nature portfolio

Peer Review File

Exactly defined molecular weight poly(ethylene glycol) allows for facile identification of PEGylation sites on proteins

Corresponding Author: Professor Andrew Livingston

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Livingston and coworkers synthesized monodisperse 5 KDa PEGylation agents on a gram scale using a membrane-filtration method for purification. The PEGylation agent was used to PEGylate BSA and illustrated the benefits of using monodisperse PEG in the identification of the PEGylation site. The study is interesting and would be of value to protein-based biopharmaceutical development. However, there are still some minor issues that should be addressed before it is accepted for publication.
1. In the introduction, the authors raised many points of view but without references to support these points, such as “the exact binding site of the PEG in a PEGylated protein often remains unclear”, “therapeutics PEGylated with defined PEG could potentially exhibit an improved biodistribution profile” and many others. Please provide references regarding these points in the introduction.
2. The UPLC-MS system is actually equipped with an ELSD detector as specified in the methods part. Why does MeO-EG112-OH still need derivatization with anisyl chloride? ELSD can detect the PEGs without UV absorption.
3. In the NMR data, the 1 and 13 in 1H and 13C should be upper-case. Also the 3 in CDCl3.
4. In the synthesis of MeO-Eg112-SP, why ethyl 3-bromopropionate was used, which is prone to eliminate hydrogen bromide and lead to ethyl acrylate? It should be explained in the manuscript.

Reviewer #2

(Remarks to the Author)
This manuscript describes the chemical synthesis of monodisperse PEG derivatives with precise molecular weight and their use for modifying proteins to precisely determine the modification site. There are two key highlights in this work. Firstly, the authors have successfully synthesized monodisperse PEG with a molecular weight of over 5 kDa at a purity of over 95% using their originally developed iterative liquid-phase approach, called Nanostar Sieving The resulting PEG was methylated to afford monodisperse mPEG, which was further derivatized into succinimidyl ester for protein modification. Second, using monodisperse mPEG, the accurate modification positions in the PEGylated proteins could be determined. Both of these results greatly impact the relevant fields and strongly appeal the usefulness of the monodisperse-PEG, particularly in the pharmaceutical industry. The manuscript is well-organized, and the discussion is based on sufficient, reliable data. The introduction part comprehensively summarizes the problems of current PEG-modified drugs and the past development of synthetic methods for monodisperse-PEGs, helping to understand the importance of the present work. Considering all this, the manuscript is basically recommended for publication in Nature Communications, after addressing the following points.

(1) Since the new method allows identification of the position of PEG modification on the protein, it would be interesting to explore the relationship between the position of PEG modification and the protein's structure. Why would monodisperse PEG of >5 kDa be introduced to the inaccessible internal residues? Does the distribution of hydrophobic and hydrophilic residues on the protein surface influence PEG modification?
(2) For fragment B, PDB ID: 3V03 showed -(E)KVLTSSA(R)- instead of -(E)KVLASSA(R)-. Is this a possible mutation or an identification error? If it is an identification error, the theoretical value of MW would also change, so please carefully verify the sequence for accuracy.

(3) Page 8, the first paragraph: "... We could equally have started with an oligomer for which n<28, but this would have required a greater number of iterative steps to reach n = 112."
Is it possible to improve the overall purity by using a higher purity short oligomer and increasing the number of reaction cycles? Could this help reduce the presence of byproducts generated from the impurities in the starting materials shown in Figure 3b?

Minor comments
(1) On page 4, the last sentence of the first paragraph:
"Could we get data on this after we submit?" seems to be a typo?
(2) The second paragraph on page 4:
This section introduces previous reports on the synthesis and purification of monodisperse PEGs. References #15 and #16 are both documents, such as datasheets, published by Polypure. Do these references meet the criteria of Nature Communications?
(3) Please clarify the meaning of "DF limiting species" in the Table in Figure 3a.
(4) In the caption for Figure 3b, it seems that "bottom" and "top" may have been mistakenly interchanged.
(5) In the caption for Figure 4, “M+5NH]45+ and M+6NH]46+”, the parenthesis before the “M” are missing.
(6) In Figure 4, it is necessary to identify all peaks in the spectrum of mPEG-SP. While five m/z peeks (1301.5, 1044.8, 873.7, 751.6, 659.8) are found in the figure, only three peaks (([M+4 NH4]4+, [M+5 NH4]5+, [M+6 NH4]6+)) were identified.
(7) It is necessary to show the condition of ultracentrifugation for purification of digested peptide samples.
(8) The vertical values in the central figure of Figure 5a appear to be partially missing.
(9) It is necessary to clarify the meaning of "AS" in Table in Figure 5c. Also, "OSN" the “keywords” needs explanation.
(10) The m/z attributions in the synthesis section of the main text and in the Supplementary Information contained numerous typographical errors, such as [M+nNH]4+n+ and [M+n.NH]4+n+. Please check and correct them carefully.
(11) Some figures in the Supplementary Information should be referenced in the main text. For instance, Figures S23 and S24 should be cited in the section describing chain extension.
(12) The statement "Error! Reference source not found." on Page 22 does not seem to be correct.

Reviewer #3

(Remarks to the Author)
Livingston and co-workers have reported on a new methodological approach to synthesise true monodisperse (5 kDa) PEG using the Nanostar Sieving Approach. The results relating to the synthetic approach are noteworthy, and methodology well described and sound. However, the work presented in its current state does not support the initial claims nor conclusions, and is not suitable for publication without the inclusion of additional evidence.

The authors claim that some of the problems with the current PEGs used are the presence of PEGamers and positional isomers. Although the nanostar sieving approach does generate monodisperse PEGs, the digestion studies presented in this study still show the presence of positional isomers, thus not bypassing the initial problem but merely facilitating the identification of those isomers. It is suggested that the authors use functional group transformations to site-specifically target protein residues, such as cysteines, disulfides, histidines, to truly show the potential of this methodology and generate precisely defined bioconjugates.

Furthermore, in this study the authors have synthesized 5 kDa PEGs. While significant in terms of synthesizing monodisperse PEGs, several bioconjugates are synthesized with larger MW PEGs (e.g., Neulasta). It is suggested that the authors expand the range of MWs synthesized to be more widely applicable.

The authors have also stated that defined PEGylation may result in a more efficient PEGylated therapeutic, with a decreased volume of distribution. It is suggested that the authors provide some supporting evidence for this, experimentally if possible or at the least, by reference through literature.

Additional comments:

Page 4 (introduction section): end of first paragraph, the authors included the statement "could we get data on this after we submit?" that does not belong / is a editing comment.

Page 8 (results and discussion section); The authors state that they have selected an Eg28 as the building block as this is the highest Mw of defined PEG is commercially available. Given that most PEGylated proteins on the market are PEGylated with PEGs > 5 kDa, could this also be used as the building block for higher MW defined PEGs? if so, how many iterations are expected to generate, for example a 20 kDa PEG and would the yields be comparable?

Page 8 (results and discussion section); In this work, the authors yield mPEG-OH that is then transformed into an amine reactive group. While this is a commonly used chemistry in PEGylation, recent PEGlyated proteins have moved towards more site specificity. Have the authors considered generated defined PEGs with for example thiol reactive moieties?

Page 9 (results and discussion section); It is unclear what DBX is?

Page 10 (results and discussion section): The authors discuss the cycles of rejection; however this is not clearly explained and is unclear what the rejection refers to and why 90% is positive. The supporting information does not provide additional

clarity.

Page 12 (results and discussion section): The authors discussed the price of production using the Nanostar Sieving process, and considered the yield an important factor - and rightfully so! However, when considering the cost associated with developing PEGylated products, would the authors be able also to comment on how commercially feasible it would to use such costly starting materials (with implications to cost of production and overall drug product for the patient?).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my concerns have been properly addressed. I recommend it to publish in current form.

Reviewer #2

(Remarks to the Author)
The authors' responses were very sincere and addressed the concerns raised by this reviewer. The manuscript was revised to include appropriate additional data and discussion to support the author's claims. Therefore, this manuscript is recommended for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
Thank you to the authors for the response and clarifications. The response to reviewers addresses my concerns adequately and upon reviewing the updated manuscript, I recommend the paper be published.

Reviewer #1

Comment 1:

In the introduction, the authors raised many points of view but without references to support these points, such as **"the exact binding site of the PEG in a PEGylated protein often remains unclear**", "**therapeutics PEGylated with defined PEG could potentially exhibit an improved biodistribution pro**file" and many others. Please provide references regarding these points in the introduction.

Thanks for pointing this out. We have provided references to these points as follows:

p.3, l. 12-14: "*The disperse nature of these PEGylation agents results in batch-to-batch variations and difficulties in purification and characterisation of the PEGylated therapeutics"*

→ Added 3 more publications to support statement.

p.3, l. 17: *"Despite the availability of advanced analytical methods, the exact binding site of the PEG in a PEGylated protein often remains unclear, or PEG-related impurities remain unidentified."*

→ Added citation for FDA report on MYL-1401H, which shows evidence for problems during characterisation of PEGylated filgrastim (this is also explained in more detail within p.3 ll.20-35 of the manuscript)

→ Added *Anal.Chem. 2016, 88, 10848- 10853,* in which it says "The dispersity of PEG produces conjugates with different molecular sizes. This may create difficulties in the chemical characterization and purity control along with variability in activity, safety and pharmacokinetic properties of the products."

→ Added *S. Gerislioglu et al., Analytica Chimica Acta 1004 (2018) 58-66,* in which it says "PEGylation converts a monodisperse protien into a polydisperse macromolecule. Ananlysis of such samples by ESI-MS is challenging due to the formation of multiple charge states with different types of charges [...]."

→ Added *International Journal of Pharmaceutics 454 (2013) 553– 558,* in which it says "Despite the growing interest in PEGylation of peptides and proteins, challenges for detailed structural characterization are created at the same time […], such as PEG polydispersity, degree of PEGylation and especially the PEGylation sites."

p.4, l.3-4: *"Furthermore, we postulate that therapeutics PEGylated with defined PEG could potentially exhibit an improved biodistribution profile compared to the disperse derivatives.*"

→ We would like to make clear that this is a hypothesis. The foundation for this is hypothesis is described in the text, with suitable references.

Comment 2:

The UPLC-MS system is actually equipped with an ELSD detector as specified in the methods part. Why does MeO-EG112-OH still need derivatization with anisyl chloride? ELSD can detect the PEGs without UV absorption.

Explanation (added on p.10/l. 25-30): Even though the available UPLC-MS system was equipped with an ELSD detector, this could not be used to quantify the PEG polymers of different chain lengths, in their unfunctionalised form (mPEG-OH). This is because the ELSD signal is non-linear across molecules of different sizes, so a calibration with a pure sample of every chain length would have been required. Hence, we employed UPLC-UV instead, using the MS trace for identifying each species, and the UV trace for their quantification.

Comment 3: In the NMR data, the 1 and 13 in 1H and 13C should be upper-case. Also the 3 in CDCl3.

This has now been changed in the manuscript.

Comment 4:

In the synthesis of MeO-Eg112-SP, why ethyl 3-bromopropionate was used, which is prone to eliminate hydrogen bromide and lead to ethyl acrylate? It should be explained in the manuscript.

Thank you, this is a useful comment. Indeed, we experienced problems with the completion of this reaction, when performed with 5 eq. of Sodium Hydride and at room temperature, most likely because the 3-bromopropionate underwent elimination and reacted to the acrylate. With the given reaction conditions, which include:

1. Reduced equivalents of sodium hydride (2.1 eq.)
2. Adding the sodium hydride to the PEG starting material first
3. Adding the 3-bromopropionate in large excess (5 eq.) and at 0 °C

The reaction reached full completion.

An explanation has been added to the manuscript (p. 14, l.4).

Reviewer #2:

Comment 1:

Since the new method allows identification of the position of PEG modification on the protein, it would be interesting to explore the relationship between the position of PEG modification and the protein's structure. Why would monodisperse PEG of >5 kDa be introduced to the inaccessible internal residues? Does the distribution of hydrophobic and hydrophilic residues on the protein surface influence PEG modification?

These are interesting questions, but besides identifying the PEGylation sites, further structural investigations were beyond the scope of this work. Our focus was to meet the challenge of finding WHERE the PEG bound – these questions perhaps belong to the class of questions about WHY the PEG bound where it did, which, now that we can identify WHERE it bound, is certainly an interesting possible topic in future work

Comment 2:

For fragment B, PDB ID: 3V03 showed -(E)KVLTSSA(R)- instead of -(E)KVLASSA(R)-. Is this a possible mutation or an identification error? If it is an identification error, the theoretical value of MW would also change, so please carefully verify the sequence for accuracy.

We appreciate the Reviewer's comment and are grateful for their attention to detail. We have double-checked our underlying data. The mass spectrum leading to the mentioned sequence exhibited the m/z series: 1015.0, 872.4, 765.8, 682.8, which corresponds to a mass of 5981.92 Da. After subtracting the mass of the PEG residue (5021.03 Da), we're left with the mass of the peptide fragment being ~960.98 Da. The only fragment matching from the tryptic digest of bovine serum albumin is –EKVLASSAR- with a mass of 960.55 Da.

The Reviewer mentions that the sequence for PDB ID 3V03 is –EKVL**T**SSAR- and not –EKVL**A**SSAR-.

The sequence we have used for analysing our data is the Uniprot sequence ID P02769 (https://www.uniprot.org/uniprotkb/P02769/entry#sequences), which shows the discussed fragment to be –EKVL**A**SSAR-.

We have double-checked the literature and found two publications which are also cited within PDB entry with the ID 3V03 and have again found the sequence to be –EKVL**A**SSAR-. Please find the according citations and screenshots below. We believe this is enough evidence for the fragment sequence to be –EKVL**A**SSAR- and have added the reference in the manuscript accordingly.

[figure redacted]

Figure 1. Part of BSA sequence (and others), copied from K.A. Majorek et al. / Molecular Immunology 52 (2012) 174– 182.

[figure redacted]

Figure 2. Part of BSA sequence (and others), copied from Acta Cryst. (2012). D68, 1278–1289.

Comment 3:

Page 8, the first paragraph: "... We could equally have started with an oligomer for which n<28, but this would have required a greater number of iterative steps to reach n = 112."

Is it possible to improve the overall purity by using a higher purity short oligomer and increasing the number of reaction cycles? Could this help reduce the presence of byproducts generated from the impurities in the starting materials shown in Figure 3b?

This is a fair comment. Indeed the -Eg impurity (MeO-Eg_{111}-OH, shown in Figure 3b) could be reduced/ avoided by using a building block with even higher chain length purity. The Eg_{28} building block used within this work is only available from one supplier and with the given purity. Using a shorter building block might offer access to higher purity building blocks. Nonetheless, when using a shorter building block, the number of cycles needed to get to a certain chain length (e.g. 112) would increase and with every chain extension/ deprotection cycle, the risk for the introduction of impurities derived through incomplete chain extension or incomplete deprotection would increase, i.e. there is a trade-off.

For example, within our research group an Eg_{12} building block was used at the beginning of the work within this project. With this, 7 cycles were needed to go from the initial Eg_{28} to Eg_{112}. Changing from the Eg_{12} to the Eg_{28} building block, not only reduced the number of cycles needed from seven to three, but also decreased the amount of Eg_n impurities (± Eg_{12} in case of the Eg_{12} building block, and ± Eg_{28} in case of the Eg_{28} building block). This can be attributed to a statistically lower chance of forming these impurities when less cycles are needed.

Hence, the Eg_{28} building block represents the optimal balance of economic viability (through a faster synthesis) and purity of the final product.

Comment 4:

p.4: the last sentence of the first paragraph:
"Could we get data on this after we submit?" seems to be a typo?

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 5:

p.4: This section introduces previous reports on the synthesis and purification of monodisperse PEGs. References #15 and #16 are both documents, such as datasheets, published by Polypure. Do these references meet the criteria of Nature Communications?

The datasheet from Polypure has been removed from the reference list and included in a Figure in Supplementary Materials (**Figure S 32**).

Comment 6:

Fig 3a: Please clarify the meaning of "DF limiting species" in the Table in Figure 3a.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 7:

Fig 3b: In the caption for Figure 3b, it seems that "bottom" and "top" may have been mistakenly interchanged.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 8:

Fig 4: In the caption for Figure 4, "M+5NH]45+ and M+6NH]46+", the parenthesis before the "M" are missing.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 9:

Fig 4: it is necessary to identify all peaks in the spectrum of mPEG-SP. While five m/z peeks (1301.5, 1044.8, 873.7, 751.6, 659.8) are found in the figure, only three peaks (([M+4 NH4]4+, [M+5 NH4]5+, [M+6 NH4]6+)) were identified.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 10:

It is necessary to show the condition of ultracentrifugation for purification of digested peptide samples.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 11:

Fig 5a: The vertical values in the central figure of Figure 5a appear to be partially missing.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 12:

Fig 5c: It is necessary to clarify the meaning of "AS" in Table in Figure 5c. Also, "OSN" the "keywords" needs explanation.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 13:

The m/z attributions in the synthesis section of the main text and in the Supplementary Information contained numerous typographical errors, such as [M+nNH]4+n+ and [M+n.NH]4+n+. Please check and correct them carefully.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 14:

Some figures in the Supplementary Information should be referenced in the main text. For instance, Figures S23 and S24 should be cited in the section describing chain extension.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 15:

The statement "Error! Reference source not found." on Page 22 does not seem to be correct.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Reviewer #3

Comment 1:

The results relating to the synthetic approach are noteworthy, and methodology well described and sound. However, the work presented in its current state does not support the initial claims nor conclusions and is not suitable for publication without the inclusion of additional evidence.

Our "initial claims" are described in the second paragraph of the manuscript's introduction. Here, we are describing how "the disperse nature of […] PEGylation agents results in […] difficulties in purification and characterisation of the PEGylated therapeutics". We now cite relevant literature, supporting these claims, as described in response to Comment 1 Reviewer 1. In this revised version, using the new references, we now make clear what we claim regarding how the characterisation problems, especially when trying to identify the PEGylation sites in PEGylated proteins, could be overcome by using defined molecular weight PEGylation agents. We then present experimental evidence to support the initial claims, by first synthesising a defined MW PEG, then using this to PEGylate the protein BSA to show how the defined nature of the PEG allows for straightforward identification of the PEGylation sites through mass spectrometry. To be clear, we claim that identifying which peptide fragments are PEGylated is not straightforward with disperse PEGs but can easily be achieved with defined MW PEG. We then demonstrate this experimentally.

Comment 2:

The authors claim that some of the problems with the current PEGs used are the presence of PEGamers and positional isomers. Although the nanostar sieving approach does generate monodisperse PEGs, the digestion studies presented in this study still show the presence of positional isomers, thus not bypassing the initial problem but merely facilitating the identification of those isomers.

Whilst we acknowledge, that the presence of PEGamers and positional isomers can indeed be problematic when producing PEGylated therapeutics, addressing this problem was not part of this work. There are already many "selective" PEGylation agents available, such as mPEG aldehydes. Because the characterisation of a PEGylated protein is even more difficult when more than one positional isomer is present, being able to analyse fragments from a mixture of positional isomers is more challenging than when PEGylation is selective. So, for our study we purposely intended to

produce a mixture of different positional isomers, by using a "random" PEGylation agent (an active ester, which binds to primary amines within a protein). Distinguishing these one from another is more challenging than if we had used a selective PEGylation agent. We have then shown in our study, that when defined MW PEG is used, even a mixture of multiple different positional isomers (ie the more difficult challenge) can easily be analysed, supporting the claim that defined PEGs make identification of PEGylation sites possible.

Comment 3:

It is suggested that the authors use functional group transformations to site-specifically target protein residues, such as cysteines, disulfides, histidines, to truly show the potential of this methodology and generate precisely defined bioconjugates.

The synthesis and application of so-called selective PEGylation agents was not intended to be part of this study, as research on this has been done extensively in the past years. Hence, we are not addressing the problem of the PEGylation agents' selectiveness, but their dispersity. Nevertheless, we acknowledge that in future, selective and defined MW PEGylation agents might become of more interest, and we are currently working on developing these within our research group.

Comment 4:

Furthermore, in this study the authors have synthesized 5 kDa PEGs. While significant in terms of synthesizing monodisperse PEGs, several bioconjugates are synthesized with larger MW PEGs (e.g., Neulasta). It is suggested that the authors expand the range of MWs synthesized to be more widely applicable.

We appreciate the Reviewer's comment and agree, that synthesising defined MW PEG of higher molecular weight (≥ 20 kDa) is of high interest, looking at the currently marketed PEGylated drugs. We are currently receiving funding for this purpose through an IAA grant from QMUL and the experimental work is ongoing.

Comment 5:

The authors have also stated that defined PEGylation may result in a more efficient PEGylated therapeutic, with a decreased volume of distribution. It is suggested that the authors provide some supporting evidence for this, experimentally if possible or at the least, by reference through literature.

The potentially increased efficiency and decreased volume of distribution of defined MW PEGylated therapeutics, is a hypothesis that we make based on data that we cite in the manuscript (citation 18). The data shows a correlation curve of PEG-half life in blood and the PEG molecular weight. This curve (please find below) is particularly steep between 0 and 40 kDa, suggesting that a small change in the PEG molecular weight creates a significant change in the PEG-half life. The trend of this curve might be transferable to PEGylated therapeutics (shifted to higher molecular weights). This would mean that a sample composed of species of different molecular weight (so a disperse sample), would have a wider range of half lifes, hence a less defined character, compared to a defined molecular weight species.

We acknowledge, that this is an assumption and producing data on this is of high interest for future work. Nevertheless, this will include the conduction of *in vivo* experiments and exceeds the scope of this manuscript.

[figure redacted]

Figure 3. Correlation curve of half live in blood (min) dependant on PEG molecular weight (kDa), copied from [18] in manuscript (P. Caliceti, F.M. Veronese / Advanced Drug Delivery Reviews 55 (2003) 1261–1277 1265)

Comment 6:

p.4 (introduction section): end of first paragraph, the authors included the statement "could we get data on this after we submit?" that does not belong / is a editing comment.

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 7:

p.8 (results and discussion section); The authors state that they have selected an Eg28 as the building block as this is the highest Mw of defined PEG is commercially available. Given that most PEGylated proteins on the market are PEGylated with PEGs > 5 kDa, could this also be used as the building block for higher MW defined PEGs? if so, how many iterations are expected to generate, for example a 20 kDa PEG and would the yields be comparable?

We appreciate the Reviewer's comment, this is also something we are currently working on. Whilst it is entirely possible to use the Eg28 building block and carry the synthesis through to the 20 kDa PEG (Eg_{448}), this would require 12 chain extension cycles starting from the Eg_{112}. Hence, we are currently working on using a Eg_{56} building block (derived through first doubling the Eg_{28} chain), which would reduce the number of cycles needed to 6 cycles and make the synthesis economically more viable. We are currently in the process of investigating the kinetics and potentially usable membranes and the results if successful will be part of a future publication.

Comment 8:

p.8 (results and discussion section); In this work, the authors yield mPEG-OH that is then transformed into an amine reactive group. While this is a commonly used chemistry in PEGylation,

recent PEGlyated proteins have moved towards more site specificity. Have the authors considered generated defined PEGs with for example thiol reactive moieties?

In our work, we have not transformed the mPEG-OH into an amine, but an active ester (mPEG-succinimidyl propionate), which is reactive towards primary amine groups within a protein. This was the suitable choice for this work, but we acknowledge that there is a range of other interesting reactive groups, especially site-selective ones, which are beyond the scope of this manuscript and is part of experimental work currently undergoing in this group.

Comment 9:

p.9 (results and discussion section); It is unclear what DBX is?

We appreciate the Reviewer's comment, this has now been changed in the manuscript.

Comment 10:

p.10 (results and discussion section): The authors discuss the cycles of rejection; however, this is not clearly explained and is unclear what the rejection refers to and why 90% is positive. The supporting information does not provide additional clarity.

The measurement and calculation of the rejection is explained in detail in the supporting information (p.6/ "Membrane Screening" and p.7/ "Calculation of rejections") and the corresponding equation is referred to in the main text. This has now been made clearer (p.10: "For the first two cycles the rejection (calculated according to Eq. S1 in the supplementary information […]).

Comment 11:

p.12 (results and discussion section): The authors discussed the price of production using the Nanostar Sieving process, and considered the yield an important factor - and rightfully so! However, when considering the cost associated with developing PEGylated products, would the authors be able also to comment on how commercially feasible it would to use such costly starting materials (with implications to cost of production and overall drug product for the patient?).

Thanks for this comment. We are at an early stage and have been mainly concerned with the science, rather than the economics. In an academic institution it is hard to make meaningful estimates of the costs of drugs on the market. What we want to say is that the major materials cost of the synthesis was the PEG monomers, and that a higher yield, for given inputs, is usually desirable for economic and sustainability reasons. That is, Nanostar Sieving is superior to chromatography in this context. We have altered the text to reflect this point.