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Cell-free expression and synthesis of viruses and bacteriophages: applications to medicine and nanotechnology

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Abstract

The efficacy of cell-free gene expression (CFE) has been considerably improved in the last decade. As a consequence, CFE systems have now the capacity to express DNAs composed of tens of genes encoding for complex self-assembly processes. A recent example is a demonstration that infectious bacteriophages can be synthesized in one-pot CFE reactions from their genomes. This landmark result opens new perspectives for producing *in vitro* large biological systems from natural or synthetic DNA. In this article, we review the recent progress in the synthesis and self-assembly of viruses in one-pot CFE reactions, primarily covering bacteriophages. We discuss the advantages and potential of producing virus-like particles and phages in CFE systems for biomedical applications. Because they are the most abundant and diverse life forms on Earth, dynamically synthesizing whole or parts of bacteriophages in test tubes could facilitate uncovering novel biological functions and exploiting their self-assembly properties for material sciences applications.

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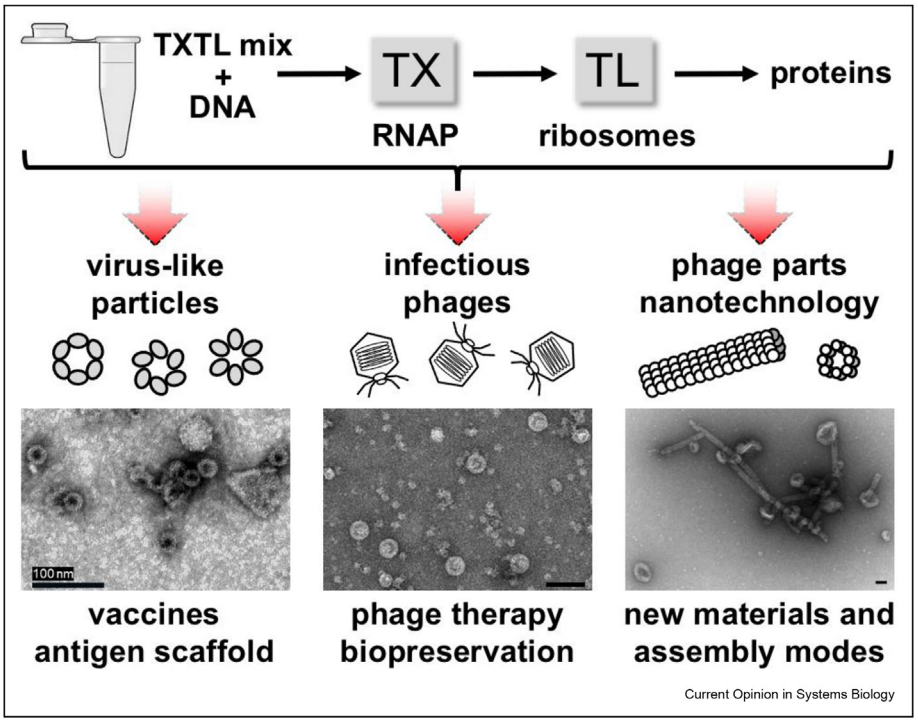
Introduction

Cell-free gene expression (CFE) is forging ahead as a highly versatile and multipurpose technology [1,2]. CFE was originally developed as an alternative approach to living cells, in particular, for the synthesis and labeling of proteins in support of structural biology and proteomics studies [3,4]. In the last decade, CFE has become a convenient environment for rapidly prototyping DNA regulatory elements and executing gene circuits encoding, for example, complex dynamical processes [5–8]. CFE systems are also effective platforms for biomanufacturing biologics and chemicals, such as biofuels [9], vaccines [10], and creating affordable and fast diagnostic tools [11]. As the protein synthesis strength of CFE systems increases, challenging their capabilities will enable an expansion in their scope of applications to new territories. DNA programs larger than simple circuit motifs can now be expressed *in vitro*. Enzymatic pathways composed of several genes, for instance, are being developed in CFE reactions to reconstruct the synthesis pathway of high-value chemicals such as the anticancer molecule violacein [10,12] or essential functions of living cells [13]. The strength of the new CFE systems is also exploited to recapitulate the self-assembly of active macromolecular structures. In particular, using CFE to synthesize parts or whole infectious viruses and bacteriophages is a research area that explores untapped properties of CFE systems with a broad extent of potential applications in biotechnology and medicine. By their abundance and diversity, bacteriophages represent a formidable reservoir of biological functional properties waiting to be discovered and exploited in CFE systems.

In this article, we review the recent efforts to leverage CFE as a means to synthesize entire viruses and phages or their cognate components (Figure 1). The cell-free synthesis (CFS) of virus-like particles (VLPs) to be used as vaccines has become potentially advantageous compared with their production *in vivo* [14] by facilitating the rapid screening of protein variants that still self-assemble into VLPs. The CFS of whole infectious bacteriophages from their genomes has been recently demonstrated [12,15,16] and has not been fully exploited yet. Bacteriophages represent a vast area of living systems with enormous possibilities for medical applications, such as phage therapy to treat multidrug-resistant microbial infections [17,18]. Phages also carry a lot of potential as tailorable bioactive material that could be used beyond medical applications. In this direction, the recent efforts to control the synthesis of phage elements reveal that the design of biological

Figure 1



Overview of cell-free expression and synthesis of components or whole viruses and phages. TXTL: transcription-translation. The major applications are listed in the following electron microscopy (EM) images. The EM images are from the study by Colant et al. [35] (left, VLPs for influenza vaccines candidates) [15], (center, scale bar 100 nm, phage T7), and [38] (right, scale bar 40 nm, self-assembly of gp15 and gp18 proteins, from phage T4, into tubes and doughnuts).

machine assembly modes could be exploited for nanotechnology applications [19].

VLPs

VLPs are non-infectious, genome-free capsids that have received considerable attention for the development of vaccines [20]. They consist of one or more capsid proteins from viruses or bacteriophages that self-assemble into particles of diameters ranging from several nanometers up to 100 nm. The principal usage of VLPs is to elicit an immune response when injected into organisms, such as mice or humans. With a half-life that can be on the order of several months in serum [21], VLPs present some advantages as potential vaccines or drug delivery carriers. VLPs are mostly produced by *in vivo* synthesis. There are limitations to this approach, however, especially the cost of production. The yield of synthesis is typically on the order of 1−5 mg/l [22,23], which is smaller than needed for vaccine biomanufacturing. *In vivo* production of VLPs are hard to scale up [24] and purification is often difficult [25,26]. Prophylactic human papillomavirus vaccines, for instance, composed of VLPs of the L1 capsid protein cost on the order of $360 for the full treatment [27]. CFE has been considered early on as an alternative approach to the production of VLPs [28] (Table 1). As an open environment, CFE enables better control of the reaction conditions, such as the pH and reduction-oxidation state, for the formation of disulfide bonds, a critical aspect of VLPs formation. In CFE reactions, VLPs are purified in a single step, which significantly decreases the cost of production compared to *in vivo* purification. To date, CFE has been primarily used to produce single-protein single-layer non-enveloped VLPs.

The first VLPs synthesized in an *Escherichia coli* CFE system was based on the MS2 phage coat protein and the C-terminally truncated human hepatitis B core antigen protein (HBc) [28]. Both VLPs were produced at yields of 10^{13}−10^{14} VLP/ml (ten-fold greater than *in vivo* methods) from a protein synthesis concentration around 0.5 mg/ml with a solubility greater than 92%. The CFE-produced VLPs were found to have comparable characteristics, such as the size and the stability, to those produced *in vivo*. The scalability of this technology was tested from 30 µl to 1 ml without loss in production yields paving the way towards the mass production of VLPs *in vitro*. In an effort to also decrease the cost and time of production *in vivo*, norovirus VLPs have been recently synthesized in a standard *E. coli* CFE system

Table 1

Components of or whole viruses and bacteriophages synthesized in cell-free expression systems.

Particle - Concentration	DNA program	Main results	Ref.
Virus-like particles (VLPs)			
• HBc, MS2 and Qβ 50 nm, 300 μg/ml	Linear, T7 promoter 2 kbp, 3 genes	First efficient disulfide bond formation in VLPs structures that increases stability of VLPs	[30]
• MS2 and Qβ 10–100 nm, 300 μg/ml	Plasmid, T7 promoter 2 kbp, 2 genes	Surface functionalization of VLPs by direct conjugation using azide–Alkyne click chemistry	[31]
• HBc, flagellin 25 nm, 300 μg/ml	Plasmid, T7 promoter 1.5 kbp, 2 genes	Covalent attachment between flagellin and VLPs using ncAA, 10-fold increase in bioactivity	[34]
• HBc 35 nm, 35 μg/ml	Plasmid, T7 promoter 0.5 kbp, 1 gene	Engineering of HBc VLPs, decrease of intrinsic immunogenicity, greater solubility and assembly	[14]
• HuNoV 10 nm, 600 μg/ml	Plasmid, T7 promoter 3 kbp, 2 genes	Synthesis and assembly of HuNoV VLPs Serious candidate for vaccine development	[29]
• HBc 25 nm, NA	Plasmid, T7 promoter 0.5 kbp, 1 gene	Synthesis of HBc VLPs Improved vaccine development efficiency	[55]
Whole bacteriophages and viruses			
• T7, phage 60 nm, 3.3 10^{11} PFU/ml	Linear dsDNA 40 kbp, 60 genes	First time a whole infectious phage is synthesized in a CFE system, plaque assay, genome replication	[15]
• MS2, phage 27 nm, 4.2 10^{12} PFU/ml	Linear RNA 3569 bp, 4 genes	Complete synthesis of the phage MS2, concentration measured by the plaque assay	[12]
• Phi X174, phage 32 nm, 1.9 10^{12} PFU/ml	Linear dsDNA 5386 bp, 11 genes	Complete synthesis of the phage Phi X174, concentration measured by the plaque assay	[12]
• T4, phage 60 nm, 10^{9} PFU/ml	Linear dsDNA 169 kbp, 289 genes	Complete synthesis of the phage T4, concentration measured by the plaque assay	[16]
• EMCV, virus 30 nm, 10^{9}-10^{10} PFU/ml	Linear RNA 7.8 kbp, 13 genes	Complete synthesis of the non-enveloped virus EMCV, concentration measured by plaque assay	[51]
Nanotechnology – controlled self-assembly			
• T4 gp15 and gp18 20 * 500 nm, 50 nm, NA	Plasmid, T7 promoter 3 kbp, 2 genes	Formation of tubes, rings, and synthetic donuts by cell-free synthesis of two T4 structural proteins	[38]
• T4 gp18 20 * (0–500) nm, NA	Linear, T7 promoter 2 kbp, 1 gene	Gradient of captured protein on a biochip, formation of nanotubes trapped on a biochip	[53]
• T4 gp6, 7, 8, 10, 11 60 nm, NA	Linear, T7 promoter 9 kbp, 5 genes	Formation of the phage T4 wedge on a biochip, controlled self-assembly in space	[19]

Table 1. The **left column** includes information about the type of particles or structures synthesized. The **column in the middle** provides the genetic information (DNA or RNA, linear or plasmid), the total length of the coding sequences composing the DNA program (promoters, operators, UTRs, genes, terminators), the number of genes. The **column on the right** provides a short description of the work.

[29], shortening the production to 4 h instead of more than 50 h *in vivo*. The two norovirus capsid proteins GII.3 and GII.4 were produced at a concentration of 0.5–0.6 mg/ml.

Although VLPs produced in *E. coli* CFE systems have rather good stability in time and complex physiological media, advantages offered by CFE have been exploited to further improve the stability of VLPs and the yields of synthesis. One strategy consisted of adjusting the redox potential of the CFE reaction to control the formation of disulfide bonds both during CFE and post-production [30]. This method resulted in an increase in the VLPs self-assembly yield.

As proof of concept of VLPs cell-free synthesis is being established, research is now also geared towards engineering the particles to expand their bioactivity. The major effort consists of modifying the surface of VLPs to either increase the immune response of future vaccines or add new functions at the surface of VLPs. The surface of MS2 and Qβ VLPs, for instance, can be functionalized by click chemistry via a surface exposed methionine [31]. This approach offers excellent control of the surface abundance of the attached species. Another asset of CFE over cell-based approaches is the possibility to synthesize cytotoxic proteins. This advantage was exploited to co-synthesize the A2 cytotoxic protein from Qβ with the phage coat protein. VLPs containing a single A2 protein were produced [32], which also enabled a greater synthesis of the poorly soluble cytotoxic protein. Flagellin, one of the most potent vaccine adjuvants for enhancing immune response [33], was successfully attached to cell-free synthesized HBc. The chemical binding was achieved by inserting a non-canonical amino acid into the flagellin to carry out a specific biorthogonal coupling reaction. When coupled to the surface of VLPs, the specific TLR5 stimulation activity of flagellin was increased by approximately 10-fold [34]. In the same spirit, CFE was used to synthesize a tandem core Hepatitis B VLPs that enabled displaying two different influenza antigens [35]. The

titer and quality of the VLPs were improved by modifying different part of the genetic constructs, thereby providing another demonstration that CFE can be effective screening platforms of VLPs for universal vaccines candidates. A more advanced work was performed on the model HBc VLP to increase its stability and antigen fusion and reduce its intrinsic immunogenicity [14]. To achieve this, the HBc protein was engineered to incorporate artificial disulfide bridges and a new surface spike region from a naturally occurring viral mutant, whose reduced negative electric surface charge was revealed to be essential to improve the conjugation efficiencies of the molecules coupled to the VLPs. Although the CFE of VLPs is still a serious alternative to producing particles with high biomedical potential, substantial work remains to be carried out to enable biomanufacturing ready-to-use VLP-based vaccines.

Whole infectious phages

Phages are a virtually limitless resource of bioactive materials that are exploited in diverse fields, including biotechnology [36,37], nanotechnology [38], personalized medicine [39], bioremediation [40], biosensing [41], and vaccine development [42]. Over the last century, phages have been instrumental in the discovery of many ground-breaking biotechnologies, including CRISPR-Cas9 [43]. Incredibly, metagenomics has revealed that we have barely scratched the surface of phages' true genetic diversity, and therefore, many biological marvels are likely awaiting to be discovered [44]. As bioactive materials, phages are invaluable owing to (i) highly specific binding to target bacteria, (ii) efficient DNA insertion into bacteria, (iii) potent and diverse enzymes and proteins, (iv) modular genomes that can be engineered, and (v) organized protein structures that can serve as scaffold matrixes. Their natural function is to infect specific host bacteria, including those harmful to the environment, animals, plants, and humans.

The coliphage T7 was the first to be fully synthesized from its linear dsDNA genome in an *E. coli* CFE system [15] (Figure 1, Table 1). Composed of about 60 genes encoded by a genome of 40-kbp, the phage T7 was produced at a concentration of 10^{11} PFU/ml (plaque-forming unit) in test tubes. In a cell viability assay, T7 phages produced via *E. coli* or in a cell-free reaction were comparably capable of infecting *E. coli* cells. As importantly, T7 DNA replication was functional during CFS when dNTPs were supplied as evidenced by the production of more infectious T7 phages than genomes initially added to the cell-free reaction. Subsequently, three other phages were synthesized in *E. coli* CFE systems. The CFS of infectious MS2 (10^{12} PFU/ml) and Phi X174 (10^{12} PFU/ml) particles showed that coliphages encoded by a small mRNA and a small circular dsDNA can also be carried out *in vitro* via CFE [12]. More significant was the synthesis of the coliphage T4 from its 169-kbp linear dsDNA genome [16], at a concentration of 10^{9} PFU/ml. To date, T4 remains the largest natural genome expressed in a CFE reaction that was capable of the synthesis of a functioning biological entity. Whether all the 289 genes encoded in the T4 genome were expressed was not determined.

Several conclusions can be drawn from these results, all obtained in batch mode cell-free reactions. Specifically, the capability of CFE systems to process a genome of 169-kbp, encoding for a total of 289 genes of which 62 are structural genes, was unexpected on diverse aspects. It revealed that the amount of biochemical energy contained in a batch mode CFE reaction is sufficient to achieve the synthesis of rather large protein sets, which was unanticipated even considering that phage genome regulation is highly efficient. The importance of molecular crowding was clearly revealed for each of the four coliphages MS2, Phi X174, T7 and T4. To reach such phage synthesis levels, the concentration of PEG8000, used to emulate molecular crowding in CFE reactions, was increased from 1.5 to 2% (2−2.5 mM) to 3.5−4% (4.5−5 mM) for the four synthesized phages [12,16,45]. Increases of PFUs by factors of up to 10^{3}-10^{4} were observed upon such a small change in the concentration of PEG8000. The synthesis of viable infectious phages in the absence of a phospholipid membrane and a closed compartment shows that these elements are not required. For some of these phages, such as T4, the self-assembly process of new phage particles inside the host relies on the lipid membrane *in vivo* [46]. The addition of phospholipid membranes to the cell-free reaction did not improve the titer of T4, suggesting that phages have alternative assembly modes.

Although experimentally proven and acknowledged as a potentially pivotal technology [47], the CFS of infectious phages has not been subsequently exploited primarily because of a lack of basic research. In particular, the development of cloning methods specific to CFE systems to facilitate the modification of phage genomes would create a completely *in vitro* approach to phage engineering and production. Such tools would eliminate the requirement of living cells as chassis for phage engineering, and thus accelerate the design−build−test cycle, and enable, for instance, cytotoxic constructs. The potential advantages of synthesizing infectious phages by CFE compared to current methods are several, (1) it avoids the use of dangerous pathogens, (2) it could expand the range of phages that can be produced, (3) it could accelerate phage characterization and engineering and (4) it could reduce the cost of phage production for applications as various as nanotechnologies, food processing aids to control foodborne diseases and phage therapy to combat the scourge of antibiotic-resistant infections [48]. Producing phages without living cells could also be advantageous to limit

the emergence of host resistance that is typically observed during large-scale amplification and manufacturing of phages, which requires a specific reactor setup that can only be run for a few days [49]. Finally, owing to their ubiquitous nature and specificity for bacteria, several phages have already been recognized by the Food and Drug Administration as generally regarded as safe for use in the food industry and present relatively low levels of safety concerns [50].

The synthesis of eukaryotic viruses has also been demonstrated albeit more challenging because of safety and security issues. To date, the encephalomyocarditis RNA virus, capable of infecting humans, is the only virus that was fully synthesized in a lysate [51] (Table 1). For this, the virus genome was encoded into dsDNA, circular or linear, under a T7 promoter. In a lysate prepared from Hela cells and supplemented with the T7 RNA polymerase, infectious encephalomyocarditis RNA viruses were produced after a few hours of incubation at concentrations ranging from 10^8 to 10^{10} PFU/ml.

Phage parts and nanotechnology

By means of their small genome size and modularity, phage parts can also be isolated, characterized, and repurposed for specific applications. For instance, the tail fibers alone of some phages have antimicrobial properties and have been co-opted by certain bacterial species as a class of bacteriocins called 'tailocins' [52]. Taking apart a phage and dynamically synthesizing some of its components in a CFE reaction can promote novel interactions and the self-assembly of non-natural structural motifs. This concept was demonstrated using only two structural genes from the phage T4, gp15 and gp18 [38] (Figure 1, Table 1). When each gene was expressed separately in test tubes, the expected structures, rigid tubes of 20 nm diameter for gp18 and hexameric rings for gp15, were observed. When both proteins were expressed in the same CFE reaction, a new structure in the form of nano-doughnut with an outer diameter of 50 nm and thickness of 20 nm was observed. This work showed that out of their natural biological context, viral building blocks can exhibit novel interactions and assemblies that otherwise would not exist. CFE enables making any combination of parts and tuning the rate of synthesis, thus creating a broad range at which the onset for self-assembly can occur, thereby demultiplexing the combinatorial aspect of the approach. In such conditions, the discovery and creation of new bionanostructures are not limited by the experiment but by the ability to rapidly analyze and characterize interesting reaction products. The creation of a biochip specifically engineered to localize and visualize such cell-free generated nano-structural motifs could help in rapidly prototyping a wealth of new materials [53].

In an effort to add spatial control to this approach, a two-dimensional chip that enables patterning of the genes encoding the viral parts was used to demonstrate the programming of protein assembly lines by local synthesis [19]. The autonomous synthesis and assembly of the phage T4 wedge, encoded by five genes, was achieved. With such a platform capable of emulating assembly lines, potentially hundreds of separate nanostructures, natural or synthetic, can be reconstructed on a single chip. This experimental setup could also be used to decipher the assembly modes and order of unknown protein sets.

Eukaryotic virus parts have also been synthesized in *E. coli* CFE systems, but in general, they require prior protein engineering and additional steps after cell-free synthesis to assemble protein complexes having medical potential. The cell-free synthesis of an engineered trimeric influenza hemagglutinin stem domain, for instance, has to be followed by a refolding procedure [54].

Perspectives and concluding remarks

Although ground-breaking works have been recently achieved using CFE systems to synthesize parts or whole phages, it remains to be broadly exploited. Phages represent an almost unlimited source of genetic diversity. The direct integration of molecular tools into CFE reactions to engineer phage genomes would create a completely new approach to produce synthetic phages with properties specifically designed to address emerging societal issues, such as antibiotic-resistant microbes. Coupled with functional genomics, the CFS of phages could elucidate phage gene functions, gene essentiality and permissive loci necessary to guide the design of new phages. Considering that only a few phages have been synthesized in one type of CFE system (*E. coli*), the possibilities for producing CFE of phages *in vitro* have been barely explored. In particular, determining phage and virus synthesis across CFE systems prepared from different eukaryotic cells or bacteria, including Gram-positive and Gram-negative ones, would provide substantial new information on the possibilities to bio-manufacture new viruses with broad host ranges. The synthesis of HBc VLPs from a *Pichia pastoris* CFE system is the first step in this direction [55]. Besides research, CFE is well-suited for hands-on practices in molecular biology and bioengineering at many different levels [56,57]. The CFS of phages could be integrated into teaching modules as it is a rather safe practice that offers unique settings for training and education, providing fast, affordable, flexible and user-friendly opportunities for student research.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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