Directed Evolution in Drops: Molecular Aspects and Applications

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Cite This: ACS Synth. Biol. 2021, 10, 2772−2783

ABSTRACT: The process of optimizing the properties of biological molecules is paramount for many industrial and medical applications. Directed evolution is a powerful technique for modifying and improving biomolecules such as proteins or nucleic acids (DNA or RNA). Mimicking the mechanism of natural evolution, one can enhance a desired property by applying a suitable selection pressure and sorting improved variants. Droplet-based microfluidic systems offer a high-throughput solution to this approach by helping to overcome the limiting screening steps and allowing the analysis of variants within increasingly complex libraries. Here, we review cases where successful evolution of biomolecules was achieved using droplet-based microfluidics, focusing on the molecular processes involved and the incorporation of microfluidics to the workflow. We highlight the advantages and limitations of these microfluidic systems compared to low-throughput methods and show how the integration of these systems into directed evolution workflows can open new avenues to discover or improve biomolecules according to user-defined conditions.

KEYWORDS: droplet microfluidics, directed evolution, enzyme, antibody, aptamer, molecular engineering

FROM NATURAL TO DIRECTED EVOLUTION

Biomolecular evolution can be described as a path from one functional biomolecule to another in the space of all possible biomolecular variants, where each variant has an assigned fitness (Figure 1). In nature, the fitness is the ability of an organism to reproduce in a particular environment and consequently spread its genes. In the laboratory, the selective pressure and therefore the fitness are set by the experimenter. Directed evolution is a growing field in synthetic biology and has the capacity to provide new proteins or nucleic acids on the basis of predefined industrial or biomedical needs. It relies on the Darwinian principle of mutation and selection, where the probability of success is determined by the ability to find rare optimal variants within a large pool of sequences. All directed evolution experiments require a measurable activity that acts as a fitness indicator to drive the selection process using of multiple strategies for assay design, protein expression, and selection.

Received: July 6, 2021
Published: October 22, 2021
evolution experiments, codons are often not fully randomized and more restricted codon sets are used, such as NNK (N = A/C, G/T, K = G/T), which covers all possible amino acids and one stop codon. In addition, one can also use codons to encode a minimal set of amino acids, representing the main chemical types. The method of choice for generating mutants will depend on prior knowledge of the key residues of a biomolecule, the size of the gene, and the screening capacities. As an example, in a NNK library, all 20 amino acids will be represented at each mutated codon, and the theoretical number of full-length protein variants is $20^n$, where $n$ indicates the number of targeted sites. Therefore, if this is the chosen strategy, one should carefully choose the residues to mutate, as it will be virtually impossible to test all of the possible amino acid variants at each position for most proteins. If the key residues responsible for the function of a biomolecule are not known or the researcher simply wishes to explore different evolutionary paths, it may therefore be more effective to use a randomized library, bearing in mind that, the bigger the biomolecule, the lower the likelihood that a key residue will be targeted.

### VARIANT PRODUCTION

Protein variants may be expressed either inside a host or within a cell-free system. The use of a host for protein expression is well established in research and in the pharmaceutical industry and has historically been the most used method to amplify and produce candidates for directed evolution. The two most common prokaryotic and eukaryotic hosts for protein production are the model bacterium *E. coli* and the yeast *S. cerevisiae*, respectively. In both cases, a plasmid carrying the gene of interest is transformed into the host cells for expression by the translation machinery. Cells are then propagated and induced to express the protein in a variety of ways, most commonly inside the cytoplasm (Figure 2) but also inside the membrane or secreted into the periplasm or into the surrounding medium. The advantages of using a cellular expression system lie in its simplicity and in the reliance on the cell environment and translational machinery, which enable the correct folding and post-transcriptional modification of the target proteins. Moreover, cell expression provides a tighter genotype–phenotype linkage, which is useful in the context of directed evolution.

An alternative to protein expression inside a host is a cell-free system, in which an improved variant can emerge. In directed evolution, genetic diversity is obtained by introducing mutations within the gene of interest to yield genetic libraries with up to tens of thousands of variants. Methods to generate genetic diversity are broadly divided into two groups: random and semirational. Random mutagenesis methods are based on the use of an error-prone polymerase chain reaction (epPCR), a process whereby PCR reaction conditions are altered to facilitate the misincorporation of nucleotides, resulting in randomly incorporated variations along the sequence. This approach is widely used when the key residues responsible for a given function are not known. In contrast, semirational design relies on *a priori* biochemical or structural knowledge of the system to create constraints in the design of the mutant library, such that residues that influence a biomolecule’s function are preferentially targeted and consequently the resulting library is more likely to contain variants with enhanced properties. The most widely used technique for semirational library design is saturation mutagenesis, where the targeted residues are randomized to introduce all possible amino acids or nucleotides in the final product. For protein
template into an RNA transcript that is subsequently translated into protein without the environment of a cell.\textsuperscript{16} (Figure 2). Cell-free systems allow the user to have direct control over the transcription and translation machinery of the cell without the constraints of the cell envelope. In this way, it is possible to co-translationally produce and solubilize membrane proteins,\textsuperscript{17,18} translate toxic or difficult-to-express proteins,\textsuperscript{19} introduce site-specific labels, or incorporate nonstandard amino acids\textsuperscript{20–23} or other monomers\textsuperscript{24} into the polypeptide chain. However, the use of these systems results in lower yields of protein compared to host organisms. The first effective cell-free transcription–translation system dates back to 1961,\textsuperscript{25} when Nirenberg and Matthaei successfully synthesized proteins with the translational machinery of \textit{E. coli} in order to decipher the genetic code, but it was not until the 2000s when the applications of this technology began to be exploited with notable advances in the field of protein synthesis,\textsuperscript{26} production of pharmaceutical compounds,\textsuperscript{27,28} or screening of protein and peptide libraries.\textsuperscript{29}

Finally, DNA variant libraries for directed evolution are typically synthesized by solid-phase synthesis and/or amplified by PCR, while RNA libraries are generally produced enzymatically from DNA templates using T7 RNA polymerase.

Once the choices for generating diversity and expressing variants are made, the microfluidics workflow must be adapted accordingly (Figure 3). Microfluidics technologies are implemented into each directed evolution scenario mainly depends on the expression system used. Common modules included in a droplet-based microfluidic system are droplet makers\textsuperscript{30} and sorters, on the basis of either dielectrophoresis\textsuperscript{31} or acoustic waves.\textsuperscript{32} In addition to these key modules, various modules allow droplet manipulation, such as droplet fusion,\textsuperscript{33} splitting,\textsuperscript{34} and picojection,\textsuperscript{35} making microfluidics a versatile and adaptable tool. Commonly, droplets are collected and incubated off-chip and later reinjected for an end-point measurement. Alternatively, incubation can take place on-chip in incubation channels when shorter incubation times are needed.\textsuperscript{36} The latter allows precise control on the reaction’s incubation time, as it is possible to precisely control flow parameters, making it possible to measure the reaction at an end point or at a controlled time point, such as in kinetics measurements. To illustrate this, let us consider a typical microfluidic workflow for the directed evolution of enzymes with enhanced properties. In cases where the enzyme of interest is to be produced in an \textit{E. coli} host, individual cells expressing a single enzyme variant are coencapsulated with a substrate for the chosen assay. If the protein is set to remain within the cytoplasm, protein induction will be performed off-chip, and the cells will later be encapsulated together with a lysis agent to provide enzyme accessibility to the assay. In the case of protein secretion, the order would be to first encapsulate the cells and later induce them by picojection once they are inside the drops to maintain phenotype–genotype linking. Alternatively, cells can also be encapsulated in inductive medium. Finally, if the protein is displayed at the cell surface or targeted to the periplasm, fewer steps are required, as there is no need to lyse the cells, and the induction can be carried out prior to the encapsulation. If, on the other hand, the enzyme is to be produced using an IVTT system, a DNA library encoding a large number of variants will be diluted and encapsulated together with a PCR mixture, such that each drop contains no more than a single DNA molecule. After performing in-drop PCR amplification, each droplet can be fused with another drop containing IVTT reagents and incubated, making these kind of processes more complex than cell-based experiments. After the enzyme of interest has been expressed, substrate is added to perform the enzymatic assay. Finally, droplets containing cells or the IVTT mixture are (re)injected into a sorting module to select the desired variants.

**VARIANT SELECTION**

The assessment of the performance of individual variants is achieved through an assay that produces a readout signal proportional to the fitness of the variant. The choice of the assay is crucial in determining the success of the experiment and in most cases the restraining factor on the possibility to
perform directed evolution. First of all, a minimal starting activity is required to proceed with a directed evolution workflow. Second, the readout must be fast and sensitive enough for the high-throughput screening of the droplets, which are flown at high velocity through microfluidic channels. Third, the diffusion of the assay components between droplets and into the oil should be limited.57,58

Most assays performed in droplets rely on a laser-induced fluorescence readout, such as fluorescence-activated droplet sorting (FADS)31,32 or adapted versions of commercial fluorescence-activated cell sorting (FACS).39,40 This allows for highly sensitive measurements to be performed in the sub-millisecond time scale down to 1 nM of product,41 thus enabling the sorting and selection of variants in the kilohertz range. However, the implementation of a fluorogenic assay is not always trivial and has so far been limited to a narrow range of highly specific reactions that result in the activation of a fluorophore directly, by using a fluorogenic substrate41−43 through a coupled assay,44,45 or by the release of a quencher.40,46 In the first case, typically, the natural substrate of the enzyme must be chemically modified with a fluorophore, which could potentially lead to the identification of an enzyme with improved activity toward the modified fluorogenic substrate rather than the native substrate. To minimize this risk, a coupled assay with a fluorophore may be used to keep the substrate unmodified. This may require an additional enzymatic cascade reaction to be included in the assay, though it must be ensured that the side reactions do not interfere with the assayed enzyme. Certainly, these additional reactions should not be rate-limiting in order to make sure that the selection pressure is applied to the enzyme of interest. In the final case, the assay relies on the activation of a fluorophore by displacing or removing a quencher from the donor−quencher pair and in this way increasing fluorescence intensity. Finding a good donor−quencher pair for a substrate is not straightforward. The substrate needs to be chemically modified with the donor−quencher pair, which can alter enzymatic activity toward the native substrate, similar to the use of a fluorogenic substrate. Finally, with this method, the background signal tends to be higher, which makes the assay less sensitive.47,48

Recent developments in detection systems compatible with microfluidic chips have been made in order to extend the range of assays amenable to droplet microfluidics. Absorption-based methods are more universal but remain challenging because of the reduced optical path length of the microfluidic channels, which impacts the sensitivity and reliability of the measurements. However, successful absorption-based directed evolution has been achieved at a reduced throughput (300 Hz)60 with a detection limit of 10 μM of product through a coupled assay. Another method was recently developed for high-speed absorbance measurements. The method relies on the phase shift of light due to the photothermal effect and allows single-point absorption measurements at rates similar to those used in FADS.50

Recently, the first directed evolution screen based on electrochemical measurements41 and other label-free screening methods have been demonstrated in droplets. Moreover, light scattering52 and image processing53 could successfully be applied to the screening of populations based on cell growth with promising capabilities when coupled to artificial intelligence.54−56 Finally, efforts were made recently to integrate Raman spectroscopy57 and mass spectroscopy58,59 into the droplet format.

After detection, droplets are deflected toward the desired outlet mostly using electric fields. The design of the electrodes plays a key role in the optimization of the electric field gradient and in maximizing droplet displacement.50 A second key factor is the size of the droplets to be sorted. The highest sorting throughput reported was 30 kHZ,61 which is achieved for rather small droplets (8 pl). However, this throughput has yet to be reached for biological experiments. The limiting factor at this point was the data acquisition of the electronic system. Nevertheless, when investigating, for example, cell proliferation, larger drops (100 pl to 1 nL) are required to provide a sufficient amount of nutrients for the longer incubation time.62 Sorting of such large drops has been performed at decreased throughputs since these require a larger deflective force to be displaced, tend to break easily due to the electric field, or split at the sorting junction when the flow velocity is too high. Recently, a novel method that utilizes an array of electrodes that can be triggered sequentially has been introduced. This method allows one to sort larger drops at increased throughputs55 (850 Hz for 1 nL droplets). The relationship between droplet size and throughput is shown in Table 1 using selected studies and their novelties.

| Table 1. Achieved Sorting Throughputs for Various Droplet Sizes44 |
|---|---|---|---|---|
| drop size (pl) | sorting throughput (kHz) | novelty | ref |
| 12 | 2 | fluorescence-activated droplet sorting | 31 |
| 110 | 0.2 | concentric sorting electrode | 63 |
| 8 | 30 | layered sorting junction | 61 |
| 100−1000 | 0.85−4.4 | sequentially addressable electrode array | 55 |

*Different technical advances have been implemented in order to enhance the final throughput.*

Several standard analytical techniques have been compartmentalized, such as PCR,64 ELISA,65 MDA,66 and cell transfection,67 giving rise to “digital” techniques and resulting in improvements in accuracy, sensitivity, and throughput. Further, droplet microfluidics can be integrated with benchtop flow cytometers for droplet sorting. However, since FACS is not compatible with the oil phase of droplet microfluidics, additional steps are necessary to do so. For example, it is possible to create a double water−oil−water emulsion68,69 or to create hydrogel beads to compartmentalize the aqueous phase56,70 to allow droplet sorting. As described above, directed evolution in droplets is a multistep process and often requires a complex workflow with numerous handling steps where temperature and pressure control are vital. Consequently, efforts to automate these processes have been made, such as developing a platform, based solely on integrating temperature control, to automate transformation, culture, and expression of recombinant proteins inside a host microorganism.71

**Newly, the possibility of full automation and integration of the microfluidic workflow was demonstrated using a system composed of 3 components: (i) a robotic liquid handler; (ii) syringe pumps with valves, which can withdraw and pump fluid; (iii) microfluidic unit operations, such as droplet generation, merging, and sorting.**72 Such a system can perform all of the steps required for directed evolution and shows a high level of flexibility.
ENZYME ENGINEERING BY DIRECTED EVOLUTION IN DROPS

Natural enzymes have been optimized over billions of years of evolution to effectively perform an enormous variety of catalytic reactions in a selective manner. For industrial or therapeutic needs, this process needs to be accelerated and must be adapted to reactions that do not normally occur in nature. Enzyme engineering and directed evolution allows one to improve the natural activity of an existing enzyme, change its preferred substrate toward an activity of interest, or even design enzymes by computational methods.73

Native Substrates. The evolution of enzymes for their activity on their natural substrate is a challenging game. Since the enzyme has already been improved through the multiple cycles of natural evolution, it is reasonable to expect, at best, a low magnitude improvement from the starting molecule in the final hit. However, the high number of variants that can be sorted using microfluidics increases the chances of finding an enhanced variant that will entail an enzyme with improved catalytic activity toward their original substrate. Usually, the generation of genetic variation includes a step of random mutagenesis to cover different evolutionary pathways, as the positions that need to be changed in the protein are not always known to the protein engineer.

One example of an enzyme that has been evolved to improve its activity toward their original natural substrates is the enzyme phenylalanine dehydrogenase. In this case, the enzyme, which catalyzes the NADH-dependent deamination of amino acids, was improved to yield 2.7-fold higher activity than the wild-type.39 To do so, genetic diversity was first introduced by epPCR followed by DNA shuffling of the resulting amplicon. The originality of this work, however, lies in the use of an absorbance detection module for the subsequent selection of protein variants in contrast with the FADS approach, which is often used in microfluidics. The protein expression system of choice was the prevalent cytoplasmic expression, where the cell is encapsulated with a lysis agent and the drop is used as a compartment to link the genotype of a variant to its phenotype, usually expressed as the level of fluorescence.

One interesting alternative to cytoplasmic cell-expression involves the generation of hydrogel beads. These beads are surrounded by a polyelectrolyte shell to link phenotype with genotype, where the compartmentalization is robustly preserved. They can therefore function as a compartment containing lysate from a single cell, allowing the use of a benchtop flow cytometer for droplet sorting. The potential of this system was demonstrated using phosphotriesterase (PTE), a bioremediation catalyst, where a single round of mutagenesis mentioned in previous examples. Interestingly, from the top five mutants, three had previously been discovered by the same group using FACS, while two were isolated for the first time using microfluidics, including a variant with kcat increased by 2.1-fold, demonstrating the robustness, sensitivity, and efficiency of the microfluidics strategy.

Successful microfluidics droplet sorting using surface display in E. coli has also been achieved.75 In this case, a homodimeric aryloxygenase was evolved for improved sulfatase activity toward two different substrates, since the enzyme shows considerable activity toward phosphoesters, compounds that are derived from the primary activity of catalyzing the hydrolysis of aryloxides. Two libraries generated by epPCR were screened, initially against the first substrate, fluorescein disulfate, and then also against the second substrate, 4-nitrophenyl sulfite. The experiments resulted in the identification of 25 unique SpAS1 variants with up to 30-fold and 6.2-fold improved activity, respectively, after a single round of mutagenesis.

Non-native Substrates. Since minimum levels of activity must be detected to start the cycle of mutagenesis, expression,
and selection, a common strategy to identify an enzyme variant with activity toward a non-native substrate by directed evolution is to improve an existing promiscuous enzymatic activity. Nearly a decade ago, droplet-based microfluidics was used to successfully screen a promiscuous sulfatase with hydrolytic activities toward the nonnative substrate phosphate. Genetic diversity was generated by epPCR, and the library was expressed in the cytoplasm of E. coli with the corresponding cell lysis. The top 4% of the most active clones, displaying at least 4-fold improved activity, was selected in each of a total of 3 rounds of sorting. The final candidate presented a 6-fold increase in $k_{cat}/K_m$ for the desired function after purification of the enzyme. This work demonstrated that rare variants with small improvements in activity could be detected and selected by microfluidic droplet screening.

When targeting non-native substrates, another common strategy is to apply serendipital library design to directly target residues in the substrate binding site. This approach was used to completely remodel the active site of cyclohexylamine oxidase, an enzyme used in the industrial production of chemicals and active pharmaceutical ingredients. Genetic diversity was generated by targeting 8 residues close to the bound cyclohexanone and randomizing them with either DYT codons (encoding for A, S, T, V, I, and F) or BYT codons (encoding for A, S, P, V, L, and F), followed by selection of the top 0.1% of the most active variants for each of 3 consecutive rounds of directed evolution. Notably, the three most active variants obtained after the third round of sorting had identical amino acid sequences despite having been obtained independently. This variant had five amino acid changes compared to the wild-type and after purification presented an impressive 960-fold improvement in catalytic efficiency for the same substrate.

Non-native substrates also include synthetic substrates, such as xeno nucleic acid (XNA) polymers. As these synthetic polymers increasingly show potential for synthetic biology and future applications in molecular medicine, nanotechnology, and materials science, the development of efficient synthetic polymerases by directed evolution is gradually gaining importance. One example is the evolution of a polymerase that replicates an unnatural genetic polymer composed of repeating units of $\alpha$-L-threofuranosyl nucleic acid (TNA) sugars. This approach was used to develop a manganese-independent TNA polymerase that functions with 99% template-copying fidelity after making the hypothesis that the presence of manganese was making the polymerization unspecific. To achieve this, three key residues known to affect substrate specificity were altered by saturation mutagenesis. The resulting enzyme variants were encapsulated in double emulsion droplets and sorted by FACS based on their ability to elongate a full-length product, which produced a fluorescent signal by donor–quencher pair disruption. Presumably, it should be possible to evolve other polymerase functions provided that the optical detection of the product can be achieved.

Computer-Designed Enzymes. Computational design can give rise to de novo biocatalysts with a function not found in nature. However, newly designed enzymes typically show very low catalytic activity and must subsequently be improved through directed evolution. A striking example of the successful optimization of a computer-designed enzyme is that of retro-aldolase, an enzyme capable of cleaving a specific carbon–carbon bond in a non-native substrate using amine catalysis. A retro-aldolase slightly modified from the original computer design was used as a starting point and reoptimized using a microfluidics-based system able to detect enzyme activities as low as $k_{cat}/K_m = 0.5 \text{ M}^{-1} \text{ s}^{-1}$. For this optimization, six libraries were generated by saturation mutagenesis using NNK codons. The targeted residues were close to the binding pocket and varied from four to five simultaneously randomized residues. After a first round of selection, two variants with >10-fold improved activity were chosen for DNA shuffling, obtaining a final variant with a 73-fold increase in $k_{cat}/K_m$ compared to the initial enzyme. The same group had previously evolved by microtiter plate the starting point retro-aldolase. Notably, the best variant after 13 rounds of directed evolution in microplates was not as active as the one obtained from only two rounds of FADS, highlighting the importance of screening a higher diversity of variants with a high-throughput method. A year later, the microtiter plate-evolved enzyme was reoptimized using FADS, leading to the identification of a new complex catalytic center, which featured a Lys-Tyr-Asn-Tyr tetrad and was 30-fold more active. When variants with low activity are detected in a high-throughput manner, FADS is a powerful tool for tuning the properties of computationally designed enzymes.

Optimization of Ribozymes. Although there are tens of examples of RNA molecules with catalytic properties (RNAzymes or ribozymes) in nature, scarce attempts have been made to improve these biomolecules using droplet microfluidics. In one of the few studies to date, the catalytic properties of an X-motif capable of RNA cleavage via an internal phosphoester transfer reaction were improved using a complex microfluidic workflow. First, a DNA library and PCR reagents were injected into droplets, and PCR was performed off-chip. Then, the droplets were mixed with a T7 polymerase-based in vitro transcription mixture. The authors discovered that T7 RNA polymerase interfered with the fluorogenic nuclease assay. However, picoinjection of the assay mixture with a high NaCl concentration allowed them to inactivate the T7 RNA polymerase and stop the transcription reaction of the ribozyme. Finally, droplets were selected with a FADS device on the basis of a fluorogenic RNA substrate comprising a fluorophore and a quencher at the 5’ and 3’ ends, respectively. After 9 rounds of selection, the catalytic properties of the ribozyme were enhanced 28-fold. Several mutations could be shown to improve the activity of the ribozyme.

### NONCATALYTIC BIOMOLECULES

**Antibody Optimization with Droplet Microfluidics.** Antibodies like immunoglobulin G (IgG) are ~150 kDa, Y-shaped, globular proteins that form an essential component of the immune system used to fight invading pathogens, such as bacteria or viruses. Although their overall structure is very similar, the region used to specifically recognize a given epitope on an antigen varies greatly from one antibody to the next. Substantial advances have been made over the past 20 years in the research, development, and clinical application of therapeutic monoclonal antibodies. Monoclonal antibodies have become one of the fastest growing sectors of human therapeutics for treating various pathologies, such as cancer, inflammation, infections, or autoimmune diseases. The selection of antibodies using microfluidic systems provides a low-cost and high-throughput approach for disease diagnosis, phenotyping of tumor cells, and biomarker detection.
Although many methods have been devised to screen for specific antibodies, each with its distinctive advantages and limitations, the identification of antibodies that bind to cell-surface receptors or target specific cells remains challenging. Starting from hybridomas producing nonspecific antibodies, more than 80,000 hybridoma-clone secreting antibodies with specific binding properties to the transferrin receptors on leukemic K562 cells could be selected.\(^8\) Remarkably, very low amounts of IgG were used per assay (33 fg), and the enrichment of specific hybridoma cells could be achieved thanks to the selection system. This promising work could be transposed to further therapeutic antibody discovery. For instance, a new microfluidic method for single-cell deep phenotyping of IgG-secreting cells was developed, in which thousands of droplet-encapsulated cells arranged as a two-dimensional droplet array were screened using a fluorescence relocation-based immunoassay.\(^8\) A comprehensive step-by-step description of this method was also published recently.\(^8\)

Historically, one of the most used methods to generate new antibody variants by directed evolution has been yeast display. When these methods were combined with microfluidic tools, a substantial increase in the number of tested variants could be achieved, resulting in a jump from medium (10² to 10³ variants) to high throughput (10⁶ to 10⁹). In 2017, Adler et al.\(^8\) combined microfluidics, yeast single-chain variable fragment (scFv) display, and deep sequencing to build an alternative to hybridoma-based antibody discovery. With this system, mouse antibody repertoires could be selected against the programmed cell-death protein 1 (PD-1), a checkpoint protein used as a target in cancer immunotherapies. A droplet-based microfluidic system was used to encapsulate B cells from mice with oligo-dT beads and a lysis solution. Polyadenylated transcripts released from cells and bound to the beads were purified from the droplets and injected into a second emulsion with a multiplexed overlap extension reverse transcriptase polymerase chain reaction mix. Finally, DNA amplicons encoding scFv with native pairs of heavy and light chain Ig were generated. These libraries were used for scFv display and screened by FACS, resulting in the identification of high-affinity scFvs against human PD-1 immunogen by deep sequencing. Two rounds of FACS produced populations of scFv with an average enrichment of 800-fold. Seventeen of these anti-PD-1 binders were synthesized as full-length monoclonal antibodies. Among them, 15 specifically bound surface-expressed PD-1 in a FACS assay, while 9 antibodies acted as checkpoint inhibitors. This approach could further be used to screen for other functional monoclonal antibodies.

**Aptamer Development by Directed Evolution in Drops.** Aptamers are biomolecules that possess the capacity of specifically binding another molecule. Their chemical nature can be proteinogenic or nucleic acid based (both DNA and RNA). RNA aptamers named riboswitches can be found in nature and are involved in the metabolite-dependent control of gene expression.\(^8\) The discovery and development of aptamers by SELEX methods started in 1990, when Tuerk and Gold identified various RNA ligands against T4 polymerase.\(^8\) In another study, also focused on discovering ligands against T4 polymerase, the term aptamer was coined by Ellington and Szostak.\(^8\) Since then, a plethora of new aptamers have been developed with the SELEX methodology.

Aptamer evolution in droplets took two more decades to become prominent. Droplet-based systems provide the advantage that every mutant can be studied individually inside drops, in contrast to classical SELEX systems. Fluorogenic aptamers are an alternative to classical fluorescent proteins, such as GFP, and are widely used for biochemistry, cell biology, and biomedical applications, making the exploration of new fluorogenic aptamers a particularly promising area of research. In particular, G-quadruplex RNA aptamers and, in some cases, their corresponding biosensors have been the focus of extensive optimization, resulting in the development of highly fluorescent iSpinach\(^8\),\(^9\),\(^10\) aptamer-based fluorogenic biosensors (Figure 4a), MangoIII\(^11\),\(^12\),\(^13\) aptamer, and the Gemini–o-Coral fluorogenic dimer.\(^14\) All of these aptamers were improved by directed evolution using the same microfluidic workflow (Figure 4b). First, a variant library was encapsulated in drops with no more than one molecule per drop. Second, the drops were amplified by PCR. Third, the drops containing the amplified variants were re-injected into a microfluidic device and fused with another drop containing in vitro transcription reagents. Fourth, the fused drops were collected and incubated to produce RNA, and fifth, the drops were sorted on the basis of their fluorescence and collected for sequencing. Step 2 was performed off-chip in a thermocycler. Step 4 can be carried out either on- or off-chip.

Spinach\(^8\) is an artificial fluorogenic RNA aptamer whose ligand emits green fluorescence similar to eGFP upon binding. The screening of Spinach gene libraries led to an improved Spinach (iSpinach) aptamer\(^8\) thanks to microfluidic-assisted in vitro compartmentalization. Two pairs of enrichment rounds were separated by a mutagenesis round. The main goal was to develop iSpinach mutants with higher thermal stability and a wider salt tolerance because none of the known DFHBI-binding aptamers are optimal for in vitro application. On the basis of this development, a new biosensor capable of detecting theophylline was built\(^8\) by randomizing the communication module of the biosensor. This region links the sensor region with the fluorogenic G-quadruplex aptamer, which in the case of iSpinach binds the fluorophore 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). After 5 rounds of selection, the fluorescence of the biosensor was improved by 5-fold.

Like Spinach, Mango is another fluorogenic RNA aptamer with a complementary emission wavelength in the red region of the spectrum. A complex was formed with thiazole orange...
CONCLUSIONS AND FUTURE PERSPECTIVES

Directed evolution is a field of growing importance with an increasing number of successfully improved molecules, from enzymes being improved for therapeutic applications to those used in self-replicating nucleic acids. Droplet microfluidics provides an additional compartment in which to perform chemical assays and enzymatic reactions while linking the activity of the molecule of interest to the genetic variant it represents. In this way, as the assay is not limited to the cell, one can broaden the spectrum of analytical tools available compared to classical cell sorting approaches like FACS.

We have highlighted here some of the most recent advances in the engineering of functional biomolecules (enzymes, antibodies, aptamers, and ribozymes) using droplet microfluidics. Over the past years, experimental procedures have been miniaturized and automated in the droplet format showing the long-term potential of the technology for molecular engineering. Improved variants have already been successfully obtained from a variety of experiments (Table 2). Most of the examples use a cell-based system for the expression of the molecules. Usually, the choice to express the molecule of interest in a single-cell organism is merely economical, since nowadays the cost of using commercial IVTT systems for expressing proteins is orders of magnitude more expensive than using a host organism. In the case of cell display, the choice of this system has an added justification, using the cells not only as a compartment in which to link phenotype and genotype but also as a means to replicate the genetic variants after sorting since lysis is not necessary and the cells can be regrown. Moreover, the related microfluidic workflow is simpler compared to the IVTT-involving platform. In addition, since the scaling up needed for production of a biological protein will usually be done in cell-based systems, it is convenient to use this system from the beginning of the experimental workflow and avoid unexpected difficulties. However, due to the more generalized use of IVTT systems, one can forecast a progressive lowering of the price. In this sense, the idea of generating in-house methods to produce cell-free systems is gaining importance. Complete methods for the whole process and plasmid designs (One-pot system) are already avail-

Table 2. Summary of the Evolved Biomolecules Described in This Review

| selected | library design | reference |
|----------|----------------|-----------|
| E. coli  | other          |           |
| S. cerevisiae |

"A total of 8 enzymes, 5 aptamers, 1 ribozyme, and 2 antibodies have been reviewed. We have also dissected the key components underlying these directed evolution experiments.
able.99,99 We foresee that this strategy will be widely deployed in the coming years.

It is interesting to highlight as well that peptides are gaining importance as therapeutic agents. As their use is becoming common to treat acute infections, chronic diseases, and even some types of cancer, droplet microfluidics can help one to discover peptides with novel properties. Droplet-based strategies could be adopted to identify antimicrobial peptides,100 discover novel antiviral peptides to treat respiratory diseases,101 or find anticancer peptides.102

Research in self-replicating nucleic acids is another field that is gaining importance. The search for the molecular origins of life and the construction of a minimal cell have found a versatile toolbox in droplet-based microfluidics. Research in template-directed self-replicating systems or replicators can also lead to the development of artificial ribozymes103 or control of protocell compartmentalization and reproduction.104 Using water-in-oil emulsions, the replication versatility, such as DNA aptamers or DNA structures has been favored due to some of the intrinsic advantages of ssDNA over RNA. For instance, ssDNA generally has higher chemical stability compared to RNA due to the absence of the 2′-hydroxyl group on the ribose moiety. The potential of studying the directed evolution of these molecules could lead to new biomedical and industrial applications due to their different chemical nature and versatility, such as DNA aptamers or DNA−metal nanoclusters for biosensing.

Nonetheless, despite the many examples discussed and the increasing speed in which the field of microfluidics for directed evolution is growing, the technology is far from being fully mature and further improvements are to be expected. For instance, DNA recovery and cell viability are still challenges to take into account and can be the limiting step in biological workflows. Moreover, greater access of nonspecialist laboratories to microfluidic setups is necessary to maximize the impact of this technology. Microfluidic technology will most likely gain in miniaturization, automation, and parallelization capabilities, pushing further the throughputs of selection with the development of new instruments by technology developers. Furthermore, the development of novel surfactant formulations may increase the scope of the assays possible in droplets and solve some of the current limitations such as the leakage of molecules through the drop or the interaction of surfactant with the content of the droplet. Finally, new and emerging approaches in molecular programming will provide interesting functions to further increase the selection throughputs and will provide new methods for the biomimetic selection of improved variants of practical interest.105 We believe that the combination of these tools will lead in the future to a whole new range of approaches for the discovery and improvement of chemicals of fundamental and practical interest in therapeutics and industrial applications.

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Author Contributions

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Funding

A.M., P.C., M.G.-B., and C.A.I. have received funding for this project from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation program (Grant Agreement No. 724040). A.M. has received funding from the EMBO Postdoctoral Fellowship Program (Grant number: 1033-2017). C.A.I. is an EMBO YIP and has received funding from the Fondation Bettencourt-Schueller. A.G., D.V.A., T.B., and J.-C.B. acknowledge financial support from Horizon 2020 EVDrops project (Grant agreement ID: 813786), the Region Aquitaine, and from the French Government ‘Investments for the Future’ program, part of the University of Bordeaux Initiative of Excellence (IDEX Bordeaux; ANR-10-IDEX-03-02).

Notes

The authors declare no competing financial interest.

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