Chemistry of SPOT Synthesis for the Preparation of Peptide Macroarrays on Cellulose Membranes

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Abstract: The SPOT technique is one of the most frequently used methods for synthesis and screening of peptides on arrays. Materials such as polypropylene and glass are used for the preparation of peptide arrays, however the most commonly used material for SPOT membranes is cellulose. This paper focuses on materials and procedures used in the SPOT synthesis on cellulose membranes as a special type of solid phase peptide synthesis. In particular, different strategies for the modification of cellulose are described which make it more suitable for solid-phase peptide synthesis. This review also provides a short overview of the synthesis procedures including some important types of peptide modification.

Keywords: Cellulose membranes, linker, membrane modification, peptide array, solid phase peptide synthesis, SPOT synthesis.

1. INTRODUCTION

The SPOT synthesis of peptides, developed by Ronald Frank and co-workers, was first presented at the 21st European Peptide Symposium in 1990 [1]. Today, this technique has become one of the most frequently used methods for synthesis and screening of peptides on arrays. SPOT synthesis is a powerful tool for screening solid-phase and solution-phase assays with the size of the arrays varying from a few peptides up to approximately 8000 peptides and other organic compounds [2, 3].

Several hundred papers regarding applications of the SPOT method have been published to date [4-7]. The SPOT method was introduced as "an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support" [8]. SPOT synthesis of peptides on membranes is a special type of solid phase peptide synthesis (SPPS) with each spot considered as a separate reaction vessel. Since the publication of the first full paper in 1992, several protocols for synthesis of peptide macroarrays using the SPOT technique have been presented [9-12]. This paper focuses on materials and procedures used in the SPOT synthesis (see Fig. (**1**)). With such large numbers of publications using the SPOT technique, citations mentioned here are either publications with protocols of distinct methods or selected examples of applications of those methods.

Cellulose membranes, the most commonly used material for SPOT membranes, are porous, hydrophilic, flexible and stable in the organic solvents used during peptide synthesis. The membranes, in the form of filter or chromatography paper, are a very inexpensive material, which makes them very useful for biochemical and biological studies in aqueous and organic media. However, since cellulose is not stable against harsh chemical conditions, the SPOT synthesis was developed for the milder type of the two major SPPS strategies, the Fmoc synthesis [13]; all protecting groups used are supposed to be compatible with this strategy [14, 15]. Besides cellulose, other materials are used for the preparation of peptide arrays such as polypropylene, polyvinylidenfluoride (PVDF), glass and gold surfaces [16-19]. Even synthesis methods on planar surfaces such as compact discs [10, 20] or by using an ink-jet or laser printing system [21, 22] have been described.

2. MODIFICATION OF THE CELLULOSE MEMBRANE

Cellulose membranes largely consist of filter or chromatography papers. The most common types of filter or chromatography paper used for SPOT synthesis are Whatman 50, Whatman 540 and CHR1 [23-26]. Cellulose is a polysaccharide containing free hydroxyl groups. These functional groups are the only groups accessible for the build-up of peptide chains on cellulose. However, since the hydroxyl groups are less reactive than amino groups, the cellulose must be modified in order to present amino functions. The amino functionalization of cellulose can be achieved by different approaches. The first described and easiest method is the coupling of Fmoc protected amino acids such as Fmoc-β-Ala-OH or Fmoc-Gly-OH [7] by using activating reagents such as *N*,*N*'-diisopropylcarbodiimide (DIC), 1,1'-carbonyldiimidazole (CDI) in presence of a base, e.g. *N*-methyl-imidazole (NMI) [27, 28]. The reactions lead to an ester bond between the amino acid and the cellulose (see A in Fig. (**2**)). A more recent report suggests the use of 1,1'-carbonyl-di-(1,2,4-triazole) (CDT) instead of CDI that could cause the deprotection of Fmoc-amino acids during the coupling reaction [29].

Besides the esterification of cellulose membranes with amino acids, many publications describe the use of epibromohydrin as an activating reagent to introduce a reactive bromine moiety attached to the cellulose via an ether bond (see B in Fig. (**2**)). The bromine moiety is able to react with amines and the ether bond is stable with no loss of peptide observed during synthesis and probing [30]. An amino functionalization of the membrane can be achieved by using diamines [17] such as DAP (1,3-diaminopropane) [31] or TOTD (4,7,10-**t**ri**o**xa-1,13-**t**ridecane**d**iamine), an aminated polyethylene-3 (PEG-3) [32, 33]. DAP modified cellulose membranes are described as *N*-CAPE (***N***-modified **c**ellulose 3-**a**mino-2-hydroxy**p**ropyl**e**ther) membranes [30, 34], while membranes modified with TOTD, are referred to as trioxa or TOTD membranes [7, 35]. Unmodified CAPE membranes can be prepared by the treatment of the cellulose paper with *N*-protected 2,3-epoxypropylamine (see C in Fig. (**2**)) [36, 37].

Another approach for the amino functionalization of a cellulose membrane is the activation of the membrane by treatment with tosyl chloride under common heating or microwave irradiation. The consecutive reaction with a diamine, such as TOTD, leads to a stabile amino functionalization (see D in Fig. (**2**)) [38, 39].

Several companies already offer already modified cellulose membranes. Specially prepared cellulose membranes with a stably attached aminated spacer of 8 to 12 PEG units (PEG300-500) were developed by AIMS (Braunschweig, Germany) and distributed by Intavis (Cologne, Germany) [40, 41]. In contrast to common cellulose membranes, this material is stable under strong acidic and basic conditions [16].

Often spacer molecules such as β-alanine were introduced in order to improve the accessibility and conformational flexibility of the growing peptide chain on the membrane [38, 42]. As an alterna-

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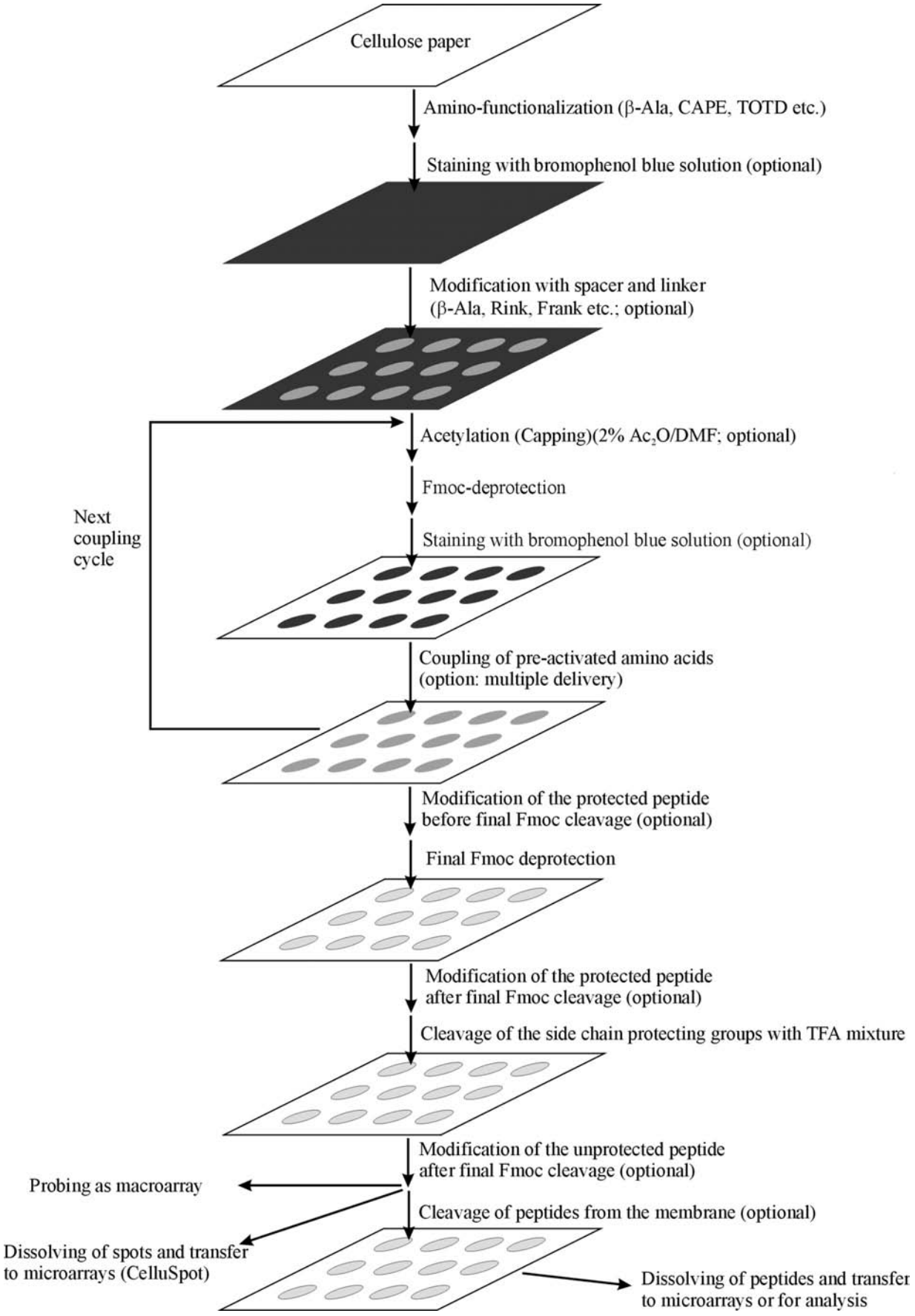

Fig. (1). Schematic process of the SPOT synthesis.

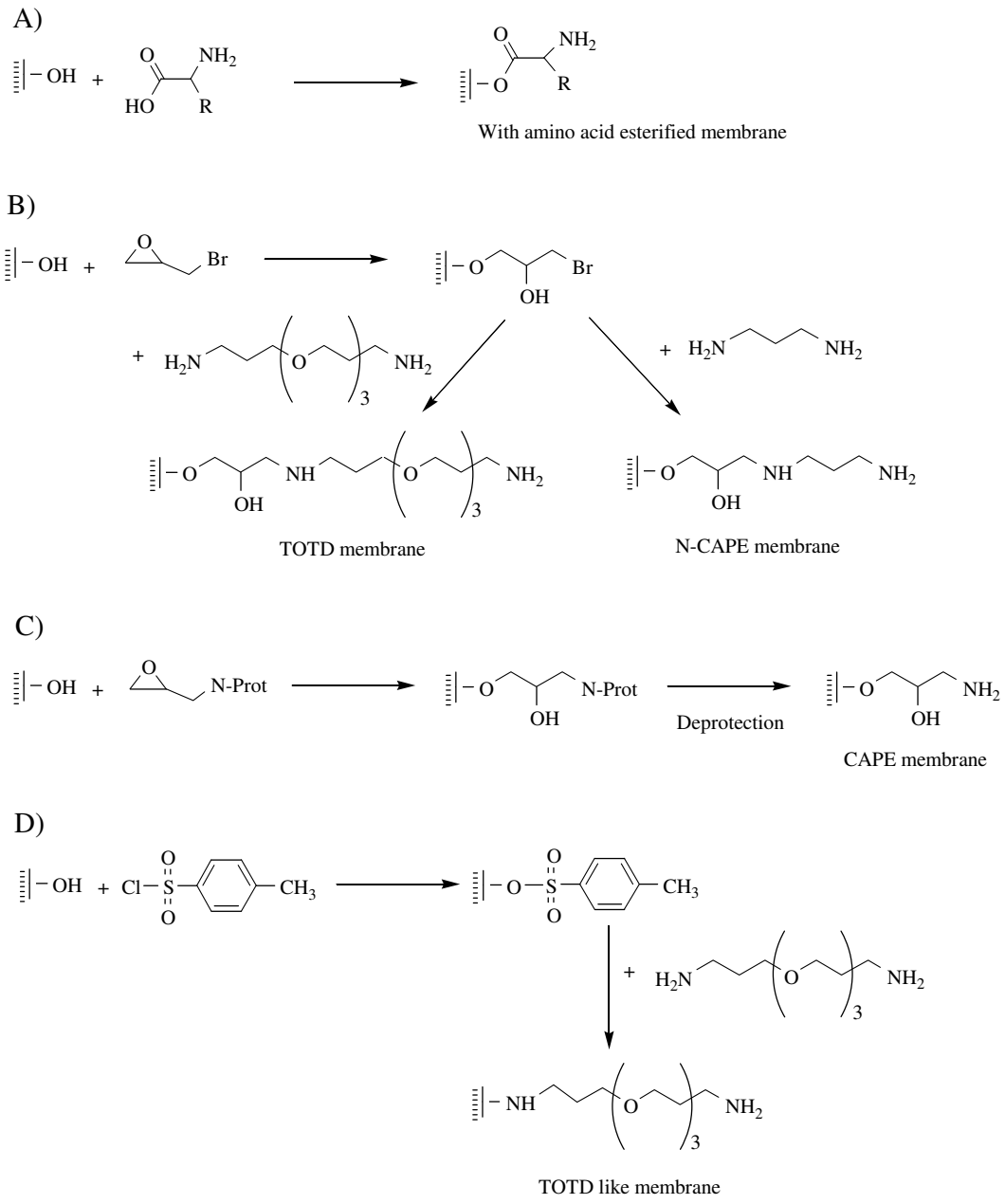

Fig. (2). Different methods of amino functionalization of cellulose membranes: **A**) Esterification of the membrane with amino acid (R = side chain, H); **B**) Treatment with epibromohydrin and subsequent reaction with 4,7,10-trioxa-1,13-tridecanediamine (left) or 1,3-diaminopropane (right) giving TODT or N-CAPE membranes; **C**) Treatment with *N*-protected 2,3-epoxypropylamine (Prot = protecting groups Fmoc, phtaloyl), followed by deprotection to obtain CAPE membrane; **D**) Treatment with tosyl chloride and subsequent reaction with 4,7,10-trioxa-1,13-tridecanediamine giving a TOTD like membrane functionalization.

tive to β-alanine, the use of ε-aminohexanoic acid [43] and 11-aminoundecanoic acid [44] has been described. In order to adjust the density of accessible amino groups, it is possible to couple a solution of Fmoc-β-alanine containing a distinct amount of acetylated β-alanine [24, 45, 46].

Another development in SPOT synthesis is the use of trifluoroacetic acid (TFA)-soluble cellulose membranes. Peptides synthesized as a SPOT macroarray on those membranes can be dissolved as peptide-cellulose conjugates and transferred onto large number of copies of microarrays on glass slides. The re-arrayed spots preserve their three-dimensional architecture which allows for good accessibility of the cellulose-bound peptides (CelluSpots™, Intavis AG, Cologne, Germany) [47-49]; see also chapter IV.

3. LINKER STRATEGIES

For experiments in solution [23, 50], for quality control [33], or for the preparation of peptide microarrays [51] free peptides are necessary. Therefore, it is required to release those peptides from the cellulose matrix. Two major ways are described: the cleavage of the peptides directly from the cellulose or the cleavage from a linker molecule attached to the membrane.

If the peptide is coupled via an ester bond, the peptides can be cleaved by aminolysis or alkaline hydrolysis directly from a membrane. Several reagents are reported, such as aqueous solutions of sodium hydroxide [29] or triethylamine [52], however the most frequently used method for releasing peptides from a membrane is the treatment of dry membranes with ammonia vapor [53]. For all

Table 1. Linker Types Used for SPOT Synthesis Including the Cleavage Methods Used to Release the Peptides from the Membrane

Linker Type	Cleavage Conditions	C-Terminus	References
Boc-imidazol linker (Frank linker)	TFA + aq. buffer	free carboxyl group	[55, 56]
HMB linker	ammonia gas	amide	[36, 57]
Photolabile linker	UV irradiation	amide	[32, 58, 59]
Rink-amide linker	TFA	amide	[34, 60]
	TFA vapor		[61, 62]
Hydroxymethylphenoxy linker	TFA	free carboxyl group	[63, 64]
Thioether (thiol + 1st amino acid coupled as bromoethyl ester)	ammonia gas	amide	[31]
	aq. NaOH solution	free carboxyl group	[31]
Wang linker	TFA vapor	free carboxyl group	[65]

types of membranes functionalized without an ester bond to the cellulose, an additional modification with linker molecules is necessary to yield free peptides (see Table **1**) [15, 54]. If linkers were used it might be possible to release the peptide from the membrane during the final deprotection step (see Section 6.).

A special type of ester-linkage system represented by the Boc-Lys-Pro sequence, which in lieu of β-alanine or glycine, is subsequently directly built up on the unmodified cellulose membrane. The first amino acid of the peptide sequence is coupled to the side-chain amino group of the Nα-protected lysine. After final deprotection peptides can be released by forming diketopiperazines (DKPs) in aqueous buffers at a pH around 7.5 [15, 66]. However, DKPs can be biologically active and may influence the probing results [67, 68].

An interesting approach is the use of linkers for the synthesis of cellulose-bound peptides with a free C-terminus. In these cases, the peptides are synthesized on scaffold, which consists of an amino acid with a reactive group on its side chain (e.g. glutamic acid or lysine) and a linker molecule. After the build-up of the peptide chain, a cyclization towards the additional amino acid is carried out. After final cleavage from the linker, the cellulose-bound peptides have switched their direction by presenting a free accessible C-terminus [57, 64].

4. COUPLING OF AMINO ACIDS AND OTHER BUILDING BLOCKS

Coupling of amino acids onto the growing peptide chain involves using small volumes of solutions of activated amino-acid derivatives dissolved in low volatility solvents such as 1-methyl-2-pyrrolidinone (NMP), or *N*,*N*-dimethylformamide (DMF). The absorption of the delivered liquid by cellulose forms spots with a distinct size. This size depends on the volume applied, the physico-chemical properties of the solution, and the type of cellulose membrane used [11, 54].

Like all other chemical peptide syntheses, SPOT synthesis is not restricted to the 20 common amino acids. Many publications have reported SPOT synthesis using D-amino acids [69], unnatural and phosphorylated amino acids [42, 70], or peptoidic moieties [3], as well as peptide nucleic acids [71], glycopeptides [61] and other natural product-derived building blocks [59].

Cellulose also shows a high thermostability of temperatures up to 180 °C, which opens the possibility to use cellulose membranes for reactions at elevated temperatures [72]. SPOT syntheses of organic compound libraries have been published [26, 62].

The couplings of building blocks are mostly carried out either by *in situ* activation using activating reagents, or by using pre-activated derivatives of building blocks. The most common approach of *in situ* activation is the use of DIC and 1-hydroxy-benzotriazole (HOBt) forming the corresponding HOBt esters of protected building blocks, which are able to react with free amino groups on the cellulose [40]. Some publications describe the use of other activating reagents in the presence of a base like *N*,*N*-diisopropylethylamine (DIPEA) [36, 38]. For difficult couplings of building blocks such as phosphorylated amino acids [70], or pseudoproline dipeptides [73], the use of 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) seems to be favorable [63].

For the other major coupling strategy, the most commonly used pre-activated amino acid derivatives are pentafluorophenyl (OPfp) esters [50, 74]. Additional pre-activated amino acid derivatives used are 2,4-dinitrophenyl (ODNp) esters [24], and 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (ODhbt) esters [9]. Usually, activated solutions are delivered twice to each spot, but a delivery of solutions up to four times per cycle has been reported [57]. Molina *et al*. investigated the coupling efficiency of selected activation methods and found a higher coupling yield using the DIC/HOBT strategy [75].

In order to reduce the number of side products caused by incomplete coupling, remaining free amino groups can be blocked by acetylation after amino acid coupling (capping). The capping is generally performed using acetic anhydride at various concentrations with or without added base like DIPEA [9, 11, 46].

The cleavage of the amino protecting Fmoc group is most frequently carried out by treatment with 20% piperidine/DMF [45, 54]. Recently, the use of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) as Fmoc-deprotecting reagent in SPOT synthesis has been published [68].

Due to the large number of peptides on a membrane, a direct monitoring of all spots is not possible. The most convenient tool for monitoring the coupling process is staining the spots with bromophenol blue after Fmoc removal [43, 76].

Since a large number of different peptide sequences can be synthesized on a macroarray, coupling rates and yields, as well as the purity of the peptides may vary. SPOT synthesis of peptides is comparable to other SPPS methods until a length of 15 amino acids. Although the synthesis of longer peptides may lead to significant lower quality and quantity [17], peptides with 31 amino acids can be synthesized with purities between 40 and 85% [77]. The use of pseudoproline building blocks allows the synthesis of 37mers with up to 65% purity [73]. The reliability of the SPOT technology regarding coupling efficiency, peptide purity and detection was investigated by several authors [24, 37, 45].

Another interesting approach to synthesize large peptides is the combination of SPOT synthesis with native chemical peptide ligation [78]. Using this technique, Toepert *et al.* synthesized 38-mers

by coupling pre-synthesized fragments to peptides synthesized on the membrane [70]. Similarly, the TASP strategy (template assembled synthetic proteins [79]) was applied in order to couple peptide building blocks onto cyclic 10mer peptide templates synthesized on cellulose membranes using the SPOT technique [60, 80]. Due to the coupling of the peptides onto the membrane attached template, and the interaction between the assembled peptide chains, a highly structured molecule could be built.

5. SELECTED PEPTIDE MODIFICATIONS

The most common modification of synthesized peptides is N-terminal acetylation that can improve the proteolytic stability of the peptides. N-terminal acetylation is carried out like capping by treatment of the protected peptide with acetic anhydride at various concentrations [27, 54] and often with DIPEA as a basic additive [69] after final Fmoc deprotection. The N-terminal amino group can also be used for the attachment of other molecules like fluorescence labels [31] or biotin [81].

One very important modification is the cyclization of peptides. Most common cyclization reactions are cyclizations forming an amide bond [82] or a disulfide bridge [83]. Most cyclizations, like those by the way of forming an amide bond and thioether bridge, are carried out with the protected peptide using orthogonal protecting groups in order to selectively remove the protection from functional groups which are involved in the formation of the cyclic structure. The amide cyclization could be achieved similar to common coupling using an activator in presence of a base such as *N*-methylmorpholine [84]. The thioether bridge is formed via a cysteine side chain and a bromine moiety by treatment with Cs_2CO_3 [64]. Single disulfide bridges are usually formed after the side-chain deprotection by treatment with aqueous dimethyl sulfoxide (DMSO) at a pH of about 7.5 [84].

6. FINAL SIDE CHAIN DEPROTECTION METHODS

The side chain deprotection of the peptides bound to the cellulose is carried out by treatment with TFA. In contrast to other materials like polypropylene, common cellulose membranes are limited in treatment with high concentrated TFA [16]. The first published and widely applied cleavage procedure is the treatment of membranes with a mixture of about 50% TFA and additional scavengers (e.g. water, triisopropyl- or triisobutylsilane, m-cresol, thioanisole, ethanedithiol) in dichloromethane (DCM) for 1 to 3 hours [15, 54]. But the cleavage of some protecting groups like those for arginine requires higher TFA concentrations. That is why several research groups described the use of cleavage mixtures with 80% TFA [44]. The optimum treatment of a common cellulose membrane is to use 90% TFA for 30 minutes up to 1 hour and subsequent treatment with 50-60% TFA for another 2.5 to 3 hours [31, 74]. Under these conditions, the best peptide purity can be obtained [45]. The use of commercially available acid-stable cellulose membranes allows the treatment with approximately 90% TFA for 16 hours and more [16, 46].

7. CONCLUSION

The SPOT synthesis is a multiple solid-phase synthesis of large number of peptides. Additionally, it is a powerful tool for screening peptides in solid-phase as well as solution-phase assays. There are large numbers of publications reporting modifications of this method, but unfortunately due to the limited space it was not possible to refer to all of them. The synthesis chemistry is similar to other SPPS methods and, with the use of common filter paper, the SPOT synthesis is a convenient and inexpensive method of generating peptide arrays. Since the membranes are relatively stable to most organic and aqueous solutions, and are biocompatible, this method has a permanent place in the repertoire of researchers in chemistry and biology for the synthesis and screening of peptides.

ABBREVIATIONS

CAPE	=	cellulose 3-amino-2-hydroxypropylether
CDI	=	1,1'-carbonyldiimidazole
CDT	=	1,1'-carbonyl-di-(1,2,4-triazole)
DBU	=	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	=	dichloromethane
DIPEA	=	*N*,*N*-diisopropylethylamine
DIC	=	*N*,*N'*-diisopropylcarbodiimide
DKP	=	diketopiperazine
DMF	=	*N*,*N*-dimethylformamide
DMSO	=	dimethyl sulfoxide
EEDQ	=	2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
HATU	=	*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate
HBTU	=	O-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate
HOBt	=	1-hydroxybenzotriazole
MSNT	=	1-(2-mesitylenesulfonyl)-3-nitro-*1H*-1,2,4-triazole
NMI	=	*N*-methyl-imidazole
NMP	=	1-methyl-2-pyrrolidinone
ODhbt	=	3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl
ODNp	=	2,4-dinitrophenyl
OPfp	=	pentafluorophenyl
PVDF	=	polyvinylidenfluorid
PyBOP	=	(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
SPPS	=	solid phase peptide synthesis
TASP	=	template assembled synthetic protein
TBTU	=	*O*-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate
TOTD	=	trifluoroacetic acid TFA; 4,7,10-trioxa-1,13-tridecanediamine

REFERENCES

[1] Frank, R.; Güler, S.; Krause, S.; Lindenmaier, W. In *Peptides 1990. Proc. 21st Eur. Pept. Symp.*; Giralt, E.; Andreu, D. Eds.; ESCOM: Leiden, **1991**, pp. 151-152.

[2] Schneider-Mergener, J.; Kramer, A.; Reineke, U. In: *Combinatorial Libraries: Synthesis, Screening and Application Potential*; Cortese, R. Ed.; Walter de Gruyter: Berlin, **1996**, pp. 53-68.

[3] Heine, N.; Ast, T.; Schneider-Mergener, J.; Reineke, U.; Germeroth, L.; Wenschuh, H. Synthesis and screening of peptoid arrays on cellulose membranes. *Tetrahedron*, **2003**, *59*, 9919-9930.

[4] Reineke, U.; Volkmer-Engert, R.; Schneider-Mergener, J. Applications of peptide arrays prepared by the SPOT-technology. *Curr. Opin. Biotechnol.*, **2001**, *12*, 59-64.

[5] Reineke, U.; Schneider-Mergener, J.; Schutkowski, M. In *BioMEMS and Biomedical Nanotechnology, Micro/Nano Technologies for Genomics and Proteomics*; Ozkan, M.; Heller, M.J. Eds.; Springer: New York, **2006**, Vol. *II*, pp. 161-282.

[6] Hilpert, K.; Winkler, D.F.H.; Hancock, R.E.W. Cellulose-bound peptide arrays: Preparation and applications. *Biotechnol. Genetic Eng. Rev.*, **2007**, *24*, 31-106.

[7] Volkmer, R. Synthesis and application of peptide arrays: Quo vadis SPOT technology. *ChemBioChem*, **2009**, *10*, 1431-1442.

[8] Frank, R. Spot-synthesis: An easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron*, **1992**, *48*, 9217-9232.

[9] Kramer, A.; Schneider-Mergener, J. Synthesis and application of peptide libraries bound to continuous cellulose membranes. *Methods Mol. Biol.*, **1998**, *87*, 25-39.

[10] Frank, R. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports-principles and applications. *J. Immunol. Meth.*, **2002**, *267*, 13-26.

[11] Hilpert, K.; Winkler, D.F.H.; Hancock, R.E.W. Peptide arrays on cellulose support: SPOT synthesis - a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nat. Protoc.*, **2007**, *2*, 1333-49.

[12] Winkler, D.F.H.; Andresen, H.; Hilpert, K. SPOT synthesis as a tool to study protein-protein interactions. *Methods Mol. Biol.*, **2011**, *723*, (in press).

[13] Chan, W. C.; White, P.D. *Fmoc solid phase peptide synthesis. A practical approach*, Oxford University Press: Oxford, **2000**.

[14] Fields, G. B.; Noble, R. L. Solid phase synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Prot. Res.*, **1990**, *35*, 161-214.

[15] Zander, N.; Gausepohl, H. In *Peptide Arrays on Membrane Supports. Synthesis and Applications*; Koch, J.; Mahler, M. Eds.; Springer: Heidelberg, **2002**, pp. 23-39.

[16] Zander, N. New planar substrates for the in situ synthesis of peptide arrays. *Mol. Divers.*, **2004**, *8*, 189-195.

[17] Wenschuh, H.; Volkmer-Engert, R.; Schmidt, M.; Schulz, M.; Schneider-Mergener, J.; Reineke, U. Coherent membrane supports for parallel microsynthesis and screening of bioactive peptides. *Biopolymers (Pept. Sci.)*, **2000**, *55*, 188-206.

[18] Fodor, S.P.A.; Read, J.L.; Pirrung C.; Stryer, L.; Lu, A.T.; Solas, D. Light-directed, spatially addressable parallel chemical synthesis. *Science*, **1991**, *251*, 767-773.

[19] Laurent, N.; Haddoub, R.; Voglmeir, J.; Wong, S.C.C.; Gaskell, S.J.; Flitsch, S.L. SPOT Synthesis of peptide arrays on self-assembled monolayers and their evaluation as enzyme substrates. *ChemBioChem*, **2008**, *9*, 2592-2596.

[20] La Clair, J.J.; Burkart, M.D. Molecular screening on a compact disc. *Org. Biomol. Chem.*, **2003**, *3*, 3244-3249.

[21] Antohe, B. V.; Cooley P.W. In situ synthesis of peptide microarrays using inkjet microdispensing. *Methods Mol. Biol.*, **2007**, *381*, 299-312.

[22] Stadler,V.; Felgenhauer, T.; Beyer, M.; Fernandez, S.; Leibe, K.; Güttler, S.; Gröning, M.; König, K.; Torralba, G.; Hausmann, M.; Lindenstruth, V.; Nesterov, A.; Block, I.; Pipkorn, R.; Poustka, A.; Bischoff, F.R.; Breitling, F. Combinatorial synthesis of peptide arrays with a laser printer. *Angew. Chem. Int. Ed.*, **2008**, *47*, 7132-7135.

[23] Petter, C.; Scholz, C.; Wessner, H.; Hansen, G.; Henklein, P.; Watanabe, T.; Höhne, W. Phage display screening for peptidic chitinase inhibitors. *J. Mol. Recognit.*, **2008**, *21*, 401-409.

[24] Weiser, A. A.; Or-Guil, M.; Tapia, V.; Leichsenring, A.; Schuchardt, J.; Frömmel, C.; Volkmer-Engert, R. SPOT synthesis: Reliability of array-based measurement of peptide binding affinity. *Anal. Biochem.*, **2005**, *342*, 300-311.

[25] Kopecky, E.-M.; Greinstetter, S.; Pabinger, I.; Buchacher, A.; Römisch, J.; Jungbauer, A. Mapping of FVIII inhibitor epitopes using cellulose-bound synthetic peptide arrays. *J. Immunol. Methods*, **2006**, *308*, 90-100.

[26] Bowman, M. D.; Jacobson, M. M.; Blackwell, H.E. Discovery of fluorescent cyanopyridine and deazalumazine dyes using small molecule macroarrays. *Org. Lett.*, **2006**, *8*, 1645-1648.

[27] Deswal, R.; Singh, R.; Lynn, A.M.; Frank, R. Identification of immunodominant regions of Brassica juncea glyoxalase I as potential antitumor immunomodulation targets. *Peptides*, **2005**, *26*, 395-404.

[28] Ay, B.; Streitz, M.; Boisguerin, P.; Schlosser, A.; Mahrenholz, C.C.; Schuck, S.D.; Kern, F.; Volkmer, R. Sorting and Pooling Strategy: A novel tool to map a virus proteome for CD8 T-cell epitopes. *Biopolymers (Pept. Sci.)*. **2007**, *88*, 64-75.

[29] Ay, B.; Volkmer, R.; Boisguerin, P. Synthesis of cleavable peptides with authentic C-termini: An application for fully automated SPOT synthesis. *Tetrahedron Lett.*, **2007**, *48*, 361-364.

[30] Hilpert, K.; Elliott, M.; Jenssen, H.; Kindrachuk, J.; Fjell, C.D.; Körner, J.; Winkler, D.F.H.; Weaver, L.L.; Henklein, P.; Ulrich, A.S.; Chiang, S.H.Y.; Farmer, S.W.; Pante, N.; Volkmer, R.; Hancock, R.E.W. Screening and characterization of surface-tethered cationic peptides for antimicrobial activity. *Chem. Biol.*, **2009**, *16*, 58-69.

[31] Licha, K.; Bhargava, S.; Rheinländer, C.; Becker, A.; Schneider-Mergener, J.; Volkmer-Engert, R. Highly parallel nano-synthesis of cleavable peptide-dye conjugates on cellulose membranes. *Tetrahedron Lett.*, **2000**, *41*, 1711-1715.

[32] Ast, T.; Heine, N.; Germeroth, L.; Schneider-Mergener, J.; Wenschuh, H. Efficient assembly of peptomers on continuous surfaces. *Tetrahedron Lett.*, **1999**, *40*, 4317-4318.

[33] Reineke, U.; Ivascu, C.; Schlief, M.; Landgraf, C.; Gericke, S.; Zahn, G.; Herzel, H.; Volkmer-Engert, R.; Schneider-Mergener, J. Identification of distinct antibody epitopes and mimotopes from a peptide array of 5520 randomly generated sequences. *J. Immunol. Methods*, **2002**, *267*, 37-51.

[34] Tapia, V.; Ay, B.; Triebus, J.; Wolter, E.; Boisguerin, P.; Volkmer, R. Evaluating the coupling efficiency of phosphorylated amino acids for SPOT synthesis. *J. Pept. Sci.*, **2008**, *14*, 1309-1314.

[35] Winkler, D.F.H.; Hilpert, K. Synthesis of antimicrobial peptides using the SPOT technique. *Methods Mol. Biol.*, **2010**, *618*, 111-124.

[36] Volkmer-Engert, R.; Hoffmann, B.; Schneider-Mergener, J. Stable attachment of the HMB-linker to continuous cellulose membranes for parallel solid phase spot synthesis. *Tetrahedron Lett.*, **1997**, *38*, 1029-1032.

[37] Landgraf, C.; Panni, S.; Montecchi-Palazzi, L.; Castagnoli, L.; Schneider-Mergener, J.; Volkmer-Engert, R.; Cesareni, G. Protein interaction networks by proteome peptide scanning. *PLoS Biol.*, **2004**, *2*, 94-103.

[38] Akita, S.; Umezawa, N.; Kato, N.; Higuchi, T. Array-based fluorescence assay for serine/threonine kinases using specific chemical reaction. *Bioorg. Med. Chem.*, **2008**, *16*, 7788–7794.

[39] Bowman, M.D.; Jeske, R.C.; Blackwell, H.E. Microwave-accelerated SPOT-synthesis on cellulose supports. *Org. Lett.*, **2004**, *6*, 2019-2022.

[40] Gausepohl H.; Behn, C. In *Peptide Arrays on Membrane Supports. Synthesis and Applications*; Koch, J.; Mahler, M. Eds.; Springer: Heidelberg, **2002**, pp. 55-68.

[41] Mandrika, I.; Prusis, P.; Bergström, J.; Yahorava, S.; Wikberg, J.E.S. Improving the affinity of antigens for mutated antibodies by use of statistical molecular design. *J. Pept. Sci.*, **2008**, *14*, 786-796.

[42] Wildemann, D.; Erdmann, F.; Alvarez, B.H.; Stoller, G.; Zhou, X.Z.; Fanghänel, J.; Schutkowski, M.; Lu, K.P. Fischer, G. Nanomolar inhibitors of the peptidyl prolyl cis/trans isomerase Pin1 from combinatorial peptide libraries. *J. Med. Chem.*, **2006**, *49*, 2147-2150.

[43] Gausepohl, H.; Behn, C. In *Peptides 1996: Proceedings of the 24th European Peptide Symposium*, Ramage, R.; Epton, R. Eds.; Mayflower Scientific: Kingswinford, **1998**, pp. 409-410.

[44] Kato, R.; Kaga, C.; Kunimatsu, M.; Takeshi Kobayashi, T.; Honda, H. Peptide array-based interaction assay of solid-bound peptides and anchorage-dependant cells and its effectiveness in cell-adhesive peptide design. *J. Biosci. Bioeng.*, **2006**, *101*, 485-495.

[45] Kramer, A.; Reineke, U.; Dong, L.; Hoffmann, B.; Hoffmüller, U.; Winkler, D.; Volkmer-Engert, R.; Schneider-Mergener, J. Spot-synthesis: observations and optimizations. *J. Pept. Res.*, **1999**, *54*, 319-327.

[46] Gail, R.; Frank, R.; Wittinghofer, A. Systematic peptide array-based delineation of differential β-catenin interaction with Tcf4, E-cadherin, and adenomatous Polyposis coli. *J. Biol. Chem.*, **2005**, *280*, 7107-7117.

[47] Dikmans, A.; Beutling, U.; Schmeisser, E.; Thiele, S.; Frank, R. SC2: A novel process for manufacturing multipurpose highdensity chemical microarrays. *QSAR Comb. Sci.*, **2006**, *25*, 1069-1080.

[48] Winkler, D.F.H.; Hilpert, K.; Brandt, O.; Hancock, R.E.W. Synthesis of peptide arrays using SPOT-technology and CelluSpots-method. *Methods Mol. Biol.*, **2009**, *570*, 157-174.

[49] Beutling, U.; Städing, K.; Stradal, T.; Frank, R. Large-scale analysis of protein–protein interactions using cellulose-bound peptide arrays. *Adv. Biochem. Eng. Biotechnol.*, **2008**, *110*, 115-152.

[50] Kamradt, T.; Volkmer-Engert, R. Cross-reactivity of T lymphocyctes in infection and autoimmunity. *Mol. Divers.*, **2004**, *8*, 271-280.

[51] Nahtman, T.; Jernberg, A.; Mahdavifar, S.; Zerweck, J.; Schutkowski, M.; Maeurer, M.; Reilly, M. Validation of peptide epitope microarray experiments and extraction of quality data. *J. Immunol. Methods*, **2007**, *328*, 1-13.

[52] Tapia, V.; Bongartz, J.; Schutkowski, M.; Bruni, N.; Weiser, A.; Ay, B.; Volkmer, R. Michal Or-Guil Affinity profiling using the peptide microarray technology: A case study. *Anal. Biochem.*, **2007**, *363*, 108-118.

[53] Bray, A.M.; Valerio, R.M.; Maeji, N.J.; Cleavage of resin-bound peptide esters with ammonia vapour. Simultaneous multiple synthesis synthesis of peptide amides. *Tetrahedron Lett.*, **1993**, *34*, 4411-1414.

[54] Frank, R.; Overwin, H. SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. *Methods Mol. Biol.*, **1996**, *66*, 149-169.

[55] Hoffmann, S.; Frank, R. A new safety-catch peptide resin linkage for the direct release of peptides into aqueous buffers. *Tetrahedron Lett.*, **1994**, *35*, 7763-7766.

[56] Gonzalez-Gil, G.; Volkmer-Engert, R.; Germeroth, L.; Piossek, C.; Schleuning, W.-D.; Schneider-Mergener, J. In: *Peptides 1996: Proceedings of the 24th European Peptide Symposium*, Ramage, R.; Epton, R. Eds., Mayflower Scientific Ltd.: Kingswinford, **1998**, pp. 427-428.

[57] Hoffmüller, U.; Russwurm, M.; Kleinjung, F.; Ashurst, J.; Oschkinat, H.; Volkmer-Engert, R.; Koesling, D.; Schneider-Mergener, J. Interaction of a PDZ Protein domain with a synthetic library of all Human protein C termini. *Angew. Chem. Int. Ed. Engl.*, **1999**, *38*, 2000-2004.

[58] Scharn, D.; Germeroth, L.; Schneider-Mergener, J.; Wenschuh, W. Sequential nucleophilic substitution on halogenated triazines, pyrimidines, and purines: A novel approach to cyclic peptidomimetics. *J. Org. Chem.*, **2001**, *66*, 507-513.

[59] Niggemann, J.; Michaelis, K.; Frank, R.; Zander, N.; Höfle, G. Natural product-derived building blocks for combinatorial synthesis. Part 1. Fragmentation of natural products from myxobacteria. *J. Chem. Soc., Perkin Trans. 1*, **2002**, 2490-2503.

[60] Haehnel, W. Chemical synthesis of TASP arrays and their application in protein design. *Mol. Divers.*, **2004**, *8*, 219-229.

[61] Ziegler, T.; Schips, C. An efficient Mitsunobu protocol for the one-pot synthesis of S-glycosyl amino-acid building blocks and their use in combinatorial spot synthesis of glycopeptide libraries. *Nat. Protoc.*, **2006**, *1*, 1987-1994.

[62] Scharn, D.; Wenschuh, H.; Reineke, U.; Schneider-Mergener, J.; Germeroth, L. Spatially addressed synthesis of amino- and amino-oxy-substituted 1,3,5-triazine arrays on polymeric membranes. *J. Comb. Chem.*, **2000**, *2*, 361-369.

[63] Ay, B.; Landgraf, K.; Streitz, M.; Fuhrmann, S.; Volkmer, R.; Boisguerin, P. Using hydroxymethylphenoxy derivates with the SPOT technology to generate peptides with authentic C-termini. *Bioorg. Med. Chem. Lett.*, **2008**, *18*, 4038-4043.

[64] Boisguerin, P.; Ay, B.; Radziwill, G.; Fritz, R.D.; Moelling, K.; Volkmer, R. Characterization of a putative phosphorylation switch: Adaptation of SPOT synthesis to analyze PDZ domain regulation mechanisms. *ChemBioChem*, **2007**, *8*, 2302-2307.

[65] Bowman, M.D.; Jacobson, M.M.; Pujanauski, B.G.; Blackwell, H.E. Efficient synthesis of small molecule macroarrays: optimization of the macroarray synthesis platform and examination of microwave and conventional heating methods. *Tetrahedron*, **2006**, *62*, 4715-4727.

[66] Bray, A.M.; Maeji, N.J.; Valerio, R.M.; Campbell, R.A.; Geysen, H.M. Direct cleavage from a solid support into aqueous buffer. Application in simultaneous multiple peptide synthesis. *J. Org. Chem.*, **1991**, *56*, 6659-6666.

[67] Prasad, C. Bioactive cyclic dipeptides. *Peptides*, **1995**, *16*, 151-164.

[68] Campbell, J.; Blackwell, H.E. Efficient construction of diketopiperazine macroarrays through a cyclative-cleavage strategy and their evaluation as luminescence inhibitors in the bacterial symbiont *Vibrio fischeri*. *J. Comb. Chem.*, **2009**, *11*, 1094-1099.

[69] Kramer, A.; Stigler, R.-D.; Knaute, T.; Hoffmann, B.; Schneider-Mergener, J. Stepwise transformation of a cholera toxin an a p24 (HIV-1) epitope into D-peptide analogs. *Protein Eng.*, **1998**, *11*, 941-948.

[70] Toepert, F.; Knaute, T.; Guffler, S.; Pires, J.R.; Matzdorf, T.; Oschkinat, H.; Schneider-Mergener, J. Combining SPOT synthesis and native peptide ligation to create large arrays of WW protein domains. *Angew. Chem. Int. Ed.*, **2003**, *42*, 1136-1140.

[71] Weiler, J.; Gausepohl, H.; Hauser, N.; Jensen, O.N.; Hoheisel, J.D. Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. *Nucleic Acids Res.*, **1997**, *25*, 2792-2799.

[72] Blackwell, H.E. Hitting the SPOT: small-molecule macroarrays advance combinatorial synthesis. *Curr. Opin. Chem. Biol.*, **2006**, *10*, 203-212.

[73] Przezdziak, J.; Tremmel, S.; Kretzschmar, I.; Beyermann, M.; Bienert, M.; Volkmer-Engert, R. Probing the ligand-binding specificity and analyzing the folding state of SPOT-synthesized FBP28 WW domain variants. *ChemBioChem*, **2006**, *7*, 780-788.

[74] Bleeke, M.; Fest, S.; Huebener, N.; Landgraf, C.; Schraven, B.; Gaedicke, G.; Volkmer, R.; Lode, H.N. Systematic amino acid substitutions improved efficiency of GD_2-peptide mimotope vaccination against neuroblastoma. *Eur. J. Cancer*, **2009**, *45*, 2915-2921.

[75] Molina, F.; Laune, D.; Gougat, C., Pau, B.; Granier, C. Improved performances of spot multiple peptide synthesis. *Pept. Res.*, **1996**, *9*, 151-155.

[76] Krchnák, V; Vágner, J; Safar, P.; Lebl, M. Noninvasive continuous monitoring of solid phase peptide synthesis by acid-base indicator. *Collect. Czech. Chem. Commun.*, **1988**, *53*, 2542-2548.

[77] Portwich, M.; Keller, S.; Strauss, H.M.; Mahrenholz, C.C.; Kretzschmar, I.; Kramer, A.; Volkmer, R. A Network of coiled-coil associations derived from synthetic GCN4 leucine-zipper arrays. *Angew. Chem. Int. Ed.*, **2007**, *46*, 1654-1657.

[78] Kent, S. Total chemical synthesis of enzymes. *J. Pept. Sci.*, **1997**, *9*, 574-593.

[79] Mutter, M.; Vuilleumier, S. A chemical approach to protein design – template assembled synthetic proteins (TASP). *Angew. Chem. Int. Ed.*, **1989**, *28*, 535-554.

[80] Rau, H.K.; DeJonge, N.; Haehnel, W. Combinatorial synthesis of four-helix bundle hemoproteins for tuning of cofactor properties. *Angew. Chem. Int. Ed.*, **2000**, *39*, 250-253.

[81] Winkler, D. F. H.; McGeer, P. L. Protein labeling and biotinylation of peptides during spot synthesis using biotin p-nitrophenyl ester (biotin-ONp). *Proteomics*, **2008**, *8*, 961-967.

[82] Kramer, A.; Schuster, A.; Reineke, U.; Malin, R.; Volkmer-Engert, R.; Landgraf, C.; Schneider-Mergener, J. Combinatorial cellulose-bound peptide libraries: Screening tools for the identification of peptides that bind ligands with predefined specificity. *Methods: Comp. Meth. Enzymol.*, **1994**, *6*, 388-395.

[83] Otte, L.; Knaute, T.; Schneider-Mergener, J; Kramer, A. Molecular basis for the binding polyspecificity of an anti-cholera toxin peptide 3 monoclonal antibody. *J. Mol. Recognit.*, **2006**, *19*, 49-59.

[84] Hahn, M.; Winkler, D.; Welfle, K.; Misselwitz, R.; Welfle, H.; Wessner, H.; Zahn, G.; Scholz, C.; Seifert, M.; Harkins, R.; Schneider-Mergener, J.; Höhne, W. Cross-reactive binding of cyclic peptides to an anti-TGFα antibody Fab fragment. An X-ray structural and thermodynamic analysis. *J. Mol. Biol.*, **2001**, *314*, 293-309.

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