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Cell-free biology: Exploiting the interface between synthetic biology and synthetic chemistry

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Just as synthetic organic chemistry once revolutionized the ability of chemists to build molecules (including those that did not exist in nature) following a basic set of design rules, cell-free synthetic biology is beginning to provide an improved toolbox and faster process for not only harnessing but also expanding the chemistry of life. At the interface between chemistry and biology, research in cell-free synthetic systems is proceeding in two different directions: using synthetic biology for synthetic chemistry and using synthetic chemistry to reprogram or mimic biology. In the coming years, the impact of advances inspired by these approaches will make possible the synthesis of nonbiological polymers having new backbone compositions, new chemical properties, new structures, and new functions.

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Introduction

Biology is unparalleled in its ability to produce complex molecules and polymers from simple building blocks. By harnessing biosynthetic pathways and macromolecular machines, biological systems convert monomer precursor molecules into a variety of products with atomic-scale resolution over composition, architecture, and functionality. These products are not only precise (defined here as being stereo-specific and regio-specific), but are also produced with remarkable fidelity, efficiency, and yield. Both the extraordinary synthetic capability of biological systems and their incredible versatility provide tremendous opportunities for making products that serve society. For instance, biological ensembles have already been used as factories to produce polyketide antibiotics, fatty acid fuels, and protein therapeutics [1–3].

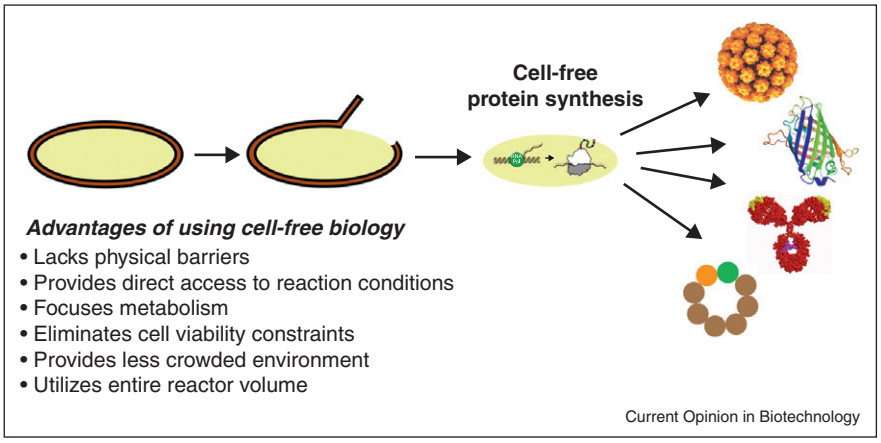
The products of biological systems are governed by the chemistry of life. This chemistry is limited to natural building blocks (e.g. nucleic acids, ~20 naturally occurring amino acids, etc.), from which the structures of living organisms are constructed. Strikingly, while biological polymers are structurally narrow, they are functionally diverse. In contrast, chemical polymers have much more structural diversity, but limited functionality (relative to the size of the monomer repertoire) [4]. The potent functionalities and higher order architectures that arise from the relatively simple monomers found in biology inspires us to expand the chemistry of life to include diverse, synthetic monomers that are not limited to those found in nature [5,6].

Indeed, efforts to incorporate non-natural monomers in biological systems are expanding at an accelerated pace. Dominant amongst frontier applications are both the manufacturing of proteins with noncanonical amino acids [5,7] and efforts to expand the genetic alphabet [8]. While the lion's share of this work has been conducted *in vivo*, we focus this review on the role of cell-free systems in these synthetic biology efforts. We begin by defining cell-free biology and its advantages. We then describe recent applications that exploit the cell's protein synthesis machinery for synthetic chemistry. Finally, we consider how synthetic chemistry is used for cell-free synthetic biology.

Cell-free biology introduction

Cell-free biology is the activation of complex biological processes without using intact living cells [9–11]. Bypassing cell walls, one can access and manipulate biology directly. In contrast to *in vivo* engineering efforts, this direct relationship with biocatalytic enzymes provides the ability to focus metabolism on the production of a single compound, removes physical barriers (allowing easy substrate addition, product removal, and rapid sampling), and eliminates the requirement for cellular viability. Furthermore, removal of native genomic regulation means that microbial growth and engineering design objectives do not conflict. Figure 1 depicts the basic concept of cell-free biology and lists key advantages. The most prominent example of cell-free biology is cell-free protein synthesis (CFPS). Originally used to decipher the genetic code [12], CFPS has emerged as a powerful platform for microscale to manufacturing scale synthesis of complex proteins, opening the way to new applications, such as expanding the chemistry of life [10].

Figure 1



Cell-free biology is a rapidly developing technology for making advanced materials, sustainable biomolecules, and life-saving medicines. This approach provides numerous advantages for exploiting the interface between synthetic biology and synthetic chemistry.

Using cell-free synthetic biology for synthetic chemistry

The synthesis of proteins with an expanded repertoire of nongenetically encoded amino acids (ngeAAs) requires (re-)assignment of codons to ngeAAs, ngeAA-transfer RNA (tRNA) substrates (typically created through an orthogonal tRNA–aminoacyl-tRNA synthetase pair), and ribosome accommodation of these non-natural substrates into the catalytic center [13]. Currently, ~70 ngeAAs have been site-specifically incorporated into proteins [5]. In one exemplary example, incorporating a uniquely reactive ngeAA for site-specific conjugation of polyethylene glycol resulted in a modified human growth hormone with increased potency and reduced injection frequency [14]. This work and others, like a recent breakthrough showing the synthesis of phosphoproteins [15•], illustrates the utility of ngeAA incorporation.

Efforts to use CFPS for site-specific incorporation of ngeAAs are beginning to grow [10,16•]. The driving force behind this recent growth is two-fold. First, a technical renaissance in CFPS has inspired new applications [10]. Underpinning these developments has been game-changing increases in cost-effective, high-level protein synthesis in both crude extracts and in the Protein synthesis Using Recombinant Elements (PURE) system [10,17]. Jewett *et al.*, for example, demonstrated the ability to activate central metabolism and oxidative phosphorylation to synthesize up to 1.2 g L^{-1} protein in two hours [18]. More recently, Dong-Myung Kim's lab used a polymeric carbohydrate to enable the synthesis of 1.7 g L^{-1} protein in an *Escherichia coli* CFPS system over 10 hours, the highest known reported batch yield to our knowledge [19]. Second, CFPS has now been demonstrated at the manufacturing scale [20•]. Zawada *et al.* synthesized 700 mg L^{-1} of human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) in 10 hours at the 100 l scale [20•]. This pioneering advance has transformed CFPS systems from a foundational tool used solely for bench-top research into an enabling technology at the industrial scale.

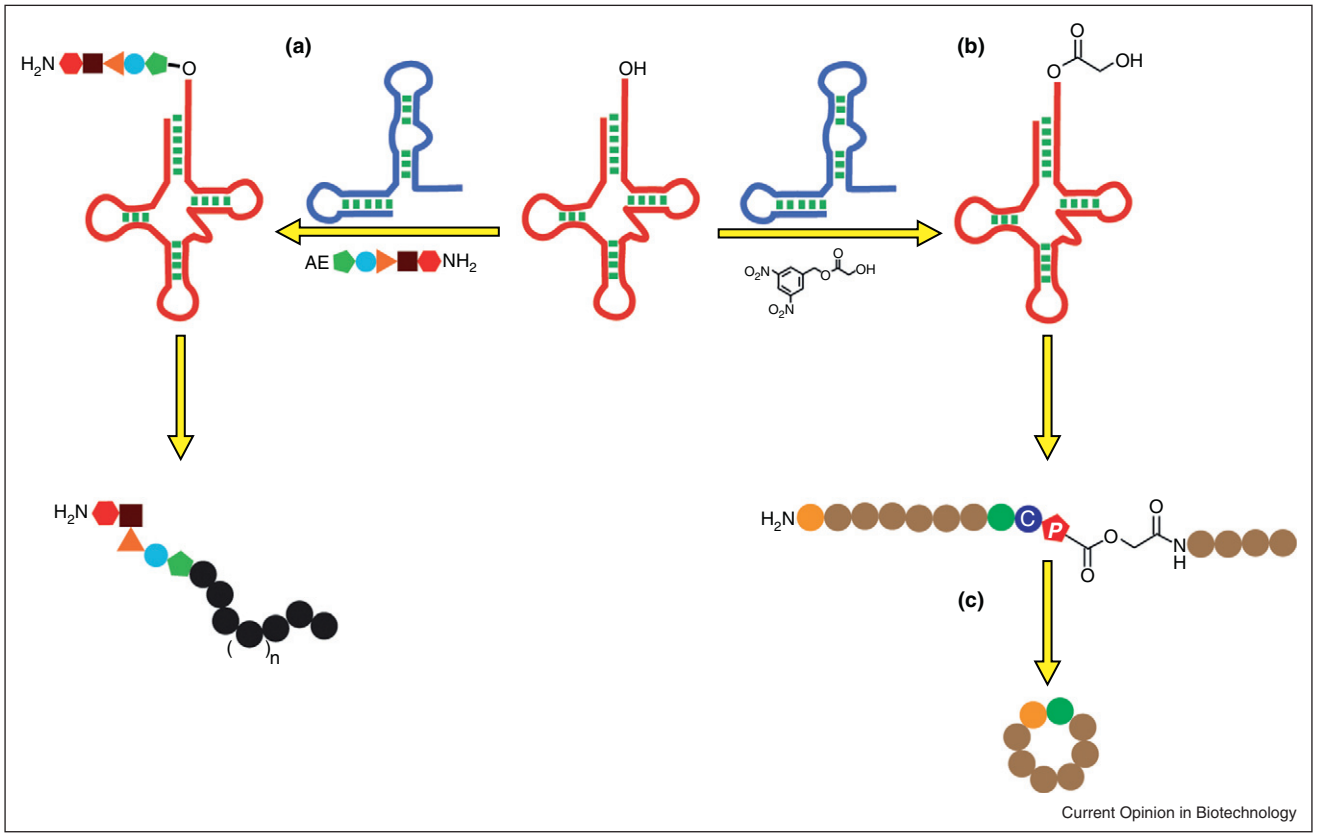
Technological improvements in CFPS have opened the way to exploiting advantages over *in vivo* methods for incorporating ngeAAs into proteins (e.g. avoiding transport limitations for getting ngeAAs into the cell). The first design decision is choosing between a crude extract or a purified system [11,17]. Typically, this decision considers trade-offs between cost and productivity versus freedom of design. For example, the cost of the PURE system is prohibitive for most commercial applications, leaving crude extract systems the clear current choice [11]. In addition, cell lysate systems produce more protein per ribosome [21]. On the other hand, the PURE system opens a whole new world of opportunities for co-opting nearly any codon for the incorporation of ngeAAs into a single peptide or protein without recoding organisms because of the ability to omit components (e.g. tRNAs) [7]. Equally important, the PURE system allows for mutating and engineering the components of the translation machinery to enhance ngeAA incorporation without background native components present. Efforts such as a recent report from Wang *et al.* may help to close the cost gap between crude extract and purified systems [22]. Here, the authors report the use of an advanced genome engineering technology [23] to simultaneously tag multiple components from the translation apparatus in a single strain for copurification and reconstitution *in vitro*.

The Swartz lab has recently demonstrated the power of using CFPS crude extract systems for site-specific

incorporation of ngeAAs. In one case, efficient incorporation of p-azido-L-phenylalanine (pAz) was accomplished with high-level production of active chloramphenicol acetyltransferase and dihydrofolate reductase, ~400–600 mg L^{-1} [16•]. Showcasing the freedom of design in adjusting cell-free system components by direct addition to the reaction, both the orthogonal synthetase and tRNA were synthesized in the extract. More recently, Bundy and Swartz used cell-free systems to avoid solubility and transport limitations typically encountered *in vivo* to incorporate the tyrosine analog *p*-propargyloxyphenylalanine (pPa) as well as pAz for demonstrating a one-step, site-specific direct protein–protein conjugation using copper(I)-catalyzed azide–alkyne [3 + 2] cycloaddition (CuAAC) [24]. In another example from the same group, cell-free systems were used to show global replacement of a natural amino acid with an ngeAA analog without requiring an auxotrophic strain [25•]. This approach enabled the synthesis of decorated virus-like particles that could function as potential vaccines and imaging agents. CFPS systems have also been used to site-specifically incorporate ngeAAs at multiple sites in a protein without having to modify the source strain [26] or by selectively removing a tagged release factor-1 (RF-1) to remove competition for the amber codon [27]. Moving forward, recent advances to improve ngeAA incorporation efficiency *in vivo*, such as deletion of RF-1 [28•,29] or reassigning natural codons to expand the number of possible co-opted codons [30••], should advance *in vitro* efforts that use crude extracts.

Transitioning to the PURE system avoids some key limitations observed in crude extracts [31]. Indeed, genetic code reprogramming in purified systems is revolutionizing our ability to incorporate multiple and diverse types of ngeAAs into proteins [7]. In pioneering works, Suga and co-workers have demonstrated the ability to synthesize a variety of peptides featuring ngeAAs that would be difficult to prepare by traditional translation or synthetic techniques [32,33,34•,35,36]. In a landmark report, they demonstrated the ability to synthesize backbone-cyclized peptides (Figure 2) [34•]. This was the first report of ribosomal synthesis of such peptides and highlights the utility of being able to incorporate multiple ngeAAs in a single peptide.

Figure 2



Exploiting cell-free protein synthesis for producing non-natural peptides. **(a)** Flexizyme (blue) charges an initiator tRNA (red) with an Xpep (multicolored), which is expressed by the PURE system. **(b)** Flexizyme (blue) charges a tRNA (red) with glycolic acid (black). Glycolic acid is then expressed adjacent to a proline (red pentagon) and cysteine (blue circle) residue. **(c)** An intramolecular rearrangement occurs, resulting in the formation of a dkp-thioester and loss of the glycolic acid portion of the peptide. The resulting dkp-thioester can then react with the N-terminal amine to form a backbone-cyclic peptide.

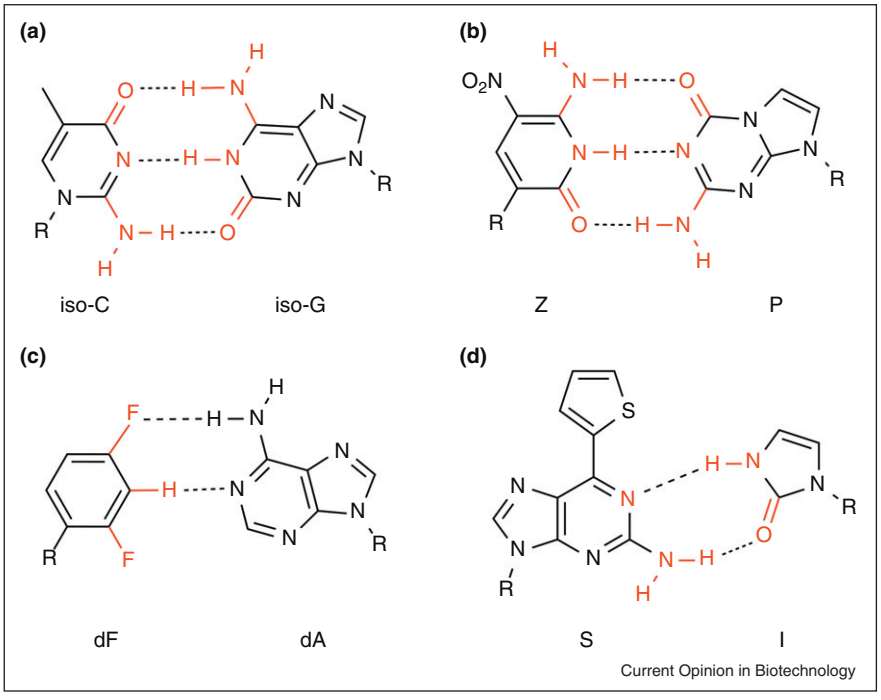
In another exemplary example from the same group, exotic amino acids were expressed by reprogramming of the translation initiation system [33]. Specifically, Flexizyme (a synthetic ribozyme that enables a ngeAA to be charged onto a desired tRNA [35]) was used to charge initiator tRNAs with short peptides, termed Xpeps, comprised of D-amino acids, β-amino acids, and N-methyl amino acids (Figure 2), rather than individual amino acids. Then, the Xpep–tRNA complex was used to initiate ribosomal protein synthesis. Eleven different Xpep sequences (with 2–5 different amino acids) were used, with varying efficiencies of incorporation (12–133%) relative to the natural starting residue for all proteins, formyl-methionine. This report opens the way to the synthesis of a wide variety of unusual peptides. Further developments of CFPS coupled with *in vitro* display technologies promise the ability to screen vastly diverse libraries using Darwinian evolution to accelerate the discovery of peptidomimmetic drugs. However, low efficiencies of incorporation of non-natural monomers by the ribosome must be addressed [37]. Ribosome engineering methods could be one solution [38].

Using synthetic chemistry for cell-free synthetic biology

Complementary to efforts to synthesize protein products containing synthetic monomers, are efforts to reprogram interactions of biology using synthetic chemistry. In pushing the limits of chemical design for natural biology, chemists are yielding a deep understanding of natural biology and providing opportunities for expanding the capabilities of canonical systems. Central to this endeavor is the expansion of the genetic alphabet by increasing the capacity of nucleic acids to store, recall, and propagate information. This is a rich and diverse field [39]. Here, we focus our discussion on modulating biological function by exploring base composition (Figure 3). While many researchers, such as Kool and colleagues, have tried to develop a genetic alphabet on a nucleic acid-like system that lacks hydrogen bonding altogether [39], we focus on work that diverges minimally from natural biology. Indeed, such efforts have provided a powerful approach to overcome the limits of natural translational biochemistry for making new codons for ngeAAs. For example, the creation of post-transcriptionally modified bases [40] or other nonstandard bases [41–43] has been used to expand the genetic code (see Figure 3).

Now 20 years old, groundbreaking work by Benner and coworkers showed that novel Watson–Crick base pairings could be realized by re-orientating the hydrogen-bond donors and acceptors of cytosine and guanine [44]. This led to the creation of a '65th' codon:anticodon pair that efficiently supported the incorporation of a ngeAA in

Figure 3



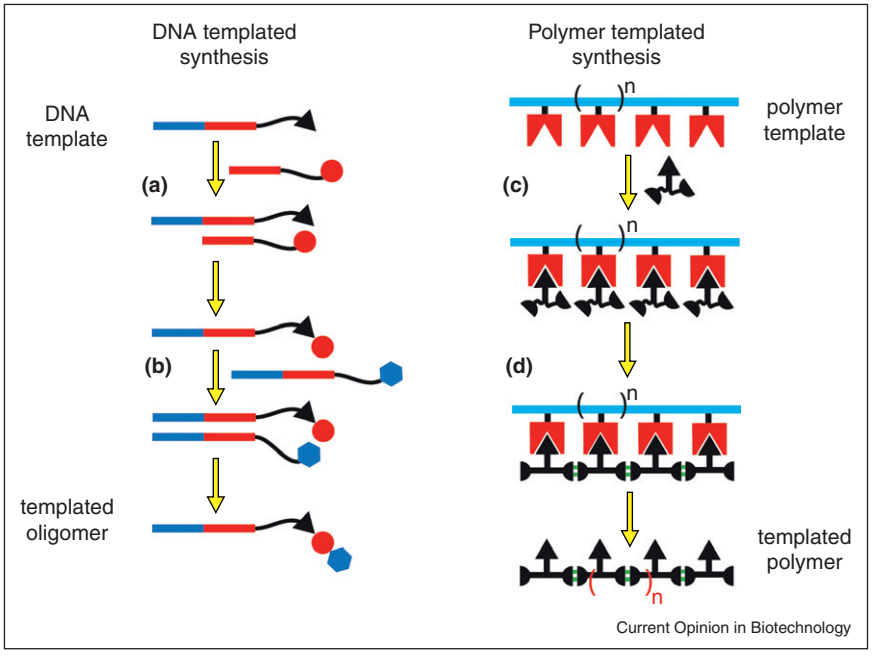
An expanded genetic alphabet for synthetic biology. **(a)** Iso-C and iso-G with shuffled hydrogen-bond donors and acceptors in red [43]. **(b)** Z and P nucleobases, note electron rich substituents on minor groove face [49]. **(c)** The thymidine isostere dF, with weak hydrogen-bond acceptor fluorine and hydrogen-bond donating carbon–hydrogen bond in red [39]. **(d)** Alternate base-pairing thiophene–purine derivative S and imidazolinone derivative I with hydrogen-bond donors and acceptors in red [42].

CFPS from the resulting nucleobases, iso-Cytosine (iso-C) and iso-Guanine (iso-G) (Figure 3) [43]. However, it was observed that during polymerase chain reactions featuring the iso-C and iso-G base pairs, tautomerization of iso-G caused occasional mispairings with thymidine [45]. Furthermore, it was noted that when iso-G and iso-C were assembled into a DNA duplex, they lacked electron rich substituents in the minor-groove, which is important for polymerase function [46].

Recently, the structures of Benner's synthetic nucleobases have been optimized with respect to their chemical stability and ability to be accepted by various enzymes routinely used in molecular biology for a variety of applications [47]. Specifically, these issues were addressed by the creation of an 'Artificially Expanded Genetic Information System (AEGIS)' that deviates less from existing biological design [8]. AEGIS increases the number of independent Watson–Crick bases from four to twelve by rearranging hydrogen-bond donor and acceptor groups on the nucleobases [48•]. A key recent focus has been on the development of the Z and P nucleobase pair [49]. Z features an electron rich, exocyclic oxygen attached to C2 and P features a nitrogen at the position analogous to N3 of purines. Additionally, Z and P are not susceptible to oxidation and epimerization. Just last year, an expansion to a six base genetic alphabet (G:C, A:T, and Z:P) has been demonstrated with polymerases capable of PCR amplification of a virtually unlimited number of sequences including sequences with multiple, consecutive non-natural Z and Ps [50]. It is tantalizing to consider an unnatural genetic system for cell-free synthetic biology (or even an artificial form of life) with six different nucleotides (GACTZP), having a possible 216 codons.

Beyond new genetic languages, another approach that exploits synthetic chemistry for synthetic biology focuses on DNA-templated synthesis. DNA-templated synthesis combines the self-assembly properties of nucleic acids with traditional organic synthesis [51,52,53•,54,55••,56]. DNA oligonucleotides are functionalized with reactive synthetic precursors. Thereby, hybridization of complementary oligonucleotides places the reactants in close proximity, boosting their effective molarity (Figure 4). In this manner, multiple reactions can be controlled in a 'single pot.' An added benefit of this technique is that large libraries of compounds can be generated by successive addition of assorted complements. Examining the template sequence can then identify the identity of each compound. Looking forward, scalability issues must be addressed for larger adoption of DNA templating technologies in the commercial arena.

Figure 4



(a) DNA-templated synthesis of small-molecules. An oligonucleotide with a terminus functionalized with a reactive precursor (black triangle) is subjected to a partially complementary oligonucleotide bearing a second reactant (red circle). **(b)** After the first reaction is complete, a fully complementary oligonucleotide bearing a third reactant (blue hexagon) is introduced. In this manner, small-molecules can be synthesized with precision. **(c)** A polymer template featuring thymine nucleobase repeat units (red polygons) is subjected to adenine nucleobase monomers (black). **(d)** Upon self-assembly of the template–monomer complexes, a polymerization reaction yields a polymer with similar structure.

Templating synthetic molecules with biology has received much attention in the field of polymer chemistry, where chemists are on a relentless pursuit to exert control over polydispersity and sequence. Once again, nucleobase recognition is being investigated as a mechanism to bridle polymerization reactions [57,58]. In one embodiment, a polymer template featuring thymine nucleobases is synthesized (Figure 4). Monomer featuring adenine is then introduced and subsequently self-assembles with the template. A polymerization reaction then yields a polymer with a narrow molecular weight distribution and chain length equivalent to the template [57]. Future efforts in synthetic polymer chemistry will likely continue to focus on producing more precise templates, with the aim of producing polymers of predefined, exact molecular weights with controllable sequences.

Conclusion and outlook

Synthetic chemistry and synthetic biology have been major driving forces in advancing biotechnology products beyond their natural limitations. For this to continue, the interface between synthetic chemistry and synthetic biology must continue to be explored and tinkered with. Immediate challenges include methods for improving the efficiency of incorporation of ngeAAs by the ribosome and the development of robust strategies for expanding the number of available codons for engineering in extract-based systems. Two key areas for growth are likely to include efforts to coordinately tune and evolve the translation apparatus for ngeAA incorporation and strategies to enable the chemically templated synthesis of arbitrary polymers of defined sequence. Addressing these challenges will enable new technologies with applications in therapeutics, practical diagnostics, and advanced materials.

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