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Synthesis at the Interface of Virology and Genetic Code Expansion

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Abstract

How a virus efficiently invades its host cell and masterfully engineers its properties provides valuable lessons and resources for the emerging discipline of synthetic biology, which seeks to create engineered biological systems with novel functions. Recently, the toolbox of synthetic biology has also been enriched by the genetic code expansion technology, which has provided access to a large assortment of unnatural amino acids with novel chemical functionalities that can be site-specifically incorporated into proteins in living cells. The synergistic interplay of these two disciplines holds much promise to advance their individual progress, while creating new paradigms for synthetic biology. In this review, we seek to provide an account of the recent advances at the interface of these two research areas.

Introduction

Viruses employ highly sophisticated strategies to infect host cells with the ultimate goal of using the host machinery and resources for their self-replication. The virus capsid, as well as the proteins and RNAs encoded in the viral genome, have evolved to possess numerous fine-tuned functions to facilitate various stages of this process including viral entry, altering the physiology of the host cell for optimal viral replication, packaging of progeny virus, and their escape back to the environment. In addition to their importance in fundamental virology and the development of new antiviral therapeutics, understanding the molecular processes associated with viral infection can provide important lessons and resources to further enrich the toolbox for synthetic biology. Indeed, genetic elements derived from viruses (e.g., promoters, nucleic acid polymerases, internal ribosome-entry site elements, etc.) are frequently borrowed in numerous synthetic biology applications. Additionally, the efficient cellular entry process of different viruses provides an attractive roadmap to create next-generation delivery vectors for a variety of therapeutic agents, including ‘gene circuits’.

The genetic code expansion (GCE) technology enables site-specific incorporation of unnatural amino acids (Uaas) into proteins expressed in living cells, using engineered aminoacyl-tRNA synthetase (aaRS)/tRNA pairs that suppress a repurposed nonsense codon (Figure 1A).[1–3] This technology has experienced remarkable progress over the last decade, facilitating its application to various domains of life.[1–3] Using this approach, over 150 different Uaas with novel sidechains have been genetically encoded to date, providing an exciting new toolset in the arsenal of synthetic biology.

In recent years, there have been exciting developments at the interface of the GCE technology and virology. It has become increasingly clear that the tools from each of these disciplines are particularly well-suited to expand the scope of the other. In this review we attempt to capture the relevant body of work demonstrating how this synergistic interplay is accelerating the progress of these two disciplines and creating new opportunities for synthetic biology.

Chemical modification of virus-like particles (VLPs)

Virus-like particles (VLPs) are protein cages resembling viruses, assembled from capsid proteins of viruses (e.g., MS2, Q β , TMV) or other proteins.[4,5] Despite lacking the highly sophisticated properties of live viruses for efficient cell-entry, they have been developed as vehicles to deliver small-molecule cargo *in vitro* and *in vivo*, including imaging agents and cytotoxic drugs, as well as for vaccine development.[6–9] Since several excellent reviews have already been published on this topic, here we briefly summarize such efforts that take advantage the GCE technology. Chemical modification has been widely used to functionalize VLPs with both targeting agents and cargo.[10–12] However, functionalizing canonical amino acid residues on VLPs intrinsically lacks site-specificity, unless a restricted number of target residues are surface-accessible. The GCE technology provides an attractive alternative by enabling site-specific incorporation of Uaas, which can be subsequently functionalized using chemoselective labeling strategies. Finn, Tirrell *et al.* used a global methionine replacement strategy to introduce azidohomoalanine (Aha) and homopropargylglycine (Hpg) into the coat protein of Q β derived VLPs on both the inner and outer surfaces, which were later functionalized with fluorophores, cell-targeting glycans, etc., through azide-alkyne click chemistry (Figure 2B; Table 1).[10,13] VLPs have also been generated incorporating Aha or Hpg using a bacterial cell-free protein expression system and subsequently functionalized with PEG, nucleic acids, and proteins.[14] Taking advantage of a previously developed engineered aaRS/tRNA pair that charges *p*-aminophenylalanine (pAmF) in response to the UAG nonsense codon,[15] the Francis group has site-specifically introduced this Uaa onto the surface of Q β VLPs. Using its chemoselective oxidative coupling with electron-rich aromatic amines (Figure 2B, Table 1), the pAmF residues were subsequently labeled with PEG[16] and cell-targeting groups including peptides,[17] aptamers,[18] proteins,[19] and antibodies.[20] This labeling strategy has been further combined with cysteine functionalization on the interior of the capsid to deliver PET[21] and MRI[22] imaging agents and cytotoxic photodynamic therapy agents[23] to cancer cells *in vivo*, and for *in vivo* imaging of fibrin clots[24]. Plant-derived tobacco mosaic virus (TMV) VLPs, which assemble into rod or disc shapes depending on conditions, have also been functionalized with Uaas using the GCE technology.[25]

Using genetic code expansion to probe and engineer viruses

The ability to selectively modify the proteins associated with viruses is important both for understanding their roles in viral replication through the attachment of biophysical/biochemical probes, and from the perspective of engineering viral vectors with desirable traits (e.g., cell selectivity, immune evasion, etc.). Unlike VLPs, viruses employ a series of complex molecular maneuvers to enter their host cells with remarkable efficiency.[26–30] To ensure that these delicate processes are not perturbed, extra care must be taken when

introducing modifications on the virus particle. Indeed, the proteins associated with various mammalian viruses are remarkably complex and multifunctional.[26–30] Consequently, these are often refractory to traditional genetic manipulations, such as incorporation of peptide or protein tags, the large size of which can significantly perturb their native function. In one of the first examples of chemoselective modification of mammalian viruses, the Carrico group generated azide-labeled adenovirus particles through metabolic incorporation of azido-containing carbohydrates as well as global replacement of methionines with azidohomoalanine (Aha).[31,32] The resulting azide groups were used to attach fluorophores, as well as retargeting agents such as folate. However, metabolic incorporation of modified sugars is restricted to native glycosylation sites, and viruses that do not undergo glycosylation are not amenable to this strategy. The scope of the global methionine replacement strategy is also restricted by a limited selection of sites as well as the kinds of Uaas that can be incorporated.

Production of mammalian viruses incorporating genetically encoded Uaas.—

The ability to incorporate Uaas into virus-associated proteins using engineered nonsense-suppressing aaRS/tRNA pairs (Figure 1B) dramatically improves both the diversity of chemical functionalities that can be precisely incorporated, and the choice and selectivity over the site of their incorporation. Using HDV as the model system and the well-established pyrrolysyl-tRNA synthetase (PylRS)/tRNA pair as the nonsense suppressor, the Chen group first demonstrated the feasibility of incorporating several different Uaas into the capsid of a mammalian virus, as well as their subsequent chemoselective labeling.[33] It is important to note that the biology of different viruses is widely divergent, and so are the associated genetic systems for their recombinant production. Consequently, to apply the GCE technology to a particular virus, the Uaa incorporation machinery must be optimally interfaced with the unique genetic system used for its recombinant production. Successful development of such a system requires both a thorough understanding of the unique nuances of the particular virus, as well as the Uaa incorporation system. Nonetheless, the GCE technology has been extended to several additional mammalian viruses in the last few years (Table 1), including human immunodeficiency virus (HIV) and recombinant lentiviruses, [34,35] adeno-associated virus (AAV)[36–41] and influenza virus,[42,43] and it has spurred many powerful applications that are summarized below.

Development of engineered viral vectors for gene therapy.—Various mammalian viruses hold great promise as vectors for human gene therapy.[44–46] However, current approaches largely rely on the native tropism of naturally occurring viral serotypes, which frequently does not align with the therapeutic need. Additionally, our immune system poses a major challenge for the development of successful gene therapy vectors based on human viruses. Uaa-directed chemoselective labeling of the virus surface with retargeting agents has recently emerged as a powerful strategy to overcome these challenges, by providing precise control over site and stoichiometry and the ability to attach molecules of theoretically unlimited chemistry and size. Adeno-associated virus (AAV) has emerged as one of the most promising candidates for human gene therapy.[47] Using the GCE technology, our group and others have shown that an azide-containing Uaa (AzK) can be incorporated into distinct sites of the AAV capsid, and that these functionalities are tolerated

well by the virus.[36–41] The resulting Uaa group has been further used to site-specifically attach retargeting agents (e.g., a cyclic-RGD peptide) onto the virus capsid that successfully alter its receptor specificity and, consequently, its tropism.[36,38] Similar approaches have also been used to attach retargeting agents onto a lentivirus.[34] In this case, the Uaa residue was incorporated into the vesicular stomatitis virus glycoprotein (VSVG) that non-specifically incorporates into the viral envelop upon co-expression, a feature that is widely used to expand the tropism of recombinant lentiviruses. The ability to precisely modify virus capsids also provides new approaches for creating immune-evading viral vectors by attaching functionalities known to reduce immunogenicity (e.g. PEG) without significantly perturbing infectivity.[48] Site-specific PEGylation of AAV using a Uaa residue indeed showed modest reduction of antibody response and improved viral titer in the blood in rats.[37]

Probing the biology of viruses.—Genetically encoded Uaas also have provided powerful new ways to interrogate the complex biology of viruses. Due to their complex nature, viral proteins are often not compatible with traditional genetically encoded probes such fluorescent proteins. Uaas provide a facile strategy to attach small biophysical probes onto predefined sites on the virus capsids. Indeed, the Zhou group has used this strategy to label AAV capsid with fluorophores to monitor its cellular entry using fluorescence microscopy.[39] The Lemke group has used this strategy to fluorescently label VLPs derived from hemagglutinin and matrix protein 1 of influenza virus for super-resolution imaging.[42] Envelope glycoproteins of HIV have also been fluorescently labeled using the GCE approach.[35] This modification was small enough to not disrupt native protein function, enabling the use of super-resolution microscopy and fluorescence recovery after photobleaching (FRAP) experiments to image the dynamics of the HIV budding process.[35] Viruses employ numerous interactions with different host factors to establish successful infection, the majority of which remain elusive for even some of the most well-studied viruses. Uaas harboring photoaffinity probes provide an exciting avenue to covalently trap these molecular interactions. The feasibility of incorporating such Uaas that harbor the popular diazirine photoaffinity probe has been demonstrated in lentivirus[34] and hepatitis D virus (HDV).[33] Efforts are currently under way to identify unknown host-virus interactions using this approach. In another example of how the GCE technology can be used to manipulate host-virus interactions, our group has created photo-activable AAV particles by replacing conserved amino acid residues on the virus capsid, which are essential for binding the primary receptor for cell entry, with their photocaged counterparts.[36] While the resulting viruses are non-infective, brief irradiation removes the caging group and restores the native infectivity, providing a precise way to perturb distinct host-virus interactions.[36]

Live replication-incompetent viruses as vaccines.—The GCE technology has also provided a novel strategy to create live but replication incompetent virus-based vaccines, where nonsense codons are incorporated into essential genes in the viral genome, such that the virus can only replicate in the presence of the nonsense suppressor aaRS/tRNA pair and their substrate Uaa.[43,49,50] This principle has recently been applied to the HIV-1[49,50] and influenza A[43] viruses to generate replication-incompetent viruses for use as “live-

attenuated” vaccines. These represent an attractive middle ground as vaccines between inactivated viruses, which are safe but not always antigenically representative, and live-virus vaccines, which are prohibitively unsafe. To avoid escape through the reversion of the nonsense codon, a legitimate concern for rapidly mutating viruses such as HIV and influenza, it was necessary to insert nonsense codons at multiple different sites that do not alter major viral antigens.

Virus-based tools to expand the scope of the GCE technology

Despite the exciting progress in recent years, there are still significant challenges that limit the scope of the GCE technology, particularly for its application in mammalian cells.[51] One central limitation comes from the suboptimal efficiency of the engineered aaRS/tRNA pairs at the core of this technology. Additionally, the overwhelming majority of applications of this technology in mammalian cells rely on transient transfection for the delivery of the genetic components, which has significant limitations.[51,52] The factors that limit the Uaa incorporation efficiency, and the principles for the design of an optimal mammalian expression system also remain incompletely understood.[51,52] Virus-based tools have played a significant role in overcoming these challenges in recent years.

Delivery and optimization of the Uaa incorporation machinery.—Various viral vectors have been adapted for efficient delivery of genetic cargo into mammalian cells. Adapting these vectors for the delivery of the Uaa incorporation machinery is associated with some unique challenges. A rather large cargo-capacity is needed to accommodate the engineered aaRS/tRNA pair and the nonsense mutant of the target gene in the same vector such that these can be efficiently co-delivered into target cells. Additionally, to achieve optimal expression levels of the suppressor tRNA, multiple copies of its expression cassette must be encoded in the viral genome [52,53], which can be challenging due to its recombination-prone nature. So far, several viral vectors has been developed for this purpose, including lentivirus,[54] adenovirus,[55] AAV[56] and baculovirus[52,57]. The efficiency of Uaa incorporation observed with lentiviral and adenoviral systems appears to be low. The low cargo capacity of AAV forces the use of two separate vectors to deliver all the necessary components for Uaa incorporation. However, using high dosage of these two vectors, Uaa incorporation has been demonstrated in cell culture, *ex vivo* tissue slice culture, as well as in mouse brain for the first time.[56] Although baculovirus is an insect virus, its VSVG-pseudotyped counterpart has been used for efficient delivery of the Uaa incorporation machinery in mammalian cells and tissues.[57] Due to its large cargo-capacity and stable double-stranded DNA genome, the entire Uaa incorporation machinery – including multiple copies of the tRNA expression cassette – could be incorporated into a single baculovirus vector.[52,57] Owing to the more homogeneous gene delivery facilitated by viral vectors, it has been shown that the expression of the transgene can be systematically modulated by simply changing the virus-to-cell ratio.[52] Taking advantage of this feature, the effect of altering the expression levels of aaRS, tRNA and the target gene on Uaa incorporation has been investigated, enabling the design of a significantly improved expression system that expresses each component at optimal levels.[52]

Evolution of improved Uaa-selective aaRS variants.—The Liu group has recently developed the powerful phage-assisted continuous evolution (PACE) platform that enables rapid multi-round directed evolution of target proteins, provided its performance can be coupled to the expression of the essential phage protein pIII.[58] As mentioned earlier, some of the engineered Uaa-selective aaRS variants show poor activity, which limits their utility. PACE provides an attractive platform to rapidly improve the activity of such suboptimal aaRS variants. Indeed, the Liu group has recently subjected PylRS – one of the most widely used platforms in GCE – to PACE, and isolated mutants with remarkably improved activities.[59] A variation of the same approach, where the phage-assisted directed evolution was performed in a discontinuous manner, also led to improved mutants.[60]

Evolution of new functions utilizing the novel chemistries encoded in Uaas.—One of the grand promises of the GCE technology is the ability to create novel biological functions using expanded chemical space. One such application involves the development of novel affinity reagents (e.g., antibodies) using non-natural functionalities that provide significant advantage for binding specific targets. Indeed, the phage display technology based on the filamentous bacteriophage M13 has been used to demonstrate that the incorporation of Uaas with such privileged “chemical warheads” provides a distinct advantage when evolving novel protein or peptide-based affinity reagents.[61–64] Using this strategy, a cyclic peptide harboring metal-binding Uaa bipyridyl alanine (BpyA) was developed with strong affinity for Ni^{+2} -NTA.[64] It has also been used to develop antibody fragments harboring a boronate-containing Uaa that selectively interacts with 1,2-diols,[62] and those harboring an O-sulfotyrosine with high affinity for the HIV gp120 protein, which naturally binds a sulfated receptor.[63] In each case the Uaa residue significantly contributed to the binding affinity. In another interesting example of virus-assisted evolution with non-natural chemical handles, Hpg-containing peptide sequences (generated through global methionine replacement) were evolved that participate in accelerated palladium-catalyzed Sonogashira coupling.[65]

Conclusion

As the above examples attest, virology and the GCE technology are mutually beneficial and research at their interface is creating new opportunities in synthetic biology. Despite the initial technical challenges associated with applying the GCE technology to a particular virus, developments of such platforms can be highly empowering, as demonstrated by the early studies reviewed here. The ability to label distinct viral proteins at predefined sites with biophysical probes, attach photoaffinity probes that help uncover the numerous host-virus interactions that facilitate viral entry and replication, and achieve other ingenious maneuvers by precisely installing unique chemical functionalities into virus capsids has enormous potential for interrogating their complex biology in powerful new ways. It is also clear that the ability to precisely decorate the virus capsid with a wide variety of entities provides opportunities to create next generation vectors with fine-tuned tropism and the ability to evade the immune system. Virus based tools are also being used to expand the scope of the GCE technology through the development of novel delivery vectors and virus-assisted directed evolution platforms that can be used to improve the intrinsic performance

of the engineered aaRS/tRNA pairs that drive the GCE technology. Virus assisted evolution schemes – such as phage display – also provide exciting opportunities to generate novel affinity reagents and other useful biological functions that take advantage of the expanded chemical space offered the genetically encoded Uaas.

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Figure 1:

A) A general scheme demonstrating the site-specific incorporation of Uaas into proteins expressed in living cells using a nonsense suppressing engineered aaRS/tRNA pair. B) Production of virus particles site-specifically labeled with Uaas.

A) Uaas used to modify viruses

B) Bio-orthogonal reactions used to modify viruses

Figure 2.

A) Genetically encoded Uaas that have been used to modify viruses and VLPs. Side-chains are color-coded according to their application. B) Various bio-orthogonal reactions used to modify Uaa-labeled viruses and VLPs.

Table 1.

Examples of site-specific Uaa incorporation in viruses and virus-like particles. Please see Figure 2 for the structure of Uaas and the chemical reactions.

Virus or VLP	Produced in	Uaa(s)	Site(s)	Purpose(s)	Reference
AAV-2	Mammalian cells	AzK	Capsid (all VPs) positions T454, R447, N587+1, R588	SPAAC click chemistry for targeting, PEGylation, imaging	[36–40]
		NBK	Capsid (all VPs) positions R585, R588	Photocaging primary receptor binding	[41]
Hepatitis D virus	Mammalian cells	PenK, ACPK, DiZPK, ONBK, BCNK	Various sites on envelope proteins	Proof of concept, CuAAC click chemistry to label with biotin	[33]
Lentivirus	Mammalian cells	AzK, DiZPK	Envelope protein VSVG positions Y116, K242, A246	SPAAC click chemistry for targeting and PEGylation; photo-crosslinking	[34]
HIV-1	Mammalian cells	AzF	Envelope protein Gag, nonstructural protease	Biocontainment	[49,50]
		BCNK, SCOK, TCOK	Envelope glycoprotein position 407+1	SPIEDAC click chemistry for super-resolution imaging	[35]
Influenza A	Mammalian cells	AzK	Non-structural proteins polymerase subunits PA, PB1, PB2, nucleoprotein	Biocontainment	[43]
Influenza VLP	Mammalian cells	TCO*K, SCOK	Envelope protein HA	SPIEDAC click chemistry, dual-color labeling, and super-resolution microscopy	[42]
Phage QB VLP	Bacteria	pAmF	Capsid protein position T19	Attach targeting molecules	[16–24]
Tobacco mosaic virus VLP	Bacteria	KPN	Capsid protein position F11	Attach biotin (proof of concept)	[25]
Phage M13	Bacteria	AzK, AcK	Minor capsid pIII	PACE	[59,60]
	Bacteria	pAzF, pAcF, BpyA, pBOF, sY	Minor capsid pIII - peptide or antibody fusion	Phage display	[61–64]