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Cell-free translation of peptides and proteins: from high throughput screening to clinical production

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In the past decade, *in vitro* transcription/translation technologies have emerged as discovery tools for screening large protein expression libraries, for the selection of engineered polypeptide libraries, and as alternatives to conventional heterologous expression for protein production. Therapeutic proteins and peptides discovered using ribosome-based display methods that link genetic information to the encoded polypeptide generated by cell-free extracts, or purified translation components, are beginning to move forward into human clinical trials. This review details the significant progress in *in vitro* translation for novel protein and non-natural amino acid containing peptide discovery platforms, as well as advances in the clinical-scale production of therapeutic proteins using cell-free transcription/translation.

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Introduction

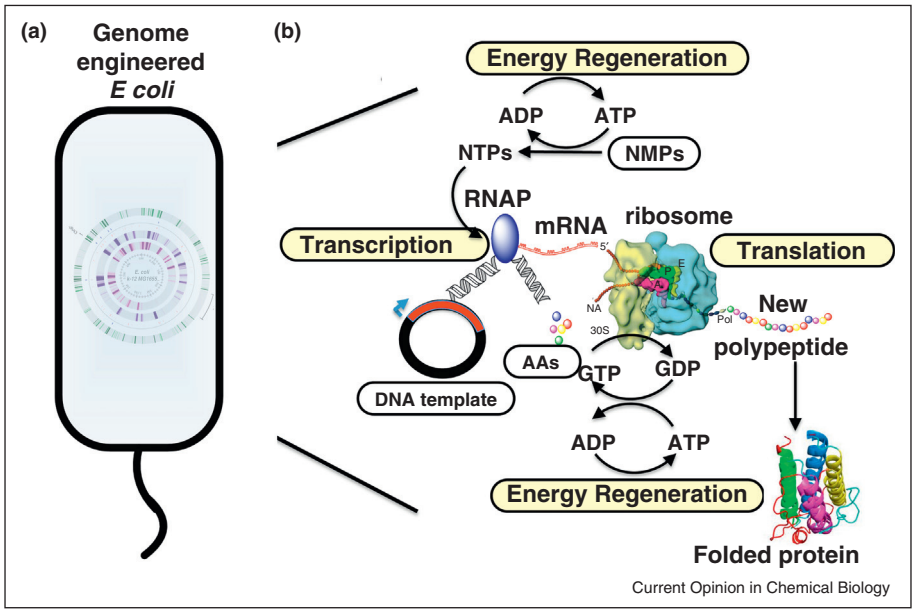
The early demonstration that cell integrity is not required for protein synthesis [1] has led to recent breakthroughs in our understanding of ribosomal protein synthesis [2,3], enabling researchers to reengineer the intricacies of protein synthesis for various biotechnology applications. The results of these efforts are now beginning to converge in highly efficient bottom-up synthetic biology approaches to reconstruct [4,5] and reengineer [6,7] ribosomal translation for high-throughput (HT) applications in structural genomics and functional protein discovery [8–10], for ribosome-based selections [11–13], and for fully integrated scalable protein production [14••] using cell-free extracts.

E. coli cell-free extracts — or lysate-derived systems for protein synthesis — are commonly used because of their ease of preparation and relatively high productivity. Though often considered a 'black-box', genome and process engineered cell-free extracts allow exquisite design and control. They contain the necessary components for transcription (template DNA and recombinant T7 RNA polymerase) and protein translation (e.g. initiation, elongation, and release factors; aminoacyl-tRNA synthetases (AARSs), and enzymes for energy generation to co-activate multiple biochemical networks) in a single integrated platform (Figure 1) [7]. Alternative systems based on eukaryotic wheat germ [15] or rabbit reticulocytes have recently been extended with Leischmanii [16], *Thermus thermophilis* [17], and HeLa [18] cell-free expression systems. In addition, the development of a reconstituted highly purified *E. coli* cell-free translation system (PURE system) has revolutionized the fit for purpose redesign of the ribosomal translational machinery (*vide infra*) [4,19•]. In general, proteins made in cell-free systems are soluble and functional and the open, flexible nature of the systems permits addition (or subtraction) of components, providing an adjustable environment for protein folding, disulfide bond formation, modification, or activity.

Cell-free translation of large encoded libraries allows researchers to explore diverse phenotypic protein sequence spaces in multiplexed array-based formats in such diverse fields as systems biology [10,20,21] and medical diagnostics [22]. More commonly in drug discovery, pooled DNA/mRNA libraries are linked to their phenotype via cell-free transcription/translation for selection of engineered polypeptides with high affinity toward drug targets.

In this review, we first highlight the two most widely used HT cell-free translation technologies — ribosome and mRNA display — with respect to their use in discovery of therapeutic proteins and peptides. We detail their individual characteristics and explain how they have been exploited for the successful and efficient generation of potent lead biologics now entering clinical trials, as well as novel cyclic non-natural amino acid (nnAA) peptides with small-molecule drug-like properties. Finally, we summarize recent advances demonstrating the potential for integrating these cell-free display-based discovery platforms with cell-free protein synthesis for cGMP manufacture of clinical drug product.

Figure 1



Combined transcription and translation using *E. coli* cell extracts to conduct cell-free protein synthesis [7,14••]. **(a)** A culture of genome engineered *E. coli* cells harvested during exponential growth is used to prepare **(b)** a cell-free lysate that provides much of the transcription and translation machinery for protein expression and folding.

Ribosome and mRNA display

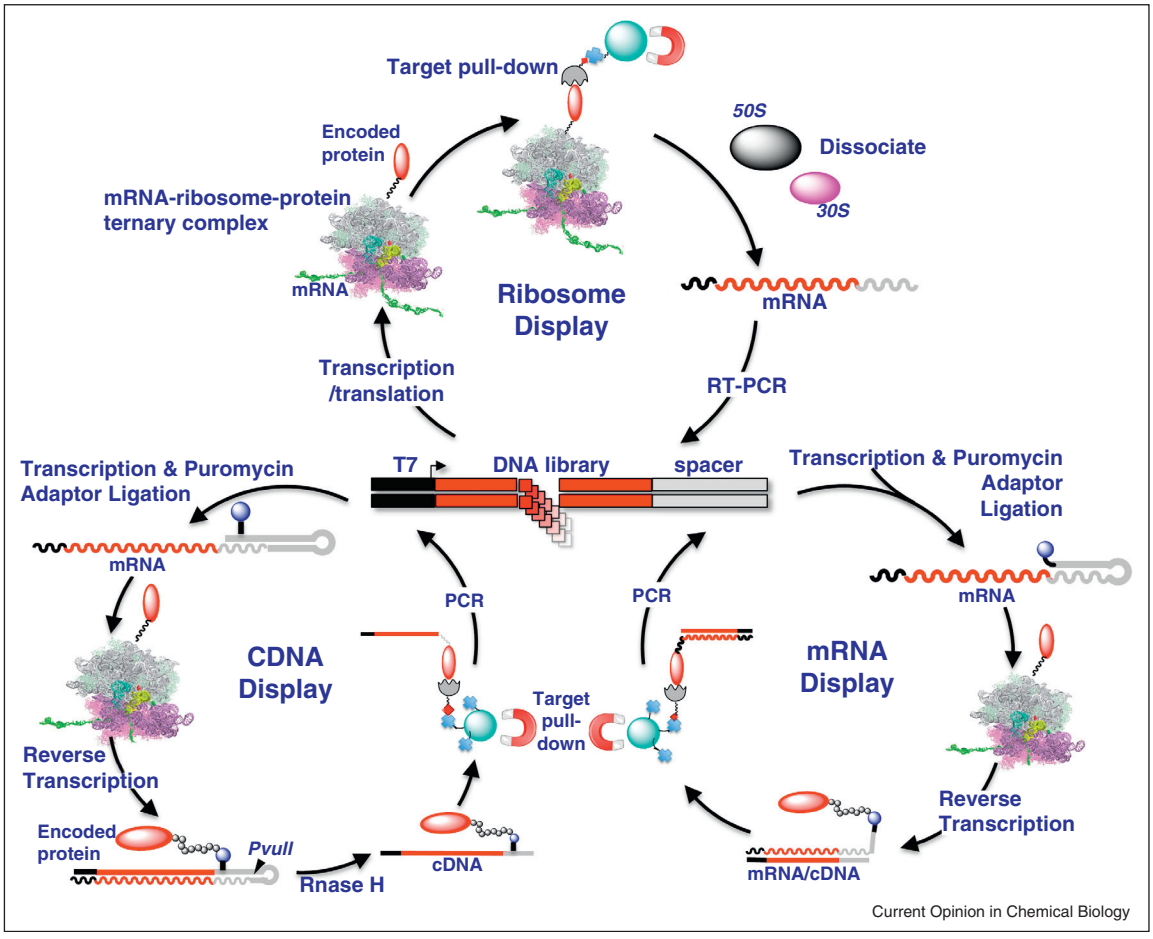
The concepts and steps of ribosome and mRNA display are explained in Figure 2. Although related ribosome-based technologies such as cell-free protein arrays [20], and *in vitro* compartmentalization (IVC) [8] have been successfully applied to protein and peptide optimization, we incorporate only some selected articles and refer to [23,24], and references therein, for more information.

Ribosome display was among the first techniques utilized for fully '*in vitro*' directed evolution of peptide and protein sequences [11]. In ribosome display, a DNA library that encodes peptides or proteins is transcribed/translated using prokaryotic or eukaryotic cell-free expression systems. In the absence of a translational stop codon, high concentration of magnesium ions, antisense knockdown of tmRNA levels [25], and low temperature efficiently stall the ribosome at the end of the mRNA while the tethered, fully folded protein is presented outside the ribosome exit tunnel for functional selection of the mRNA-ribosome-protein ternary complex. After selective enrichment, ternary complexes are dissociated by addition of EDTA, the mRNA is recovered, reverse transcribed, and PCR amplified in order to identify the genotype associated with the functionally selected proteins. Additional cycles of mutagenesis and selection can be applied to favor the accumulation of beneficial mutations in the pool of selected variants. Ribosome display has been used to rapidly map areas of antibody surfaces that are tolerant of amino acid replacement [26,27], leading to the development of tralokinumab an anti-IL-13 IgG_4 antibody now in clinical trials (Table 1).

In mRNA display [12] (and a related variation, cDNA display [28]; Figure 2), separately transcribed mRNA is covalently ligated to a 3′ terminal puromycin DNA adaptor molecule (or 3′ internal puromycin and biotin for cDNA display) that, upon translation, stalls in the A-site of the ribosome while covalently linked to the polypeptide. The covalent mRNA–puromycin–protein adduct is reverse transcribed to form a stable mRNA-cDNA hybrid tethered protein. Selection for target binders is conducted before hydrolyzing the mRNA (or after mRNA hydrolysis and digestion in cDNA display) and recovered cDNA is amplified by PCR. mRNA display has been used to develop high affinity immunoglobulin-like protein scaffolds (Adnectins^{TM}) [29–32], several of which are now in clinical trials (Table 1). mRNA and ribosome display have been limited to single-chain polypeptides such as single chain Fvs, Adnectins, and DARPins. However, Doi and colleagues [33] have recently demonstrated the potential of selections with heterodimeric Fab antibody fragments by combining mRNA display and IVC in order to limit cross-talk between separately encoded heavy and light chain genes.

Four key features make these completely *in vitro* techniques highly efficient for directed evolution of proteins. First, they are not limited by transformation or phage-based infections of cells in order to generate and select

Figure 2



Common protocols for directed evolution cycles using *in vitro* cell-free translation display. Starting with transcription of a DNA library of 10^{12} to 10^{14} sequences encoding variants of a protein or peptide, the translated product is trapped either non-covalently as a ternary complex with mRNA and ribosomes (ribosome display), or covalently tethered to the mRNA transcript using puromycin attached to a DNA oligonucleotide (cDNA and mRNA display). After the selection of the desired peptide/protein, the encoded sequence information is recovered by reverse transcription and PCR amplification.

libraries; the library size and diversity is only limited by the number of ribosomes present in an *in vitro* translation reaction and can be as large as 10^{13} to 10^{14}. Second, linear template DNA libraries can be rapidly constructed and designed to comprehensively monitor selections using massively parallel DNA sequencing [34••,35,36]. Third, the reverse transcriptase and PCR amplification steps can be used to easily introduce additional diversity between

Table 1

Representative examples of therapeutics and diagnostics that have reached the preclinical or clinical stages of development using *in vitro* cell-free translation technologies

Technology	Organization/company	Molecule (target)	Status	References
Ribosome display	CAT (Medimmune)	Tralokinumab (IL-13)	Phase II	[26,50]
Ribosome display	Molecular Partners; Allergan	MP0112 DARPin (VEGF-A)	Phase II	[51,52]
mRNA display	Adnexus (BMS)	Pegdinetanib (VEGFR-2)	Phase I/II	[31,32]
mRNA display	Adnexus (BMS)	FGF21-PKE Adnectin (human serum albumin)	Phase I	
In vitro display	Ra Pharmaceuticals	Cyclomimetic nnAA peptide (kallikrein)	Preclinical	www.rapharma.com
Cell-free protein synthesis	Sutro Biopharma	GM-CSF	Preclinical	[14••]
Cell-free protein synthesis	Stanford University	Anti-CD19-lymphoma idiotype diabody	Preclinical	[53]
Cell-free protein synthesis	RIKEN Innovation Center	scFv, MR1-1-[^{11}C]	Preclinical	[54•]

generations (e.g. by error-prone PCR and recombination [37]). For example the CT-322 Adnectin contains stability mutations outside the designed randomized loops mutated during affinity maturation [29]. Fourth, chemical additives or protein factors can be added to manipulate folding and stability of the displayed protein, important properties for favorable production of biotherapeutics. For example chaperones can be added to tune proper formation of disulfide bonds in antibody fragments [38••]. In a recent study, Buchanan *et al.* [39•] were able to isolate functional G-CSF variants with increased levels of soluble expression in *E. coli* after four rounds of ribosome display selection in the presence of DTT with hydrophobic resin-based removal of aggregated variants.

Cell-free display of non-natural peptides using genetic code reprogramming

Another key advantage of using an *in vitro* transcription/translation approach in HT discovery is the ability to expand the structural and chemical diversity of amino acids beyond the 20 natural amino acids by designed manipulation of the genetic code and translation machinery. Early work using *E. coli* extracts showed the possibilities of genetic code expansion *in vitro* (hijacking the UAG stop codon [40] or introduction of four-base codons [41]). Re-engineering translation by the subtraction of competing endogenous protein release factor RF1 that recognizes the UAG stop codon in normal translation termination, allows efficient production of site-specific nnAA containing proteins *in vitro* [4,42,55].

More recently, the PURE system has enabled reprogramming of the genetic code with simultaneous complete reassignment of multiple codons to different nnAAs. By removing certain cognate amino acids and AARSs and adding separately nnAA aminoacylated-tRNAs to recognize the vacant codons, the translation apparatus of *E. coli* has been shown to be remarkably tolerant of a wide range of amino acid derivatives [6]. For example, ribosomal incorporation of *N*-substituted amino acids, a modification that may increase cell permeability, allows selection of complex peptides with drug-like properties [13,43••]. Some key advantages of this approach are that the large peptide library sizes accessible via the ribosome may yield higher-affinity ligands faster than traditional lead optimization by chemical synthesis and nnAAs sample a different functional and chemical space than even large libraries can sample with the 20 proteinogenic amino acids.

Genetic code reprogramming does not require an orthogonal tRNA body (some endogenous AARSs are not present), but does require efficient methods for generating charged nnAA-tRNAs. Early tRNA ligation strategies with chemical synthesis are not efficient [6,40]. Suga and colleagues [43••] addressed this issue by using an RNA ribozyme aptamer to aminoacylate the 3′ end of tRNAs with nnAAs [44]. As the aminoacylation reaction is independent of amino-acid side chain structure, including *N*-methylation of the alpha-amino group, a wide variety of nnAAs-tRNAs can be generated. Szostak and colleagues [45,46] have shown that native and engineered AARSs can charge a wide array of non-standard amino acids onto tRNAs under conditions lacking the cognate amino acids.

Once nnAA-tRNAs are produced and genetically reprogrammed DNA libraries are designed, they may be selected using cell-free translation display in the PURE system to enrich for highly modified peptides with high affinity toward drug targets. Yamagishi *et al.* [47] performed mRNA display selections for *N*-methylated cyclic peptide binders of a formerly nondruggable ubiquitin ligase enzyme E6AP. The selectant with the highest binding affinity contained the non-canonical features found in Cyclosporine A, four *N*-methyl groups, a D-amino acid and a macrocyclic backbone. It had a K_d of 0.6 nM and showed micromolar inhibition of polyubiquitinylation activity of E6AP *in vitro* on its physiological targets p53 and Prx1. The Szostak group has also identified a single-digit nM macrocylic peptide inhibitor of human thrombin using an optimized PURE/mRNA-display system [48•].

Cell-free protein synthesis for clinical manufacture

The ability to integrate HT discovery using cell-free translation with cell-free protein synthesis at manufacturing scale would have distinct advantages over traditional cell-based methods of biotherapeutic discovery and development. For many expression systems, identifying cell lines that stably synthesize high protein titers of a lead candidate is a time-consuming and labor-intensive process and creates one of the major bottlenecks in protein drug discovery and development. In the case of antibody discovery, scFvs and Fabs identified in HT discovery campaigns often require reformatting into IgGs with uncertain results in mammalian cell expression systems. Integrating cell-free translation at the discovery, preclinical, and manufacturing scales, all using the same cell-free extracts, should increase the speed and developability of new leads continuing through the drug development pipeline. Some examples of cell-free produced biologics in preclinical development are summarized in Table 1.

Until recently cell-free protein production at the multigram and kilogram scale, an essential starting requirement for biotherapeutics, has been hampered by the lack of scalable systems amenable to standard bioreactor configurations at large scale. Zawada *et al.* [14••] showed that scalable cell-free protein synthesis is possible using standard microbial fermentation and process equipment that are known to scale to thousands of liters under cGMP manufacturing processes. Efficient, scalable execution of the bioproduction process starts with high density cell culture of a highly engineered *E. coli* strain with fast

growth rates to optimize the yield of ribosomes. After cell-lysis and extract activation, the process validated at 100-L scale showed high protein synthesis yields of GM-CSF in a 10-hour batch reaction (Table 1). Yin *et al.* [38••] used the scalable cell-free protein synthesis system to produce antibody fragments and an aglycosylated IgG antibody containing 16-disulfide bonds. Although the system is limited to producing proteins with only a few post-translational modifications, recent demonstration of a glycoengineered *E. coli* cell-free system [49] suggests that with further engineering even more complex post-translationally modified proteins may be produced in cell-free systems at scale. Yin *et al.* [38••] also showed the ability to rapidly produce expression libraries of engineered antibodies in high density formats using cell-free translation. Using scanning mutagenesis libraries produced by *de novo* DNA synthesis with automated parallel cell-free synthesis and micropurification in plates with off-the shelf robotics equipment and disposables, it is now possible to rapidly screen designed libraries of several thousand variants in a two week 'make-test cycle' from DNA design/synthesis to cell-biology assays. Integrating such HT cell-free protein arrays with cell-free translation display methods, and next-generation sequencing [21,36] should prove particularly valuable for antibody engineering studies exploring *in vitro* antibody affinity evolution and as a rapid way of identifying variants with interesting properties that can then be rapidly scaled for more detailed structural and functional characterization.

Conclusions

Cell-free protein synthesis has emerged as a promising approach to progress novel biologics to clinical testing. The tremendous speed and diversity of cell-free translation selection methods has led to their application to the entire range of polypeptide libraries, from novel nnAA-containing peptides, to alternative scaffold proteins and antibodies now in clinical trials. With the advent of scalable cell-free synthesis for clinical drug manufacture, we anticipate further improvements in our ability to discover and deliver safe and efficacious drug candidates using cell-free protein synthesis.

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