Biotechnology

DOI: 10.1002/anie.200801616

Combinatorial Synthesis of Peptide Arrays with a Laser Printer**

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In memory of Annemarie Poustka

More than forty years ago, Merrifield described the consecutive coupling of amino acid monomers to a growing peptide immobilized on a solid support.[1] His approach was later expanded to establish the field of combinatorial chemistry, whereby multiple reactions are carried out in parallel to synthesize many different peptides.[2] The aim of these methods is to synthesize and analyze as many peptides as possible, for example, to identify individual peptides that bind to a target protein. The one-bead–one-compound method generates many different peptides readily;[3] however, the decoding of peptide binders is labor intensive. Furthermore, problematic peptides, for example, hydrophobic peptides that bind nonspecifically to any protein, are also synthesized during library preparation by these methods.

Arrays do not have these drawbacks. The position of a given peptide on an array corresponds directly to its sequence, and problematic peptides can be omitted in subsequent arrays. Peptide arrays were first described by Frank, whose spot synthesis dominates the field because of its reliability and wide applicability.[4] High peptide densities can be achieved with the SC^2 method, whereby the individual peptide–cellulose conjugates synthesized in a first array are separated and spotted in high density on a secondary support, for example, glass slides. This variant of the spot synthesis is particularly useful for the production of multiple replicas of densely spaced peptide arrays.[5] Arrays with thousands of oligonucleotides per cm^2 can be synthesized by lithographic methods;[6] however, with these methods only one kind of monomer can be coupled at a time to spatially defined regions on the solid support. Thus, 20×10 coupling cycles are required to synthesize an array of peptides of 10 amino acids in length. In contrast, only 4×10 coupling cycles are required to generate an analogous array of oligonucleotides. This peptide-specific drawback makes it difficult to generate arrays with longer peptides by lithographic methods.[7]

To generate customized peptide arrays at high density, high speed, and low cost, we used a modified color laser printer to "print" the 20 amino acids in the form of solid amino acid toner particles at defined positions on a glass support. After printing, an entire layer of all the different amino acid toners is melted at once to release hitherto immobilized amino acids and initiate the coupling reaction. Washing and deprotection steps between the printing processes complete the cycle, the repetition of which results into the combinatorial synthesis of a peptide array (Figure 1). An advantage of this method is that conventional Fmoc (9-fluorenylmethoxycarbonyl) chemistry[8] can be used. The method differs from standard solid-phase synthesis only in the use of a "solid solvent" (at room temperature), which immobilizes the amino acids within toner particles until the beginning of the coupling reaction.

Our peptide laser printer is based conceptually on the color laser printer OKI C7400, but accommodates 20 instead of four printing units (each of which contains a particular amino acid toner), as well as a drive and mounting that enable the repeated exact positioning (± 5 μm) of the solid support (Figure 2a). A row of approximately 10000 light-emitting

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[**] We thank Dorothea Freidank, Daniela Rambow, Thorsten Kühlwein, and Jürgen Kretschmer for technical assistance, Martina Schnölzer (DKFZ) for mass spectrometry, Hans Lehrach for helpful discussions and encouragement, Kazuhiro Ohmori (Sekisui) for advice in particle composition, Alexander Küller, Michael Grunze, and Reiner Dahint (University of Heidelberg) for surface-analytical methods, and the BMBF (grant nos. 03N8710 and NGFN-0313375), the Helmholtz Society (grant no. VH-VI-108), the HFSPO (grant no. RGP5/2006), the European Union (grant no. 508399), and the Volkswagenstiftung (grant no. 79 466) for financial support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200801616.

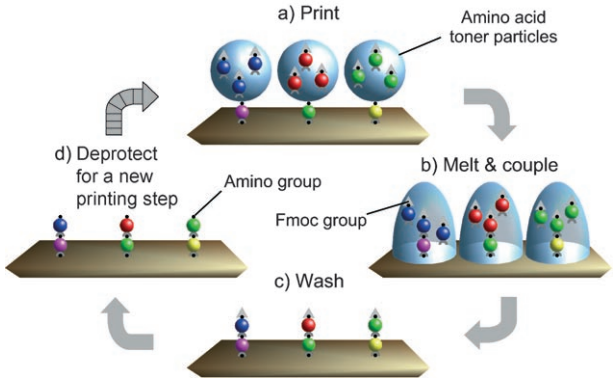

Figure 1. Particle-based Merrifield synthesis. a) A laser printer delivers Fmoc amino acid–OPfp esters embedded within toner particles to specific locations on a solid support, on which b) the particles are melted after transfer. Melting enables the amino acid derivatives to diffuse and undergo coupling to the support. A synthetic cycle is completed when c) excess monomers are washed away, and d) the Fmoc protecting group is removed. Repeated coupling cycles generate a peptide array.

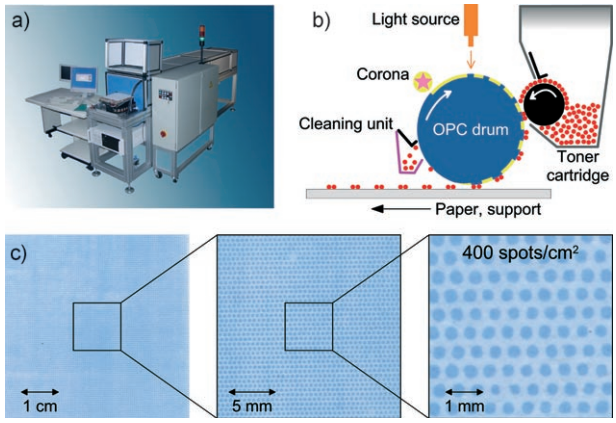

Figure 2. Laser printing: a) The peptide laser printer with 20 different printing units aligned; the mounting for the support is visible at the front of the printer. b) A light source (LED row, orange) illuminates and thereby neutralizes selected areas of an OPC drum (blue), which is first uniformly charged (yellow) by a corona. Triboelectrically charged toner particles are transferred to these neutralized areas and from there by a strong electric field to a solid support. c) Amino acid toner was printed by the peptide laser printer onto a glass slide derivatized with free amino groups. The esters (in this case: Fmoc-Ile-OPfp) embedded in the particles were released by heat, the residual material was washed away with DMF, and the remaining free amino groups were blocked with 10% acetic anhydride in DMF. Finally, the Fmoc protecting groups were removed with 20% piperidine in DMF, and the newly introduced free amino groups were stained with 0.1% bromophenol blue in methanol.

diodes (LEDs) per 20 cm generates a light pattern on the surface of a uniformly charged organic photoconducting (OPC) drum, which rotates with approximately 10000 steps per 20 cm. The resulting two-dimensional light pattern comprises around 100 million pixels per $(20\times20)\ cm^2$. The OPC material translates this light pattern into the corresponding electrostatic pattern of around 100 million pixels per $(20\times20)\ cm^2$: The OPC drum is charged evenly by a corona, and then the pixels that are irradiated with light are discharged. This process is facilitated by the properties of the OPC material. The drum coating, which is insulating in the dark, becomes conductive upon exposure to light, so that the illuminated areas of the drum are neutralized rapidly by grounding.[9] Subsequently, charged toner particles are transferred only to those areas previously neutralized by irradiation with light. Thus, the electrostatic pattern is transformed into the corresponding particle pattern. Finally, the particles delivered by the OPC drum are collected by a strong electric field ($4\ kV\,mm^{-1}$) on a solid support, on which a printout is assembled from the 20 different amino acid toners (Figure 2b).

The amino acid toner particles are charged triboelectrically within their cartridges by mild friction of particles against a foam-rubber drum, whereby a negative charge is generated on the surface of the particles. We adjusted the physical properties of the particles to those of commercial toner particles in terms of size distribution (see Figure S1 in the Supporting Information), toner transfer (see Figure S3 in the Supporting Information), melting behavior (see Figure S2 in the Supporting Information), their morphology (see Figure S4 in the Supporting Information), and, finally, printing performance (Figure 2c, and Figure S8 in the Supporting Information). We embedded the 20 different Fmoc amino acid–OPfp esters (OPfp = pentafluorophenyl), which were chosen for their commercial availability and relative stability as activated amino acid derivatives for peptide synthesis, into the corresponding amino acid toner particles (see Table S1 in the Supporting Information). The component designated to serve as a solvent in our particle-based Merrifield synthesis was chosen from higher homologues of standard solvents, for example, *N*,*N*-diphenylformamide. Most such solvents that are solid at room temperature gave the coupling products in similar yields to those observed with the standard solvent *N*,*N*-dimethylformamide (DMF) for liquid-phase synthesis, but some resin components of commercial toners also performed as well. When we melted the printed particles on amino-derivatized glass slides,[10] we observed a pattern of coupled amino acids in the same resolution as that of the original printout (Figure 2c). Thus, the printing performance of our amino acid toners in terms of spot resolution and the amount of toner transferred is nearly indistinguishable from that of commercial color toners (see Figure S3 in the Supporting Information). The driver software of our first peptide laser printer limits the printing resolution to 160000 spots in an area of $(20\times20)\ cm^2$ (Figure 2c): many fewer than the millions of different peptides that such a method could generate.

To explore combinatorial peptide synthesis in more detail, we synthesized the HA (hemagglutinin) and Flag epitopes, each on a larger area of our glass surface. We determined the yield for repetitive coupling by our particle-based method to be about 90% on average (see Figure S6 in the Supporting Information) and confirmed the integrity of the synthesized peptides by mass spectrometry (see Figures S9–11 in the Supporting Information). This low yield for repetitive coupling relative to yields observed for standard Fmoc synthesis

on polymer beads can be explained partly by the fact that our particle-based method is not compatible with the use of preswollen PEG-derivatized glass slides, as DMF vapor harms the OPC drums of the laser printer (see the Supporting Information; PEG = poly(ethylene glycol)). We ruled out the possibility that the nonstandard solvent or elevated coupling temperatures induce the racemization of the L-amino acids (see Figure S7 in the Supporting Information). Our observations are in agreement with the results of Schüttler et al., who detected no racemization of the closely related Fmoc amino acid–OPcp esters at coupling temperatures of 90 °C (Pcp = pentachlorophenyl).[11] Although neither mass spectrometry nor HPLC data indicate that any other heat-induced side reaction occurs to a significant extent (see Figures S7 and S9–S11 in the Supporting Information), this possibility has to be investigated in more detail in the future. To our surprise, none of the Fmoc amino acid–OPfp esters showed a measurable decay rate when stored at 25 °C inside our toner cartridges, except for Fmoc-Arg(Pbf)-OPfp, which decayed at a moderate rate of 5% per month (see Figure S5 in the Supporting Information). This finding is remarkable given the notorious instability of carboxy-activated Fmoc–arginine derivatives in other solvents.[12] Finally, we synthesized a peptide array with 5500 different permutated Flag and Myc epitopes on a microscope slide (400 peptides per cm^2; Figure 3). When we incubated the array with the corresponding antibodies, all of the permutated peptide epitopes showed the expected staining pattern, which correlated with previously published results (Figure 3 and Figure S8 in the Supporting Information).[13]

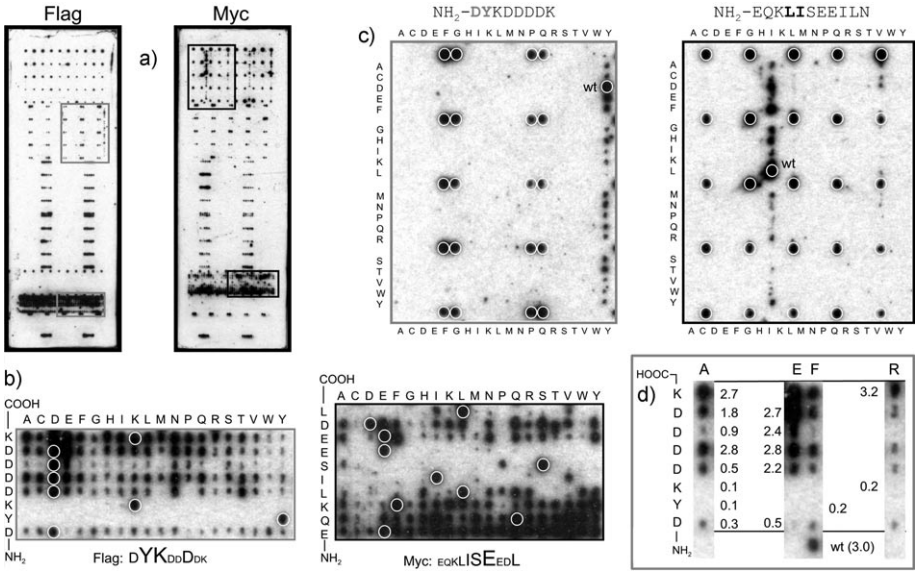

Figure 3. Combinatorial synthesis of a peptide array. Permutated Flag and Myc epitopes were detected by the consecutive addition of epitope-specific mouse monoclonal antibodies (enhanced chemoluminescence luminescence (ECL, Pierce) readout). a) Glass slides with approximately 5500 peptides generated by combinatorial synthesis; the resulting slides were stained with Flag or Myc antibodies. Regularly interspersed positive controls (encircled spots in (b,c) correspond to wild-type epitopes) and some reactive peptide variants are clearly visible. Frames delineate the enlargements shown in (b,c). b) Characterization of Flag and Myc epitopes. Every amino acid position of each epitope was exchanged for all 20 different amino acids to characterize the binding requirements of epitope-specific antibodies. The epitope specificity deduced from individual experiments is given in the sequence written underneath, whereby the size of an amino acid symbol reflects the importance of that amino acid for antibody binding. The epitope specificities of 9E10 and Flag M2 antibodies agree with published results.[13] c) Epitopes were permutated at the two positions highlighted in bold in the peptide sequence. Interspersed wild-type sequences are encircled. The N-terminal aspartic acid residue of the Flag epitope can be substituted for other amino acids, whereas the adjacent tyrosine residue is mandatory for the binding of the Flag M2 antibody. The highlighted isoleucine residue of the Myc epitope can only be substituted for valine, whereas the adjacent leucine residue is less important for antibody binding. The results of these more complex permutations all agree with the experiments in (b). d) Correlation of the staining intensity of the Flag epitope variants in (b) with published relative affinities,[13] which are written next to the spots stained with the Flag M2 antibody.

In summary, we have described the truly combinatorial synthesis of high-density peptide arrays with a custom-made peptide laser printer that addresses 20 different amino acid toners within consecutive combinatorial layers. A variant of this method, in which a microchip is used to attach amino acid particles to individual pixel electrodes, has so far enabled us to address only regular patterns of amino acid particles.[14] Through the temporary "freezing" of the activated amino acids within solid particles (Figure 1), the method described herein enables the separate production and rigorous purification of the different amino acid toner particles. These "postal packages" can be stored for months within the laser printer, addressed spatially on demand, and only then melted to initiate combinatorial peptide synthesis. Thus, our approach adds speed (seconds of printing versus hours of spotting), flexibility, cost effectiveness, and robustness (see Figure S5 in the Supporting Information) to the combinatorial synthesis of peptide arrays, and at the same time avoids the drawbacks of other methods. Our peptide laser printer should make it possible to translate entire genomes of pathogenic viruses and bacteria into sets of overlapping peptides, which can be screened for antipathogen antibodies.

Received: April 7, 2008
Revised: May 9, 2008
Published online: July 31, 2008

Keywords: amino acids · biotechnology · combinatorial chemistry · high-throughput screening · peptides

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