been no reports of successfully expanding the number of building blocks by further assigning another amino acid in the same codon box and simultaneously and accurately discriminating a total of three amino acids. Furthermore, versatility of the selectable amino acids may be low for the already reported methods. Moreover, when an aminoacyl tRNA prepared outside the translation system is used for translation, a higher concentration of aminoacyl tRNA is required than aminoacylated tRNA prepared by ARS in the translation system, in which case, it has been shown mathematically that discrimination between the NNA and NNG codons will become difficult (NPL 5).

[0005]

In this technical field, assigning various amino acids to a codon table, and translating by accurately discriminating the individual codons determines the quality of the display library. It is very important that the amino acids assigned to the individual codons are specifically translated from the respective codons.

[Citation List]

[Patent Literature]

[0006]

[PTL 1] WO2016/154675

[Non-Patent Literature]

[0007]

[NPL 1] Shimizu et al., Nat Biotechnol. 2001; 19(8): 751-755.

[NPL 2] Iwane et al., Nat Chem. 2016; 8(4): 317-325.

[NPL 3] Mukai et al., Nucleic Acids Res. 2015; 43(16): 8111-8122.

[NPL 4] Cui et al., J Am Chem Soc. 2015; 137(13): 4404-4413.

[NPL 5] Frenkel-Morgenstern et al., Mol Syst Biol. 2012; 8: 572.

[Summary ~~of Invention~~]

[Technical Problem]

[0008]

Various attempts have so far been made for codon expansion; however, there is no report of assigning different amino acids to the NNA and NNG codons and clearly showing accurate discrimination of these codons. Furthermore, there is no report of assigning 3 amino acids within the same codon box and clearly showing their accurate discrimination. The present ~~invention~~disclosure was achieved in view of such circumstances. An objective of the present ~~invention~~disclosure is to provide novel means for enabling discrimination of codons.

[Solution to Problem]

[0009]

This time, the present inventors succeeded in discrimination of the NNA and NNG codons in specific codon boxes, which had been difficult to achieve due to the presence of wobble base pairing. Furthermore, the inventors assigned another amino acid to the NNU or NNC codon in the same codon box. When a sequence containing these 3 codons was actually translated to evaluate the discrimination ability, the codons of interest were specifically translated into only the corresponding amino acids, and the successful accurate discrimination was also confirmed numerically.

In one aspect, the present disclosure refers to a translation system, comprising a tRNA having an anticodon complementary to a codon represented by M_1M_2A and a tRNA having an anticodon complementary to a codon represented by M_1M_2G; wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and wherein each of the two tRNAs is attached to an amino acid or an amino acid analog that is different from each other.

In another aspect, the present disclosure refers to a translation system, comprising (i) a tRNA having an anticodon complementary to a codon represented by M_1M_2U, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G, or (ii) a tRNA having an anticodon complementary to a codon represented by M_1M_2C, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G; wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and wherein each of the three tRNAs in the above-mentioned (i) and (ii) is attached to an amino acid or an amino acid analog that is different from each other.

In another aspect, the present disclosure refers to a method for producing the translation system as disclosed herein, comprising attaching the amino acid or the amino acid analog to a tRNA outside the translation system.

In another aspect, the present disclosure refers to a peptide produced by the method as disclosed herein.

In another aspect, the present disclosure refers to a method for producing a peptide library, comprising translating a nucleic acid library using the translation system as disclosed herein or a translation system obtained by the method as disclosed herein.

In another aspect, the present disclosure refers to a peptide library produced by the method as disclosed herein.

In another aspect, the present disclosure refers to a method for identifying a peptide having binding activity to a target molecule, comprising contacting the target molecule with the peptide library as disclosed herein.

[0010]

The present disclosure is based on such findings, and specifically encompasses the ~~embodiment~~examples exemplified in [1] to [32] below:

[1]

A translation system, comprising a tRNA having an anticodon complementary to a codon represented by M_1M_2A and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;

wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and

wherein each of the two tRNAs is attached to an amino acid or an amino acid analog that is different from each other.

[2]

The translation system of [1], wherein at least two amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G.

[3]

A translation system, comprising

(i) a tRNA having an anticodon complementary to a codon represented by M_1M_2U, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G, or

(ii) a tRNA having an anticodon complementary to a codon represented by M_1M_2C, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;

wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and

wherein each of the three tRNAs in the above-mentioned (i) and (ii) is attached to an amino acid or an amino acid analog that is different from each other.

[4]

M_1M_2G.

[0011]

Furthermore, the present disclosure also encompasses the ~~embodiment~~examples exemplified in [101] to [120] below:

[101]

A method for producing a peptide, comprising translating a nucleic acid using a translation system,

wherein the translation system comprises a tRNA having an anticodon complementary to a codon represented by M_1M_2A and a tRNA having an anticodon complementary to a codon represented by M_1M_2G, and

M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U), and

wherein each of the two tRNAs is attached to an amino acid or an amino acid analog that is different from each other.

[102]

The method of [101], wherein at least two amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G.

[103]

A method for producing a peptide, comprising translating a nucleic acid using a translation system, wherein the translation system comprises

(i) a tRNA having an anticodon complementary to a codon represented by M_1M_2U, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G, or

(ii) a tRNA having an anticodon complementary to a codon represented by M_1M_2C, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;

wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and

wherein each of the three tRNAs in the above-mentioned (i) and (ii) is attached to an amino acid or an amino acid analog that is different from each other.

[104]

The method of any one of [101] to [103], wherein at least three amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G.

The method of any one of [101] to [110], wherein the anticodon comprises one or more types of nucleosides selected from adenosine (A), guanosine (G), cytidine (C), and uridine (U).

[112]

The method of any one of [101] to [111], wherein more than 20 types of amino acids can be translated from one genetic code table.

[113]

The method of any one of [101] to [112], wherein the translation system is a cell-free translation system.

[114]

The method of [113], wherein the translation system is a reconstituted cell-free translation system.

[115]

The method of [113] or [114], wherein the translation system comprises an *Escherichia coli*-derived ribosome.

[116]

The method of any one of [101] to [115], wherein the tRNA has been attached to the amino acid or the amino acid analog outside the translation system.

[117]

The method of any one of [101] to [116], wherein among the tRNAs, the tRNA having an anticodon complementary to a codon represented by M_1M_2A and the tRNA having an anticodon complementary to a codon represented by M_1M_2G have been attached to the amino acid or the amino acid analog outside the translation system.

[118]

The method of any one of [101] to [117], wherein the tRNA is obtained by the pCpA method, the pdCpA method, a method using an artificial RNA catalyst (flexizyme), or a method using an aminoacyl-tRNA synthetase (ARS).

[119]

The method of any one of [101] to [118], wherein the nucleic acid comprises a codon represented by M_1M_2A and a codon represented by M_1M_2G.

[120]

The method of any one of [103] to [118], wherein the nucleic acid comprises (i) a codon represented by M_1M_2U, a codon represented by M_1M_2A, and a codon represented by M_1M_2G, or (ii) a codon represented by M_1M_2C, a codon represented by M_1M_2A, and a codon represented by M_1M_2G.

[0012]

Furthermore, the present disclosure also encompasses the ~~embodiment~~examples exemplified in

[201] to [218] below:

[201]

A kit or a composition for producing a peptide,

which comprises a tRNA having an anticodon complementary to a codon represented by M_1M_2A and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;

wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and

wherein each of the two tRNAs is attached to an amino acid or an amino acid analog that is different from each other.

[202]

The kit or the composition~~method~~ of [201], wherein at least two amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G.

[203]

A kit or a composition for producing a peptide, comprising

(i) a tRNA having an anticodon complementary to a codon represented by M_1M_2U, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G, or

(ii) a tRNA having an anticodon complementary to a codon represented by M_1M_2C, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;

wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and

wherein each of the three tRNAs in the above-mentioned (i) and (ii) is attached to an amino acid or an amino acid analog that is different from each other.

[204]

The kit or composition of any one of [201] to [203], wherein at least three amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G;

[205]

The kit or composition of any one of [201] to [204], wherein M_1 and M_2 are any one of the following:

(i) M_1 is uridine (U) and M_2 is uridine (U);

(ii) M_1 is uridine (U) and M_2 is adenosine (A);

(iii) M_1 is uridine (U) and M_2 is guanosine (G);

amino acid with an amino acid having low translation efficiency other than methionine (initiation read-through; skipping the start codon). Another method includes, for example, removing methionine at the N-terminus of the peptide by allowing enzymes such as peptide deformylase and methionine aminopeptidase to act (Meinnel et al., Biochimie (1993) 75: 1061-1075). A library of peptides starting from methionine is prepared, and the above enzyme is made to act on the peptide library to prepare a library of peptides starting from a random amino acid at N-terminus.

[0185]

In another aspect, the present disclosure provides a peptide produced by the method for producing a peptide in the present disclosure. Peptides obtained by further chemically modifying the peptide produced by the method in the present disclosure are also included in the peptides provided by the present disclosure.

[0186]

In one aspect, the present disclosure provides a method for producing a peptide library, comprising translating a nucleic acid library using the translation system in the present disclosure. By preparing a plurality of nucleic acid molecules each encoding a peptide and rich in nucleic acid sequence diversity, and then translating each of them into a peptide, a plurality of peptide molecules rich in amino acid sequence diversity can be produced. The size of the library is not particularly limited, and may be, for example, 10^6 or more, 10^7 or more, 10^8 or more, 10^9 or more, 10^{10} or more, 10^{11} or more, 10^{12} or more, 10^{13} or more, or 10^{14} or more. The nucleic acid may be DNA or RNA. RNA is usually mRNA. DNA is translated into a peptide via transcription into mRNA. Such a nucleic acid library can be prepared by a method known to those skilled in the art or a similar method. By using a mixed base at a desired position when synthesizing a nucleic acid library, a plurality of nucleic acid molecules rich in nucleic acid sequence diversity can be easily prepared. Examples of codons using mixed bases are, for example, NNN (where N represents a mixture of 4 bases, A, T, G, and C), NNW (where W represents a mixture of 2 bases, A and T), NNM (where ~~W~~ M represents a mixture of two bases, A and C), NNK (where K represents a mixture of two bases, G and T), and NNS (where S represents a mixture of two bases, C and G). Alternatively, by limiting the base used in the third letter of the codon to any one of A, T, G, and C, a nucleic acid library in which only some specific amino acids are encoded can be synthesized. Furthermore, when a codon containing mixed bases is prepared, it is possible to arbitrarily adjust the appearance frequency of amino acids obtainable from the codon by mixing a plurality of bases at different ratios rather than in equal proportions. By taking a codon such as that mentioned above as one unit to prepare a plurality of different codon units, and then linking them in the desired order, a library in which the appearance position and appearance frequency of the contained amino acids are controlled

saturated aqueous sodium bicarbonate solution. The organic layer was dried over anhydrous sodium sulfate, then filtered, and concentrated under reduced pressure to remove the solvent, thus obtaining (9H-fluoren-9-yl)methyl (S)-5-oxo-4-(pyridin-3-ylmethyl)oxazolidin-3-carboxylate as a crude product.

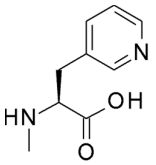
The obtained crude product (9H-fluoren-9-yl)methyl (S)-5-oxo-4-(pyridin-3-ylmethyl)oxazolidin-3-carboxylate (18 g, 44.95 mmol) was dissolved in dichloroethane (100 mL), and triethylsilane (Et_3SiH) (47 g, 404.20 mmol) and trifluoroacetic acid (100 mL) were added at room temperature. The reaction solution was stirred under nitrogen atmosphere at 70°C for 16 hours, and then concentrated under reduced pressure. The obtained residue was dissolved in isopropyl acetate, and then a mixed solution of t-butylmethyl ether and hexane (9:1) was added to it. The resulting solution was stirred at room temperature for 20 minutes, and then it was left to stand at 4°C for one hour. The precipitates that formed were collected by filtration, and they were washed with a cooled mixed solution of t-butylmethyl ether and hexane (9:1) to obtain (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)-3-(pyridin-3-yl)propanoic acid compound 2,2,2-trifluoroacetic acid salt (Compound SS52, Fmoc-MeA3Pyr-OH·TFA) (19 g, 95%).

LCMS(ESI) m/z = 403 (M+H)+

Retention time: 0.77 minutes (analysis condition SMD method 1)

[0224]

Synthesis of (S)-2-(methylamino)-3-(pyridin-3-yl)propanoic acid (Compound SS53, MeA3Pyr-OH)



[0225]

DMF (72 mL) and piperidine (28.5 mL) were added at room temperature to (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)-3-(pyridin-3-yl)propanoic acid 2,2,2-trifluoroacetic acid salt (Compound SS52, Fmoc-MeA3Pyr-OH·TFA) (19 g, 47.21 mmol), and the reaction mixture was stirred at room temperature for three hours. Diethyl ether (140 mL) and hexane (280 mL) were added, and this was stirred at room temperature for another three hours. The precipitates that formed were collected by filtration to obtain (S)-2-(methylamino)-3-(pyridin-3-yl)propanoic acid (Compound SS53, MeA3Pyr-OH) (7 g) quantitatively.

LCMS(ESI) m/z = 181 (M+H)+

Retention time: 0.15 minutes (analysis condition SMD method 2)

[0226]

[CLAIMS]

1. A translation system, comprising a tRNA having an anticodon complementary to a codon represented by M_1M_2A and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;
wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and
wherein each of the two tRNAs is attached to an amino acid or an amino acid analog that is different from each other.

2. The translation system of claim 1, wherein at least two amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G.

3. A translation system, comprising
(i) a tRNA having an anticodon complementary to a codon represented by M_1M_2U, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G, or
(ii) a tRNA having an anticodon complementary to a codon represented by M_1M_2C, a tRNA having an anticodon complementary to a codon represented by M_1M_2A, and a tRNA having an anticodon complementary to a codon represented by M_1M_2G;
wherein M_1 and M_2 represent nucleosides for the first and second letters of the codon respectively, and each of M_1 and M_2 is independently selected from any of adenosine (A), guanosine (G), cytidine (C), and uridine (U); and
wherein each of the three tRNAs in the above-mentioned (i) and (ii) is attached to an amino acid or an amino acid analog that is different from each other.

4. The translation system of claim 1 or 3, wherein at least three amino acids or amino acid analogs can be translated from a codon box constituted by M_1M_2U, M_1M_2C, M_1M_2A, and M_1M_2G.

5. The translation system of any one of claims 1 to 4, wherein M_1 and M_2 are any one of the following:
(i) M_1 is uridine (U) and M_2 is uridine (U);
(ii) M_1 is uridine (U) and M_2 is adenosine (A);
(iii) M_1 is uridine (U) and M_2 is guanosine (G);

(iv) M_1 is cytidine (C) and M_2 is adenosine (A);
(v) M_1 is cytidine (C) and M_2 is guanosine (G);
(vi) M_1 is adenosine (A) and M_2 is uridine (U);
(vii) M_1 is adenosine (A) and M_2 is cytidine (C);
(viii) M_1 is adenosine (A) and M_2 is adenosine (A);
(ix) M_1 is adenosine (A) and M_2 is guanosine (G); or
(x) M_1 is guanosine (G) and M_2 is adenosine (A).

6. The translation system of claim 5, wherein M_1 and M_2 are any one of the following:
(i) M_1 is uridine (U) and M_2 is uridine (U);
(ii) M_1 is uridine (U) and M_2 is guanosine (G);
(iii) M_1 is cytidine (C) and M_2 is adenosine (A);
(iv) M_1 is cytidine (C) and M_2 is guanosine (G);
(v) M_1 is adenosine (A) and M_2 is adenosine (A);
(vi) M_1 is adenosine (A) and M_2 is guanosine (G); or
(vii) M_1 is guanosine (G) and M_2 is adenosine (A).

7. The translation system of any one of claims 1 to 6, wherein the concentration of the tRNA included in the translation system per codon is any one of (i) 0.8 μM to 1000 μM, (ii) 1.6 μM to 500 μM, (iii) 3.2 μM to 250 μM, (iv) 6.4 μM to 150 μM, or (v) 10 μM to 100 μM.

8. The translation system of any one of claims 1 to 7, wherein the amino acid is a natural amino acid or an unnatural amino acid.

9. The translation system of any one of claims 1 to 8, wherein the tRNA is an initiator tRNA or an elongator tRNA.

10. The translation system of any one of claims 1 to 9, wherein the tRNA is derived from a prokaryote or a eukaryote.

11. The translation system of any one of claims 1 to 10, wherein the anticodon comprises one or more types of nucleosides selected from adenosine (A), guanosine (G), cytidine (C), and uridine (U).

12. The translation system of any one of claims 1 to 11, wherein more than 20 types of amino acids or amino acid analogs can be translated from one genetic code table.

13. The translation system of any one of claims 1 to 12, which is a cell-free translation system.

14. The translation system of claim 13, which is a reconstituted cell-free translation system.

15. The translation system of claim 13 or 14, comprising an *Escherichia coli*-derived ribosome.

16. The translation system of any one of claims 1 to 15, wherein the tRNA has been attached to the amino acid or the amino acid analog outside the translation system.

17. The translation system of any one of claims 1 to 16, wherein among the tRNAs, the tRNA having an anticodon complementary to a codon represented by M_1M_2A and the tRNA having an anticodon complementary to a codon represented by M_1M_2G have been attached to the amino acid or the amino acid analog outside the translation system.

18. The translation system of any one of claims 1 to 17, wherein the tRNA is obtained by the pCpA method, the pdCpA method, a method using an artificial RNA catalyst (flexizyme), or a method using an aminoacyl-tRNA synthetase (ARS).

19. A method for producing the translation system of any one of claims 1 to 18, comprising attaching the amino acid or the amino acid analog to a tRNA outside the translation system.

20. The method of claim 19, wherein attaching the amino acid or the amino acid analog to a tRNA outside the translation system is carried out by the pCpA method, the pdCpA method, a method using an artificial RNA catalyst (flexizyme), or a method using an aminoacyl-tRNA synthetase (ARS).

21. A method for producing a peptide, comprising translating a nucleic acid using the translation system of any one of claims 1 to 18 or a translation system obtained by the method of claim 19 or 20.

22. The method of claim 21, wherein the peptide has a cyclic portion.

23. A peptide produced by the method of claim 21 or 22.

24. A method for producing a peptide library, comprising translating a nucleic acid library using

the translation system of any one of claims 1 to 18 or a translation system obtained by the method of claim 19 or 20.

25. A peptide library produced by the method of claim 24.

5

26. A method for identifying a peptide having binding activity to a target molecule, comprising contacting the target molecule with the peptide library of claim 25.