec. The following text has thus been added to the manuscript (pages 11-12, Lines 254-266):

"NNC2215 exposure and plasma glucose concentration after s.c. versus i.v. administration of NNC2215 to female LYD pigs are shown in Extended data fig. 4. The mean (±SD) bioavailability of NNC2215 after s.c. administration was estimated to 73±24%. A small acute plasma glucose lowering effect was observed after i.v. administration of 0.3 nmol/kg, while after s.c. administration of 2 nmol/kg, the drop in plasma glucose was less, in line with the exposure of NNC2215. A protracted pharmacokinetic profile was observed following s.c. administration with a harmonic mean half-life of 19 h. This is longer than previously observed for insulin detemir (6.5 h) (Pedersen KM et al. Transl. Res. 2022;239:71-84) and insulin degludec (7.2 h) (Kurtzhals P et al. Nat. Rev. Drug Discov. 2023;22:59-80) in pigs and would suggest that NNC2215 could be suitable for once-daily dosing based on this animal model. The difference in s.c. half-life between the different insulin analogues highlights the rationale for using i.v. infusion as the route of administration in the pig studies to demonstrate in vivo glucose sensitivity of NNC2215, which was to circumvent the confounding effects of the different s.c. pharmacokinetic profiles."

The pharmacokinetic results for NNC2215 after intravenous administration in LYD pigs originally shown in Extended data table 1 were from another study than the study of subcutaneous vs. intravenous administration now included in the new Extended data fig. 4. To ensure consistency, we have therefore updated Extended data table 1 to show pharmacokinetic results for NNC2215 after intravenous administration in LYD pigs from the same study as presented in Extended data fig. 4. This update has no impact on any of the points made in the manuscript.

**Referee 3 Comments**

**This is a revised and improved manuscript. Glucose-stimulated insulin suitable for treating patients would be useful clinically and this work is of interest to diabetes researchers. The authors have adequately addressed most of my prior concerns. A few items require attention.**

RESPONSE: Thank you very much for your assessment of our revised version of the manuscript and the overall positive evaluation. We have now further improved the manuscript based on your input. Below, we provide point by point responses to your specific comments.

**1. The text (line 30) "12.5-fold when glucose……from 0 to 20 mM and" should be removed from the Abstract.**

RESPONSE: This part of the sentence has now been deleted (Page 2, Lines 29-30).

**2. P. 6, line 130. The hypoglycemic range in humans is not 0-5 mM. The goal of therapy in individuals with diabetes is to minimise time at glucose 3.0-3.9. The text needs to be corrected for the non-expert reader.**

RESPONSE: We excuse the direct "translation" of the *in vitro* conditions to human hypoglycaemia. We have now changed this text to read "The steepest part of the binding curve is consistent with what would be considered as hypoglycaemia, below 4 mM glucose." (Page 6, Lines 130-131)

**3. Extended data Fig. 1. The data for insulin at 0 mM glucose appear to have been omitted.**

RESPONSE: Thank you for bringing this up. Actually, the reason is that the two grey curves are essentially superimposable. To address this, we have now changed the symbols to open triangles for 0 mM D-glucose and open circles for 20 mM D-glucose.

**4. P. 12. I did not see is the reference interval ("normal range") for blood glucose in the pigs they studied. Is the "physiological range"3-15 mM as stated?**

RESPONSE: We have removed the word "physiological" from the text as it is not clear if this relates to pigs or humans (Page 13, Line 282). The quoted range is relevant to people with diabetes, and our investigations show glucose sensitivity of NNC2215 in that range both in vitro and in animal models.

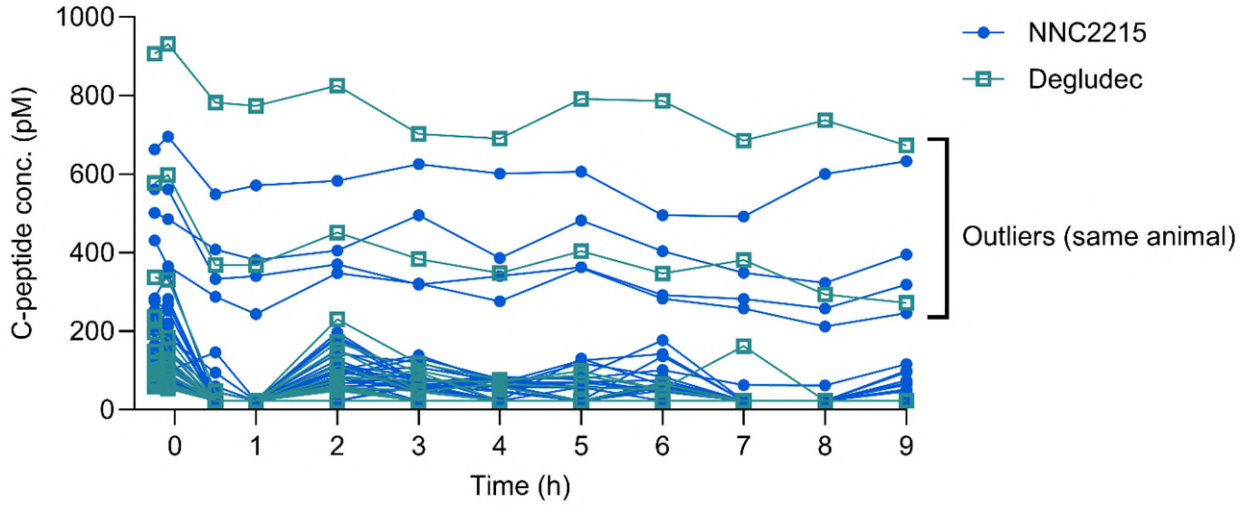
**5. Fig. 4a. The authors should show control without NNC2215, as previously requested.**

RESPONSE: In the first revision, we had already provided these data (using insulin degludec as control). They can be found in Extended data fig. 2.

**6. Extended data Fig. 3. The data are not convincing. The enormous SDs cast doubt on the interpretation. Note that the colours used make it very difficult to distinguish the two conditions. What is the reason for the very poor replicates? What are the accuracy and imprecision (analytical variation) of the assay?**

RESPONSE: The reviewer is correct that the error bars are large in Extended data fig. 3d. For the C-peptide assay, the inter-assay variation was 5.1% and the accuracy was +/-10%. The primary reason for the large error bars is the fact that one of the animals exhibited unusually high C-peptide levels, both at basal and during somatostatin infusion on all six experimental days (Source data, Sheet 'Extended data fig. 3d'; NNC2215 rows 5, 9, 20 and 24; Insulin degludec rows 42 and 50). We occasionally see LYD pigs with very high C-peptide levels despite the fact that they have normal glucose levels. As we do not have fasting C-peptide or insulin as inclusion criteria, it is not possible for us to know about the high C-peptide levels until we measure the samples after the study. In the below figure, the individual C-peptide data are shown, clearly illustrating the outlier. Omission of the six data sets from the single animal with high C-peptide levels does not alter the conclusion: Please, refer to the revised Extended data figure 3, showing the plot with (Extended data fig. 3b) and without (Extended data fig. 3c) the outlier. It is also important to note that in all animals, C-peptide levels during the experiment were lower at time 360 min (when the glucose infusion was stopped to evaluate glucose sensitivity) than at baseline even though glucose was considerably higher than at baseline. Both with and without the outlier, C-peptide levels were not different between NNC2215 and insulin degludec groups. Our model is one of relative insulinopenia and not complete ablation of endogenous insulin secretion. The degree of suppression is not different between the groups. Therefore, any effect of endogenous insulin was similar between NNC2215 and insulin degludec. We have now included the information about the outlier in the Results section 'Hypoglycaemic study in LYD pigs' (Page 11, Lines 246-249) and the sensitivity analysis in Extended data fig. 3c. In the Discussion section (Page 15, Lines 349-350), we have also

altered the description of our model from ‘a novel, acute model using diabetic pigs’ to ‘an acute diabetic-like pig model induced by somatostatin and glucagon replacement infusions’.



**Reviewer Reports on the Second Revision:**

Referee #1 (Remarks to the Author):

Thank you enormously for the efforts made in improving the quality of the data gathered, analyzed and made available. In addition the caveated use / interpretation of the MD is welcomed; I agree with the limits of what might be discovered by extensive additional work.
I am very happy to recommend publication 'as is' of this excellent work.

Referee #2 (Remarks to the Author):

The authors fully addressed all my remaining comments. I have nothing to add and look forward to seeing the work published.

Referee #3 (Remarks to the Author):

The authors have satisfactorily addressed all my comments.