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Particle-Based Synthesis of Peptide Arrays

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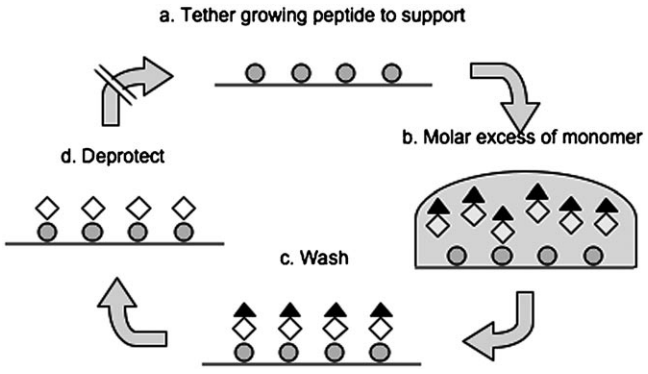
Dedicated to the memory of Annemarie Poustka

Lithographic methods allow for the combinatorial synthesis of $>50\,000$ oligonucleotides per cm^2, and this has revolutionized the field of genomics. High-density peptide arrays promise to advance the field of proteomics in a similar way, but currently lag behind. This is mainly due to the monomer-by-monomer repeated consecutive coupling of 20 different amino acids associated with lithography, which adds up to an excessive number of coupling cycles. Combinatorial synthesis based on electrically charged solid amino acid particles resolves this problem. A color laser printer or a chip addresses the different charged particles consecutively to a solid support, where, when completed, the whole layer of solid amino acid particles is melted at once. This frees hitherto immobilized amino acids to couple all 20 different amino acids to the support in one single coupling reaction. The method should allow for the translation of entire genomes into sets of overlapping peptides to be used in proteome research.

Introduction

Solid-phase peptide synthesis, later to win its creator a Nobel prize, was invented more than forty years ago, when Merrifield consecutively coupled amino acid monomers to a growing peptide chain immobilized on a solid support.[1] His approach allowed for especially easy purification of the growing peptide products, which remained tethered to the support throughout the synthesis, while all the unreacted monomers were simply washed away. Even more importantly, he was able to use an excess molar amount of a given amino acid monomer over the growing peptide chains to drive the coupling reaction close to completion (Scheme 1). This basic principle today routinely allows for repetitive coupling yields of $>95\,\%$ during peptide synthesis, and as a result the affordable peptides many laboratories use today.



Scheme 1. Merrifield synthesis. a) The growing peptide chain is fixed to a solid support. b) Each free N-terminal amino group at the tip of the growing chain reacts with excess quantities of a C-terminally activated amino acid derivative to drive the coupling reaction close to completion. c) Thanks to the linkage of the peptide to the solid support, excess quantities of monomer are easily washed away. d) A cycle of synthesis is completed when the transient N-terminal protecting group is removed. Repetitive coupling cycles generate a peptide that is finally deprotected of permanent side chain protecting groups and cleaved from the solid support.

Merrifield's approach was later expanded to establish the field of combinatorial chemistry, with processing of multiple reaction spheres in parallel to synthesize many different peptides.[2] These methods, always based on the principle of solid-phase synthesis, allow as many peptides as possible to be 1) synthesized, and 2) analyzed for—for example—individual peptides that bind to a target protein. An especially cheap, easy, and elegant procedure to generate huge libraries of different peptides is the one-bead-one-compound method, with many published reports of successfully screened peptide binders.[3] These peptide libraries are built by consecutive cycles of dividing the beads for a solid-phase synthesis between 20 different reaction vessels, in each of which one of the 20 different amino acids is added to the growing peptide chains on individual beads, and afterwards pooling them again before the next cycle begins. This procedure gives every bead its individual history of sequential stopovers in one of the 20 vessels, where this history is translated into a sequence of amino acids added to the growing peptide chain on the bead. Thereby, with the synthesis finished, nearly every bead displays a different peptide, but always only one kind of peptide per bead

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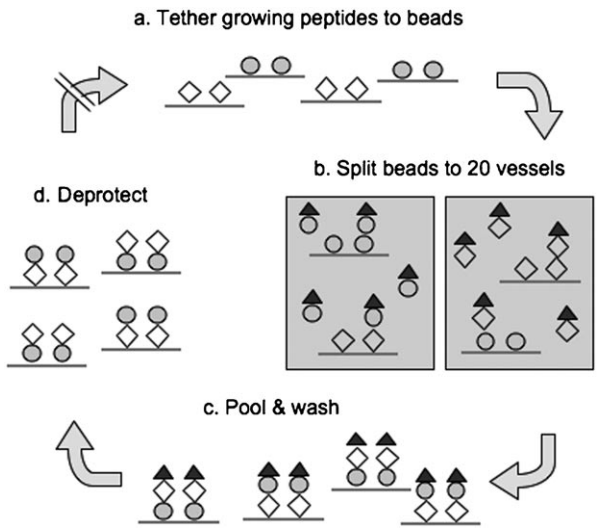
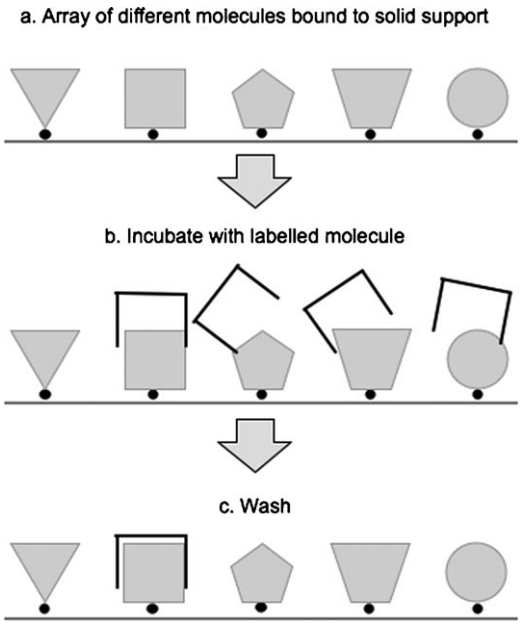


Scheme 2. One-bead-one-compound method. a) The growing peptide chains are fixed to a solid bead support (shown as a flat surface to illustrate similarities to the other methods). b) The beads are randomly distributed over 20 different reaction vessels (only two different vessels are shown). In each of the vessels the free N-terminal amino group is allowed to react with a different C-terminally activated amino acid derivative. c) Excess quantities of monomers are washed away, and the beads are afterwards pooled. d) A cycle of synthesis is completed when the transient N-terminal protecting group is removed from all of the beads. Repetitive coupling cycles generate a library of peptides with every bead displaying a different peptide. It is necessary to retrieve the sequence information of binding peptides from identified bead-binders.



Scheme 3. The array concept. a) Many different oligomers are linked to a two-dimensional solid support, each at a known position. b) When the array is incubated with a labeled molecule, the diffusing labeled molecule probes all the different oligomers on the array and eventually sticks to those with complementary surfaces. c) Excess quantities of labeled molecules are washed away and the labeled areas on the array are identified. Thereby, one single experiment reveals the oligomers that specifically bound to the labeled molecule (and in addition, also those oligomers that did not bind).

(Scheme 2).[4] Because of the random distribution of beads over the 20 different reaction vessels, however, it is nearly impossible to avoid problematic peptides during library preparation. These include, for example, insoluble peptides or those that bind to any protein, and thus elicit a strong background of false positive binders. Another drawback of this particular method is the labor-intensive encoding/decoding that is needed to obtain the sequences of those peptides that have bound to, for example, a target protein.

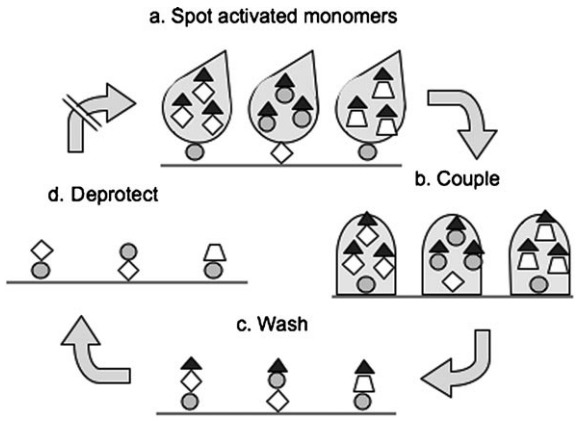
Peptide Arrays

SPOT synthesis

When an experimenter arrays different oligomers on a two-dimensional surface, he or she knows exactly the positions and the sequences of all of these different molecules. If such an array is then incubated with, for example, a labeled protein, the diffusing protein probes all the different molecules on the array and eventually sticks to those with a complementary surface: that is, it specifically binds them (Scheme 3). Thereby, with one single experiment the experimenter immediately knows the sequences of those molecules that bound to that particular protein (and in addition, also of those that did not bind). Moreover, the information increases with the number of arrayed molecules. This is the underlying reason why, ever since the invention of the array by Robert Ekins,[5] scientists have been striving to develop ever higher-density peptide or oligonucleotide arrays. Another advantage of the array concept over, for example, the one-bead-one-compound method is that those oligomers that bind nonspecifically to any protein are easily identified and can simply be omitted in the next array generation.

To achieve the goal of very-high-density arrays, Edwin Southern introduced the concept of in situ combinatorial synthesis of oligonucleotide arrays,[6] which was adapted by Ronald Frank to the synthesis of peptide arrays.[7] They paralleled the Merrifield synthesis shown in Scheme 1 by adding not one base or amino acid to the support, but instead patterning the four different bases or 20 different activated amino acid derivatives as small droplets on a flat two-dimensional surface. There, the bases or amino acids react with the solid support, with each droplet defining a small reaction sphere. Consecutively printed layers result in the parallel growth of many different oligonucleotide or peptide chains, in which the number of different oligomers is dependent only on the achievable miniaturization of individual spots (Scheme 4). Frank's SPOT synthesis has earned a reputation for reliability and wide applicability over the years and thus still dominates the field. High peptide densities of peptides synthesized in situ exceeding 25 peptides per cm^2, however, are difficult to obtain with this method, mainly due to the difficulties inherent in the handling of tiny droplets, which tend to evaporate or spread over the array's surface.

High-density peptide arrays can be generated with the SC^2 method.[8] This variant SPOT synthesis first involves the synthe-



Scheme 4. SPOT synthesis. a) A spotter addresses the 20 different C-terminally activated amino acid derivatives in liquid droplet form to defined areas on a solid support, where b) they couple to the support in parallel. A cycle of synthesis is completed when c) excess quantities of amino acid derivatives are washed away, and d) the transient protecting group is removed. Repetitive coupling cycles generate a peptide array, where the peptide sequence and peptide address on the support for each of the individual peptides is known.

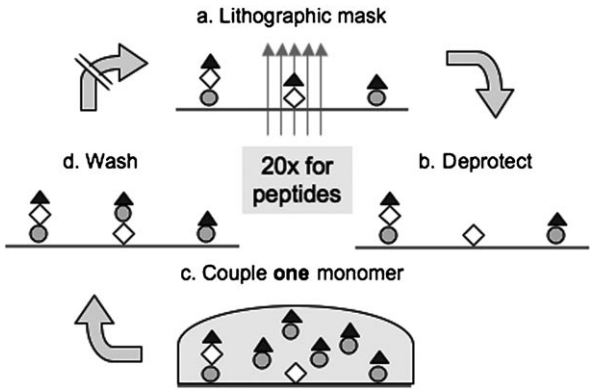
sis of individual peptides each in a larger area and in larger quantities. Deprotection of the peptides with concentrated TFA simultaneous breaks down the cellulose, which leads to small peptide–cellulose conjugates that are each collected individually and stored in, for example, 96-well plates. From there, the peptide conjugates are spotted in high density on a secondary support—glass slides, for example—where they are immobilized due to their sticky cellulose moieties. In particular, this procedure allows for the production of multiple densely spaced peptide array replicas of constant quality. Efficient methods for printing pre-synthesized peptides or compounds in high-density microarray format have also been advanced by Kit S. Lam's group[9] and by the company JPT Peptide Technologies.[10]

Interestingly, high-density oligonucleotide arrays manufactured by an ink-jet printer approach are commercially available through Agilent's SurePrint technology,[11] whereas high-density peptide arrays generated in an analogous way have not been reported, although they were patented as early as 1994.[12] This striking discrepancy might be due to the solvents needed for peptide synthesis. These are usually viscous, which makes it difficult to print them with inkjet or piezoelectric printers.

Lithographic synthesis

Lithographic techniques were originally developed for the manufacture of computer chips with very small structures. At the heart of this method is a lithographic mask that defines a pattern of very small dark or illuminated areas on a two-dimensional surface. For the manufacture of computer chips these patterns are used to remove a light-sensitive protective surface coverage, which is then followed by the spatially defined removal/deposition of material from/on those areas no longer shielded by the protective layer. Several consecutive steps of this kind finally result in a computer chip structured with very small features.

When this technique was modified for the selective removal of a light-sensitive transient protecting group, it became possible to define very small areas of growing oligomers that would react with an added monomer, while all the other dark areas would not (Scheme 5). With this approach, truly high-density



Scheme 5. Lithographic synthesis. a) A pattern of light defines one first set of areas on a two-dimensional solid support. b) There, the transient protecting group at the tip of the growing oligomer chain is removed through irradiation. c) Next, the whole array is uniformly covered with one of the 20 different C-terminally activated amino acid derivatives. These couple only to growing oligomers located in those areas of the support that were deprotected by the previous lithographic step. d) Excess quantities of monomers are washed away. These steps are repeated four times with the four different nucleotides, or 20 times with the 20 different amino acids to add one layer during the synthesis of oligonucleotides or peptides, respectively. Repetitive coupling cycles generate an array of oligomers.

arrays were produced for the first time,[13] revolutionizing the whole field of genomics.[14] In the interim, oligonucleotide arrays with thousands of oligonucleotides per cm^2 have become commercially available[15] and are used, for example, to detect genome-wide transcription activity[16] or to link gene variants with diseases.[17] Interestingly, the seminal publication by Fodor et al., describing the combinatorial synthesis of an array by lithographic methods for the first time, showed the synthesis of a peptide array, albeit only a few cycles of synthesis were detailed.[11] Soon after this publication, this group and their spin-off company Affymetrix completely shifted their focus to the lithographic synthesis of oligonucleotide arrays.

The principle of lithographic synthesis is shown in Scheme 5: a lithographic mask is used to define a first class of very small areas to be irradiated by light (Scheme 5 A). The action of the light removes a photolabile transient protecting group at the ends of the oligomer chains, but only in those areas selected by the lithographic mask (Scheme 5 B). The whole array is then incubated with a solution of activated monomer that reacts with the deprotected oligomer only in the first class of areas (Scheme 5 C), and unreacted monomer is then washed away (Scheme 5 D). This whole process is repeated until one whole layer of monomers has been added to the array: that is, a second class of areas is defined by use of another lithographic mask, a second type of monomer is coupled

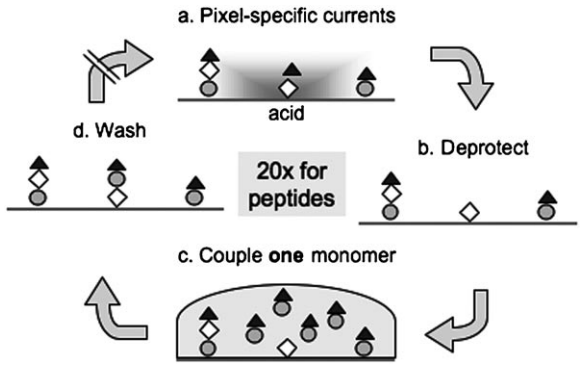
to deprotected oligomers from this second class of areas, and eventually all the different monomers have been coupled to the support to add one layer of monomers to the growing oligomer chain. The whole procedure then starts again until finally, for example, ten layers of monomers have been coupled to generate an array of decameric oligomers.

A peptide-specific drawback of this method is due to the peptide-specific large number of coupling cycles intrinsic in all lithographic synthesis, in which only one kind of monomer at a time couples to spatially defined regions on the solid support. This adds up to 20×10 coupling cycles to synthesize a decameric peptide array, compared to only 4×10 coupling cycles to generate a decameric oligonucleotide array (Scheme 5). Besides the peptide-specific large number of coupling cycles (200 for an array of decamers), 200 expensive lithographic masks are also necessary to allow for combinatorial freedom. In addition, with such a large number of coupling cycles it is difficult to avoid the accumulation of unwanted side reactions. These difficulties are probably the reason why the inventors of lithographically produced arrays completely shifted their focus to oligonucleotide arrays.

Yet another peptide-specific difficulty associated with lithographic synthesis methods is due to the available photolabile transient protecting groups needed for lithographic synthesis. In terms of repetitive coupling yield, these perform less well than conventional *tert*-butyloxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) protecting groups. This difficulty was resolved by Pellois et al., who for the first time used a photo acid for the combinatorial synthesis of a high-density peptide array by lithographic methods.[18] Photo acids are neutral precursor molecules that are transformed into acids when illuminated with light. In the process, a two-dimensional pattern of light with very small feature sizes is translated into a corresponding pattern of acidic or neutral areas. Pellois' clever approach combines the very small feature size of lithographic techniques and the use of conventional acid-sensitive Boc protecting groups with their good repetitive coupling yields in standard peptide synthesis. It remains to be seen, however, if the more fundamental peptide-specific drawback of too many coupling cycles also associated with this variant lithographic synthesis method can be overcome to synthesize arrays that display long peptides in good yields.

Chip-based synthesis

Yet another approach directly uses the very small feature sizes of available computer chips for the combinatorial synthesis of high-density arrays. A normal memory chip allows the experimenter either to store electrical charges in individually chosen chip electrodes (status 1) or to ground them (status 0). Usually such a chip's electrodes are insulated from the environment to shield them from heat due to leakage currents, but with direct connection of the electrode surface and conductive aqueous surroundings, freely chosen patterns of currents linked to individual electrodes are easily induced. These currents decompose water molecules through electrolysis, which transforms a pattern of electrode currents into a corresponding pattern of acidic or neutral areas with very small feature sizes. These essentially depend only on the distance between individual electrodes. In this process, and similarly to the photo-acid-based approach described above, acid-sensitive Boc protecting groups are removed in the vicinities of individual chip electrodes, which allows for the combinatorial synthesis of oligomer arrays with very small feature sizes (Scheme 6).[6,19]



Scheme 6. Chip-based synthesis. a) A pattern of currents defines one first set of areas on a chip's surface. b) There, through electrolysis, the transient protecting group at the tip of the growing oligomer chain is removed. c) Next, the whole array is uniformly covered with one of the 20 different C-terminally activated amino acid derivatives. These couple only to those areas on the chip deprotected by the previous lithographic step. d) Excess quantities of monomers are washed away. These steps are repeated four times with the four different nucleotides, or 20 times with the 20 different amino acids to add one layer during the synthesis of oligonucleotides or peptides, respectively. Repetitive coupling cycles generate an array of oligomers.

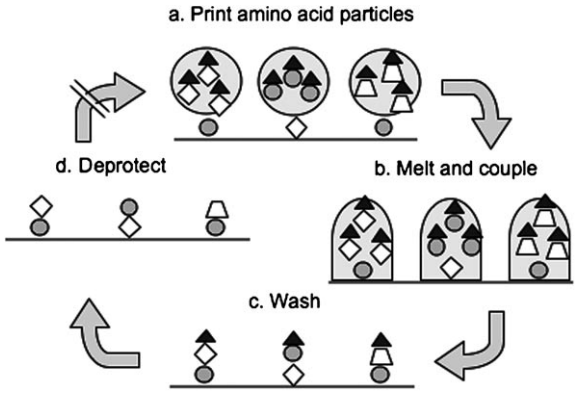
In a variant method, Heller and Tu directly attracted charged monomers for combinatorial synthesis from solution to discrete electrodes on a microchip's surface to induce a combinatorial synthesis.[20] Electrolysis in the vicinities of those electrodes chosen to attract charged monomers also ensues in this approach, and eventually interferes with the coupling reaction. In addition, both variants of the chip-based combinatorial synthesis suffer from the peptide-specific drawback described in the precedent section: 20×10 coupling cycles are needed to synthesize a decameric peptide array, in comparison with only 4×10 coupling cycles to generate a decameric oligonucleotide array. Indeed, this kind of chip-based synthesis has so far been reported only for the synthesis of oligonucleotide arrays.

Particle-based synthesis

Our laboratory has recently developed a method for the combinatorial synthesis of peptide arrays that is based on solid particles containing derivatives of 20 different amino acids.[21] In a normal laser printer, small solid toner particles are triboelectrically charged (by mild friction) by, for example, grinding the particles against a rubber foam drum inside the toner cartridge. Depending on the materials involved, this procedure leads to very strong electrical charges on the surfaces of the toner particles, which in state-of-the-art toner particles can come close to electrical breakdown in air. Because of these

charges toner particles can be moved within electrical fields, as a result of which, for example, a laser printer eventually delivers different color toners to different addresses on a two-dimensional surface.

To do this, either a laser or a row of ~10000 light-emitting diodes [LEDs] per 20 cm is used to generate a light pattern on the surface of a uniformly charged organic photoconducting [OPC] drum that rotates in ~10000 steps per 20 cm. This results in a two-dimensional light pattern that in state-of-the-art laser printers comprises ~100 million pixels per $(20\times20)\ cm^2$. The OPC material translates this light pattern into the corresponding electrostatic pattern of ~100 million pixels per $(20\times20)\ cm^2$ through rapid neutralization of illuminated areas by grounding.[22] Subsequently, charged toner particles are transferred only to those areas previously neutralized by irradiation with light, which transforms the electrostatic pattern into the corresponding particle pattern. Finally, the particles delivered by the OPC drum are transferred onto a solid support by means of a strong electric field ($4\ kV\,mm^{-1}$), with a printout being assembled (Scheme 7A).



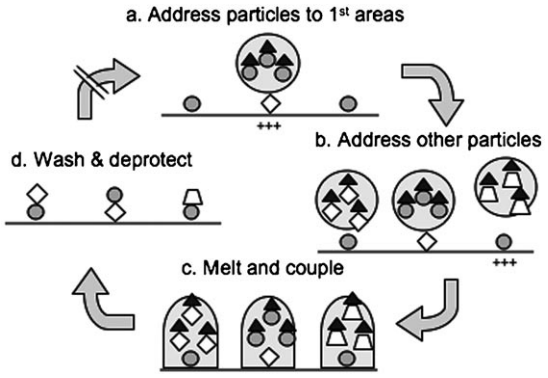
Scheme 7. Addressing amino acid particles with a laser printer. a) A laser printer addresses Fmoc-amino acid OPfp esters embedded within solid toner particles onto a solid support that displays reactive amino groups, where b) the particles are melted after transfer. This frees the chemically activated amino acid derivatives to diffuse and to couple to growing peptide chains on the support in parallel. Different reaction spheres are separated from each other because of surface tension, which constricts melted particles to small individual hemispheres. A cycle of synthesis is completed when c) excess quantities of monomers are washed away, and d) the Fmoc protecting group is removed. Repetitive coupling cycles generate a peptide array with one coupling cycle per layer.

This technique is thus ideally suited for the main challenge all different kinds of combinatorial synthesis must face: to deliver different building blocks flexibly and with very high accuracy. Because a color laser printer prints only four different color toners, but a peptide is made out of 20 different building blocks, we have had to adapt laser-printing technology to the combinatorial synthesis of peptide arrays: the peptide-laser-printer we developed accommodates 20 printing units rather than four. In addition, the drive and mounting of that machine allows for repeated exact positioning of consecutively printed layers, which is the prerequisite for the parallel elongation of growing peptide chains by combinatorial synthesis (Scheme 7A). Apart from these modifications, the peptide-laser-printer prints in exactly the same way as its parent C7000 series OKI laser printer does, but with 20 different kinds of amino acid particles instead of four different color toners.

The remaining task was to reconcile the use of solid particles with the requirement for a solvent that is needed for a chemical reaction. This was done in order to benefit from one of the main advantages of this technique: once addressed, the whole layer of amino acid particles is melted in a single step to initiate the coupling reaction for all reactive monomers in parallel (Scheme 7B). Washing and deprotection steps finish the cycle, and result, if repeated, in the combinatorial synthesis of a peptide array. The method uses conventional Fmoc chemistry[23] and differs from the SPOT synthesis only in the solid (at room temperature) solvent employed; this allows for the intermittent immobilization of amino acids within particles (compare Schemes 4 and 7).

Although the laser-printing technique is the most prominent method for directing charged particles to their addresses on a two-dimensional support through the use of electrical fields, variations of this theme have described even nanoscale deposition of particles on a surface.[24] To explore an alternative route that should lead to miniaturization beyond the resolution of a laser printer, we have also used the electrical fields of individual pixel electrodes of a computer chip to direct amino acid particles to very small synthesis areas. Currently, the miniaturization achieved by this variant particle-based combinatorial synthesis method stands at 40000 peptides per cm^2 (Scheme 8A and B).[25]

The intermittent "freezing" of a chemical reaction in solid particles is the main novel element of the method. Chemically highly reactive amino acid derivatives used for peptide synthesis are embedded within a solid particle matrix, which completely blocks their diffusion and concomitant chemical cou-



Scheme 8. Addressing amino acid particles with a chip. a) Electrical fields generated by individual pixel electrodes address the first type of amino acid particles onto a first set of areas on the surface of a chip. b) Different patterns of pixel electrodes are switched to voltage to direct all 20 different amino acid particles onto the chip surface consecutively until a whole layer of all 20 different amino acid particles is complete. c) The whole layer of consecutively addressed amino acid particles is melted in one step to induce the coupling reaction at all electrodes in parallel. d) Excess quantities of monomers are washed away, and the Fmoc protecting group is removed. Repetitive coupling cycles generate a peptide array with one coupling cycle per layer.

pling to a reaction partner. Indeed, all the 20 different Fmoc-amino acids with C-terminal pentafluorophenyl ester activation proved to be stable for months at room temperature when embedded in particles, except for Fmoc-arginine-OPfp, which decayed at a moderate 4% per month.[6 19] This is a remarkable finding in view of the notorious instability of carboxy-activated Fmoc-arginine derivatives in other solvents.[26] This feature gives the experimenter plenty of time to manufacture different particles, to purify them rigorously, to store them over months, and to address them consecutively to different areas on a two-dimensional support (Scheme 8 A and B), before the coupling reaction is induced simply by melting the printed particles.

When we scrutinized the novel method in detail, we found a surprising robustness with respect to undesirable side reactions that might have been induced by the use of the non-standard solvent or the elevated coupling temperatures. Even with extended coupling times of 90 min at temperatures of 90 °C we observed no aspartimide formation or racemization at all, nor any major unexplainable peaks in mass spectrometric analysis of synthesized peptides.[6 19]

Conclusions

Particle-based synthesis introduces a novel concept into chemistry: a reactive chemical that is "canned" into particles and sent as "postal packages" to different addresses where the chemical is freed simply by melting. This procedure is especially advantageous if more than just a few chemical building blocks have to be separated from each other in a densely spaced and chemical saving arrangement, such as in the combinatorial synthesis of high-density peptide arrays. The solid particle matrix shields reactive chemicals from each other and from their environment, and thus contributes robustness, long-term storage, and easy handling. Moreover, with electrically charged particles the experimenter can profit from the very small feature sizes of a computer chip or the good printing resolution of a laser printer to space very small reaction spheres densely and to address them repeatedly.

These features, together with the reduced numbers of coupling cycles intrinsic to all printing methods, make the novel particle-based method particularly well suited for automation. This in turn should drastically reduce the cost per peptide spot in the near future, and thus bring affordable high-density peptide arrays into laboratories. These might have an impact similar to that of high-density oligonucleotide arrays: genome-wide screening for peptidic T-cell epitopes might bring rationally designed vaccines into reach, or a panel of peptides that comprehensively diagnoses a patient's or even a population's antibody response should bring clues to hitherto enigmatic diseases, or an easy screen for (better) binding molecules might have a big impact on novel therapeutics. Screening for and combination of different peptide modules might also speed up the new field of synthetic biology that in an engineering approach combines gene modules to build living systems with specially tailored properties. Beyond the life sciences, a screen for peptidic semiconductors or catalysts might be feasible.

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