Chapter 5

Synthesis of Peptide Arrays Using SPOT-Technology and the CelluSpots-Method

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Abstract

Peptide synthesis on cellulose using the SPOT technology follows the standard Fmoc-chemistry and can be performed manually or automated. This method allows the synthesis of low-cost peptide arrays containing around 900 large spots of addressable peptides on a cellulose sheet of 19 cm × 29 cm. These peptides can be cleaved from the cellulose support by ammonia gas and afterward spotted on glass microchips. Alternatively, the peptides can be synthesized on modified cellulose discs and CelluSpot microarrays can be produced.

Key words: Spot synthesis, peptide array, screening.

1. Introduction

Solid-phase peptide synthesis has been automated for tBoc- as well as for Fmoc-chemistry. Established methods can be used to synthesize large numbers of peptides for screening procedures, including tea bag synthesis (1), digital photolithography (2), pin synthesis (3), and SPOT^{TM} synthesis on cellulose (4). Additionally, biological techniques such as phage (5), bacterial (6), or ribosome display (7) can be applied to synthesize and screen large numbers of peptides.

The success of DNA and protein microchips has inspired researchers to develop microchips for peptides arrays (8) (*see* **Table 5.1**). The small amounts of peptides used to fabricate microchips, together with existing technologies to analyze binding events on chips, has opened up new directions for peptide libraries. Several hundreds of different peptides can be placed on a microchip. The effort of synthesizing all these different peptides is high, but is balanced by the large number of chips that can be synthesized with the same peptide set. In particular for medical screens, this type of screening strategy is required since the purity of the peptides needs to be

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Table 5.1
Recommended literature

Description	References
Handbook about spot synthesis and applications	(45)
Detailed protocol for spot synthesis	(30)
Characterization of some useful types of filter paper and description of some linkers	(44)
Investigations on the quality of spot syntheses	(14)
Detailed review about synthesis and application using spot technique	(12)
Review about peptide arrays	(8)

very high. However, synthesis of peptide sets on resin is often very expensive. One strategy to overcome this issue is to synthesize the required peptide sets on cellulose membranes. The SPOT synthesis method described here permits the synthesis of multiple peptides for less than 1% of the cost compared to common synthesis on resin. In addition, we describe the synthesis of peptides on cellulose discs using a conventional peptide synthesizer. The synthesized peptides can then either be released as free peptides to produce common peptide microarrays on glass chips (**Fig. 5.1**), or remain bound to cellulose for direct screening on that membrane as well as for the production of CelluSpots microarrays (9, 10).

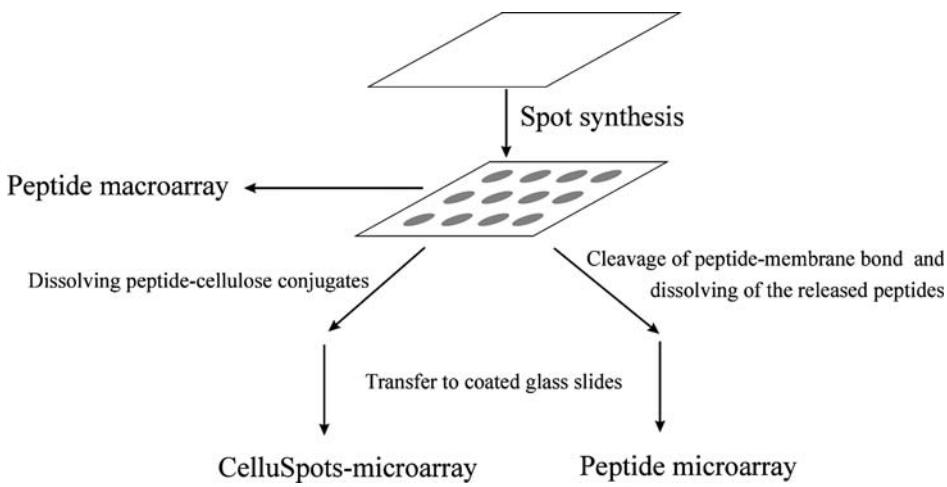

Fig. 5.1. Outline of the preparation of peptide arrays using spot synthesis.

SPOT synthesis was developed by Frank and co-workers in 1990 (11). This special type of solid-phase peptide synthesis follows standard Fmoc-chemistry using cellulose as the solid support (12).

Initially the method was performed manually. Although it is easy to perform and no special equipment is necessary, it is quite laborious to perform by hand and, therefore, is only feasible for a small number of peptides. Automation of the method permitted the synthesis of up to 8000 different peptides on a single sheet (19 cm × 29 cm) in a highly parallel and addressable manner (13), and this improvement has led to a great variety of applications of SPOT synthesis (14) (**Fig. 5.2**). The large number of different peptides synthesized in a parallel manner results in differences in coupling yields, and, therefore, in the quality and quantity of the different peptides. Nevertheless, the SPOT technology has been frequently reported to be very reliable (14–16). For transferring peptides synthesized on cellulose onto a microchip, a larger peptide amount is required than for direct screening on cellulose membranes. A sufficient amount of peptide can be obtained by synthesis on cellulose membranes with larger peptide spots or by multiple syntheses on cellulose discs. Using large cellulose sheets (19 cm × 29 cm) around 900 large peptide spots can be synthesized.

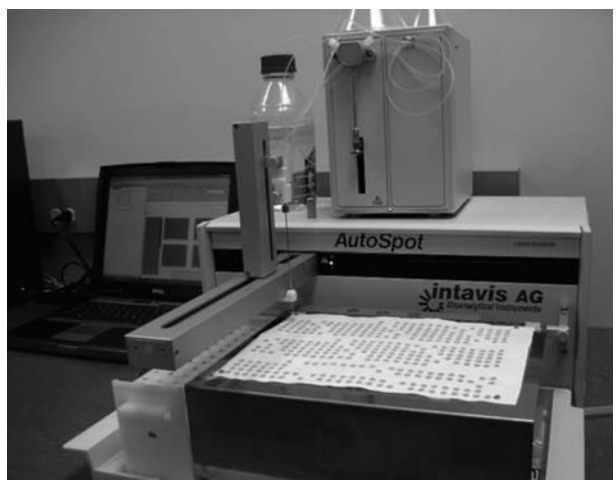

Fig. 5.2. Semi-automated synthesis of large spots using AutoSpot robot (INTAVIS).

Peptide synthesis on cellulose has several advantages: cellulose is inexpensive and withstands the organic solvents and acids used during peptide synthesis. In addition, cellulose is stable in aqueous solutions and, because it is non-toxic, it is appropriate for screening biological samples. Furthermore, peptides up to a length of about 40 amino acids can be synthesized using the SPOT method (e.g., 17, 18), although the optimal range is between 6 and 15 amino acids. With shorter peptides, the affinity for binding reactions might be too low. For the synthesis of long peptides, the following must be considered: with the increase of peptide length the likelihood of difficulties during coupling increases, such that the number of possible side reactions might increase and purity of the desired peptide will correspondingly decrease.

In contrast to biological peptide synthesis, SPOT technology is not restricted to gene-encoded amino acids, and more than 600 commercially available building blocks can be used (e.g., 19, 20). Another advantage of using SPOT synthesis on cellulose is the possibility of modifying the peptide. For detection purposes peptides can be labeled with, e.g., biotin (21, 22), dyes (23), or fluorophores (24). The biotinylation of peptides is also useful for the directed immobilization of peptides to streptavidin-coated glass slides to produce peptide microarrays (25). Maleimide-functionalized peptides can be attached to thiol-modified array surfaces (26). Various types of cyclization strategies can also be performed using this technology (e.g., 27, 28). This allows the synthesis and screening of more rigid molecules that might be more resistant to proteolytic cleavage and heat denaturation.

Peptides synthesized using the SPOT technology can be released from the cellulose support by treatment with ammonia gas or other strong bases such as tertiary amines, and transferred to glass slides. Some peptide arrays have the disadvantage that the peptide molecules can interact with the glass surface in a fashion that might interfere with the screening process. Alternatively, a TFA-soluble cellulose membrane can be used to obtain soluble peptide–cellulose conjugates (29). The resulting solutions of individual peptides covalently linked to macromolecular cellulose can then be spotted onto planar surfaces such as coated glass slides, to produce CelluSpots microarrays. After evaporation of the solvent, a three-dimensional layer is formed that is not soluble in the aqueous reagents used for standard assays (**Fig. 5.3**). It is possible to use detection methods such as autoradiography, chemiluminescence, or enzymatic color development which can be performed without expensive instrumentation.

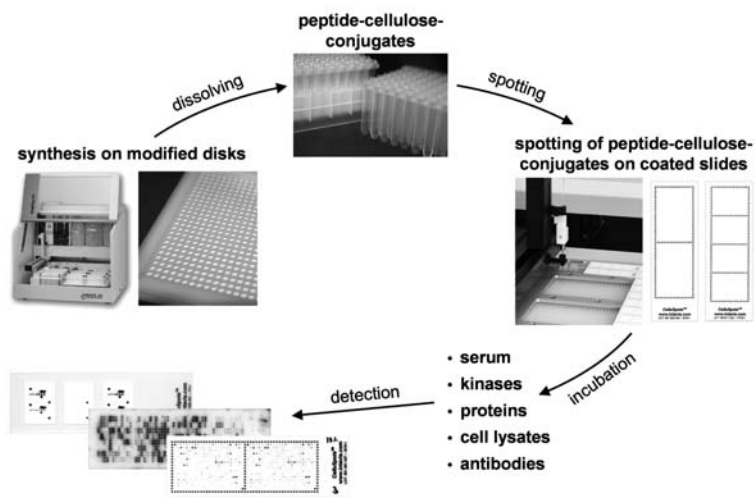

Fig. 5.3. Scheme of preparation cycle for CelluSpots peptide microarrays.

2. Materials

2.1. Solvents

1. *N,N′*-dimethylformamide (DMF; VWR).
2. Methanol or ethanol (MeOH or EtOH; VWR).
3. *N*-methylpyrrolidone (NMP; Fluka).
4. Diethyl ether (DEE; VWR) or *t*-butyl methyl ether (MTBE, VWR).
5. Dichloromethane (methylene chloride, DCM; VWR) (*see* **Note 1**).

2.2. Spot Synthesis of Macroarrays

2.2.1. Preparation of the Membrane

1. Cellulose membranes for releasing peptides from membrane for the preparation of peptide arrays are prepared from filter paper Whatman 50, Whatman 540, or Chr1 (Whatman, Maidstone, UK) (4, 30). Ready-to-use modified membranes for synthesis of peptides attached onto soluble cellulose for the preparation of CelluSpots microarrays are available from INTAVIS (Köln, Germany).
2. Diisopropylcarbodiimide (DIPC, DIC; Fluka, *see* **Note 2**).
3. *N*-methylimidazole (NMI; Sigma).
4. Fmoc-β-alanine (GL Biochem or EMD Biosciences, *see* **Note 4**).

2.2.2. Preparation of Activated Amino Acid Solutions

1. Coupling reagents: diisopropylcarbodiimide (DIPC, DIC; Fluka) and *N*-hydroxybenzotriazole (HOBt; EMD Biosciences). Coupling reagents are necessary only if non-pre-activated amino acid derivatives are used (*see* **Note 2**).
2. For in situ activation of amino acids and other building blocks, utilize derivatives compatible with the Fmoc-protection strategy (31, 32) (GL Biochem or EMD Biosciences). Pre-activated amino acid derivatives must also be derivatives with protecting groups according to the Fmoc-protection strategy. These are usually pre-activated as pentafluorophenyl esters (OPfp ester; EMD Biosciences or Bachem) (33) (*see* **Note 2**).

2.2.3. Spot Synthesis on Planar Cellulose Membranes

1. Solution for Fmoc-deprotection: 20% (v/v) piperidine (Sigma) in DMF.
2. Capping solution: 2% (v/v) acetic anhydride (Sigma) in DMF. To support the reaction, 2% (v/v) ethyl-diisopropylamine (DIPEA, DIEA; VWR or Sigma) should be added.
3. Staining solution: 0.002% bromophenol blue (BPB; Sigma) in MeOH (20 mg in 1 L).
4. Cleavage solution I: 90% trifluoroacetic acid (v/v) (TFA; VWR) + 5% (v/v) dist. water + 3% (v/v) triisopropylsilane or triisobutylsilane (TIPS or TIBS; Aldrich) + 1% (w/v) phenol (Sigma) + 1% (v/v) DCM.

5. Cleavage solution II: 60% (v/v) TFA + 3% (v/v) TIPS or TIBS + 2% (v/v) dist. water + 1% (w/v) phenol + 44% (v/v) DCM (*see* **Note 3**).
6. Ammonia gas (for releasing peptides from cellulose membrane).

2.3. Preparation of the Peptide Microarrays (CelluSpots Arrays)

2.3.1. Synthesis of Peptide–Cellulose Conjugates for CelluSpots Microarrays

1. Modified membranes (INTAVIS) or multi-well plates holding 384 modified membrane discs (INTAVIS) for the synthesis of peptide–cellulose conjugates.
2. Solution for Fmoc-deprotection: 20% (v/v) piperidine (Sigma) in DMF.
3. Cleavage solution (CelluSpots): side-chain cleavage solution CA: 80% (v/v) trifluoroacetic acid (TFA; VWR) + 5% (v/v) dist. water + 3% (v/v) triisopropylsilane (TIPS; Aldrich) + 12% (v/v) DCM.
4. Cleavage solution CB: 88.5% (v/v) trifluoroacetic acid (TFA; VWR) + 4% (v/v) trifluoromethanesulfonic acid (TFMSA; Fluka) + 5% (v/v) dist. water + 2.5% (v/v) triisopropylsilane (TIPS; Aldrich) (*see* **Note 3**).
5. Dimethyl sulfoxide (DMSO; Fluka).
6. SSC buffer (20x): dissolve 175 g sodium chloride (Sigma) + 88 g tribasic sodium citrate dihydrate (Fluka) in 1 L dist. water, adjust to pH 7.0 with 1 M HCl.

3. Methods

3.1. Spot Synthesis of Macroarrays

Spot synthesis of peptides can be carried out manually, semi-automated, or fully automated. Manual spot synthesis is useful for the synthesis and screening of a limited number of peptides (up to 100) with a pipetting volume per spot of about 1 µL or more. For all other situations, a semi-automated or fully automated synthesis is recommended. Spot synthesis with pipetting volumes per spot of 0.5 µL and more can be performed by most of commercially available *x*–*y*–*z* programmable pipetting robots. For synthesis of smaller spots with a pipetting volume less than 0.5 µl, spot synthesizers from INTAVIS AG (Köln, Germany) are recommended (34). In principle, for all types of spot synthesis, the preparation and synthesis procedure and final treatment of the membranes are similar and can be carried out according to the protocol below.

When not noted, all methods correspond to the preparation of an approximately 19 cm × 29 cm cellulose membrane, which can accommodate about 400 large spots with a diameter of about 7 mm. The synthesis on smaller membranes follows the same procedures. When the phrase "use of non-pre-activated or in situ

activated amino acids" appears, it includes the use of other organic building blocks (e.g., unnatural amino acids, PNA monomers, peptoidic elements) (35, 36), which can be used under conditions where spot synthesis is performed. Here we describe only the basic procedures for spot synthesis of linear conventional peptides to yield free peptides for the preparation of peptide arrays. The synthesis of modified peptides including cyclizations or side-chain modifications was described previously (30).

3.1.1. Preparation of a Cellulose Membrane for Spot Synthesis

1. Cut a piece of filter paper to the size required to accommodate all peptide spots including controls (here 19 cm × 29 cm).
2. For amine functionalization of filter paper, dissolve 0.64 g Fmoc-β-alanine in 10 mL amine-free DMF (*see* **Notes 1** and **4**). Add 374 μL DIC and 317 μL NMI, mix well and transfer the reaction solution into a chemically resistant box with a lid. To achieve homogeneous functionalizations of the cellulose membrane, avoid the presence of air bubbles under the paper during placement of the filter paper in the box and ensure that the surface of the membrane is slightly covered by the solution. Close the box and leave the membrane in the reaction mixture for at least 2 h, or overnight.
3. Wash the membrane three times with DMF for at least 30 s each. If the membrane needs to be stored for a limited time, wash the modified membrane at least twice with methanol or ethanol and dry it in an air stream in a fume hood, or by using a hair dryer without heat (*see* **Note 5**). The membrane can be stored at −20°C (if the membrane is stored, warm up the membrane to room temperature and wash the membrane once with DMF for at least 20 min prior to step 4).
4. For Fmoc-deprotection treat the membrane twice with 20% piperidine in DMF for at least 5 min each.
5. Staining (optional) (37): wash the membrane four times with DMF and then at least twice with methanol or ethanol for at least 30 s each. Treat the membrane with staining solution until the filter paper shows a homogeneous blue color. After staining wash the membrane at least twice with methanol or ethanol, until the wash solution remains colorless. Dry the membrane in air.

3.1.2. Preparation of Activated Amino Acid Solutions

Preparation of the coupling solutions can be performed according to two different protocols. One method involves synthesis using preactivated Fmoc-protected amino acids (e.g., pentafluorophenyl ester). The advantage of this method is the use of only one reagent for each solution, making the preparation of the amino acid solutions very simple and the likelihood of mistakes low. A disadvantage is the higher price of the amino acid derivatives; however, due to the small amounts of activated amino acids used, the absolute difference is only

a few dollars for the synthesis of an entire peptide membrane array. Another disadvantage is the fact that activated esters are only commercially available for the standard amino acids.

The second procedure is based on the use of in situ activated amino acids. Activation of the amino acids is carried out by adding an activator and coupling reagent to the non-activated Fmoc-protected amino acid derivative. Although the preparation according to this method is more laborious than the first described method, it has the advantage that it can be applied for all derivatives of amino-acid-like building blocks that can be protected according to the Fmoc-protecting group strategy.

Both methods can also be used for automated synthesis of peptide–cellulose conjugates on modified cellulose discs for the preparation of CelluSpots arrays (**Section 3.2**). For that purpose one must use commercially available CelluSpots membranes (INTAVIS).

Method 1: preparation of coupling solutions using pre-activated amino acid derivatives

Except for the arginine derivative, all solutions of pre-activated amino acids can be prepared and used for a week. Dissolve all amino acid derivatives, except serine pentafluorophenyl ester, in amine-free NMP at a concentration of 0.3 M (*see* **Table 5.2**). Due to poor solubility, serine pentafluorophenyl ester must be dissolved in amine-free DMF. Solutions can be stored in sealed tubes at –20°C for at least one week. At room temperature, the solutions are stable for about 1 day. Therefore, before starting the first synthesis cycle of a day, always discard the solutions from the previous day and replace

Table 5.2
Weights (mg) of amino acid pentafluorophenyl esters for 0.3 M solutions for spot synthesis using pre-activated derivatives

Amino acid		MW (g/mol)	0.5 mL	0.75 mL	1.0 mL
	Fmoc-β-Ala-OPfp	477.39	71.6	107.4	143.2
A	Fmoc-Ala-OPfp	477.39	71.6	107.4	143.2
C	Fmoc-Cys(Trt)-OPfp	751.79	112.8	169.2	225.5
D	Fmoc-Asp(OtBu)-OPfp	577.5	86.6	129.9	173.3
E	Fmoc-Glu(OtBu)-OPfp	591.50	88.7	133.1	177.5
F	Fmoc-Phe-OPfp	553.49	83.0	124.5	166.0
G	Fmoc-Gly-OPfp	463.36	69.5	104.3	139.0
H	Fmoc-His(Trt)-OPfp	785.78	117.9	176.8	235.7

(continued)

Table 5.2 (continued)

Amino acid		MW (g/mol)	0.5 mL	0.75 mL	1.0 mL
I	Fmoc-Ile-OPfp	519.47	77.9	116.9	155.8
K	Fmoc-Lys(Boc)-OPfp	634.60	95.2	142.8	190.4
L	Fmoc-Leu-OPfp	519.47	77.9	116.9	155.8
M	Fmoc-Met-OPfp	537.51	80.6	120.9	161.3
N	Fmoc-Asn(Trt)-OPfp	762.74	114.4	171.6	228.8
P	Fmoc-Pro-OPfp	503.43	75.5	113.3	151.0
Q	Fmoc-Gln(Trt)-OPfp	776.77	116.5	174.8	233.0
R	Fmoc-Arg(Pbf)-OPfp	814.84	122.2	183.3	244.5
S	Fmoc-Ser(tBu)-OPfp	549.5	82.4	123.6	164.9
T	Fmoc-Thr(tBu)-OPfp	563.52	84.5	126.8	169.1
V	Fmoc-Val-OPfp	505.44	75.8	113.7	151.6
W	Fmoc-Trp(Boc)-OPfp	692.70	103.9	155.9	207.8
Y	Fmoc-Tyr(tBu)-OPfp	625.60	93.8	140.8	187.7

them by transferring the appropriate amount for that day from the stock solutions. Due to the instability of dissolved pre-activated arginine derivatives, this solution must be prepared fresh every day.

Method 2: Preparation of coupling solutions using in situ activated derivatives of amino acids or other building blocks

Prepare a 0.9 M solution of HOBt in amine-free NMP. Dissolve the Fmoc-amino acids or protected building blocks to a concentration of 0.45 M using the HOBt-solution (*see* **Table 5.3**). Except for the arginine derivatives, these solutions can be stored at –20°C for at least a week. Each day prepare a 20% mixture of DIC in amine-free NMP (*see* **Notes 1** and **2**). Replace the coupling solutions of the previous day by preparation of a fresh one. Then take the desired amount of the amino acid/HOBt stock solution and add to those 20% DIC/NMP at a ratio of 3:1 (e.g., 75 µL amino acid solution and 25 µL of fresh prepared DIC).

3.1.3. Spot Synthesis on Planar Cellulose Membranes

1. Definition of the spot pattern: place the dry membrane on a planar surface. Deliver the required volume of activated Fmoc-β-alanine/DMSO solution to all spot positions (*see* **Note 4**). Repeat the delivery after 20 min, and allow the reaction to proceed once more for at least another 20 min.

Table 5.3
Weights (mg), prior to adding DIC solution, of amino acid derivative solutions used in spot synthesis with non-pre-activated derivatives. If after dissolving of amino acids in the corresponding amount of HOBt solution the volume does not achieve the value described in the table, make up the volume with NMP

Amino acid		MW (g/mol)	0.5 mL	0.75 mL	1.0 mL
	Fmoc-β-Ala-OH	311.3	70.0	105.1	140.1
A	Fmoc-Ala-OH	311.3	70.0	105.1	140.1
C	Fmoc-Cys(Trt)-OH	585.7	131.8	197.7	263.6
D	Fmoc-Asp(OtBu)-OH	411.5	92.6	138.9	185.2
E	Fmoc-Glu(OtBu)-OH	425.5	95.7	143.6	191.5
F	Fmoc-Phe-OH	387.4	87.2	130.7	174.3
G	Fmoc-Gly-OH	297.3	66.9	100.3	133.8
H	Fmoc-His(Trt)-OH	619.7	139.4	209.1	278.9
I	Fmoc-Ile-OH	353.4	79.5	119.3	159.0
K	Fmoc-Lys(Boc)-OH	468.5	105.4	158.1	210.8
L	Fmoc-Leu-OH	353.4	79.5	119.3	159.0
M	Fmoc-Met-OH	371.5	83.6	125.4	167.2
N	Fmoc-Asn(Trt)-OH	596.7	134.3	201.4	268.5
P	Fmoc-Pro-OH	337.4	75.9	113.9	151.8
Q	Fmoc-Gln(Trt)-OH	610.7	137.4	206.1	274.8
R	Fmoc-Arg(Pbf)-OH	648.8	146.0	219.0	292.0
S	Fmoc-Ser(tBu)-OH	383.4	86.3	129.4	172.5
T	Fmoc-Thr(tBu)-OH	397.5	89.4	134.2	178.9
V	Fmoc-Val-OH	339.4	76.4	114.5	152.7
W	Fmoc-Trp(Boc)-OH	526.6	118.5	177.7	237.0
Y	Fmoc-Tyr(tBu)-OH	459.6	103.4	155.1	206.8
Added 0.9 M HOBt-solution			0.38 mL	0.56 mL	0.75 mL

2. Blocking free areas: add an appropriate amount of capping solution to the box and place the membrane face down into the solution. Avoid air bubbles under the surface. Do not shake! After a minimum of 5 min of reaction time, take out

the membrane and pour out the used capping solution. Add an appropriate amount of capping solution with 2% DIPEA and place the membrane face up in this solution for 20 min.

3. Fmoc-deprotection: wash four times with DMF for at least 30 s each. Treat the membrane twice with 20% piperidine/DMF for 5 min each. Wash three times with DMF for at least 30 s each. Wash at least twice with MeOH or EtOH.
4. Staining (optional) (37): shake the membrane with staining solution in a box. If the staining solution changes to blue very quickly, renew the solution. Let the reaction proceed for at least 15 s until spots are visible (*see* **Note 6**). Wash at least twice with MeOH or EtOH until the wash solution remains colorless.
5. Dry the membrane in an air stream. If faster drying is necessary, additionally wash the membrane once or twice with DEE before drying. The membrane is now ready for a coupling cycle.
6. Coupling step: Deliver the prepared coupling solutions of desired activated amino acids to the corresponding positions on the membrane with the required volumes (*see* **Note 7**). Repeat the spotting after 20 min.
7. Blocking unreacted free amino groups (capping): place the membrane face down for at least 5 min in an appropriate amount of capping solution. Do not shake! Take out the membrane and pour out the used capping solution. Add an appropriate amount of capping solution with 2% DIPEA and place the membrane face up in this solution for at least another 5 min.
8. Building up the peptide chain: peptides are built up step by step (one amino acid at a time), starting from the C-terminus to the N-terminus. Except for the last coupling cycle, repeat steps 3–7. At the last coupling cycle carry out the steps above without capping and staining!
9. Final Fmoc-deprotection: Wash four times with DMF for at least 30 s each. Treat the membrane twice with 20% piperidine/DMF for 5 min. Wash again four times with DMF followed by three times washing with DCM for at least 30 s each.
10. Final side-chain deprotection (*see* **Note 3**): treat home-made membranes with cleavage solution I for 30 min. For each small membrane (96-well plate size) use at least 25 mL cleavage solution and for a large membrane (19 cm $\times$ 29 cm) at least 80 ml. The surface of the membrane must be well covered by the cleavage solution. Keep the box closed. Do not shake! Pour off the solution very carefully. Wash the membrane five times with DCM for at least 1 min each. Treat home-made membranes with cleavage solution II for 3 h. Use at least the same volume as for cleavage solution I. The surface of the membrane must be well covered by the cleavage solution. To avoid evaporation keep the

box tightly closed. Do not shake! Pour off the solution very carefully. Commercially available membranes are usually more stable than the homemade, which is why most of them can be treated with cleavage solution I for 3.5 h. Wash the membrane five times for at least 1 min each with PBS or TBS until the pH value is around 7 (*see* **Note 8**). Wash the membrane three times with water and then three times MeOH or EtOH for at least 1 min each. Dry the membrane in the air stream of a fume hood or with a hair dryer without heat.

11. For the preparation of CelluSpots arrays punch out the spots, transfer all single discs into deep-well plates or 96-well tube racks with a minimum holding capacity of 1 mL and proceed with the preparation according to the instructions of **Section 3.2.2**.

12. For the preparation of common peptide arrays, it is necessary to release the peptides from the membrane. One method is to treat the dry membrane or punched-out spots overnight in a glass desiccator containing ammonia gas. The strong basic environment leads to a cleavage of the ester bond to the cellulose by forming a C-terminal amide (*see* **Note 9**). If not done previously, on the next day punch out the spots and place the discs into wells of a microtiter plates or into vials in which one can dissolve the released peptides (*see* **Note 10**). If the peptides need to have a free carboxyl group at the C-terminus, another treatment is necessary. In this case punch out the spots from the membrane and transfer the discs into wells of microtiter plates or into vials. Then treat them with an aqueous basic solution, for example ammonium hydroxide, aqueous solution of triethylamine (38), or sodium hydroxide (39).

Another method is to release the peptide by forming C-terminal diketopiperazines. Therefore, modify the membrane with Boc-Lys(Fmoc)-OH instead of the Fmoc-β-Ala-OH, followed by coupling of Fmoc-proline as first coupled amino acid. After TFA treatment, punch out the spots and transfer them into microtiter plates or vials (*see* **Note 10**). The peptides are then released by treatment with basic aqueous buffers (pH $\geq$ 7.5) overnight (1).

3.2. Preparation of the Peptide Microarrays (CelluSpots Arrays)

Peptide–cellulose conjugates for the preparation of CelluSpots microarrays on coated microscope slides can be synthesized on modified membranes or in multi-well plates holding up to 384 modified discs in a semi-automated or fully automated manner. For semi-automated synthesis on CelluSpots membranes using the SPOT-technique follow the instructions under **Section 3.1**.

3.2.1. Synthesis of Peptide – Cellulose Conjugates for CelluSpots Microarrays

1. 384-Well filter-plates containing modified cellulose discs are placed into the synthesizer.
2. Fmoc-deprotection: to each well with a disc add 2X 4 μL of a 20% piperidine/DMF solution for 5 and for 10 min, respectively. Wash seven times with DMF (25 μL per well/disc) for at least 10 s each. Wash six times with EtOH or MeOH (25 μL per well/disc).
3. Dry the discs in a steady air stream for at least 12 min. The synthesis plate with the discs is now ready for the first coupling cycle.
4. In situ activation of amino acids and coupling: for each cycle all necessary amino acids are to be in situ activated with HOBt and DIC. To each disc add 1.2 μL of a coupling mix of 0.33 mole eq. DIC, 0.45 mole eq. HOBt, and 0.3 mole eq. Fmoc-amino acid (*see* **Note 11**). Repeat the distribution after a reaction time of 20–40 min.
5. Blocking un-reacted free amino groups on peptide fragments: add to each disc, 4 μL of capping solution. Allow the reaction to occur for at least 5 min. Remove the liquid and wash seven times with DMF (25 μL per well/disc) for at least 10 s each. Wash six times with EtOH or MeOH (25 μL per well/disc) and dry the discs in a steady air stream for at least 12 min.
6. Building up the peptides on modified cellulose discs: repeat steps 2–5.
7. Final Fmoc-deprotection: treat each disc with 4 μL of a 20% piperidine/DMF for 5 min. Repeat the treatment twice for 10 min and then once for 15 min. Wash seven times with DMF (25 μL per well/disc) for at least 10 s each. If N-terminal acetylated peptides are needed add three times 4 μL capping solution and let it react for at least 5 min. Wash another seven times with DMF, followed by six washing steps with EtOH or MeOH (25 μL per well/disc). After drying in a steady air stream for at least 12 min the discs are ready for the side-chain deprotection and dissolving (*see* **Note 12**).
8. Transfer all single discs from the synthesis plate into deep-well plates or 96-well tube racks with a minimum holding capacity of 1 mL.
9. Side-chain deprotection: add 150 μL of the side-chain cleavage solution CA to each well and incubate for 1–2 h in a fume hood (*see* **Note 13**). Remove the cleavage solution by suction, e.g., with a multi-channel device connected to a water jet pump or by a multi-channel pipette (*see* **Note 3**).

3.2.2. Preparation of Peptide–Cellulose Conjugate Solutions

1. Cleavage and dissolving of discs: add 250 μL of the cleavage solution CB to each well and close the plate with a lid or with stopper stripes (*see* **Note 13**). Incubate overnight in a hood,

until the cellulose discs are completely dissolved (*see* **Note 3**). Add 750 µL of ice-cold ether to each well and close the plate with a lid or with stopper stripes. Shake the plate gently for 10 s and let it cool down at –20 or –70°C for 1 h. Leave the plate in the freezer over night or spin down the precipitated peptide–cellulose conjugates (*see* **Note 14**).

2. Remove the supernatant and wash the pellet one or two times with fresh ether. Remove the remaining ether by evaporation under a fume hood until the residue remains as a gel (*see* **Note 15**).
3. To dissolve the conjugates, add 500 µL DMSO to each well. Close the plate with caps and store the stock solutions at –20°C.

3.2.3. Preparation of CelluSpots Microarrays

1. Spotting of peptide–cellulose conjugates onto coated microscope slides: take a new plate (e.g., 384-well plate for spotting) and dilute the peptide–cellulose conjugates from stock solutions in DMSO or in a mixture of DMSO and SSC buffer (2 parts DMSO and one part 1X SSC buffer). Dilutions of 1:2–1:6 can be tested (*see* **Note 16**). Deliver about 80 nL of that solution to coated microscope slides. Different types of slides can be used. The spotting of identical CelluSpots slides can be done by using a conventional contact printer (e.g., Slide Spotting Robot, INTAVIS). For detection via chromogenic substrate reactions, white-coated glass slides (e.g., INTAVIS) are favored.
2. Let the spots dry in air and put the slides into an oven at 75°C for 1 h. Until use, CelluSpots peptide arrays should be kept in a dry and dark place at 4°C. Under these conditions the arrays are stable for several months.

4. Notes

1. Solvents for dissolving reagents must be amine and water free. To check whether the solvent is sufficiently amine free, add to 1 mL of the solvent few microliters of the methanolic BPB solution. If it remains yellow or turns only to yellow green, the solvent can be used (34). All other solvents should be at least ACS or synthesis grade. Organic solvents should be stored in the dark.

2. All synthesis reagents must be protected from moisture. Reagent bottles and vials with solutions must be sealed with parafilm before storage. To avoid condensation, reagent bottles stored in the fridge or freezer should not be opened until they are warmed up to room temperature for about 30 min.
3. Safety: for all handling with TFA lab coat, gloves, safety glasses and fume hood are mandatory!
4. Due to the flexibility and linear structure of the molecule, β-alanine is mostly used for modification of the cellulose. However, the use of other amino acids is also possible for the functionalization of the membrane (40). For example, if all peptides have the same C-terminal amino acid, the corresponding derivative of that amino acid can be used for the amine functionalization of the membrane. The necessary amount of that derivative must be calculated according to its molecular weight. After cleavage from the cellulose, the amino acid used for functionalization remains on the peptide.
5. The membrane can be stored at –80°C for several months until use. For longer storage a loss of peptide is possible. Storage of the membranes at –80°C is recommended. The membranes can also be stored at –20°C or +4°C; but the risk of losing activity over a long storage time increases with the storage temperature and the length of the storage.
6. Do not stain the spots too much. If the absorbed amount of bromophenol blue is too high, some of the dye could become incorporated in the peptide. The removal of that incorporated dye is almost impossible and might affect the detection. Since bromophenol blue only turns blue in a basic environment, e.g., in the presence of primary amino functions, due to different acidity of the coupled amino acids and the built-up peptide chain, a difference in the intensity and type of the color of the spots is normal.
7. To ensure that the whole spot area is covered by the coupling solution, it is recommended to use at least 20% more amino acid solution volume than in step 1 of **Section 3.1.3**.
8. After TFA treatment, the home-made membranes might become very soft and can be easily damaged due to strong agitation. Be careful and shake very gently! Do not try to lift the membrane out until it becomes harder and less likely to break apart during the washing steps (at around the time of the MeOH washing step)!
9. Release of peptides with ammonia might work only on membranes prepared according to the instructions of **Section 3.1.1**. Commercially available amino-functionalized

membranes often have bonds other than ester bonds between the peptide and cellulose. For such membranes their modification with additional linkers is necessary (e.g., thioester (41), HMB linker (42), and Rink linker (43)).

10. Due to chromatographic effects during the transfer of the coupling solution to the spots, the concentration of side products increases toward the edge of the spots. To reduce the amount of side products in the released peptides, the discs should be punched out in the center of the spot and their diameter should be smaller than the spot diameter.
11. It is also possible to use solutions of pre-activated amino acids. For preparation of those, please follow instructions in method 1 in **Section 3.1.2**.
12. The discs must be completely dry. Otherwise, remaining traces of EtOH or MeOH can lead to partial esterification of the peptides during TFA treatment.
13. If the cellulose discs or solutions turn from yellow to red, add a few more microliters of TIPS to the cleavage mix.
14. It is prohibited to spin open tubes with ether solutions in a non-explosion-proof centrifuge!
15. Take care that the pellet is not completely dry before adding the DMSO at a subsequent step – its consistency should be like a gel. If the pellet is dried completely, it will not dissolve in DMSO!
16. Especially if aqueous solutions like SSC buffers are used, some amount of conjugates may precipitate and can be centrifuged down. There will still be sufficient conjugate in solution.

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