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Miniaturized and Automated Synthesis of Biomolecules—Overview and Perspectives

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Chemical synthesis is performed by reacting different chemical building blocks with defined stoichiometry, while meeting additional conditions, such as temperature and reaction time. Such a procedure is especially suited for automation and miniaturization. Life sciences lead the way to synthesizing millions of different oligonucleotides in extremely miniaturized reaction sites, e.g., pinpointing active genes in whole genomes, while chemistry advances different types of automation. Recent progress in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) imaging could match miniaturized chemical synthesis with a powerful analytical tool to validate the outcome of many different synthesis pathways beyond applications in the life sciences. Thereby, due to the radical miniaturization of chemical synthesis, thousands of molecules can be synthesized. This in turn should allow ambitious research, e.g., finding novel synthesis routes or directly screening for photocatalysts. Herein, different technologies are discussed that might be involved in this endeavor. A special emphasis is given to the obstacles that need to be tackled when depositing tiny amounts of materials to many different extremely miniaturized reaction sites.

1. Introduction

Miniaturized structures in computer chips have reached the lower nanoscale and have thereby revolutionized our world. Chemical synthesis is another field that is suited for automation and radical miniaturization. Synthesis is done by mixing different chemical building blocks in a solvent and in exactly defined stoichiometry, while meeting additional conditions, e.g., temperature and reaction time. Synthesis usually starts with a planning phase to select suitable mechanisms and procedures, followed by a trial-and-error phase. This was also true some 100 years ago when Haber[1] asked his technicians at the Technical University in Karlsruhe to manually mix and process thousands of different mixtures of nanoparticles to eventually find the catalyst that transforms nitrogen and hydrogen into ammonia—to manufacture the fertilizer that a fast growing population needed so desperately. Some 80 years later, the Heidelberg University spin-off company high-throughput experimentation (HTE),[2] now a subsidiary of BASF with some 300 employees) automated Fritz Haber's screening procedure by employing pipetting robots and by adding a professional analytical pipeline made of liquid chromatography (LC)-coupled and gas chromatography (GC)-coupled mass spectrometers to screen for all kinds of catalysts.[2b]

Although impressive, this type of miniaturization only moderately increases the number of different chemicals that can be screened, e.g., for the novel types of fuel cell needed nowadays. In organic chemistry, one obstacle to more radical miniaturization has been the absence of an analytical method that: i) can handle very small amounts of synthesized molecules, and, in addition, ii) would do so for tens of thousands, maybe millions of reaction sites in a few minutes. There are certainly additional points on the wish list: iii) synthesized molecules should be analyzed quantitatively, iv) a method to follow the reaction kinetics would be helpful, and v) above all, synthesis sites should be reliably supplied with extremely miniaturized amounts of materials that are needed for chemical synthesis (chemical building blocks, catalysts, scavengers, acids, or bases).

Herein, we summarize recent examples that promise to miniaturize and automate organic synthesis, with an emphasis on the challenges of handling tiny amounts of chemicals in defined stoichiometry. In addition, recent progress in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[3] imaging, which could indeed match miniaturized chemical synthesis with at least one important analytical tool to validate the outcome of a very large number of different synthesis routes, is briefly discussed. If combined, automation, miniaturization, and analytical methods that can handle many different reaction sites promise to drastically reduce the efforts that are needed to find novel chemical synthesis pathways or even novel reaction mechanisms. When mated to the material sciences, and similar to Fritz Haber's screening for novel catalysts, strong economic, scientific, and societal interests will presumably advance these efforts.

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2. Automated, Autonomous Synthesis for Organic Chemistry

The (fully) automated, autonomous synthesis of organic molecules is a challenge that many research groups in academia and industry have tried to address for many decades. In comparison to the fully automated solutions in engineering, experimental molecule chemistry, in particular for research purposes or synthesis on a small scale, has never reached this progress in automation. In essence, there are in general two synergistic approaches to reach this aim: some projects intend to automate parts of a chemical synthesis to enhance the throughput of a chemistry lab; other projects aim for the machine-driven handling of all traditionally human-based procedures with the focus on autonomous synthesis, which does not necessarily have to lead to a high-throughput procedure.

2.1. Automated Combinatorial and High-Throughput Synthesis (of Submilligram Entities)

Automated combinatorial synthesis arose as a well-known procedure for half-automated synthesis of small organic compounds in the 1990s. In particular, the chemical industry searching for large libraries of different compounds for biological screening campaigns, operated departments that were able to synthesize, analyze, and purify dozens of thousands of compounds in a few months. Combinatorial chemistry is supported and enhanced using automated systems like pipetting robots (liquid handlers), automated synthesis stations, automated tracking systems, automated purification stations, and the generation of possible libraries via virtual design. Many of those approaches use solid-phase organic synthesis (invented by Merrifield in the 1960s)[4] as a methodology to be able to deal with the large number of different compounds in combination with an easy handling of batches in special resin-containing vessels. As an example, systems like the IRORI technique,[5] in combination with the use of reaction mini blocks, have been shown to be efficient for the upscaling of compound numbers. As a result, the amount of isolated reaction product per reaction had to be decreased to allow a reduction of reagent costs and to ensure the fast purification of the obtained compounds via high-performance liquid chromatography (HPLC).

While combinatorial synthesis was very successful in terms of synthesis of small molecules in a high-throughput manner, the procedure was criticized due to the low impact of the resulting libraries with regard to biological activity and the identification of novel pharmaceutical blockbusters. Therefore, the efforts in combinatorial chemistry have been drastically reduced in the chemical and pharmaceutical industry during the last 20 years. Only for very specific applications was combinatorial chemistry continued. These efforts were accompanied by manifold academic developments in combinatorial chemistry and solid-phase chemistry.[6]

Recently, modern applications[7] of combinatorial chemistry were presented, but unfortunately, these modern applications[8] suffer very often from manual handling and are not supported by automation techniques for chemical synthesis and compound purification, as demonstrated by the industry in former times.

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Nicole Jung studied chemistry at the University Frankfurt am Main and did her Ph.D. thesis with Stefan Bräse at the Karlsruhe Institute of Technology. She works on the development of novel methods for organic syntheses on solid phases and heterocycle syntheses applied in biological systems and for materials sciences. Besides synthetic chemistry, she is involved in software development for research data management and cheminformatics. She works in the group of Stefan Bräse and coordinates the work of the Compound Platform (ComPlat).

Frank Breitling studied biochemistry at the University of Heidelberg. He developed the technology of recombinant antibodies at the German Cancer Research Centre (DKFZ), before he switched to solid-material-based, high-density peptide arrays. Since September 2009, he has headed the department of peptide arrays at IMT at the Karlsruhe Institute of Technology.

2.2. Approaches to Fully Automated Synthesis by Robots (Automation Platforms)

The fully automated synthesis of compounds by the substitution of human work and handling offers many benefits with respect to the efficiency of chemical synthesis, safety issues, and reproducibility. A fully automated synthesis must cover all necessary steps of a synthetic procedure and should be as

flexible as possible with respect to the adaptation of the synthetic procedure (plants in the chemical industry designed for large-scale production of one particular synthesis in tons are not considered here). Therefore, systems for a fully automated synthesis of pure organic compounds differ in many aspects from systems that focus only on high-throughput synthesis. High-throughput procedures, as described for the synthesis of large libraries of different compounds in combinatorial chemistry, often focus on selected reactions that are optimized to give the target compound classes for a variety of different starting materials. In contrast to the aims of a fully automated single reaction, the yield and purity of the single compound in a high-throughput synthesis are less important than the generation of many derivatives in parallel. Another important difference is the scale of the reactions and the obtained products: while high-throughput procedures usually get along with a few milligrams of product or less, fully automated reaction systems and synthesis platforms are often designed to produce the target compound in multi-milligram to gram scale, offering options for full analysis of the compounds. Automated systems for fully automated chemical synthesis have been developed by companies and also in academia, while many of these systems claim a full automation, which is, in most cases, not given. Examples for partially automated chemical reactions, offering diverse recording and observation opportunities are, among others, the synthesis machines Integrity[9] or the microwave systems of Anton Paar[10] and Biotage.[11] Other systems for special applications are, for example, the automation systems for peptide or oligosaccharides synthesis (see Section 3.2), which offer, despite a limitation in automation processes, a good applicability to certain chemical procedures with less-complicated procedures. Systems that include liquid-handling stations, powder dosing modules, vial transfer options, and other options to add helpful modules that can be incorporated, are for example the ChemSpeed[12] automation systems, Zinsser analytics platforms, or research plants from companies like Hightec Zang.[13] In particular the ChemSpeed automation platforms are widely used in industry and in academia, as they can be combined with many modules necessary for standard organic and inorganic chemistry. The platform itself can be modified according to the needs of the scientific project and can serve as a synthesis platform for single reactions with detailed control of the reaction parameters, or can be used for the parallel synthesis of dozens of compounds.[14] All of the abovementioned systems suffer from the disadvantage that the full replacement of human interaction has still not been reached, as the described commercial platforms are still independent systems with partly flexible but still limited preparation, synthesis, and analysis capacity. The missing parts of these systems are, in general, an automated storage system for all chemicals, being those that are accessible to the scientist, and a suitable dosing station for liquids, solids, and oily substances. Also, these systems, in general, do not offer procedures that most often have to follow an automated synthesis, like the purification of the compounds. The purification of the compounds is always necessary if filtering is not sufficient to analyze the compounds via suitable techniques like NMR, HPLC, or GCMS. Very impressive platforms that try to solve these bottlenecks of the synthesis stations have been developed in the past by researchers at Sanofi-Aventis.[15] The team in Germany installed the automation pipeline SynCar for automated solution-phase synthesis (10–100 mg scale) covering almost all aspects of common organic chemistry, like synthesis (temperature control and liquid-reagent handling), filtration, liquid–liquid extraction, evaporation, weighing, solid-phase extraction, and HPLC/MS analysis (**Figure 1**). The concept was realized by seven modular workstations, connected by a shuttle transfer. The system was suitable for a parallel treatment of four reactions, of which each can follow a different reaction workflow through the shuttle service.

Other companies also created automated systems to reach a fully integrated automated synthesis solution. For example, Lilly built a platform that covers not only most of the important steps of an organic chemical synthesis but is also globally accessible via a special program. This program (Open Innovation Drug Discovery Synthesis (OIDD))[16] allows other external researchers to propose target structures and libraries to be synthesized with the remote-controlled platform. The architecture of the platform was constructed in a way that allows flexible adaptations to diverse synthesis strategies and several applications, such as the synthesis of heterocycles, like benzodiazepine-diones or pyrazoles.[17] Recently, Burke and co-workers[18] presented a procedure for solution-phase synthesis of very diverse compound classes via an automated process. The concept relies on the selection of reactions that allow a conversion in high yields via the use of building blocks, very similar to a combinatorial synthesis approach. The automation protocol

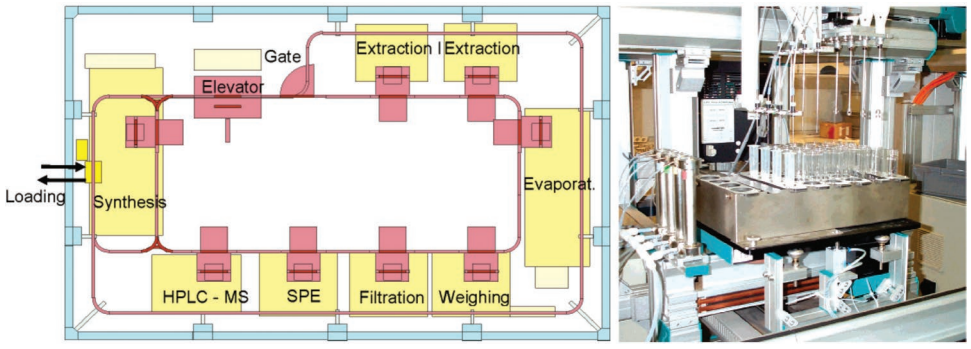

Figure 1. Automated synthesis platform SynCar. One example is shown for an automated synthesis platform developed in an industrial project. Left: the layout of SynCar with transfer system in red, workstations in yellow, and cabins in blue. Right: the liquid–liquid extraction module of SynCar. Reproduced with permission.[15] Copyright 2005, American Chemical Society.

contains the synthetic steps of coupling, deprotection, as well as purification, and is fully controlled via a self-written script. While Burke and co-workers use a cartridge-based system based on commercially available plastic cartridges for almost all steps of the preparation, synthesis, and purification, approaches from Cronin and co-workers[19] focus on the use of common glassware and standard equipment of a chemistry lab or a redesign of common glassware by 3D printing. Automated solutions for the manufacture of fine chemicals and pharmaceuticals in a self-contained plastic reactionware device were successful. A chemical to computer-automated design (ChemCAD) approach was used, which translates bench-scale synthesis into a platform-independent digital code. Additionally, printed devices were constructed that manage the operation of the synthetic route internally via simple operations. This approach was demonstrated for the γ-aminobutyric acid receptor agonist (±)-baclofen, which was synthesized in a three-step synthesis procedure including five modules (**Figure 2**a–e).[20] The reaction modules cover: a) a module for a Michael addition, combined with evaporation and ether extraction; b) a module that combines solvent exchange and a reduction step; c) a module for phase separation and filtration; d) a module for solvent exchange and hydrolysis; and e) a module for a final filtration step. The overall procedure includes processes to add reagents in solution (by Luer syringes), the stirring of the reactions with PTFE-coated stirring bars, and cooling or heating of the reactions in the cartridges via transfer of the reaction vessels to an ice or a sand bath on a stirring hotplate.

In another example, Cronin and co-workers constructed an automated synthesis robot by modifying an open-source 3D-printing platform. The resulting automated system was used to print polypropylene reaction vessels of differing internal volumes and subsequently make use of these fabricated vessels to synthesize the nonsteroidal anti-inflammatory drug ibuprofen via a consecutive one-pot three-step approach.[19] The same group could show some interesting examples to apply automated synthesis procedures to a parallel reaction mode.[21] The development of a networked reaction station consisting of up to four simple and affordable (<$500) robots was demonstrated. The robots were built with a standard set of hardware and software protocols that can be networked to coordinate chemical experiments in real time using a cloud-based workstation. During the last years, automation technologies attracted more and more attention in organic synthesis for materials

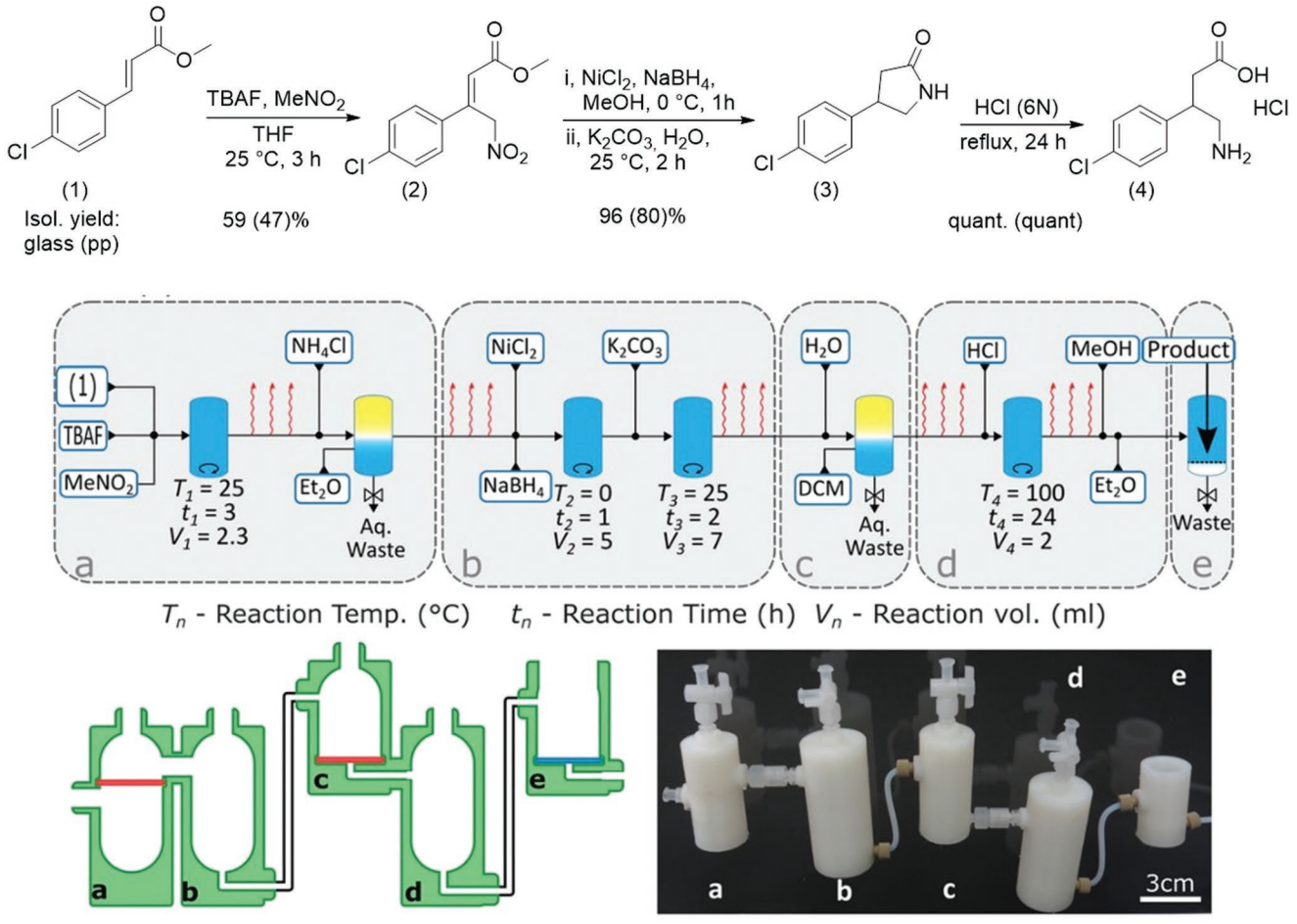

Figure 2. Concept of the cartridge-based solution of Cronin and co-workers shown for the synthesis of baclofen. The identified synthetic processes are split into different modules (a–e), which are used as model for the fabrication of cartridges in which the reaction steps and further steps like separation processes take place. Reproduced with permission.[20] Copyright 2018, AAAS. The reaction scheme is newly drawn according to ref. [20].

science. Also, researchers coming from physics are interested in the design of chemistry automation, as this might accelerate breakthroughs in research areas like organic light-emitting diode (OLED) construction and photovoltaics, only mentioning two particular applications.[22] Hein,[23] Aspuru-Guzik,[24] and Berlinguette[24] and their co-workers demonstrated smart solutions to design concepts for materials development via AI, combined with automation technologies in chemistry and automated analysis tools focusing on materials development. Their concept of a materials acceleration platform (MAP)[24] aims for the establishment of self-driving laboratories that may solve key questions in materials design by combining the most important steps for new developments. As not all developments can be highlighted here, we refer to additional reviews for further reading.[25]

2.3. Automated Synthesis by Flow Chemistry

Flow chemistry offers different advantages for the automation of chemical synthesis in contrast to traditional flask chemistry. First of all, processes in flow chemistry are very often digitally controlled and the systems are connected to sensors. As most of the processes take place in tubes and coils or miniaturized reactors and are connected by valves, no special hardware needing manual handling or adaption of automated transfer by grippers is required. Additionally, the scale-up is facilitated in continuous flow chemistry, which makes it a very useful alternative to common flask chemistry especially in the chemical industry. Many processes for automated reactions via flow chemistry have been published, describing one-step syntheses, that are used for the synthesis of many different compound types and reaction classes[26] in academia (see publications of the key players and their co-workers[27]) and in industry.[28] Missing key steps of these approaches are in almost all cases procedures belonging to the chemical-synthesis workflow, like preparation of the reactants (dissolution) and the purification of the resulting compounds. However, the combination of different techniques recently allowed also complex, multistep syntheses. It is beyond the scope here to comprehensively summarize all of them, but some highlights of the current literature are given in the following paragraph.

As one example, the use of Cu-pipes as the catalytic medium for a Cu-catalyzed alkyne–azide cycloaddition (CuAAC) reaction yielding triazoles was shown as an interesting example for flow chemistry recently.[29] The authors could show that the use of Cu-pipes yielded the target compounds with very low content of Cu, which allowed use in biological systems that are sensitive to the amount of copper impurities usually obtained in CuAAC reactions. Flow reactions have also been applied to low-temperature reactions that are needed for individual processes within one reaction, like cooling and the addition of further reagents. A recent example was presented by Newby et al. who combined several flow reactors to a continuous flow chemistry platform allowing the subsequent reaction of organic compounds with temperature-sensitive organometallic reagents followed by the reaction with an electrophile.[30] The procedures were applied, for example, to the synthesis of a 20-compound library of polysubstituted, fluorine-containing aromatic substrates. In particular, photochemistry is suitable for application in flow synthesis,[31] as several examples for fast reactions in small reaction (irradiation) volumes[32] or applications to reactions with visible-light laser sources for flow-chemistry-integrated vessel reactors show.[33] Although, many flow reactors need chemical engineering to solve chemistry-related synthesis challenges,[34] photochemistry-based flow reactors are also commercially available,[35] allowing a practical ready-to-use automation in chemistry labs. Systems, including the purification of the obtained products are very beneficial in terms of a full automation of a chemical procedure. The purification is particular important for compounds that are used for materials sciences, which are very sensitive to impurities. A downstream processing in a flow reactor or platform representing the processing in a routine chemistry lab (e.g., the purification of the obtained compounds) is therefore very important. Examples for purification techniques based on phase separation were recently demonstrated by Lévesque et al.[36] In-line techniques for the purification of aryldiazomethanes were used to allow their direct application in subsequent reactions. In the latter case, the target reagents needed to be deprotected in situ from stable sulfonylhydrazones and needed to be reused directly, as they are often unstable and toxic. The purification was established by an aqueous washing step yielding a clean and base free diazo target compound. Another approach was shown by Dai et al., who reported the synthesis of atropine, including the separation of the target compound from several by products with structural similarity to atropine. The purification of atropine to >98% purity was achieved by pH control in three sequential liquid–liquid extractions and a functionalized resin.[37] As this is true for an automated procedure in general, flow-chemistry approaches allow the highest benefit, if several steps can be done sequentially. Some platforms indeed enable a combination of several reactors without the need of manual interaction.[27j,28b,38] Examples for those multistep reactors or platforms are known for pharmaceutical applications, but, if the system offers flexible reaction modes, the use for other applications such as in materials science is, in principle, possible. A very impressive example for an automation of flow-chemistry processes for the synthesis of complex molecules was shown by the DARPA project SynFini realized by SRI international.[39] The developed platform automates the design, reaction screening, and optimization (RSO), as well as the production of the target molecules. The platform is a nice example how synthesis can work, if several parts of the chemist's activities are combined in an automated workflow. The heart of the platform is a benchtop multistep synthesizer (AutoSyn) that can reproduce many different reaction routes, which are based on solution-phase chemistry. AutoSyn is composed of unit operation modules to accomplish reagent delivery, mixing, heating, cooling, separation, and analysis. Other examples of multistep reactions showed the compatibility of batch synthesis with flow synthesis as an example for a flexible approach to combine the best methods for each synthetic procedure in a multistep reaction.[40] The platform was developed by Fitzpatrick and Ley, who applied open-source technologies to automate, control, and monitor the individual process in the batch or flow part of the synthesis. They also showed how other standard laboratory tasks of a chemical synthesis, like continuous extraction and solvent switching steps, can be included in

such a platform. Although the procedures and opportunities of flow-chemistry applications already have a high impact in synthetic chemistry and allow a high level of automation for the production of a wide range of compound types, drawbacks of many single-step continuous-flow systems and multistep systems are, for example, missing steps for: 1) the preparation of the reactants (dissolution) and 2) comprehensive methods for the purification of the intermediates or resulting compounds, such as chromatography. In addition, automation in flow chemistry is applied mainly to pharmaceutical synthetic challenges, while materials science, being a suitable application for existing automation techniques, has almost not benefited at all from the flow technologies so far.

2.4. Scope of Artificial Intelligence on Automation in Chemical Synthesis

Many automation procedures are nowadays combined with artificial intelligence (AI) to enhance the productivity of the designed systems. A combination of AI and automation is very beneficial, in particular in those cases where either, even automated chemical synthesis, is time consuming and expensive or the analysis of high-throughput procedures cannot be managed by humans. AI is used for the analysis of the synthesis result and is also used for reaction design and compound selection. Many procedures have been developed and applied; among them are computer-aided reaction design, and reaction prediction based on high-level quantum chemical methods, machine learning for retrosynthesis, and product prediction. Different models for the optimization of chemistry processes have been described recently. Zare and co-workers describe the optimization of chemical reactions by deep reinforcement learning, to obtain the optimal reaction conditions in microdroplet reactions;[41] meanwhile, deMello and co-workers presented a constrained optimization procedure for self-optimizing reactors that are trained to produce certain target compounds in parallel to suppressing the formation of unwanted by products.[42] Aiming for the generation of self-driving chemistry laboratories, Aspuru-Guzik and co-workers showed the use of Phoenics and Chimera in the context of chemistry and experimentation. Phoenics, a probabilistic global optimization algorithm, was used to identify the set of conditions of an experimental chemical reaction by proposing conditions and updating of those proposed conditions after the experimental feedback to Phoenics.[43] Chimera was used as a multitarget optimization method for automation techniques like the autocalibration of a virtual robotic sampling sequence for direct injection.[44] Cronin and co-workers[45] presented an example to predict the reactivity by a reaction system that is controlled by a machine-learning algorithm. The machine-learning system was able to predict the reactivity of about 1000 reaction combinations with accuracy higher than 80%. A similar approach[46] demonstrated the design of an autonomous reaction network that included the evaluation of the reactions' outcome by in-line spectroscopy and real-time feedback and analysis with an algorithm that is able to distinguish and select the most reactive pathways. Machine learning was proven to be very suitable to allow alternatives to human experience and expertise for a fast suggestion of a possible reaction outcome or the suggestion of the right starting materials and conditions for reaction design and retrosynthesis. During the last few years, different procedures have been proposed, including work of the group of Jensen,[47] Baldi,[48] Doyle,[49] and Waller,[50] and their co-workers. The most prominent approach was contributed by Waller and Segler, who developed a template-based machine-learning model using the training of a neural network with chemical fingerprints. As the dataset, 3.5 million curated reactions of the Reaxys database were used. The reaction data were translated via the creation of extended connectivity fingerprints (ECFP) in machine-readable input formats for a neural network. The training of the neural network was done with the help of reaction rules that were extracted algorithmically (for details for the automatic generation of reaction rules please, see the information in ref. [51] and references therein). Retrosynthetic challenges are then submitted to the system with the fingerprint of the reaction product (see **Figure 3**a). By using the previously trained neural network, the fingerprint is then assigned to the most relevant reaction rules, which, in consequence, define the necessary starting materials. The use of fingerprints for the training of the neural network and the retrieval of the retrosynthetic information enables the system to learn the context (e.g., functional groups) of the applied rules (Figure 3b). If the procedure is applied successively to all starting materials that are identified by a retrosynthetic approach, the procedure of Waller and co-workers can be used to design a full retrosynthetic multistep procedure. Such an example was shown by the reconstruction of a recently published synthetic procedure, for that the theoretical retrosynthesis pathway was fully reconstructed by a step-by-step prediction of the most probable reaction rules for each step (Figure 3c).

The authors combined their previously developed procedure[51] (applied to 12.5 million reactions) with additional AI in form of a Monte Carlo tree-search model to build a filter network to preselect the most promising retrosynthetic steps.[50]

Also, large companies invest in machine-learning applications for chemists and offer developments to the community. Recently,[52] IBM launched a seq2seq-based machine-learning approach based on a training of their network with a patent dataset of 2 million reactions. The results of the project are offered as a web application in the IBM cloud. So far, the application of machine learning, independent of the applied neural network is not a suitable method to invent reactions or to plan reactions beyond the established knowledge. Options to achieve such an ability were proposed by an approach to model chemical reasoning.[53] The method uses a formalism for reaction prediction to find missing links in a knowledge graph. A knowledge graph, containing 14.4 million molecules and 8.2 million binary reactions, was created, allowing generalization beyond known reaction types, and is thus capable of discovering novel transformations within an experimentally shown similarity of reaction behavior.

3. Miniaturized Synthesis and Molecule Libraries

Miniaturization in organic chemistry means from a technical point of view: tiny amounts of different materials must be

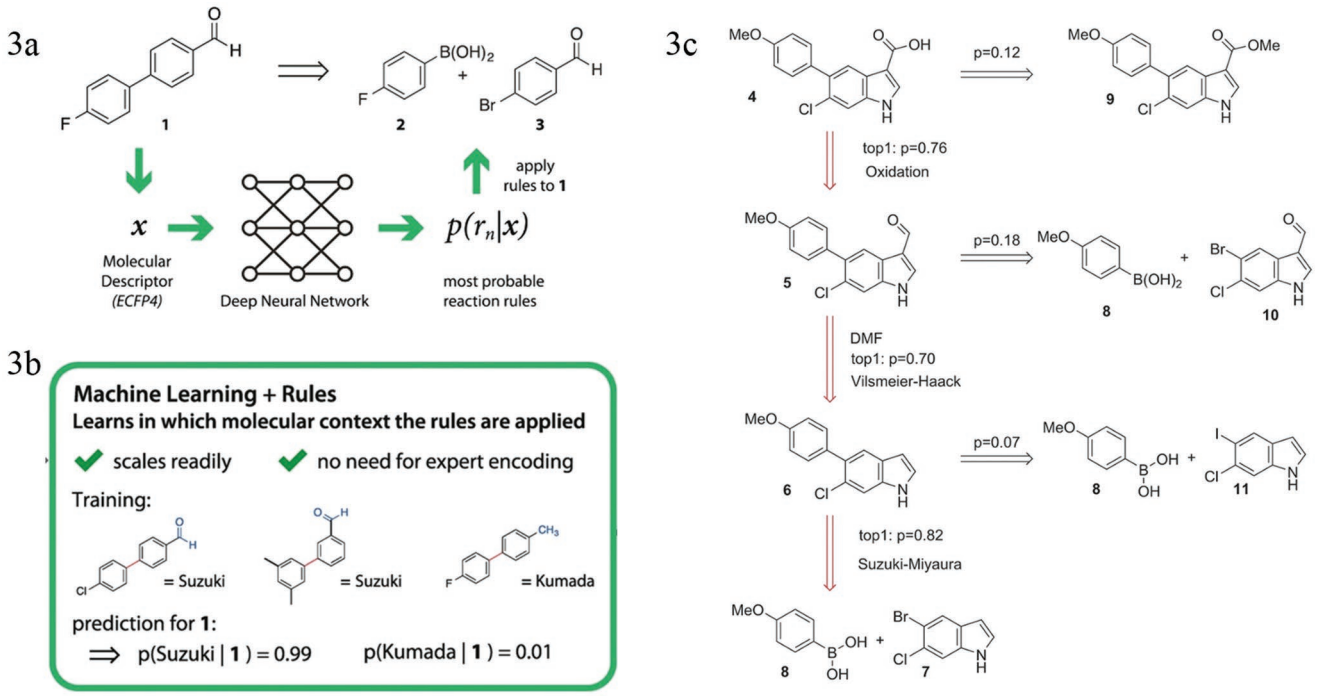

Figure 3. Automated retrosynthesis via the application of neural networks. a,b) Schematic description of the procedure to gain realistic retrosynthetic procedures and necessary starting materials. c) Example showing the capability of the presented procedure with a published synthetic strategy according to ref. [51a]. a–c) Reproduced with permission.[51b] Copyright 2019, Wiley-VCH.

reliably deposited at many different reaction sites, in exactly defined stoichiometry, and in a suitable formulation, before these molecules are reacted. All these tiny reaction mixtures should not decay over time and start and stop their reactions at defined time points. Moreover, they should not be snatched away by absorbing surfaces. Clearly, the smaller the reaction sites, the more challenging these tasks are. Finally, miniaturized synthesis must be matched by suitable analytical tools.

Life sciences have been the drivers in the invention of high-throughput methods that came along with miniaturized chemical synthesis. Sparked by Merrifield's invention of solid-phase synthesis of peptides,[4a] the automated synthesis of biomolecules such as oligonucleotides,[54] peptides,[55] and oligosaccharides[56] was developed. In parallel, the question arose of how to handle a large number of such molecules in meaningful scientific experiments. It was Ekins[57] who advanced the concept of the "array" in the mid 1980s with an argument that is still valid today: Driven by Brownian motion, an antibody, or any other molecule that binds to another molecule, will probe a potential binding partner similar to a key that tests a lock. Only if the "antibody key" exactly fits to the "binding molecule lock", these two molecules come close enough to develop hydrogen bonds, electrostatic, hydrophobic, or van der Waals forces. Then, these exert mutual attraction over very short distances, and binding ensues. Moreover, if one binding partner is tethered to a known position on the 2D surface, then the site where binding is detected immediately informs the experimenter about the type of molecule that did the binding. The revolutionary nature of this concept is based on the fact that a binding antibody or deoxyribonucleic acid (DNA) molecule would probe not just one, but rather thousands, or even millions of arrayed molecules in a single experiment. Ekins' concept convinced Boehringer Mannheim (now Roche Diagnostics) to develop such arrays, but these arrays never materialized as a commercial product. Obviously, too many resources were needed to synthesize thousands of different molecules and reliably spot and link them to a 2D-support in an array format.

More successful proved to be strategies for the in situ synthesis of oligonucleotides,[58] oligosaccharides,[59] and peptides.[60] While Ekins had to individually synthesize and handle thousands of different molecules, in situ synthesis drastically reduced these efforts by using only a small number of building blocks. Especially successful proved to be strategies for the synthesis of oligonucleotides[58]and peptides,[60] while the large-scale in situ manufacturing of a third important biological class of molecules—oligosaccharide[59] arrays—is still challenging. The basic idea in all of these methods is to do highly parallelized solid-phase synthesis in many different drastically miniaturized synthesis sites on a 2D support. The main technical difficulty in all of these methods is to reliably supply these many separated miniaturized reaction sites ("spots") with tiny amounts of different monomers to elongate the growing oligomers with "their" monomers. This was done by using different printing techniques,[61] or by using lithography[62] to define those reaction sites.

It should be noted that quality control in array synthesis is still problematic. Usually, the amount of synthesized molecules (pmol–fmol) per spot is too small for most of the standard analysis methods. In this respect, the invention and commercialization (Shimadzu, Bruker, Applied Biosystem) of matrix-assisted laser desorption–ionization (MALDI)[63]–mass spectrometry

(MS) imaging[64–66] was a milestone for the quality control of synthesized molecules in the array format.

3.1. Synthesis of Oligonucleotide Arrays

In the 1990s, the human genome project revealed the existence of some 20 000 different human genes, and immediately the question arose as to which ones of these genes were active when comparing, for example, a tumor cell to a normal cell. DNA arrays were obviously the tool to answer this type of crucial questions. All you had to do was to collect messenger ribonucleic acid (mRNA) from the different cells, and hybridize labeled mRNA (or, for technical reasons, complementary DNA (cDNA)) to arrayed DNA spots that represent aforementioned 20 000 human genes.[67] These mRNA molecules would then automatically find their complementary DNA on the array to reveal, in one single experiment, which ones of these genes were transcribed to mRNA. Experiments of this type coined the word "genomics," which was meant to reveal the activity of the whole genome, for example, to find those genes that were only active in a certain cell type.

Southern[68] was the first to synthesize a dense oligonucleotide array by repetitively spotting the four different building blocks for DNA synthesis onto a microscope slide that was functionalized with an aliphatic linker containing a free hydroxy group (**Figure 4**). They used the phosphoramidite method[69] for their highly parallelized solid-phase synthesis method, which allowed them to miniaturize synthesis sites down to a spot diameter of ≈0.9 mm. Only three steps (coupling of the four different β-cyanoethylene phosphoramidite, oxidation, and detritylation) were necessary for the coupling of one monomer

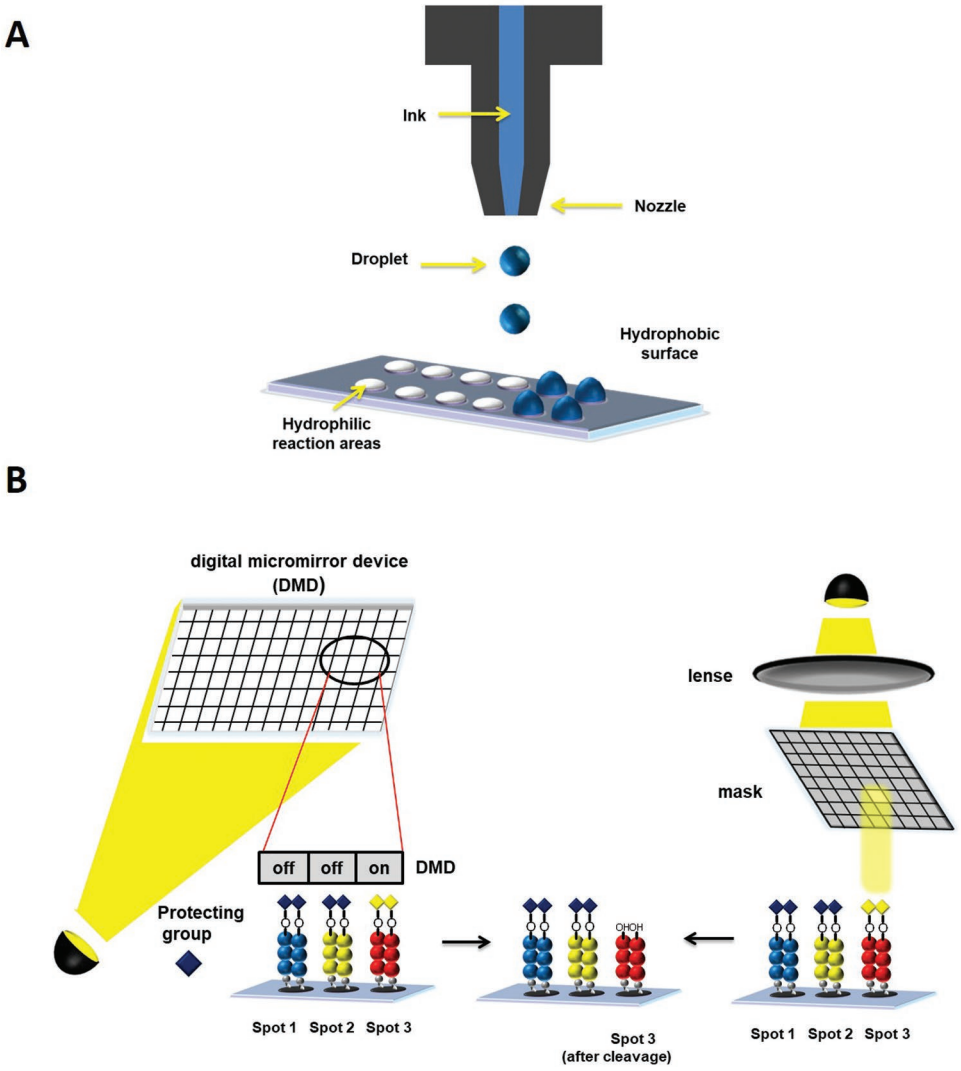

Figure 4. A) Printing versus B) lithographic synthesis methods.[62a] In all of the printing methods, such as inkjet printing, individual synthesis sites (e.g., hydrophilic reaction areas on a hydrophobic surface, to confine chemicals) are supplied with tiny amounts of the different building blocks that are needed for array synthesis, while lithographic methods, e.g., consecutive micromirrors (left) or lithographic masks (right) are used to deprotect the molecules (chain of spheres: immobilized molecules, rhomb: protecting group) in the synthesis to define 1st, 2nd ... *n* sites that are all reacted at once with one type of building block.

layer, which drastically reduced the resources that were needed to generate a high-density array.

Shortly afterward, in 1991,[62a] the Fodor group developed an even more radical approach to miniaturize individual synthesis sites with spot sizes down to the low-micrometer range. They used lithographic masks—that were known from computer-chip technology—to illuminate selected areas with ultraviolet (UV) light in order to remove UV-sensitive protecting groups in those illuminated areas. This elegant approach allowed them to circumvent the basic problem that comes with any type of miniaturized parallel synthesis of many different chemicals, namely how to supply individual synthesis sites with "their" building blocks. They simply reacted all of these deprotected sites at once with one of the building blocks for array synthesis, and afterward defined second sites that were reacted with another monomer (Figure 2). However, this elegant approach comes at a cost: these lithographic methods can always couple only one type of chemical to lithographically defined synthesis sites, which means that $20 \times 15 = 300$ coupling cycles and the same number of expensive lithographic masks are needed to synthesize an array with 15meric peptides with its 20 different amino acid building blocks. Moreover, such a large number of processing steps certainly hamper the quality of synthesized arrays due to the accumulation of unwanted side reactions.

Indeed, although Fodor and co-workers[62a] demonstrated their seminal approach by synthesizing very high-density peptide arrays, they used only a very limited number of amino acid building blocks to synthesize quite short peptides. In order to attenuate this basic drawback of lithographic synthesis methods, the Fodor group and their spin-off company Affymetrix shifted to the synthesis of oligonucleotides that were made of only four different building blocks. In 1994[70] Affymetrix used this technology to generate first octanucleotide arrays (256 octanucleotides; 157 molecules per 1 cm^2) that were hybridized to single-stranded DNA.

Soon afterward, this company commercialized very high-density oligonucleotide arrays with up to ≈1 million octanucleotides per 1 cm^2. The standard length of these oligonucleotides was limited to 25 bases, probably due to the modest yield in each synthesis circle during the light-induced deprotection step. Still, this was a major technical breakthrough and a big commercial success. The biggest manufacturer of oligonucleotides—Illumina Inc.—reported revenues of $666 million for the year 2009.[71] These sales from Illumina and Affymetrix reflect the many different types of scientific questions that could be answered by high-density oligonucleotide arrays: they were used in sequencing,[72] gene-expression analysis,[73] transcription factor binding analysis,[74] and genotyping.[75] It should be noted that this type of array could be used to synthesize complete genomes that are assembled by PCR. Further information can be found in the reviews of Bumgarner[58a] and Heller.[76] In recent years, sales of oligonucleotide arrays were replaced by next-generation sequencing, which, in a sense, is also an example of extremely miniaturized and highly parallelized chemical reactions. These techniques are reviewed elsewhere.[77] It should be noted that many additional developments were needed to make these very high-density arrays a commercial success. Extremely miniaturized synthesis sites necessitated the development of fluorescence scanners that could analyze millions of very small spots that were labeled with fluorescently labeled cDNA. Meanwhile, several companies developed scanners with a resolution that is compatible with synthesis sites as small as 500 nm (e.g., Innopsys 1100,[78] GeneChip Scanner,[79] and SureScan Dx Microarray).[80]

Also, the quality of lithographically produced arrays was insufficient due to the low cleaving efficiency of light-sensitive protecting groups. The group from Gao were the first to publish a practical solution. They added a photoacid to "translate" the incoming UV light into a local acidification, which allowed them to replace the photolabile protecting group with its notorious low cleaving efficiency (α-methyl-2-nitropiperonyl)oxy]carbonyl (MeNPOC)) with an acid-cleavable group that had been used for many years in standard solid-phase synthesis (dimethoxytrityl (DMT)). Xeotron, later Invitrogen, commercialized this type of array. Pellois and co-workers[81] adapted this principle for the synthesis of peptide arrays by using a *tert*-butyloxycarbonyl (Boc)-group.

Yet another drawback was the large number of expensive lithographic masks that were needed to define synthesis sites for each building block and for all of the coupling circles. In 2002, Nuwaysir and co-workers[82] used an array of switchable micromirrors to illuminate selected areas with UV light, which made expensive lithographic masks redundant. This maskless photolithographic approach allowed them to synthesize ≈80 000 oligonucleotides per cm^2. Roche NimbleGen[83] commercialized such arrays with up to 2.1 million synthesized oligonucleotides per wafer.

Also, the printing method that was invented by Edwin Southern was considerably advanced in the following years. Blanchard and co-workers[84] used inkjet printing to synthesize oligonucleotide arrays via the standard phosphoramidite method. They generated high-density oligonucleotide arrays by confining individual synthesis sites with the help of a hydrophobic/hydrophilic pattern on the solid glass slide support that was used to grow the oligonucleotides. Thereby, the four different phosphoramidate building blocks could be printed into hydrophilic areas without diffusing them to neighboring reaction sites (Figure 2). In 2001, Hughes and co-workers[85] published a second-generation inkjet oligonucleotide synthesizer that allowed him to synthesize ≈1300 oligonucleotides per 1 cm^2, which was commercialized by Rosetta Inpharmatics.[86] Moreover, the inject technology (POSaM arrays) was used from Lausted and co-workers in 2004, to generate 10 000 molecules per microscope slide.[87] Meanwhile Agilent offers high-quality inkjet-printed oligonucleotide arrays with ≈1 million different oligonucleotides per glass slide[88] (≈100 000 oligonucleotides per cm^2, with an estimated 50 attomoles per reaction site.[89] If compared to lithographic synthesis methods, these arrays feature longer oligonucleotides with up to 100 bases, presumably due to the fact that all the printing techniques do not have the technical drawback of reacting one building after the other, but rather do it for all of them at once.

3.2. MALDI-MS-Based Analysis

While the binding of labeled DNA is easily detected with a fluorescence scanner, a chemist certainly prefers to use more

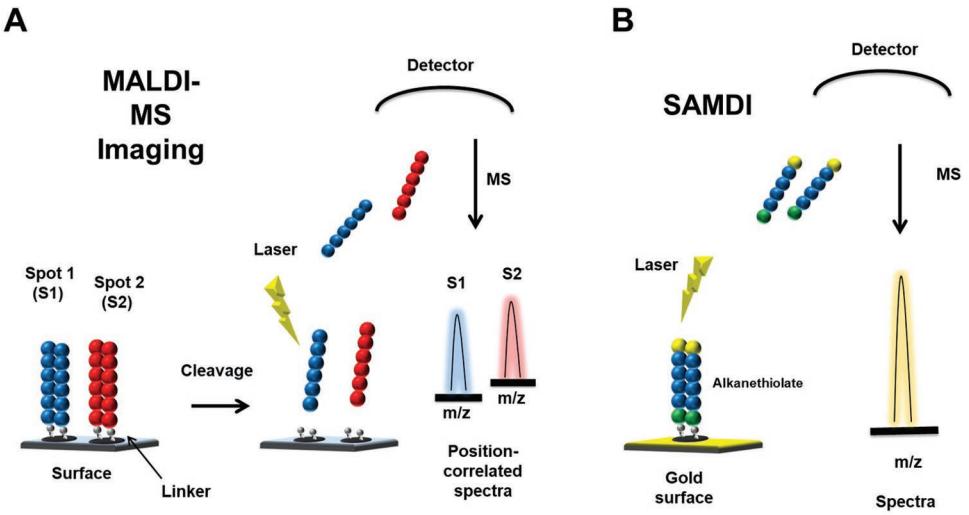

Figure 5. MALDI-MS imaging and SAMDI-MS.[96] A laser scans a 2D molecule array surface, and *m*/*z*-spectra for every position on the array are generated. A) To-be-analyzed molecules in spot (S1) (blue) or S2 (red) either have to be cleaved from a linker (gray sphere) without lateral diffusion from the solid support before the measurement to receive spatially resolved spectra, or B) the functionalized (yellow spheres, e.g., polysaccharide) alkanethiolates (blue spheres), which were bound via a thiol (green sphere) group to the gold surface, can be directly cleaved via laser during the measurement due to lower binding energy of thiol-groups to gold surfaces.

sophisticated methods for analysis. The invention of MALDI-MS imaging and the development of commercially available machines was a milestone in this respect. These machines can be used to find out the precise masses of synthesized molecules in high-density array format,[90] and in formidable sensitivity (**Figure 5**). MALDI requires a matrix material to absorb the incoming laser light that causes the ionization of the molecules. Moreover, the solid support should be conductive to avoid artifacts due to light-induced charging. Synthesized molecules must be cleaved from the solid support to detect them by MALDI, which was done, for example, with a photolinker.[91] Due to the low cleavage rate, different groups have tried to carry out MALDI-MS imaging without employing such linkers. Su and Mrksich[92] synthesized arrays on a gold surface, where the relatively weak covalent bond via oligoethylene thiolates is directly cleaved through laser irradiation. This method is known as self-assembled monolayers and desorption–ionization mass spectrometry (SAMDI).[93] Beloqui and co-workers[94] developed a variant of this method on indium tin oxide (ITO) glass. They immobilized the molecules via a noncovalent bidentate lipid linker that interacted with hydrophobic chains on the ITO glass. However, MALDI gives no definitive information about product purity and product distribution. LeProust and co-workers[95] used a phosphorus-32 (^{32}P) gel electrophoresis of the cleaved oligonucleotides to provide product distribution.

In addition, MALDI-MS imaging can also be used to find out the masses of the binding molecules, while a fluorescently labeled DNA would only deliver the information that a labeled molecule is detected. In 1996, Köster and co-workers[97] used MALDI to determine the masses of bound DNA molecules that were hybridized to a DNA array. In this type of analysis, mass spectrometry was used for sequencing;[98] for example, exons of the p53 gene and for genotyping.[99] In 2003, Cheran et al.[100] used imaging time-of-flight secondary-ion mass spectrometry (TOF-SIMS) for the recognition of the spatial distribution of DNA strands. Meanwhile, MALDI-MS imaging is the method of choice in obtaining much of information for many different reaction sites.

Indeed, Lin and co-workers[3d] used plate-based microreactors to prove that MALDI-MS can be used to validate the outcome of many chemical reactions that were miniaturized down to the nanomole scale. They employed four different catalytic reactions to react 192 different N-heterocycle-containing aryl bromides with 192 different secondary amines in 1536 experiments and in nanomole scale. They showed that the yield of this nanomole-scale synthesized product can be reliably quantitated by MALDI-MS by comparing it to a known amount of added standard (deuterated reaction product). Next, they added closely related reaction partners to determine in 1536 experiments an influence of the added reagents on the synthesis yield, and once again employing the internal standard. Thereby, they could quantitate a "poisoning effect" of added molecules. However, Lin and co-workers had a much more ambitious goal: they wanted to do simple prescreens with many different reaction partners and in drastically miniaturized chemical reactions to find out which ones would or would not yield a product. Especially, the unproductive chemical reactions ("the dark space of chemical reactions") are largely missing in the literature, but they are surely needed to train programs that predict promising synthesis routes, or, more simple, to avoid the design of synthesis routes that are doomed to failure. Obviously, it does not make sense to synthesize thousands of different deuterated standards to do as many miniaturized chemical reactions. Therefore, Lin and co-workers used only one internal standard to correct, for example, for losses during handling. They performed four different catalytic reactions to synthesize molecules and they painstakingly compared the strength of the reaction product signals in MALDI-MS versus UV-spectroscopy quantification of chromatography-purified reaction products. As expected, the signals in MALDI-MS did not match

exactly to UV-spectroscopy-based quantification of the same sample, but, by defining a pass/failure threshold (20% average MALDI signal), they could indeed correlate MALDI signals to failed versus productive synthesis. In other words, Lin and co-workers showed that indeed MALDI-MS can be used to explore the chemical space with the help of miniaturized chemical reactions.

3.3. Glycan Array Synthesis

Oligosaccharides are biomolecules with an important role in living organisms, such as cell recognition and cell adhesion.[101] However, their functions are barely studied, due to the difficulties to synthesize them. In the 1970s the solid-phase synthesis of oligosaccharides was developed.[102–104] In 2014, Seeberger and co-workers commercialized an automated synthesizer that was based on a peptide synthesizer (Glyconeer 2.1).[105] This machine enabled the automated synthesis of single glycans (up to a 50mers), and, thereby, made complex glycans accessible to the scientific community. These aspects were reviewed by Wen and co-workers and Panza and co-workers in 2018.[56b,106] For the synthesis, Seeberger and co-workers[107] used a mannosyl phosphate with a temporary C6 fluorenylmethoxycarbonyl protecting group (Fmoc), while benzoyl groups (Bz) served as permanent protecting group for hydroxy groups (**1** in **Figure 6**). Similar to peptide synthesis, the growing oligomers were immobilized on a solid-phase resin. Repetitive glycosylation via trifluoromethanesulfonic acid trimethylsilyl ester (TMSOTf) in DCM (low temperature) and Fmoc-deprotection via piperidine in DMF resulted in the synthesis of polysaccharides (Figure 6).

After the generation of the desired chain length, the protecting groups were removed, and the product was cleaved from the solid support. In 2003, Calin and co-workers[108] demonstrated the synthesis of a 30mer mannoside by this method.

Similar to DNA-arrays, glycan-arrays can be synthesized in situ or with presynthesized glycans by contact or noncontact printing. This procedure is laborious, but the in situ synthesis of glycan arrays is still challenging. Moreover, pure glycans are certainly preferred to study interactions,[59b] which requires either high synthesis yields on the surfaces or a final purification of the glycans on the array generated in situ.

In 2002, Wang et al. printed glycan arrays with ≈20 000 spots. These oligosaccharides were immobilized on glass surfaces[109] or nitrocellulose membranes[110] by hydrophobic interactions. Adams and co-workers[111] and Fukui and co-workers[110] synthesized glycan arrays to study the protein–glycan interactions that are relevant for the scrutinized infectious disease.[112] For the immobilization on the surfaces, different covalent linkage methods were used,[113] for example, aldehyde/hydrazide chemistry,[114] maleimide/thiol chemistry,[115] and amino/*N*-hydroxysuccinimide (NHS) ester or amino/epoxy chemistry.[116]

Fluorescence spectroscopy and MALDI were chosen not only for the quality control of the glycan synthesis, but also to

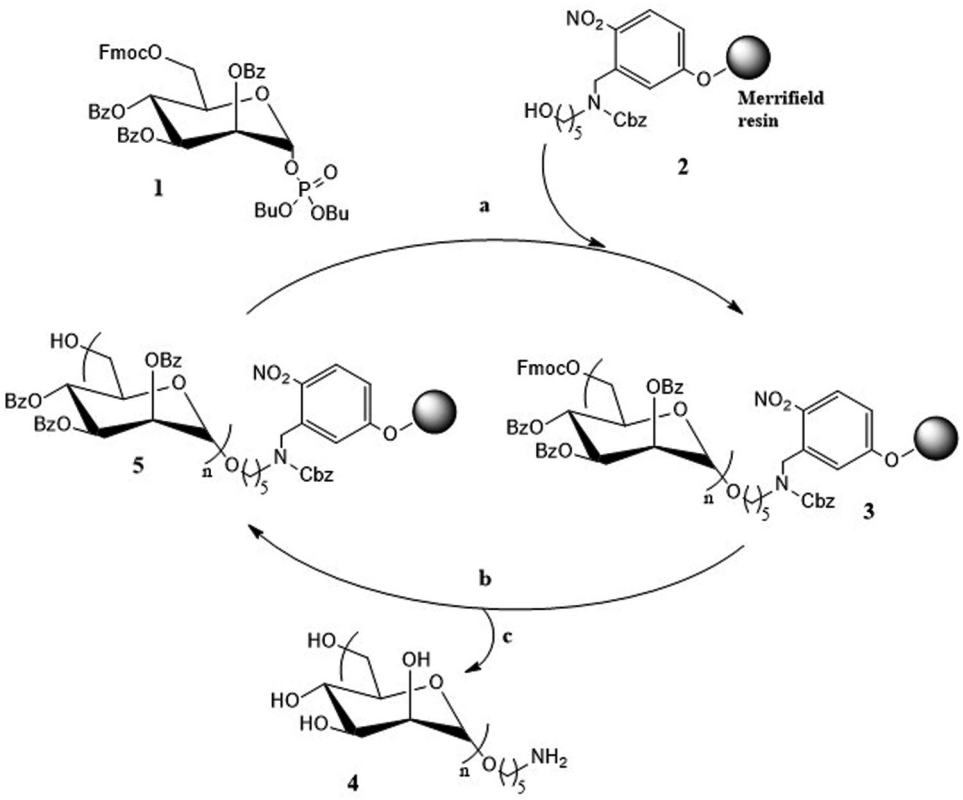

Figure 6. Automated synthesis of α-(1,6)-polysaccharides (**4**): a) **1**, TMSOTf, dichloromethane (DCM), –15 (45 min) –0 °C (15 min); b) Fmoc-deprotection of **3**: piperidine in DMF, 25 °C, 5 min; c) cleavage: *hν*, DCM; sodium methoxide (NaOMe), methanol (MeOH); Pd/C, H_2, H_2O. (Bu = Butyl: Cbz = benzyloxycarbonyl).

detect binding molecules. Numerous groups have used MALDI to investigate the interactions of proteins to glycans immobilized on arrays[94,117] and also to measure enzyme activity; for example, studies with glycosyltransferase on an array.[94,118,119]

In 2008, Ban and Mrkisch[96] synthesized in situ an oligosaccharide array on a robust gold surface to study enzyme activity. The surface was functionalized with an alkanethiolates with 10% phenol groups to couple the first building block. The monomer was activated as trichloroacetimidates for the immobilization on the surface and contained a temporary C5 levulinate (Lev) group. The remaining hydroxy groups were protected with acetyl (Ac) groups. The repetition of the two steps: glycosylation and Lev group deprotection via hydrazine allowed the synthesis of the oligosaccharide. The deprotection of the base-labile groups and the cleavage of the product were done via sodium methoxide. The discovery of SAMDI (Figure 3) enabled the direct reaction control after each coupling step. However, the method certainly needs to be optimized further, as the configuration of the anomeric coupling is not easily predetermined.

3.4. Peptide and Protein Arrays

Life sciences have been the drivers in the development of oligonucleotide arrays, and a similar desire to analyze millions or even billions of different proteins or peptides for biological activity has motivated scientists to advance techniques that would satisfy this desire. Indeed, proteins fulfill most of the tasks in a living organism, for example, as transporters, antibodies,[120] structural elements,[121] and catalysts[122] and in DNA replication.[123] Usually, the first step toward a better understanding of these biological functions is to study protein–protein interactions. Again, Ekins' concept of the array has guided many researchers. Büssow and co-workers[124] were the first to grow bacteria that expressed protein fragments on nitrocellulose sheets to generate protein arrays. Bacterial clones were simply lysed, and no further purification was done to enrich the expressed protein fragments. The simplicity of this method comes at a cost: the expressed proteins must compete with the host's proteins for binding on the nitrocellulose. Moreover, human serum antibodies must be preabsorbed with *Escherichia coli* proteins to remove anti-*E. coli* antibodies. Zhu and co-workers[125] modified this approach by growing bacterial clones that each expressed a different yeast protein in 96-well plates. Thereby, 5800 yeast open reading frames were expressed (93,5% of the total genome), and the expressed proteins were purified with the help of an N-terminally fused GST tag. They used these "purified protein microarrays" to screen for calmodulin and phospholipid-interacting proteins. Crompton and co-workers[126] produced similar arrays to display 1200 from a total of 5400 proteins from *Plasmodium falciparum*, the causative agent of malaria. When they stained these arrays with ≈200 different malaria sera they found a stunning number of 491 immunogenic proteins (≈40% of tested proteins reacted with at least one serum). Interestingly, when Jaenisch[127] and co-workers stained high-density arrays with *P. faliciparum*-derived peptides, they also found a very high percentage of immunogenic peptides, which might explain the observation that many malaria patients have considerably elevated levels of IgG antibodies in their blood—obviously this pathogen reliably re-infects humans, although it induces a plethora of different antibodies that are directed toward the erythrocyte stage of the pathogen. Meanwhile, the Arrayit[128] corporation offers arrays that display >20 000 different human proteins (an estimated 89% of full-length human proteins) and a set of different pathogen protein arrays that all cover the majority of the different pathogen's proteins. Similar to oligonucleotide arrays, spotting high-density protein arrays demands the generation of individual clones. Each of these clones expresses a different protein, but expression levels vary widely depending on the expression system, codon usage, background proteases, proper folding, post-translational modification, generation of inclusion bodies, and intrinsic stability of expressed proteins. Therefore, these "purified protein microarrays" are usually stored at –80 °C, and must be used directly after thawing. Plasmid or oligonucleotide arrays do not have these stability problems, which has sparked the idea of first producing an array of plasmids, double-stranded PCR bands, or mRNA that code for the different proteins, and then employ in vitro transcription/translation systems to produce the corresponding protein arrays on demand (reviewed by He et al.[129] and Yu et al.[130]) Different systems have been developed to capture in vitro translated proteins nearby their arrayed coding DNA. One system coarrays different PCR products, which all include a T7 promoter and a tag, for example, the his-tag with the capturing agent, e.g., Ni-NTA surfaces, before an in vitro transcription/translation system starts to translate the different DNA molecules to the corresponding proteins. Thereby, the newly formed proteins diffuse only a limited distance before they are trapped by the capturing agent.[131] This elegant approach offers some clear advantages: arraying DNA molecules is a standard technology by now; these arrays are stable when stored over long time periods; usually in vitro translated proteins are less prone to aggregation; and a wide variety of different in vitro transcription/translation systems are available, for example, from *E. coli*, yeast, and rabbit reticulocytes, to mention a few. Using such an approach, Hufnagel and co-workers[132] translated the proteome from the bacterial pathogen *Chlamydia trachomatis* into protein arrays to find immune dominant antigens in infected women. They tested the quality of their in vitro translation process by staining two different N- and C-terminally added tags in every expressed protein, and, indeed, thereby they could show that nearly all of the PCR fragments yielded full-length proteins. The pathogen they scrutinized is a nicely example of the strength of protein-array method: it was suspected before that *C. trachomatis* infection and cervix carcinomas influence each other, but it was not known if there was a differential humoral immune response, and which of the pathogen's proteins were differentially targeted. Using their arrays, they could clearly define *C. trachomatis* proteins that diagnose general infections, persistent infections, or an additional cervical cancer. A variant of this method has been developed by LaBaer and co-workers. They coined the expression "nucleic acid programmable protein array" (NAPPA) for a method that would further limit lateral diffusion of in vitro translated proteins. They structured the glass slide with different biotinylated coding plasmids that were captured and fixed at the spotting site by immobilized avidin. Thereby, they could add the in vitro transcription/translation system without diffusing the different

plasmids. Again, in vitro translated proteins were captured near their coding DNA via an expressed GST-tag, this time using an anti-GST tag antibody.[133] Yet another method transcribed different mRNAs from a library of plasmids, and spotted these different mRNAs onto a glass slide, where they were captured via a single-stranded DNA that hybridized to the mRNA's 3′-end. An in vitro translation system was then used to translate the different mRNAs at "their" spots to a protein. Tao and Zhu employed a brilliant trick to fix the in vitro translated proteins directly to aforementioned single-stranded DNA: When reaching the RNA/DNA hybrid region, the ribosome stalls and transfers the nascent protein to a puromycin that was coupled before to the single-stranded DNA.[134] Early reviews from the pioneers in protein arrays were given by Walter and co-workers[135] and Zhu, Snyder, Sutandy, and co-workers.[136]

Smith[137] developed the phage display technique to fuse the to-be-expressed gene to a protein that is anchored on the phage's surface. Thereby, the expressed protein or peptide carries its own gene as a backpack. Meanwhile, and commercialized by Dyax Corp., peptide libraries are available that display billions of different peptides that could be screened for binders. Finally, McCafferty et al. and Breitling et al. varied that approach to display billions of different antibody fragments on a phage[138] or phagemid[139] to screen for recombinant antibodies. In 2018, George P. Smith and Gregory Winter were awarded the Nobel prize for these achievements. However, the focus of this review is on highly parallelized and miniaturized chemical synthesis, and phage display has been reviewed elsewhere.[140]

Ronald Frank invented peptide arrays some 30 years ago.[141] In 1988, small pieces of paper were immersed consecutively in the 20 different solutions that contained the different building blocks for peptide synthesis to generate unique peptides per piece of paper with only 20 different solutions, and in 1990—similar to Edwin Southern's invention of oligonucleotide arrays—he modified that approach by spotting these 20 different Fmoc-amino acid pentafluorophenol (Pfp)-esters in *N*-methyl-2-pyrrolidon (NMP) at discrete locations onto a larger piece of paper that was derivatized with amino groups.[141c]

All the other processing steps were identical to standard Merrifield synthesis: acetylation of the residual, unreacted amines was carried out with 2% acetic anhydride in DMF, and Fmoc-deprotection with 20% piperidine in DMF. Thereby, Ronald Frank's SPOT synthesis technique supplied the scientific community with high-quality peptide arrays, even though the level of miniaturization was moderate with only 25 spots per cm^2.[141b] This technique dominated the field of peptide arrays for the next 20 years. Compared to phage display it had two decisive advantages: binding peptides could be immediately identified, and, contrary to phage display, it was easy to also build in unnatural building blocks, as well as other organic compounds.[141a] In 1993, the first SPOT-synthesizer (ASP222, 25 000 spots on 30 cm × 30 cm) was commercialized by ABIMED Analysen-Technik, making array synthesis feasible to any research laboratory.[141a] Interestingly, although patented as early as 1994,[142] and contrary to oligonucleotide arrays, high-density peptide arrays that were in situ synthesized with an inkjet printer did not materialize for a long time period. Only very recently, Lam and co-workers published the combinatorial synthesis of peptide arrays with the help of a modified inkjet printer.[143]

Still, high-density, high-quality peptide arrays are needed for many different applications, especially when precious biological samples are only available in low quantities. To name a few: peptide arrays were used to find binding partners for patient- and for monoclonal antibodies,[60,144] interaction partners for double-stranded DNA,[145] metal ions,[146] to detect enzyme activity,[147] and cell adhesion (**Figure 7**).[148] In order to detect

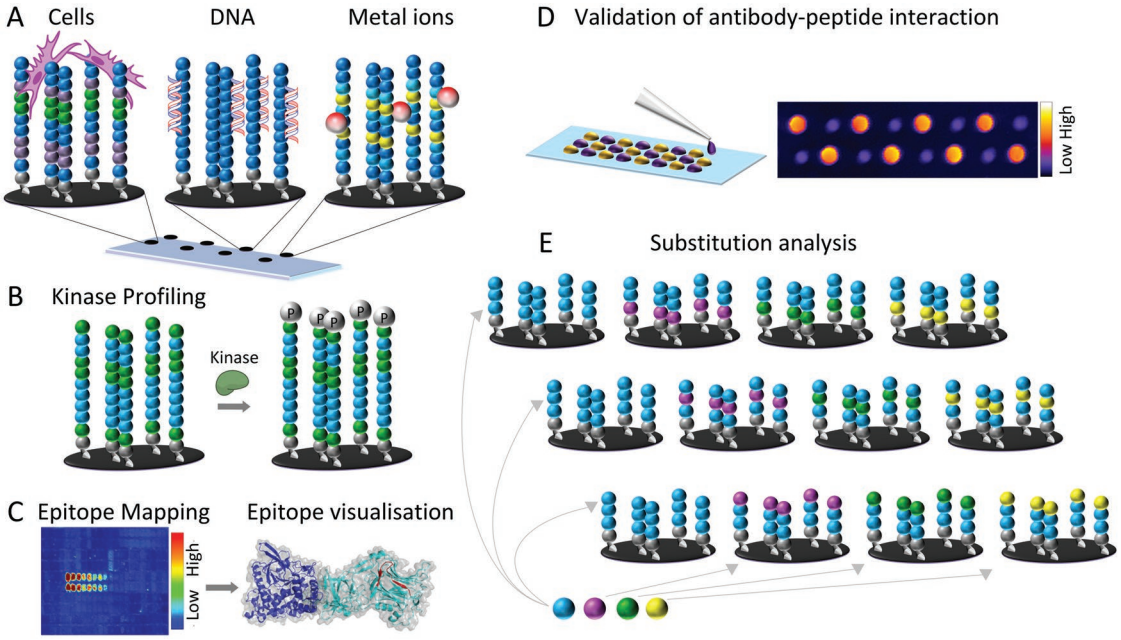

Figure 7. Applications for peptide arrays. A) Interactions via metal ions, cells and DNA. B) Enzyme activity such as phosphorylation via measuring kinase activity. C) Epitope mapping and visualization. D) Validation of antibody peptide interactions. E) Substitution analysis. C–E) Reproduced with permission.[153] Copyright 2017, Wiley-VCH.

these biological activities, for example, radioactivity,[148b,149] fluorescence spectroscopy,[148b,150] surface plasmon resonance, and MALDI-MS[147a] were used. To generate such high-density arrays, individual peptides were synthesized by SPOT synthesis in a low-density format and with amino-derivatized cellulose as solid support. Next, individual peptides were solubilized by partially degrading the cellulose support before they were spotted in densities of up to 10 000 peptides per cm^2.[62b,151,152]

Due to the cellulose moieties, these peptides were easily immobilized onto a glass surface by physical absorption.[141a,154] Alternatively, biological interactions (biotin/streptavidin[155] or avidin/biotin[156]) or covalent linkage (amine/aldehyde,[157] maleimide/thiol,[158] or amine/carboxy[159] chemistry) were used to tether these peptides to a surface and in array format. When synthesized by SPOT synthesis, chemical costs are estimated to be ≈1€ per peptide. Spot sizes of 100 pL were achieved by Pan and co-workers[160] with a printing platform that integrated microfluidic elements. In 2018, the group[161] presented an automated microdisk array preparation and screening platform that allowed them to synthesize 2500 peptides per 15 cm^2. The different reaction sites all form individual reservoirs that are supplied with the different amino acid building blocks via microfluidic channels (30 nL per cycle). As proof of concept, Pan and co-workers synthesized a peptide library and investigated the interaction of integrin proteins with the printed peptides.

In 2018, Atwater, Mattes, and co-workers[162] proved that very high-density peptide arrays indeed could be in situ synthesized by preparing a peptide array with a pitch of 40 µm (62 500 per cm^2) via microchannel cantilever spotting (µCS). However, µCS and other known printing technologies (e.g., dip-pen nanolithography,[163] or polymer-pen lithography)[164] are too slow to produce affordable very high-density peptide arrays.

Legutki and co-workers[90] and others[165] combined the approach from Fodor and co-workers with the photoacids that were introduced by Pellois and co-workers[62a,81] and with maskless photolithographic techniques[62b,165] to synthesize very high-density peptide arrays. Moreover, the company LC Science[166] commercializes such peptide arrays (30 000 per chip). It should be mentioned that the use of photoacids to cleave standard protecting groups comes with another drawback: it is difficult to remove the locally generated acids without hampering neighboring synthesis sites. Therefore, several of these modern lithographic synthesis methods use sophisticated microfluidic systems to remove light-generated acids in the *z*-direction away from the synthesis sites. Another challenge is to demonstrate the quality of peptides in these very high-density peptide arrays. Legutki and co-workers[90] did that by MALDI-MS-imaging.

These very high-density peptide arrays have some very big advantages compared to phage display techniques. While it is very difficult to do phage display with posttranslational modified peptides—that seem to play a role especially in autoimmune diseases—Price and co-workers[167] synthesized silicon-based peptide arrays with 10 000 posttranslational modified peptides for mapping antibody-binding sites. Legutki and co-workers[90] synthesized 660 000 peptides per cm^2. If stained with patient serum, these are so many peptides that simply, by chance, many of the patient's antibodies will find a binding partner. Legutki and co-workers[90] and others[152b,165b,168] used these arrays to correlate found "immune signatures" with the patient's diseases to eventually get a clue as to the cause of the investigated disease.

Experiments of this type will certainly pave the way for the commercial success of peptide arrays that still considerably lags behind oligonucleotide arrays. Currently, such arrays are either too expensive due to low density, or the quality of lithographic synthesized arrays is questionable. Therefore, the Breitling group developed yet another method to synthesize high-density and high-quality peptide arrays.

This solid-material-based synthesis method[61,144,169] employs a matrix material that is solid at room temperature with the different amino acid building blocks embedded within. Solid materials do not spread or diffuse laterally on a surface. If compared to spotting tiny liquid droplets, it is much easier to structure a surface with small spots of different solid materials. Moreover, chemicals that are embedded within these solid materials do not diffuse, and these spots do not evaporate, which gives an experimenter plenty of time to complete the structuring without the fear that solvents will evaporate or some of the building blocks will start to couple to the surface before the structuring is completed. Another big advantage is that the employed matrix material obviously shields quite reactive chemical building blocks from decay when stored in solid form.[169b] The basic trick of solid-material-based synthesis is simple: only when the structuring with the different chemical building blocks is completed, a heating step suddenly mobilizes hitherto immobilized amino acid building blocks for all of the reaction sites at once. They are then free to diffuse to the solid support where they couple to free amino groups on the solid support (**Figure 8**).

There are many different ways to structure a solid support with different solid materials. One approach employed the pixel electrodes of a computer chip to either attract or repel charged "amino acid particles" from an aerosol. Thereby, peptide arrays with 40 000 different peptides per cm^2 were synthesized.[169a] Even more peptides could be synthesized when first spreading a particle layer over the solid support, and then melting and gluing these particles with single laser pulses at selected areas to "their" synthesis sites.[61b] However, neither of these two methods led to a commercial product. Much more successful in this respect was another approach that used a 24-color laser printer to deposit the different amino acid particles at their designated synthesis sites.[169b] These laser-printed peptide arrays have a density of 1000 peptides per cm^2. They are commercialized by PEPperPRINT GmbH. The machine is equipped with 24 printing units, each containing another type of amino acid particle. The printing mechanism in this machine is based on a standard laser-printing process: Each printing unit has an organic photoconductor drum (OPC) that is heavily charged with the help of a corona that repels triboelectrically charged amino acid particles. This OPC drum is illuminated at selected areas with a light source, in order to neutralize selected areas on the surface of the OPC drum. The amino acid particles then stick to these neutralized areas. Next, the resulting particle pattern is transferred to a solid functionalized carrier, once again employing a strong electrical field. At the center of this machine is the light source that is used to generate the electrostatic pattern on the OPC drum: it consists of a ≈20 cm long row with

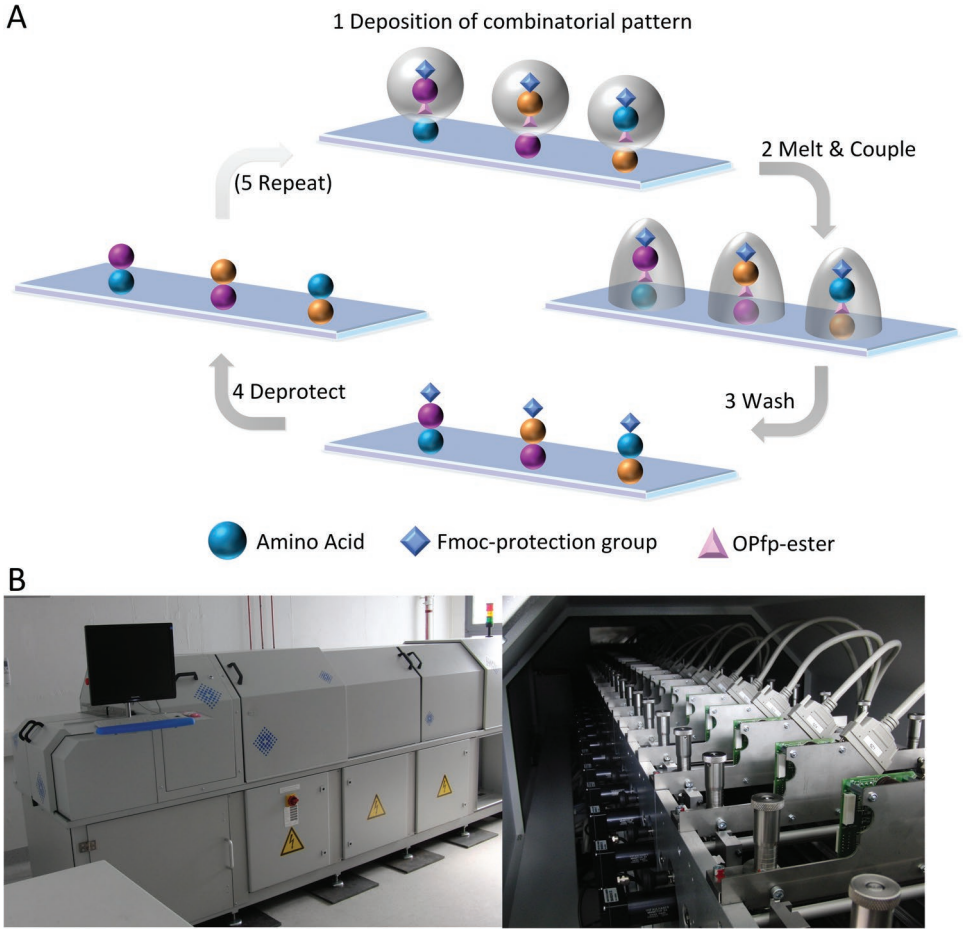

Figure 8. Solid-material-based synthesis. A) A laser printer deposits toner particles consisting of a matrix material, OPfp-activated and Fmoc-protected amino acid derivatives, at designated synthesis sites. After deposition of all 20 amino acid particles (example illustrated with three colors for three different amino acids) of each layer, these are all melted at once to let hitherto-immobilized monomers diffuse and couple to the surface. After coupling, residues of the matrix material are washed away, remaining coupling sites are capped and the growing peptide chain is deprotected to be prepared for the next layer to be coupled. B) View of the peptide laser printer and a detailed look at its 24 printing units. A) Reproduced with permission.[169b] Copyright 2008, Wiley-VCH.

10 000 individually steerable light-emitting diodes (LEDs) from the OKI C7400 color laser printer.

Yet another method to structure a solid support with matrix-embedded amino acid building blocks was published by Loeffler and co-workers in 2016.[61a] This time, the same solid material that was used in the "peptide laser printer" was spin-coated onto a thin Kapton foil as a 1–2 µm thin material layer. A 2D laser scanning system was developed to illuminate selected areas on this "donor foil" with individual laser pulses. These pulses are absorbed by the Kapton foil, and, thereby, "translated" to site-specific heat packages that propagate to the material layer on the surface to site-specifically "punch out" tiny material spots from the material layer. This material is deposited as a thin layer onto an acceptor slide where peptide synthesis takes place, with a maximal distance of 40 µm between donor slide and acceptor slide. With this approach, Löffler and co-workers synthesized arrays with >17 000 peptides per cm^2 and in good quality. Moreover, this method is especially easy to automate: donor and acceptor slides are easily exchanged by a robot, while the structuring is done by simply writing a second pattern with the 2D laser-scanning system that would transfer a second material on top or next to previously transferred materials.

At least for those materials that were analyzed in more detail, Löffler, Förtsch, and co-workers realized that transferred material spots were nanoscale in the *z*-direction (**Figure 9**). Moreover, the energy of the laser pulse determines the amount of transferred materials. In other words, the amount of chemical building blocks that are deposited in their respective synthesis sites is easily tuned by adjusting the laser's pulse energy, and this can be done with many different chemicals, and in any combination. Indeed, Löffler and co-workers[61a] clearly showed that a nanoscale activation layer (*N*,*N*′-diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt)) stacked over nanoscale matrix-embedded nonactivated Fmoc-protected amino acid building blocks could couple these in situ activated amino acids to the solid support. In 2018, Mattes et al.[170] used the 2D laser-scanner system to stack up to four different nanoscale material layers on top of each other to generate peptoid arrays via the monomer and submonomer method.

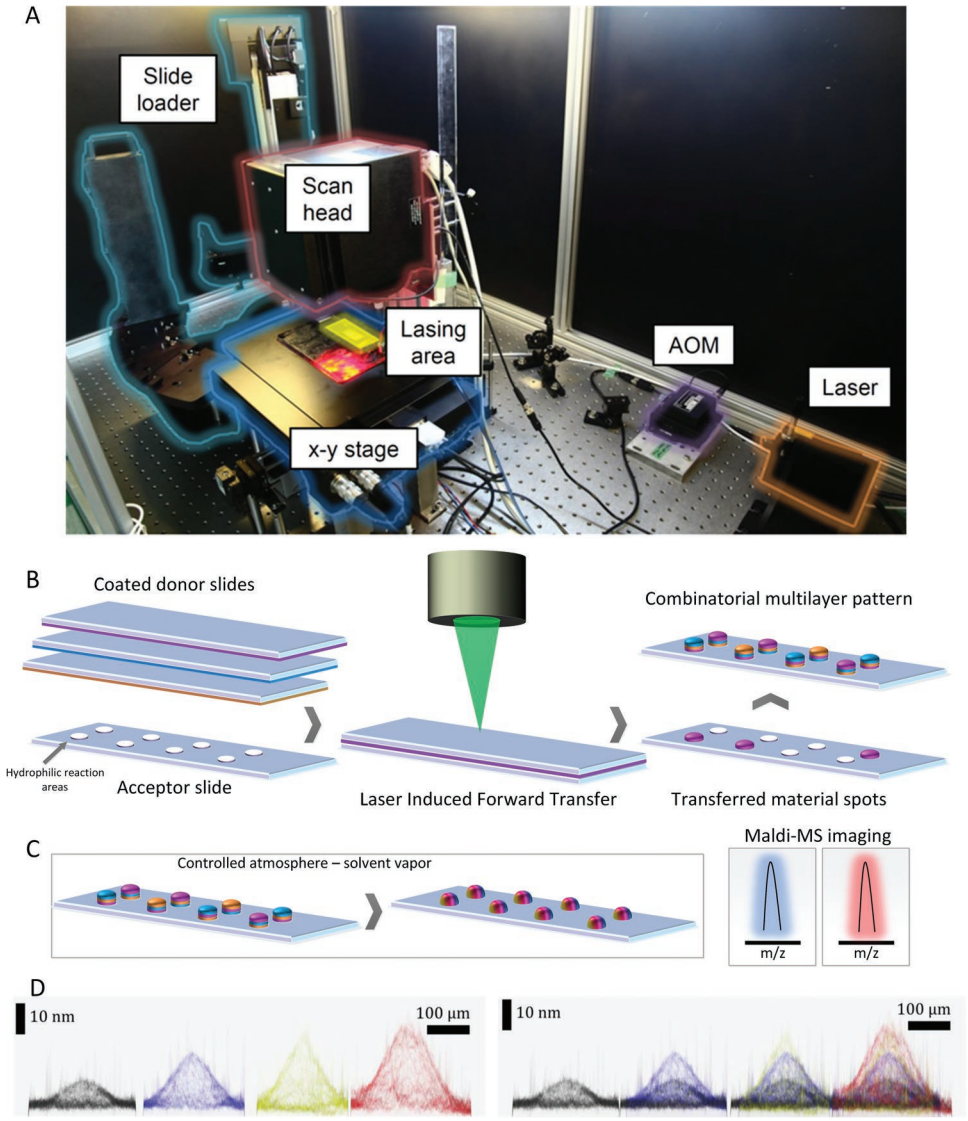

Figure 9. Laser-induced nanoprinting (in the *z*-direction). A) View of the nano-3D-printer with its different functional units (AOM = acousto-optic modulator). B) A coated donor slide is positioned over an acceptor substrate. Next, single laser pulses from a 2D laser scanning system "punch out" tiny material spots that are transferred to the acceptor slide. Simply by repeating this processing step with additional donor slides leads to the structuring of the acceptor slide with many different nanolayers that comprise polymer-embedded, and, thereby, stabilized chemicals. These solid nanolayers are stacked by the robot in freely chosen combinations and stoichiometry. C) When melted by solvent vapor in a controlled atmosphere, extremely miniaturized chemical reactions are started in the array format. Removal of solvent vapor stops these chemical reactions, and additional chemicals can be added to do multistep one-pot reactions. MALDI MS imaging can be used to analyze reaction products. D) Transferred material spots are nanoscale (measured with vertical scanning interferometry). The transfer of a first, a second, a third, and a fourth layer of matrix material (the material that is used to embed Fmoc-amino-acid building blocks) is shown. Stacked layers from 1×, 2×, 3×, 4× printing are depicted in different colors to highlight added material. A) Reproduced under the terms of the CC-BY 4.0 Creative Commons Attribution International License (http://creativecommons.org/licenses/by/4.0/).[61a] Copyright 2016, Springer Nature. D) Provided by and used with the permission of Dr. Tobias Förtsch.

Upon melting, Fmoc-protected N-substituted glycine derivatives, embedded in matrix material, diffused through three nanometer-thin layers into an immobilized polymer film on a glass slide. These nanolayers harbored different chemicals to activate the building blocks for peptoid synthesis during their passage through these activation layers. The verification of peptides was done via MALDI-MS imaging. The necessary MALDI matrix material was laser-transferred to ablate a thin amount of the matrix on top of the peptide spot to generate good m/z signals. Future experiments certainly will try to implement this approach beyond peptide, peptoid synthesis, and solid-phase synthesis, for example, by doing multistep one-pot reactions in many different reaction sites that are isolated from each other with the help of hydrophilic/hydrophobic patterns.

3.5. Microfluidics

Microfluidic devices find wide applications in nanoparticle synthesis,[171] organic chemistry,[172] combinatorial chemistry,[161,173]

and cell[174] and biological[175] assays. In 2006, Whitesides[176] predicted a prominent role for microfluidics in miniaturizing chemical reactions, but this prediction has not yet come true. Indeed, nowadays, microfluidics chips exist that reliably generate and move thousands or even millions of monodisperse droplets in the micrometer range. These oily droplets are immersed in an immiscible aqueous carrier medium (or vice versa) to create many confined microreactors, whereby the interface is usually stabilized by surfactants. They can be loaded with building blocks for chemical synthesis, reagents for analytical assays, or even cells for biological assays (**Figure 10**a). These droplet-based microreactors have some unique features when compared to batch chemical synthesis in a flask: they are tiny (≈10 µm-sized droplet translates to a reaction volume of ≈1 pL), which translates to low costs per chemical reaction. Moreover, better heat and mass transfer should lead to faster chemical reactions. Finally, scientists have developed methods to fuse selected droplets, which could be used to start a chemical reaction. There are a few drawbacks though: surfactants restrict the use of microreactors; for example, a reaction product should not destabilize the interface, nor should the surfactant interfere with the chemical reaction. Another restriction comes from the surrounding liquid that should not be a solvent for any of the reaction partners or products.

Indeed, in digital microfluidics,[177] individual microdroplets can be manipulated via electric voltage such as droplet transfer, droplet mixture, and separation in multistep processes. Such a technique could be used to freely combine different building blocks for chemical synthesis.[178] However, to date, digital microfluidics is not yet suitable to setup thousands of different miniaturized reaction mixtures, due to the too slow handling of thousands of droplets in a short time period.

Obviously, one important feature is still missing: procedures are needed to first generate, and then store many different kinds of droplets in the microfluidic system, before different kinds of droplets with their different chemicals inside are fused to initiate many different chemical reactions. Kaminski and Garstecki[179] proposed a concept to do that: larger reservoirs inside the microfluidic system could be filled with the different chemicals dissolved in a suitable solvent. The individual reservoirs are then used to form small droplets that are transported to the reaction site, where they are fused with other droplets to start the chemical reaction.[180] However, since the chemicals inside influence the droplet's surface tension, it is

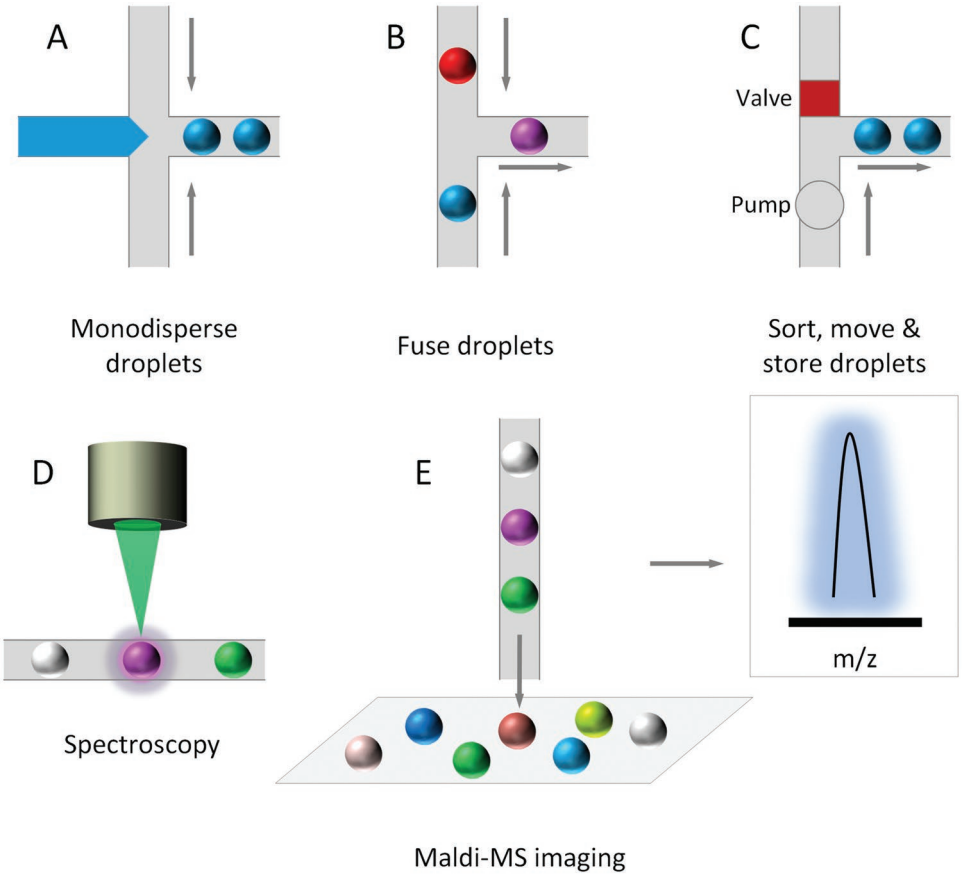

Figure 10. Microfluidics offers all the elements that are needed to do many extremely miniaturized chemical reactions while efficiently shielding reactive chemicals from the environment. A) Cross-junctions or T-junctions can generate monodisperse droplets that harbor exactly defined amounts of chemicals. B) Selected microdroplets can be fused to start a chemical reaction in a defined reaction sphere, before they are moved to a "parking place." C) In-chip-integrated microactuators and valves will allow the movement of and sort selected microdroplets at will. D) Reaction spheres can be monitored over time by spectroscopic methods. E) Reaction products can be patterned onto a slide for MALDI-MS imaging.

difficult to create droplets of similar size from different reservoirs and using only one pressure pump.

This difficulty could be solved by the integration of several microactuators inside the chip.[181] These actuators could tune the applied pressure for the individual reservoirs to generate monodisperse droplets from each reservoir. The near future will certainly see chips with extremely miniaturized pumps that are integrated into microfluidic systems,[182] and one application certainly will be to form droplets of defined sizes from different reservoirs inside the microfluidic system. Fusing two of these droplets would then create a unique mixture of chemicals to start a chemical reaction (Figure 10b). Next, one of the in-chip micropumps could move these fused droplets to a side branch of the microfluidic system and store them there to let the chemical reaction proceed (Figure 10c). For monitoring the reaction spheres, spectroscopic methods can be used (Figure 10d). Finally, the pump could resume its activity; for example, to spot the individual droplets in array format to a gold-covered glass chip, add the MALDI matrix, and determine the masses of synthesized molecules by MALDI-MS imaging (Figure 10e).

Currently, most microfluidic chips are made of an elastomer such as poly(dimethylsiloxane) (PDMS)[183] or off-stoichiometry thiol-enes (OSTEs).[184] Compared to sophisticated chip technology, such PDMS chips are easily manufactured,[185] and, therefore, have dominated the research area in recent years. These PDMS-based chips are not rigid, which translates to significant pressure decline over distance. That in turn makes it difficult to guarantee repeatable flow conditions in sophisticated chip designs. In addition, the completely different ways to manufacture them make them incompatible with the in-chip-micropumps that were described by Uhlig et al.[182] Moreover, PDMS is unstable for many organic chemicals, which certainly makes borosilicate glass microfluidic chips the preferable choice for chemical synthesis. These glass chips are synthesized via wet[186] or dry[187] etching, which are procedures that are available to only a few laboratories. Meanwhile such chip designs are commercially available, but their production used to be too expensive to rival the flexible design of PDMS-chips. In 2016, Kotz et al.[188] invented a procedure to print glass, and, thereby, opened a route to easily design and manufacture glass-based microfluidics systems that is as flexible as the production of PDMS chips. The combination with a method to store different types of microdroplets, for example, by printing microfluidic channels onto a chip with integrated micropumps, means that these glass-chips indeed could be used to set up tens of thousands extremely miniaturized chemical reactions.

4. Conclusion and Outlook

We have described selected examples in the automation and in the miniaturization of chemical synthesis. We think that the future will see a merger between these two different trends, i.e., automated synthesis of many different chemicals and in many miniaturized reaction sites. While miniaturization of chemical reactions was initially driven by a desire in the life sciences to synthesize and analyze many different oligonucleotides or peptides, the driving forces that have advanced the automation of chemical reactions are manifold.

4.1. Automation in Chemistry

In the past, chemical companies invested into the automation of chemical reactions to improve the time and cost efficiency of chemical synthesis. This was and is important to meet the need for new compounds and large libraries of different compounds that are not available with reasonable effort by standard synthesis techniques. Other benefits of advances in automation techniques are an improvement of safety standards, which is especially important for reactions that include hazardous substances and an improvement of the reproducibility of the reactions in contrast to a manual synthesis. In particular, the latter arguments are relevant in academia as well, and have led to developments and applications of automated synthesis in universities. Especially flow chemistry gives the opportunity to do an upscaling of synthesized chemicals in an automated fashion, which was yet another reason that drove automation in chemical synthesis. While in the past, automation in particular in pharmaceutical research was driven due to the need of chemical compounds for biological screenings, the past developments are now ready to be used and advanced by the need of chemical compounds for materials development. The growing numbers of cheminformatics tools and prediction models allow the virtual design and evaluation of virtual compound libraries that are supposed to provide suitable structures for future developments. For those new virtual libraries, novel retrosynthesis models allow the automated analysis of the most suitable reaction paths without the need for a design by a chemistry expert. In combination, these AI-driven developments enforce the need for efficient synthesis methods and automation for materials sciences in the future to be able to evaluate the virtually predicted applications. At the moment, the necessary amount of chemical substance for the testing of properties in materials sciences is still high and does not correspond to the substance efficiency that has been achieved in biological screenings in the last decades. Therefore, to date, automation techniques resulting in the isolation of substances in multi-milligram to gram scale are of high importance for materials science. In future, approaches in miniaturization of the testing procedures and techniques will most likely result in a drastically reduced amount of necessary substance for a validation and evaluation of properties. Miniaturization strategies that are already known for the synthesis of peptides, oligonucleotides, sugars, and other substances or are in development at the moment may have high impact on the future development of materials sciences.

4.2. Miniaturized Chemical Synthesis

Some very impressive examples from the life sciences prove that chemical synthesis indeed can be drastically miniaturized: i) based on the seminal work of Edwin Southern and Stephen Fodor, nowadays whole genomes are assembled from synthesized oligonucleotides, and ii) based on the work of Ronald Frank, we are not too far away from translating whole

proteomes into arrays of overlapping peptides, while others have employed cells to synthesize whole genome protein arrays. Certainly, we can expect this type of approach to be used in the near future to in situ synthesize arrays that display tens of thousands of oligosaccharides, peptides, proteins, peptoids, and other oligomers that are of interest for the life sciences. Such arrays can be used, for example, to synthesize whole genomes, or pinpoint antibody-binding structures that are especially good vaccine candidates.

However, simple screening for binding antibodies or complementary cDNA—and ignoring sites where synthesis failed—is not sufficient if the task is to find and optimize novel synthesis routes. For such a task, an experimenter must first find optimized conditions for the different steps in a synthesis route, and then combine several of these synthesis steps; for example, in solid-phase synthesis of oligomers, or in sophisticated multistep one-pot reactions. Clearly, the experimenter must find out which of the different reaction conditions gave the desired product(s) for the different synthesis steps. Otherwise, false-positive reaction conditions would drastically augment the number of erroneously designed multistep reactions, and, thereby, invalidate the advantages of high-throughput experimentation with miniaturized synthesis sites. Therefore, one important task for highly parallelized and miniaturized synthesis is to find out for thousands or even millions of reaction sites, if a molecule of the expected molecular weight has been synthesized or not. Indeed, the seminal work of Lin et al.[3d] showed that MALDI-MS could deliver that information for miniaturized chemical reactions.

Another task is to mix different chemicals in freely chosen and exactly tunable stoichiometry, and then have them reacting for a defined time span. Doing that in extremely miniaturized chemical synthesis adds yet another challenge: reactive chemicals must be shielded from the environment during the processing steps. The nano-3D printer (Figure 9) and microfluidics (Figure 10) are especially suited to perform this task. The nano-3D-printer stacks the different chemicals in solid, i.e., protective nanoscale layers on top of each other, and then starts the many different chemical reactions by either melting the solid material, or by adding solvent vapor in a controlled environment. Microfluidics can be used to create and fuse droplets with exactly defined size, which are used as miniaturized reaction spheres that are efficiently shielded from the environment. A very charming feature of this approach is that all of these individual droplets can be analyzed by spectroscopic methods while still inside the microfluidic system. The recent invention of in-chip actuators that could be used as micropumps could supply the only thing that was missing so far: an easy method to store different sorts of droplets within the system before they are spotted to discrete sites on a glass slide to analyze the outcome of the different chemical reactions by MALDI-MS imaging.

Certainly, and similar to the life-sciences-driven approaches, the driver for miniaturized chemical reactions will be screening for function. The reason is simple: funding agencies and venture capital will increasingly fund only those projects that promise to solve major challenges that our world faces in the near future. Nearly always chemistry is and will be needed to tackle these challenges as some important examples show that: i) for more efficient regenerative energy, we need to improve organic or inorganic solar cells, ii) to transform our transportation systems, we need novel batteries and fuel cells, iii) to synthesize all kinds of raw materials (methanol, sugar, building blocks for polymers), we need novel catalysts that directly harvest the energy of the sun, and iv) for energy-efficient light-emitting diodes, we need innovative chemistries. All these examples demand that chemistry mates with the material sciences to synthesize as many different molecules as possible and screen them for function, which can only be done in miniaturized form.

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Conflict of Interest

Frank Breitling holds shares from SME PEPperPRINT GmbH.

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