Alternative Setups for Automated Peptide Synthesis

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Abstract: Nowadays, lithographic methods facilitate the combinatorial synthesis of >50.000 oligonucleotides per cm^2, an achievement that revolutionized the whole field of genomics. High-density peptide arrays might spark a similar development for the field of proteomics, but all lithographic methods have a peptide specific disadvantage that impairs their use for peptide synthesis: Each monomer must be coupled separately to the solid support. This adds up to an excessive number of coupling cycles, especially when comparing the 4 x 20 coupling cycles that would generate an array of 20meric oligonucleotides, to the 20 x 20 cycles that would yield an array of 20meric peptides. This review mainly discusses one recent development that leads to very high-density peptide arrays: the combinatorial chemical synthesis based on electrically charged solid amino acid particles. Either a colour laser printer or a chip addresses the different charged amino acid particles to a solid support, where the whole layer of solid amino acid particles is melted. Hitherto immobilized amino acids then start to diffuse to the support, where all the 20 different amino acids couple in a spatially defined manner, and in one single coupling reaction to the support. The method should allow for the translation of entire genomes into sets of overlapping peptides to be used in proteome research.

Keywords: Biotechnology, combinatorial chemistry, high-throughput screening, particle-based chemistry, peptide array, peptides.

INTRODUCTION

Scientists strive to get hold on many different peptides or proteins, especially if they want to develop pharmaceutically relevant assays. This is also the main incentive for the development of high-density protein and peptide arrays. Proteins and peptides are obtained by four different procedures: (1) They are extracted from natural sources, or (2) recombinantly expressed from genes "smuggled" into microorganisms, or (3) synthesized with the help of a cell lysate (and a cell's ribosomes) outside a cell, or they are (4) chemically synthesized. This review discusses methods that yield high-density peptide arrays by chemical synthesis (procedure #4).

Compared to the other procedures, chemical synthesis has one big advantage: it allows for the incorporation of unnatural amino acids, D-amino acids, and other building blocks. Another advantage is that usually large quantities of peptides can be produced in high quality. Chemistry also helps to synthesize natural peptides that are difficult to express in living systems. The breakthrough in chemical synthesis of peptides came with the solid phase peptide synthesis (SPPS) that was invented by Bruce Merrifield more than forty years ago. He consecutively coupled amino acid monomers to a growing peptide chain immobilized on a solid support [1]. This allowed for an especially easy purification of the growing peptide products because these remain tethered to the support throughout synthesis, while everything else is simply washed away. Even more importantly, the excess molar amount of amino acid monomer over the growing peptide chains drives the coupling reaction near completion (Scheme **1**). Meanwhile, this basic principle routinely gives a repetitive coupling yield of >95% during peptide synthesis, which is a prerequisite for the affordable peptides that many laboratories use today.

Even more reliable is the peptide synthesis that was invented by evolution. The synthesis apparatus inside a cell yields very long peptides that are called proteins. On the ribosome, the amino acids are exactly positioned in subnanometer dimensions according to the sequence of neighboring anticodons on the mRNA that these tRNAs recognize. Thus, potentially reactive side chains of the growing peptide are kept away from reactive groups of the activated amino acid while the amino acid is being coupled to the growing peptide chain by the formation of a peptide bond. The elucidation of this digitally programmed nanomachine was awarded with the Nobel prize in 2009 [2]. Thereby, a cell can be used to synthesize any non-toxic protein, provided the corresponding gene is "smuggled" into this cell that is then grown to a clone. Protein arrays that depend on Nature's ribosomes are not in the focus of this review. They are described elsewhere [3].

Compared to Nature's sophisticated ribosomal nanopositioning tools, chemical synthesis must rely on simply diffusing the monomers to the growing oligomer chain. Therefore, these chemically activated molecules would couple to any other reactive group, unless all the reactive side chains on the growing peptide and also on the monomers are protected. This extra difficulty is one of the reasons for the lower performance of chemical synthesis *vs.* ribosomal synthesis because the chemist always has to choose between incomplete side chain protection, which is accompanied by unwanted side reactions, and incomplete deprotection, which also yields unwanted molecules. This nuisance sparked the development of many different chemical protection and activation regimes. However, a detailed description of these is beyond the scope of this review. They are discussed elsewhere [4]. Despite these drawbacks, soon after the invention of SPPS the demand for peptides exploded, which eventually culminated into the Nobel prize that was awarded in 1984 for Merrifield's ingenious invention. The strong demand also sparked the idea to slightly modify SPPS, and process many different reaction spheres in parallel in order to synthesize many different peptides. Since that procedure uses the same 20 different solutions with activated amino acid building blocks that are needed for SPPS, many different peptides could be synthesized with only a moderately increased workload [5]. These endeavors established the whole new field of combinatorial chemistry, which intends (1) to synthesize and (2) to analyze as many peptides as possible. The resulting "libraries" of peptides are then used to screen for, e.g. individual peptides that bind to a target protein. Interestingly, although 40 years have passed since Merrifield's invention, all of

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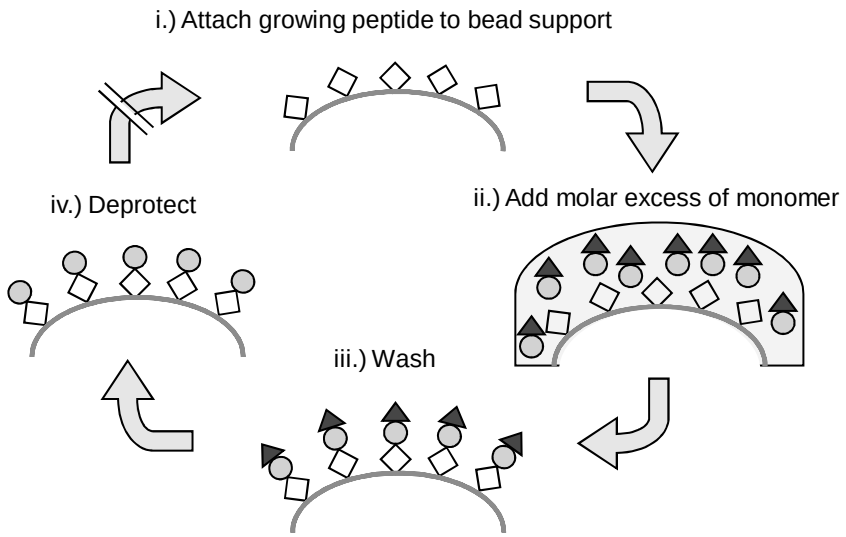


Scheme 1. Solid phase peptide synthesis (SPPS; Merrifield synthesis); i) The growing peptide chain is attached to a solid phase during peptide synthesis. The bead support is denoted by a curvature; ii) C-terminally activated amino acid monomers couple to the free N-terminal amino groups at the tip of the growing peptide chain. A molar excess of these monomers drives the coupling reaction near completion, iii) Due to the linkage of the peptide to the solid support excessive monomers are easily washed away, iv) The removal of the transient N-terminal protection group completes one synthesis cycle. Repetitive coupling cycles yield a peptide that must be deprotected from permanent side chain protection groups (not shown in the scheme) and cleaved from the solid support.

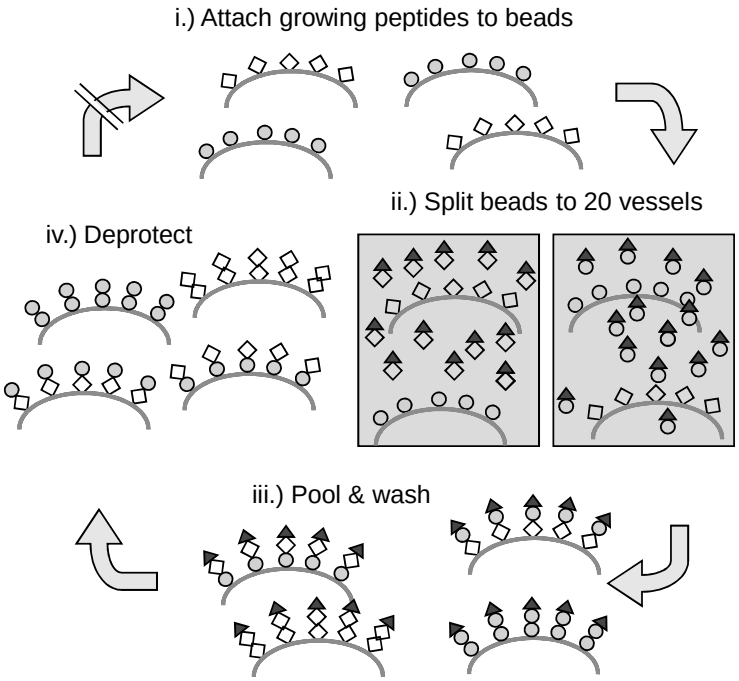
these chemical methods are still based on the principle of solid phase synthesis.

An especially easy, elegant, and cheap procedure to generate huge libraries of different peptides is the one-bead-one-compound method, which was invented by Kit S. Lam in the early nineties. By then, the Merrifield synthesis routinely was done on a meshwork of beads (mostly made of cross-linked polystyrene (PS), polyamide, or poly ethylene glycol (PEG) based resins) with a diameter of 200 - 500 μm. These beads swell in the solvents used for peptide synthesis (Dimethylformamid (DMF) or N Methyl Pyrolidone (NMP)), whereby they gain a nearly 3-fold volume. The bead form is ideal for such a swelling procedure because a bead expands in all three dimensions, and, thereby, minimizes any shear stress during this expansion. Pre-swelling the solid support for the growing peptides considerably enhances the repetitive coupling yield during synthesis cycles, which is the main driving force to use these beads. For his one-bead-one-compound method Kit S. Lam simply split the synthesis beads for a solid phase synthesis to 20 different reaction vessels that each add one of the 20 different amino acids to the growing peptide chains on individual beads. Afterwards, the beads are pooled again, the transient protecting group is removed (9-fluorenylmethyl carbamate (Fmoc) or di-tert-butyl dicarbonate (Boc)) before the beads are distributed again to the 20 different reaction vessels for the next synthesis cycle. This procedure gives every bead its individual history of sequential stopovers in one of the 20 vessels, where always one amino acid is added to the growing peptides. Thereby, this history is translated into a sequence of added amino acids, and, at the end of the synthesis procedure, nearly every bead displays a different peptide, but always only one kind of peptide per bead (Scheme **2**) [6]. Meanwhile, based on the one-bead-one-compound method many reports of successfully screened peptide binders have been published, which is excellently reviewed by Liu *et al.* [6]. Although unrivalled in the synthesis cost per peptide and also in the incredible number of peptides that are easily synthesized, there are two drawbacks attached to this procedure. First, due to the random distribution of beads when splitting them to the 20 different reaction vessels it is nearly impossible to avoid problematic peptides during library preparation. Examples for such problematic peptides are insoluble peptides or those that bind to any protein, and thus elicit a strong background of false positive binders. The second drawback of the one-bead-one-compound method is the labor-intensive encoding/decoding that is needed to access the sequences of those peptides that bound to a target protein. Both of these drawbacks are resolved by peptide arrays.

THE ARRAY CONCEPT

The array was invented by Robert Ekins, who was the first to spot different known molecules onto a two-dimensional surface, and, thereby, "arrayed" them into a regular pattern. If such an array then is incubated with a protein, the diffusing protein probes all the different molecules on the array and eventually sticks to those with a complementary surface. This process where a "protein-key" that is driven by Brownian motion probes many different "protein-locks" until it finally fits to its specific lock is called "specific binding" (Scheme **3**). The non-covalent and rather weak forces that are responsible for this specific binding (hydrogen bonds, electrostatic, hydrophobic, van der Waals, stacking forces) exert their influence on a binding partner only over very short distances, which makes a complementary surface mandatory for specific binding. From a technical point of view this procedure is very simple because it involves only the incubation of the array with the sample. If the binding protein from the sample carries a label, all those regions on the two-dimensional surface that display a specifically binding molecule are stained. Obviously, the intensity of the signals depends on the concentrations of the molecules that are arrayed per area, while a better contrast to neighbouring spots is achieved by strictly confining the arrayed molecules within uniform small areas. Since the experimenter arrayed all the molecules before at known positions, he can translate the staining pattern directly into the identities of all of the binding molecules, and, in addition, of those that didn't bind [7]. Moreover, the information retrieved with one single experiment of this kind increases with the number of arrayed molecules. This is the reason why ever since the invention of the array scientists strive to develop higher density arrays that display as many different oligonucleotides, proteins, peptides, sugars, or small molecules as possible.

This incredible amount of information retrieved by a single easy experiment is the one big advantage of the array concept when comparing it to those molecule libraries where the information on the identity of individual molecules is not linked to a spatial information. One single experiment with an array attributes to each and



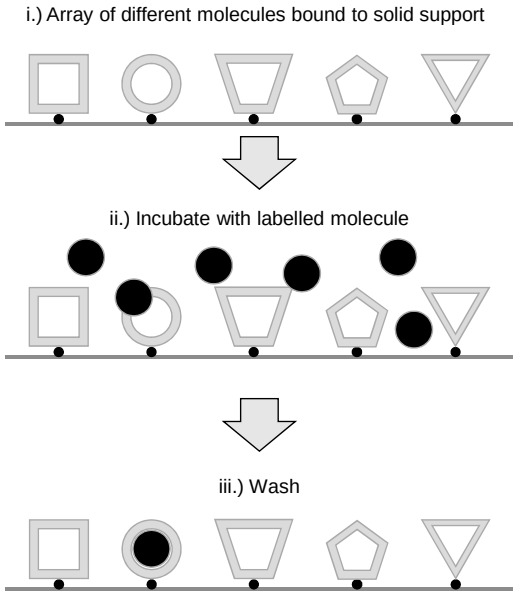
Scheme 2. The one-bead-one-compound method; i) The growing peptide chains are fixed to a solid bead support. The bead support is denoted by a curvature, ii) The beads are randomly distributed to 20 different reaction vessels. In order to simplify the scheme, only two different vessels are shown. Each of the vessels contains a different C-terminally activated amino acid derivative that couples to the free N-terminal amino group from the growing peptide, iii) Excessive monomers are washed away and the beads are pooled afterwards, iv) A synthesis cycle is completed when the transient N-terminal protection group is removed from all of the beads. Repetitive coupling cycles generate a library of peptides with every bead displaying a different peptide. The sequence information of bead-coupled peptides is lost due to random distribution of the beads to the vessels. Therefore, the peptide sequence information from identified bead-binders must be retrieved.

every molecule on the array the information that it either does or does not bind to the sample, compared to only a very limited number of binders from the one-bead-one-compound library where the identity of the binding peptides eventually is determined by a labor- and cost-intensive sequencing procedure. This huge amount of information translates into yet another advantage of the array concept over the one-bead-one-compound method. Those molecules on the array that non-specifically bind to any protein are easily identified and simply omitted in the next array generation.

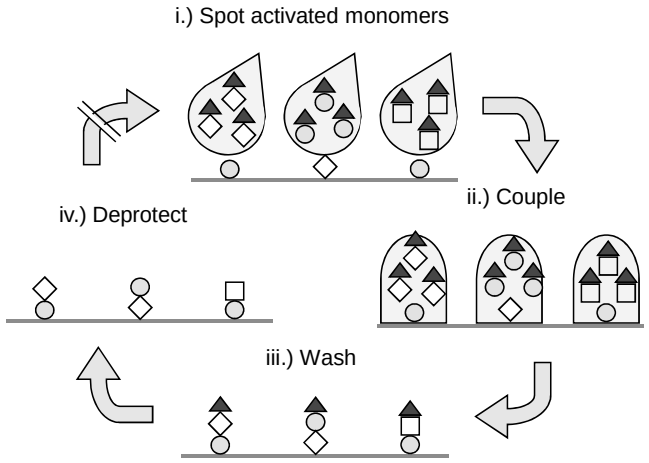
PEPTIDE ARRAY TECHNOLOGIES

SPOT Synthesis

The early experiments from Ekins revealed that spotting many different pre-formed molecules in the array format is technically difficult and laborious. It is also very expensive because it involves the synthesis and handling of a very large number of different molecules. In order to overcome this drawback, Edwin Southern mated Ekins' array concept with the combinatorial synthesis of oligomers. The basic idea behind this endeavor was to use a very limited number of chemically activated monomers (four different bases or twenty different amino acids) to synthesize many different oligomers on neighboring places. Edwin Southern introduced this novel concept first for the *in situ* combinatorial synthesis of oligonucleotide arrays [8]. Thereby, he came very close to the goal of cheap and very high-density arrays. A few years later, Ronald Frank adapted this concept to the synthesis of peptide arrays [9]. They both parallelized the Merrifield synthesis schematically depicted in Scheme **1** by adding not one base or amino acid to the support, but instead patterning the 4 different bases or 20 different activated amino acid derivatives as small droplets on a flat two dimensional surface. There, the chemically activated monomers 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, whereby the number of different oligomers is only dependent on the achievable miniaturization of individual spots (Scheme **4**). Ronald



Scheme 3. The array concept; i) Many different molecules are linked to a two-dimensional solid support, each at a known position, ii) A diffusing labeled potential binder probes all the different molecules on the array and eventually sticks to those with a complementary surface, iii) Unbound labeled molecules are washed away and the labeled areas on the array are identified. Thereby, one single experiment reveals those of the many different molecules on the array that specifically bound to the labeled potential binder, and also those that didn't bind.



Scheme 4. SPOT synthesis; i) The 20 different C-terminally activated amino acid derivatives are spotted in parallel within liquid droplets to defined areas on a solid support, where ii) they couple to the support. A cycle of synthesis is completed when iii) excessive amino acid derivatives are washed away, and iv) the transient protection group is removed. Repetitive coupling cycles generate a peptide array, where the peptide address on the support and peptide sequence for each of the individual peptides is known.

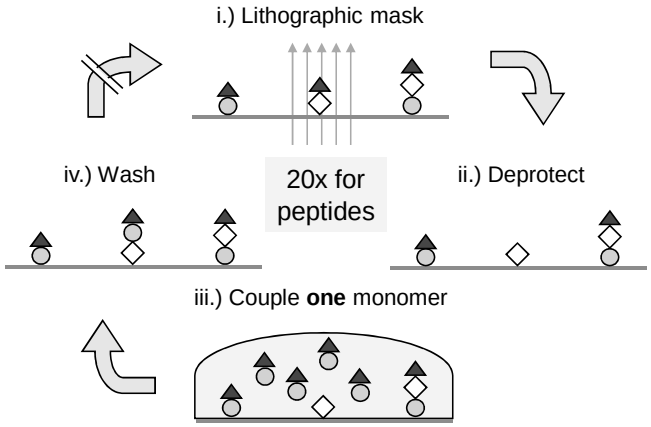
Frank's SPOT synthesis over the years earned a reputation of reliability and wide applicability and thus still dominates the field [10]. It is described in detail in an accompanying paper.

High peptide densities of *in situ* synthesized peptides that exceed 25 peptides per cm^2, however, are difficult to obtain by SPOT synthesis, mainly due to the difficult handling of tiny droplets that tend to evaporate or spread over the array's surface. Interestingly, high-density peptide arrays manufactured with an ink jet printer were patented as early as 1994 [11]. While oligonucleotide arrays meanwhile are commercially available through Agilent's SurePrint technology [12], corresponding high-density peptide arrays have not been reported yet. 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 ink jet or piezoelectric printers.

Lithographic Synthesis

Lithographic masks are used to illuminate very small areas on a two dimensional surface while shielding other sites from light. They are used to manufacture computer chips with very small structures. At the heart of this method is a photosensitive protective surface coverage, which is called "photoresist". The action of light either sensitizes or, alternatively, stabilizes the photoresist. Either way, the photoresist translates the light pattern from the lithographic mask into areas were the protective photoresist coverage is selectively removed, while still protecting the other areas. This procedure then results into the spatially defined removal or deposition of material to those areas no longer shielded by the protective layer. Several consecutive steps of this kind finally result into a computer chip structured with very small features.

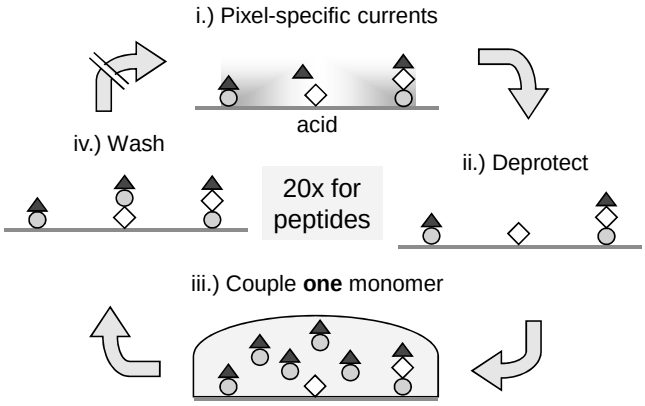
The seminal publication from Fodor *et al.* described a variant lithographic method that for the first time yielded truly high-density arrays by combinatorial synthesis [13]. The authors used a lithographic mask to selectively remove a light sensitive transient protection group instead of photoresist. Thereby, very small areas of growing oligomers could be defined that would react with an added monomer, while all the other dark areas wouldn't (Scheme **5**). Meanwhile oligonucleotide arrays with thousands of oligonucleotides per cm^2 are commercially available [14] that are used, e.g. to detect genome wide transcription activity [15], or to link gene variants with diseases [16]. Thereby, this novel technique revolutionized the whole field of genomics [17]. Interestingly, this powerful novel method was first demonstrated for the synthesis of high-density peptide arrays, albeit only a few cycles of synthesis were shown [18]. Soon afterwards, however, the Fodor group and also their spin-off company Affymetrix completely shifted their focus from peptide to oligonucleotide arrays. The reason for this shift is quite obviously due to a peptide specific drawback inherent to all lithographic synthesis methods that is explained in Scheme **5**:



Scheme 5. Lithographic synthesis; i) A pattern of light defines one first kind of areas on a two dimensional solid support, ii) There, through irradiation, the transient protection group at the tip of the growing oligomer chain is removed, iii) 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 that are in those areas of the support that were deprotected by the previous lithographic step, iv) Excessive monomers are washed away. These steps are repeated 4x with the 4 different nucleotides, or 20x 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.

During lithographic synthesis, the action of light selectively removes a photolabile transient protection group at the end of the growing oligomer chains, but only in those areas defined by the lithographic mask (Scheme **5ii**). The whole array then is incubated with a solution of chemically activated monomers that react with the deprotected oligomer (Scheme **5iii**). After the coupling reaction, unreacted monomers are washed away (Scheme **5iv**). This cycle adds only one monomer to the growing oligomer chains, and, therefore, must be repeated until all the different monomers from one synthesis layer have been added to the array. Then the whole process is repeated to elongate the growing ologomers by another monomer. This consecutive addition of different monomers is the peptide-specific drawback of this method because the number of monomers is much larger in peptides when compared to oligonucleotides. Peptides are made of twenty different amino acids while oligonucleotides are composed of only four different bases. This fact leads to a peptide-specific large number of coupling cycles intrinsic in all lithographic synthesis. While only 4 x 10 coupling cycles are needed to generate a 10meric oligonucleotide array, the twenty different amino acid monomers demand 20 x 10 coupling cycles to synthesize a 10meric peptide array (Scheme **5**). Such a large number of coupling cycles usually is accompanied by the accumulation of an intolerable amount of unwanted side reactions. In addition, 200 expensive lithographic masks are necessary to allow for combinatorial freedom. These difficulties probably are the reason why the inventors of lithography-made-arrays completely shifted their focus to oligonucleotide arrays.

Yet another peptide-specific difficulty associated with lithographic synthesis methods is due to the photolabile transient protec-



Scheme 6. Chip-based synthesis; i) Pixel-specific currents define a pattern of first areas on a chip's surface, ii) There, through a pH-shift induced by electrolysis, the transient protection group at the tip of the growing oligomer chain is removed, iii) Next, the whole array is incubated 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, iv) Excessive monomers are washed away. These steps are repeated 4x with the 4 different nucleotides, or 20x with the 20 different amino acids to add one layer during the synthesis of oligonucleotides or peptides. Repetitive coupling cycles generate an array of oligomers.

tion groups that are mandatory for lithographic synthesis. All protection groups that are available for the transient N-terminal protection of amino acid building blocks perform poorly in terms of repetitive coupling yield when compared to conventional *t*-butyloxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) protection groups. This difficulty was resolved by Pellois *et al.* They used a photo acid in combination with standard Boc protection instead of a photolabile protection group. Photo acids are neutral precursor molecules that are transformed into an acid when illuminated with light. Thereby, a two dimensional pattern of light with very small feature sizes is translated into a corresponding pattern of acidic *vs*. neutral areas. This leads to selective and very efficient cleavage of standard Boc groups in those areas illuminated by light. Thus, Pellois' clever approach combines the very small feature size of lithographic techniques and conventional acid sensitive transient Boc protection groups with the good repetitive coupling yield in standard peptide synthesis [18]. However, the more fundamental peptide-specific drawback of too many coupling cycles associated also with this variant lithographic synthesis remains unsolved. It remains to be seen if this variant lithographic synthesis method indeed yields arrays that display peptides of normal length and in good yield.

Chip-based Synthesis

Yet another approach directly uses the very small feature sizes of computer chips for the combinatorial synthesis of high-density arrays. A normal memory chip stores electrical charges in individually chosen chip electrodes (status 1) or, alternatively, discharges its electrodes by grounding (status 0). Usually these chip electrodes are insulated from the environment to shield them from heat due to leakage currents, but with a direct connection of the electrode surface and conducting liquid surrounding, freely chosen patterns of currents linked to individual electrodes are easily induced. These currents decompose water molecules through electrolysis, which results into a tiny acidic environment around a small electrode and a basic environment around the counter electrode. Thereby, a pattern of electrode currents is transformed to a corresponding pattern of acidic (or, alternatively, basic) *vs.* neutral areas with very small feature sizes. Thereby, and similar to the photo acid based approach described above, acid sensitive Boc protection groups are removed in the vicinity of individual chip electrodes (Scheme **6**) [19]. As discussed above for lithographic synthesis, this patterning step then allows for the combinatorial synthesis of oligomer arrays with very small feature sizes. These essentially depend only on the dimensions of individual electrodes.

In a variant method Heller and Tu directly attracted charged monomers from solution to discrete electrodes on a microchip's surface in order to do a combinatorial synthesis [20]. However, unavoidable electrolysis ensues also in their setting in the vicinity of those electrodes chosen to attract charged monomers. Thereby, these monomers are exposed to an acidic, or, alternatively, a basic environment that eventually leads to premature deprotection. Heller and Tu tried to alleviate a negative interference on the coupling reaction due to this setting by depositing a buffered solution in the vicinity of the electrodes connected to voltage, which, however, due to its salt character also weakens the attractive force on the charged monomers.

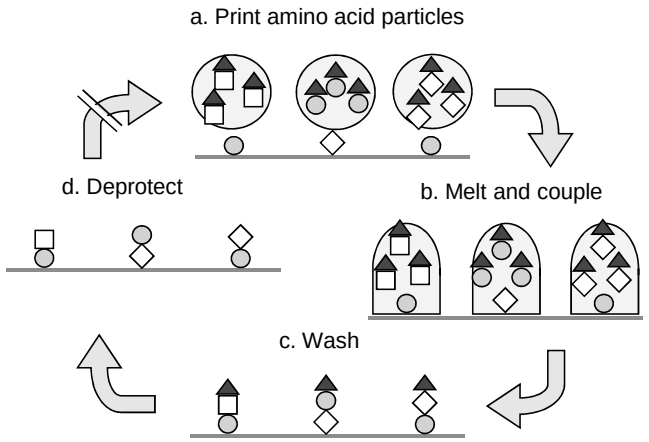
Similar to the lithographic synthesis methods, both variants of the chip-based combinatorial synthesis also suffer from the peptide-specific drawback put forward in the precedent chapter: 20 x 10 coupling cycles are needed to synthesize a 10meric peptide array, compared to only 4 x 10 coupling cycles to generate a 10meric oligonucleotide array. Indeed, so far this kind of chip-based synthesis has been reported only for the synthesis of oligonucleotide arrays.

Particle-based Synthesis

As discussed above, lithographic synthesis methods yield very high-density arrays, but suffer from the peptide specific drawback of too many coupling cycles that are needed for a combinatorial synthesis involving 20 different amino acid monomers. SPOT synthesis, on the other hand, yields high quality peptide arrays, but these reach a density of only 25 peptides per cm^2. The only way out of this dilemma would be a high resolution printing technology that delivers the 20 different amino acid monomers simultaneously to a two dimensional support (*only one coupling reaction per layer*), and, at the same time confines the lateral diffusion of the monomers (*to achieve a very high density of synthesis sites*).

In principle, laser printing is such a high resolution printing technology. A modern color laser printer delivers within seconds to more than 100 million individual pixels (20 x 20 μm^2) per DinA4 page the tiny amount of ~4ng black, cyan, magenta, or yellow toner particles. Moreover, such a color laser printer sends different kinds of particles to precisely defined areas, and the printing pattern is easily changed, which is exactly the kind of machine that is needed for combinatorial synthesis. However, these particles are solid, while at least one from two chemical reaction partners must be diffusible in order to find each other for a chemical reaction. In other words: a chemical reaction doesn't proceed in a solid particle, instead a solvent is needed. To reconcile combinatorial synthesis with solid toner particles our laboratory developed a method to first embed amino acid monomers within solid particles, print these particles onto the array support, and then transform the matrix material of these solid particles into a solvent simply by melting them. This procedure diffuses all the amino acid monomers embedded within the particles, and, thereby, induces the chemical coupling reaction.

In order to achieve this goal, one of the first tasks was to develop the 20 different solid amino acid particles that are needed for such an endeavor. We did that by first dissolving commercially available standard amino acid monomers that are suitable for peptide synthesis (*Fmoc transient protection group; OPfP ester activation of the C-terminus*), a resin that stabilizes the particles against aggregation induced by pressure, a higher homologue of standard solvents for peptide synthesis (*e.g. diphenyle formamide*), and "charge transfer agents" (*these stabilize the electrical charge on the surface of toner particles*). Then, we removed the solvent and



Scheme 7. Combinatorial synthesis with a peptide laser printer; i) A laser printer addresses different Fmoc-amino acid-OPfP esters in parallel to a solid support. These chemically activated amino acid derivatives are embedded within solid toner particles. The support displays reactive amino groups that would react with these C-terminally activated amino acid derivatives. ii) Once printed, the particles are melted. This frees the monomers to diffuse and couple to growing peptide chains on the support. Different reaction spheres are separated from each other due to surface tension that constricts melted particles to small individual hemispheres. A cycle of synthesis is completed when iii) excessive monomers are washed away, and iv) the Fmoc protection group is removed. Repetitive coupling cycles yield a peptide array. Different from lithographic synthesis methods, this is done with only one coupling reaction per layer.

milled the resulting dry mass to small particles with an air mill. These were finally covered by silica nanoparticles to prevent them from aggregation. The detailed procedure for particle production is published elsewhere [21].

The second task was to build a laser printer that would deliver not four different color toners, but twenty different amino acid particles to a solid support, and, moreover, would reliably find the synthesis site of the growing peptide also when printing consecutive layers of amino acid toner particles (see Scheme **7**). We based this machine on the construction principle of the Oki C7000 series where a row of ~10,000 light emitting diodes [LEDs] per 20 cm generates a light pattern on the surface of a uniformly charged organic photo conducting [OPC] drum that rotates in ~10,000 steps per 20 cm (*other laser printers scan the OPC drum's surface with a laser beam and a rotating mirror*). This results into a two dimensional light pattern that comprises ~100 million pixels per (20 x 20) cm^2. The OPC material translates this light pattern into the corresponding electrostatic pattern of ~100 million pixels per (20 x 20) cm^2, as illuminated areas are rapidly neutralized by grounding [22]. Subsequently, charged toner particles jump only to those areas previously neutralized by irradiation with light, which transforms the electrostatic pattern to the corresponding particle pattern. Finally, the particles delivered by the OPC drum are transferred onto a solid support by a strong electric field (1kV/mm), where a printout is assembled (Scheme **7i**) [22].

As mentioned before, the pixel specific delivery of the laser printer's toner particles is due to strong pixel specific electrical fields, which can only work if the toner particles are electrically charged. This charging is done triboelectrically (*by mild friction*), e.g. by 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 surface of toner particles, which for state of the art toner particles comes close to electrical breakdown in air. Due to these charges, toner particles can be reliably moved within electrical fields, whereby, e.g. a laser printer eventually delivers different color toners to different addresses on a two dimensional surface.

The third task was to develop a surface coating that is suited for the combinatorial synthesis of peptide arrays. The solid support must provide free amino groups that react with pre-activated amino acid derivatives (Scheme **7**). In addition, it must stand harsh conditions during peptide synthesis (*solvents, bases, strong acids during final cleavage of side chain protecting groups*) and it must allow for the incubation of arrays with an analyte, e.g. an antibody solution. Examples for such surfaces are published in detail elsewhere [23, 24]. To summarize, a glass surface was thoroughly cleaned and activated with KOH/2-propanol, then silanized with a tertiary bromo silane which was finally used to coat the surface with poly(ethylene glycol)methacrylate (PEGMA) in an atom transfer radical polymerization (ATRP). The PEGMA coating is a covalently linked, three dimensional brush polymer film providing free hydroxyl groups at each side chain. These hydroxyl groups were esterified with Fmoc-β-alanine which, finally deprotected, resulted in up to 40 nmol/cm^2 of free amino groups.

As shown schematically in Scheme **7** these three elements were then combined for a particle-based combinatorial synthesis of peptide arrays: First, a laser printer addresses the twenty different amino acid particles to a surface with free amino groups (Scheme **7i**), then the whole layer of amino acid particles is melted at once to initiate the coupling reaction for all the twenty different amino acid monomers in parallel (Scheme **7ii**). Washing and deprotection steps finish the cycle that results into the combinatorial synthesis of a peptide array, if repeated. Our particle-based method uses conventional Fmoc chemistry [25] and differs from the SPOT synthesis only in the - at room temperature - solid solvent employed that allows for the intermittent immobilization of amino acids within particles (compare Schemes **4** & **7**).

The intermittent "freezing" of a chemical reaction within solid particles is the main novel element of the method. Chemically very reactive amino acid derivatives used for peptide synthesis are embedded within a solid particle matrix that completely blocks their diffusion and concomitant chemical coupling to a reaction partner. Unexpectedly, the solid matrix that was used to embed the amino acid monomers not only blocked their diffusion to the solid support, but also very efficiently shielded these very reactive chemicals from decay. Indeed, all the 20 different Fmoc-amino acids with a C-terminal OPfp-ester activation proved to be stable for months at room temperature when embedded in particles. The only exception in our hands proved to be Fmoc-Arginine-OPfp that decayed a moderate 4% per month [21]. This is a remarkable finding with regard to the notorious instability of carboxy-activated Fmoc-Arginine derivatives within solvents [26]. Another advantage of the particle-based method is less obvious. The surprising stability of chemically activated amino acid derivatives within solid particles gives the experimenter plenty of time to manufacture, rigorously purify, store over months, and consecutively address different particles to different areas on a two dimensional support, before the coupling reaction is finally induced. This uncoupling of particle production, storage, printing, and chemical reaction is a decisive advantage in the automation and standardization of the whole procedure, which can be elucidated by comparing SPOT synthesis and the particle-based method. An amino acid monomer spotted within a liquid solvent immediately starts to diffuse to the surface of the solid support and eventually reacts with free amino groups, while the printing procedure is still going on, eventually for many more minutes. Therefore, the reaction conditions for those monomers printed first are quite different from those that are printed later. No such bias is found in the particle-based method, where the coupling reaction starts at the same time for all the different amino acid particles because the whole layer of particles is melted at once.

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 non-standard solvent or elevated coupling temperatures. Even with extended coupling times of 90 min at

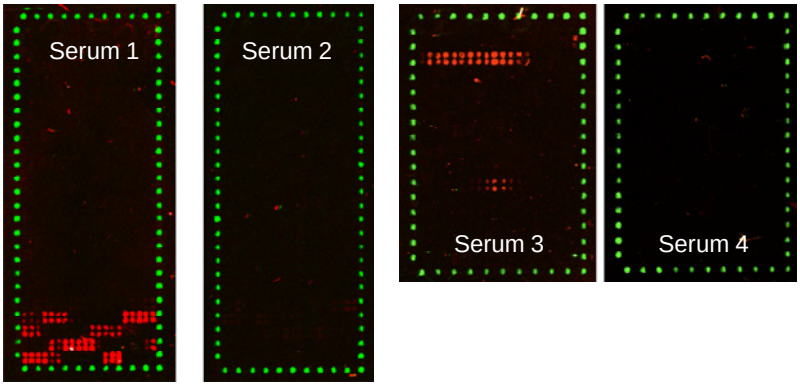


Fig. (1). Peptide-specific antibodies in rabbit serum; i) Two different rabbits were immunized with protein #A and their sera collected (serum 1 & 2). Another two rabbits were immunized with protein #B, and serum 3 & 4 collected, ii) Using the peptide laser printer we synthesized an array of closely overlapping peptides derived from protein sequence #A (left) and protein #B (right). Overlapping 15meric peptide neighbors were shifted in their sequence by one amino acid, iii) Protein arrays #A were stained with serum 1 & 2 (left), while protein arrays #B were stained with serum 3 & 4 (right), iv) The staining pattern revealed that rabbit 1 developed several antibodies that are directed against peptides from the C-terminal end of protein #A, while rabbit 3 developed antibodies that are mainly directed against the N-terminal end of protein #B. Rabbits 2 & 4 didn't develop peptide-specific antibodies.

temperatures of 90°C, we observed no aspartimide formation or racemization at all, nor any major unexplainable peaks in mass spectrometry analysis of synthesized peptides [21]. These advantages meanwhile allow for the production of high-density, high-quality peptide arrays that give clear signals even for unpurified peptides that are stained with complex mixtures of analytes. As an example, Fig. (**1**) shows the differential staining of two different peptide arrays that were stained with four different sera. These sera were derived from rabbits that were immunized with protein #A (rabbit 1 and 2) and protein #B (rabbit 3 and 4). Peptide array #A displays densely overlapping peptides derived from protein #A, while peptide array #B displays peptides derived from the sequence of protein #B. The staining pattern clearly shows that rabbit 1 developed different antibody species that specifically reacted with C-terminal peptides of protein #A, while rabbit 2 didn't produce such antibodies upon immunization. A similar picture emerges when staining the array that displays peptides related to protein #B. Here it is only rabbit 3 that developed antibodies against peptide sequences from the N-terminal region of protein #B.

Chip Printer

Today, laser printers are present in many households, making the laser-printing technique the most prominent method to direct charged particles by electrical fields to their addresses on a two dimensional support. The feature size achieved by the Oki 7000 LED printer series is around ~20µm. Obviously, the manufacturers of laser printers don't feel the pressing need to improve the resolution of their printers beyond that because a feature size of 20µm is well below the resolution of a human eye. Thus, they don't expect additional profits due to a higher-resolution laser printer. Moreover, due to the expansion to 20 cartridges, our custom-made peptide laser printer is an expensive and bulky machine. This machine would grow even bulkier if it should accommodate in addition to the 20 different L amino acid particles other non-natural building blocks. Such additional monomers could be used, e.g. to also synthesize peptoids that might be useful for therapeutic applications. However, even nanoscale deposition of particles to a surface is described in the literature [27], which compares favorable to state of the art laser printers.

We therefore explored a chip-based variant procedure of our particle-based synthesis method that should further increase the density of different peptides on the arrays, easily accommodate more monomeric building blocks, avoid the expensive and bulky peptide laser printer, and, as a result, save costs per peptide. In order to achieve these goals, we used the electrical fields of individual pixel electrodes of a programmable complementary metal oxide semiconductor [CMOS] chip to direct our charged amino acid particles to defined patterns on the chip's surface due to pixels that were switched to voltage (Scheme **8**). Adhesion forces keep sticking these particles to "their" pixel electrodes when the pattern of pixels that is switched to voltage is changed and further kinds of particles are addressed to other pixels (Fig. **2**). Thereby, this variant particle-based synthesis method deposits many different solid amino acid particles at easily programmable very small synthesis sites. After addressing a whole layer of the different amino acid particles, and very similar to the peptide laser printer described above, chemically pre-activated amino acids are then diffused by simply melting the whole layer of particles, which induces the coupling reaction to the support's free amino groups. However, this time the precision of particle deposition is built into the chip itself, and, thus, can do without an expensive and bulky peptide laser printer, but instead use a cheap disposable chip. The chip design we used allows for the application of relatively high voltage (50-100V) to individual pixel electrodes. It is described in detail elsewhere [28]. Currently, the arrays synthesized with this procedure accommodate up to 40.000 peptides per cm^2, which comes near the density of oligonucleotide arrays synthesized with lithographic methods (see also Fig. **3**) [17].

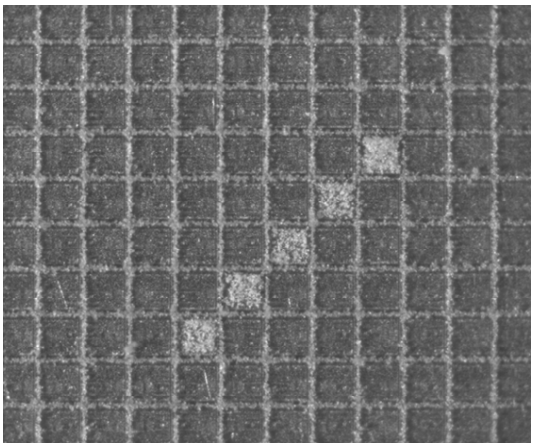
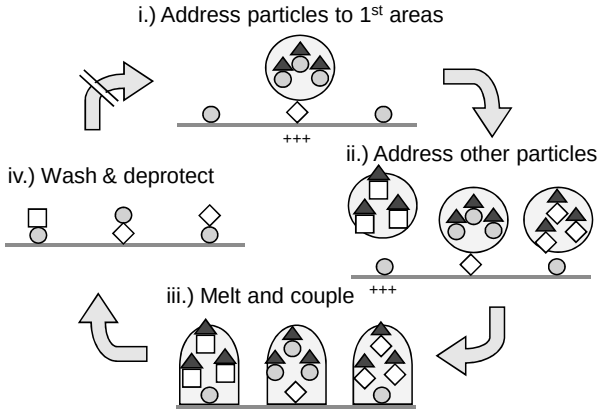


Fig. (2). Adhesion forces keep particles sticking to the surface; i) Fmoc Asp OPfP esters were embedded in Asp-amino acid particles, ii) These particles were consecutively addressed to five different single pixles, iii) Particles keep sticking to the surface due to adhesion forces although "their" pixel is no longer switched to voltage. In addition, hardly any misplaced particles are visible.



Scheme 8. Combinatorial synthesis with a computer chip; i) Individual pixel electrodes are switched to voltage, which generates a pattern of electrical fields on the surface of the chip. This pattern of electrical fields addresses one first kind of amino acid particles onto one first kind of areas on the surface of a chip, ii) Different patterns of pixel electrodes are switched to voltage to consecutively direct all 20 different amino acid particles onto the chip surface until a whole layer of all 20 different amino acid particles is completed, iii) The whole layer of consecutively addressed amino acid particles is melted at once to induce the coupling reaction in parallel, iv) Excessive monomers are washed away, and the Fmoc protection group is removed. Repetitive coupling cycles generate a peptide array with one coupling cycle per layer.

One drawback of the method schematically shown in Scheme **8**, however, is that combinatorial synthesis is done directly on the chip's surface. One difficulty associated with that procedure is the vulnerability of 10µm thin bonding wires that must be shielded from mechanical stress to assure proper functioning of the chip. Normally, this is done by embedding the bonding wires into glue ("glob top"), but none of the commercially available brands we tested resisted standard solvents for peptide synthesis. Therefore, we designed specialized fittings that separate the bonding wires from aggressive chemicals at the expense of a relatively large area on the chip that is occupied by the O-ring seal. Another difficulty relates to trace amounts of unknown metal atoms eventually associated with chip production. These interfere with the grafting of the PEG layer onto the chip surface.

To add even more flexibility to the method we very recently constructed a "chip printer" (Fig. **4**) that picks up particles to defined pixel areas, and, in addition, "prints" these amino acid particles in high resolution to a support that is suitable for peptide synthesis (Fig. **5**) [23]. A microscope allows to implement an automated quality control of particle loading (Fig. **6**). In order to closely

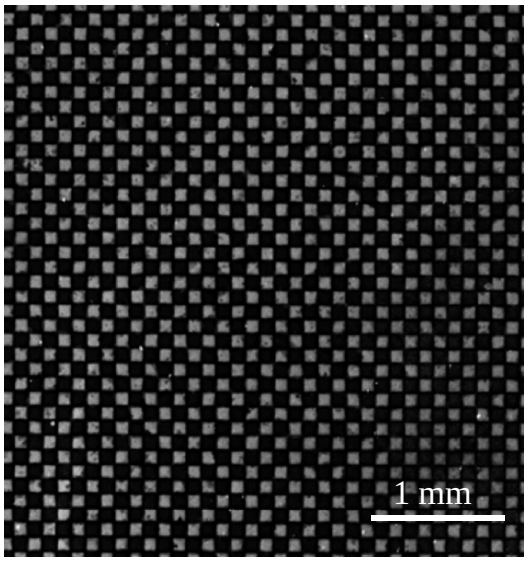


Fig. (3). Array with 10.000 peptides per cm^2; i) Two different peptides were synthesized on top of a chip's surface according to Scheme 8 at a density of 10.000 peptides per cm^2, ii) The resulting array was stained with a mixture of anti Flag and anti HA antibodies that were labeled with different fluorescent chromophores.

align the chip's pixels in parallel with the substrate, where synthesis of peptides ensues after the printing of particles, we mounted the chip on a print head that is adjustable in x, y, and z directions (±0.1µm; ±5µrad). The print head with mounted chip then moves in x-direction to pick up particles on selected pixels that are switched on voltage (Fig. **5**, upper left). Then, the chip moves to the "substrate position" and the particles are printed by a strong external electrical field (1kV per mm) due to additional positive voltage applied to the opposite side of the substrate (Fig. **5**, upper right). Thereby, we can print the amino acid particles in high resolution onto freely chosen substrates. Moreover, the observed high quality of particle deposition (Fig. **5**) opens the road for the consecutive addition of >20 different kinds of particles, which should help in the combinatorial synthesis of peptoid arrays. In order to assess the compatibility of our chip printer with our particle based synthesis we finally melted the particles (Fig. **5**, lower left) to induce the coupling reaction to free amino groups on the PEG grafted glass slide [23], blocked non-reacted free amino groups with a large excess of acetic anhydride, removed the Fmoc protecting group, and stained the amino acids that were coupled to the surface with bromophenole blue (Fig. **5**, lower right). Since the observed blue staining hints to a concentration of >0,5 nmoles per cm^2 of free amino groups, we think that this variant particle based method soon will

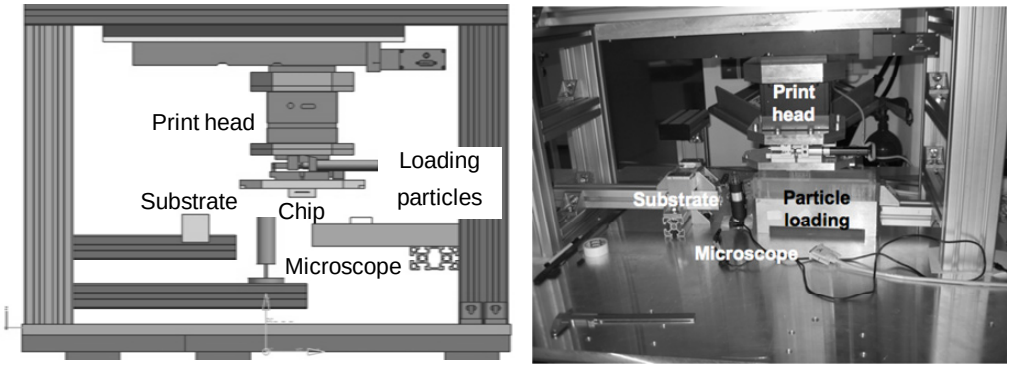


Fig. (4). Chip printer. Schematic drawing (left) and photograph of the machine (right); i) A chip is mounted underneath a tilt stage that is used to align the chip surface and the substrate in parallel, ii) The chip's pixels are switched to voltage and a dust cloud of electrically charged particles is used to load specific pixels with amino acid particles (loading area), iii) Then the print head moves to a glass substrate (substrate area) grafted with amino groups and prints the particles to defined areas; iv) A microscope is used to monitor the quality of particle loading.

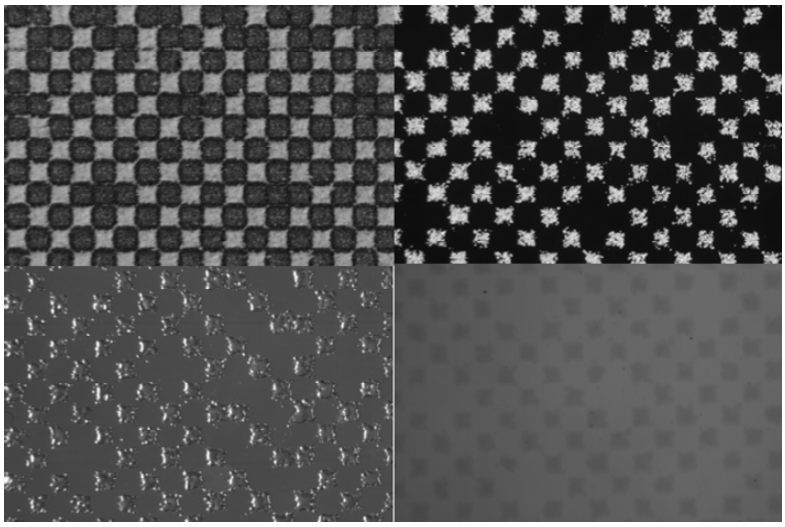


Fig. (5). Handling of amino acid particles with the chip printer; i) Particles were loaded on the specific areas of the chip print head (upper left), ii) These were then printed onto a glass substrate (upper right), iii) Particles were melted to induce the coupling reaction (lower left), iv) Finally, free amino groups were blocked with acetic acid anhydride, the Fmoc protecting group removed, and newly introduced free amino groups stained with bromophenole blue (lower right).

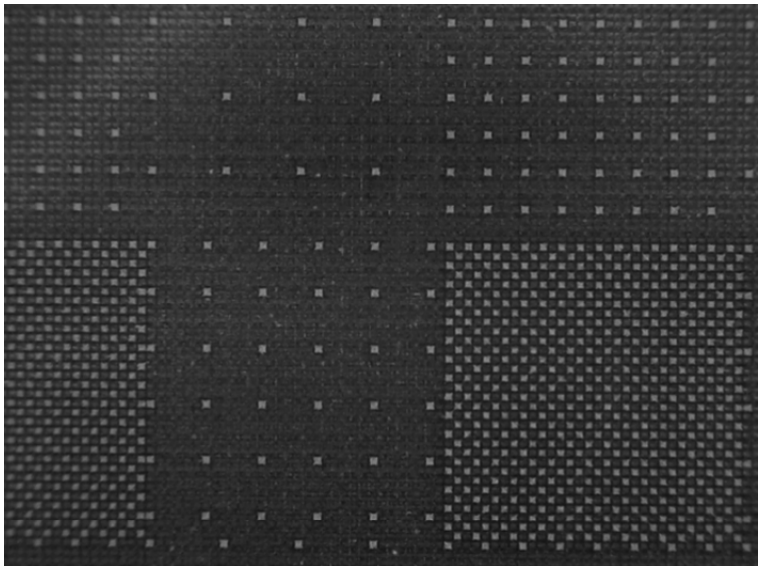


Fig. (6). View from the microscope on the chip loaded with amino acid particles.

generate high-density peptide arrays at an unrivaled prize and density.

CONCLUSION

The SPOT synthesis method only yields 25 peptides per cm^2 at relatively high costs, but due to its reliability SPOT synthesis still dominates the field of peptide arrays. The accompanying papers in this issue of Minireviews in Organic Chemistry describe a plethora of applications for this beautiful and robust technology, which are not further detailed here. Instead the focus of this review is on methods that yield very high density peptide arrays, and the scientific opportunities such methods might bring. Obviously, and for principle reasons, lithographic techniques don't work for the synthesis of high-density peptide arrays due to the inherent consecutive addition of (too many) monomers. However, recently the authors of this article developed a particle based combinatorial synthesis of peptide arrays that yields cheap high-density peptide arrays that are commercially available [29]. 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 for the combinatorial synthesis of high-density peptide arrays where twenty different chemical building blocks must be separated from each other in a densely spaced and chemical saving arrangement. 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 densely space and repeatedly address very small reaction spheres. These features, together with the reduced number of coupling cycles intrinsic to all printing methods, make the novel particle-based method particularly suited for automation. In turn, this should drastically reduce the cost per peptide spot in the near future, which should bring affordable high-density peptide arrays into laboratories.

What impact might affordable high-density peptide arrays bring with them? One field of applications certainly would be to extract

the information that is deposited in a patient's immune system. Challenged by many different pathogens, the immune system from every human individually decides which antibodies and which T-cells it should amplify to tackle these infections. As a result, in every patient a few hundred different antibodies and a similar number of different T-cells patrol the blood in elevated levels, and these amplified antibodies and T-cells are different in every human. The question which antibodies and which T-cells have been amplified and actually confer immunity to a pathogen is of utmost importance for therapy, diagnosis, prognosis, and vaccine development, but especially the antigens that protective T-cells recognize are very difficult to identify. These T-cell antigens are short peptides that are only 9 or 10 amino acids long, but they are only recognized by a T-cell if they are presented in the context of a Major Histocompatibility Complex (MHC) Class I protein. Inside every cell MHC proteins are constantly loaded with peptides derived from digested cellular proteins, and then the MHC proteins with their peptide load migrate to the outer membrane where they are presented to the outside world. There, the peptide presented by the MHC protein eventually is recognized by a specifically binding T-cell that constantly scans every cell for alarming non-self peptides that are derived from a pathogen. The most interesting question is: which peptides from a pathogen are recognized because once identified this peptide could be used in a vaccine to protect us from the pathogen.

High-density peptide arrays might help to answer this question because, as an example, only ~30.000 different 9 or 10 amino acids long peptides possibly could be coded for by a pathogenic Hepatitis C Virus (HCV). This number is well in the range of the methods described above, but it used to be nearly unaffordable at a prize of 10€ per peptide, not to mention the many more peptides that a pathogenic bacterium could code for. The methods discussed above could deliver the peptides that are needed, and recently also the other major technical challenge has been solved: Soen *et al.* successfully loaded peptides in the array format onto MHC proteins and thereby identified those peptides that were specifically recognized by T-cells [30].

It should also be possible to nearly completely diagnose all of the patrolling antibodies even without knowing the original antigen that induced them. Simply depending on the number of displayed peptides, eventually any antibody would find a binding partner. Moreover, peptide binders found in the first screen could be systematically varied in their sequence for consecutive screening rounds to eventually identify better binders. If done with many different serum antibodies, such a procedure would identify an increasingly overlapping panel of peptides that comprehensively diagnoses a population's antibody response. Assembling several thousands of such antibody diagnosing peptides onto an array would then result into a formidable new research tool because the traditional diagnostic question could be widened to ask: What pattern of antibodies (if any) correlates to the sera, e.g. of Parkinson's diseased (or any other enigmatic disease)?

A technically very similar screen might help to find novel therapeutics that, e.g. block a signaling pathway by inhibiting a target protein that a cancer cell needs for continued growth. Not only antibodies, but any protein should bind to some peptides, if only the number of peptides presented on the array is large enough. As discussed in the preceding paragraph, these peptide binders could be varied in their sequence, screened again, and eventually be stabilized by the incorporation of non-natural amino acids. However, all the endeavors listed above have one absolute requirement: affordable, high-density peptide arrays.

CONFLICT OF INTEREST

Some of the authors hold shares in the DKFZ spin off company PEPperPRINT that commercializes the particle based combinatorial synthesis method. This method is covered by the patent family EP1140977B1, which is also authored by Dr. Frank Breitling.

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LIST OF ABBREVIATIONS

ATRP	=	atom transfer radical polymerization
Boc	=	di-tert-butyl dicarbonate
CMOS	=	complementary metal oxide semiconductor
DMF	=	dimethylformamid
Fmoc	=	9-fluorenylmethyl carbamate
HCV	=	Hepatitis C Virus
MHC	=	major histocompatibility complex proteins
NMP	=	N methyl pyrolidone
PEG	=	polyethylene glycol
PEGMA	=	poly(ethylene glycol)methacrylate
PS	=	polystyrol
SPPS	=	solid phase peptide synthesis

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