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Review

# Cotranslational incorporation of non-standard amino acids using cell-free protein synthesis

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ABSTRACT

**Over the last years protein engineering using non-standard amino acids has gained increasing attention. As a result, improved methods are now available, enabling the efficient and directed cotranslational incorporation of various non-standard amino acids to equip proteins with desired characteristics. In this context, the utilization of cell-free protein synthesis is particularly useful due to the direct accessibility of the translational machinery and synthesized proteins without having to maintain a vital cellular host. We review prominent methods for the incorporation of non-standard amino acids into proteins using cell-free protein synthesis. Furthermore, a list of non-standard amino acids that have been successfully incorporated into proteins in cell-free systems together with selected applications is provided.**



## 1. Introduction

Nature has evolved a set of 20 primary building blocks – the proteinogenic or standard amino acids (sAAs) – serving as substrates in ribosome-mediated protein synthesis in almost all living organisms. Exceptionally, this natural repertoire is expanded by selenocysteine [1] and pyrrolysine [2,3] that are translationally incorporated only in certain species. Based on their biochemical and biophysical characteristics this finite number of amino acids limits the ability to directly study proteins but thereby defines the possible chemical space of protein chemistry [4]. Chemical synthesis bears the potential to generate a vast variety of non-standard amino acids (nsAAs) with a tremendous amount of possible characteristics exceeding the natural repertoire of primary protein building blocks [5,6]. With the objective of supporting biological investigations as well as creating proteins with novel characteristics scientists have developed methods in order to cotranslationally incorporate a variety of different nsAAs into proteins. In the following we review some of the most prominent approaches with special emphasis on their use in the context of cell-free protein synthesis (CFPS). Although the methods presented here have mainly been applied in *Escherichia coli* based cell-free systems (for a more concise presentation please refer to [7] and [8]) this article aims at pointing out key features that support their transfer to eukaryotic CFPS. Finally we provide an overview on diverse nsAAs that have been successfully employed in different CFPS systems (Fig. 1 and Table 1) together with a presentation of selected applications.

## 2. Cell-free protein synthesis

CFPS also termed in vitro protein translation has emerged as a versatile technology to complement cell-based protein expression [9–12]. CFPS utilizes the translational machinery of cells obtained either by preparation of crude cell extracts or the reconstitution of purified components. By addition of an appropriate template that can be either DNA or mRNA protein synthesis is directed towards the predominant production of a desired protein. Until today CFPS based on a variety of different sources including *E. coli* [13–16], *Leishmania* [17], *Thermus* [18], wheat germ [19,20], tobacco [21], insect [22–24], yeast [25], rabbit reticulocytes [26], CHO [27], mouse [28] and human cells [29,30] has been demonstrated. Different reaction modes are available that enable transcription and translation to be performed separately (linked

*Abbreviations:* aatRNA, aminoacyl-tRNA; CECF, continuous-exchange cell-free; CFCF, continuous-flow cell-free; CFPS, cell-free protein synthesis; CuAAC, copper-catalyzed alkyne-azide cycloaddition; eRF1, eukaryotic release factor 1; IRES, internal ribosome entry site; nsAA, non-standard amino acid; oRS, orthogonal aminoacyl-tRNA synthetase; otRNA, orthogonal tRNA; OTS, orthogonal translation system; Pyl, pyrrolysine; PylRS, pyrrolysyl-tRNA synthetase; RF, release factor; sAA, standard amino acid

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**Table 1**
List of nsAAs that have been incorporated using cell-free protein synthesis. The entry numbers correspond to numbers given in Fig. 1. It should be noted that this list is not intended to be exhaustive. *Abbreviations used:* rs = residue-specific; pc = precharged tRNA; op = orthogonal tRNA/synthetase pair; fs-tRNA = frame-shift-tRNA; s-tRNA = stop-codon-suppressor-tRNA; RR = rabbit reticulocyte; WG = wheat germ.

| Entry | Non-standard amino acid | Methodology |
|---|---|---|
| **1** | 4-Fluorotryptophan | rs (*E. coli* [80]) |
| **2** | 4-Methyltryptophan | rs (*E. coli* [80]) |
| **3** | 5-Fluorotryptophan | rs (*E. coli* [80]) |
| **4** | 5-Hydroxytryptophan | pc s-tRNA (*E. coli* [181]) |
| **5** | 5-Methyltryptophan | rs (*E. coli* [80]) |
| **6** | 6-Fluorotryptophan | rs (*E. coli* [80]) |
| **7** | 6-Methyltryptophan | rs (*E. coli* [80]) |
| **8** | 7-Azatryptophan | rs (*E. coli* [80]); pc fs-tRNA (*E. coli* [162]); pc s-tRNA (*E. coli* [126,181]) |
| **9** | Azidohomoalanine | rs (*E. coli* [130,178]) |
| **10** | 2-Naphthylalanine | pc fs-tRNA (*E. coli* [111]; *Sf*21 [115]) |
| **11** | 2-Anthrylalanine | pc fs-tRNA (*E. coli* [132]) |
| **12** | Acetyl-lysine | op s-tRNA (*E. coli* [182]) |
| **13** | Dansyl-lysine | pc s-tRNA (*E. coli* [126,181]) |
| **14** | Homophenylalanine | pc s-tRNA (*E. coli*[125]) |
| **15** | para-Nitrophenylalanine | pc s-tRNA (*E. coli* [125]); pc fs-tRNA (*E. coli* [111,113,164]; RR [113]; *Sf*21 [115]) |
| **16** | para-Fluorophenylalanine | pc s-tRNA (*E. coli* [125]) |
| **17** | para-Trifluoromethylphenylalanine | op s-tRNA (*E. coli* [127]) |
| **18** | para-Iodophenylalanine | pc s-tRNA (*E. coli* [183]) |
| **19** | para-Azidophenylalanine | op s-tRNA (*E. coli* [63,108,128,169,171,172,178,179]; *Sf*21 [107]) |
| **20** | para-Azidomethyl-phenylalanine | op s-tRNA (*E. coli* [177]) |
| **21** | para-Acetylphenylalanine | op s-tRNA (*E. coli* [95,128,171]) |
| **22** | para-Benzoylphenylalanine | pc s-tRNA (*E. coli* [126,128]; RR [175]); pc fs-tRNA (*E. coli*/RR [113]) |
| **23** | para-Bipyridylphenylalanine | op s-tRNA (*E. coli* [128,145]) |
| **24** | 2-Methyltyrosine | op s-tRNA (*E. coli* [171]) |
| **25** | 3-Iodotyrosine | op s-tRNA (WG [154]) |
| **26** | 3-Chlorotyrosine | pc s-tRNA (WG [124]) |
| **27** | 3-Azidotyrosine | pc s-tRNA (WG [170]); op s-tRNA (*E. coli* [173]) |
| **28** | Propargyloxyphenylalanine | op s-tRNA (*E. coli* [63,95,108,174,176,178]) |
| **29** | Allylglycine | pc s-tRNA (*E. coli* [184]) |
| **30** | Homopropargylglycine | rs (*E. coli* [130,178,179]) |
| **31** | Selenomethionine | rs (*E. coli* [155]) |
| **32** | Biocytin and derivatives | pc s-tRNA (RR [185]); pc fs-tRNA (*E. coli* [186] |
| **33** | L-3,4-Dihydroxyphenylalanine | rs (*E. coli* [187]) |
| **34–39** | Glycosylated serine derivatives | pc s-tRNA (*E. coli*/RR [167]) |
| **40–45** | Glycosylated tyrosine derivatives | pc s-tRNA (*E. coli*/*RR* [167]) |
| **46** | Dabcyl-diaminopropionic acid | pc s-tRNA (*E. coli* [162]) |
| **47** | β-Anthraniloyl-L-α,β-diaminopropionic acid | pc fs-tRNA (*E. coli* [133,164]) |
| **48** | (7-Hydroxy-coumarin-4-yl)ethylglycine | op s-tRNA (*E. coli* [127,128,158]) |
| **49** | Bodipy-FL-aminophenylalanine and derivatives | pc fs-tRNA (*E. coli* [156,159,188]); s-tRNA (*E. coli* [188]) |
| **50** | Bodipy558-aminophenylalanine | pc fs-tRNA (*E. coli* [188]) |
| **51** | 4-[(6-[Tetramethylrhodamine-5-(and-6)-carboxamido]hexanoyl)amino]phenylalanine | pc fs-tRNA (*E. coli* [156,163]) |

mode) or simultaneously (coupled mode) in one reaction vessel [8]. Even linear PCR products can be used as gene templates significantly reducing time expenses by circumventing laborious cloning steps [31–33]. Lacking the cellular plasma membrane the open environment of CFPS enables the direct manipulation of the protein translation process to achieve a tailored protein production. Therefore, CFPS is a promising tool for the synthesis of difficult-to-express proteins [8,34]. For example, the over-expression of toxic and membrane proteins is strongly facilitated due to the fact that CFPS lacks constrains accompanied by cell vitality. In general, the choice of appropriate synthesis conditions has to be determined in accordance with the individual protein to be expressed in order to obtain maximum yields of properly folded and functional products. In this context, prokaryotic systems are characterized by comparatively high protein yields but due to the limited capability to perform sophisticated post-translational modifications e.g. phosphorylation, glycosylation and signal peptide cleavage, the synthesis of functional eukaryotic proteins can be improved using cell-free systems based on extracts derived from cultured eukaryotic cells.

### 2.1. Unique features of eukaryotic cell-free systems

Over the last years, systems derived from wheat germ embryos have become the most recognized eukaryotic CFPS systems (for a detailed overview please refer to [35]). A multitude of different proteins have been successfully synthesized using wheat germ extracts, illustrating its versatility and in particular the convenience of a eukaryotic translation and folding machinery. Nevertheless, cell-free wheat germ systems lack the capability to perform post-translational modifications, which can clearly have a significant impact on protein folding and corresponding functionality. Interesting developments in the extract preparation procedure have enabled the production of eukaryotic cell-free systems based on crude extracts from cultured tobacco BY-2 [21], *Sf*21 [36,37], CHO and human K562 [38] cells that contain endogenous microsomal structures. These lipid bilayers have been found to carry out some of the functions of the endoplasmic reticulum thereby providing PTMs such as protein N-glycosylation [37,39], lipid modifications [40] and signal peptide cleavage [41]. They further support active cotranslational translocation of proteins

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