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The good of two worlds: increasing complexity in cell-free systems

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In vitro biocatalytic systems have moved far beyond established uses in food, diagnostic, and chemical applications. As new strategies to construct and manage multiple enzymes in ever more complex systems are developed, novel applications emerge. In the field of chemistry, complex protein networks are applied to enable the production of fine chemicals, such as dihydroxyacetone phosphate, and even bulk chemicals, such as biofuels, from cheap sugars. Cell-free protein synthesis is applied to expanding protein and nucleic acid biochemistry and enabling novel assay formats, while programmable DNA-circuits can be exploited to engineer sensitive detection methods. Novel developments in chemical analytics such as real-time mass spectrometry to follow the metabolism online, directed physical assembly of network members facilitating substrate channeling, and encapsulation forming biofunctional subunits enable a better control and potential for optimization.

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Introduction

Framed by the astonishing advances in the engineering of living cells [1] and of enzymes [2] for the manufacturing of fine and bulk chemicals and fueled by the emerging system-wide scope of analytic [3] and synthetic technologies [4], cell-free methods shift into a new focus. On the one hand cell-free systems are attractive due to their reduced complexity when compared to living cells, as for example the need to maintain a cell's viability or to transport substrates across cellular or other compartmental boundaries is omitted. On the other hand it was exactly this limitation in complexity which used to focus applications typically to single-step or two-step enzyme chemistry. However, together with general advances in a multitude of relevant biotechnological methods, the above mentioned overarching trends change this situation and enable novel ways how to build, apply, and manage more complex cell-free systems, capitalizing on the degrees of freedom afforded by the absence of cells without any longer accepting the most severe restrictions on complexity. This enables the exploration of novel fundamental concepts, such as the generation of novel informational polymers, as well as of novel avenues in established fields of applications, such as chemistry. In this contribution, we will illustrate this development with a number of examples from the recent literature.

Building complex cell-free systems

One of the major challenges and limitations for cell-free systems is assembling the required multiple components, typically enzymes and cofactor regeneration and energy conversion systems [5]. Even though various examples exist where an impressive number of system components was assembled by separate purifications from bacterial strains, as illustrated by the assembly of a complete *de novo* purine biosynthesis pathway or of the complete protein translation machinery [6,7], more direct methods are desirable. One such method is the repetitive engineering of changes in the chromosomal DNA sequence by allelic replacement with single stranded DNA oligonucleotides to introduce the same affinity tag into a large set of genes. The automation of this serial mutagenic process as 'MAGE' ('Multiplex Automated Genome Engineering') allows in principle a nearly intervention-free modification of multiple genes [8] and was used to engineer His6-tags into a set of 38 genes representing the entire translation machinery. The 38 genes were engineered in only nine strains, so that the system is now better suited for simple affinity purification from a reduced number of strains [9••]. In the future, such extensive re-engineering tasks should also be possible by straightforward *de novo* DNA synthesis, as the technological hurdles for inexpensive template-free large-scale DNA synthesizing are being overcome [4]. An alternative to expensive affinity chromatography is the application of a cell-free extract (CFX), which offers direct and inexpensive access to potentially very large reaction networks but retains the problem of potential interference of background functions like energy-draining or substrate-consuming side reactions. The complexity of the CFX has traditionally limited its use to analytical scale-experiments, for example in the cell-free protein synthesis (CFPS) of proteins with noncanonical amino acids [10]. This notion is however no longer valid: After a period of continuously improving cofactor regeneration procedures in CFXs, including elimination of disturbing background activities

and improvement of protein folding [11], up to 70 g of a cytokine with disulfide bonds in a 100 L reactor (700 mg/L) were produced in 10 hours with an optimized *Escherichia coli* CFX [12•]. Even though this number is still small when compared to optimized *in vivo* expression systems, it needs to be seen against the fact that such a system might enable a standardized expression even of genes that are usually toxic or impossible to functionally express in a microbial expression host such as *E. coli*. Clearly, this experiment illustrates that CFXs can be successfully applied beyond the analytical scale. Such advances raise anew the question how the indisputable limitations of CFX-based production systems can be addressed to advance these systems even further. Such efforts might be facilitated in the future by the availability of streamlined bacterial strains that already have lost a part of their metabolic capacity [13] and detailed knowledge on the essentiality of remaining genes [14,15]. Furthermore, identification of interfering activities should be facilitated by the availability of genome-wide computational tools such as genome-scale metabolic models. As these were built for living cells, such models need to be adapted in order to reflect a reaction system without membranes, but can then serve to generate easily testable hypotheses for the sources of undesired sinks for reaction intermediates [16]. Finally, also simple measures such as preconditioning the background in the CFX by the growth conditions of the cells that give rise to the CFX can be very effective. For example, by growing *E. coli* cells in the presence of glucose, carbon catabolite repression prevents the transcription of the genes for the consumption of lactose and galactose, which allowed the efficient production of globotriose from these two sugars in a metabolic coupling experiment with high yields on both substrates [17].

Applying complex cell-free systems

Cell-free systems have a long history in the realization of preparative complex chemical multistep reactions with substrates or products that are difficult to traffic across the cytoplasmic membrane [17,18]. The typical products of such reactions are intended for pharmaceutical or fine chemical use, thus rather low volume but not low price [19]. Efforts in this direction continue, for example in implementing multistep reaction routes to the stereocontrolled synthesis of vicinal diols via the aldolase donor substrate dihydroxyacetone phosphate. As this donor is expensive and instable, it can be produced *in situ*, either starting from glycerol-3-phosphate by the use of glycerolphosphate oxidase and catalase to remove the side-product H_2O_2 [20], by phosphorylation of dihydroxyacetone with polyphosphate as a cheap phosphate donor [21], or from glucose using the enzymes of glycolysis for dihydroxyacetone formation and cofactor regeneration [22]. Another example is the successful production of *N*-acetylneuraminic acid in an 11-step reaction from glucose and *N*-acetylglucosamine, to impressive overall concentrations of over 50 g/L [19]. The approach can also be extended to compounds outside the field of carbohydrates, for example for the production of triketide lactones [23]. Even though biocatalytic precursor and product generating reactions were carried out one after the other but under compatible conditions, the work is still an interesting application of multienzyme catalysis for the generation of pharmaceutically relevant chiral synthons. Remarkably, the cascade reaction approach is currently also explored for completely different applications, for example, the large scale production of bulk chemicals such as hydrogen in highly complex cell-free systems [24]. In a series of elegant proof-of-principle experiments, it could be shown that NADPH (or, as an intermediate storage, xylitol) can be generated as a precursor to biohydrogen from cellobiose in reaction cascades with up to 13 enzymes and including cofactor regeneration cycles [25••] (Figure 1). Economic estimations show that such systems might indeed be economical, provided some development goals in enzyme and cofactor stability are met [26]. As already mentioned above, a second major domain for the application of cell-free systems is CFPS, in particular for proteins that are otherwise difficult to produce, such as membrane proteins or hydrogenases [27] or proteins containing noncanonical amino acids [28,29]. One interesting development is the use of CFX of bacterial origin in order to overcome the slow translation rates of mammalian systems. These bacterial CFXs can be adapted to allow, for example, disulfide-bond formation [10] and might become interesting for the rapid production of personalized therapeutics and vaccines [30]. Next to these preparative applications, CFPS can be exploited to overcome bottlenecks in advanced screening efforts. For example, screening for improved elements in complex signaling cascades can be difficult as the implementation of large expression libraries in mammalian cells and their application in reliable and meaningful assays is nontrivial.

Many of the associated problems can elegantly be overcome with CFPS, as demonstrated recently for the engineering of epidermal growth factor (EGF) variants with enhanced mitogenic activities [31•]. First, a library of monoclonal EGF variants was produced by PCR, dilution to single molecule level in microtiter plate wells, reamplification by single molecule PCR, and gene expression in a bacterial CFPS-system engineered for disulfide bridge formation. Importantly for the use in signaling cascade assays, the *in vitro* production of the EGF variants allowed determination of the amount of produced protein (in this case by including a 14C-labeled mixture of amino acids during CFPS) and subsequent equalizing of the EGF variant amount, thus excluding influences of varying mitogen concentrations on the assay result. *In vitro* screening platforms would lend themselves particularly easily to nonstandard detection processes, as they are, for example, currently developed in the field of *in vitro* DNA-circuits, where catalyzed hairpin assembly [32] is used to