Selection, Addiction and Catalysis: Emerging Trends for the Incorporation of Noncanonical Amino Acids into Peptides and Proteins in Vivo

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Expanding the genetic code of organisms by incorporating noncanonical amino acids (ncAAs) into target proteins through the suppression of stop codons in vivo has profoundly impacted how we perform protein modification or detect proteins and their interaction partners in their native environment. Yet, with genetic code expansion strategies maturing over the past 15 years, new applications that make use—or indeed re-purpose—these techniques are beginning to emerge. This Concept article highlights three of these developments: 1) The incorporation of ncAAs for the biosynthesis and selection of bioactive macrocyclic peptides with novel ring architectures, 2) synthetic biocontainment strategies based on the addiction of microorganisms to ncAAs, and 3) enzyme design strategies, in which ncAAs with unique functionalities enable the catalysis of new-to-nature reactions. Key advances in all three areas are presented and potential future applications discussed.

Background: Stop Codon Suppression

The site-selective incorporation of ncAAs into peptides and proteins in vivo can be achieved through the suppression of a stop codon by the action of an orthogonal translation system (OTS, Figure 1A). An OTS is comprised of an engineered aminoacyl tRNA synthetase (aaRS), which loads a ncAA onto its corresponding tRNA, while none of the OTS components interacts with endogenous amino acids, aaRSs or tRNAs (α-orthogonal, Figure 1B). The incorporation of ncAAs by an OTS is achieved if: 1) protein engineering efforts have changed the cognate substrate preference of the aaRS to a ncAA of choice, and 2) the tRNA features the anticodon sequence (e.g., CUA) to a stop codon, for example, UAG (Figure 1A). Charged tRNAs are then recruited to the ribosome, where in-frame UAG stop codons in mRNAs are suppressed, resulting in the site-selective incorporation of a ncAA into the nascent peptide chain. Despite typically modest suppression efficiencies and the fact that not every ncAA is genetically encodable (metabolic stability, limited uptake, etc.), this strategy has proven exceptionally versatile.[1,2] Indeed, more than 150 ncAAs have been successfully incorporated into peptides and proteins of interest in a variety of model organisms. Since its conception more than 15 years ago, stop codon suppression as a strategy has predominantly been applied for introducing ncAAs with functional groups that enable site-selective protein modification and/or elucidating, altering or regulating protein function.[3,5] However, more recently, OTSs have also been repurposed for other tasks. This Concept article will highlight recent developments for which ncAA incorporation has proven particularly impactful and is divided into three sections: 1) Selection, 2) Addiction, and 3) Catalysis.

Selection: ncAAs in Peptide Macrocyclization

Macrocyclic peptides (MPs) are privileged scaffolds for the development of chemical probes and therapeutics.[6,7] Combining a high degree of functional complexity with a restricted conformational flexibility make MPs well-suited to achieve tight binding to notoriously difficult targets, such as biomolecular interfaces.[8] Moreover, peptide macrocyclization is a straightforward means to reduce protease degradation and can facilitate cellular uptake.[9] Lastly, peptides are genetically encodable and lend themselves to massive parallel screening and selection efforts that allow for identifying tight binders from randomized populations. As a result, methods to genetically encode MP libraries are sought after, yet the number of cyclization strategies—and therefore, the accessible ring geometries—remain limited.[10]

Bioactive MPs found in nature often feature ncAAs and utilize unique functionalities placed in their side chains to promote macrocyclization.[11] In an effort to mimic such natural products, the Suga group has led efforts to encode MPs containing a wide variety of ncAAs in vitro.[12] Taking advantage of well-defined, reconstituted translation systems, in which natural aaRS/tRNA pairs are replaced with synthetically pre-charged tRNAs featuring non-standard building blocks,[13] the group...
Figure 1. A) A ncAA is taken up into the cell, where it is charged onto an orthogonal tRNA through the action of an engineered aaRS. Once charged, this tRNA, which features the complementary sequence of a stop codon (e.g., CUA), is recruited to the ribosome, where it suppresses an in-frame stop codon (UAG) located on an mRNA. B) Schematic representation of orthogonality with respect to the OTS. Note that engineered aaRS and tRNA (orange) do not interact (dashed arrows) with the endogenous aaRS/tRNA pairs (blue).

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in vivo and showed a 120-fold increased affinity, when compared to the parent, linear peptide.

Lastly, the Fasan group also demonstrated that O2beY-mediated macrocyclization is compatible with split intein circular ligation of peptides and proteins (SICLOPPS), a widely employed technique to obtain MPs in vivo.\textsuperscript{17,25,26} When combined with SICLOPPS, O2beY incorporation allowed for the biosynthesis of bicyclic peptides. A proof-of-principle that the resulting bicyclic peptides are attractive scaffolds for identifying bioactive MPs is provided by the biosynthesis of bicyclo-Z8C (Figure 2D), which was based on the streptavidin-binding peptide described above. Critically, bicyclo-Z8C showed a 2.5-fold lower IC\textsubscript{50} value, when compared to a monocyclic variant, which was attributed to the lower conformational flexibility of bicyclo-Z8C.\textsuperscript{26}

Together, these examples demonstrate that ncAAs are a promising means to access and identify bioactive MPs with novel ring geometries. As the presented strategies enable peptide macrocyclization in vivo, they are now readily available for interfacing with phenotypic screens and selection strategies. Thus, future efforts will focus on expanding this methodology to select mono- and bicyclic peptides for biologically relevant targets. Moreover, the introduction of ncAAs other than MeaF and O2beY is likely to make other cyclization strategies available in the near future. Specifically, ncAAs that enable macrocyclization strategies with side chains other than cysteines and transiently formed thiosters are desirable to access “natural product-like” MPs with new ring geometries.

**Addiction: ncAAs in Biocontainment**

Synthetic biology aims to deploy genetically modified organisms (GMOs) as common and valued solutions in clinical, industrial and environmental settings.\textsuperscript{27} However, such real-world applications necessitate the development of biocontainment strategies, reminiscent to those outlined in the 1975 Asilomar conference for recombinant DNA.\textsuperscript{28} Generally, effective biocontainment strategies must protect against GMO escape mechanisms, including mutagenic drift, environmental supplementation and horizontal gene transfer.\textsuperscript{29}

In principle, constructing GMOs with an alternative genetic code by introducing a ncAA into an essential gene (Figure 3A) would advance the barrier between an engineered and a natural organism, as survival of the former would depend on an
Such addiction of protein function to a synthetic amino acid can be achieved by various means. For example, Church and co-workers designed synthetic auxotrophs by computationally redesigning hydrophobic interactions in protein cores to exclusively accommodate 4,4′-biphenylalanine (bipA, Figure 3B, C,[31]) For the genes of adenylate kinase and tyrosyl-tRNA synthetase this redesign resulted in two separate organisms with low EFs (≈10⁻⁴). Moreover, engineering a single GMO that harbored the identified mutations for both genes amplified the effect and resulted in a synthetic auxotroph for which no escape variants could be detected (EF < 10⁻⁴).

Directed evolution provides another means to redesign the hydrophobic packing of protein cores to make it depend on a synthetic amino acid. Specifically, the Ellington group selected TEM-1 β-lactamase variants, the ability of which to confer carbenicillin resistance was dependent on the introduction of 3-nitrotyrosine (3nY) or 3-iodotyrosine (3iY, Figure 3B).[29] For a promising engineered variant, TEM-1-B9, phenylalanine was the only canonical amino acid that could rescue the activity in absence of the ncAA. However, codons for phenylalanine (UUU and UUC) cannot be accessed by a single mutation from UAG, thus making this reversion unlikely. The group confirmed that this is an unlikely escape mechanism by culturing E. coli strains, which harbored TEM-1.B9 and the OTS on a single plasmid, continuously in liquid or solid media without detecting any escape variants (EF < 10⁻⁴). The single plasmid setup is particularly notable, as it allowed for transformation of other enterobacteria, which all became dependent on 3nY in the presence of ampicillin and did not escape the containment (EF < 10⁻⁴).

Another means for making protein function dependent on the presence of a ncAA involves replacement of a natural active site residue with a non-standard one. For example, the Schultz group reported the incorporation of N₁-acetylated (AcK) (Figure 3B) into the essential branched chain aminotransferase (BCAT) of E. coli (Figure 3D).[13] Specifically, replacing a catalytic lysine with AcK will first produce an inactive BCAT variant, which is then activated upon deacetylation by endogenous acetyltransferases in E. coli. As such, synthetic auxotrophs can only breach this containment through mutations that allow for the incorporation of lysine in response to the UAG nonsense codon. Indeed, the authors identified this mechanism as the common feature in escape mutants (EF > 10⁻⁴). Se-
sequence analysis of escape variants revealed a point mutation in the anticodon of an *E. coli* lysine tRNA (3'-UUU-5' to 3'-AUU-5'), which resulted in an anticodon that could suppress UAG stop codons through G/U wobble pair formation. To counter this escape mechanism, a barnase gene featuring two in-frame UAA stop codons in its mRNA was added to the plasmid. Barnase production is lethal and full-length protein will only be produced in escape variants featuring the mutated lysine tRNA anticodon (3'-AUU-5'), which is complementary to the in-frame UAA stop codons. With this conditional kill switch in place escape mutants effectively committed suicide, which resulted in a tight biocontainment (EF < 10⁻¹¹).

While making protein function dependent on the presence of a ncAA is desirable, it requires a certain degree of design or evolution. An alternative strategy involves the replacement of conserved residues with a ncAA across multiple essential genes. This strategy was showcased by the Isaacs group, who employed multiplex automated genome engineering (MAGE) to identify a synthetic auxotroph that featured three TAG sites in the MurG, DnaS and SerS genes, as well as an OTS decoding p-azidophenylalanine (pAzF, Figure 3B). In the identified GMO, addition to pAzF is achieved by the ability of the ncAA to replace conserved aromatic residues in these three genes. Similar to the escape mechanism for Akc-dependent organisms mentioned above though, all escape mutants in this work featured point mutations in the anticodon of one of the three endogenous tyrosine tRNAs. The identified mutations converted them into suppressor tRNAs, resulting in the incorporation of tyrosine instead of pAzF in response to UAG codons. To overcome this mechanism, the Isaacs group deleted two of the three *E. coli* tyrosine tRNAs; the remaining one was therefore essential for tyrosine incorporation in the GMO, preventing it from accumulating mutations and becoming a suppressor tRNA. In addition to a stringent biocontainment (EF < 10⁻¹²), the authors demonstrated that the resulting synthetic auxotroph could not be rescued by cross-feeding and that the alternative genetic code indeed impeded horizontal gene transfer.

Related to these efforts, introducing multiple stop codons in the genome of viruses is an emerging strategy to generate live but replication-deficient virus (Figure 3E). These can serve as live-attenuated vaccines, as they retain their full infectivity and elicit a strong immune response. Zhou and co-workers, for example, have recently created a replication-deficient influenza A virus, in which multiple UAG codons were introduced at conserved residues throughout the viral genome. Viruses created by this strategy were highly productive in transgenic cell lines that featured an OTS to decode the ncAA to replace conserved aromatic residues in these escape mutants effectively committed suicide, which resulted in a tight biocontainment (EF < 10⁻¹¹).

Overall, the highlighted examples demonstrate that addition to ncAAs is a promising means to create tight biocontainment for synthetic biology applications. Future efforts are likely to expand the number of ncAAs that can be used to create such GMOs and will see their integration into real-world applications. To avoid the use of an expensive ncAA, it is conceivable that endogenous metabolic pathways could be taken advantage of in order to shift the addiction to a more cost effective, yet still exogenously-supplied synthetic precursor of a ncAA.

**Catalysis: ncAAs in Designer Enzymes**

The enzyme orotidine-5'-phosphate decarboxylase accelerates its target transformation by a factor of 10¹⁷ with respect to the uncatalyzed reaction and does so by using only canonical amino acids. However, the scope of enzyme catalysis accessible by standard amino acids is fundamentally limited by the diversity of functional groups present in their side chains. Therefore, enzymes in nature routinely recruit electrophiles, redox equivalents, metal ions, transfer agents, etc., in the form of co-factors or co-substrates. Similarly, natural side chains in active sites can undergo posttranslational modifications to install uniquely reactive functional groups that promote a desired transformation (for example, converting cysteine to formylglycin in type I sulfatases). Enzyme design, in part, aims to create proficient protein catalysts with new-to-nature activities. In analogy to the natural strategies described above, the incorporation of ncAAs into protein scaffolds has emerged as an attractive means to expand the reaction scope of designer enzymes beyond what is possible with canonical side chains. For example, ncAAs that feature bioorthogonal handles in their side chains can be employed for covalent modification with abiological, transition metal catalysts (an artificial metalloenzyme, Figure 4A). Amongst others, the Lewis group has reported the creation of a designer cyclopropanation enzyme by first introducing pAzF into the binding pocket of prolyl oligopeptidase (POP) and then recruiting a dirhodium catalyst through a strain-promoted azide–alkyne cycloaddition. Introducing a bioorthogonal handle (i.e., the azide in pAzF) for protein modification is more desirable than other bioconjugation strategies that rely on natural side chains (for example, cysteines), as the modification reaction can be carried out in complex mixtures. This aspect is critical when attempting to evolve artificial metalloenzymes, as anchoring strategies are typically not compatible with the complex cellular milieu. Indeed, the Lewis group has demonstrated the utility of pAzF in the directed evolution of the BOP-derived cyclopropanases. By performing the cofactor anchoring reaction in bacterial lysates, the group was able to screen libraries of BOP-variants that were generated by random mutagenesis and could successfully identify more proficient cyclopropanases. Additionally, these engineered designer enzymes also showed superior reactivity and selectivity in related N–H, S–H and Si–H insertion reactions.
Instead of relying on a biorthogonal handle to recruit a synthetic cofactor, the incorporation of metal-chelating ncAAs that can directly bind metal ions or complexes is another means to create artificial metalloenzymes (Figure 4C,[49]) For example, the Schultz group installed 2,2′-bipyridin-5-ylalanine (bpyA, Figure 4B) into the E. coli catabolite activator protein, which upon binding of copper or iron ions endowed the protein with nucleic activity.[50] More recently, the Roelfes group has expanded this approach by introducing bpyA into the promiscuous, hydrophobic binding pockets of multidrug resistance regulators (MDRs). Upon binding copper ions, a number of MDR-based artificial enzymes were created that could catalyze abiological Friedel–Crafts alklylation[51, 52] and hydration reactions.[53] The high activities and selectivities observed in these designer enzymes are the result of embedding the copper ion (through coordination to bpyA) into the MDR binding pockets, which aids in recruiting hydrophobic substrates. Incorporating genetically encodable, metal-chelating ncAAs, such as bpyA, into protein binding pockets is also an attractive strategy for future directed evolution campaigns. A metal-chelating nCAA alleviates the need for a posttranslational synthetic step to recruit the catalysts species and therefore could facilitate artificial metalloenzyme formation in complex media or even living cells. As a result, this strategy could significantly increase the throughput, when screening for improved designer enzymes.

In a variation of this theme, the Hilvert and Schultz groups have replaced histidine with N$_2$-methyl histidine (NmH) to position a heme prosthetic group in myoglobin[54] and an essential zinc ion an mannose-6-phosphate isomerase.[55] While the latter resulted in a GMO addicted to NmH (EF $< 10^{-11}$), for the former the introduction of NmH was shown to subtly alter the electronic properties of the bound heme. Notably, installing NmH as axial heme ligand in a previously engineered myoglobin[56] did not only boost the peroxidase activity[57, 58] but also its promiscuous cyclopropanation activity.[59] Moreover, in the presence of NmH, cyclopropanation reactions could be carried out in absence of a reducing agent, conditions under which the parent histidine variant was largely inactive.[56]

Besides recruiting, positioning and fine-tuning of (synthetic) metal cofactors, the Roelfes group has recently described a new strategy for enzyme design, in which a nCAA with a unique functionality is incorporated to act as a catalytic residue (Figure 4E). Specifically, the incorporation of p-aminophenylalanine (pAF) at position 15 in the MDR from Lactococcus lactis (LmrR), resulted in LmrR_V15pAF, which promoted hydrazone and oxime formation reactions.[60] This activity was ascribed to the unique ability of the anilne side chain of pAF to form an iminium ion (covalent catalysis) with a carbonyl moiety in the substrate.

Overall, these examples demonstrate that the incorporation of ncAAs into protein scaffolds has already begun to significantly expand the reaction scope of designer enzymes. In the future, placing metal-chelating amino acids into protein scaffolds will continue to create new and/or improved metal-binding environments that will give access to new reactivities. For
the incorporation of ncAAs that directly promote a target transformation, the creation and directed evolution of LmrV153pAF opens up questions that need to be answered. For example, is it a general phenomenon that the performance of organocatalysts, which are versatile yet notoriously slow, is boosted by placing them in protein scaffolds by ncAA incorporation?\cite{79, 80} And, are the resulting designer enzymes generally privileged starting points for directed evolution efforts, as it was demonstrated for LmrVpAF?\cite{81} Independent of the answers to these questions, designer enzymes will continue to make use of ncAAs to catalyze new-to-nature transformations.

**Future Directions: Orthogonal Ribosomes**

With each section closing with a brief outline of future directions, this section aims to highlight developments that could impact all areas discussed in this Concept article. As mentioned briefly in the **Selection** part, the simultaneous incorporation of multiple, chemically distinct ncAAs is typically not efficient.\cite{82} This stems from the fact that adding codons to the existing genetic code is not straightforward and the number of codons readily available for recoding is limited. For example, universal reassignment of more than one nonsense codons is difficult, due to their native function to signal for termination of translation. To meet this challenge, the Chin group has begun to install a parallel genetic code into model organisms, by making use of orthogonal ribosomes (ORs).\cite{83} In brief, ORs feature mutations in the 16S ribosomal RNA that enables them to recognize mRNAs, which are not translated by endogenous ribosomes.\cite{84} Conversely, ORs do not recognize native mRNAs and as a result, do not participate in the synthesis of endogenous proteins. Being now not essential for the survival of the organism, an OR is free to accumulate mutations that alter its interaction with tRNAs and/or release factors.\cite{85} Such engineering efforts have allowed for the addition of codons,\cite{86} the selection of new OTS that are specific for ORs,\cite{87} and the efficient encoding of two ncAAs simultaneously.\cite{88, 89} Thus, it is conceivable that applying ORs to the strategies discussed in this article could allow for novel peptide macromolecularization strategies, reinforce synthetic biocatalysis, and further expand the scope of reactions catalyzed by designer enzymes.

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**Conflict of Interest**

The authors declare no conflict of interest.

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