Future prospects for noncanonical amino acids in biological therapeutics

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Highlights

- Use of noncanonical amino acid (ncAAs) in therapeutics requires careful evaluation.
- NcAA-mediated strategies enable important routes to precisely defined conjugates.
- Viral and cell replication can be controlled using ncAA-dependent protein function.
- “Protein medicinal chemistry” with ncAAs can generate more druglike proteins.
- Improved incorporation technologies will further advance therapeutic applications.

Abstract

There is growing evidence that noncanonical amino acids (ncAAs) can be utilized in the creation of biological therapeutics ranging from protein conjugates to cell-based therapies. However, when does genetically encoding ncAAs yield biologics with unique properties compared to other approaches? In this review, we attempt to answer this question in the broader context of therapeutic development, emphasizing advances within the past two years. In several areas, ncAAs add valuable routes to therapeutically relevant entities, but application-specific needs ultimately determine whether ncAA-mediated or alternative solutions are preferred. Looking forward, using ncAAs to perform "protein medicinal chemistry," in which atomic-level changes to proteins dramatically enhance therapeutic properties, is a promising emerging area. Further upgrades to the performance of ncAA incorporation technologies will be essential to realizing the full potential of ncAAs in biological therapeutics.

Introduction

Genetically encoding noncanonical amino acids (ncAAs) enables powerful strategies for manipulating the properties of proteins, viruses, and cells. In addition to their rapidly expanding uses in basic research [1-3], ncAAs exhibit considerable potential in therapeutic applications (ncAAs, defined as amino acids beyond the 20 canonical amino acids (cAAs), are also referred to as unnatural amino acids (uAAs), nonstandard amino acids (nsAAs), and nonnatural amino acids (nAAs)). Two recent reviews summarized what therapeutic applications are feasible with ncAAs [4,5]. Here, we attempt to place these advances within a broader context: when does genetically encoding ncAAs yield biologics with unique properties compared to other approaches? And what opportunities lie ahead for utilizing ncAAs in therapeutic settings? Researchers are addressing these questions using several methods of genetically encoding ncAAs, including stop codon suppression in cells [2], residue-specific canonical amino acid replacement in cells [6], and drastic alterations to the genetic code in vitro [7].

The majority of this review focuses on three areas of application (Figure 1), drawing upon examples from the last two years whenever possible. In the areas of conjugates and limiting cell and viral replication, we highlight ways in which ncAA-mediated approaches provide valuable new routes for the creation of therapeutic candidates within these crowded spaces. Next, we show how the emerging concept of "protein medicinal chemistry" enables the use of atomic-level protein perturbations to drastically improve therapeutically relevant polypeptide properties; this area appears ripe for further exploitation. Finally, we emphasize the ongoing need to rigorously characterize and improve ncAA incorporation technologies, ranging from orthogonal translation systems (OTSs; Figure 1) to the translation apparatus and the genome itself [8-10]. Realizing nativelike translation efficiencies with alternative genetic codes will enable ncAAs to reach their full potential in therapeutic applications.

Conjugates

Selectively addressing individual chemical groups in proteins supports the generation of conjugates with therapeutic value. This includes utilizing ncAAs to link proteins to cytotoxic compounds, polymers, and additional classes of molecules via bioorthogonal functional groups including azides, alkynes, ketones, tetrazines, and cyclopropenes [11,12]. However, how do conjugates formed utilizing ncAA-mediated approaches compare to conjugates produced with alternative approaches? Comparisons between methods (Table 1) indicate that while the answer to this question is application-specific, careful exploitation of ncAAs significantly expands the range of therapeutically relevant conjugates.

Antibody-drug conjugates. Utilizing antibodies to deliver cytotoxic compounds to specific cells is reaching maturity [11,13,14]. Four antibody-drug conjugates (ADCs) are clinically approved, and more than 175 clinical trials utilizing ADCs are ongoing [13]. At the present time, approximately 4% of clinical trials in this area involve ADCs produced using an ncAA-mediated conjugation strategy (NIH U.S. National Library of Science Clinical Trials Database; URL:

https://www.clinicaltrials.gov). NcAAs appear to offer important, although nuanced, advantages for generating clinically relevant ADCs.

First-generation ADC chemistries targeting lysine residues or disulfide bonds in IgGs offer viable routes to ADCs (Table 1) [11,13]. Although these strategies have resulted in multiple clinically approved products, challenges with manufacturing, characterization, and product stability resulting from heterogeneous conjugation products have prompted intense research into site-specific conjugation approaches. ADCs with precisely positioned payloads exhibit comparable potency, improved pharmacokinetics, and an expanded therapeutic window relative to ADCs with heterogeneous payloads [15**-17**]. Table 1 depicts key technologies for producing homogeneous ADCs [18**]; each approach exhibits distinct advantages and limitations. For example, THIOMABs, which contain an unpaired cysteine residue for conjugation, lead to ADCs exhibiting excellent therapeutic properties [17**]. However, the site-dependent stability of thiol-reactive linkers in plasma requires careful conjugate engineering and selection of chemistry [11,19,20]. Enzyme-mediated installation of bioorthogonal groups at specific peptide sequences is another efficient route to site-specific ADCs, and resulting linkages do not typically exhibit site-dependent changes in stability [11]. A drawback of this approach is that scale-up of enzymatic transformations requires careful optimization [21,22]. These selected considerations highlight the tradeoffs inherent in choosing and ADC construction methodology.

NcAA-mediated approaches to ADC preparation (Table 1) [16] provide access to diverse, therapeutically relevant ADC structures, including some that are difficult to access with antibodies containing only cAAs [15]. Conjugation methods with ncAAs also avoid known challenges with other chemistries, such as additional reduction and oxidation steps to conjugate a payload at engineered cysteine residues [23], without sacrificing product yield [24]. Historically, challenges with large-scale production of proteins containing ncAAs has posed a significant barrier to clinical applications of ncAA-containing ADCs, but recent reports indicate

that these challenges are being addressed [4,15**,24]. With clinical-scale production feasible, are there other potential benefits of utilizing ADCs formed with ncAAs, or are the benefits limited to distinct manufacturing routes? We are aware of only a single published study that directly addresses this question [15**]. Tian et al. used the same anti-Her2 antibody and payload to prepare ADCs via cysteine- or ncAA-mediated (ketone) chemistries at identical antibody locations and evaluate the resulting ADCs in preclinical mouse studies. Intriguingly, in both tumor control and pharmacokinetic studies, ADCs produced via the ncAA-mediated route exhibited improved potency and longer half-lives, respectively, in comparison to ADCs produced via cysteine modification. These tantalizing results indicate that simply changing chemistries to an ncAA-mediated approach led to improved ADC properties; such observations should prompt further study to evaluate the extent of their generality. Thus, even in the highly competitive area of ADCs, the distinct structures, manufacturing routes, and possibly enhanced efficacy, afforded by ncAA-mediated ADC production significantly expands the range of clinically relevant ADCs.

Protein-polymer conjugates. Attaching polymers to proteins can enhance therapeutically relevant properties such as half-life and stability while reducing undesirable immunogenicity [25]. As a result, a number of protein-polymer conjugates are clinically approved [26]. Interestingly, lysine-mediated PEGylation strategies have led to several approved drugs, despite the range of bioactivities found in the heterogeneous conjugation products (Table 1) [25]. Site-specific conjugation strategies for producing protein-polymer conjugates extend beyond those used for ADCs to include N-terminal modifications and genetically encoded “PEG-like” stretches of cAAs (Table 1). Along with lysine- and cysteine-mediated approaches, these strategies have led to clinically approved products or candidates in ongoing clinical trials [27*-30]. All site-specific strategies, including ncAA-mediated approaches, are subtly distinct. N-terminal modifications and recombinant “polymers” enable modifications to native proteins, but are limited to positioning at protein termini; in some cases, biological activity can be abrogated upon modifying termini [30-32**]. In contrast, modifications utilizing cysteines or ncAAs enable access

to a much larger range of sites [25]. Fibroblast growth factor 21 (FGF21) provides an interesting case study highlighting the advantages of this versatility and a basis for comparing cysteine- versus ncAA-mediated conjugation strategies [31,32**]. Both Xu et al. (cysteine) [31] and Mu et al. (ketone-containing ncAA) [32**] found that PEGylated FGF21 variants exhibited a range of activities that depended upon the location of the PEGylation site. While several substitution positions resulted in bioactive variants in each study, Xu et al. reported that some cysteine substitutions appeared to interfere with native disulfide bond formation, while the Mu et al. study did not report any such effects. These findings indicate, in this case, a distinct advantage for ncAA-containing FGF21 because ketones are not known to interfere with native disulfide bond formation. Clinical studies with PEGylated FGF21 emerging from Mu et al. (Pegbelfermin; BMS-986036) indicate favorable safety profiles and, in Phase II studies in the indication of type II diabetes, patients receiving the drug experienced improved metabolic parameters including reduced cholesterol and triglycerides [33]. These and other findings [34] validate the feasibility of large-scale manufacture and clinical administration of protein-polymer conjugates produced via an ncAA-mediated strategy (It is important to note that these findings do not rule out the development of an FGF21 conjugate produced via an alternative approach; in the PEGylated FGF21 space, development of BMS-986036 is simply the most advanced). The crowded nature of protein-polymer conjugate development indicates the need to carefully consider preferred technologies for lead candidate generation. As highlighted by FGF21 conjugates, ncAA-mediated approaches are particularly well-suited for preserving native disulfide bonding patterns and positioning of attachment sites away from protein termini.

Other types of conjugates. Many additional well-defined conjugates are accessible with ncAA-mediated strategies that could be therapeutically relevant. For example, both bispecific antibodies [35] and antibody-nucleic acid conjugates can be produced utilizing ncAAs (Table 1) [36]. As discussed above, advantages of utilizing ncAAs to prepared these conjugates is likely to be nuanced. While numerous protein conjugates are formed by joining macromolecules or

cytotoxic drugs to a protein, chemical introduction of small molecules into proteins with medicinal chemistry-like precision is an appealing but understudied area of opportunity. The *Protein medicinal chemistry* section below explores how ncAA-mediated approaches, which include conjugations, facilitate powerful, unique approaches to therapeutic discovery.

Vaccines and cell-based therapies

Researchers are exploring ways in which ncAA incorporation can augment the functions of vaccines and cell-based therapies. Both conjugation strategies and control over protein translation are possible with ncAAs in cells and viruses. To date, ncAA-mediated conjugation strategies in this area tend to focus on fundamental virology studies. Examples include utilizing fluorescent conjugates to investigate the roles of envelope glycoproteins in HIV-1 viral budding [37], and modifying adeno-associated viruses with different receptor targeting agents to infect distinct cell types [38,39]. Though intriguing, these approaches are not yet mature enough to have direct therapeutic applications. Thus, the remainder of this section focuses on using codon suppression with ncAAs to control virus or cell replication. Stringent regulation of protein synthesis is effective for controlling replication events in patients or the environment (biocontainment); ncAA-mediated methods offer unique advantages for doing so.

Limiting viral replication. Although live or attenuated vaccines mimic pathogens and elicit robust immune responses, these vaccine formats do not completely eliminate the risk of viral replication. Conversely, synthetic vaccines based on conjugates, toxoids, subunits, or viral-like particles eliminate replication risks at the cost of native structure [40,41]. Genetic code expansion enables virus attenuation by making viral replication dependent on the incorporation of ncAAs in response to stop codons [38,42] or even four-base codons [43*]. For example, Chen et al. reported a genomically recoded HIV-1 that uses a quadruplet codon to encode Nε-(tert-butyloxy-carbonyl)-L-lysine (BocLys) in essential genes. The resulting engineered viruses require an OTS, a ncAA, and a successful frameshift to produce their full complement of

proteins, providing extremely stringent regulation of translation. Control over protein synthesis via codon suppression can be defeated by aberrant misincorporation of cAAs [10,44**]. However, engineering higher fidelity OTSs or making protein function dependent on ncAA side chain structure can largely alleviate these concerns (see also below). Thus, the ability to produce native-like viral structures and carefully control replication with ncAAs appears to offer unique advantages in vaccine production over alternative methodologies.

Limiting cell replication. Genetically modified microorganisms (GMMs) are an attractive platform for generating live bacterial vaccines and cell-based therapeutics [45]. Several complementary strategies for limiting the replication of GMMs now exist, including auxotrophy, gene circuits, unnatural nucleotides, and the use of ncAAs with OTSs. These biocontainment approaches all perform at similar levels, each reaching escape frequencies of 10^{-10} to 10^{-12} [45-47*]. However, with ncAAs an additional level of control is achievable: Mandell et al. redesigned enzymes in genomically recoded E. coli to require the incorporation of L-4,4′-biphenylalanine (bipA) to perform essential catalytic functions. Thus, not even cAA misincorporation in these proteins, which occurs with some OTSs, breaks containment [46*]. Finally, redundancy in biocontainment systems is important in ensuring that escape frequencies remain below detectable limits. Thus, ncAA-mediated biocontainment is a valuable addition to the biocontainment toolkit.

Protein medicinal chemistry

In principle, ncAA incorporation enables atom-by-atom control over protein function in ways that are not possible with cAAs. This precise control is routinely exploited in medicinal chemistry to identify small molecule drug leads [48]. There is growing evidence that “protein medicinal chemistry” supports the discovery of more druglike proteins and peptides (Figure 2) [4,7,49**]. Peptide-based discovery strategies (Figure 2a) already utilize this concept effectively [7], and basic studies with ncAAs in full-length proteins (Figure 2b) point the way to leveraging

these concepts in larger structures [1,50]. Several additional areas (Figure 2c-f) appear ripe for further exploration of this concept. While examples below exploit functionalities found in genetically encoded ncAAs, conjugation strategies are also expected to support the practice of protein medicinal chemistry.

Peptide-based discovery. The unique properties of peptides enable interactions with traditionally "undruggable" features of therapeutic targets, but peptides containing only cAAs exhibit short circulating half-lives and proteolytic sensitivity. In vitro translation systems utilizing ncAAs support genetically encoded macrocyclic constraints, altered backbone structures, and altered side chain structures during discovery and optimization [51,52]. In particular, the use of mRNA display to screen libraries of up to 10^{13} ncAA-containing peptides has led to the identification of numerous leads that could not have been identified with other approaches. For example, Passioura et al. used a 23-amino acid genetic code containing a set of hydrophobic ncAA side chains to identify anti-interleukin-6 receptor macrocyclic peptide ligands (Figure 2a). The resulting hits exhibited more druglike hydrophobicities than hits from control cAA-containing libraries [53**]. This and other successes highlight the expanded range of properties accessible when ncAAs are incorporated into genetically encoded peptide discovery.

Atomic-level perturbations in proteins. Historically, many studies employing ncAAs in full-length proteins have leveraged atomic-level changes to elucidate fundamental insights, especially in the area of membrane protein function [50]. For example, replacing aromatic cAAs with fluorinated aromatic analogs has validated numerous interactions between aromatic rings and positively charged chemical groups. These cation-pi interactions are strongly affected by the electron-withdrawing properties of even single-fluorine substitutions [54]. Subtle but powerful effects such as these lay the foundation for broader practices of protein medicinal chemistry.

Some work directly demonstrates how minute side chain alterations modulate properties of therapeutically relevant proteins. Using a model antibody fragment, Van Deventer et al. serendipitously discovered that replacing methionine residues with an azide-containing ncAA

resulted in enhanced antigen binding [55]. This finding implies that ncAA side chains support altered modes of molecular recognition, which may be valuable in tailoring the interactions of proteins with therapeutic targets. In a groundbreaking study on insulin, Lieblich and Fang et al. discovered that a single proline-to-*4S*-hydroxyproline substitution facilitated more rapid monomer dissociation and slower fibrillation compared to the parent insulin molecule (Figure 2b); these enhancements are both highly clinically desirable [49**]. These studies clearly indicate the potential for ncAAs to support the discovery of more druglike proteins; the following subsections highlight additional areas ripe for exploitation.

Post-translational modifications. Naturally occurring protein post-translational modifications (PTMs) can drastically alter protein properties. NcAA incorporation provides the means of producing proteins with precisely defined patterns of “post-translational” modifications [56]. Moreover, some PTMs modulate the functions of extracellular targets, raising the possibility of near-term therapeutic applications. For example, sulfation events at tyrosines play important roles in mediating extracellular protein-protein interactions [57,58*]. However, sulfation occurs when enzymes recognize amino acid motifs that may not be optimal for target recognition. Recent work by Li et al. used genetic code expansion in an anti-HIV antibody to quantify the potency of all combinations of tyrosine sulfations within an antibody region that mediates interactions with gp120 (Figure 2c) [58*]. This excellent work indicates clear opportunities for producing and screening proteins containing precisely defined sets of PTMs in search of proteins with more druglike properties. There are numerous additional ways in which naturally occurring PTMs could be harnessed in therapeutic applications. The reader is referred elsewhere for potential opportunities in areas including breaking immune tolerance [59], crosslinking (see also the following section) [60], glycosylation (requires chemical synthesis) [61,62], and intracellular PTMs (requires efficient intracellular protein delivery technologies) [63].

Covalent crosslinkers. NcAAs also facilitate the presentation of crosslinkable groups within proteins [64,65]. Covalent therapeutic target engagement has long been a part of

medicinal chemistry. Researchers have demonstrated several advantages of covalently bonding with active site residues. For example, covalent inhibitors support potent inactivation even during substrate buildup, which noncovalent inhibitors cannot [66,67]. However, covalent inhibitors can quickly become toxic if they engage off-targets. The exquisite specificities of antibodies and other binding proteins offer a potential solution to this challenge, provided that crosslinkable functionality can be presented within these scaffolds without the need for ultraviolet light or exogenous catalysts (Figure 2d) [68*,69*]. Several recent reports have demonstrated the use of ncAAs containing functional groups that undergo proximity-based crosslinking, but are not reactive enough to interfere with protein translation or other biochemical processes [68*,69*]. Expanding these approaches beyond model systems has strong therapeutic potential. In addition, proximity-based crosslinking enables formation of intramolecular crosslinks, leading to stabilized or constrained protein structures that may be more druglike [70,71].

Metal binding groups. The introduction of metal-binding groups (MBGs) into proteins [72] is another enticing approach to enhancing therapeutic protein properties. This includes: 1) the creation of artificial metalloproteins with enhanced stability or nonnatural catalytic functions; and 2) the creation of metalloproteinase function-disrupting proteins by binding active site metal ions. In one strategy for enhancing stability, Luo et al. inserted an ncAA containing an MBG into the HIV gp41 trimeric coiled-coil (Figure 2e). Placement of the MBG at the N-terminus led to a highly stabilized trimer [73*], which could lead to viral inhibitors or vaccine components with better pharmacological properties.

Recent work in medicinal chemistry has led to the reemergence of MBGs as compelling elements of therapeutic molecules. In particular, fragment-based drug discovery efforts have revealed a diversity of MBGs that interfere with metalloprotein function [74,75*]. However, modulation of the specificities of these groups remains a significant challenge [76]. Introduction of MBGs within binding protein scaffolds could lead to inhibitors that eliminate the off-target

effects of previous generations of metalloprotein inhibitors. Thus, judicious use of MBGs could lead to multiple new types of macromolecular therapeutics.

Glycan targeting. Specific glycosylation patterns modulate important protein and cell regulation events [61,62]. Because these patterns are known to be disregulated in cancer and other diseases, glycan targeting is a tantalizing but largely unrealized therapeutic approach [61]. NcAAs offer potential ways to generate proteins that target specific glycan structures in ways that natural sugar-binding proteins cannot. Most notably, boronic acids, which covalently bind to diols in sugars, can be directly genetically encoded within proteins [77] (Figure 2f). Early work in this area includes the presentation of boronates within antibody variable regions [77] and other binding proteins [78*]. Realizing these approaches in therapeutic settings will require additional design and engineering to enhance the affinity and specificity of boron-containing proteins.

Underlying technologies for encoding ncAAs in proteins

The machinery required to genetically encode ncAAs must exhibit efficiencies and fidelities rivaling those of natural protein translation machineries in order to fully realize potential therapeutic applications. Some early successes include protein expression systems that support clinical-scale production of proteins containing a narrow range of ncAAs [4] and peptide-based discovery with mRNA display [7,51]. However, these examples are exceptional. In contrast, the integration of ncAAs with additional display technologies (yeast display [79], phage display [80], and bacterial display [55]) and selection-based approaches [71] remains in early stages.

We address here ongoing challenges in generating efficient genetic code expansion systems in cells. Examples of cell-free systems for efficient genetic code alteration and *E. coli*-based systems for residue-specific ncAA incorporation are described elsewhere [6,7]. The lack of efficient orthogonal translation systems (OTSs) is one of the largest barriers to establishing robust genetic code expansion. In addition, suppression events at noncognate codons tend to be inefficient compared to cognate codon readthrough. Characterizing the performance of

engineered translation systems is crucial for understanding and improving all aspects of ncAA incorporation.

Quantifying stop codon readthrough efficiencies and fidelities with robust reporters is a critical but frequently overlooked aspect of evaluating genetic code manipulation systems. Reporters have the throughput needed to conduct the dozens of measurements of OTS performance needed to identify promising ncAA-containing protein expression conditions, and even the millions of measurements needed to screen for improved ncAA incorporation systems. These do not replace other important characterizations: soluble protein yields and mass spectrometry experiments on purified proteins are the ultimate proof of precisely defined ncAA substitution(s). Furthermore, enzymatic and structural characterizations of OTSs provide insights that may lead to OTSs with native-like properties. However, tuning the *in vivo* expression levels of aminoacyl-tRNA synthetases and tRNAs can result in efficient, high fidelity protein synthesis even when aaRS/tRNA properties are known to be poor [10,81]. Thus, reporters have an important role to play in evaluating ncAA incorporation events in cells.

Historically, single fluorescent protein reporters containing one or more stop codons have been detected in plate readers to characterize ncAA incorporation in cells [82]. However, these reporters ignore cell-to-cell variability and other potential changes in protein synthesis that accompany ncAA incorporation events. Advanced reporters that monitor both initiation of protein synthesis and codon readthrough events overcome limits of single-fluorescent protein reporters. Monk et al. utilized a dual fluorescent protein reporter in *E. coli* to measure ncAA incorporation efficiency and fidelity while controlling for cell-to-cell variability and cell viability [83**]. This work also established quantitative metrics of ncAA incorporation efficiency and fidelity that suit any dual reporter system. Beranek et al. implemented the use of a similar format in mammalian cells [84], although efficiency and fidelity metrics were not reported. Building off of Monk et al.'s approach, our lab recently described yeast-based measurements of ncAA incorporation in a dual reporter format [44**]. The yeast display-based reporter enabled flow cytometry-based

evaluation of OTS performance on a single-cell basis while eliminating potential problems with fluorescent reporter folding kinetics. These types of reporters support evaluations of ncAA incorporation while controlling for stop codon position within a construct, nonsense-mediated decay (in eukaryotes), genome composition, and other factors.

Robust reporters can aid the discovery of improved ncAA incorporation platforms in engineering efforts ranging from OTS screening to genomic recoding. Many efforts to identify OTSs for new or improved ncAA incorporation are still conducted with selection systems where stringency is difficult to tune [10]. In contrast, screens utilizing fluorescence-activated cell sorting (FACS) provide quantitative cell-by-cell comparisons while readily supporting changes in sorting stringency. Even with single fluorescent protein reporters, FACS can lead to OTSs with excellent properties [85**,86]. Next-generation selection systems such as phage-assisted continuous evolution are also promising tools for evolving OTSs [87]. Finally, many aspects of the translation apparatus can be engineered to enhance ncAA incorporation efficiencies. The most sophisticated of these efforts have yielded *E. coli* in which the amber stop codon has been removed and recoded for a 21st amino acid [88] and *E. coli* with a third DNA base pair to provide additional codons [89]. Reporter systems are important for evaluating these impressive systems so that the process of ncAA incorporation rivals that of canonical amino acid incorporation.

Conclusions

There is no doubt that ncAAs will play important roles in future generations of therapeutically relevant proteins, viruses, and cells. NcAA-mediated approaches expand the routes available for discovery and development of entities including conjugates, vaccines, and cell-based therapies. Careful exploitation of ncAAs supports generation of a broader range of therapeutic leads, some which may even exhibit superior properties, compared to those produced with alternative methods. Integrating ncAAs into genetically encoded peptide discovery has yielded peptides with structures and functions that would be nearly impossible to

duplicate with only canonical amino acids. Hints of the power of utilizing medicinal chemistry concepts in larger proteins, i.e. protein medicinal chemistry, are also coming into focus. However, the full potential of protein medicinal chemistry and other applications of ncAAs in biotherapeutics will be only realized with the availability of high-performing ncAA incorporation systems in bacterial and eukaryotic expression systems. Given the power of platforms for discovering biologics that utilize only canonical amino acids, providing these platforms with full access to the chemical cabinet is sure to lead to entirely new classes of therapeutics.

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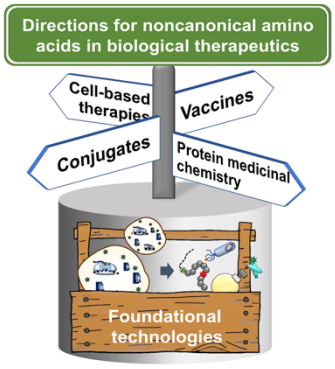
Table 1. Summary of advantages and limitations of selected conjugation strategies.

		Conjugation Strategy	Advantages	Limitations
Antibody Drug Conjugates (ADCs)	ncAA-mediated technologies	*Strain-Promoted Cycloaddition:* genetically encoded azide, exogenous strained alkyne	*Reaction:* fast kinetics; various payloads compatible *Clinical:* larger therapeutic window; improved stability *Location:* site-specific	*Manufacture*: advanced translation engineering required (cell line/cell free system must support efficient protein translation with ncAAs)
		Copper-Catalyzed Cycloaddition: genetically encoded azide, exogenous terminal alkyne*	*Reaction:* see above *Clinical:* see above *Location:* site-specific	*Reaction:* complex kinetics *Manufacture:* see above
		Oxime Ligation: genetically encoded ketone, exogenous hydroxylamine	*Reaction:* robust; compatible with large range of payloads *Clinical:* see above *Location*: site-specific	*Reaction*: slow reaction kinetics *Manufacture:* see above
	Other competing technologies	*Lysine Conjugation:* surface-accessible lysine, exogenous amine-reactive compound	*Manufacture*: established production strategies	*Clinical*: lower bioactivity; low plasma stability *Location:* not site-specific
		Disulfide Conjugation: thiols from disulfide bond reduction, exogenous electrophile	*Manufacture*: established protein production strategies	*Manufacture:* carefully controlled reaction conditions *Location:* not site-specific
		Single-Cysteine Conjugation: single engineered cysteine, exogenous thiol-reactive compound	*Clinical:* larger therapeutic window; improved stability *Manufacture:* established protein production strategies	*Reaction:* careful control to avoid reduction of native disulfides; not compatible with some payloads
		Enzyme-Mediated Conjugation: encoded peptide substrate, exogenous compatible payload	*Reaction:* robust; compatible with large range of payloads *Location:* site-specific	*Reaction:* conditions particular to enzyme and conjugation site
Protein-Polymer Conjugates	ncAA	*Copper-Catalyzed Cycloaddition*	See above	See above
		Oxime Ligation	See above	See above
	Other	*Lysine Conjugation*	See above	See above
		N-terminal Amine Group Modification: free N-terminus, exogenous reactive compound	*Location:* site-specific	*Reaction:* tight control needed to avoid side products
		PEG Mimetics: flexible, genetically encoded amino acid chains	*Location:* N- or C-terminus	*Clinical*: free terminus may be important for bioactivity
Bispecific Antibodies	ncAA	*Strain-Promoted Cycloaddition*	See above	See above
	Other	*Bispecific T-cell Engagers*: genetically fused antibody fragments	*Clinical:* efficacy can be high at low concentrations	*Clinical*: some fusions have short half-lives
Other Conjugates	ncAA	*Antibody-Nucleic Acid:* genetically encoded ketone, exogenous aminooxy-nucleic acid	See above	See above
	Other	*Lysine Conjugation*	See above	See above

*The alkyne can also be genetically encoded and azide added exogenously.

Figures

Graphical abstract



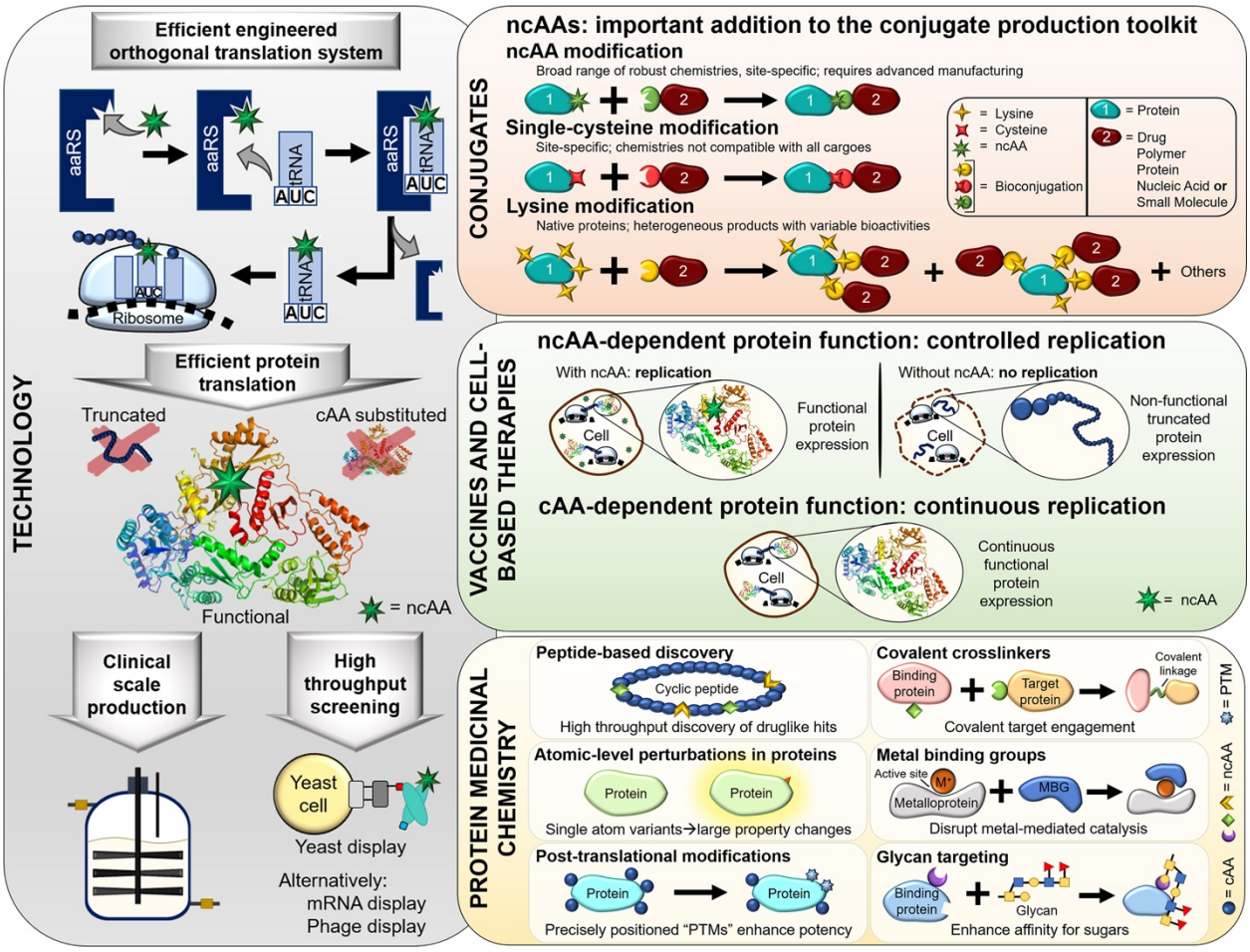


Figure 1. Overview of topics covered in this review. Technology: underlying successful applications of ncAAs in therapeutic settings are high efficiency, high fidelity platforms for genetically encoding ncAAs in proteins. The performance of orthogonal translation systems (OTSs), comprised of aminoacyl-tRNA synthetase (aaRS)/suppressor tRNA pairs, remains a limiting factor in many platforms. Conjugates: ncAA-mediated conjugations are important additions to the conjugate production toolkit. Vaccines and cell-based therapies: ncAA-dependent protein function allows for precise control over viral and cell replication. Protein medicinal chemistry: utilizing ncAAs to precisely alter protein structure and function provides many opportunities for discovering new classes of therapeutics. Protein structure taken from PDB ID 1DLO. Glycan structure taken from reference [61].

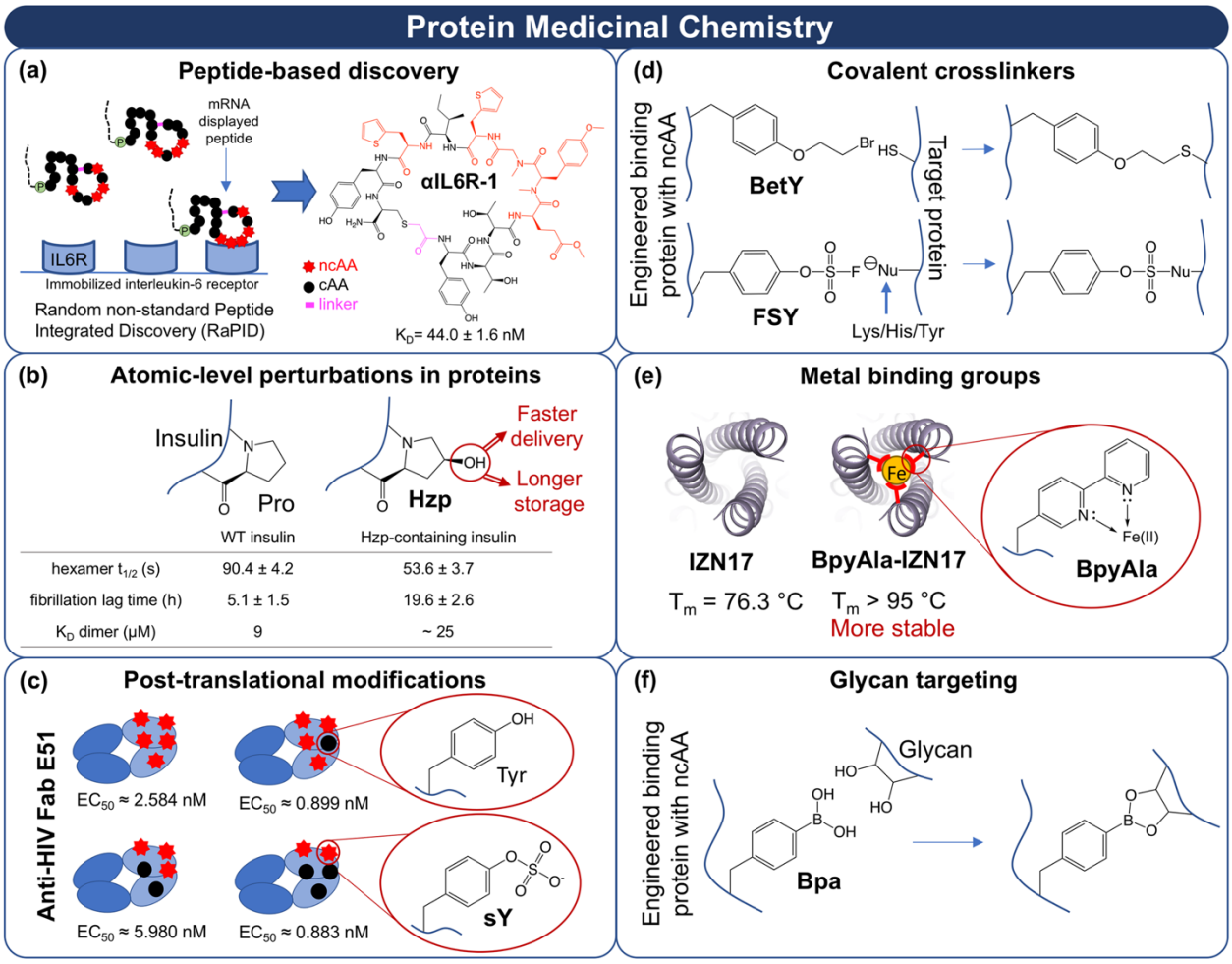


Figure 2. Early examples of protein medicinal chemistry. **(a)** NcAA-containing macrocyclic peptide synthesis and screening. The portion of the genetic code encoding hydrophilic cAAs was reassigned to a set of hydrophobic ncAAs to make encoded peptides more druglike. A macrocyclic library generated with this altered genetic code was screened against the interleukin-6 receptor (IL6R) in mRNA display format. Identified hits exhibited hydrophobicities that would be extremely challenging to achieve utilizing only canonical amino acids [53**]. **(b)** A single-atom substitution at position 28 in the insulin B-chain significantly shortened the dissociation time of monomeric insulin (hexamer $t_{1/2}$) while prolonging its shelf life (fibrillation lag time). **Hzp:** (4*S*)-hydroxyproline [49**]. **(c)** Precise control of posttranslational modifications using ncAA incorporation. Uniformly sulfated antibodies were produced by genetically encoding sulfotyrosine (**sY**) in response to a stop codon. By evaluating all possible sulfation patterns in an

antibody region important for binding, combinations leading to the highest potencies were identified [58*]. **(d)** Two representative examples of ncAA-based, proximity-activated covalent crosslinkers that function in mammalian cell culture [68*,69*]. **BetY**: *O*-(2-bromoethyl)tyrosine; **FSY:** fluorosulfate-L-tyrosine. **(e)** The trimeric coiled-coil structure of the N-peptide of HIV surface protein gp41 (**IZN17**) can be stabilized by encoding a metal chelating ncAA within its structure. **BpyAla:** (2,2'-Bipyridin-5-yl)alanine [73*]. PDB: 2R3C. **(f)** The boronic acid group in 4-borono-L-phenylalanine (**Bpa**) is capable of forming a covalent bond with the diol structures found in many glycans.