nature portfolio

Peer Review File

A super versatile flexizyme system with phenol esters for genetic code reprogramming

Corresponding Author: Professor Hiroaki Suga

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 reports a new method for tRNA aminoacylation using a versatile flexizyme system with phenol esters as acyl-donors. This system improves the efficiency of aminoacylation compared to traditional benzyl(thio)ester-based methods, enabling higher yields and better access to diverse npAAs. The use of 3-nitrophenol (3NP) and eFx enhances the process, allowing the synthesis of a wide variety of nonstandard peptides, including those with macrocyclic structures, without extensive optimization. Overall, the work is original and solid, and the method developed is valuable. The manuscript is suitable for publication, and this reviewer only has one comment for the authors.

Can authors discuss whether npAA-phenol esters can completely replace npAA-benzyl(thio)esters with superior results, or if the two systems complement each other? For example, might benzyl(thio)esters be more effective for the aminoacylation of certain npAAs, while phenol esters perform better for others?

Reviewer #2

(Remarks to the Author)
The Suga lab invented and pioneered the use of flexizymes to charge tRNAs. Flexizymes are extremely versatile ribozymes that can charge both naturally occurring as well as exotic amino acids to virtually any tRNA since only the 3'-CCA tail is recognized. Unlike aminoacyl tRNA synthetases, flexizymes utilize chemically activated amino acids in reactions typically conducted in an ice bath. This technology has been used to develop bioactive peptides, to generate mischarged tRNAs for mechanistic studies, and to prepare stably charged tRNAs wherein the amino acid is linked to the tRNA via an amide linkage.

Despite the availability of different flexizymes and the use of different activating groups, some non-proteinogenic amino acids (npAAs) suffer from low acylation yields. In this study the Suga lab describes the use of a new 3-nitophenol (3NP) activating group which dramatically improves the yield of acylation. This is a significant advance which will expand the number of npAAs that can be attached to tRNAs.

Traditionally, benzyl (thio)ester (BZE) leaving groups have been used to activate amino acids for the flexizyme reaction. In this study five phenol leaving groups, each with a different pH ranging from 7.6 to 8.8, were tested in conjunction with or without the eFx flexizyme for their ability to improve the acylation of problematic npAAs. The charging of microhelix tRNAs was monitored by acid denaturing PAGE gel electrophoresis while the charging of full length tRNA(Tyr) was monitored by MALDI-TOF MS of the 3'-RNA fragment resulting from RNase T1 digestion. Carefully documented studies in which time, temperature, and pH of the charging reactions were varied demonstrated that the 3-nitrophenol leaving group performed the best. As a final measurement of performance, 17 structurally different npAAs were conjugated to 3NP. In each case the percent charging was greater than 20% when carried out in the presence of the eFx flexizyme.

Several of the charged tRNAs from this study were successfully incorporated either alone or in combination into short oligopeptides by in vitro translation. In the final in vitro translation a cyclic oligopeptide containing six different npAAs was prepared. MALDI-TOF MS was used to characterize all the oligopeptide products.

Overall, the paper is easy to read, and the experiments logically ordered and meticulously executed. I think this is a well-crafted study representing a major advance in the genome expansion field.

I suggest that the authors should include a short section in the Material and Methods where the chemistry for conjugating 3NP to an amino acid is described. It would also be nice if the authors could comment on whether this advance would be applicable to the preparation of stably charged tRNAs.

Other minor suggested revisions . . .

1) On lines 368 and 372 correct the entry “100 u3NPAA-“
2) On lines 292 and 316 give a pH range

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
After reading their revised manuscript, this reviewer felt that the revised version is suitable for publication.

Reviewer #2

(Remarks to the Author)
I approve the revisions made by the quthors/

Response to the reviewers' comments

RE: NCOMMS-24-80479-T

Choi et al.

"A super versatile flexizyme system with phenol esters for genetic code reprogramming"

Followings are our point-by-point responses to the comments and requests provided in the decision letter we received on Feb 7th.

Requests/comments are in *black and italicized* and our responses are in red.

Reviewer #1 (Remarks to the Author):

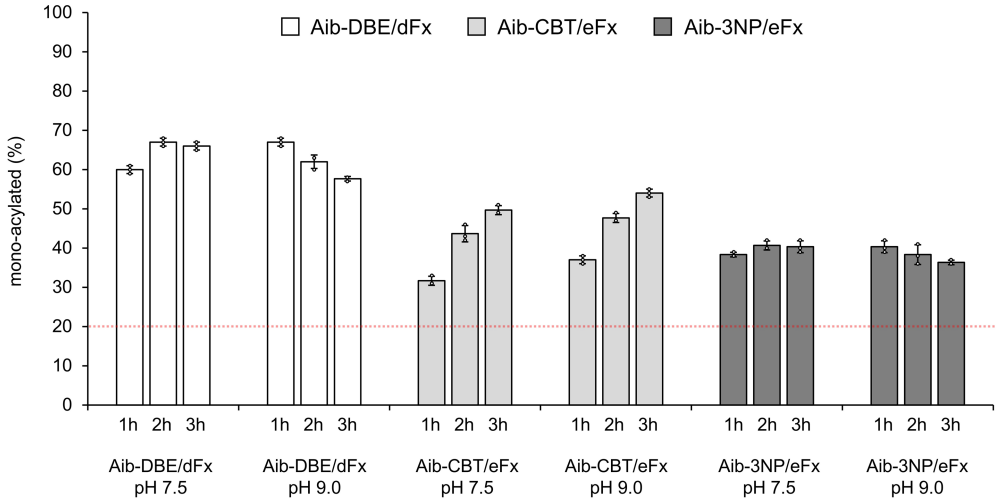
This manuscript reports a new method for tRNA aminoacylation using a versatile flexizyme system with phenol esters as acyl-donors. This system improves the efficiency of aminoacylation compared to traditional benzyl(thio)ester-based methods, enabling higher yields and better access to diverse npAAs. The use of 3-nitrophenol (3NP) and eFx enhances the process, allowing the synthesis of a wide variety of nonstandard peptides, including those with macrocyclic structures, without extensive optimization. Overall, the work is original and solid, and the method developed is valuable. The manuscript is suitable for publication, and this reviewer only has one comment for the authors.

We thank the reviewer very much for evaluating the manuscript thoroughly. Please find our responses to the reviewer #1's comment below.

Q: Can authors discuss whether npAA-phenol esters can completely replace npAA-benzyl(thio)esters with superior results, or if the two systems complement each other? For example, might benzyl(thio)esters be more effective for the aminoacylation of certain npAAs, while phenol esters perform better for others?

A: In order to respond to the reviewer's comment, we evaluated aminoacylation of an efficient npAA in the standard BZE/flexizyme system, which is Aib. Aminoacylation of Aib can be done quickly and efficiently in 2 h reaction using Aib-DBE/dFx pair (Nucleic Acids Res. 2017, 45, 12601-12610). For comparison, we have tested three different substrate/flexizyme pairs, Aib-DBE/dFx, Aib-CBT/eFx, and Aib-3NP/eFx, at various pHs and time points. The aminoacylation yield was determined by acid denaturing PAGE

analyses (Supplementary Fig. 15).



Even though the Aib-3NP/eFx pair was slightly more efficient than the Aib-CBT/eFx pair in the 1 hour reaction by reaching 40% yield, the Aib-CBT/eFx pair showed higher efficiencies for >2 hour reaction. Furthermore, the Aib-DBE/dFx pair showed the highest yield under all tested conditions. Therefore, we concluded that standard BZE/flexizyme pairs can be more efficient than the 3NP/eFx pairs for certain npAAs, as the reviewer expected. As a reflection of this result, we have added the discussion, “Even though some BZEs of certain npAAs (e.g. Aib) are more effective for the aminoacylation than the eFx/npAA-3NP system (Supplementary Fig. 15),…” , in the discussion section of the manuscript (line 274).

The gel data of the abovementioned comparison experiments are shown in Supplementary Fig. 15a–c. Since we newly synthesized Aib-DBE and Aib-CBT for the aminoacylation evaluation, we included the synthesis procedures and NMR data (Supplementary Figs. 61–62) in the Supplementary Information. Thank you so much again for giving us the opportunity to evaluate our system with the balanced viewpoint.

Reviewer #2 (Remarks to the Author):

The Suga lab invented and pioneered the use of flexizymes to charge tRNAs. Flexizymes are extremely versatile ribozymes that can charge both naturally occurring as well as exotic amino acids to virtually any tRNA since only the 3’-CCA tail is recognized. Unlike aminoacyl tRNA synthetases, flexizymes utilize chemically activated amino acids in reactions typically conducted in an ice bath. This technology has been used to develop

bioactive peptides, to generate mischarged tRNAs for mechanistic studies, and to prepare stably charged tRNAs wherein the amino acid is linked to the tRNA via an amide linkage.

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Several of the charged tRNAs from this study were successfully incorporated either alone or in combination into short oligopeptides by in vitro translation. In the final in vitro translation a cyclic oligopeptide containing six different npAAs was prepared. MALDI-TOF MS was used to characterize all the oligopeptide products.

Overall, the paper is easy to read, and the experiments logically ordered and meticulously executed. I think this is a well-crafted study representing a major advance in the genome expansion field.

We really appreciate the reviewer's detailed and thorough evaluation of our 3NP/eFx system. Please find our responses to the reviewer #2's comment below.

Q: I suggest that the authors should include a short section in the Material and Methods where the chemistry for conjugating 3NP to an amino acid is described.

A: Based on the reviewer's suggestion, we have included a short section which describes the synthesis method of phenol ester derivatives in line 295. We also added further

information in "chemical synthesis" section of supplementary information, where the detailed synthesis procedures and characterization results are presented.

Q: It would also be nice if the authors could comment on whether this advance would be applicable to the preparation of stably charged tRNAs.

A: On line 284, we have added discussion related to the further potential of the 3NP/eFx system in the preparation of 3′-aminoacyl-NH-tRNAs, which are hydrolytically stable analogs of 3′-aminoacyl-OH-tRNAs. Considering the aminoacylation reaction of 3′-NH-tRNA is relatively slower than that of 3′-OH-tRNA, the 3NP/eFx pair can become a potential solution for the acceleration of 3′-NH-tRNA aminoacylation. We thank this reviewer for giving us the chance to consider the further potential of the eFx/3NP system.

Other minor suggested revisions . . .

1) On lines 368 and 372 correct the entry “100 u3NPAA-“

Based on the reviewer’s comment, we have changed the typo “100 u3NPAA-” to “100 µM npAA-” on line 380 and 383. We appreciate the reviewer’s thorough review.

2) On lines 292 and 316 give a pH range

Following the reviewer’s comment, we have added the detailed information related to pH buffer such as “HEPES-KOH (pH 7.0 or 7.5) and CHES-KOH (pH 9.5) for respective pH” on line 303 and line 328.