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Synthesis and Application of Peptide Arrays: Quo Vadis SPOT Technology

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Dedicated to Dr. Ronald Frank on the occasion of his 60th birthday.[+]

Introduction

In 1990, at the 21st European Peptide Symposium in Barcelona, Ronald Frank presented a method for the rapid and effective simultaneous parallel synthesis of multiple peptides on a filter paper.[1] Two years later the first seminal paper on this method was published; this manuscript defined the approach as SPOT synthesis, an easy technique for positionally addressable, parallel chemical synthesis on a membrane support.[2] The SPOT synthesis technique established itself in a series of investigations beginning with Mario Geysen's visionary concept of peptide synthesis on derivatised plastic pins.[3] A few years later the first oligonucleotide array synthesis on a planar glass surface was reported.[4,5] Peptide library methods such as Richard Houghten's[6,7] tea-bag approach and combinatorial peptide library techniques[8–10] emerged almost simultaneously. Progress in library and array technologies was driven by a paradigm change in the life sciences, through the application of a more empirical search rather than an iterative rational approach to the solving of complex biological questions. Indeed, combinatorial chemical libraries, together with biological libraries such as phage display[11] and yeast-two-hybrid libraries,[12] were the trail-blazing technologies, and it is likely that all parallel handling techniques found their inspiration from these pioneering concepts.

The array format is a high-throughput device for binding assays and involves the spatially addressable presentation of different molecules immobilised in discrete areas on a planar support surface. The device is simultaneously analysed with one or more purified or crude biological samples used to probe the array. This principle has been fruitfully applied in RNA hybridisation experiments.[13,14] In particular, the genomic field—the human genome project, for example—has driven the progress of array technology. However, the distribution of cellular labour between DNA and proteins may make measurements of genetic change insufficient to explain emergent phenotypes[15] involved in normal or pathological cellular behaviour. Moreover, RNA levels do not correlate with protein abundance,[16,17] and it is proteins rather than genes that are the immediate operational agents of cellular functions. Here, assays at the protein level are required in order to identify gene function at the phenotypic level, and protein arrays are ideally suited to support proteomic research. However, critical factors such as native folding stability or functionality have been an enormous challenge for the production of protein arrays. It is therefore not surprising that the first reports on protein arrays came just in time for the new millennium.[18–20]

Peptides, in contrast, are easier to handle and retain partial aspects of protein function, and so peptide arrays are suitable to support proteomic research. Two techniques for the preparation of peptide arrays were published almost simultaneously. Firstly, Frank presented the SPOT synthesis technique[1,2] and, secondly, Fodor and co-workers[21] reported the concept of light-directed, spatially addressable chemical synthesis. Both techniques are milestones in the advancement of peptide array technologies. However, the majority of peptide arrays reported to date have been produced by use of the SPOT synthesis concept. This is due to the fact that SPOT synthesis is a very simple but extremely robust method for the highly parallel synthesis of peptides on planar surfaces. Not surprisingly, the technology has been successfully commercialised, by, for example, the company JPT Peptide Technologies GmbH (http://www.jpt.com).

The SPOT synthesis technique was initially applied to molecular recognition events based on synthetic peptides in the immune system. Over a period of 18 years, the SPOT synthesis technique—or SPOT technology for short—has become a widespread and essential tool in biology and biochemistry. The technique has been used for a broad spectrum of protein–protein or protein–ligand interactions. From 1990 until now, more than 400 original, peer-reviewed papers relevant to SPOT technology have been published. In the bibliography compiled by Frank and co-workers, 167 original publications published between 1992 and 2001 are mentioned.[22] A comprehensive bibliography on peptide array and SPOT technology can be found in the impressive review of peptide arrays in proteomics and drug discovery published by Reineke and co-workers.[23] The authors collected all kinds of literature on peptide array technologies and applications from 1991 up to 2004.

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[+] *Ronald Frank introduced the concept of the SPOT technology for studying protein–ligand interactions. His pioneering work has been a source of inspiration for many research groups. I wish him a happy and full life and several years of active research ahead.*

Here I would like to give a basic overview of the technology, subdivided for demonstrative and didactical reasons into fundamental and advanced SPOT technology sections. Finally I will focus on recently published applications of SPOT technology. To those interested in more details about peptide array preparation and applications I recommended the extensive review by Reineke and co-workers.[23]

Fundamental SPOT Technology

SPOT synthesis (for further reviews refer to refs. [22–37]) is one player on the array technology field and fulfils the main characteristics of array technology. These are: 1) spatially addressable immobilisation of huge numbers of different capture molecules, 2) probing of the array with a biological sample in a simultaneous and highly parallel manner to detect binding, 3) tendency towards miniaturisation of the arrays, and 4) software supported read-out and data analysis. Fundamental SPOT technology makes use of peptides as capture molecules. Those are synthesised in a stepwise and parallel manner directly on a planar cellulose support. The basic principle of SPOT technology involves the positionally addressed delivery of small volumes of activated reagent solutions (for example, activated amino acid derivatives) directly onto a cellulose membrane sheet. The areas wetted by the resulting droplets can be considered microreactors, provided that a nonvolatile solvent system is used. The functional groups fixed on the membrane surface react with the pipetted reagents as in conventional solid-phase synthesis. The physical properties of the membrane surface, the solvent system and the applied volumes define the size of the resulting spots. Both immobilised and soluble peptides can be prepared by SPOT technology. Figure 1 A demonstrates the synthesis of six different peptides, but with each peptide equally distributed as 1000 reiterations on membrane A and used for testing antibody binding.[38] Small-scaled spots are typically used for such an approach. In contrast, large-scaled spots are used for preparation of soluble peptides (Figure 1 B). On membrane B, 949 peptides were synthesised to generate soluble peptides, here subsequently used in a cell-based assay.[39]

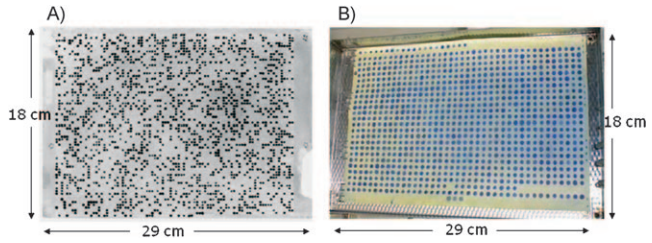


Figure 1. Left: A cellulose-membrane-bound peptide array for protein binding studies (binding results in a black spot). Right: A cellulose membrane with a peptide array useful for generating soluble peptides (spots are stained for visualisation). On membrane A, 6000 spots with an average diameter of 1.2 mm were generated with the goal of synthesizing huge numbers of peptides for an on-support binding study. On membrane B, 949 spots with diameters of 8 mm were produced and used for the synthesis of soluble peptides subsequently applied in a cell-based assay (spots are coloured with bromophenol blue).

In situ peptide synthesis on cellulose membranes

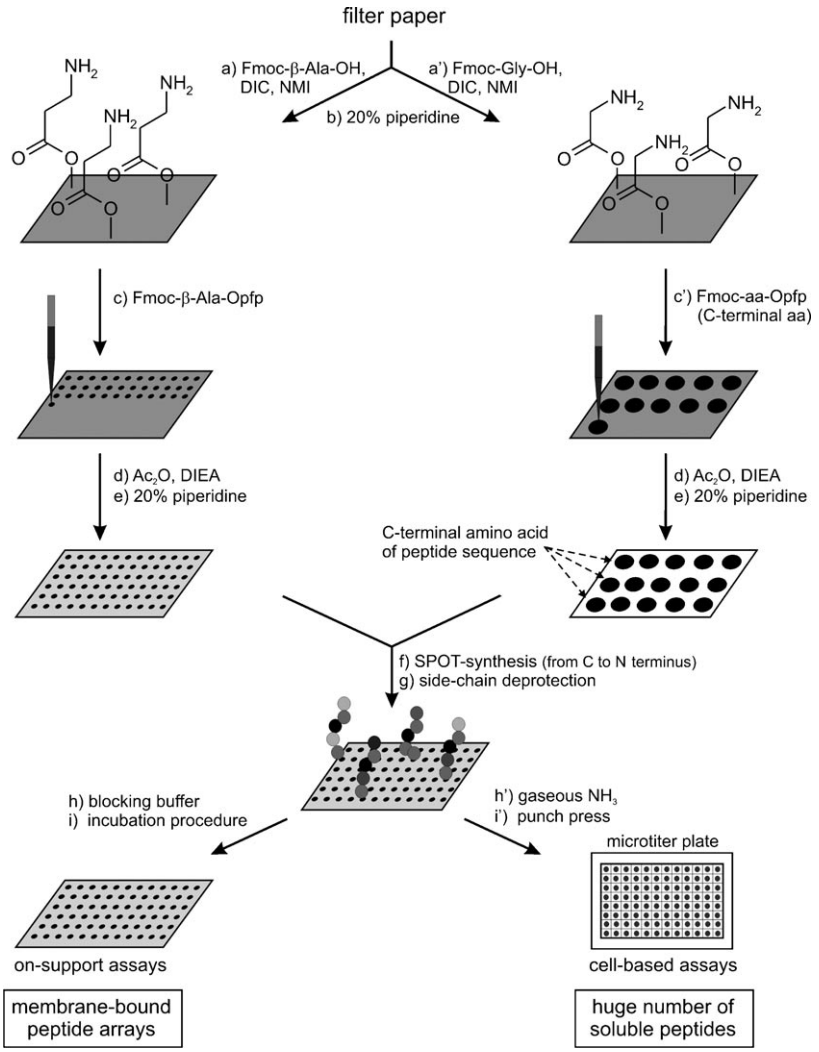
Whatever the intended application of SPOT technology, the general strategy for parallel peptide assembly on a cellulose membrane is the same, as shown in Scheme 1. In a first step, hydroxy groups in the cellulose membrane are used as anchoring points for the attachment of the more reactive amino functions (Scheme 1 a, a′, b). The most frequently used procedure results in ester-type cellulose membranes.[2,32] Treatment of a cellulose membrane with a solution either of activated Fmoc-β-alanine or of Fmoc-glycine gives modified membranes (for working protocols refer to refs. [2, 40–43]). The commercially available filter paper types Whatman 50, Chr1 and 540, as well as the chromatography paper type 3MM (Whatman, Maidstone, UK) are the preferred supports. Glycine ester-type cellulose membranes are suitable and inexpensive supports for the synthesis of soluble peptides,[39,44–46] whereas β-alanine ester-type membranes are mostly used for binding studies performed directly on the support.[22,23,33,34,37]

Spots are defined next (Scheme 1 c, c′, d, e) with droplets of an activated Fmoc-β-alanine solution.[40–43] Activation can be achieved with *N*-hydroxybenzotriazole ester (HOBt ester) intermediates[26,37,43] generated in situ or by use of commercially available pentafluorophenyl esters (OPfp esters)[24,28,30,31,40] available from several suppliers (Novabiochem/Merck Biosciences, Schwalbach, Germany, or Bachem, Bubendorf, Switzerland). For the preparation of soluble peptides (Scheme 1 c′), spots are defined with solutions of activated amino acids corresponding to the given C-terminal residue.[42,44–45]

In the next step, peptides are assembled from the C to the N terminus (Scheme 1 f), exclusively by Fmoc-/*t*Bu-chemistry.[46] In the first standard SPOT synthesis protocol, Frank described a manual process.[2] The subsequently published protocols take into account the development from a manual[2,47] to semiautomatic[25,48,49] and then fully automated systems.[49] The published SPOT synthesis protocols (e.g., refs. [31,37,41–43]) vary slightly in the procedures for amino acid activation or coupling steps and also in the use of different solvents. We have recommended the use of amino acid OPfp ester derivatives because this simplifies the synthesis process, particularly if SPOT robots are used.[49] SPOT synthesis protocols for research and practical courses at the Volkmer lab can be found on the internet.[42]

During the last synthetic step (Scheme 1 g) side chains of the completed peptides are deprotected. The limited stability of standard cellulose membranes against the deprotection reagent trifluoracetic acid (TFA) has to be taken into account. We recommend our two-step deprotection procedure,[40,42] which starts with immersion of the membrane in 90% TFA in dichloromethane (DCM) for 0.5 h and, after several washing steps, further treatment with 50% TFA in DCM for another 3 h. Details of this working protocol have been published.[40–42]

Peptides synthesised on large-scale spots can be cleaved from the cellulose membrane after side chain deprotection (Scheme 1 h′ i′) by dry aminolysis[50] of the peptide-ester bond with ammonia gas.[31,32,44,45,51,52] The compounds are released from the cellulose membrane as carboxamides and remain physically adsorbed on the membrane. The spots can be



Scheme 1. Standard SPOT technology procedure. One the one hand, thousands of immobilised cellulose-membrane-bound peptides can be synthesised rapidly and inexpensively especially for on-support binding studies. On the other hand, large numbers of soluble peptides can be easily generated in sufficient quality and in yields suitable for several kinds of solution- and cell-based assays.

punched out and the adsorbed compounds can be extracted with water or any suitable buffer system. Use of glycine ester-type membranes as solid supports results in peptides with C-terminal glycinamide modifications. The amount of peptide recovered from each spot (0.23 cm^2) is typically around 40–70 nmol.

The qualities of SPOT-synthesised peptides have been investigated by several authors. Takahashi and co-workers[53] reported peptide purities higher than 92%, whereas Kramer and co-workers[31] reported lower purities. An extended HPLC analysis has shown that purities of SPOT-synthesised short peptides of up to 15 amino acids are similar to those synthesised by solid-phase methods in reactors.[32] Ay and co-workers analysed a huge number of SPOT-synthesised cytomegalovirus-derived nonameric peptides by HPLC–MS and found peptide purities in the 50–85% range.[39] This is in good agreement with Molina and co-workers, who reported peptide purities in the 74.4–91.3% range.[54] Even longer peptides such as the 34-meric FBP28 WW domain could be SPOT-synthesised in good quality (65% purity).[55] However, optimised SPOT protocols must be used in such special cases (see advanced SPOT technology sections).

Assays for cellulose-membrane-bound peptide arrays

After side chain deprotection, peptide arrays are ready to be used for protein binding experiments (Scheme 1 h, i). These are carried out by incubating membranes with solutions of the protein partners, such as an antibody, a bodily fluid, a cell extract, a recombinant protein, an enzyme, a synthetic protein domain or a peptide, etc. Planar cellulose supports have proven to be compatible and excellent materials for various screening methods. In general, prior to incubation with a putative interaction partner, unspecific binding sites on the membranes must be blocked by incubation with blocking solutions. It is reported that signal-to-noise ratios can be varied by use of different blocking solutions. Bräuning and co-workers[56] investigated the influence of six blocking solutions on an antibody-peptide interaction and listed some general considerations, such as 1) polyclonal sera require strong blocking systems, 2) horse serum, BSA and Tween 20 are not very effective in reducing unspecific reactions, and 3) superblock [a mixture of horse serum (50%), blocking buffer concentrate (Genosys, Cambridge, UK, 10%), Tween 20 (0.2%) and sucrose (150 mM) in TBS] acts as the most effective blocking system. Dürauer and co-workers[57] investigated the influence of salt concentrations on the signal-to-noise ratios, and Beutling and co-workers[37] published a ranking of blocking solutions in terms of increasing "stringency". For a long time in our lab we used a blocking buffer solution made up of sucrose (2.5 g), blocking buffer concentrate (Sigma, 5 mL) and TBS concentrate (10×, 5 mL) diluted with water to a solution volume of 50 mL. This blocking solution works best for most cellulose membrane array applications.[31,40,58–61] As well as the influence of blocking buffers, we recognised that both peptide density[38] and membrane type[58,59] also strongly influence signal-to-noise ratios.

The quality of an assay system used for probing peptide arrays depends on the well-balanced combination of screening and read-out methods. Both should address either the molecular recognition event or the means of observing which peptide was bound or modified by an interaction partner. In general, both screening and read-out are carried out directly on the peptide array. Screening and read-out can be achieved simultaneously if the soluble interaction partner is labelled with a detectable moiety, such as a fluorescent dye, a radioactive isotopes (^{35}S, ^{125}I, $^{32/33}$P etc.) or an enzyme such as a peroxidase or phosphatase.[55,60,62,63] Published protocols for protein labelling can be found either online or in a handbook.[64,65] Fortunately, in situ labelling of immobilised peptides with radioactive isotopes [$^{32/33}$P] can occur while probing them for enzyme-substrate interactions with protein kinases.[66,67] The resulting enzymatically converted $^{32/33}$P-phosphopeptides can be detected by autoradiography or scintillation counting.[66,68] Rathert and coworkers[69,70] recently published an analogous screening/read-out procedure for the enzyme–substrate interaction of methyltransferases. Here, immobilised peptides are enzymatically methylated and simultaneously labelled with [^{3}H] by use of [methyl-^{3}H]AdoMet.

In practice, screening and read-out are mostly performed as separated procedures. The visualisation of peptides binding the interaction partner is carried out in an additional step, in which the probed peptide array is subsequently immersed with a labelled moiety that recognises the actual interaction partner. Antibody-based immunoblotting with the additional use of labelled secondary antibodies[65] is a common approach mostly used for antibody or serum profiling studies.[71] Labelled proteins A or G represent alternatives to secondary antibodies.[72,73] Peptide-interacting proteins can be detected on the array by use of an antibody either against the protein itself or against a purification tag (poly-His-, Strep-, S-tag, etc.) or a fused region (for example, GST fusion). If biotinylated proteins are used for array probing, the array read-out is achieved with a streptavidin conjugate.[57,74]

False-positive results can occur in all cases while probing a peptide array. This is due to the fact that peptides may interact directly with any of the detection agents, such as a protein-fusion moiety, a protein-tag moiety, an anti-tag antibody, a labelled secondary antibody or a streptavidin conjugate. Control incubations using only the detection agents for the read-out procedure are always required. Fortunately, peroxidase itself shows almost no detectable binding to peptides. Discussions continue about the use of horse radish peroxidase or alkaline phosphatase.[37] The detection of peptide–protein binding with a chemiluminescence read-out (peroxidase-labelled moieties with a chemiluminescence substrate) is highly sensitive, but oxidation of peptides due to the use of hydrogen peroxide may occur, so some authors recommend alkaline phosphatase rather than peroxidase as an enzyme label. In our lab we prefer the peroxidase label, due to its higher sensitivity and variable imaging system, with easy quantification of spot signal intensities.[38,58] Chromogenic read-out and densitometry involve methods such as 1) precipitating nitroblue tetrazolium (NBT)/bromochloroindolylphosphate (BCIP) catalysed by alkaline phosphatases,[2,24,37] 2) binding of dye-coupled moieties[60,75] or 3) metal ion detection with chromogenic chelators.[24,76] The advantage is that no expensive imaging system is required and documentation only requires a scanner. However, the sensitivity is much poorer than with chemoluminescence or radioactivity.

Fluorescence read-out and quantification on cellulose membranes is hampered by the intrinsic background of the cellulose membrane. Preferably, fluorescent dyes with longer emission wavelengths should be applied for binding studies.[60,77] In contrast, aminobenzoic acid was used as a fluorescent dye in protease assays.[78,79]

Peptide library types for fundamental SPOT technology

A great variety of library types have been designed and evolved since Mario Gysen's revolutionary concept.[3,80] The design principles of libraries can be classified into approaches based on protein sequences and de novo approaches.[32,23]

Libraries derived from protein sequences provide the basic tools to elucidate interactions between a protein and a ligand. Scans of overlapping peptides (synonyms: peptide scan, pepscan, SPOTscan analysis[26]) signify that the entire protein sequence is synthesised as short, linear, overlapping peptides that are tested for ligand binding, usually as hexa- to 15-mer overlapping peptides.[2,26,32] These lengths are sufficient for antibody–protein interactions because linear epitopes do not exceed this range.[81] In contrast, pepscans comprising overlapping peptides of up to 20-mers in length have been synthesised to elucidate the binding sites of Pex,[82–84] Tat[62,85] and maltose importer proteins.[86,87] Besides peptide length, the number of overlapping amino acids between the consecutive peptides defines a peptide scan. In general, peptides are shifted by one to three positions along the linear sequence. Simple peptide scans are sufficient for revealing linear binding motifs, but often fail for discontinuous motifs. Therefore, the so-called hybritope scan, duotope scan[88] and matrix scan[89] were developed.

After elucidation of a binding motif, details can be elaborated by amino acid substitution scans and substitution analyses. Key residues of a binding motif are those amino acids that are effectively in contact with the binding partner. Critical residues are those residues that facilitate adoption of a certain binding conformation prior to or upon binding. These residues define the specificity and binding free energy. The concept of alanine scanning[90] (synonyms: alanine walk, alanine scan) was developed to identify these residues and has been successfully applied in SPOT technology.[91] Residues that cannot be exchanged by alanine without loss of binding are regarded as key residues for the interaction. In this sense further scans such as glycine scans,[92,93] tyrosine scans[94] and proline scans[95] have also been reported. All these scans reveal effects that depend on the amino acid side chains, with the exception of proline scans, which influence peptide conformation. Further modifications of amino acid substitution scans have been reported by Podolnikova and co-workers[96] and by Espaniel and co-workers.[97,98]

Applying all genetically encoded amino acids for an amino acid substitution scan results in a so-called complete amino acid substitution scan or (complete) substitution analysis (synonyms: substitutional analysis, mutational analysis, replacement analysis). Here, each amino acid of the original sequence (synonym wild-type—or wt—sequence) is replaced by all the other 19 gene-encoded amino acids. This approach has been used quite often in SPOT technology since its beginning.[26,30,32,99] Substitution analyses are well suited for SPOT technology (synonym: SPOT analogue analysis[26]) for rapid and effective mapping of the structure–activity relationships of binding motifs, or even of a complete protein domain.[55,60,100]

Further sequence-derived libraries are truncation and deletion libraries. These types are constructed to determine the minimum lengths of binding motifs. Truncation libraries (synonyms: size scan, window scan, length analysis, SPOTsize analysis[26]) display binding site sequences with omission of one or more N- or C-terminal amino acids, or of both simultaneously.[23,26,32] Deletion libraries involve the deletion of one or more consecutive amino acids at all possible sequence positions.[23] As an alternative to truncation, substitution with one or more alanine residues can be used.[97,98,101] Cyclic libraries are designed to optimise the free energy of a binding peptide by stabilisation of the binding conformation.[102,103] The most widely applied type is the disulfide-cyclisation scan, which comprises all possible combinations of two cysteine residues throughout the original sequence.

Peptide libraries designed de novo are used if no natural protein binding partner is known or if (novel) peptide ligands need to be identified without any prior information. In such cases one has to use combinatorial libraries or randomly generated libraries. Combinatorial library types were pioneered in the 1990s, predominantly for peptide libraries on beads[104,105] (for review see refs. [106] and [107]), and the principles were translated to SPOT technology.[76] The synthesis and application of cellulose-membrane-bound combinatorial peptide libraries have been reviewed extensively several times,[24,27,28,34] and so, because of limitations of space here, the interested reader is referred to these reports. As an alternative to combinatorial libraries, random peptide libraries consist of individual peptide sequences generated by a random algorithm. This approach works well for identifying distinct antibody epitopes and mimotopes.[108,109]

Advanced SPOT Technology

This section focuses on expansion and improvements to SPOT technology. Improvements to SPOT technology include employment of nonpeptidic capture molecules, construction of diverse membrane supports, development of novel membrane modifications, elaboration of useful library and assay types and efforts to quantify read-out of spot signals. In consequence, these improvements allow the application of SPOT technology to answer a broad range of biological and biochemical questions.

Special membranes and membrane modifications

As well as cellulose membranes, researchers have once in a while used polypropylene membranes[32,73] for SPOT technology. Polypropylene membranes are more stable to certain reaction conditions than cellulose membranes and could therefore be used for the synthesis of organic compounds. However, the labour- and time-consuming manufacturing processes for polypropylene membranes are drawbacks for common use. Fortunately, though, commercially available functionalised polypropylene membranes for SPOT technology can now be purchased: from AIMS Scientific Products GmbH,[110] for example, who also offer several types of cellulose membranes with adjusted stability and/or functionality.[111]

Historically, amino-functionalised ether-type membranes were developed five years after the first reported classical amino-functionalised ester-type cellulose membranes.[2,32] Unlike ester-type membranes, ether-type membranes guarantee stable membrane-anchoring of peptides or other compounds, thanks to the chemical stability of the ether bond. Furthermore, such chemically resistant anchoring allows more demanding chemistry on planar supports.[112,113] The first report on an ether-type membrane appeared in 1997 and described the synthesis and application of a cellulose-aminopropyl ether membrane (CAPE membrane).[52] Since then, CAPE membranes have been successfully used for several biological studies (e.g., refs. [58, 82–84, 114–116]). They are distinguished by excellent signal-to-noise ratios during on-support assays, due to the extremely low background signal of the membrane itself.[58] Unfortunately, CAPE membranes cannot be used in fully automated SPOT synthesis processes[49] because of their mechanical brittleness. However, semiautomatic synthesis methods have successfully been adopted.[58] The mechanical stabilities of the more recently developed *N*-CAPE-[77] and trioxa-ether-type membranes[117] look much better. Epibromhydrin and diamine compounds such as trioxa- or 1,3-diaminopropane are used for their preparation. These ether-type membranes open up the opportunity for more demanding chemistry such as the syntheses of peptoids[118–120] triazine libraries,[112] inverted peptide arrays[59,121] and soluble peptides with authentic C termini.[77,122] Continuing towards more challenging nonpeptide arrays, Helen Blackwell's lab developed a robust and linker-functionalised planar cellulose support.[123] Direct tosylation of the support was found to be an effective method to activate cellulose prior to introduction of a diamine compound.[124] Densities of several amino-functionalised cellulose membranes are shown in Table 1.

Another idea advancing SPOT technology involves the use of linker strategies to enable cleavage of peptides from the support. In contrast with ester-type glycine membranes, incorporation of special linker molecules allows the release of peptides with authentic C termini. An interesting linker moiety is the Carboxy-Frank-Linker[125,126] (commercially available from IRIS Biotech, GmbH; http://www.iris-biotech.de). This type of linker allows peptide release from the resin in aqueous solution (pH 7–8); this makes in situ tests possible.[127] Other linker types used in SPOT technology nowadays are the *p*-hydroxy-

Table 1. Amino-modified cellulose membrane types including loading capacity.

Cellulose membrane type 1	Capacity [nmol cm^{-2}]	Refs.[a]
ester-type: β-alanine	400–600	[2, 26, 40–43]
ester-type: β-alanine reduced	10–300	[31, 57]
ester-type: glycine	800–1800	[44, 45]
ester-type: different amino acids	200–1700	[39]
ether-type: CAPE	30–120	[58, 52]
ether-type: trioxa, *N*-CAPE	200–1200	[77, 117]
amine-type: TsCl, diamine	450–4000	[123, 130]

[a] Selected references.

methyl-benzoic acid (HMB) linker,[52] the Rink-amide linker,[113,128,129] photolabile linker moieties,[112,117,130] the Wang linker,[124] thioether moieties[77,122] or 4-hydroxymethyl-phenoxy acetic acid (HMPA) and 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linkers.[131]

An interesting approach based on the use of the C-terminal amino acid of a peptide as a linker moiety was reported by Ay and co-workers,[39,132] who sorted peptides according to their C termini and modified membranes with the corresponding C-terminal amino acids, either spot- or surface-wise.

Due to the fact that peptide density is crucial for probing peptide arrays with a protein of interest,[31,58] several authors have reported on amino functionality adjustment in order to optimise synthesis or screening.[31,57,133–135]

Advanced in situ synthesis on planar supports

Normally, with the exception of combinatorial approaches, the rule "one spot, one peptide" is valid. Espanel and co-workers enlarged this rule and called the result the SPOT-DS method (one spot, two peptides).[136,137] Two different peptides could be synthesised on one spot by an orthogonal protection group strategy through the use of a mixture of Fmoc-β-alanine and Alloc-β-alanine for spot definition. In consequence, this method allowed synergistic components of interacting protein–protein motifs to be mapped. A similar concept called IANUS (induced organisation of structure by matrix-assisted togetherness) was introduced by Yu and co-workers.[138] Here, two different peptides could be synthesised on an orthogonally protected template.

In addition to the standard coupling procedures of the basic SPOT synthesis approach, activators such as 2-(1*H*-9-azobenzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HATU), benzotriazol-1-yl-*N*-tetramethyl-uronium tetrafluoroborate (TBTU) or benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) have been used in combination with bases.[52,59,138] Recently it was reported that 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) is the activator of choice for incorporation of phospho-amino acids in a growing peptide chain by the SPOT synthesis approach.[139] This is in good agreement with other reports in which EEDQ has been applied as a satisfactory coupling reagent for incorporation of different building blocks and residues.[55,75,122]

As mentioned above, ether-type and amine-type cellulose membranes provide opportunities for more demanding SPOT synthesis chemistry. In particular, Helen Blackwell's group has made several important contributions, such as microwave-accelerated SPOT synthesis,[123,124] the construction of small-molecule macroarrays through Ugi four-component reactions[130,140] or the discovery of fluorescent cyanopyridines.[141] As well as the peptoids, peptomers or triazines mentioned above, hydantoins[142] and glycopeptides[143,144] have recently been synthesised by the SPOT technology approach.

SPOT synthesis of peptides is not restricted only to short peptides. This has been demonstrated by Toepert and co-workers in the synthesis of a complete substitution analysis of the *h*YAP WW domain.[100] In a similar approach using pseudoproline building blocks, Przezdziak and co-workers managed to perform a complete substitution analysis of the difficult-to-synthesise FBP28 WW domain.[55] A successful SPOT synthesis that enabled the complete substitution analysis of the GCN4 leucine zipper has been reported more recently by Portwich and co-workers.[60] Even native chemical peptide ligation has been applied for SPOT synthesis.[75] An interesting adaptation of the template-assembled synthetic proteins (TASP) concept to SPOT technology has been shown by Haehnel and co-workers.[128,129]

A widespread strategy through which to optimise the binding free energy of a peptide interacting with a binding partner is to stabilise the binding conformation; this is mostly achieved by cyclisation. On-support cyclisation of peptides is predominantly achieved by disulfide bridge formation (see, for example, refs. [103, 145, 146]). More sophisticated approaches involve the formation of amide bridges (see, for example, ref. [102]), thioether bridges[59] or cyclic peptidomimetics.[112]

Reports on nonporous planar supports used for in situ SPOT synthesis are rare, due to difficulties in generating addressable spots on such surfaces. An interesting approach to SPOT synthesis of peptide arrays on self-assembled monolayers coated on gold surfaces was reported very recently by Laurent and co-workers.[147] A further possibility was reported by Kim and co-workers, who designed a glass slide for SPOT synthesis by patterning with photoresist and perfluorination followed by amination.[148]

Advanced assays and peptide library types

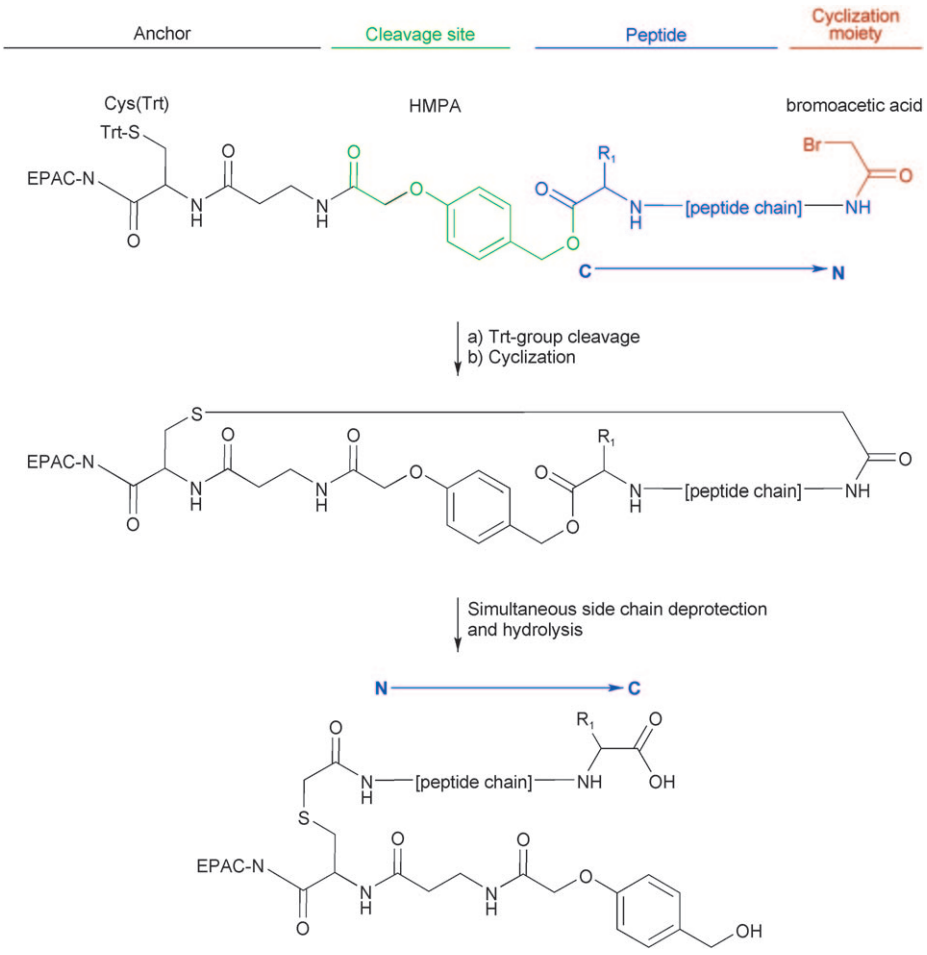
Obviously, function initially requires an interaction. This is why proteome research aims to provide a complete description of the network of protein interactions within a cell, an organism or a tissue. Both the quantitative analysis and the time-dependent description of protein–protein networks is still a great challenge today. Proteins interact through surface-accessible interaction sites, which involve amino acid side chain and backbone contacts. Such contact residues are presented along a linear (not necessarily contiguous) segment of the protein chain, or are parts of two or more regions of the protein chain brought together by the folded conformation.

The strength of SPOT peptide assays is their unbiased, comprehensive and systematic approach to evaluation of a given protein for binding linear peptide sequences, which may also

be additionally modified. The clear advantage of the array format can be fully exploited for study of protein interactions in which one of the partners participates in complex formation by docking with a relatively short peptide within a receptor protein. In fact, a fairly large set of protein interactions are mediated by families of protein binding domains such as the SH2, SH3, PH, EVH1, PDZ or WW domains. Those domains, also called peptide recognition modules (PRMs), act as receptors to accommodate short peptides in their binding pockets, in extended conformations.[149,150] In an ideal scenario, unique peptides representing the entire proteome of an organism would be synthesised on an array and assayed individually for interactions with a PRM of interest. In practice, however, a filtering step is required to generate an array of manageable size. One approach, involving a strategy named WISE (Whole Interactome Scanning Experiment), has been described by Landgraf and co-workers.[58] All sequences within the yeast proteome matching a relaxed consensus of a given SH3 domain were identified by computational methods. The consensus sequences were deduced through screening of random peptide repertoires such as phage display libraries. Next, all of the matching peptides were synthesised on a cellulose membrane and probed for binding of the yeast SH3 domains. In very recently finished work, the complete SH3 domain interactome of yeast was analysed in more detail by computational and mathematical methods in combination with orthogonal experimental proteomic tools such as phage display, yeast two-hybrid and SPOT technology. The results will be published soon elsewhere.

There are considerably more modular protein domains, especially PRMs, in the human proteome than there are in the yeast model organism. The human proteome harbours approximately 300 SH3 domains, whereas yeast contains only 28 copies of the same domain family. Nevertheless, Wu and co-workers have started a systematic study to identify SH3 domain-mediated human protein–protein interactions by synthetic peptide array target screening.[151] A SPOT-synthesised peptide array of 1536 potential peptide ligands was used to probe a group of 12 human SH3 domains.

PDZ domains anchor transmembrane proteins to the cytoskeleton and hold signalling complexes together.[152,153] In general, PDZ domains recognise four to seven residues of their protein binding partners. In other words, PDZ domains are PRMs known to recognise short linear peptides containing free C termini. Unfortunately, SPOT-synthesised peptides lack free C termini, due to their C-terminal fixation to the cellulose support. Boisguerin and co-workers developed a robust SPOT synthesis concept for synthesising inverted peptide arrays with free C termini,[52] which was recently adjusted to incorporate phosphorylated amino acids.[121] Briefly, a library of peptides was synthesised on a solid support. Upon completion of the peptide sequence, the N termini were further modified such that they could loop back and attach to the solid support through a thioether. The C termini were then liberated from the solid support, leaving the peptides attached only by the N termini. Scheme 2 > shows the key SPOT synthesis steps of the procedure actually used to prepare an inverted peptide



Scheme 2. Key steps during the synthesis of inverted peptides. Top row: It is recommended that the synthesis be performed on an ether-type membrane (on a *N*-CAPE membrane, for instance). The sequence β-Ala-Cys(Trt)-β-Ala represents the anchor molecule (black) and is coupled under standard SPOT synthesis conditions. The cleavage site (green) is formed by [4-(hydroxymethyl)phenoxy]acetic acid (HMPA) and the C-terminal amino acid. Peptide completion (blue) is carried out under standard SPOT synthesis conditions, and finally bromoacetic acid is attached as the cyclisation moiety (crimson). Second row: After selective cleavage of the Trt group, cyclisation occurs upon treatment with cesium carbonate. Bottom row: Hydrolysis and side chain deprotection occurs simultaneously, and the inverted peptide sequence with the free C terminus is the result.

array. Meanwhile, this kind of library has been applied to map the specificities of several PDZ domains.[52,121,154,155]

A genome-wide peptide screening approach should directly address functional protein interaction sites; this would lead to detailed insights into the discovered molecular recognition events, and place them in context in the whole genome. It even allows one to rapidly decipher the chemical natures of these interactions.[156] In this context, Bialek and co-workers developed a peptide array-based epitope-targeted proteome analysis.[157,158] The concept involves screening of a library of peptide fragments with a library of protein domains displayed on a bacteriophage library. Because phage particles are easy to propagate and analysis by modern DNA microarray analysis is feasible, these are chosen for the read-out.

Cellulose-membrane-bound peptide arrays are ideal tools with which to investigate both protease–substrate and protease–inhibitor interactions. Peptides labelled at their N terminus with a chromophore and/or fluorescent dye are used, and protease specificity around the cleavage site is mapped as a function of time by measuring the absorbance or fluorescence of the liberated dyes.[78,79,159–161] Protease–inhibitor interactions are analysed by determination both of binding to and inhibition of a given protease. Binding has been studied directly on a cellulose membrane through the use of HRP-labelled proteases, for example, whereas inhibition has been measured in microtitre plates with punched-out peptide spots and a chromogenic substrate (see, for example, refs. [145,162]).

In general, cellulose-membrane-bound peptides are probed for binding with a protein of interest. Otte and co-workers turned it around and probed 42 presynthesised WW domains immobilised onto a cellulose membrane with HRP-labelled peptide ligands.[163]

Ay and co-workers have developed an assay to map an entire virus proteome for putative CD8 T cell epitopes. Based on a sorting and pooling strategy, it was used with the proteome of human cytomegalovirus.[39]

Spot technology is an ideal tool with which to initiate and control transformation of a peptide into a nonpeptide analogue with retention of biological activity. Starting with a biologically active peptide, Hoffmann and co-workers applied an iterative transformation process involving a sequence of successive complete substitution analyses, which finally resulted in a peptoid with comparable biological activity.[120]

Recent Applications of SPOT Technology

This section presents a selection of very recently published papers relevant to SPOT technology. For reasons of space, however, a complete overview of SPOT technology papers over the past five years cannot be presented here.

Bleeding disorders can be caused by an antibody that inhibits the human clotting factor VII. Kopecky and co-workers screened cellulose-membrane-bound combinatorial peptide libraries for peptides capable of neutralising these antibodies.[164] The study demonstrated for the first time that short peptides can be used to compete for polyclonal inhibitory antibodies from various patients. Subsequently, these peptides were converted into different PEGylated forms with the aim of using them as therapeutic substances.[165]

Nuclear pore complexes (NPCs) are large organelles that bridge the double membrane of the nuclear envelope and mediate all macromolecular exchanges between the nucleus and cytoplasm. A vertebrate NPC is made up of approximately 30 different proteins called nucleoporins or Nups. Most nuclear proteins contain targeting signals and are actively transported into and out of the nucleus by transport carriers that bind both the transport cargo and the NPC. Cushman and co-workers used the SPOT technology approach to identify short amino acid sequences within Nups that bind the nuclear carrier importin-β.[166]

Bacterial infections are commonly treated with antibiotics. Unfortunately, though, the effectiveness of antibiotics has become limited for several reasons. To bring antibiotic resistance under control, it is a challenge for scientists to design novel antimicrobial candidates. Hilpert and co-workers started to analyse the modes of action and sequence requirements of host defence peptides.[45,167] Substitution analyses, scrambled peptides and random peptide libraries prepared by the SPOT technology approach helped to reveal sequence requirements and an optimisation strategy for short antimicrobial peptides. More recently, *N*-CAPE membranes[77,122] were used to synthesise peptides that remained covalently bound during biological assays. Out of 122 tested sequences, the best surface-tethered nona-, dodeca- and 13-mer peptides were found to be highly antimicrobial against bacteria and fungi, as was confirmed by use of alternative surface materials and coupling strategies, as well as by coupling through the C and N termini of the peptides.[168] Cherkasov and co-workers[169] very recently created two large random nine-amino-acid peptide libraries based on the amino acid compositions of the most antibiotically active peptides. The resulting data were used together with "Artificial Neural Networks", a powerful machine learning technique, to create quantitative in silico models of antibiotic activity. On the basis of random testing, these models proved remarkably effective in predicting the activity of 100 000 virtual peptides. The best peptides, representing the top quartile of predicted activities, were effective against a broad array of multidrug-resistant bacteria. Blackwell's lab extended the approach to small-molecule macroarrays synthesised on a planar cellulose support and probed for antimicrobial activity against *Staphylococcus aureus*.[170] Macroarrays of chalcones and heterocycles were constructed and subjected to a suite of antibacterial assays conducted either on or off the macroarray support.

Surprisingly, it took a long time before SPOT technology was applied to chromatin research. One of the primary mechanisms regulating access to DNA is the post-translational modification of histone proteins.[171] Many of these modifications occur on the N-terminal tails of histone proteins that protrude from the nucleosome. Nevertheless, there are huge gaps in our knowledge of which proteins read and write the histone code. Modules in proteins that recognise the modified histones include the BROMO and CHROMO domains, malignant brain tumour repeats, plant homeodomain zinc fingers and tudor domains. Arrays of peptides immobilised on a cellulose membrane seem

to be a perfect tool for investigation of the specificity of these histone code readers. This has very recently been demonstrated by Nady and co-workers, who used synthetic peptide arrays in a screening approach to characterise macromolecules that interact with specific covalent modifications of histone tails.[172] Looking at the writers of the histone code, Rathert and co-workers developed a novel assay based on SPOT technology that could determine the specificity profiles of protein methyltransferases.[69,70] The authors found that the methyltransferase G9a mostly recognises an Arg-Lys sequence and that enzyme activity is inhibited by methylation of the arginine residue. Furthermore, the authors were able to identify new nonhistone protein targets of the enzyme G9a.

Data relating to peptide–ligand interactions gained from approaches based on extensive screening of synthetic peptide arrays could be used for modelling studies. This has recently been demonstrated by two contributions from Mandrika and co-workers, who applied chemometric techniques and a statistical molecular design to model an antigen–antibody interaction on the basis of SPOT-synthesised peptide arrays.[173,174]

Besides other functions, short linear peptides are also known to regulate various cellular events, such as phagocytosis, smooth muscle contraction and lymphocyte activation. Investigation of such peptide–cell interactions is important for further understanding of cellular regulatory mechanisms and should also lead to the design of biologically functional materials for clinical applications.[175] Synthetic peptide arrays were applied by the Honda lab[176] to assay peptide–cell interactions between cellulose-membrane-bound peptides and anchorage-dependant cells.[173] As a model case, they investigated cell-adhesive peptides that could enhance cell growth as tissue engineering scaffold materials. In a further study from the same lab, Okochi and co-workers screened for human mesenchymal stem cell-adhesive peptides derived from fibronectin type III domains.[177]

Outlook: Quo Vadis SPOT Technology

I hope this review has shown that SPOT technology is today a well-established screening tool for biologically active peptides. Starting from simple antibody-peptide binding assays, the methodology is nowadays applied to sophisticated enzyme assays, comprehensive proteomic studies and recently for studies with living microbes or cells. A multitude of investigations are possible with this technology, which has now become integrated into many biochemical laboratories. The equipment for SPOT technology is commercially available and does not require special conditions. It is likely that SPOT technology will be adapted and used in new fields over the next few years.

One aspect of SPOT technology needs to be improved. The quantitative read-out of the SPOT technology approach is not as good as might be expected.[38,58] Further development of the method is required to obtain highly accurate quantitative signals. And there is one aspect of SPOT technology that remains controversial. Although several protocols for membrane regeneration are reported in the literature, this process seems to be a crucial point. From personal experience there is no robust and reliable protocol for reproducible membrane regeneration available. This is a severe limitation of SPOT technology, especially from the viewpoint of proteomics. It would be very welcome if a given peptide array could be screened several times without any loss of quality—with, for example, different representatives from a protein domain family. Furthermore, comprehensive serum profiling studies depend on reliable membrane regeneration. One way out of this dilemma could be the use of peptide microarrays that could be prepared for a multitude of replicas. Presynthesised soluble peptides have been immobilised on glass slides by several methods.[37,178–180] However, the peptide microarray technique requires expensive equipment and special laboratory conditions. At the moment, production of peptide microarrays involves highly parallel and high-throughput peptide synthesis, as well as robotic-supported immobilisation of pre-synthesised peptide derivatives on glass slides. High peptide densities can be achieved, for example, with the SC^2 method,[37] in which the individual peptide–cellulose conjugates synthesised in a first array are separated and spotted in high density on a secondary support. Hence, SPOT synthesis is a prerequisite for the preparation of peptide microarrays.

In situ synthesis of high-density peptide microarrays is still a great challenge. Arrays with thousands of peptides per square centimetre can be synthesised by photolithography on a glass surface[21] and the technology has been commercialised by LC Sciences (http://www.lcsciences.com). One further possible route uses chargeable amino acid particles that are guided step by step onto a computer chip's surface by electric field patterns.[181] In continuation, and with the aim of generating customised peptide arrays at high density, high speed and low costs, Stadler and co-workers used a modified colour laser printer to "print" the 20 amino acids in the form of solid amino acid toner particles at defined positions on a glass support.[182] The peptide laser printer opens up the opportunity to translate entire proteomes into huge sets of immobilised peptides that can be screened with ligands of interest. The technology of particle-based synthesis of peptide arrays has been commercialised by the company PEPperPRINT (http://www.pepperprint.com).

It is quite evident that generation of peptide microarrays is a task for highly specialised laboratories, due to the high investment costs. However, one can assume that peptide microarrays have the potential to become commercialised in future.

In conclusion, the capability to prepare high-quality peptide arrays efficiently and at low cost, as well as the implementation of cost-effective and rapid analytical techniques to generate and process data from peptide arrays, are milestones for the future development of both peptide macro- and peptide microarrays. It has become apparent that SPOT technology meets most of these requirements. However, quality has to be improved and array miniaturisation on cellulose membranes would be preferable. Nevertheless, SPOT technology has established itself as a highly flexible, robust and reliable research method that could be implemented in nearly every biochemical laboratory.

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