nature portfolio

Peer Review File

Improving the biosynthesis of eukaryotic P450 system via architectural organization in Escherichia coli

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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)
This manuscript describes the use of different approaches to enhance the function of plant and human cytochrome P450/P450 reductase enzyme pairs in E. coli. Direct genetic fusion, SH3/peptide and Spy/Snoop Tag/catcher ligation are tested. The leading system they test leads to an increase in biosynthetic yield. The strength of this paper lies in the good novelty of the application of the isopeptide covalent ligation for this enhancement of enzyme function and the substantial improvements obtained. Only a few examples are tested so it is a little premature to say if this will be a general solution to a broad problem, but the results are certainly promising and could generate substantial interest. The weakness of the manuscript is that the extensive literature for spatial organisation of interacting enzymes is not really explained and the results are not sufficiently put in the context of this. In addition, a common weakness of the field is that it is often assumed that the effects on product generation are a result of spatial relationships between two enzymes, when often the differences were subsequently found to relate to differences in expression levels or catalytic activities of each individual unit, which can be surprisingly sensitive to specific fusion proteins (see for example The role of dynamic enzyme assemblies and substrate channelling in metabolic regulation. Lee J. Sweetlove & Alisdair R. Fernie. Nature Communications volume 9, Article number: 2136 (2018)). There is insufficient data in this manuscript to exclude this alternative explanation.
Error bars are suitably described but statistical tests are not performed on the differences seen.

“The results indicated that the isopeptide bond 379
in the covalent N-termini-bridged heterodimers, such as covalent heterodimer (IV), 380
improved the biosynthetic performance of the eukaryotic P450 enzyme in E. coli and 381
suggested that the mechanical stability of self-assembled peptide biomachinery for 382
spatially organizing the multireaction-involved enzymes should have a crucial effect on the biosynthetic efficiency.”
Mechanical stability suggests that there is a specific force leading to separation of the two components, but no description of such a force is provided. Is not a more likely hypothesis that the covalent interaction leads to the two components being more often found associated together?

Minor points:
Line 78, Taxol, not Toxal
Line 84: originally derived from cow, or
originally derived from the bovine sequence
Fig. 1b,c: it might be worth drawing in the outer membrane of E. coli
“The recombinant E. coli strain without the 149
chimera accumulated 590.63 μM trans-cinnamic acid with no p-coumaric acid at 48 h
postinduction (hpi) (Fig. 2c), and its cell growth was severely inhibited (Fig. S1). As
shown in Fig. 2c, the recombinant strain with chimera (i) produced 201.31 μM
p-coumaric acid”
Often numbers are presented here to 5 significant figures, but given the error bars on all measurements, it is unlikely that the values should be presented so precisely.
Fig. 2a Draw chirality of phenylalanine
It might help readers to provide the common name of 4-acetamidophenol: Paracetamol/acetaminophen.
It would be more conventional to draw in the hydrogen on the amide nitrogen in phenacetin and 4-acetamidophenol.
Fig. 5c: the error bars on the inset graph are large, making a firm conclusion about the effects difficult?

“The weak electrostatic interaction between the FMN domain of CPR and the heme domain 500
of P450 27,28 may pledge a mutual recognition of these two domains, by which an orientation-dependent interface fitness would presumably be conducted between CPR 502
and P450 for the correlative reactive activity.”
The English would benefit from editing here.

“The nitrogen-free buffer contained 100 mM 3-(N-morpholino) propane sulfonic acid (MOPS)”
MOPS itself contains a nitrogen- please rephrase.

Fig. S3: The methods give insufficient detail on Western blotting. Similarly the legends don’t make it clear what antibody was used. This looks like a direct visible light image rather than chemiluminescent detection?

Fig. S6: image resolution should be improved

It looks like there was no spacer between the fusion peptides and the enzymes. Often a spacer can improve folding/expression yield. Also the residue after the initiating methionine in some constructs is K, which can destabilize the protein inside cells, so adding a further stabilizing residue may help expression.

Reviewer #2

(Remarks to the Author)
The manuscript by Li et al. reports the construction of N-termini-bridged eukaryotic P450-CPR heterodimer for improved catalytic efficiency in Escherichia coli. Using a plant P450 hydroxylase (Arabidopsis thaliana CYP73A5), the authors demonstrated this type of architecture shows better biosynthetic performance than the free enzymes or tandem fused chimeras. Additional constructs were made to compare the efficiency. This approach was also applied to a human P450 enzyme, which also showed enhanced biosynthetic performance in E. coli. While this work is interesting and provides a new method to engineer P450 and CPR for improved efficiency, the lack of the structural insights into the phenomenon and overall design of the experiments lowered the enthusiasm. Below are some comments and suggestions:
Major:
1. The authors claimed that the better efficiency of the N-termini-bridged heterodimer is due to improved electron transfer. It is unclear whether the formation of this heterodimer would affect other important factors such as the substrate binding.
2. Trans-cinnamic acid was used to test the hydroxylation efficiency of CYP73A5-related constructs in E. coli. Why did the authors choose to co-express PAL with the tested enzymes? It is hard to determine the expression level of each enzyme after IPTG induction in a multiple-enzyme system, especially in a glucose-containing medium. Would it be easier and more accurate to compare the efficiency by just feeding the substrate into the broth of E. coli?
3. It would be more appropriate to say “generalizable” after testing more P450 enzymes.
4. Statistical significance should be analyzed for the comparison of the biosynthetic performance.
5. Page 16, lines 307-311: “…SnoopSystem could manage the architectural conformation… in a more flexible mode”; Why?
6. Page 22, Fig. 5c: It seems that there is no statistically significant difference in the molar yield between the free floating enzymes and SnoopSystem-mediated N-termini-bridged heterodimer.
7. Page 18, lines 356-361: “…produced a protein orientation more favorable for electron transfer…”; Would be better to show a structural model to support this statement.

Minor:

8. Abstract: Provide the full name of “E. coli”
9. Page 4, line 78: fix the typo “Toxal”
10. Page 14, legend of Fig. 4, line 275: “site-direct” should be “site-directed”

Reviewer #3

(Remarks to the Author)
What are the major claims of the paper?

The paper claims that P450 mediated oxofuctionalization in the endomembrane free chassis E. coli can be substantially improved compared to existing strategies by an N-to-N fusion of P450 and CPR. They also claim that the strategy can be used for type II P450/CPR pairs of diverse origin.

Will the paper be of interest to others in the field?

P450s are wide spread in natural product pathways and are notoriously difficult to express well, particularly in prokaryotes. The new engineering strategy presented in the paper will be of interest to many others in synthetic biology.

Will the paper influence thinking in the field?

The paper presents a novel and effective engineering strategy for P450 expression in prokaryotic hosts. Even though self-

assembling peptides are not novel, they use them in a new way. The findings draw attention to the importance of fusion topology topology in protein engineering and that mimicking the native topology of interaction partners can be beneficial.

Are the claims convincing? If not, what further evidence is needed?

The results clearly demonstrates that N-to-N P450-CPR fusion (achieved through self-assembling peptide tags) results in the highest improvement in titer/specific productivity when comparing the four possible fusion topologies: C-to-N, N-to-C, N-to-N, and C-to-C.
They show that an N-to-N topology improves the titer from both a plant and human P450/CPR pair. However they only show that this orientation is the best for the plant P450/CPR enzymes.
They assume that any improvements in titer/specific productivity are due to improved interaction between P450 and CPR, even though enzyme abundance and solubility are also important for product formation. Since they only show limited data on protein abundance (Western blots of 5 samples in fig S3) they cannot exclude that the differences in titer are at least partially because of differences in enzyme abundance/solubility.

Are there other experiments that would strengthen the paper further? How much would they improve it, and how difficult are they likely to be?

As the authors state in the introduction, many biosynthetic pathways require more than one P450. It would strengthen the paper further if they showed that their strategy simultaneously can be applied to at least two P450 steps in the same pathway. This should not be difficult to achieve for a lab experienced in synthetic biology.
If the manuscript is unacceptable in its present form, does the study seem sufficiently promising that the authors should be encouraged to consider a resubmission in the future?
To be acceptable the author's must include a proper statistical analysis for comparing titers and specific productivities of the different P450/CPR designs.
They must also show protein abundance of all constructs, preferably with a quantitative method to show that differences in titer/specific activity are due to difference in enzymatic activity and not just enzyme abundance. They should also show the ratio of dimerization for all heterodimeric constructs, like they did for one construct in the western blot in fig S3. This is to clearly show that the observed differences in titers are not caused by difference in protein abundance/solubility or dimerization ratio.

Is the manuscript clearly written? If not, how could it be made more accessible?

It would aide understanding to include a brief explanation of the SpySystem and SnoopSystem used for post-translational dimerization.
The manuscript is mostly understandable, but sometimes the language is unclear. See the comment below for specific examples.

Is the statistical analysis of the data sound?

The authors did not present any statistical analysis of the data. This is highly problematic when they then go on to compare the effectiveness of their different P450/CPR designs. They should include a statistical analysis of both the reported titers and specific activities.

Specific comments:
Page 4: It's not clear why it should be more labor intensive to express chimeric P450-CPR fusion enzymes.

Page 11: "This N-termini-bridged heterodimer of P450 and CPR might be considered a counterpart of the eukaryotic membrane-bound P450 system" It's not clear what is meant by "counterpart". It's not known if the forced spatial orientation of the P450-CPR interaction partners is the main reason why these enzymes are membrane bound in eukaryotes.

Page 14: Fig. 4. They need to add a statistical analysis to this figure. It would also help understanding if the specific productivity for each construct was also plotted in this figure, since the authors use both measurements to compare the different P450-CPR designs.

Page 15: What is the difference between the SpySystem and SnoopSystem? Can the difference explain why there is no difference in titer when the SnoopCathcer and SnoopTag are swapped between P450 and CPR? Does the two systems perform equally well in dimerization. They should include a western blot showing the dimerization of the four different designs: SpyCatcher-CYP/SpyTag-ATR, SpyTag-CYP/SpyCathcer-ATR, SnoopCatcher-CYP/SnoopTag-ATR, & SnoopTag-CYP/SnoopCatcher-ATR
Page 17: They don't show any evidence that 8RP increases p-coumaric acid production through increased P450/CPR aggregation.

Page 17 + Page 18: On both pages the authors speculate on "turnover rate" and "biosynthetic performance" with reference to the band intensities of the western blot in fig S3. This is quite speculative and subjective. If wants to state anything on turnover rate for specific P450/CPR designs they should first quantify the soluble and insoluble protein abundance with some proteomic method e.g. SRM.

Page 19: When comparing the three designs with mutated SpySystem tags they only focus on SpyCatcherCYP73A5Δ2-

28/SpyTagmutATR2Δ2-77 and fail to address that third design SpyCatchermutCYP73A5Δ2-28/SpyTagmutATR2Δ2-77 basically has the same specific activity (0.89±0.04 μM/OD600/h vs. 0.82±0.2 μM/OD600/h).

Page 19: They can't conclude in general terms that the iso-peptide bond formation is crucial for the improvement seen by using self-assembling peptides they also show that the non-covalent heterodimer SH3CYP73A5Δ2-28/SH3ligATR2Δ2-77 has the same specific activity (1.45±0.05 μM/OD600/h) as the covalent heterodimer SpyCatcherCYP73A5Δ2-28-SpyTagATR2Δ2-77 (1.5±0.12 μM/OD600/h).

Page 19: What is meant by "multireaction-involved enzymes"?

Page 20: The description of the results for the human P450/CRP is confusion. It is not clear from the text which of the peptide tag systems perform the best.

Page 21: What is meant by "human pharmaceuticals"? Pharmaceuticals human use of pharmaceuticals made by human enzymes?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript has been substantially strengthened by a series of changes.
My comments have been addressed.
My only remaining recommendation is that it should be a small k and not a large k for each use of kcat, consistent with this being a rate and the common usage in the literature.
I am happy to recommend publication.

Reviewer #3

(Remarks to the Author)
Summary:
In this manuscript, Li et al. report that linking the N-termini of eukaryotic CYP450s and CPRs increases their catalytic activity in vitro as well as in prokaryotic E. coli. The CYP450-CPR system with N-N termini linkage architecture outperformed individual proteins and all other combinations of CYP450-CPR linkage architectures (N-C, C-N, and C-C). The authors discovered that this phenomenon is generalizable across two CYP450-CPR systems (plant and human) and three linkage types (covalent SpyTag/SpyCatcher, covalent SnoopTag/SnoopCatcher, and non-covalent SH3/SH3lig). This is an exciting result that will be interesting to the broad field of synthetic biology. While the authors have addressed many of the reviewer comments from the last round of reviews, they did not address the comment from Reviewer 2 that the lack of structural characterization/models explaining this phenomenon lowered the enthusiasm for this discovery. While I agree with this comment from Reviewer 2, I support the publication of this manuscript in Nature Communications after the following comments and suggestions have been addressed.

Major comments:
1. The central hypothesis of this work is that the relative orientation of CYP450s and CPRs has a significant impact on their catalytic activity. However, there is no structural characterization/models to support claims that the N-N, N-C, C-N, and C-C linked proteins have different relative orientations. The flexible linkers in the CYP450-CPR systems mean that different relative orientations could be adopted in each system. The authors should limit claims in their manuscript to say that the linkage architecture of CYP450s and CPRs has a significant impact on their catalytic activity.
2. The only evidence provided for the concentrations of free proteins and linked proteins in E. coli experiments were from two Western blot gels in Figure S3. The author's claims would be strengthened with densitometry analysis of their Western blot gels and/or quantitative proteomics.
3. The only structural characterization of N-N, N-C, C-N, and C-C CYP450-CPR linkage architectures were the two Western blot gels in Figure S3. The author's claims would be strengthened with additional structural characterization such as analytical SEC, MALDI-TOF MS, and/or native PAGE.
4. I agree with a comment from Reviewer 1 in the previous round of reviews that the manuscript be edited to remove meaningless significant figures from experimental numbers. For example, on line 142, a tier is reported of 201.31 ± 59.21 μM. There is no way that the titer is precisely measured to two decimal places when the standard deviation is nearly 60. Please revise the manuscript to use appropriate numbers of significant figures.
5. While it is not necessary for the publication of this manuscript, It would be interesting to see if this result is generalizable to many more CYP450-CPR systems or many more microorganisms.

Minor comments:
1. The abbreviation of SOPA for spatial orientation-guided protein assembly is unnecessary. Please remove this abbreviation from the paper.
2. In Figure 2C, there are data points with no error bars. If there are error bars behind these data points, can the caption be

revised to indicate this?
3. In the schematic representations of CYP450s (large blue arrows) and CPRs (large green arrows), it is unclear what the small arrows deformations represent. Please clarify their meaning in figure captions.
4. The abbreviations NADPH, FAD, and FMN (lines 56-57) are used without introducing what they mean.
5. There should be a "." following E in E. coli (line 61).
6. The sentence "[t]hese existing mismatches lead eukaryotic P450 systems suffering from poor solubility, weak functionality and low turnover rate in microbes, especially in prokaryotic host s such as E coli" (lines 63-65) is a fragment.
7. The statement "no less than eight P450s involved in Taxol biosynthesis" (lines 75-76) should be revised to "no less than eight P450s are involved in Taxol biosynthesis".
8. The use of "[i]n consistent" (line 191) is confusing. Replace this with whichever of "consistent" or "inconsistent" is correct.
9. It is not clear what "oriental orchestration" (line 587) means. Please replace this term.
10. In section 5.3, "ml" should be rewritten to "mL" in multiple instances.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The revised manuscript adequately addressed my major concerns from the previous draft. Overall, I am pleased with the changes and would gladly recommend this manuscript for publication in Nature Communications.

Responses to Reviewer #1 (Remarks to the Author):

> This manuscript describes the use of different approaches to enhance the function of plant and human cytochrome P450/P450 reductase enzyme pairs in *E. coli*. Direct genetic fusion, SH3/peptide and Spy/Snoop Tag/Catcher ligation are tested. The leading system they test leads to an increase in biosynthetic yield. The strength of this paper lies in the good novelty of the application of the isopeptide covalent ligation for this enhancement of enzyme function and the substantial improvements obtained. Only a few examples are tested so it is a little premature to say if this will be a general solution to a broad problem, but the results are certainly promising and could generate substantial interest.

We thank the reviewer for her/his positive comments.

> The weakness of the manuscript is that the extensive literature for spatial organisation of interacting enzymes is not really explained and the results are not sufficiently put in the context of this.

We appreciate the reviewer for the critical comment. In the revised manuscript, we reorganized the discussion, and focused on in detail the spatial organization of interacting enzymes besides eukaryotic P450 system. And also, we discussed the results in the context of the spatial organization of interacting enzymes in the revised manuscript.

> In addition, a common weakness of the field is that it is often assumed that the effects on product generation are a result of spatial relationships between two enzymes, when often the differences were subsequently found to relate to differences in expression levels or catalytic activities of each individual unit, which can be surprisingly sensitive to specific fusion proteins (see for example

The role of dynamic enzyme assemblies and substrate channelling in metabolic regulation. Lee J. Sweetlove & Alisdair R. Fernie. Nature Communications volume 9, Article number: 2136 (2018)). There is insufficient data in this manuscript to exclude this alternative explanation.

We appreciate the reviewer for pointing out this professional problem.

In the revised manuscript, we supplemented and performed the *in vitro* enzyme kinetics experiment to circumvent the potential differences in the intracellular expression levels and to explore the effect of the peptide-based spatial organization on the function of eukaryotic P450. As shown in **Section Results 2.3** in the revised manuscript, we purified the components of the four SpySystem-based CYP73A5$^{\Delta 2\text{-}28}$-ATR2$^{\Delta 2\text{-}77}$ heterodimers as well as the free-floating individuals (CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$). We then quantified the proteins, mixed the components *in vitro* at an equimolar ratio to organize the architecture of CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$ pair, and measured the enzymatic activities of the reconstructed CYP73A5 enzymes and the catalytic efficiencies of the heterodimers in the tube.

In the present of excess electron donor NADPH, SpyCatcher-appended CYP73A5 in the heterodimer (I), (II), (III) and (IV) showed a K_{cat} value of 11.00-, 4.72-, 1.11- and 48.94-fold higher than the free-floating truncated CYP73A5$^{\Delta 2\text{-}28}$ for *trans*-cinnamic acid, respectively (**Table 1** in the revised manuscript). While at increasing concentrations of NADPH, heterodimer (II) and (III), containing ATR2$^{\Delta 2\text{-}77}$SpyTag component, presented a significantly reduced K_m for NADPH (**Table 2** in the revised manuscript). In terms of the catalytic efficiency (K_{cat}/K_m), the heterodimer (I), (II), (III) and (IV) were 2.84-, 2.76-, 0.52- and 10.88-fold of the dissociative form, respectively (**Table 2** in the revised manuscript).

The results demonstrated that, the appended peptide bio-machinery for the architectural organization had an effect on substrate affinity and catalytic efficiency of the reconstructed protein assemblies. As is evident from **Table 1** and **Table 2** in the revised manuscript, heterodimer (I) with the CYP102A1-like architecture and heterodimer (IV) with the N-termini-bridged architecture possessed higher turnover number, shown as K_{cat}. In detail, the turnover number and the catalytic efficiency of heterodimer (IV) were 4.45 and 3.83 times higher than those observed in heterodimer (I).

Thus, the *in vitro* quantitative results clearly illustrated that the nature-mimicking N-termini-bridged architecture of heterodimer (IV) contributed to the reconstructed eukaryotic P450 system the highest turnover number and catalytic efficiency, even though the peptide-based bio-machinery for architecture generation had an influence on substrate access and electron transfer between CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$.

> Error bars are suitably described but statistical tests are not performed on the differences seen.

Thank you very much for your critical comment. To test whether the differences among different recombinant strains were significant, a one-way analysis of variance (ANOVA) was further performed. We presented the statistical tests in **Figure 2, 3, 4** and **5** in the revised manuscript. And the method was shown in **Section 5. Materials and Methods** as **5.8 Statistical analysis** in the revised manuscript.

> "The results indicated that the isopeptide bond in the covalent N-termini-bridged heterodimers, such as covalent heterodimer (IV), improved the biosynthetic performance of the eukaryotic P450 enzyme in *E. coli* and suggested that the mechanical stability of self-assembled peptide biomachinery for spatially organizing the multireaction-involved enzymes should have a crucial effect on the biosynthetic efficiency."

Mechanical stability suggests that there is a specific force leading to separation of the two components, but no description of such a force is provided. Is not a more likely hypothesis that the covalent interaction leads to the two components being more often found associated together?

We appreciate the reviewer for the critical comment.

In the revised manuscript, we rephrased the above paragraph (Lines 420–426 in the revised manuscript). The two components of the self-assembled peptide bio-machinery SpySystem, used in this study, can spontaneously form a covalent isopeptide bond between the carboxyl group in the side chain of Asp7 in SpyTag peptide and the amino group in the side chain of Lys34 in SpyCatcher peptide [1] (Lines 174–176 in the revised manuscript). In this process, the Glu80 in SpyCatcher peptide is involved in the covalent side chain self-assembly of SpyTag^{D7} and SpyCatcher^{K34} [1] (Lines 408–411 in the revised manuscript).

We performed the site-directed mutation of these participant amino acids to abrogate the formation of the covalent isopeptide bond in SpySystem and test the role of the covalent isopeptide bond in the improvement of CYP73A5 function. The production of *p*-coumaric acid dropped to 39.77% (SpyCatcher^{E80Q}), 67.35% (SpyTag^{D7A}) and 60.05% (both SpyCatcher^{E80Q} and SpyTag^{D7A}) of heterodimer IV (SpyCatcher^{E80} and SpyTag^{D7}), respectively (**Figure 4 panel a** and **e** in the revised manuscript). Besides, the noncovalent N-termini-bridged heterodimers (VIII) and (IX), based on the SH3/SH3lig affinity system [2], displayed an output of 82.91% and 54.67% of heterodimer (IV), respectively (**Figure 4 panel a** and **d** in the revised manuscript).

Furthermore, with the other covalent self-assembled peptide bio-machinery SnoopSystem, the highest *in vivo* output of N-termini-bridged heterodimers (VI) and (VII) was quantitatively comparable to that of heterodimers based on SpySystem (**Figure 4 panel a** and **b** in the revised manuscript). In SnoopSystem, the amino group

in the side chain of Lys9 in SnoopTag peptide is able to spontaneously form a covalent isopeptide bond with the amide group in the side chain of Asn106 in SnoopCatcher peptide [3] (Lines 338–341 in the revised manuscript).

These results indicated that, among the reconstructed N-termini-bridged plant P450 systems, the N-terminal covalent joint of plant P450 and the redox partner was superior to the N-terminal noncovalent tethering in improving the biosynthetic performance of eukaryotic P450 enzyme in *E. coli*. The interaction between SpyTag peptide and SpyCatcher peptide of SpySystem via an amide linkage has been proved to be irreversible and bear a force exceeding 1 nN where covalent bonds snap [1]. However, the noncovalent affinity of the interacting peptide pair is susceptible to the changes in the environmental conditions (pH, temperature, concentration, folding, ionic strength, redox potential, etc.). And also, the P450-involved oxyfunctionalization is an oxidation-reduction reaction. Hence, we suggested that the mechanical stability of the self-assembled peptide bio-machinery, employed for spatially organizing a multienzyme cascade bioreactor, could have a considerable effect on the efficiency of biosynthesis.

Minor points:

Line 78, Taxol, not Toxal

Thank you very much for your critical suggestion. We have fixed the typo “Toxal” to “Taxol” in the revised manuscript (Line 76).

Line 84: originally derived from cow, or originally derived from the bovine sequence

Thank you very much for your critical suggestion. The sentence has been rephrased to “an eight-residue peptide (8RP) originally derived from the N-terminus of bovine

CYP17A1 has recently been found to promote CYP725A4-based taxadiene oxyfunctionalization" in the revised manuscript (Line 81-85).

Fig. 1b,c: it might be worth drawing in the outer membrane of *E. coli*

Thank you very much for your critical suggestion. We redrew the *E. coli* cell diagrams with the outer membrane in the revised manuscript (**Figure 1 b** and **c**).

"The recombinant *E. coli* strain without the chimera accumulated 590.63 μM *trans*-cinnamic acid with no *p*-coumaric acid at 48 h postinduction (hpi) (Fig. 2c), and its cell growth was severely inhibited (Fig. S1). As shown in Fig. 2c, the recombinant strain with chimera (i) produced 201.31 μM *p*-coumaric acid"

Often numbers are presented here to 5 significant figures, but given the error bars on all measurements, it is unlikely that the values should be presented so precisely.

Thank you very much for your critical suggestion. All the constructs in this study were performed at least three independent experiments, and the data shown in the figures were calculated as mean ± SEM (standard error of mean). According to your advice, we presented all experimental data as mean ± SEM in **Section Results** in the revised manuscript.

Fig. 2a Draw chirality of phenylalanine

Thank you very much for your critical suggestion. The chirality in the structural formula of phenylalanine in **Fig. 2a** has been drawn.

It might help readers to provide the common name of 4-acetamidophenol: Paracetamol/acetaminophen.

Thank you very much for your critical suggestion. According to your advice, 4-acetamidophenol was replaced with its common name, acetaminophen, throughout the revised manuscript.

It would be more conventional to draw in the hydrogen on the amide nitrogen in phenacetin and 4-acetamidophenol.

Thank you very much for your critical suggestion. According to your advice, the hydrogen on the amide nitrogen in phenacetin and 4-acetamidophenol (rephrased as its common name, acetaminophen, in the revised manuscript) in **Fig. 5b** has been drawn in.

Fig. 5c: the error bars on the inset graph are large, making a firm conclusion about the effects difficult?

Thank you very much for your critical suggestion.

To further stress the importance of the architecture on the biosynthetic performance of human P450, we redesigned the expression pattern of human P450 system in the revised manuscript. In details, we additionally constructed the CYP102A1-like tandem fusion and the other three SpySystem-based heterodimers of CYP1A2 and HsCPR according to the architectural organization of plant CYP73A5 and ATR2. The results indicated that the heterodimer with an N-termini-bridged architecture, SpyCatcherCYP1A2$^{\Delta 2\text{-}37}$-SpyTagHsCPR$^{\Delta 2\text{-}52}$, had superior biosynthetic performance among the tandem fusion, the SpySystem-based assemblies as well as the free-floating individuals.

With regard to the yield of acetaminophen to the substrate phenacetin, the results showed that there was no significant difference among all the reconstructed CYP1A2s,

because the same host, *E. coli* BL21(DE3), was used for the expression of the reconstructed CYP1A2s.

"The weak electrostatic interaction between the FMN domain of CPR and the heme domain of P450 may pledge a mutual recognition of these two domains, by which an orientation-dependent interface fitness would presumably be conducted between CPR and P450 for the correlative reactive activity."

The English would benefit from editing here.

Thank you very much for your critical suggestion. According to your advice, we have re-organized the Discussion Section in the revised manuscript.

"The nitrogen-free buffer contained 100 mM 3-(N-morpholino) propane sulfonic acid (MOPS)"

MOPS itself contains a nitrogen- please rephrase.

Thank you very much for your critical suggestion. According to your advice, we have rephrased "nitrogen-free buffer" to "biotransformation buffer" in the revised manuscript (Line 673-676).

Fig. S3: The methods give insufficient detail on Western blotting. Similarly the legends don't make it clear what antibody was used. This looks like a direct visible light image rather than chemiluminescent detection?

Thank you very much for your critical suggestion. According to your advice, more details about the method for Western blotting were given in the revised manuscript (Lines 686-694). And the antibodies used in Western blotting were also shown in the legend of the **Fig. S3** in the revised supplementary file.

Fig. S6: image resolution should be improved

Thank you very much for your critical suggestion. According to your advice, the image resolution of **Fig. S6**, renumbered as **Fig. S9** in the revised supplementary file, was improved.

> It looks like there was no spacer between the fusion peptides and the enzymes. Often a spacer can improve folding/expression yield.

Thank you very much for your critical suggestion.

In this study, we attempted to investigate the effect of the reconstructed architecture of eukaryotic P450 system on the biosynthetic performance of eukaryotic P450 in the prokaryotic host *Escherichia coli*. In practice, the self-assembled peptide bio-machinery was employed for the architectural organization of eukaryotic P450 system. It is indeed that, during the initial trials for biosynthesis *in vivo*, we did not add any additional spacer between the peptide biobricks and the component of eukaryotic P450 system. Our motivation for doing so was based on two considerations.

The first was that the engineered N-terminus has an effect on the level of expression, the solubility and the activity of eukaryotic P450s [4]. Considering that as a part of the terminus-modified component, and based on that the goal of this study was to investigate the effect of the architectural organization on the biosynthetic performance of eukaryotic P450 system in *E. coli*, the additional spacer would make the architectural organization complicated.

The second was that we noticed that, when we modeled the structures, there are disordered regions in both N-terminus and C-terminus of the truncated P450 and the redox partner as well as the self-assembled peptide bio-machinery. In details, the N-terminal 16 amino acids and the C-terminal 3 amino acids of CYP73A5$^{\Delta 2\text{-}28}$ are

likely disordered. And the N-terminal 26 amino acids and the C-terminal 1 amino acid of ATR2$^{\Delta 2\text{-}77}$ are also likely disordered. With regard to the SpySystem, the N-terminal 6 amino acids and the C-terminal 11 residues of SpyCatcher are disordered; the small SpyTag peptide only contains one β-sheet while the C-terminal 7 amino acids are disordered [1].

Indeed, we found that the appended peptide bricks, rigidly fused to the C-terminus of eukaryotic P450 system, were detrimental to the reconstructed protein in terms of the turnover number, the catalytic efficiency and the biosynthetic performance (**Table 1** and **2**, and **Figure 3** in the revised manuscript).

It would be beneficial to the spatial fitness and the conformational dynamics during the electron transfer between CPR and P450 via adding a suitable spacer between eukaryotic P450 system and the self-assembled peptide bricks.

It should be pointed out that, when we performed the *in vitro* enzyme kinetic assays, the hexahistidine tags added for protein purification were sandwiched by two flexible linkers (6×His-tagged CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$ in **Figure S10** in the revised supplementary file) to circumvent the potential effect of the affinity tag on the enzymatic activity. During the Western blotting, two octapeptides rich in Gly and Ser were added between the eukaryotic P450 system and the C-terminus-appended tags (C-terminus-tagged proteins for Western Blotting in **Figure S10** in the revised supplementary file) in order to stretch the tags as far as possible.

> Also the residue after the initiating methionine in some constructs is K, which can destabilize the protein inside cells, so adding a further stabilizing residue may help expression.

Thank you very much for your critical suggestion.

Considering that eukaryotic P450s as well as their shared redox partner usually reside in inner membranes in eukaryotic cell, we truncated the N-terminal inner-membrane anchors and expressed the cytoplasmic regions of eukaryotic P450 system in prokaryotic *E. coli* cells. N-terminal 28 amino acids of CYP75A5 were predicted to participate in the subcellular localization, as the inner-membrane anchor of a CYP73A protein from *Lycoris aurea* characterized in protoplasts of *Arabidopsis thaliana* [5].

As you pointed out, the second residue of CYP73A5$^{\Delta 2\text{-}28}$ and the self-assembled peptide bio-machinery (SnoopTag and SnoopCatcher) used in this study is K. The basic Lys amino acid may facilitate the hydrophilicity of the reconstructed N-terminus. And we also retrieved the Met-Lys initiating protein from the protein bank encoded by the genome of *E. coli* BL21(DE3), and found 611 proteins, accounting for 14.78% of the whole proteome.

Based on the fact that the N-terminus of a protein plays a universal role in the expression, the solubility and the stability, etc., the bio-bricks for the N-terminal reconstruction are required further optimization not only in amino acid sequence but also in coding sequence.

1. Zakeri B, Fierer JO, Celik E, Chittock EC, Schwarz-Linek U, Moy VT*, et al.* Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(12)**:** E690-697.
2. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV*, et al.* Synthetic protein scaffolds provide modular control over metabolic flux. *Nature biotechnology* 2009, **27**(8)**:** 753-759.
3. Veggiani G, Nakamura T, Brenner MD, Gayet RV, Yan J, Robinson CV*, et al.* Programmable polyproteams built using twin peptide superglues. *Proceedings of the National Academy of Sciences* 2016, **113:** 1202-1207.
4. Biggs BW, Lim CG, Sagliani K, Shankar S, Stephanopoulos G, De Mey M*, et al.* Overcoming heterologous protein interdependency to optimize P450-mediated Taxol precursor synthesis in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 2016, **113**(12)**:** 3209-3214.
5. Li Y, Li J, Qian B, Cheng L, Xu S, Wang R. De Novo Biosynthesis of *p*-Coumaric Acid in *E. coli* with a trans-Cinnamic Acid 4-Hydroxylase from the Amaryllidaceae Plant *Lycoris*

aurea. *Molecules* 2018, **23**(12).

Responses to Reviewer #2 (Remarks to the Author):

The manuscript by Li et al. reports the construction of N-termini-bridged eukaryotic P450-CPR heterodimer for improved catalytic efficiency in *Escherichia coli*. Using a plant P450 hydroxylase (*Arabidopsis thaliana* CYP73A5), the authors demonstrated this type of architecture shows better biosynthetic performance than the free enzymes or tandem fused chimeras. Additional constructs were made to compare the efficiency. This approach was also applied to a human P450 enzyme, which also showed enhanced biosynthetic performance in *E. coli*. While this work is interesting and provides a new method to engineer P450 and CPR for improved efficiency, the lack of the structural insights into the phenomenon and overall design of the experiments lowered the enthusiasm. Below are some comments and suggestions:

We thank the reviewer for her/his critical comments.

Major:

1. The authors claimed that the better efficiency of the N-termini-bridged heterodimer is due to improved electron transfer. It is unclear whether the formation of this heterodimer would affect other important factors such as the substrate binding.

We appreciate the reviewer for the critical comment.

In the revised manuscript, we supplemented and performed the *in vitro* enzyme kinetics experiment to explore the effect of the appended peptide bricks and the resulting spatial architectures on the enzyme properties. As shown in **Section Results 2.3** in the revised manuscript, we purified the components of the four

SpySystem-based CYP73A5$^{\Delta 2\text{-}28}$-ATR2$^{\Delta 2\text{-}77}$ heterodimers as well as the free-floating individuals (CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$). We then quantified the proteins, mixed the components *in vitro* at an equimolar ratio to organize the architecture of CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$ pair, and measured the activities of the reconstructed CYP73A5 enzymes and the catalytic efficiencies of the heterodimers in the tube.

In the present of excess electron donor NADPH, SpyCatcher-appended CYP73A5 in the heterodimer (I), (II), (III) and (IV) showed a K_{cat} value of 11.00-, 4.72-, 1.11- and 48.94-fold higher than the free-floating truncated CYP73A5$^{\Delta 2\text{-}28}$ for *trans*-cinnamic acid, respectively (**Table 1** in the revised manuscript). While at increasing concentrations of NADPH, heterodimer (II) and (III), containing ATR2$^{\Delta 2\text{-}77}$SpyTag component, presented a significantly reduced K_m for NADPH (**Table 2** in the revised manuscript). In terms of the catalytic efficiency (K_{cat}/K_m), the heterodimer (I), (II), (III) and (IV) were 2.84-, 2.76-, 0.52- and 10.88-fold of the dissociative form, respectively (**Table 2** in the revised manuscript).

The results demonstrated that, the appended peptide bio-machinery for the spatial architectural organization had an effect on substrate affinity and catalytic efficiency of the reconstructed protein assemblies.

2. *Trans*-cinnamic acid was used to test the hydroxylation efficiency of CYP73A5-related constructs in *E. coli*. Why did the authors choose to co-express PAL with the tested enzymes? It is hard to determine the expression level of each enzyme after IPTG induction in a multiple-enzyme system, especially in a glucose-containing medium. Would it be easier and more accurate to compare the efficiency by just feeding the substrate into the broth of *E. coli*?

Thank you very much for your critical comment.

In this study, our motivation was to investigate the biosynthetic performance of the reconstructed P450 enzymes in prokaryotic *Escherichia coli* via organizing in space eukaryotic P450 system, which are usually reside in the inner membrane in eukaryotic cell. Benefiting from metabolic engineering and synthetic biology, *de novo* biosynthesis of valuable compounds from the renewable biomass-derived carbohydrates such as glucose in microbes is a promising way for bio-manufacturing.

There were two else considerations for the co-expression of PAL. The first was that the oxy-functionalization catalyzed by eukaryotic P450s requires the indispensable electron donor NADPH (**Fig. 1a** in the revised manuscript), which is confined in cells and hard to permeate the cell membrane. And also, NADPH as a reduced co-factor is unstable and expensive. Therefore, we sought to the generation of NADPH from the metabolism of the carbon source. The second was that the substrate *trans*-cinnamic acid fed into the broth would be excluded by the cell due to carbon catabolite repression while a carbohydrate is also present in the broth. Hence, we investigated the biosynthetic performance of the reconstructed P450 enzymes via *de novo* biosynthesis in the cells where the enzyme PAL was co-expressed for affording the substrate of the P450.

Besides, as stated in **Table S2** in the revised supplementary file, the genes encoding eukaryotic P450 system were driven by the *T7* promoter, and the gene encoding PAL was driven by a core *trc* promoter with the consensus –10 box and –35 box of *E. coli* promoters [1]. What needs to be pointed out is that there are no binding sites for the transcriptional dual regulator CRP, cAMP receptor protein, in the upstream regions of the used promoters. So the genes encoding eukaryotic P450 system and PAL are not regulated by glucose.

3. It would be more appropriate to say “generalizable” after testing more P450 enzymes.

Thank you very much for your critical suggestion.

Based on the results of the architectural organization of plant CYP73A that N-termini-bridged protein self-assembly afforded the efficient biosynthesis of natural product in *E. coli*, we furthermore examined the effect of architectural organization on the biosynthetic performance of animal P450 system in *E. coli*. In detail, human CYP1A2, one of the major oxidative drug-metabolizing enzymes in liver, along with the redox partner HsCPR were organized with the self-assembled peptide bio-machinery SpySystem according to the spatial organization of plant CYP73A5 and ATR2. Meanwhile, the CYP102A1-like chimera was also constructed via fusing the N-terminus-truncated CYP1A2 to the N-terminus of the N-terminus-truncated HsCPR in a tandem pattern. And N-terminus-truncated CYP1A2 and HsCPR were also co-expressed individually.

The results in **Section Results 2.7** in the revised manuscript showed that the strain harboring the N-termini-bridged heterodimer, SpyCatcherCYP1A2$^{\Delta 2\text{-}37}$-SpyTagHsCPR$^{\Delta 2\text{-}52}$, produced the most drug metabolite. The results confirmed that the N-termini-bridged architecture also favored human P450 system for biosynthesis in space. Therefore, the results indicated that N-termini-bridged protein self-assembly was feasible and adaptable to organize human P450 system to improve the production of drug metabolites in *E. coli*.

We replaced “generalizable” with “adaptable” and rephrased the prior subtitle “N-termini-bridged protein self-assembly generalizable to the human P450 system in *E. coli*” to “N-termini-bridged protein self-assembly adaptable to improve the biosynthesis of human P450 system in *E. coli*” in **Section Results 2.7** in the revised manuscript.

4. Statistical significance should be analyzed for the comparison of the biosynthetic performance.

Thank you very much for your critical suggestion. We further performed a one-way analysis of variance (ANOVA) to test whether the differences among different recombinant strains were significant. We added the statistical analysis of the outputs of the P450 constructs in the revised manuscript. And the method was shown in **Section 5. Materials and Methods** as **5.8 Statistical analysis** in the revised manuscript.

5. Page 16, lines 307-311: "…SnoopSystem could manage the architectural conformation… in a more flexible mode"; Why?

Thank you very much for your critical comment.

In this study, we resorted to the self-assembled peptide bio-machinery to organize in space the architecture of eukaryotic P450 system for the biosynthesis of valuable compounds in prokaryotic *E. coli*. We found that the superior biosynthetic performance was achieved by harnessing the covalent peptide ligation system (either SpySystem or SnoopSystem) in an N-termini-bridged pattern. Quantitively, the highest *in vivo* outputs of N-termini-bridged heterodimers based on either SnoopSystem or SpySystem were comparable (**Figure 4 panel a** and **b** in the revised manuscript).

Via swapping the N-terminus-appended peptide biobricks, we further investigated the biosynthetic performance of the assemblies in terms of configuration. The results indicated that the N-terminal SpySystem swapping led to a reduction of 50.75% in the production (509.54 ± 46.64 μM & 1034.64 ± 34.86 μM) (**Figure 4 panel a** in the revised manuscript), while the N-terminal SnoopSystem swapping resulted in a margin of 13.05% (896.87 ± 102.60 μM & 1031.46 ± 60.84 μM) (**Figure 4 panel b** in the revised manuscript). The results suggested that SnoopSystem-based N-termini

reconstruction of CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$ might afford the N-termini-bridged heterodimers with an enhanced structural fitness.

SpySystem is derived from the second immunoglobulin-like collagen adhesin domain (CnaB2) of the fibronectin binding protein FbaB from *Streptococcus pyogenes* [2]. SnoopSystem is derived from the D4 Ig-like domain of the pilus-associated adhesin RrgA from *Streptococcus pneumoniae* [3]. There is an amino acid triad for the formation of the intramolecular isopeptide bond in CnaB2 domain or D4 domain. Both of CnaB2 domain and D4 domain are highly reminiscent IgG domain, and have similar folding in the structure. This may be the reason for the robust output of SpySystem-based and SnoopSystem-based CYP73A5-ATR2 heterodimers (**Figure 4 panel a** and **b** in the revised manuscript).

SpySystem and SnoopSystem were developed by splitting in the loop of the relevant domain. Nevertheless, the prominent difference between the SpySystem and the SnoopSystem is that the SpyTag peptide in the SpySystem is split from the C-terminus of CnaB2 domain while the SnoopTag peptide in the SnoopSystem is split from the N-terminus of D4 domain. This may be the reason for the different pattern that SpySystem and SnoopSystem afforded the N-termini-bridged CYP73A5$^{\Delta 2\text{-}28}$-ATR2$^{\Delta 2\text{-}77}$ heterodimers superior biosynthetic performance. In detail, the SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$-SpyTagATR2$^{\Delta 2\text{-}77}$ heterodimer is more active than the SpyTagCYP73A5$^{\Delta 2\text{-}28}$-SpyCatcherATR2$^{\Delta 2\text{-}77}$ heterodimer (**Figure 4 panel a** in the revised manuscript), while the SnoopTagCYP73A5$^{\Delta 2\text{-}28}$-SnoopCatcherATR2$^{\Delta 2\text{-}77}$ heterodimer is superior than the SnoopCatcherCYP73A5$^{\Delta 2\text{-}28}$-SnoopTagATR2$^{\Delta 2\text{-}77}$ heterodimer (**Figure 4 panel b** in the revised manuscript).

The second structures of the connectors may have a potential inherent role in the conformational dynamics of the assemblies when the self-assembled peptide bio-machinery was directly connected to eukaryotic P450 system. A larger loop

between SnoopCatcher and SnoopTag may endow a higher degree in the flexible organization of SnoopSystem-based heterodimers.

6. Page 22, Fig. 5c: It seems that there is no statistically significant difference in the molar yield between the free floating enzymes and SnoopSystem-mediated N-termini-bridged heterodimer.

Thank you very much for your critical comment.

To further stress the importance of the architecture on the biosynthetic performance of human P450, we redesigned the expression pattern of human P450 system in the revised manuscript. In details, we additionally constructed the CYP102A1-like tandem fusion and the other three SpySystem-based heterodimers of CYP1A2 and HsCPR according to the architectural organization of plant CYP73A5 and ATR2. The results indicated that the covalent heterodimer with an N-termini-bridged architecture had superior biosynthetic performance among the tandem fusion, the SpySystem-based assemblies as well as the free-floating individuals.

With regard to the yield of acetaminophen to the substrate phenacetin, the results showed that there was no significant difference among all the reconstructed CYP1A2s, because the same host, *E. coli* BL21(DE3), was used for the expression of the reconstructed CYP1A2s.

7. Page 18, lines 356-361: "…produced a protein orientation more favorable for electron transfer…"; Would be better to show a structural model to support this statement.

Thank you very much for your CRITICAL comment.

At the moment, the structure modelling of one protein is based on either homologous template having a resolved structure or evolutionary data extracted from many homologous sequences via deep learning. *Ab initio* protein folding and protein structure prediction is performed from amino acid sequence only via harnessing atomic-level knowledge-based force field, but is competent in small fragments (no more than 500 residues long). While as for the artificial chimeric proteins, especially those without template targets, the method for modelling the entire structure is scarce. And the routine method is the manual docking followed by molecular dynamics optimization.

We have attempted to model the structure of the peptide-mediated heterodimers. However, with regards to the peptide-based heterodimers containing four protein modules in this study, it is hard to model a valuable structure. Protein-protein docking modeling has showed that, among four SpySystem-tethered CYP73A5-ATR2 assemblies with distinct architectures, the distances between heme cofactor and FMN cofactor were all more than the distance (<= 14 Å) for the rapid intramolecular electron transfer, that may be due to the absence of a cocrystalline structure of eukaryotic P450 systems. Considering that there is a cocrystalline complex of P450 enzyme together with the FMN domain of the cognate redox partner, the FMN domain of ATR2 protein was subsequently docked to CYP73A5 enzyme under the architectural organization of SpySystem, and the distances of FMN and heme cofactors were reduced to about 18 Å. At present, we are working hard on the structure identification via purifying the peptide-based heterodimers as an entirety, and try to elucidate the mechanism underlying the architectural organization of eukaryotic P450 system based on the SpySystem.

The supplemental *in vitro* enzymatic assays (**Section Results 2.3** in the revised manuscript) demonstrated that the peptide-based architecture used to organize eukaryotic P450 system in *E. coli* affected not only the substrate affinity but also the catalytic efficiency for the improvement of biosynthetic performance resulted from

the peptide-based architectural organization. And the configuration of the peptide-based heterodimers also had an influence on the biosynthetic performance. And via abolishing the covalent isopeptide bond, we found that the tightening of the peptide-based heterodimers is also of influence on the biosynthetic performance.

Minor:

8. Abstract: Provide the full name of "*E. coli*"

Thank you very much for your critical suggestion. According to your advice, the full name of "*E. coli*" has been provided in the abstract of the revised manuscript (Line 20-21).

9. Page 4, line 78: fix the typo "Toxal"

Thank you very much for your critical comment. We have fixed the typo "Toxal" to "Taxol" in the revised manuscript (Line 76).

10. Page 14, legend of Fig. 4, line 275: "site-direct" should be "site-directed"

Thank you very much for your critical comment. We have modified "site-direct" to "site-directed" in the legend of **Figure 4** in the revised manuscript (Line 329-331 and 408-411).

1. Brosius J, Erfle M, Storella J. Spacing of the -10 and -35 regions in the tac promoter. Effect on its in vivo activity. *The Journal of biological chemistry* 1985, **260**(6)**:** 3539-3541.
2. Zakeri B, Fierer JO, Celik E, Chittock EC, Schwarz-Linek U, Moy VT*, et al.* Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(12)**:** E690-697.
3. Veggiani G, Nakamura T, Brenner MD, Gayet RV, Yan J, Robinson CV*, et al.*

Programmable polyproteams built using twin peptide superglues. *Proceedings of the National Academy of Sciences* 2016, **113:** 1202-1207.

Responses to Reviewer #3 (Remarks to the Author):

1. What are the major claims of the paper?

The paper claims that P450 mediated oxofunctionalization in the endomembrane free chassis *E. coli* can be substantially improved compared to existing strategies by an N-to-N fusion of P450 and CPR. They also claim that the strategy can be used for type II P450/CPR pairs of diverse origin.

We thank the reviewer for her/his critical comment.

2. Will the paper be of interest to others in the field?

P450s are wide spread in natural product pathways and are notoriously difficult to express well, particularly in prokaryotes. The new engineering strategy presented in the paper will be of interest to many others in synthetic biology.

We appreciate the reviewer for the positive comment.

3. Will the paper influence thinking in the field?

The paper presents a novel and effective engineering strategy for P450 expression in prokaryotic hosts. Even though self-assembling peptides are not novel, they use them in a new way. The findings draw attention to the importance of fusion topology in protein engineering and that mimicking the native topology of interaction partners can be beneficial.

We appreciate the reviewer for the positive comments.

4. Are the claims convincing? If not, what further evidence is needed?

The results clearly demonstrates that N-to-N P450-CPR fusion (achieved through self-assembling peptide tags) results in the highest improvement in titer/specific productivity when comparing the four possible fusion topologies: C-to-N, N-to-C, N-to-N, and C-to-C.

Thank you very much for your critical comment.

They show that an N-to-N topology improves the titer from both a plant and human P450/CPR pair. However they only show that this orientation is the best for the plant P450/CPR enzymes.

Thank you very much for your critical comment.

To further stress the importance of the architecture on the biosynthetic performance of human P450, we supplemented the expression pattern of human P450 system in the revised manuscript (**Section Results 2.7**). In details, we constructed the CYP102A1-like tandem fusion and the other three SpySystem-based heterodimers (C-to-N, N-to-C, and C-to-C) of CYP1A2 and HsCPR according to the architectural organization of plant CYP73A5 and ATR2. The results also indicated that the heterodimer with an N-termini-bridged architecture had superior biosynthetic performance among the tandem fusion, the SpySystem-based assemblies as well as the free-floating individuals.

They assume that any improvements in titer/specific productivity are due to improved interaction between P450 and CPR, even though enzyme abundance and solubility are also important for product formation. Since they only show limited data on protein abundance (Western blots of 5 samples in fig S3) they

cannot exclude that the differences in titer are at least partially because of differences in enzyme abundance/solubility.

We appreciate the reviewer for pointing out this professional problem.

In the revised manuscript, to circumvent the potential differences in the intracellular protein abundance or solubility, we further *in vitro* measured the enzymatic activities of the four SpySystem-based heterodimers in a quantitative manner. As shown in **Section Results 2.3** in the revised manuscript, we purified the components of the four SpySystem-based CYP73A5$^{\Delta 2\text{-}28}$-ATR2$^{\Delta 2\text{-}77}$ heterodimers as well as the free-floating individuals (CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$). And then, we quantified the proteins and mixed the components *in vitro* at an equimolar ratio in the tube.

In the present of excess electron donor NADPH, SpyCatcher-appended CYP73A5 in the heterodimer (I), (II), (III) and (IV) showed a K_{cat} value of 11.00-, 4.72-, 1.11- and 48.94-fold higher than the free-floating truncated CYP73A5$^{\Delta 2\text{-}28}$ for *trans*-cinnamic acid, respectively (**Table 1** in the revised manuscript). While at increasing concentrations of NADPH, in terms of the catalytic efficiency (K_{cat}/K_m), the heterodimer (I), (II), (III) and (IV) were 2.84-, 2.76-, 0.52- and 10.88-fold of the dissociative form, respectively (**Table 2** in the revised manuscript).

As is evident from **Table 1** and **Table 2** in the revised manuscript, heterodimer (I) with the CYP102A1-like architecture and heterodimer (IV) with the N-termini-bridged architecture possessed higher turnover number for the substrate *trans*-cinnamic acid, shown as K_{cat}. In particular, it is worth pointing out that the turnover number and the catalytic efficiency of heterodimer (IV) were 4.45 and 3.83 times higher than those observed in heterodimer (I).

Hence, the *in vitro* quantitative results clearly illustrated that the nature-mimicking N-termini-bridged architecture of heterodimer (IV) contributed to the reconstructed

eukaryotic P450 system the highest turnover number and catalytic efficiency, even though the peptide-based bio-machinery for architecture generation had an influence on the substrate affinity of CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$.

5. Are there other experiments that would strengthen the paper further? How much would they improve it, and how difficult are they likely to be?

As the authors state in the introduction, many biosynthetic pathways require more than one P450. It would strengthen the paper further if they showed that their strategy simultaneously can be applied to at least two P450 steps in the same pathway. This should not be difficult to achieve for a lab experienced in synthetic biology.

Thank you very much for your critical and valuable suggestion.

To test whether our peptide-based self-assembly strategy supports the biosynthesis of natural products that involve in two or more P450 enzymes, we attempted to biosynthesize caffeic acid by introducing another P450 enzyme in the established plant *p*-coumaric acid pathway in *E. coli*. In plant, *p*-coumaric acid is converted to caffeic acid by *Arabidopsis thaliana* CYP98A3 [1]. Although CYP98A3 has been used for caffeic acid production in *Saccharomyces cerevisiae* via constructing an artificial pathway [2], the plant caffeic acid biosynthetic pathway containing two P450 steps is not yet constructed in microbes.

Herein, we reconstructed the N-terminus of *A. thaliana* CYP98A3 with the SpyCatcher peptide as CYP73A5. We then introduced the N-terminus-reconstructed SpyCatcherCYP98A3$^{\Delta 2\text{-}29}$ into the strain JIB1924 harboring the superior N-termini-bridged heterodimer based on SpySystem. The fermentation results showed that the strain harboring SpyTagATR2$^{\Delta 2\text{-}77}$, SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$ SpyCatcherCYP98A3$^{\Delta 2\text{-}29}$ and AthPAL1 was confirmed to produce authentic caffeic

acid via LC-MS analysis. The result demonstrated that SpyTagATR2 could be shared by SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$ and SpyCatcherCYP98A3$^{\Delta 2\text{-}29}$, and support the function of both P450 enzymes. The sharing mechanism of the P450 redox partner in the peptide-based self-assembly strategy was resemble to the fact that there are hundreds of P450 enzymes accompanied by only one to four redox partners in eukaryotic cells [3]. We intend to publish the follow-up study as an independently story of constructing the plant caffeic acid biosynthesis pathway in prokaryote *E. coli*.

6. If the manuscript is unacceptable in its present form, does the study seem sufficiently promising that the authors should be encouraged to consider a resubmission in the future?

To be acceptable the author's must include a proper statistical analysis for comparing titers and specific productivities of the different P450/CPR designs.

Thank you very much for your CRITICAL and valuable suggestion.

We further performed a one-way analysis of variance (ANOVA) to test whether the differences among different recombinant strains were significant. We added the statistical analysis of the outputs of the P450 constructs in the revised manuscript. And the method was shown in **Section 5. Materials and Methods** as **5.8 Statistical analysis** in the revised manuscript.

They must also show protein abundance of all constructs, preferably with a quantitative method to show that differences in titer/specific activity are due to difference in enzymatic activity and not just enzyme abundance. They should also show the ratio of dimerization for all heterodimeric constructs, like they did for one construct in the western blot in fig S3. This is to clearly show that the observed differences in titers are not caused by difference in protein abundance/solubility or dimerization ratio.

Thank you very much for your critical comment.

To circumvent the differences of intracellular protein abundance and quantitatively measure the enzymatic activities of the reconstructed CYP73A5 enzymes, we further purified the SpySystem-appended CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$ to depict the enzyme kinetics *in vitro*. The *in vitro* quantitative results in **Table 1** and **Table 2** in the revised manuscript showed that the N-termini-bridged heterodimer had the highest turnover rate (K_{cat}) and catalytic efficiency, despite the appended SpySystem had an influence on substrate access (**Section Results 2.3** in the revised manuscript).

7. Is the manuscript clearly written? If not, how could it be made more accessible?

It would aide understanding to include a brief explanation of the SpySystem and SnoopSystem used for post-translational dimerization.

Thank you very much for your valuable suggestion. We stated and explained the consideration of the use of the SpySystem and the SnoopSystem for post-translational dimerization (Line 174-176 and 338-341 in the revised manuscript).

The manuscript is mostly understandable, but sometimes the language is unclear. See the comment below for specific examples.

Thank you very much for your critical suggestion. We have asked for a native English speaker to assist us make a language polishing. And we modified the phrase and the sentence mentioned in the below comments throughout the revised manuscript, and responded point-to-point.

8. Is the statistical analysis of the data sound?

The authors did not present any statistical analysis of the data. This is highly problematic when they then go on to compare the effectiveness of their different P450/CPR designs. They should include a statistical analysis of both the reported titers and specific activities.

Thank you very much for your critical and valuable suggestion. We further performed a one-way analysis of variance (ANOVA) to test whether the differences among different recombinant strains were significant. We added the statistical analysis of the outputs of the P450 constructs in the revised manuscript. And the method was shown in **Section Materials and Methods - 5.8 Statistical analysis** in the revised manuscript.

Specific comments:

9. Page 4: It's not clear why it should be more labor intensive to express chimeric P450-CPR fusion enzymes.

Thank you very much for your critical comment.

When one pathway comprises several P450s, the chimeric strategy for the functional expression of P450 enzymes requires that each P450 need to be fused with a CPR gene. In general, there are more than once digestion and ligation during the construction of the engineered pathway. In the process, the available restriction sites in the vector for the next round cloning should be considered. Besides, the existence of several identical CPR genes could elevate the chance of homologous recombination, and overconsume the intracellular deoxynucleotides. To maximize the production of the desired chemicals, the expression of the chimeras needs to be optimized at the level of transcription and/or translation. Hence, the subsequent modulation of the expression vector is not a picnic. So, a modular expression system

for P450 and CPR is demanded, especially that CPR protein is shared for P450s as the circumstance in eukaryotic cells. We also replenished the sentence to state clearly in the revised manuscript (Line 74-78).

10. Page 11: “This N-termini-bridged heterodimer of P450 and CPR might be considered a counterpart of the eukaryotic membrane-bound P450 system” It’s not clear what is meant by “counterpart”. It’s not known if the forced spatial orientation of the P450-CPR interaction partners is the main reason why these enzymes are membrane bound in eukaryotes.

Thank you very much for your critical comment.

In eukaryotic cell, P450s and their redox partner CPR are integral membrane proteins, and have a short hydrophobic anchor in N-terminus. Eukaryotic P450s and CPR generally colocalize on the cytoplasmic side of the ER membrane, and their soluble catalytic region stretch in cytoplasm. In our study, we resorted the self-assembled peptide bio-machinery to spatially organize eukaryotic P450 and CPR in prokaryote *E. coli*. Via altering the appending terminus, we found that the N-termini-bridged heterodimers showed superior biosynthetic performance when the two peptide elements of a self-assembled bio-machinery were both appended in N-termini of eukaryotic P450 and CPR. In the architecture of the heterodimer, both P450 and CPR were N-terminally reconstructed on the self-assembled peptide bio-machinery. Based on the N-terminal colocalization of P450 and CPR on the integrated peptide bio-machinery, the peptide-based N-termini-bridged P450/CPR heterodimer whether covalent or not were proposed to be considered as a counterpart of eukaryotic P450 and CPR complex, colocalizing N-terminally on the organelle membrane.

11. Page 14: Fig. 4. They need to add a statistical analysis to this figure. It would also help understanding if the specific productivity for each construct was also

plotted in this figure, since the authors use both measurements to compare the different P450-CPR designs.

Thank you very much for your critical and useful suggestion. In the revised manuscript, we added the statistical analysis throughout the text, and plotted the specific productivity for each construct with the output in Figure 4.

12. Page 15: What is the difference between the SpySystem and SnoopSystem? Can the difference explain why there is no difference in titer when the SnoopCatcher and SnoopTag are swapped between P450 and CPR? Does the two systems perform equally well in dimerization. They should include a western blot showing the dimerization of the four different designs: SpyCatcher-CYP/SpyTag-ATR, SpyTag-CYP/SpyCathcer-ATR, SnoopCatcher-CYP/SnoopTag-ATR, & SnoopTag-CYP/SnoopCatcher-ATR.

Thank you very much for your critical and useful suggestion.

SpySystem is derived from the second immunoglobulin-like collagen adhesin domain (CnaB2) of the fibronectin binding protein FbaB from *Streptococcus pyogenes* [4]. However, SnoopSystem is derived from the D4 Ig-like domain of the pilus-associated adhesin RrgA from *Streptococcus pneumoniae* [5]. There is an amino acid triad for the formation of the intramolecular isopeptide bond in CnaB2 domain or D4 domain. And, SpySystem and SnoopSystem were developed by splitting in the loop of the relevant domain. Both of CnaB2 and D4 are highly reminiscent IgG domain, and have similar folding in the structure. This may be the reason for the robust output of SpySystem-based and SnoopSystem-based CYP73A5-ATR2 heterodimers (**Figure 4 panel a** and **b** in the revised manuscript).

Nevertheless, the prominent difference between the SpySystem and the SnoopSystem is that the SpyTag peptide was the C-terminal β-strand of CnaB2 domain while the

SnoopTag peptide was the N-terminal β-strand of D4 domain. This may be the reason for the swapping of the N-terminal appendixes. In detail, the SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$-SpyTagATR2$^{\Delta 2\text{-}77}$ heterodimer is superior than the SpyTagCYP73A5$^{\Delta 2\text{-}28}$-SpyCatcherATR2$^{\Delta 2\text{-}77}$ heterodimer (**Figure 4 panel a** in the revised manuscript), while the SnoopTagCYP73A5$^{\Delta 2\text{-}28}$-SnoopCatcherATR2$^{\Delta 2\text{-}77}$ heterodimer is better than the SnoopCatcherCYP73A5$^{\Delta 2\text{-}28}$-SnoopTagATR2$^{\Delta 2\text{-}77}$ heterodimer (**Figure 4 panel b** in the revised manuscript).

Regarding to a small output gap between the SnoopTagCYP73A5$^{\Delta 2\text{-}28}$-SnoopCatcherATR2$^{\Delta 2\text{-}77}$ heterodimer and the SnoopCatcherCYP73A5$^{\Delta 2\text{-}28}$-SnoopTagATR2$^{\Delta 2\text{-}77}$ heterodimer (**Figure 4 panel b** in the revised manuscript), a larger loop between SnoopCatcher and SnoopTag may endow a higher degree in the flexible organization of SnoopSystem-based heterodimers.

13. Page 17: They don't show any evidence that 8RP increases p-coumaric acid production through increased P450/CPR aggregation.

Thank you very much for your critical and useful suggestion. All of the amino acids in 8RP (MALLLAVF) are hydrophobic, that makes the 8RP-modified proteins to be membrane-associated [6]. We rephrased the sentence in the revised manuscript.

14. Page 17 + Page 18: On both pages the authors speculate on "turnover rate" and "biosynthetic performance" with reference to the band intensities of the western blot in fig S3. This is quite speculative and subjective. If wants to state anything on turnover rate for specific P450/CPR designs they should first quantify the soluble and insoluble protein abundance with some proteomic method e.g. SRM.

Thank you very much for your CRITICAL and USEFUL suggestion.

In the revised manuscript, we purified the soluble CYP73A5$^{\Delta 2\text{-}28}$, ATR2$^{\Delta 2\text{-}77}$ and the components of the four SpySystem-based heterodimers, and measured the kinetics using *trans*-cinnamic acid and NADPH for the comparison of the turnover rate. The quantitative results in **Table 1** and **Table 2** showed that the N-termini-bridged heterodimer had a 48.94-fold higher turnover rate (K_{cat}) than the free-floating individuals (**Section Results 2.3** in the revised manuscript).

15. Page 19: When comparing the three designs with mutated SpySystem tags they only focus on SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$ and fail to address that third design SpyCatcher^{mut}CYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$ basically has the same specific activity (0.89±0.04 µM/OD600/h vs. 0.82±0.2 µM/OD600/h).

Thank you very much for your critical and useful suggestion. In the same culture condition, the strain with SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$ produced 696.79 µM *p*-coumaric acid with a productivity of 0.89 ± 0.04 µM $(OD_{600}\ h)^{-1}$, while the strain with SpyCatcher^{mut}CYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$ produced 621.30 µM *p*-coumaric acid with a productivity of 0.82 ± 0.2 µM $(OD_{600}\ h)^{-1}$. The third design SpyCatcher^{mut}CYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$ shown the production and productivity comparable to the second design SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$, that may suggest that SpyCatcher^{mut} peptide and SpyTag^{mut} still have a considerable affinity.

16. Page 19: They can't conclude in general terms that the iso-peptide bond formation is crucial for the improvement seen by using self-assembling peptides they also show that the non-covalent heterodimer SH3CYP73A5$^{\Delta 2\text{-}28}$/SH3ligATR2$^{\Delta 2\text{-}77}$ has the same specific activity (1.45±0.05 µM/OD600/h) as the covalent heterodimer SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$-SpyTagATR2$^{\Delta 2\text{-}77}$ (1.5±0.12 µM/OD600/h).

Thank you very much for your CRITICAL and USEFUL suggestion. In the same culture condition, the strain with covalent SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$-SpyTagATR2$^{\Delta 2\text{-}77}$ produced 1034.64 μM *p*-coumaric acid with a productivity of 1.5 ± 0.12 μM (OD_{600} h)$^{-1}$, while the strain with non-covalent SpyCatcherCYP73A5$^{\Delta 2\text{-}28}$/SpyTag^{mut}ATR2$^{\Delta 2\text{-}77}$ produced 696.79 μM *p*-coumaric acid with a productivity of 0.89 ± 0.04 μM (OD_{600} h)$^{-1}$. And, the strain with SH3CYP73A5$^{\Delta 2\text{-}28}$/SH3ligATR2$^{\Delta 2\text{-}77}$ produced 876.09 μM *p*-coumaric acid with a productivity of 1.45 ± 0.05 μM (OD_{600} h)$^{-1}$. It has reported that the affinity of SpyCatcher and SpyTag^{mut} is 0.2 μM [4], while that of SH3 and SH3lig is 0.1 μM [7]. The results suggested that the mechanical stability of self-assembled peptide bio-machinery, employed for spatially organizing a multienzyme cascade bioreactor, could affect the biosynthetic efficiency. We have made some modifications for this speculation in the revised manuscript.

17. Page 19: What is meant by “multireaction-involved enzymes”?

Thank you very much for your critical and useful suggestion. In the revised manuscript, we modified the phrase “multireaction-involved enzymes” to “a multienzyme cascade bioreactor” (Line 420-426). And, we also checked the full text, and re-expressed the phrase involving “multireaction” in the revised manuscript. Herein, we use “a multienzyme cascade bioreactor” to mean that an enzymatic system involves two or even more enzymes, which perform a cascade reaction.

18. Page 20: The description of the results for the human P450/CRP is confusion. It is not clear from the text which of the peptide tag systems perform the best.

Thank you very much for your critical and useful suggestion.

To further stress the importance of the architecture on the biosynthetic performance of human P450, we redesigned the expression pattern of human P450 system in the revised manuscript. In details, we additionally constructed the CYP102A1-like tandem fusion and the other three SpySystem-based heterodimers of CYP1A2 and HsCPR according to the architectural organization of plant CYP73A5 and ATR2. The results indicated that the heterodimer with an N-termini-bridged architecture had superior biosynthetic performance among the tandem fusion, the covalent heterodimers as well as the free-floating individuals (**Section Results 2.7** in the revised manuscript).

19. Page 21: What is meant by “human pharmaceuticals”? Pharmaceuticals human use of pharmaceuticals made by human enzymes?

Thank you very much for your critical comment.

Prodrugs are metabolized in body as a mechanism of bioactivation to the active pharmaceutical ingredients. In these processes, human cytochrome P450 enzymes are responsible for the biotransformation of many prodrugs, including phenacetin bioactivated by CYP1A2. And, human P450s also participate in the clearance of many drugs. However, there are limited information of some of these drug metabolites due to their unavailable. Nowadays, human P450s are harnessed for the production of drug metabolites. The N-termini-bridged assembly strategy for eukaryotic P450 system, developed in this manuscript, was demonstrated to improve the production of acetaminophen, the active metabolite of phenacetin, by 6.22 or 4.46-fold, compared to the tandem fusion method or the co-expression strategy, respectively. For precise expression, we have modified “human pharmaceuticals” to “human drug metabolites” in the revised manuscript (Line 457-459 and 477-480).

1. Nair RB, Xia Q, Kartha CJ, Kurylo E, Hirji RN, Datla R*, et al. Arabidopsis* CYP98A3 mediating aromatic 3-hydroxylation. Developmental regulation of the gene, and expression in yeast. *Plant physiology* 2002, **130**(1)**:** 210-220.
2. Qi H, Yu L, Li Y, Cai M, He J, Liu J*, et al.* Developing Multi-Copy Chromosomal Integration Strategies for Heterologous Biosynthesis of Caffeic Acid in *Saccharomyces cerevisiae*. *Frontiers in microbiology* 2022, **13:** 851706.
3. Andersen TB, Hansen NB, Laursen T, Weitzel C, Simonsen HT. Evolution of NADPH-cytochrome P450 oxidoreductases (POR) in Apiales - POR 1 is missing. *Molecular phylogenetics and evolution* 2016, **98:** 21-28.
4. Zakeri B, Fierer JO, Celik E, Chittock EC, Schwarz-Linek U, Moy VT*, et al.* Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(12)**:** E690-697.
5. Veggiani G, Nakamura T, Brenner MD, Gayet RV, Yan J, Robinson CV*, et al.* Programmable polyproteams built using twin peptide superglues. *Proceedings of the National Academy of Sciences* 2016, **113:** 1202-1207.
6. Gillam EM, Baba T, Kim BR, Ohmori S, Guengerich FP. Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Arch Biochem Biophys* 1993, **305**(1)**:** 123-131.
7. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV*, et al.* Synthetic protein scaffolds provide modular control over metabolic flux. *Nature biotechnology* 2009, **27**(8)**:** 753-759.

Responses to Reviewer #1 (Remarks to the Author):

The manuscript has been substantially strengthened by a series of changes. My comments have been addressed.

Reply 1-1: We thank the reviewer for the positive comment, and appreciate the reviewer for the recognition of our responses.

My only remaining recommendation is that it should be a small k and not a large k for each use of kcat, consistent with this being a rate and the common usage in the literature.

Reply 1-2: We thank the reviewer for pointing this out. We have lowercased the letter k of kcat in the revised manuscript. (Lines 274, 284, 294, 303)

I am happy to recommend publication.

Reply 1-3: We appreciate the referee for the valuable time spent to evaluate our work and the support for publication.

Responses to Reviewer #3 (Remarks to the Author):

Summary:

In this manuscript, Li et al. report that linking the N-termini of eukaryotic CYP450s and CPRs increases their catalytic activity in vitro as well as in prokaryotic *E. coli*. The CYP450-CPR system with N-N termini linkage architecture outperformed individual proteins and all other combinations of CYP450-CPR linkage architectures (N-C, C-N, and C-C). The authors discovered that this phenomenon is generalizable across two CYP450-CPR systems (plant and human) and three linkage types (covalent SpyTag/SpyCatcher, covalent SnoopTag/SnoopCatcher, and non-covalent SH3/SH3lig). This is an exciting result that will be interesting to the broad field of synthetic biology. While the authors have addressed many of the reviewer comments from the last round of reviews, they did not address the comment from Reviewer 2 that the lack of structural characterization/models explaining this phenomenon lowered the enthusiasm for this discovery. While I agree with this comment from Reviewer 2, I support the publication of this manuscript in Nature Communications after the following comments and suggestions have been addressed.

We are very grateful to the referee for the valuable time in evaluating our work again. We thank the reviewer for the detailed and in-depth feedback, and believe that it has helped to improve the overall quality of our manuscript. All comments are replied point-to-point.

Major comments:

1. The central hypothesis of this work is that the relative orientation of CYP450s and CPRs has a significant impact on their catalytic activity. However, there is no structural characterization/models to support claims that the N-N, N-C, C-N, and C-C linked proteins have different relative orientations. The flexible linkers in the CYP450-CPR systems mean that different relative orientations could be adopted

in each system. The authors should limit claims in their manuscript to say that the linkage architecture of CYP450s and CPRs has a significant impact on their catalytic activity.

Reply 3-1: We appreciate the reviewer for the critical comment and helpful suggestion that have greatly improved our manuscript. We have rephrased the statement according to the referee's suggestion in the revised manuscript. (Lines 29, 34, 93, 98-99, 106-107, 119, 606)

2. The only evidence provided for the concentrations of free proteins and linked proteins in *E. coli* experiments were from two Western blot gels in Figure S3. The author's claims would be strengthened with densitometry analysis of their Western blot gels and/or quantitative proteomics.

Reply 3-2: We thank the reviewer for the critical comment and helpful advice. According to those valuable suggestions, we have measured and analyzed the density of the protein bands in the Western blotting gels, renumbered as Figure S5 in the revised Supporting Information.

3. The only structural characterization of N-N, N-C, C-N, and C-C CYP450-CPR linkage architectures were the two Western blot gels in Figure S3. The author's claims would be strengthened with additional structural characterization such as analytical SEC, MALDI-TOF MS, and/or native PAGE.

Reply 3-3: We thank the reviewer for the critical comment and valuable suggestion. To further characterize the protein linkage architectures of the SpySystem-mediated P450-CPR assemblies, we have detected all the SpySystem-linked covalent CYP73A5$^{\Delta 2\text{-}28}$-ATR2$^{\Delta 2\text{-}77}$ heterodimers with four linkage architectures by SDS-PAGE, of which all showed a molecular weight (MW) of about 130 KDa (Calculated MW = 139.7 KDa) (Figure S3 in the revised Supplementary Information). We then cut off the protein bands with the desired size, and further performed the protein identification by LC-MS/MS (Section 5.6 in the revised Manuscript). The results showed that the matched peptides in the samples covered

78.47%, 80.08%, 85.65% and 78.31% of the full-length target amino acid sequence of heterodimer (I), (II), (III) and (IV), respectively; moreover, all of the four components, i.e. SpyTag, SpyCatcher, CYP73A5$^{\Delta 2\text{-}28}$ and ATR2$^{\Delta 2\text{-}77}$, were verified in each band of interest (Figure S4 in the revised Supplementary Information), indicating that the reconstructed CYP73A5 and ATR2 were post-translationally assembled into a covalent heterodimer with a designed linkage architecture by virtue of the SpySystem.

4. I agree with a comment from Reviewer 1 in the previous round of reviews that the manuscript be edited to remove meaningless significant figures from experimental numbers. For example, on line 142, a tier is reported of 201.31 ± 59.21 μM. There is no way that the titer is precisely measured to two decimal places when the standard deviation is nearly 60. Please revise the manuscript to use appropriate numbers of significant figures.

Reply 3-4: We thank the reviewer for the critical comment. It's of great help to improve our manuscript. According to your suggestion, we have checked and removed the meaningless significant figures from experimental numbers throughout the manuscript.

5. While it is not necessary for the publication of this manuscript, it would be interesting to see if this result is generalizable to many more CYP450-CPR systems or many more microorganisms.

Reply 3-5: This is a very important and interesting suggestion. In our further study, based on the constructed phenylpropanoid pathway, we will attempt to construct the *de novo* biosynthetic pathway of galantamine (a derivative of *p*-coumaric acid and an Amaryllidaceae alkaloid for Alzheimer's disease), which involves another two P450s from CYP98A and CYP96T subfamilies, not only in Gram-negative *Escherichia coli* but also in other microorganisms, such as Gram-positive *Corynebacterium glutamicum* and the model fungus *Saccharomyces cerevisiae*.

Minor comments:

1. The abbreviation of SOPA for spatial orientation-guided protein assembly is unnecessary. Please remove this abbreviation from the paper.

Reply 3-6: Thanks for the valuable suggestion. We have removed the abbreviation accordingly in the revised manuscript. (Lines 29, 107, 606)

2. In Figure 2C, there are data points with no error bars. If there are error bars behind these data points, can the caption be revised to indicate this?

Reply 3-7: Thank you for the carefully reviewing. In Figure 2C, there are error bars behind the data points except the *p*-coumaric acid production of the strain harboring the empty vector, which cannot produce *p*-coumaric acid. We have redrawn the figure to show the error bars clearly in the revised manuscript.

3. In the schematic representations of CYP450s (large blue arrows) and CPRs (large green arrows), it is unclear what the small arrows deformations represent. Please clarify their meaning in figure captions.

Reply 3-8: We thank the reviewer for the critical comment. We have clarified the orange short curves with a single arrow in the schematic diagrams, which are representatives of a flexible peptide linker, in the figure captions throughout the revised manuscript and Supplementary Information.

4. The abbreviations NADPH, FAD, and FMN (lines 56-57) are used without introducing what they mean.

Reply 3-9: Thanks for the critical comment. According to your suggestion, we have introduced the full names of the three co-factors, used by cytochrome P450 reductase for electron donor and electron transfer mediator. (Lines 54–58 in the revised manuscript)

5. There should be a "." following *E* in *E. coli* (line 61).

Reply 3-10: We thank the reviewer for pointing out the missing. We have filled in the dot of the abbreviated generic name in the revised manuscript. (Line 63)

6. The sentence "[t]hese existing mismatches lead eukaryotic P450 systems suffering from poor solubility, weak functionality and low turnover rate in microbes, especially in prokaryotic host s such as *E coli*" (lines 63-65) is a fragment.

Reply 3-11: We thank the reviewer for the critical comment. We have checked and rewritten the sentence in the revised manuscript (Lines 64-67).

7. The statement "no less than eight P450s involved in Taxol biosynthesis" (lines 75-76) should be revised to "no less than eight P450s are involved in Taxol biosynthesis".

Reply 3-12: We thank the reviewer for the critical suggestion. We have revised accordingly. (Line 77)

8. The use of "[i]n consistent" (line 191) is confusing. Replace this with whichever of "consistent" or "inconsistent" is correct.

Reply 3-13: We thank the reviewer for raising the important issue. The sentence is not clear, and causes misunderstanding for the readers. We have corrected the sentence to state the results clearly and precisely. (Line 206)

9. It is not clear what "oriental orchestration" (line 587) means. Please replace this term.

Reply 3-14: We thank the reviewer for the critical comment. In this study, by harnessing self-assembly peptide bio-machinery, we organized eukaryotic P450 systems in spatial architecture (i.e. N-C, C-N, C-C, N-N termini linkage architecture), and shown that a novel N-termini-bridged architecture can finely tune the spatial arrangement and biosynthetic performance of eukaryotic P450 systems. The N-N termini linkage architecture led to superior biosynthetic performance for the

reconstructed eukaryotic P450 systems derived from both plants and humans. It's worth noting that, the configuration of the tethered P450-CPR assemblies, derived from the self-assembly peptide bio-machinery, had an inherent effect on the biosynthetic performance of the reconstructed P450 system. At a minimum, a conformational change of the redox partner CPR is necessary for electron transfer in the P450 oxyfunctionalization. The geometric arrangement can preset the structural dynamics and, to some extent, the biosynthetic output of the engineered P450-CPR assemblies. According to your suggestion, we have replaced the sentence "a biosynthetic and orientational orchestration of eukaryotic P450 system is achieved in *E. coli*." by "an architectural and biosynthetic orchestration of eukaryotic P450 system is achieved in *E. coli*.". (Lines 603-604)

10. In section 5.3, "ml" should be rewritten to "mL" in multiple instances.

Reply 3-15: Many thanks for noticing these typos. We have rewritten the units of volume, including μL, mL and L, throughout the revised manuscript.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The revised manuscript adequately addressed my major concerns from the previous draft. Overall, I am pleased with the changes and would gladly recommend this manuscript for publication in Nature Communications.

Reply 3-1: We thank the reviewer for the positive comment, and appreciate the reviewer for the recognition of our responses. We appreciate the referee for the time spent to evaluate our work and the support for publication.