Solid-Phase Synthesis | Hot Paper

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Flow-Through Synthesis on Teflon-Patterned Paper To Produce Peptide Arrays for Cell-Based Assays**

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Abstract: *A simple method is described for the patterned deposition of Teflon on paper to create an integrated platform for parallel organic synthesis and cell-based assays. Solvent-repelling barriers made of Teflon-impregnated paper confine organic solvents to specific zones of the patterned array and allow for 96 parallel flow-through syntheses on paper. The confinement and flow-through mixing significantly improves the peptide yield and simplifies the automation of this synthesis. The synthesis of 100 peptides ranging from 7 to 14 amino acids in length gave over 60 % purity for the majority of the peptides (> 95 % yield per coupling/deprotection cycle). The resulting peptide arrays were used in cell-based screening to identify 14 potent bioactive peptides that support the adhesion or proliferation of breast cancer cells in a 3D environment. In the future, this technology could be used for the screening of more complex phenotypic responses, such as cell migration or differentiation.*

Solid-phase synthesis (SPS) is a central technique for producing libraries of lead organic compounds for the pharmaceutical and biotechnology industry. SPOT-synthesis was developed in the 1990s as a method for parallel SPS on a planar support to yield high-density arrays of peptides[1] and other organic molecules.[2] SPOT-synthesis has been adapted in academic and industrial research for the production of functional ligands, epitope mapping, cell-based screens,[3,4] and the identification of functional materials.[5] To date, the environment of chemical reactions in SPOT synthesis is sub-optimal when compared to SPS: the optimal yield and reactivity in SPS is achieved in an actively-mixed solution or in a flow-through reactor in which the solid support is exposed to a continuous flow of reagent. By contrast, in classical SPOT synthesis, a limited amount of reagent is spotted onto paper, thus forming a static spot of liquid with a defined size. Within this spot, flow-through conditions are not possible, thereby limiting mass transfer to diffusion. Furthermore, the fixed relationship between the size of the spot and the volume of the solution limits the volume that can be deposited onto the support.[4] We solved these problems by introducing solvent-repelling Teflon barriers into the paper. The patterns confine liquids and thus allow the deposition of an excess volume of reagents, enable parallel flow-through synthesis, and significantly improve the yields of the chemical reactions. While we focus on paper-based supports and peptide synthesis, we believe that an analogous approach could be applied to any planar porous support and other types of reactions.

Herein, we demonstrate that Teflon-patterned paper satisfies all of the criteria for multistep organic synthesis and downstream analysis: 1) Teflon-impregnated paper is stable to prolonged exposure to organic solvents, organic bases (e.g., piperidine), and strong acids (TFA). 2) most of the required solvents and reagents exhibit a high contact angle on paper permeated by Teflon. 3) Teflon-modified paper is suitable for downstream biochemical and cell-based assays since it is neither fluorescent nor toxic. None of the pre-existing methods for the patterning of paper[6,7] satisfy all of the above criteria. We introduced Teflon into the paper according to the protection–deprotection strategy illustrated in Figure 1; a process referred to as "sweet patterning".[8] The outlines of the patterns were first defined by using a solid ink printer[7] and the zones that should not be impregnated by Teflon were "protected" with a solution of sucrose. The entire array was then exposed to a solution of Teflon to impregnate the unprotected zones, with the Teflon forming solvent-repelling barriers upon drying. Finally, the sucrose was washed away with water. Given that it requires only two steps of liquid deposition, the process can be readily scaled: we automated it by using a liquid handling robot Precision XS, BioTek (Figure S3, Script S1, and Movie S1 in the

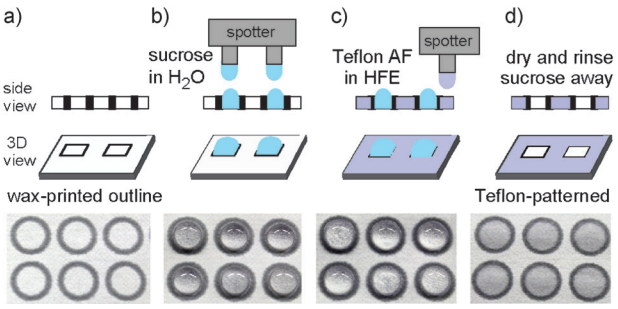

Figure 1. The Teflon-patterning process. a) The pattern is defined by wax-printing. b) Sucrose is spotted into the zones to be kept solvophilic. c) A Teflon solution is deposited onto the remaining regions to form solvophobic barriers. d) After evaporation of the hydrofluoroether (HFE) solvent, the sucrose is washed off.

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Supporting Information). We identified that a pattern with a width of at least 1 mm of Teflon is required to ensure long-term stability of the pattern to organic solvents. (Figure S4 in the Supporting Information).

Teflon-patterned paper confines most solvents that are immiscible with Teflon solution (Figure S5 in the Supporting Information), including weakly polar (dichloromethane, dioxane), polar aprotic (DMF, NMP, DMSO), and protic (alcohols) solvents, as well as aqueous solutions of proteins and surfactants (Figure 2b,c). Examples of liquids that cannot be contained are nonpolar hydrocarbons (hexane) and perfluorinated solvents (HFE-7100). We characterized the pattern by using a "confinement factor" $CF = V_1 V^{-1}_2$, where V_1 is the volume contained per unit area of patterned paper and V_2 the volume of solvent retained by the same area of nonpatterned paper. For example, when spotting 25 μL of DMF, we obtained $CF = 12$ (Figure 2a). Hydrophobic ink provided no confinement because the ink is both wetted and dissolved by DMF.

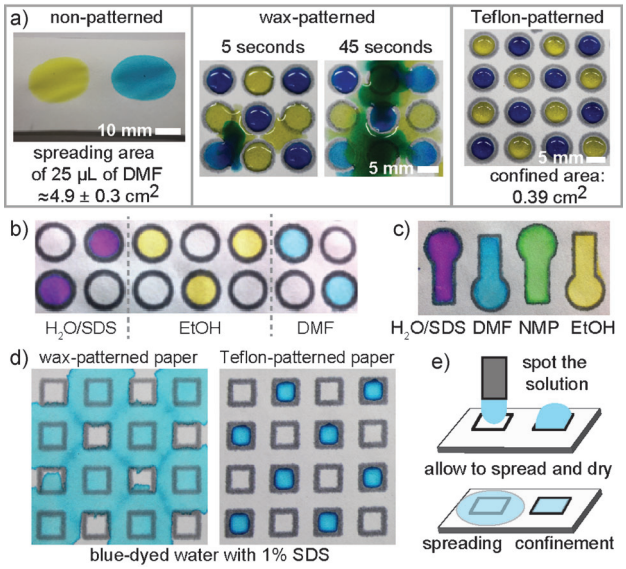

Figure 2. a) Spreading of DMF on nonpatterned and wax-patterned paper and confinement on Teflon-patterned paper. b,c) Examples of Teflon-patterned arrays that confined ethanol, DMF, and NMP. d) SDS in water destroys wax-patterned paper; the same solution is confined on Teflon-patterned paper. e) A scheme showing the process of evaluating spreading and confinement. DMF = *N*,*N*-dimethylformamide, NMP = *N*-methyl-2-pyrrolidinone, SDS = sodium dodecylsulfate, DMSO = dimethyl sulfoxide.

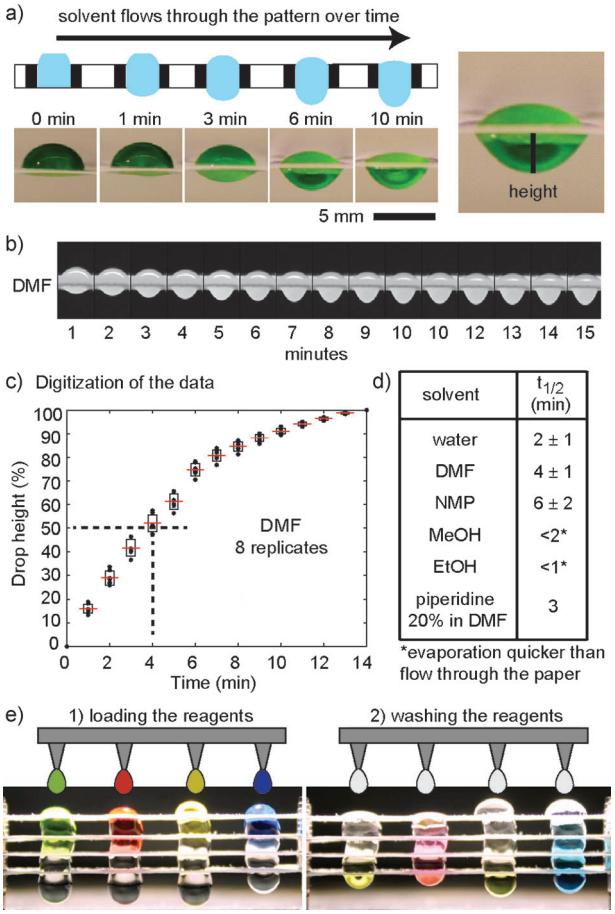

Figure 3. a) The flow of solvent through the paper. The height of hanging droplet was used for the digitization of flow-through data. b) Time-lapse images of DMF flowing through the patterned paper. c) Digitization of eight independent time-lapse experiments. d) A table summarizing the time needed for a liquid to reach half of the maximum height (see the Supporting Information). e) The flow of reagents (1) or washing solutions (2) through a stack of four arrays.

The confinement of an excess volume of reagents on each spot induced gravity-driven flow through the paper (Figure 3a,b). Flow rates were reproducible for specific types of solvent and paper (Figure 3c and Figure S6). The flow rate is nonlinear but it can be characterized by using the time required for half of the volume of the droplet to flow through the paper ($t_{1/2}$; Figure 3d). For filter paper Grade 50, the $t_{1/2}$ values for DMF and NMP solutions were approximately 5 min. This flow rate allowed approximately 90% of the solvent to flow through the paper during the typical reaction steps of peptide synthesis, such as amino acid coupling (20–40 min) or deprotection steps (10–15 min). The rate could be accelerated by using paper with a higher porosity (Figure S6), or by placing an aspirating nozzle below the paper (Figure S7). In the following examples, we used 96-zone arrays with a footprint identical to a conventional 96-well plate to perform 96 parallel flow-through syntheses. Flow-through also allows the replication of reactions in a stack of multiple patterned papers (Figure 3e and movie S7). Confinement and dynamic flow-through were advantageous for reactions on paper. The yields of the reactions in flow-through peptide synthesis conditions were higher than those from conventional peptide synthesis on paper (Figure 4). We used Fmoc quantification to demonstrate that the synthesis of the di-β-alanine linker (βAβA) and the subsequent coupling of the first amino acid (to give βAβAA) were significantly more efficient with the Teflon-patterned paper than with nonpatterned paper in multiple independent trials (Figure 4b). We used the same quantification to show that the conversion at each coupling step in the synthesis of the $(Ala)_{10}$ peptide

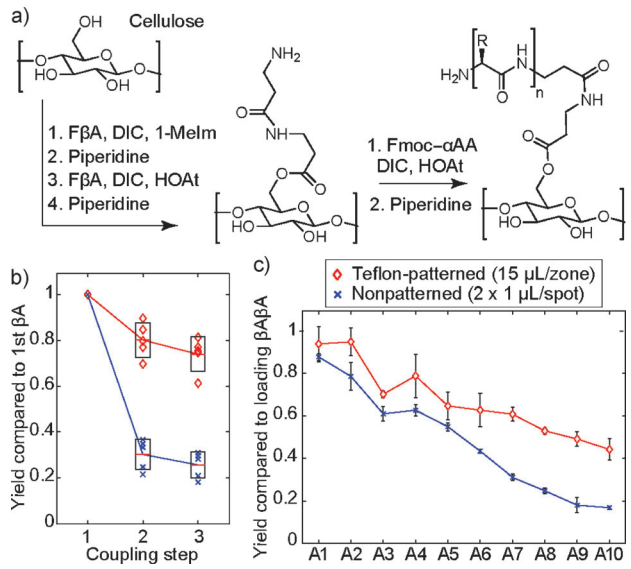

Figure 4. a) Peptide synthesis on paper modified with a βAβA linker. Yields for the synthesis of the βAβAA construct (b) and the $\beta A\beta A(A)_{10}$ peptide (c) on Teflon-patterned paper (◇) and non-patterned paper (×) are shown. In (b), the five data points represent synthesis on five separately prepared arrays. All yields were estimated as an average of Fmoc loading measured in four independent areas. DIC = diisopropylcarbodiimide, 1-MeIm = 1-methylimidazole, HOAt = 1-hydroxy-7-azabenzotriazole, Fmoc = 9-fluorenylmethyloxycarbonyl, FβA = Fmoc-β-alanine, Fmoc–αAA = Fmoc-protected α-amino acid.

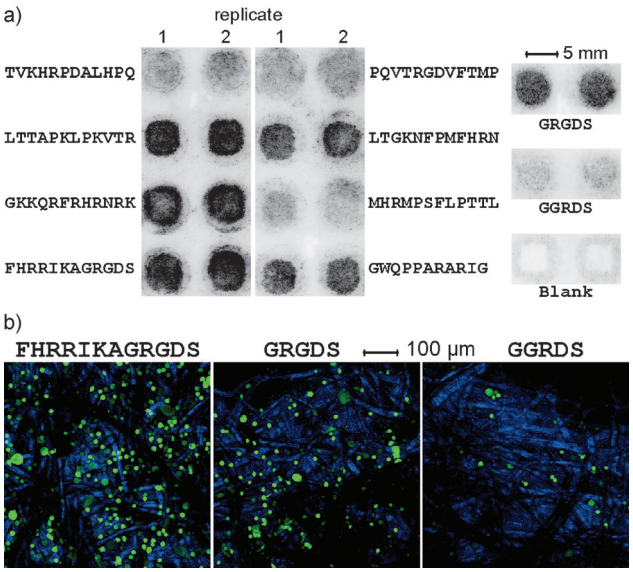

Figure 5. The adhesion of MDA-MB-231-GFP cells to known bioactive peptides synthesized on paper. a) Imaging with a fluorescence gel scanner locates GFP fluorescence (dark areas). b) We confirmed the results by confocal microscopy to validate binding to GRGDS (positive control) and no binding to GGRDS (negative control).

was higher when using the Teflon-patterned paper, with an estimated 50% conversion of a linker to the final peptide for flow-through synthesis compared to < 20% for conventional SPOT (Figure 4c). The identity and purity (correlates to conversion) of more than 100 peptides, ranging in length from 7 to 14 amino acids, that were synthesized and characterized by one technician are shown in Table S1 in the Supporting Information. Confinement of the solvent and flow-through also simplified the automation of the synthesis and improved the washing steps (Figure S8); it reduced the volume of the washing solutions from 15–30 mL, as required in conventional SPOT synthesis,[4] to 1.5 mL in flow-through washing. We used plate-to-plate transfers with the Precision XS workstation to sequentially deposit solutions of activated amino acids (Figure S9), deprotection and capping agents, and washing solvent.

We previously demonstrated that paper can be used to generate "foldable" 3D tumor models to study 3D cultures of cells in vivo and in vitro,[9] for example, to investigate migration[10] or drug resistance.[11] The use of Teflon-patterned arrays allowed us to characterize surface-immobilized peptides that can support cell adhesion, growth, or differentiation. We synthesized eight reported bioactive peptides that are known to support the self-renewal of stem cells[12,13] and induce epithelial–mesenchymal transition.[14] When immobilized on paper, five of the eight peptides supported adhesion of MDA-MB-231 breast carcinoma cells at levels similar to or higher than the positive control peptide GRGDS (Figure 5). The other three peptides supported greater adhesion than the scrambled peptide GGRDS. Unmodified cellulose and paper decorated with GGRDS supported minimal binding. Confocal micrographs of the cells in each substrate are shown in Figure S10. None of the components of the Teflon-patterned peptide array exhibited any toxicity toward the cells (Figure S11). Over the long term, the cells on some peptide-modified surfaces spread along the fibers and resumed cell division (Figure S12). We then used Teflon-patterned arrays to validate the biological properties of 30 peptides identified de novo by phage-display panning on MDA-MB-231 cells (Table S1). Imaging with a fluorescence gel scanner (Figure 6a and Figure S13) and confocal microscopy (Figure 6b and Figures S14, S15) demonstrated that 14 of the 30 peptides supported cell adhesion at levels higher than the integrin-binding peptide GRGDS (Figure 6c); four of the peptides supported adhesion at similar levels, and 10 did not support adhesion. These experiments confirmed that Teflon-patterned paper is an effective platform for the synthesis and cell-based screening of a large number of peptides. Paper is a versatile support for applications such as analytical devices or low-cost diagnostics.[15] We believe that parallel-synthesis capability and the generation of patterns resistant to organic solvents and surfactants will also be beneficial in these areas. The patterning of low-cost paper makes this technology conveniently available; however, we anticipate that future advances in materials production (lithography, 3D-printing, weaving, etc)[16] could yield similar low-cost, self-supported, patterned porous sheets suitable for organic synthesis and bioassays.

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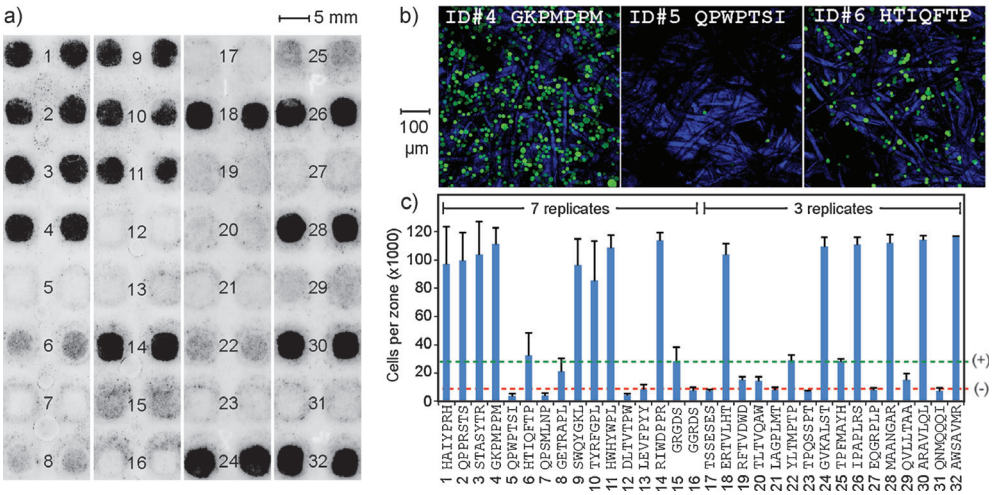

Figure 6. a) We used peptide-arrays to validate the adhesion of MDA-MB-231-GFP cells to 30 peptides identified by phage-display panning. b) Representative confocal images of peptide-modified paper supporting high cell adhesion, no cell adhesion, and moderate cell adhesion. c) The digitization of the number of cells per zone from fluorescence gel scanner images of the cell binding experiments. The (+) and (−) lines represent the intensity levels of the positive (GRGDS) and negative (GGRDS) controls, respectively.

Keywords: cell adhesion · high-throughput screening · peptide arrays · peptides · solid-phase synthesis

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