ACS SyntheticBiology

pubs.acs.org/synthbio Review

In Vitro Selection of Peptides and Proteins—Advantages of mRNA Display

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Cite This: https://dx.doi.org/10.1021/acssynbio.9b00419 Read Online

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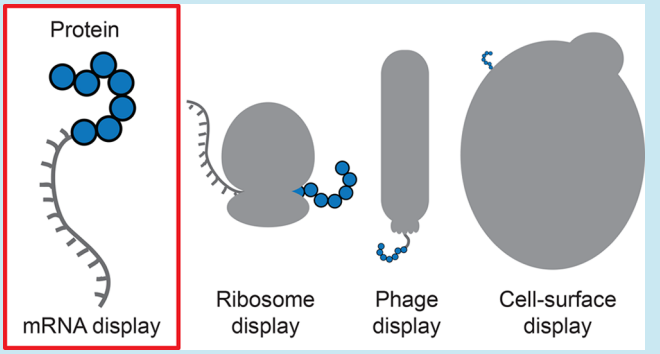
ABSTRACT: mRNA display is a robust *in vitro* selection technique that allows the selection of peptides and proteins with desired functions from libraries of trillions of variants. mRNA display relies upon a covalent linkage between a protein and its encoding mRNA molecule; the power of the technique stems from the stability of this link, and the large degree of control over experimental conditions afforded to the researcher. This article describes the major advantages that make mRNA display the method of choice among comparable *in vivo* and *in vitro* methods, including cell-surface display, phage display, and ribosomal display. We also describe innovative techniques that harness mRNA display for directed evolution, protein engineering, and drug discovery.



KEYWORDS: *protein engineering, in vitro selection, mRNA display, phage display, unnatural amino acids, ribosome display*

mRNA display is an *in vitro* selection and directed evolution technique that enables the screening of trillions of protein variants for desired functions in a single experiment. In directed evolution, researchers working to alter binding or catalytic properties of a target protein or peptide have a range of *in vitro* and *in vivo* techniques at their disposal to isolate their desired variant from large mixtures of variants. The goal of this review is to highlight the unique advantages of mRNA display that make this method superior to most other directed evolution techniques.

Other selection and screening techniques have been excellently reviewed elsewhere.[1−6] Here, we will only briefly detail other major methods as a basis for comparison to mRNA display. It is first pertinent to distinguish between screens and selections. A screen requires that every variant be individually assessed by qualitative (*e.g.*, color) or quantitative (*e.g.*, size) means. A selection couples a desired trait to survival of the organism or molecule.

mRNA display is a selection technique predicated on the formation of a covalent bond between a protein variant and its encoding mRNA molecule during *in vitro* translation (Figure 1).[7,8] First, the mRNA is modified at its 3′-end with puromycin—an antibiotic that mimics the structure of an aminoacylated tRNA.[7,8] At the end of the translation of this modified mRNA into protein, puromycin enters the A-site of the ribosome and forms a peptide bond with the C-terminus of the translated polypeptide, thus covalently linking the mRNA and peptide. The stable genotype−phenotype linkage renders the protein directly amplifiable and enables the enrichment of mRNA-displayed protein variants with desired properties.

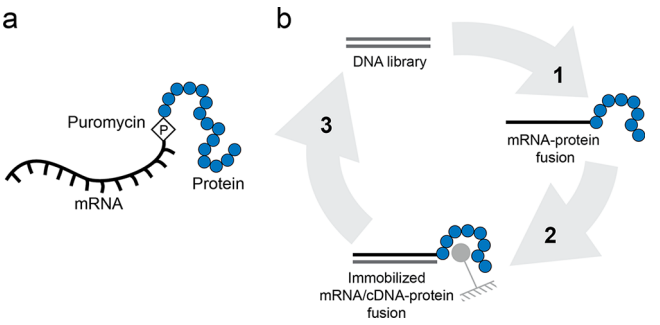


Figure 1. *In vitro* selection by mRNA display. (a) Schematic of the covalent mRNA−protein fusion. The puromycin linkage is shown by a diamond. (b) An mRNA selection for protein binding. A DNA library is transcribed, the resulting mRNA modified with puromycin, and then translated to generate the mRNA−protein fusion (1). The fusions undergo an experiment-specific selection step (2) for ligand binding or enzyme activity, in which the fusions with the desired function are immobilized and undesired fusions are removed. Either before, or sometimes after, the selection step, fusions are reverse transcribed into cDNA. The cDNA of selected fusions is amplified *via* PCR (3) in order to regenerate the DNA template library for the next selection round.

Received: October 13, 2019
Published: December 31, 2019

Ribosome display[4,9,10] is a technique most similar to mRNA display, in which protein and mRNA are linked *via* the entire stalled ribosomal complex. Phage display[5,11] and cell-surface display[12−14] fuse the protein of interest to a capsid or outer membrane protein and require the fusion protein to be transported to the outside of the viral particle or cell. The protein's coding sequence is retrieved by lysing the host. In all these techniques, proteins with desired functions are immobilized or otherwise sorted from the rest of the population. In cell-based *selections* (different from cell-surface display), the protein of interest is expressed inside the cell and its activity is coupled to growth and/or viability—surviving cells will carry sequences of desirable proteins. In cell-based *screens*, cells expressing the protein of interest are separated from the population according to a characteristic such as fluorescence.

This article will describe the advantages of mRNA display technology in comparison to these other *in vivo* and *in vitro* screening and selection techniques (Figure 2). Specifically, we

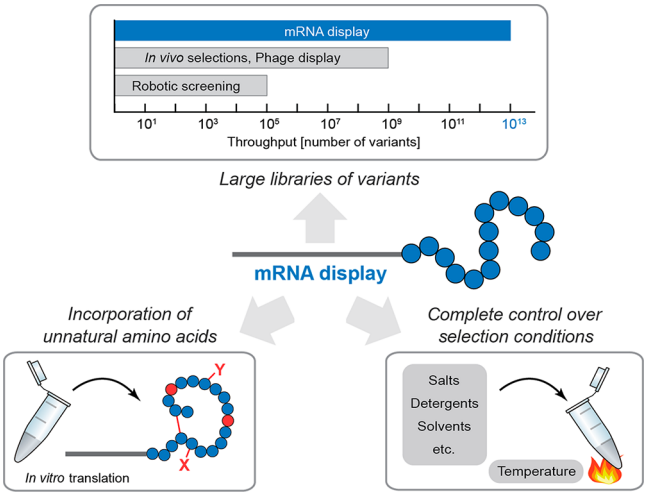


Figure 2. Advantages of mRNA display. mRNA display combines the use of very large libraries of protein variants; the ability to readily incorporate unnatural amino acids; and to perform selections under a wide range of conditions. This combination of advantages is unique to mRNA display and makes it superior to any other *in vitro* or *in vivo* selection method.

will discuss (1) the value of interrogating more protein variants than is possible with most other techniques; the benefits of an *in vitro* method that (2) frees protein expression from cellular constraints; (3) allows the incorporation of unnatural amino acids in the protein variants; and (4) permits protein screening under an unlimited range of desirable conditions. We will conclude by discussing the current limitations of the technique and how they are being overcome, as well as future prospects of mRNA display.

THE NUMBERS GAME—ADVANTAGES OF LARGE LIBRARIES

Directed evolution, as detailed above, is a protein engineering method proven to generate proteins with modified properties by mimicking the natural process of mutagenesis and selection.[3,15] The chance of identifying a protein variant with a desired function from a library of mutants is proportional to the number of protein variants that can efficiently be tested. Numerous methods have been developed to readily create large numbers of protein variants including techniques such as random mutagenesis by error-prone PCR, DNA shuffling, or the use of randomized synthetic DNA.[16−21] The generation of genetic diversity is therefore not the limiting aspect of a directed evolution approach, but instead the throughput of the subsequent screening or selection step.

The following thought experiment will demonstrate the importance of library size in directed evolution approaches. Let us assume that in a typical protein cavity—be it a binding site or enzyme active site—is lined by about a dozen amino acids, forming the first shell of contact with the ligand. Many of these residues (and more throughout the protein) will strongly influence binding affinity or enzymatic activity. A common engineering approach to modify these properties is therefore to use site-saturation mutagenesis of these first shell residues to generate diversity for a directed evolution experiment.[20−22] A simple calculation shown in Table 1 exemplifies the dilemma

Table 1. Library Size Required to Sample All Possible Protein Variants in Site-Saturation Mutagenesis, Using the 20 Canonical Amino Acids

positions randomized	number of possible variants
1	20
3	8.0×10^3
6	6.4×10^7
10	1.0×10^{13}
12	4.1×10^{15}

the researcher quickly faces: library size increases 20-fold with every additional residue mutated. The numbers demonstrate that classic cell-based screening methods with a throughput of 10^2−10^4 allow exhaustive sampling for only about up to three positions, and higher-throughput FACS-based screening or *in vivo* selection methods for up to six positions (Table 2).

Table 2. Differences in Throughput for Protein Screening and Selection Techniques

technique	typical library size	references
classic cell-based screen	10^2−10^4	3
robotic screen	10^5	24,25
FACS-based screen	10^6−10^8	26
in vivo selection (cell-based)	10^6−10^8	1,27−30
cell-surface display		14,27,29
eukaryote	10^6−10^7	
prokaryote	10^8−10^{10}	
phage display	$\sim 10^9$	31
ribosome display	10^{12}−10^{14}	4
mRNA display	10^{12}−10^{14}	32

Several engineering teams have attempted to somewhat lessen this numbers problem by synthesizing so-called "smart libraries", which sample a limited number of codons to reduce library size while mostly maintaining chemical diversity.[21−23] Unfortunately, these approaches only slightly delay the combinatorial "explosion" of possible variants. In any case, for smart libraries or not so smart ones, the chances of finding a protein variant with a potentially rare new or improved property increases proportionally with the number of variants interrogated.

mRNA display and ribosome display allow the use of protein libraries exceeding 10^{13} variants, which is several orders of

https://dx.doi.org/10.1021/acssynbio.9b00419
ACS Synth. Biol. XXXX, XXX, XXX−XXX

magnitude larger than for all other techniques (Table 2).[33] In contrast, cell-surface display and other selection methods that require expression of the protein library inside a cell[4,5,14,20] are limited by transformation efficiency, which can be as high as 10^{10} for *Escherichia coli* and 10^{7} for *Saccharomyces cerevisiae* (Table 2). More typical cell libraries contain only about 10^{6}–10^{8} unique sequences.[1] Phage display has been reported to screen up to 10^{9}–10^{12} sequences,[31,34,35] but again 10^{9} is more common because the technique is also limited by *in vivo* transformation of *E. coli* for propagation.[31] mRNA display, on the other hand, is performed entirely *in vitro*, and therefore the number of sequences is not limited by a transformation step.[32,33,36]

mRNA display selections with trillions of variant sequences have been successfully performed to isolate proteins with *de novo* functions, thereby demonstrating that large libraries can yield potentially rare protein variants.[19,37−39] The generation of *de novo* functions, *e.g.*, to instill binding or catalytic capability into a protein originally lacking those properties entirely, is still considered a great challenge in protein engineering.[40] The first example of mRNA display selecting for *de novo* protein function was the isolation of artificial ATP-binding proteins from a completely random polypeptide library.[19] The authors calculated that the ATP-binders, which exhibited high specificity and affinity, occurred within the naïve library with a frequency of about one in 10^{11} protein sequences. Such rare events could easily have been missed had a lower throughput selection method been used. mRNA display was also used to successfully select *de novo* enzymes that catalyze a reaction not observed in nature from a library based on a noncatalytic protein scaffold (Figure 3a).[33,38,41] During the selection process for these artificial enzymes, surprisingly, the original scaffold was abandoned and instead, a new fold was formed that lacks classic secondary structure elements and, like the catalyzed reaction, has not been found in nature.[42]

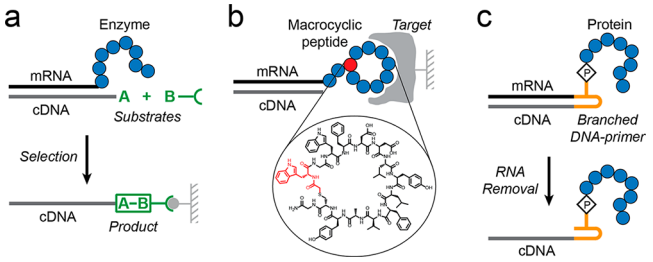


Figure 3. Three example applications demonstrate the wide adaptability of mRNA display. (a) General selection strategy for enzymes that catalyze bond-forming reactions. Substrate A is attached to the reverse transcription primer used for cDNA synthesis and substrate B contains a selectable anchor group (*e.g.*, biotin). cDNA of protein variants that catalyze the bond formation between A and B is selected, *e.g.*, by binding to streptavidin.[38] Selections for bond-breaking or other modifications can also be envisioned (not shown).[40] (b) Selection for macrocyclic drug candidates. A nonproteinogenic amino acid (red) enables the cyclization. Cyclic peptides are isolated for binding to a protein target.[58] (c) Advancement of mRNA display into cDNA display, thereby overcoming potential RNA stability concerns. A branched DNA oligonucleotide is ligated to the mRNA to covalently attach the encoded protein *via* puromycin (P) and to function as primer for the reverse transcription to cDNA.[59,60]

Large library size in mRNA display also enables the screening of an entire natural proteome, for example to identify protein−protein[6,43,44] or protein−drug interactions,[45,46] and to isolate kinase substrates[47] or protease substrates.[48,49] The length of polypeptides that have been successfully screened range from small peptides of <10 residues[8,50] to proteins of ∼650 residues.[44,51] The display efficiency and thereby the size of library produced per volume of translation reaction slightly increases with decreasing size of the displayed peptide.[6] To date, mRNA display has produced a wide range of engineered polypeptides including peptide inhibitors, antibody mimics, and antibody fragments.[51−57] Although mRNA display and ribosome display allow the experimenter to screen larger libraries than any other technique currently available, it is important to acknowledge that protein variant library sizes can rapidly exceed the display's current ceiling of 10^{14} unique variants, as demonstrated by Table 1. Therefore, the onus is still on the experimenter to be discriminating in their library design if they wish to sample all possible sequences.

Methods for screening large libraries for function are an integral part of the protein engineer's toolbox and *in vitro* techniques such as mRNA display allow the highest variety of sequences to be tested. Besides large library size, further benefits of mRNA display include a high level of control over protein translation and the conditions under which the selections can be performed (Figure 2). These advantages will be described in detail in the following sections.

WHAT'S IN A CELL?—BENEFITS OF *IN VITRO* PROTEIN EXPRESSION

All steps of the mRNA display procedure are performed entirely *in vitro*, including protein expression. This fact solves many of the potential issues associated with protein production inside a cell. The heterologous expression of proteins in *E. coli* or other cell-based systems often involves arduous optimization to obtain abundant, pure protein. Such expressions can lead to the accumulation of protein aggregates that are detrimental to cell viability.[61,62] Foreign, high copy number proteins can, in some cases, be toxic to the host cell[62,63] by disrupting normal metabolism or generating toxic metabolites.[64,65] Cell-free protein expression removes both the need to maintain the viability of the cell and the danger of other cell components confounding a selection by acting as competing enzymes, potential inhibitors, or alternative substrates. Ribosome display benefits from the same advantage of omitting an *in vivo* translation step. In contrast, cell-surface and phage display both require cellular translation and are further limited by the requirement for transport of a folded protein across a cell membrane[14] or the assembly and release of a phage from a host cell.[5,34] This effectively constitutes an additional undesired selection bias for proteins that can be successfully transported for display. mRNA display has no such requirement as the formation of the fusion is independent of a protein's sequence.

Cell-based methods will likely result in degradation of poorly folded protein library variants, but the experimenter has no real means to influence this process. In contrast, *in vitro* selections provide the option to fine-tune the removal of poorly folded proteins by selective protease treatment,[66] or improve their solubility *via* buffer conditions and the solubility of the mRNA-tag.

Cell-free translation in mRNA display is typically performed using eukaryotic cell lysates from rabbit or wheat germ,[33] allowing a wider range of post-translational modifications than would be possible with bacterial *in vivo* expression. Protein

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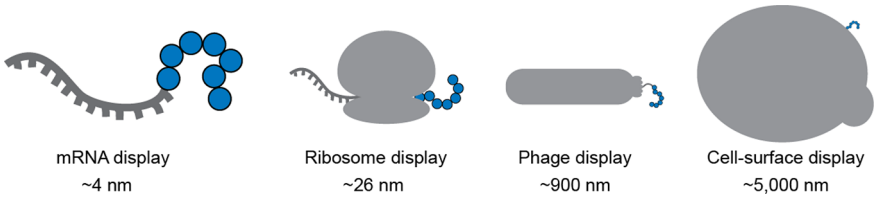

Figure 4. Display methods compared by size: mRNA is the least bulky display platform. The reported lengths or diameters are approximations based on a 300 nucleotide long mRNA;[105] prokaryotic ribosome; M13 bacteriophage; *Saccharomyces cerevisiae* yeast.[106] The graphic elements are not drawn to scale.

expression conditions can be controlled even more tightly with the use of the PURE translation system (protein synthesis using recombinant elements).[67,68] The PURE translation system is reconstituted from the purified components necessary for *E. coli* translation such as tRNAs, aminoacyl-tRNA synthetases, amino acids, ribosomes and release factors.[69] This fully chemically defined protein synthesis system allows the experimenter an even greater level of control over peptide modification, which is expanded upon in the next section.

EXPANDING THE PROTEIN ALPHABET WITH NONSTANDARD AMINO ACIDS

The use of the PURE cell-free translation system also enables the facile incorporation of diverse nonstandard amino acids (NSAA) into mRNA-displayed proteins, thereby vastly expanding the chemical properties of the selected proteins (Figure 2). NSAAs are either rare, naturally occurring amino acids different from the standard 20 residues, or completely synthetic amino acids that introduce novel chemistries to engineered proteins.[70,71] NSAAs have been used to enhance protein stability, catalysis, or detection;[70−75] enable subsequent protein modification *via* bio-orthogonal click chemistry;[70,75] and generate biologically active peptides for pharmaceutical use. To date, more than 150 different NSAAs have been genetically encoded in proteins.[76] While NSAAs can be incorporated by different approaches both *in vitro* and in some cases *in vivo*,[72] the PURE cell-free system is the most versatile method as it can use multiple orthogonal incorporation techniques in parallel by altering the composition of a user-defined translation system.[70,77] mRNA display in combination with PURE can therefore readily realize the full potential of the ever-expanding repertoire of amino acid building blocks.[70−72,78]

Protein libraries containing NSAAs have been selected by mRNA display for a wide range of activities. In a proof-of-principle study, the Roberts group reported streptavidin-binding proteins selected from a library of mRNA-displayed peptides containing biocytin, a biotin derivative of lysine.[50] In separate work, four-base codons were used to incorporate biocytin and other NSAAs to select for novel streptavidin-binding peptides.[79] In more applied studies, mRNA display of NSAA-containing peptides has been used to identify peptide drugs with improved pharmacological traits. One study selected for phosphomimetic peptides that inhibit Breast Cancer Associated protein 1 (BRCA1);[80] another study showed that protease stability can be improved through unnatural methylation.[81]

The PURE system has also been used in conjunction with mRNA display to introduce synthetic amino acids that allow further modification by click chemistry. In work on HIV, mRNA-displayed peptides were glycosylated *via* a click reaction between their alkyne-modified glycine and azide-modified high mannose glycans at either fixed or random positions. The glycopeptide libraries were then used to select for binding to the HIV broadly neutralizing antibody 2G12.[82]

NSAA incorporation has been harnessed to select macrocyclic peptide binders to pharmacologically important targets from mRNA-displayed libraries of up to 10^{13} variants (Figure 3b).[58,79,83−97] Circularized peptides are favored in drug screening due to their increased proteolytic stability, improved membrane permeability, and reduced conformational flexibility that can lead to tighter target binding.[46,98−100] For example, mRNA display has been used to select binders to thrombin with low nanomolar dissociation constants from a library of peptides comprising 12 different NSAAs and only eight standard amino acids.[90] An overview of peptide cyclization strategies can be found in several reviews.[46,98−101] mRNA display with NSAAs and click chemistry can be used to generate cyclic peptides *via* linkages between β-azidohomoalanine and *p*-ethynyl-L-phenylalanine;[102] the formation of a thioether bond between a chloroacetyl-analogue of tyrosine and a cysteine side chain;[91,103] or by the conversion of 4-selenalysine to dehydroalanine and cyclization with a cysteine residue to form a linkage similar to natural lantipeptides.[89,90] The Suga group, which uses a modified mRNA display protocol called RaPID (random nonstandard peptide integrated discovery), obtained macrocyclic peptide inhibitors of JmjC histone demethylases that are important in tumor cell proliferation.[104] In separate work, they isolated cyclic peptides that modulate degradation of ubiquitylated enzymes, also important in cancer.[58] The coupling of mRNA display with click chemistry has great potential even beyond the examples mentioned: methods could be designed to immobilize an mRNA−peptide fusion to a surface for purification; attach them to a binding partner in solution; or trap a reaction substrate or product, thereby further expanding mRNA display's applications. These chemistries would be very difficult to perform in the context of selections inside cells or other display technologies.

These examples demonstrate that the *in vitro* translation feature of mRNA display enables the experimenter to harness the continuing progress in nonstandard amino acid incorporation and promises to greatly expand the potential for protein engineering in both fundamental research and for practical applications.

COMPLETE CONTROL OVER SELECTION CONDITIONS DUE TO ROBUST *IN VITRO* FORMAT

Cell-based selections are by necessity limited to conditions that are compatible with cell growth and replication, while *in vitro* selections *via* mRNA display enable the researcher to vary selection conditions freely. Numerous parameters such as pH, temperature, and ionic strength can be precisely controlled.

https://dx.doi.org/10.1021/acssynbio.9b00419
ACS Synth. Biol. XXXX, XXX, XXX−XXX

The format also allows easy inclusion of additional components such as reaction substrates, binding targets, solvents or other chemicals (Figure 2). Unlike within the crowded cytosol of a cell where millimolar concentrations of potentially competing proteins, solutes and other factors could confound selection outcomes, all components of an *in vitro* selection system are entirely controlled by the experimenter.

In order to identify "winning" sequences by any protein screening or selection technique, each protein variant must be linked to its genetic information. For example, each gene variant is contained inside the cell or viral capsid for cell-based screening and phage display, respectively. Compared to the size of the protein variant molecule, a cell or a phage is a very large entity (>1000-fold larger than an mRNA molecule encoding a small protein, Figure 4) with numerous other proteins on their surfaces, which might interfere with the selection outcome. In mRNA display, this essential gene-protein link is dramatically miniaturized to a simple covalent bond between the protein and its encoding mRNA *via* the small molecule puromycin. Therefore, the bias that an attached cell or phage might exert on a protein selection is reduced to the bare necessity—the genetic material itself. In addition, this stable covalent linkage between the protein and mRNA further facilitates the use of stringent selection conditions. Ribosome display, which otherwise has many favorable features in common with mRNA display such as large library size and an *in vitro* format, uses the whole ribosome to keep mRNA and protein together in a noncovalent and therefore less stable manner.[4] The small size of the linkage used in mRNA display—relative to cell, phage, or ribosome display constructs—likely increases the chances of a displayed peptide maintaining similar physicochemical properties as the respective unfused peptide. For example, mRNA-displayed fusions selected for the ability to infiltrate cancer cells were demonstrated to still have cell-penetrating properties as the free peptides alone.[107,108] However, the high negative charge of the mRNA could potentially influence the selection result under some circumstances. A report from the Nemoto group showed a 10-fold reduction in a protein's binding efficiency when it was mRNA-displayed.[109] Nevertheless, in most reported mRNA display projects, the selected proteins still maintain the selected function when tested without the mRNA attachment. The functionality of isolated peptides is apparently not significantly hindered by the presence of the fused mRNA, and peptide variants selected by mRNA display are accurately represented.

The *in vitro* nature of mRNA display strongly discourages selection of false positives and other factors that can confound *in vivo* selections. In contrast, cell-based screening and selection systems can yield false positive results due to global stress responses or the unintentional up-regulation of multicopy suppressors[110] with promiscuous activity that is similar to the desired activity. Nearly 40% of all *E. coli* proteins are estimated to be promiscuous[111] and many of these functions have yet to be characterized. Therefore, selections for enzymatic function through complementation of auxotrophic strains are complicated by the risk of chromosomal mutations leading to the overexpression of existing weakly promiscuous enzymes[112,113] or by the library variant itself acting as a transcription factor to the same effect. The tight control over selection conditions afforded by mRNA display, which omits all unnecessary components, completely avoids such issues.

The stability of the covalent linkage between mRNA and protein also allows a wide variety of conditions to be used during a selection that would lead to cell death or dissociation of ribosome-displayed peptides. mRNA display can withstand solvents, ligands or salts that are toxic or difficult to traffic across a cell membrane, and high incubation temperatures (Figure 2). For example, in order to evolve artificial ATP binders toward greater structural stability, a selection was carried out in the presence of increasing concentrations of the chemical denaturant guanidine hydrochloride.[114,115] Furthermore, an artificial RNA ligase enzyme, originally selected at room temperature, was evolved for increased thermostability by performing the selection at elevated temperature. The resulting thermostable ligase variant had a melting temperature of 72 °C, which was 35 degrees more stable than the most closely related variant selected at ambient temperature.[116] In a recent example, cDNA display (Figure 3c), which is an mRNA display variant with increased stability, was used to select for peptides that bound a pH-sensitive dye; the selection included an incubation at pH 12, which could not be tolerated by comparable selection methods.[117] These examples demonstrate that the *in vitro* format and the small, covalent gene-protein linkage in mRNA display provide great freedom to choose selection conditions and selection stringency.

■ ADDRESSING POTENTIAL CONCERNS WITH mRNA DISPLAY

mRNA display is a robust method for the selection of functional proteins from large libraries. While this technique compares favorably with other selection methods in many aspects, we will here also address potential concerns associated with the technique and ways to overcome them.

RNA Stability. A common question about mRNA display involves the perceived sensitivity of RNA to degradation. This question likely originates from people's experiences isolating long RNA from lysed cells. However, due to the *in vitro* nature of the entire mRNA display procedure, RNA nuclease degradation is simply not an issue. Simple precautions such as using nuclease-free chemicals and RNase inhibitors; wearing gloves during experiments; and protecting against dust contamination are usually sufficient to avoid degradation.[33] Only when mRNA-displayed proteins are to be incubated with biological specimens does mRNA degradation become a concern due to the inherent presence of nucleases. Nevertheless, recent work on selecting cell-penetrating peptides showed that up to 60% of the mRNA-displayed library could be recovered intact after incubation with cells.[108] Moreover, through a modification of the mRNA display procedure, cDNA-displayed protein libraries can be produced which avoid mRNA stability concerns altogether (Figure 3c).[59,60]

Monomeric *vs* Dimeric Proteins. mRNA display is suited to monomeric proteins due to the technique's requirement for a direct link between peptide and mRNA. This requirement has limited most work to date to single domain proteins. Recently, however, a new strategy was developed to display dimeric proteins. The polypeptides of the heavy and light chains of an antibody Fab fragment were separately mRNA-displayed and then mixed to form heterodimers. After affinity selection, the heterodimers were individually encapsulated in water-in-oil emulsions and the winning genes were recovered by overlap-extension PCR.[55]

Selectable Functions. As with any selection technology, mRNA display relies on an effective selection step that isolates

https://dx.doi.org/10.1021/acssynbio.9b00419
ACS Synth. Biol. XXXX, XXX, XXX−XXX

desired variants from a vast pool of undesired variants. Due to the ease of implementation, the technique has most often been used to isolate proteins or peptides with binding function. Using a slightly more elaborate scheme, mRNA display can select for enzymes that catalyze bond-forming reactions (Figure 3a).[38,116] This selection scheme can be expanded to bond-breaking or other modification reactions.[40] While this is a general selection scheme, it is only applicable to reactions where either the substrates (or the product) can be modified with a selectable handle. We acknowledge that some catalytic activities may be out of reach of this technique, but in many cases, the limitation may be in the ingenuity of the experimenter. An advantage of the mRNA display enzyme selection approach is that product formation is the only selection criterion. A limitation of the current scheme is that enzymes are isolated after only single turnover, although selected enzymes have been found to show multiple turnovers.[40] While mRNA display is well-suited to finding rare new functions due to its high throughput, there are other directed evolution methods (albeit with lower throughput) that have the ability to directly select for multiple turnover. For example, *in vitro* compartmentalization would be appropriate to select for faster turnover rates in a slow enzyme initially identified with mRNA display.[118−120]

mRNA display is a cell-free system and, therefore, the selection or evolution of membrane proteins constitutes a challenge, for which other selection strategies might be better suited.[121,122] Nevertheless, it is feasible to embed membrane proteins into vesicles or liposomes, which have been successfully used in conjunction with cell-free expression systems.[123]

***In Vitro vs in Vivo* Expression and Function.** mRNA-displayed proteins are selected completely *in vitro* and can therefore be tailor-made for cell-free synthetic biology applications. While *in vitro* selection has many benefits, as detailed above, it carries the risk that selected proteins may not express well, or not function, inside a cell. Facile and affordable *in vivo* expression of larger amounts of proteins is often desired to carry out a detailed characterization of protein variants. If protein folding is suspected to be the issue, the mRNA display protocol can be modified to include a selection against poorly folded proteins, using proteases to cleave exposed loops.[66] Furthermore, solubility can often be improved by simply expressing the selected protein with a solubility tag, such as maltose-binding protein, which rarely interferes with function. We argue that selecting for the desired function is usually the biggest challenge, requiring selection from the greatest sequence diversity *via* mRNA display. Any further optimization for improved cellular performance can be conducted *via in vivo* selection methods with smaller libraries. Protein stability has been shown to be dramatically increased with just 1−2 missense mutations.[124]

Perceived Technical Challenges. mRNA display is a technique that requires precision and care, but a typical display protocol does not contain any steps that would be wholly unfamiliar to a competent biochemist or molecular biologist. We adapt commercial kits for *in vitro* transcription and translation, perform simple affinity chromatography, and analyze our results *via* SDS-PAGE and real-time quantitative PCR. mRNA display has often been used in conjunction with the facile and sensitive detection of the protein or nucleic acid moiety by radioisotope-labeling, which may be a hindrance for some users. This potential issue can easily be circumvented by replacing radioisotope with fluorescent labeling. In our laboratory, summer students and visiting scholars typically learn to produce mRNA-displayed proteins in a week.

A typical round of an mRNA display selection takes approximately 2−3 days depending on selection conditions and purification steps.[33,125] Recently, however, it has been suggested that this time can be drastically reduced to about 2 h per round. An optimized version of the mRNA display protocol successfully accomplished six rounds of selection in just 14 hours thanks to a one-pot transcription, translation, and puromycin coupling reaction.[126] This mRNA display protocol was termed TRAP display: transcription−translation coupled with association of puromycin linker. Furthermore, coupling mRNA display with continuous magnetic flow separation and subsequent high throughput sequencing has enabled the selection of IgG binders with nanomolar affinities in a single round.[127] Likewise, performing mRNA display on a microfluidic chip with immobilized target yielded single chain variable fragment antibody binders with nanomolar affinity after only 1−2 rounds of enrichment.[128] We believe that advances in library synthesis, expanded amino acids incorporations (NSAA) and separation techniques, and rapid, inexpensive deep sequencing are ever increasing the possibilities for mRNA display and its accessibility to diverse laboratories. In summary, multiple robust protocols are available,[33,129−131] and the technique is under constant improvement, to help establish mRNA display in your lab from scratch.

CONCLUSIONS

In vitro selection of polypeptides by mRNA display has proven to be an excellent tool for peptide design, protein engineering, and the investigation of protein interactions. The ability to search very large libraries of candidate sequences dramatically outperforms all *in vivo* methods and most other *in vitro* selection techniques. The convenient option of incorporating unnatural amino acids in the protein libraries expands the possibilities of obtaining new structures and activities that have no natural precedent and could not be selected by other means. This avenue is being heavily pursued in industry and academic laboratories to generate macrocyclic peptide therapeutics with unnatural modifications, opening the way to more potent protein inhibitors. The covalent genotype−phenotype linkage in mRNA display not only allows easy recovery and identification of the selected candidates but also, due to its robustness, enables the use of a wide variety of selection conditions, many of them incompatible with *in vivo* methods. This can be especially valuable in enzyme engineering in order to evolve soluble, folded, thermostable proteins that are also compatible with organic solvents, extreme pH, or high salt concentrations.

In summary, if you want to search the largest possible libraries of protein variants under the widest range of selection conditions, mRNA display technology should be your method of choice.

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https://dx.doi.org/10.1021/acssynbio.9b00419
ACS Synth. Biol. XXXX, XXX, XXX−XXX

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Fredarla Miller for comments on the manuscript. We gratefully acknowledge financial support from the US National Institutes of Health GM108703, AI113406, the US National Aeronautics and Space Administration NNX14AK29G and 80NSSC18K1277, the Simons Foundation, and the Biocatalysis Initiative of the BioTechnology Institute at the University of Minnesota.

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https://dx.doi.org/10.1021/acssynbio.9b00419
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