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Cellulose-bound Peptide Arrays: Preparation and Applications

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Introduction

The way of the development of the cellulose-bound peptide arrays leads via the synthesis of oligonucleotides on cellulose pieces (Frank *et al*., 1983) and multiple peptide synthesis on cellulose paper discs in columns (Frank and Döring, 1988). Probably inspired by the addressability of the multipin method (Geysen *et al*., 1984), Ronald Frank and co-workers developed SPOT synthesis and first presented the method in 1990 at the 21st European Peptide Symposium (Frank *et al*., 1991). Today,

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Abbreviations: Ac, acetyl; Alloc, allyloxycarbonyl; ADAMs, a disintegrin and metalloprotease; ATP, adenosine 5'-triphosphate; CD, circular dichroism; CDI, 1,1´-carbonyl-diimidazole; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethyl; DIC, N,N´-diisopropyl carbodiimide; DIPEA, diisopropylethylamine; DKP, diketopiperazine; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; Fmoc, 9-fluorenylmethoxycarbonyl; GMP, guanosine monophosphate; HATU, O-(7-azabenzotriazole-1-yl)-tetramethyluronium hexafluorophosphate; HBTU, 2-(1H-benzotriazole-1-yl)- tetramethyluronium hexafluorophosphate; HIV, human immunodeficiency virus; HOBt, N-hydroxybenzotriazole; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl; NMR, nuclear magnetic resonance; MRSA, methicillin resistant *Staphylococcus aureus*; MSNT - 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazole; ODhbt, 3-hydroxy-2,3-dihydroxy-4-oxobenzotriazolyl; ODNp – 2,4-dinitrophenyl; OPfp, pentafluorophenyl; PEG, polyethylene glycol; PNA, peptide nucleic acid; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SDS, sodium dodecyl sulfate; STOP, stable tubule only polypeptides, TBTU, 2-(1H-benzotriazole-1-yl)- tetramethyluronium hexafluoroborate; tBu, tertiary-butyl; TFA, trifluoroacetic acid; TIBS, triisobutylsilane; TIPS, triisopropylsilane; TsCl, tosylchloride.

The abbreviations, one and three letter codes for the amino acids and peptides are according the UIPAC-IUB convention (IUPAC-IUB, 1984).

SPOT synthesis is one of the most frequently used array methods for screening peptides and other compounds, and provides both solid-phase assays as well as solution-phase assays.

The first arrays applying the SPOT method were synthesized manually (Frank, 1992). This technique requires no special equipment, but because it is laborious, manual SPOT synthesis is recently only used for screening of a small number of peptides (e.g. Petersen, 2002; Santona *et al*., 2002). In order to screen larger numbers of peptides or peptide mixtures automation of the synthesis was necessary. In principle, any X/Y-programmable pipetting workstation could be used, but due to the minimum aspiration volume of 0.5 microlitres, most of these machines are only useful for large spots with a diameter of about 6 millimetres. To place thousands of spots on a single membrane sheet requires more precise pipetting machines with lower minimum aspiration volumes. This is why semi-automated (Intavis, Köln, Germany - Gausepohl and Behn, 2002) and fully automated synthesizers (Intavis, Köln, Germany; Jerini Peptide Technologies, Berlin, Germany - Zerweck *et al*., 2003) were developed (*Figure 1*). There are now publications that describe the parallel synthesis of a few peptides (e.g. Bialek *et al*., 2003) up to approximately 25,000 peptides using the SPOT method (Schneider-Mergener and Kramer, 1994). The very large number of different peptide sequences synthesized means that coupling yields can vary and therefore the amount, as well as the purity of the peptides may differ. Due to the low amount of peptide per spot, another disadvantage is that these peptides are very difficult to purify. The limitations of the reliability of the SPOT technology were described by several authors (Kramer *et al*., 1999a; Landgraf *et al*., 2004; Weiser *et al*., 2005).

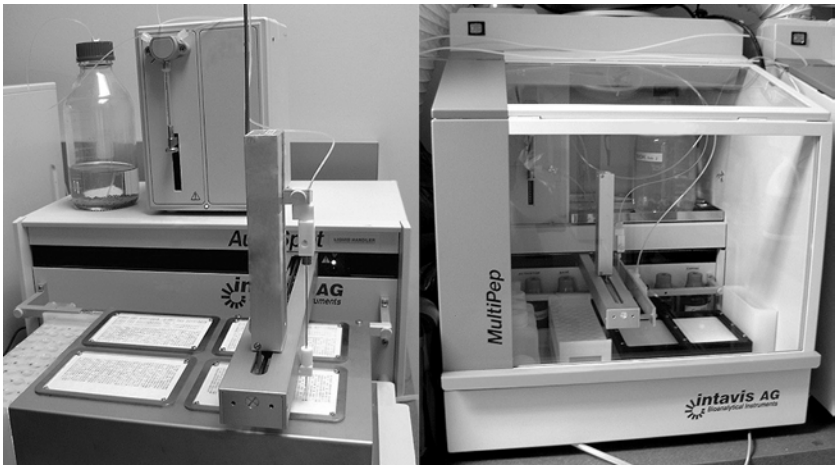


Figure 1. Semi-automatic spotting robot (left) and a fully automatic spot synthesizer (right) (Intavis AG, Köln, Germany).

SPOT synthesis of peptides on cellulose membranes is a special type of solid phase peptide synthesis. Each spot can be seen as a separate reaction vessel. Like all other chemical peptide syntheses, and in contrast to methods using biological systems, SPOT synthesis is not restricted to gene-encoded amino acids. Many publications have reported peptide synthesis with D-amino acids (e.g. Piossek *et al*., 1999b, Stigler *et al*., 1999; Rüdiger *et al*., 2001) or unnatural amino acids using the SPOT technology

(Toepert *et al*., 2003; Wildemann *et al*., 2006). In addition, insertions of non-amino acid building blocks greatly enhance the variability of the structural space that can be covered (Volkmer-Engert *et al*., 1997). When comparing a chemical (SPOT synthesis) and a biological library (phage display) for epitope characterization, Kramer and co-workers wrote that "... with regard to ease of handling and identified sequences, the chemical libraries are clearly favoured to study antibody-epitope interactions". (Kramer *et al*., 1995).

Here, we present an overview of the current state of preparation of cellulose membranes, synthesis of peptides on cellulose support and applications using the SPOT method. However, owing to the overwhelming body of literature on this topic, complete coverage is probably not feasible.

Membranes

SPOT synthesis is described as a solid-phase synthesis method on porous and planar surfaces (membranes). The classical and widely used solid support for SPOT synthesis is cellulose (Frank, 1992). Cellulose is available in almost every laboratory as filter paper, and in contrast to other screening surfaces is very inexpensive. Cellulose membranes are porous, hydrophilic, flexible and stable in the organic solvents used for the peptide synthesis. These properties make cellulose membranes very useful for biochemical and biological investigations of interactions in aqueous as well as organic media. In contrast to polymer membranes, cellulose shows high thermo-stability up to temperatures of about 180 °C, making it possible to use cellulose membranes for reactions at elevated temperatures (Blackwell, 2006). To date filter papers have been mostly used as the solid support. The preferred types of filter paper are:

- Whatman Chr1 (e.g. Adler *et al*., 1994; Santona *et al*., 2002; Bowman *et al*., 2006b)
- Whatman 50 (e.g. Dong *et al*., 2003; Hujer *et al*., 2004; Hilpert *et al*., 2005b)
- Whatman 540 (e.g. Tegge *et al*., 1995b; Cruz *et al*, 2004a; Kopecky *et al*., 2006b)

Several specially prepared cellulose membranes are commercially available (e.g. from AIMS Scientific Products, Braunschweig, Germany, and Sigma-Genosys/Sigma-Aldrich, St. Louis, USA). The latest development are TFA-soluble cellulose membranes to multiply the synthesized cellulose-bound peptide macroarray by transferring the solutions to a number of microarrays (Beutling *et al*., 2005; Zander *et al*., 2005; Dikmans *et al*., 2005).

Several materials other than cellulose can be used as solid supports, e.g. polymer membranes (Gao and Esnouf, 1996; Volkmer-Engert *et al*., 1999; Zander *et al*., 2002), nitrocellulose and PVDF membranes (Strutzberg *et al*., 1995; Papagrigoriou *et al*., 2004; Drakulovski *et al*., 2003). Other synthesis and screening methods on planar surfaces using glass slides (Fodor *et al*., 1991) or compact discs (Frank, 2002; La Clair and Burkhart, 2003) or by reagent delivery with an ink-jet printing system (Cooley *et al*., 2002) have been described.

Modifications

MEMBRANE FUNCTIONALIZATION

Cellulose is a polysaccharide with free hydroxyl groups. Since these functional groups are less reactive than amino groups, the direct attachment of amino acids often leads to very low yields. To make the cellulose suitable for the synthesis of peptides it is necessary to modify its surface and change the functionalization from hydroxyl to amino groups (see *Table 1*). Modification of the cellulose membrane often involves insertion of a spacer molecule permitting better accessibility to the amino groups on the membrane. The use of a spacer leads to an increase of the distance of the synthesized peptides from the membrane surface and thus improves their accessibility to proteins during probing of such an array.

The easiest and an often utilized derivatization of cellulose membranes is the esterification using ß-alanine with DIC, and the additional coupling of a second ß-alanine as a spacer (Frank, 1992; Dürauer *et al*., 2006). Esterification of the cellulose with glycine can increase functionalization, particularly if CDI is used instead of DIC (Kamradt and Volkmer-Engert, 2004). This higher functionalization, and potentially higher yields of peptides, is of special interest for applications requiring high peptide concentrations in soluble assays, where the peptides are to be cleaved from the membrane (e.g. Kamradt and Volkmer-Engert, 2004; Hilpert *et al*., 2005a). As an alternative to using two ß-alanines, it is possible to use ε-aminohexanoic acid (Gausepohl and Behn, 1998). One method of releasing free peptides from the membrane involves cleavage through the formation of diketopiperazines at the C-terminus (see section: Linker and cleavage from membrane support), in which case the membrane has to be modified by coupling proline directly to the cellulose as the first amino acid (Frank and Overwin, 1996).

Other possibilities for the esterification of cellulose include MSNT activation of the amino acids (Blankemeyer-Menge *et al*., 1990) or the use of amino acid fluorides (Wenschuh *et al*., 1999). The esterification of the cellulose creates ester bonds between the amino acid and the cellulose. Ester bonds are labile, and particularly at high pH the peptide can be cleaved from the membrane. A Gly-Gly modification of the cellulose is particularly unstable, and due to spontaneous diketopiperazine formation can cause the capacity of the cellulose to decrease with storage and during each Fmoc deprotection step. Additionally, in contrast to the non-proteinogenic ß-alanine, the Gly-Gly motif can cause non-specific interactions to antibodies (Frank, 1992).

In some cases, a high density of peptides can cause difficulties (e.g. “ring spot effect”; Kramer *et al*., 1999a) when assessing the interactions with the molecules of interest. If such difficulties occur, or to investigate the peptide concentration dependency of selected interactions, a reduction in amino group loading may be necessary. When using ß-alanine as spacer molecule, several methods to reduce the peptide density have been described. One possibility is through the coupling of defined mixtures of Fmoc-ß-Ala-OPfp and Ac-ß-Ala-OPfp. This results in blocking of part of the amino groups on the membrane by acetylated ß-alanine, leading to a lower functionalization of the membrane (Kramer *et al*., 1999a; Gail *et al*., 2005) Other possibilities include the funtionalization of the membrane with ß-alanine at different concentrations (Otvos Jr *et al*., 2000) or the synthesis of the peptide using lower amino acid concentrations (Dürauer *et al*., 2006).

Table 1. Types and density of amino functionalization of distinct cellulose membranes.

Cellulose type	Functionalization & treatment	Activity [µmol/cm²]	References
	ß-Alanine	0.4 – 0.6	Frank and Overwin, 1996; Frank *et al*.,1996
	TsCl + diamino-PEG-3 + microwave irradiation	~ 4.0	Lin *et al*., 2005
	TFA pre-treatment + TsCl + diamino-PEG-3 + microwave irradiation	3.8 - 10	Bowman *et al*., 2006b
Whatman Chr1	TsCl + diamino-PEG-3 + microwave irradiation + linker	~ 0.45	Lin *et al*., 2005
	TFA pre-treatment + TsCl + diamino-PEG-3 + microwave irr. + linker	1.8 – 2.6	Bowman *et al*., 2006b
Whatman 50	ß-Alanine	0.2 – 0.4	Frank and Overwin, 1996; Frank *et al*., 1996
	Ac-ß-alanine/ß-alanine	0.28 – 0.01	Kramer *et al*., 1999a
	Amino-epoxy	0.05 – 0.20	Volkmer-Engert *et al*., 1997; Wenschuh *et al*., 2000; Landgraf *et al*., 2004
	Epoxy + diamino-PEG-3	0.2 - 1.5	Ast *et al*., 1999; Wenschuh *et al*., 2000
	Glycine	0.8 – 1.9	Kamradt and Volkmer-Engert, 2004
	Different amino acids	0.2 – 1.7	Streitz *et al*., 2003
Whatman 540	ß-Alanine	0.2 – 0.6	Frank and Overwin, 1996; Frank *et al*., 1996
AIMS	amino-PEG	0.4 - 0.6	Otvos Jr *et al*., 2000; Gail *et al*., 2005
	amino-PEG	2.0 - 5.0	Zander, 2004

To achieve a chemically stable bond between the amino acid and the cellulose a bond other than an ester bond is necessary. The preparation of cellulose membranes with chemically stable bonds has been described for the attachment of the amino acid or another spacer via ether or amide bonds (Volkmer-Engert *et al*., 1997; Wenschuh *et al*., 2000; Bowman *et al*., 2006b).

The first description of a chemically stable peptide-cellulose bond reported the reaction of protected amino-epoxy derivatives to yield an ether bond between the coupled molecule and the cellulose. After cleavage of the amino protection group

from the coupled molecule, the membrane was ready to use for peptide synthesis (Volkmer-Engert *et al*., 1997). Disadvantages of this procedure are undesired side reactions and that it is labor intensive (Wenschuh *et al*., 2000).

Another method for attaching amino acids is the covalent coupling of a diamine, e.g. diaminopropane (Landgraf *et al*., 2004) or 4,7,10-trioxa-1,13-tridecanediamine, an aminated PEG-3 unit, (Ast *et al*., 1999). The stable diamine bond can be achieved either through treatment of the cellulose with epibromohydrine, followed by reaction of the diamine with the coupled epoxy unit at elevated temperatures (Licha *et al*., 2000) or by tosylation of the cellulose with tosylchloride and substitution of the tosyl group with diamine using microwave irradiation (Bowman *et al*., 2004; Lin *et al*., 2005).

Of special interest for investigating complex peptide-protein interactions are peptide spots containing more than one peptide. The SPOT-DS method (SPOT-DS = double spot; Espanel *et al*., 2003; Espanel and van Huijsduijnen, 2005) uses a mixture of Fmoc-ß-alanine and Alloc-ß-alanine. This orthogonal protecting group strategy makes it is possible to synthesize two different peptide sequences on a single spot. A similar result was achieved with the IANUS (= induced organization of structure by matrix-assisted togetherness) method by the application and successive deprotection of orthogonal protected Fmoc-Lys(Dde)-OH (Yu *et al*., 2005). A technique based on the SPOT-DS method allowed the synthesis of four distinct peptides on one spot. According to this method, which is called SPOT^4 (= 4 peptides per spot; Espanel and van Huijsduijnen, 2005), the coupling of orthogonal protected Fmoc-Lys(ivDde)-OH to the stepwise deprotected alanine molecules and successive deprotection of the lysines, permitted successive syntheses of four peptides.

Already modified cellulose membranes are also commercially available; for instance with a stably attached aminated spacer of 8 to 12 PEG units (PEG_{500}) (AIMS, Braunschweig, Germany) (Gausepohl and Behn, 2002; Zander and Gausepohl, 2002). In contrast to common cellulose membranes, these membranes are stable under strong acidic and basic conditions (Zander, 2004). This may be an advantage for regenerating membranes after probing experiments as well as for using harsher synthesis conditions.

LINKER STRATEGIES AND CLEAVAGE FROM THE MEMBRANE SUPPORT

In order to obtain free peptides from the cellulose it is necessary to cleave the C-terminal bond between the peptide and the modified cellulose surface. Also to obtain cellulose-bound peptides with a free C-terminus (see Modifications) the C-terminal part must be released from the membrane (see Peptide modifications). This is generally carried out through use of an appropriate linker molecule coupled to the cellulose support.

In cases in which the peptide is coupled via an ester bond (ß-alanine of glycine spacer), this bond can be hydrolyzed by treatment at high pH (>9). Several reagents are suitable, e.g. aqueous solutions of ammonia, sodium hydroxide, trialkylamines or lithium carbonate (see *Table 2*). A widely used method for yielding soluble peptide amides is the treatment of dry membranes with ammonia vapor (Bray *et al*., 1991a and 1993). When the cleavage is carried out with nucleophils in an anhydrous environment, the substitution of the ester bond leads to amides, hydrazides and other derivatives of the carboxy function (Ast *et al*., 2001).

Table 2. Published linker types attached to cellulose supports including the cleavage methods used to release the peptides from the membrane.

Linker type	Cleavage conditions	C-Terminus	References
	gaseous ammonia	amide	Wenschuh *et al*., 1999; Drabner *et al*., 2002; Ehrlich *et al*., 2005
	hydrazine	hydrazide	Ast *et al*., 2001
	hydroxyl amine	hydroxyl amide	Ast *et al*., 2001
	aq. NaOH	free carboxy group	Streitz *et al*., 2003
	aq. triethylamine	free carboxy group	Lizcano *et al*., 2002
	prim. alkyl/aryl amine	alkyl/aryl amide	Ast *et al*., 2001
Allyl-Linker	palladium(0)-catalyst	free carboxy group	Blankemeyer-Menge and Frank, 1988
Boc-Imidazol-Linker	TFA + aq. buffer	free carboxy group	Hoffmann and Frank, 1995; Gonzalez-Gil *et al*., 1998
Boc-Lys-Pro	TFA + aq. buffer	diketopiperazine	Geginat *et al*., 1998; Ede, 2002; Zander, 2004
HMB linker	gaseous ammonia	amide	Volkmer-Engert *et al*., 1997 and 1998
photo-labile linker	UV irradiation at 365 nm	amide	Ast *et al*., 1999; Scharn *et al*., 2001; Lin *et al*., 2005
Rink-amide-linker	TFA TFA vapor	amide	Rau *et al*., 2000; Haehnel, 2004 Scharn *et al*., 2000
Thioether (thiol + coupled by amino acid haloalkyl esters)	gaseous ammonia NaOH/H_2O/methanol NaOH/H_2O/aceto-nitrile	amide free carboxy group free carboxy group	Volkmer-Engert and Schneider-Mergener, 1999; Licha *et al*., 2000 Licha *et al*., 2000 Bhargava *et al*., 2002
Wang-Linker	TFA vapor	free carboxy group	Bowman *et al*., 2004

Another possibility to achieve defined cleavage reactions is the cleavage of the peptide from a C-terminal linker molecule that is inserted by coupling to the (modified) cellulose before coupling of the first amino acid. Several linker systems have been described: Wang-linker, allyl-linker, imidazole-linker, photolabile linker etc. (see *Table 2*).

A very simple linkage system is the Boc-Lys-Pro sequence that is directly coupled to the cellulose membrane. The first amino acid of the peptide sequence couples to the side-chain amino group of the lysine. After the final acidic side-chain deprotection, the peptide is released in aqueous buffers by forming diketopiperazines (Bray *et al*., 1991b).

The modification of the cellulose with a maleinimide-linker in order to form a covalent bond to the sulphur of the thiol group of a cysteine-containing peptide was described by Otte *et al*., 2003.

Several publications have reported the transfer of the peptides after deprotection and cleavage from the cellulose membrane to glass slides (Lizcano *et al*., 2002; Rychlewski *et al*., 2004). Due to the small volume transferred, hundreds of microarrays can be derived from a single cellulose peptide array synthesized by the SPOT method. This method thus provides the opportunity for multiple screening of the synthesized peptides.

SPOT synthesis of peptides

Peptide synthesis using the SPOT method follows a solid phase peptide synthesis protocol (see *Figure 2*). The side-chain protection strategy for the amino acids usually follows Fmoc-/tBu-chemistry (Fields and Noble, 1990). After membrane functionalization, the amino acids are coupled either as an active ester solution or as *in situ* activated mixtures (see Coupling methods). Usually a double coupling of the amino acid solution is performed. The coupling is monitored by checking the free amino groups through staining with a methanolic bromophenol blue solution (Krchnak *et al*., 1988). After coupling, the color of the stained peptide spot changes, depending on the coupled amino acid derivatives, from blue to green or yellow. After the amino acid coupling, the remaining free amino groups are blocked by acetylation (capping). Then the Fmoc group is removed by treatment with 20% piperidine/DMF to prepare the membrane for the next coupling step. The last step after the complete assembly of the peptide chain is cleavage of the side-chain protection groups by treatment with TFA (see Final side-chain deprotection methods). Further special treatment is necessary to yield free unbound peptides (see Linker strategies and cleavage from membrane support).

COUPLING METHODS

There are two principal activation routes for the coupling of amino acids, the first being *in situ* activation of the amino acids. These activations are mostly carried out with DIC and HOBt shortly before coupling (Frank and Overwin, 1996; Zander and Gausepohl, 2002; Ehrlich *et al*., 2004). In comparison to the other main activation route, reagent costs are lower and this method is applicable to all amino acids. But due to the formation of poorly soluble urea, difficulties can arise through needle blocking during automated delivery. Moreover, these reagent mixtures have to be freshly prepared every day. In addition to this method, the use of other *in situ* activation methods have been described, e.g. using activators like HATU, HBTU or TBTU with bases like DIPEA (Volkmer-Engert *et al*., 1997; Giuliani *et al*., 2005) or the use of activators like PyBOP as an additive to DIC (Pflegerl *et al*., 2002). The activation of amino acid with EEDQ was carried out by Toepert *et al*. (2003).

The other principal activation route is the use of pre-activated amino acids. The commonly used pre-activated amino acid derivatives are pentafluorophenyl esters (Stigler *et al*., 1995; Hilpert *et al*., 2005a; Kopecky *et al*., 2006b). Unfortunately, only selected pre-activated amino acids (i.e. all common L-amino acids) are commercially available. All solutions of the OPfp-derivatives, except the arginine derivative, are stable at –70°C for at least one month and at room temperature for at

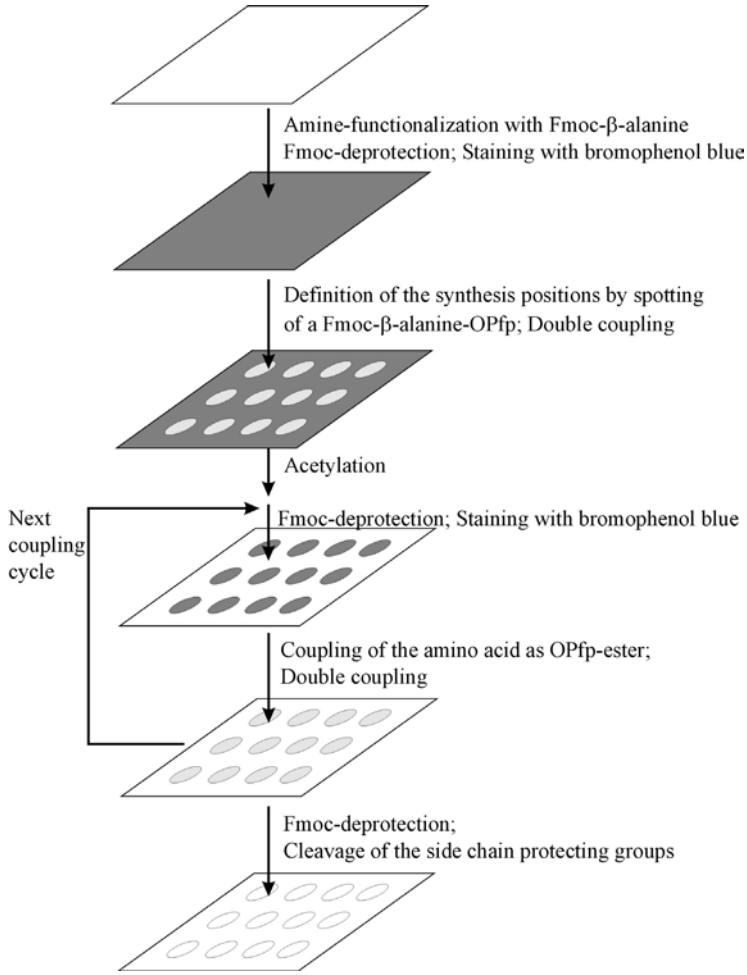


Figure 2. Schematic workflow of SPOT synthesis.

least one day (Frank, 1992). Therefore, the OPfp-amino acids (except arginine) can be prepared once for the complete syntheses. Other preactivated amino acids derivatives used are N-carboxy anhydrides (Frank, 1992), ODNp esters (Weiser *et al*., 2005) or ODhbt esters (Kramer and Schneider-Mergener, 1998b). The advantage of this method is that in contrast to the first method described, preparation of the coupling solution is easier, since only one reagent (the pre-activated amino acid) is necessary, and there are no problems caused by the formation of urea crystals in the activation solution. For the results of investigations into the efficiency of selected activation methods see Molina *et al*. (1996) and Gausepohl and Behn (1998).

In order to achieve equivalent coupling ratios of amino acids in mixtures during combinatorial synthesis of peptides, Volkmer-Engert and co-workers investigated the most useful concentration of the amino acids in a mixture (Volkmer-Engert *et al*.,

1994 and 1995). Following these results, 0.8 equivalents of the amino acid mixture were often used (Kramer *et al*., 1994).

The purity of peptides synthesized on cellulose was reported by Takahashi *et al*. (2000) to be higher than 92%. But several authors have described lower purities (e.g. Kramer *et al*., 1999a).

Molina *et al*. (1996) reported, depending on the coupling method, a coupling yield per cycle of 74.4% to 91.3%. Due to incomplete coupling and to avoid deletion sequences, the use of a capping step to block free uncoupled N-terminal amino groups is recommended. Capping was described using acetic anhydride at various concentrations (Otvos Jr. *et al*., 2000; Petersen, 2002; Gail *et al*., 2005) and alternatively by using 2% acetic anhydride with 2% DIPEA (Kramer and Schneider-Mergener, 1998b).

One interesting approach is the combination of spot synthesis with native chemical peptide ligation to synthesize large peptides (for native chemical ligation, see Kent, 1997). Toepert *et al*. (2003) described the synthesis of 38-mers using this method. A similar technique was published by Lu and Tam (2003) for coupling a dye-peptide thioester complex to a cysteine containing peptide.

A special application is the adaptation of the synthesis of TASPs (template assembled synthetic proteins; Mutter and Vuilleumier, 1989) to the SPOT technique on cellulose membranes (Haehnel, 2004). The single peptide chains were synthesized separately and then successively coupled to the membrane-bound cyclic peptide template (Rau *et al*., 2000; Schnepf *et al*., 2001 and 2004). Due to the fixation on the membrane and the interaction between the assembled peptide chains, a highly structured molecule could be assembled, which could be considered a small protein.

The latest development in SPOT synthesis is the use of elevated temperatures during coupling reactions on the cellulose support (Bowman *et al*., 2006b). In particular, heating of the membrane by microwave irradiation was carried out (Bowman *et al*., 2004 and 2006a; Blackwell, 2006). Similar to solid phase synthesis, the use of microwave irradiation during SPOT synthesis may lead to a decrease in reaction times, and yield increases are also possible (Grieco, 2004; Rybka and Frank, 2005).

PEPTIDE MODIFICATIONS

During and after assembly of the peptide chain, several modifications have been decribed. The most common modification of the synthesized peptides is the N-terminal acetylation (e.g. Torrens *et al*., 1999). N-terminal acetylation leads to improved proteolytic stability of the peptides and avoids the positive charge of the N-terminal amino group. Similar to capping, the use of acetylation solutions was described with only acetic anhydride at various concentrations (Frank and Overwin, 1996; Laune *et al*., 2002; Espanel and van Huijsduijnen, 2005) or using acetic anhydride with DIPEA (Kopecky *et al*., 2006b).

The synthesis of phosphorylated peptides was performed by using O-phosphorylated serine, threonine and tyrosine (Espanel *et al*., 2002; Wälchli *et al*., 2004; Frese *et al*., 2006). Other modified amino acids that can be incorporated into the peptide chain are biotinylated lysine (Kramer *et al*., 1999b) or lysine coupled with a fluorescent dye (Reineke *et al*., 1999c). The SPOT synthesis of glycopeptides was described using unprotected activated amino acids with a carbohydrate moiety coupled to the side-chain (Jobron and Hummel, 2000).

In case modified amino acids are not available, the required modifications can be obtained by modifying the amino acids, for example, by coupling a modifying building block either to the α-amino group of the N-terminal amino acid or to the side-chain amino group of diamino acids such as lysine or ornithine (Duan and Laursen, 1994; Volkmer-Engert *et al*., 1997, Licha *et al*., 2000). A special type of N-terminal modification was shown by Niggemann *et al*. (2002), where modification of positions 1 and 4 of an N-terminal coupled cis-4-amino proline by natural product building blocks was carried out.

An often described peptide modification after assembly of the peptide chain is cyclization of the peptides (Kramer *et al*., 1994; Winkler, 1997b; Winkler *et al*., 1998). Because of the relatively feasible reaction, this mostly involves cyclization of unprotected peptides via disulfide bridges (Winkler *et al*., 1996; Hilpert *et al*., 2000; Otte *et al*., 2006). Reineke *et al*. (1999e) reported the synthesis of disulfide-cyclized peptides with lengths of up to 32 amino acids. Cyclization is carried out by incubation of the membrane in buffer at a pH of about 7.5, or 10% to 20% DMSO in this buffer, or using charcoal at a 1:1 mass ratio of charcoal to membrane in a similar buffer. The synthesis of cyclic peptides with two distinct disulfide bonds is more sophisticated, due to the necessity for orthogonal cysteine protection (Welschof *et al*., 1999; Reineke *et al*., 1999d).

Similar difficulties occur during cyclization via an amide bridge, where orthogonal protection groups are also used (Winkler *et al*., 1995; Hahn *et al*., 2001). The amide bridge can be built via the C-terminal carboxy group and the N-terminal amino group, between two side-chains with a functional group or, by coupling between a side-chain functional group and a terminal functional group (Winkler, 1997a). A special type of cyclization was described by Scharn *et al*., 2001. The described peptides were cyclized via N-terminal coupled halogenated heterocycles and the side-chain amino group of a lysine under microwave irradiation.

The standard synthesis protocol for SPOT technology results in a free N-terminus and a C-terminus that is bound via a spacer to the cellulose support. Several proteins can only recognize peptides when a free C-terminus is present (Boisguerin *et al*., 2004). To achieve a free C-terminus, one possibility is to couple the first amino acid (Glu or Asp) to the cellulose via its side-chain. The result of this strategy is to gain the corresponding amides (Gln and Asn) and the free a-carboxy group of the C-terminus (Wildemann *et al*., 2006). Applying this procedure, the C-terminal amino acid is still bound to the membrane via its side-chain group.

In order to achieve a completely free C-terminus the coupled peptide has to change its orientation without losing the bond to the cellulose. This is possible according the principle described for solid phase synthesis on resin by Kania *et al*., 1994. The first step is cyclization of the peptide via its N-terminus to an orthogonal protected linker molecule, followed by cleavage of the C-terminal bond to the solid support. Recently, this principle was successfully adapted to SPOT synthesis and described in two methods (Hoffmüller *et al*., 1999a; Boisguerin *et al*., 2004).

FINAL SIDE CHAIN DEPROTECTION METHOD

Due to the Fmoc/tBu-protection strategy, cleavage of the side-chain protecting groups is carried out using TFA. Using the common cellulose filter paper treatment with

TFA is limited because of the lability of this cellulose material at high acid concentrations (Zander, 2004). Different cleavage procedures have been published. The first published and also widely used cleavage mixture contains 50% TFA and additional scavengers (e.g. water, trialkylsilane, phenol) in DCM (Frank *et al*., 1992; Tegge *et al*., 1995b; Giuliani *et al*., 2005). But some protecting groups need to be cleaved at higher TFA concentrations. In particular, the Pbf protecting group of the arginine has to be cleaved at TFA concentrations higher than 50%. To overcome this problem, several cleavage methods were described (see *Table 3*). According to our own experience, the use of 90% TFA for 30 minutes and an additional treatment with 50-75% TFA for another 3 hours is the most favourable deprotection method for the side-chain deprotection of the peptides synthesized on cellulose filter paper.

Table 3. Overview of published side-chain deprotecting methods.

Cellulose type	TFA treatment	Scavangers	References
Whatman 50	90% TFA, 3h	5% water + 3% phenol + 2% TIPS	Hilpert *et al*., 2000 and 2001
	90% TFA /DCM, 0.5 h + 50% TFA/ DCM, 2 h	3% TIBS + 2% water + 1% phenol	Hilpert *et al*., 2005a
	90% TFA /DCM, 0.5 h + 50% TFA/ DCM, 3 h	3% TIBS + 2% water + 1% phenol	Licha *et al*., 2000
	50% TFA /DCM	3% TIBS + 2% water	Tegge *et al*., 1995b; Münch *et al*., 1999
Whatman 540	90% TFA /DCM, 0.5 h + 50% TFA/ DCM, 2.5 h	3% TIBS + 2% water + 1% phenol	Kopecky *et al*., 2006b
Whatman*	80% TFA, 4 h	12% thioanisole + 6% ethanedithiol + 2% m-cresol	Kato *et al*., 2006
AIMS / Abimed / Intavis	50% TFA /DCM	3% TIBS + 2% water	Giuliani *et al*., 2005
	46 % TFA	13.5% m-cresol + 13.5% thioanisole + 13.5% water + 13.5% ethanedithiol	Otvos Jr *et al*., 2000

* No further specicication available.

When using commercially available acid-stable cellulose membranes, it is possible to carry out the cleavage of the side-chain protecting group with the same cleavage mixture as used for the common Fmoc solid-phase peptide synthesis, namely a treatment with approximately 90% TFA for 3.5 hours.

SPOT synthesis of non-peptidic compounds

To complete the overview of the SPOT method we include here a short introduction to the synthesis of non-peptidic compounds using this technique. The synthesis of non-peptidic compounds or peptides with non-peptidic elements has been carried out on cellulose as well as polypropylene membranes. Using the SPOT technique, one of the most frequently synthesized non-peptidic compounds is a peptoid (Zuckermann *et al*., 1992). These compounds are synthesized pure or as hybrids in peptides, so-called peptomers (Ast *et al*. 1999). Zimmermann *et al*. (2003) investigated the possibility of replacing natural amino acids by peptoidic elements. Screening of an array of 8000 hexapeptoids and peptomers was carried out by Heine *et al*. (2003). Hoffmann *et al*. (2006) described the transformation of a biologically active peptide into peptoid analogs while retaining biological activity.

Another application of the SPOT method is the synthesis of oligomers of peptide nucleic acids (Gausepohl *et al*., 1998). Weiler *et al*. (1997) described the synthesis of a PNA oligomer library, with coupling yields of >97%.

The synthesis of small organic compounds is a broad field for the application of SPOT synthesis (Blackwell, 2006). *Table 4* shows a number of types of small organic molecules synthesized by the SPOT technique. These syntheses were often carried out using elevated temperatures, in particular by microwave irradiation.

Table 4. Small organic molecules synthesized using the SPOT method.

Organic compounds	References
sec. Amides	Lin *et al*., 2005
chalcones	Bowman *et al*., 2004
cyanopyridines	Bowman *et al*., 2006a
deazalumazines	Bowman *et al*., 2006a
dihydropyrimidines	Bowman *et al*., 2004
hydantoins	Heine *et al*., 2001
2-oxopiperazine	Zander *et al*., 2002
triarylpyridines	Bowman *et al*., 2006b
trisamino-/ amino-oxy-triazines	Scharn *et al*., 2000

Regeneration of the membranes

After probing the membranes with proteins it is possible to re-use them after a regeneration process (stripping). The regeneration mixture consists of ingredients that break the structure of the bound protein (e.g. mercaptoethanol, thiourea or urea) and a detergent such as SDS to remove the protein from the cellulose (Härkönen *et al*., 2002; Kovacs-Nolan *et al*., 2003; Schmidt *et al*., 2004). Often a second regeneration step by treatment with diluted acid follows. Zander described the stripping of commercially available, chemically modified, stabile cellulose membranes by treatment with highly concentrated TFA (Zander, 2004).

Common cellulose filter papers with ester-bound peptides are unstable in strong chemical conditions, but after probing they can be regenerated up to 20 times using mild regeneration conditions (Martens *et al*., 1995).

Array strategies

The principal array strategies provided by peptide SPOT synthesis are: peptide scan, substitution analyses, length analyses, random libraries and combinatorial libraries. All these strategies were well known before the SPOT technology was developed (e.g. Geysen *et al*., 1984; Geysen *et al*., 1985) and were subsequently adapted for peptide synthesis on cellulose supports.

A peptide scan is used to detect a region of interest within a selected protein with a known sequence. The linear protein sequence is fragmented into different peptide sequences. To increase the chances of hitting the peptide with the desired function, the fragmented peptides may overlap (see *Figure 3*). As a result, the whole protein sequence may be narrowed down to one or a few peptide sequences. These peptides can then be used for further techniques, such as substitution analysis or length analysis, to gain a deeper understanding of the interaction. An additional coupling of amino acid mixtures to the N- and C-termini of the peptide sequence can lead to an enhancement of the binding strength and so to an improved detection of weak interactions (hybritope scan, Reineke *et al*., 1998a).

The terms "substitution analysis", "substitutional analysis", "replacement analysis" or "mutational analysis" have been used in the literature as synonyms; in this review "substitution analysis" is used throughout. Each amino acid of the original, or so-called wild type peptide sequence (wt) is substituted by all other 19 gene-encoded amino acids, or any other selected non-gene encoded amino acid. *Figure 4* shows the principle of this approach. This technique is a powerful tool for investigating the importance of each amino acid for the interaction of interest. This information can help identify key amino acids or important patterns for the interaction. That knowledge may lead to improvements of distinct properties (e.g. affinity, hydrophilicity, biological activity). By applying this strategy in a stepwise manner, peptides can be optimized for the desired activity (e.g. Hoffmüller *et al*., 2000; Hilpert *et al*., 2000). A simplified substitution analysis is replacement of the amino acids of the wild type sequence by only one distinct amino acid, e.g. alanine ("alanine scan", "alanine walk" "alanine substitution peptide array"; Gao and Esnouf, 1996; Hilpert *et al*., 2005b; Bolger *et al*., 2006), glycine ("glycine walk" or "glycine scan"; Kneissel *et al*., 1999; Blüthner *et al*., 2002; Liang *et al*., 2003) or tyrosine (Wälchi *et al*., 2004). Modifications of such simplified substitution analyses are AAA-Scan (Podolnikova *et al*., 2005), "Ala reverse scanning" (Espanel *et al*., 2002) and progressive Ala-Scan (Espaniel and Sudol, 2001).

A length analysis can be useful to identify the shortest possible peptide sequence for the interaction of interest. An example is given in *Figure 5*. Most commonly, a length analysis will be carried out to determine the minimal epitope sequence of an antibody (e.g. Gao and Esnouf, 1996; Blüthner *et al*., 2000). Using the minimal length for further investigations may save money and time, as well as open up a new spectrum of methods.

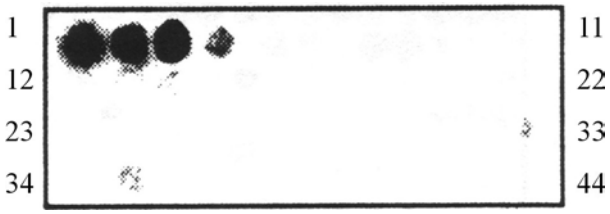
A

```
THATISAPEPTIDESCAN
THATIS
   TISAPE
      APEPTI
         PTIDES
            DESCAN
```

B

```
THATIS-XX-TISAPE
THATIS-XX-APEPTI
THATIS-XX-PTIDES
THATIS-XX-DESCAN
TISAPE-XX-THATIS
TISAPE-XX-APEPTI
TISAPE-XX-PTIDES
TISAPE-XX-DESCAN
..
PSCCAN-XX-DESCAN
```

C




```
1-VVSHFNDCPDSHTQFCFHGTCRFLVQEDKPACVCHSGYVGARCEHADLLA-50

1  VVSHFND      13 TQFCFHG      25 VQEDK
2  VSHFNDC      14 QFCFHGT      26 QEDKP
3  SHFNDCP      15 FCFHGTC      27 EDKPA
4  HFNDCPD      16 CFHGTCR      28 DKPAC
5  FNDCPDS      17 FHGTCRF      29 KPACV
6  NDCPDSH      18 HGTCRFL      30 PACVC
7  DCPDSHT      19 GTCRFLV      31 ACVCH
8  CPDSHTQ      20 TCRFLVQ      32 CVCHS
9  PDSHTQF      21 CRFLVQE      33 VCHSG
10 DSHTQFC      22 RFLVQED      34 CHSGY
11 SHTQFCF      23 FLVQEDK      35 HSGYV
12 HTQFCFH      24 LVQEDKP      36 SGYVG
```

Figure 3. A Example of a peptide scan of a 14-mer peptide sequence, using 6-mer peptide fragments with an overlap of three amino acids. **B** The resulting sequences for a duotope scan of this 14-mer peptide using 6-mer peptide fragments with an overlap of three amino acids. **C** On top is an example of a cellulose membrane probed with a monoclonal antibody. The peptide sequences stem from a peptide scan (7-mer, 6 positions overlapping) from the miniprotein sequence given in the middle. At the bottom all peptides from the peptide scan are presented, the peptides showing binding signals towards the antibody are labeled with bold type.

Libraries are powerful tools for the screening large numbers of peptides. A random peptide library contains a single peptide sequence on each spot, generated by a random algorithm (Reineke *et al*., 2002a; Lenze *et al*., 2006). An example is given in *Figure 6*. The generated sequences can also be semi-random. In the case of semi-random libraries, selected amino acids or properties (e.g. charge or hydrophobicity) can be fixed or biased within the random set. Since the introduction of automation for SPOT synthesis it is now easily possible to screen tens of thousands of random peptides. For example, this technique can be used to screen for new enzyme substrates or detect peptides with a desired function that are different from known sequences (drug design).

A

wt	A	C	D	E	F	…	…	W	Y
PDF	ADF	CDF	DDF	EDF	EDF			WDF	YDF
PDF	PAF	PCF	PDF	PEF	PEF			PWF	PYF
PDF	PDA	PDC	PDD	PDE	PDF			PDW	PDY

B

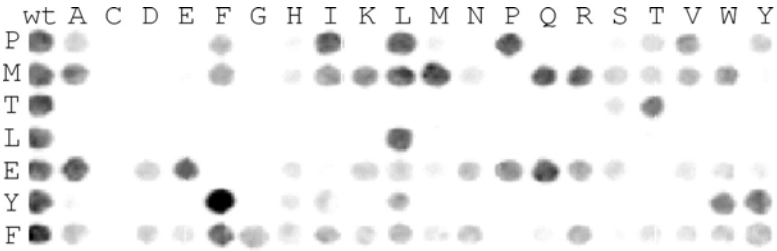


Figure 4. A Principle of a substitution analysis of the 3-mer peptide PDF. The first column represents the parent or wild type (wt) peptide, which is used to compare the effects of the substitution variants. All other columns describe the substituted amino acids at each position of the peptide. All underlined positions show the actual substituted amino acid in the sequence. The wild type sequence is labeled in bold. **B** Substitution analysis of the 7-mer peptide PMTLEYF using all 20 proteinogenic amino acids. The binding of an HRP-labeled protease was detected using a chemiluminescent substrate. For better visualization the image is in an inverted form: dark spots represent strong binding, whereas bright ones weak or no binding. All peptide sequences are given in the one letter code.

Combinatorial peptide libraries may contain all possible combinations of peptide sequences for a peptide of a certain length (see *Table 5*). The combinatorial library is highly favorable for the systematic and fast screening of millions of peptides, and therefore an ideal tool for high throughput screening of peptides. This technique, described by Houghten and co-workers (1991) for solid phase peptide synthesis on resin (Eichler and Houghten, 1995), was very quickly adapted to SPOT synthesis (Kramer *et al*., 1993; Frank, 1995). In order to screen huge numbers of peptides in a combinatorial library with a limited number of spots, it is necessary to use amino acid mixtures (e.g. Kramer *et al*., 1995). To achieve distinct single peptide sequences from the combinatorial library, the amino acid mixture at all positions within the peptide have to be substituted by the best fitting amino acid at these positions. The complete determination of these amino acids is carried out by iteration using the combinatorial approach until all mixtures are substituted ("deconvolution"; see *Figure 7*). Another possibility to define the peptide sequence is to combine the results of several positional scanning combinatorial libraries (e.g. Frank *et al*., 1995 and 2002).

A

Peptide Length	Sequence
7	PEPTIDE
6	PEPTID
	EPTIDE
5	PEPTI
	EPTID
	PTIDE
4	PEPT
	EPTI
	PTID
	TIDE
3	PEP
	EPT
	PTI
	TID
	IDE
2	PE
	EP
	PT
	TI
	ID
	DE

B

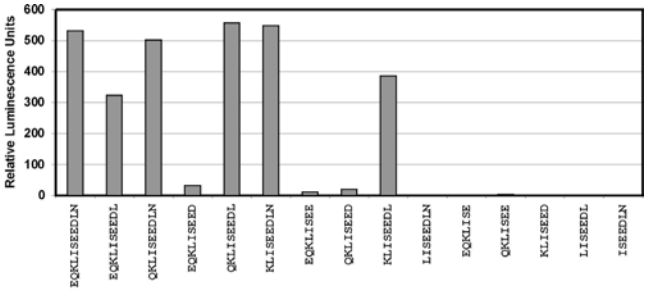


Figure 5. A. Example of the principle of a length analysis using a 7-mer peptide. Each sequence shortening step results in an additional possible peptide sequence. The peptide length is given in amino acids and the sequence uses the one letter code. **B** A length analysis of an antibody epitope comprising 15 peptides synthesized on cellulose where the binding was measured by chemiluminescence. The resulting light signal of the bound antibody was measured and converted into a bar graph.

Applications

The general workflow for the application of most screening techniques for SPOT membranes is as follows: the desired peptides are synthesized on cellulose. This cellulose membrane is washed, equilibrated, blocked and incubated together with the chosen protein. The protein bound to the peptide can be detected by a labeled antibody, for example with a horseradish peroxidase (HRP) label and a corresponding chemiluminescent substrate (*Figure 8*). Alternatively, the protein itself can be labeled. To exclude false positive signals, selected peptides should be re-synthesized as free peptides and their activity determined and compared to the membrane bound peptides. An overview of different applications is given in *Table 6*.

PEPTIDE-ANTIBODY INTERACTION (EPITOPE MAPPING)

The “classical” application for SPOT synthesis is mapping the epitope of an antibody of interest. SPOT synthesis was actually developed to address this question (Frank, 1992) and has achieved substantial success. Today, this method can be considered as a standard method for mapping the linear epitopes of antibodies, and the largest portion of publications concerning SPOT synthesis relate to this application (for alternative methods see Tribbick, 2002). Epitope mapping can be used for monoclonal as well as polyclonal antibodies (e.g. Kopecky *et al*., 2005; Mahler *et al*., 2000; Reineke *et*

A

1.	DMGNFDHPLDQFRVC	26.	RGMSIEKCENVAARI	51.	GQVFHNWAEVLMGYG	76.	WQSAVPVMTAHPRKC
2.	IVCYRYYNRYCTPCW	27.	WLLEWNMSWAATGWC	52.	QEEPYQGVFRPLMYF	77.	LEWSVKYVMSPDSNI
3.	LIEWVFMTDNFNDMM	28.	LGMYGRVPCMVSCRG	53.	RDQCTHTGWCSGHEN	78.	PTGFHSGICGKEQTM
4.	QSNQQWNNLEWFAEN	29.	PVMRFRMRIIPMSQM	54.	PCHYWHWYVEFQMLT	79.	WKGISDESHTHFMSY
5.	ELRQYEYADWSGCNN	30.	KRDFQEGSCHANQRF	55.	MWQKMYDGRYSFIGS	80.	LQKVVGDCAHAYDMM
6.	KRVYTYFPMTSVPGS	31.	SGCHDDEEQKIHPQK	56.	WIFERWAWKNHNTAT	81.	TRPWKSWQVNDKSFI
7.	NLLHHCFMKNTVFEH	32.	RSFIYYFIHGSHPIK	57.	PKCSITIYGFWLYYG	82.	TAQQHFQEKGEMMKR
8.	HSDCPQLEFEWCLHY	33.	MAQFTVKYFASNIAH	58.	YQDRTNMIMKCNRGC	83.	DQTIPRALIWCGVQD
9.	FSTVWSDAFIHPCEM	34.	GENTTVFTMCYAIIG	59.	SFHAKAKQGCEMHKT	84.	TIGYIVYMHSWFFCP
10.	KLPSKSWKNGNENNI	35.	IVLYVNRWQRGEFGS	60.	TCKSEDRIWIKSYVQ	85.	CEDGSYLCLWLMCTR
11.	RNEIDHEWRMPGYCF	36.	VQVYALQPRYGRCPV	61.	GICADIYCCAKTCVF	86.	FMKQMFEFEVYFVHQ
12.	ICQTCVEVNQVVRHK	37.	LPDCTMPARQTLSCT	62.	NGSWGSLGWLQLDTE	87.	KLGREFHPYLVVLTL
13.	KWVQKVWDIEVRFLH	38.	TLMDRDPHSRFWGNT	63.	IDFHMQWVCPQVNDH	88.	QIYLESCWTFWACNQ
14.	LWVKVLANQDYTPHP	39.	VLQIGINFCTKINAY	64.	KDENCLTYFCHKKRK	89.	TVGRPKVGRWMSREY
15.	VSSCMQFYDAYLPKA	40.	DEDFGPSMTERDWES	65.	YWYVGGNYKSANTNH	90.	GWDMMFANHEGAQSM
16.	IIKDHACRDIIEITS	41.	DNAFYFKAMFPEQIS	66.	CFIDIFMGHFTPGVE	91.	DQECYFWHFWRESAP
17.	DEVEVADHYHPALNM	42.	RSVHVVAGVTGIGCM	67.	QMASNICTADVEKRE	92.	KHNPVLPHVCCENYA
18.	VPSWWFVGATNYDYW	43.	HPAFPVYNFGCVHFC	68.	ERWGESIDWSDHRKF	93.	DFVIMQLKLHEPYFA
19.	DEYVLKFTTAQWEQP	44.	KPFFPPMSRILSRTV	69.	MQSFSRMIRVAMAIS	94.	TVKVRCDEEDGLGRF
20.	FCCALENVWMINYMA	45.	ACSQVFDRMQDTFAY	70.	NTCNGHHTFLASDCP	95.	FCGHRAWIWRTPCCH
21.	ELLYWYQHTKCLWNI	46.	HDCGQVLERVVRIIT	71.	YCLNELNYIMHDARE	96.	MRWRCSKACCDNFFI
22.	VQCENTKSIKLKDED	47.	HQIHMFEDWEEYDWL	72.	HHLWKKYSDNVELNG	97.	QNHHGGRNCIPIMPF
23.	TDMRITFQGCKPGHH	48.	CNRGSMAVQQRHIVY	73.	FPFFWGHECWTRKKY	98.	FMDACLLSFWDDEWR
24.	KPENKTFKRQMMSEI	49.	HWHHSLRSMRAHVKG	74.	FPILCRLHRNMEEHT	99.	GLEKYPGQSNWSRLF
25.	TSWLFIRHNIQVTFS	50.	GEGEFDGWTSKTLDV	75.	HYKLMTIMKPGFARV	100.	CDRDWASFKTGNREP

B

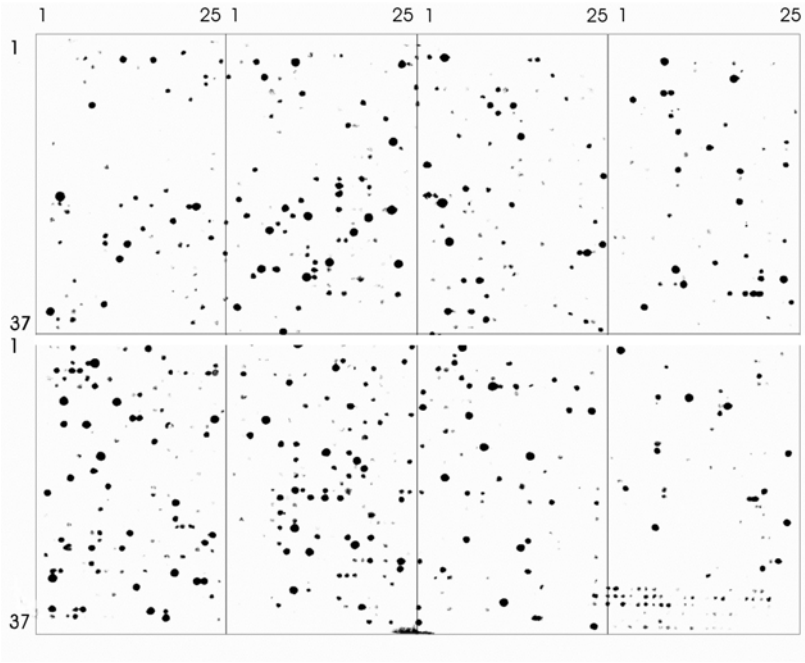


Figure 6. A Peptide sequences of a 15-mer random peptide library. **B** Example of a random library, where the chemiluminescence of the bound labeled protein was detected. For better visualization the image is in an inverted form, dark spots representing strong binding, whereas bright spots weak or no binding. Each field consists of 925 random peptides, in total 3,700 peptides were tested for binding.

Table 5. Example for the drastic increase in the number of possible peptide sequences by extention of the peptide length using a combinatorial approach with all 20 gene-encoded amino acids (B1 and B2 are positions with defined amino acids; X are position with mixtures of all 20 amino acids)

Length	Sequence	# Possibilities	# Peptides
4-mer	XB_1B_2X	20^4	1.6×10^5
6-mer	XXB_1B_2XX	20^6	6.4×10^7
8-mer	$XXXB_1B_2XXX$	20^8	2.5×10^{10}
12-mer	$XXXXXB_1B_2XXXXX$	20^{12}	4.1×10^{15}

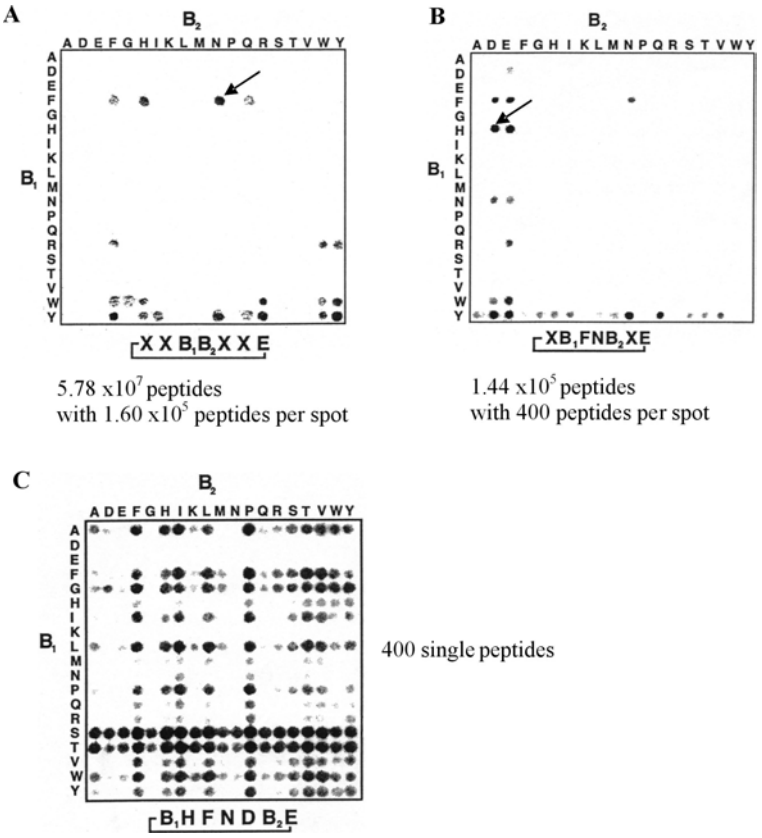


Figure 7. Example of the deconvolution of a combinatorial cyclic peptide library from an unknown hexamer to a defined peptide sequences. **A.** First round of the combinatorial library with an unknown hexapeptide sequence. The C-terminal E was inserted in order to cyclize via the side chain group of the C-terminal E and the N-terminal amino group. The amino acids at position 3 (B_1) and 4 (B_2) were arrayed as a chessboard to simplify determination of binding sequence motifs (due to the possible strong side reaction of cysteine with the substrate, this amino acid was excluded). **B.** After selection of a sequence motif for amino acids 3 and 4 (in this case F and N) the next synthesis of a combinatorial library was designed with amino acids 2 (B_1) and 5 (B_2) as a checkerboard array, flanking the motif FN. **C.** After selection of the binding motif (in this case HFND) the synthesis was repeated by deconvolution of the last unknown sequence part. Each spot now represents one distinct single sequence and can be easy defined by the checkerboard array design.

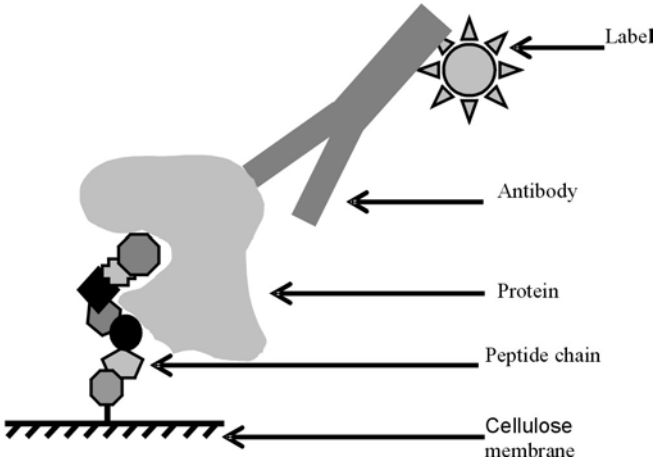


Figure 8. Schematic representation of a binding assay on a cellulose support. The peptide chain is bound to the cellulose membrane and a protein binds to the peptide. A labeled antibody recognizing this protein is used to detect the binding event, either by radioactivity, chemiluminescence or a substrate staining reaction.

al., 1996; Schultz *et al*., 1999). For a detailed protocol on epitope mapping see Koch J., *et al*. (2002). After detecting an epitope by applying the peptide scan technology, a length analysis and/or a substitution analysis can be performed to gain a deeper insight into the epitope-antibody interaction. In addition, combinatorial libraries can be used to study cross-reactivity and/or polyspecificity. In one example, 68,590 peptide mixtures, prepared by the SPOT technology, were used to identify peptides that bound to a monoclonal antibody (Kramer *et al*., 1997). In addition to peptides with sequences related to the epitope (cross-reactivity), unrelated peptide sequences were also found (polyspecificity). Screening the protein library SWISSPROT for molecules containing these sequences led to the discovery of other proteins recognized by this antibody. The molecular basis for such interactions was studied by substitution analysis and X-ray crystallography, providing a foundation for understanding cross-reactivity and polyspecificity (Kramer *et al*., 1997; Keitel *et al*., 1997). A similar approach to studying cross-reactivity, including thermodynamic analyses, was performed on another monoclonal antibody (Winkler *et al*., 1998; Hahn *et al*., 2001). For a deeper understanding of the antibody-epitope interaction, point mutations can be made in the paratope to study their effects on epitope binding (Liu *et al*., 1999; Winkler *et al*., 2000).

In molecular biology some antibody epitopes are used as protein tags (*myc*-tag, FLAG-tag) for protein purification, protein localization, immunochemistry and ELISA (Mahler *et al*., 2002). Substitution analysis and length analysis was used to characterize the epitope of a monoclonal antibody that recognizes the *myc*-tag. The results showed that it is possible to adopt and optimize the commonly used *myc*-tag for specific needs required for the research project's objectives (Hilpert *et al*., 2001). Another example for tag optimization is describes by Böldicke and coworkers (Böldicke *et al*., 2000).

Table 6. Examples of applications performed with SPOT technology[#].

Keywords	Techniques*	Reference
DNA binding site mapping		
Type II restriction endonuclease, *Eco*RII, specificity, affinity, consensus sequences	Peptide scan (b)	Reuter *et al*., 1999
Epitope mapping		
Amino acid property matrix, murine monoclonal IgG antibodies, linear epitopes	Substitution analysis (b)	Dong *et al*., 1999
Antigen binding activity, variable regions of immunoglobulins, anti-thyroglobulin antibody, HyHEL-5 anti-lysozyme antibody, anti-angiotension II antibody	Peptide scan (b) Alanine scan (b)	Laune *et al*., 1997
Anti-immunoglobulin antibodies, discontinuous epitope, IgG hinge region, auto immune disease, organ transplantation	Substitution analysis (b)	Welschof *et al*., 1999
Autoantigen, fine-specificity, anti-centromere antibodies, anti-CENP-A B cell autoimmune response	Peptide scan (b)	Mahler *et al*., 2000
Autoantigen, linear B cell epitopes, U1 snRNP-C autoantigen, U1 small nuclear ribonucleoprotein complex, systemic lupus erythematosus	Peptide scan (b, s)	Halimi *et al*., 1996
Autoimmune disease, early endosome antigen 1, EEA1, polyclonal antibody, neurological auto immune disease	Peptide scan (b)	Esposito *et al*., 1999
Autoimmune disease, human anti-thyroid peroxidase, epitope specifities, single-chain fragment variable of Ig, kinetic analysis, human sera,	Peptide scan (b) Phage display (b)	Chapal *et al*., 2000
Autoimmune disease, SmD1 protein, polyclonal antibody, systemic lupus erythematosus, autoantibody	Peptide scan (b)	Riemekasten *et al*., 1998
B-cell epitope, T- cell epitope, *Streptococcus pyogenes*, SfbI protein, fibronectin-binding domain	Peptide scan (b)	Schulze *et al*., 2003
Contraceptive, feral cat, feline pellucida protein, vaccine, antigenic epitopes	Peptide scan (b)	Ringleb *et al*.; 2004
Enterovirus, tyrosine phophatase, cross-reactivity, diabetes	Peptide scan (b)	Härkönen *et al*., 2002
Human autoantibodies, discontinuous immunodominant region,human thyroperoxidase, directed mutagenesis	Peptide scan (b) Alanine scan (b)	Bresson *et al*., 2004
Monoclonal antibody, actin, actin binding protein, gelsolin, N-terminal extension, alternative splicing	Peptide scan (b)	Fock *et al*., 2005

Table 6. Contd.

Keywords	Techniques*	Reference
Monoclonal antibody, affinity, grafting, phage-displayed peptide library, human cardiac troponin I	Alanine scan (b)	Ferrieres *et al*., 2000b
Monoclonal antibody, antigen specificity, hepatitis B, flexible docking, Fab, scFV, neutralizing anti-pre-S1 and anti-pre-S2	Peptide scan (b) Substitution analysis (b) Length analysis (b)	Küttner *et al*., 1999
Monoclonal antibody, amino acid cluster, TGFα, phage display	Combinatorial library (b)	Kramer *et al*., 1995
Monoclonal antibody, antibody paratope, cognate anti-idiotypic antibody, anti-human thyroglobulin, Tg 10	Peptide scan (b) Alanine scan (b)	Laune *et al*., 2000
Monoclonal antibody, autoantibodies, alternatively spliced, human thyroglobulin (hTg), thyroid hormones T3 and T4	Length analysis (b) Peptide scan (b) Alanine scan (b)	Molina *et al*., 1997
Monoclonal antibody, blood pressure, *E. coli*, single-chain variable fragment (scFv), angiotensin II, SPR analysis	Peptide scan (b)	Cohen *et al*., 2001
Monoclonal antibody, coat protein, beet necrotic yellow vein virus	Peptide scan (b) Alanine scan (b)	Commandeur *et al*., 1994
Monoclonal antibody, continues epitope, discontinuous epitope, herpes simplex virus type 2 (HSV-2), glycoprotein G-2	Peptide scan (b)	Liljeqvist *et al*., 2002
Monoclonal antibody, cross-reactive determinants, Chagas disease, *Tryponosoma cruzi*, *trans*-sialidase	Peptide scan (b)	Pitcovsky *et al*., 2001
Monoclonal antibody, cytoskeleton, colocalization, profilin, cortical microfilament webs, microfilament suprastructures	Peptide scan (b)	Mayboroda *et al*., 1997
Monoclonal antibody, discontinuous epitope, blood coagulation, ß-factor XIIa, polymer membrane	Peptide scan (b) Alanine scan (b) Length analyses (b)	Gao and Esnouf, 1996
Monoclonal antibody, discontinuous epitope, human complement receptor type 2C3dg, CD21	Peptide scan (b)	Prodinger *et al*., 1998

Table 6. Contd.

Keywords	Techniques*	Reference
Monoclonal antibody, DNA vaccine, Alzheimer's disease, mouse model	Peptide scan (b)	Schultz *et al*., 2004
Monoclonal antibody, *E. coli*, NhaA Na^+/H^+ antiporter, pH dependents, conformational change	Peptide scan (b)	Venturi *et al*., 2000
Monoclonal antibody, *E. coli*, p24 HIV-1, binding specificity, point mutations, scFv mutants	Substitution analysis (b)	Winkler *et al*., 2000
Monoclonal antibody, *E. coli*, site-directed mutagenesis, bacterial and archeal domains, N-terminus, *Thermus thermophilus, Mycoplasma hominis*, elongation factor Tu, EF-Tu	Peptide scan (b) Length analysis (b) Substitution analysis (b)	Baensch *et al*., 1998
Monoclonal antibody, glucan phosphorylase, organ specific expression (*Pho1*), *Solanum tuberosum*, starch metabolism	Peptide scan (b) Substitution analysis (b)	Albrecht *et al*., 2001
Monoclonal antibody, human insulin, antigenic regions, type 1 diabtes, autoimmune disease	Peptide scan(b)	Allauzen *et al*., 1995
Monoclonal antibody, human immunodeficiency virus (HIV) 1, computer model gp41/HIV, Fab fragment	Subsitution analysis (b)	Stigler *et al*., 1995
Monoclonal antibody, HIV-1, p24, peptide mixtures, solid phase-bound library	Combinatorial library (b) Substitution analysis (b)	Volkmer-Engert *et al*., 1995
Monoclonal antibody, human insulin, evolution, soluble insulin, adsorbed human insulin, antigenic regions	Peptide scan (b)	Allauzen *et al*., 1995
Monoclonal, antibody, IL-10, TNF-α, TNF-receptor, discontinuous epitope	Peptide scan (b)	Reineke *et al*., 1996
Monoclonal antibody, linear epitope, antibody 9E19, myc-tag, key positions, modification, application, protein tag	Substitution analysis (b) Length analysis (b)	Hilpert *et al*., 2001
Monoclonal antibody, *Listeria monocytogenes*, CD4 T cells, p60 protein	Peptide scan (b)	Geginat *et al*., 1998
Monoclonal antibody, M2 acetylcholin receptor, agonist like antibody, surface plasmon resonance, cardiomyocytes	Peptide scan (b)	Elies *et al*., 1997
Monoclonal antibody, microtubules, post-translational modification, tubulin, tyrosination, tubuline-tyrosine ligase (TTL)	Peptide scan (b)	Erck *et al*., 2000

Table 6. Contd.

Keywords	Techniques*	Reference
Monoclonal antibody, mitochondria, membrane bound complex I, respiratory chain, 49 kDa subunit	Peptide scan (b)	Zickermann *et al.*, 2003
Monoclonal antibody, morbillivirus, P-protein, ELISA	Peptide scan (b)	Martens *et al.*, 1995
Monoclonal antibody, *Neisseria meningitidis*, vaccine candidates, mimetic antigen, GNA33, PorA, molecular mimetic	Peptide scan (b) Length analysis	Granoff *et al.*, 2001
Monoclonal antibody, neutralizing antibody, human granulocyte macrophage-colony stimulation factor (hGM-CSF)	Combinatorial library (b) Peptide scan (b)	Eberhardt *et al.*, 2003
Monoclonal antibody, neutralizing antibody, proliferation, maturation, cytokine, granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Peptide scan (b) Length analysis (b) Combinatorial library (b)	Oggero *et al.*, 2004
Monoclonal antibody, phage display, gene-3 protein (g3p), filamentous phage M13, multifunctional g3p-peptide tag	Peptide scan (b)	Beckmann *et al.*, 1998
Monoclonal antibody, polyclonal antibody, glycoprotein G-2 (gG-2), herpes simplex virus type 2 (HSV-2), secreted amino-terminal portion (sgG-2), type-dicriminating serodiagnosis	Peptide scan (b)	Liljeqvist *et al.*, 2002
Monoclonal antibody, polyclonal antibodies, disease prognosis, autoantibodies, intervention therapies, Type I diabetes mellitus, IA-2 and IA-2ß	Peptide scan (b)	Piquer *et al.*, 2006
Monoclonal antibody, polyclonal antibodies, quantitative determination, epitope recognition pattern, epitope specificity, advanced glycation end products (AGEs), immunohistochemical localization	Combinatorial library (b)	Dukic-Stefanovic *et al.*, 2002
Monoclonal antibody, recombinant antibody, single chain, hypervariable domain, antigen, drosophila, RNA polymerase	Substitution analysis (b)	Liu *et al.*, 1999
Monoclonal antibody, reliability, binding affinity, dissociation constant, effective concentration, mass action law, competition	Peptide scan (b)	Weiser *et al.*, 2005

Table 6. Contd.

Keywords	Techniques*	Reference
Monoclonal antibody, reliability, binding affinity, dissociation constant, epitope length, peptide density	Substitution analysis (b)	Kramer *et al.*, 1999a
Monoclonal antibody, *Salmonella typhimurium*, maltose ATP-binding cassette transporter (ABC transporter), membrane-associated complex ($MalFGK_2$), periplasmic receptor (MalE)	Peptide scan (b) Substitution analysis (b)	Stein *et al.*, 2002
Monoclonal antibody, vaccine design, tumor inhibitory and stimulatory anti-ErbB-2, self-oncoprotein ErbB-2	Peptide scan (b)	Yip *et al.*, 2001
Monoclonal antiboby, vascular endothelial cadherin (VE-cadherin)	Peptide scan (b)	Corada *et al.*, 2001
Monoclonal anti-peptide antibody (2E11), peptide-affinity tag, affinity purification of recombinant proteins	Peptide scan (b)	Böldicke *et al.*, 2000
Multiple sclerosis, viral epitopes, Epstein-Barr Virus, Herpes simplex virus 1, transaldolase autoimmune disease, monoclonal and polyclonal antibody	Peptide scan (b)	Esposito *et al.*, 1999
Myasthenia gravis, NF-M, autoimmune disease, monoclonal antibody	Peptide scan (b)	Schultz *et al.*, 1999
Neonates, group B streptococci, immunoglobulin A	Peptide scan (b) Length analysis (b)	Jerlström *et al.*, 1996
Novel therapeutic approaches, liver transplants, leukocytes, neutralizing antibody, P-selectin glycoprotein ligand-1 (PSGL-1)	Peptide scan (b)	Tsuchihashi *et al.*, 2005
Polyclonal antibodies, anti-cancer drugs, inhibitors, glyoxalase I, *Brassica juncea*	Peptide scan (b)	Deswal *et al.*, 2005
Polyclonal antibodies, antigenic sites, coxsackievirus A9, heat treatment	Peptide scan (b)	Pulli *et al.*, 1998
Polyclonal antibodies, autoantibodies, ribosomal P proteins, systemic lupus erythematosus (SLE), *Trypanosoma cruzi*, *Brugia malayi*, *Pseudomonas aeruginosa*, *Bartonella henselae*	Peptide scan (b) Length analysis (b) Substitution analysis (b)	Mahler *et al.*, 2003
Polyclonal antibodies, *Babesia canis*, Bcvir25, dsRNA encoded protein	Peptide scan (b)	Drakulovski *et al.*, 2003

Table 6. Contd.

Keywords	Techniques*	Reference
Polyclonal antibodies, Borna disease virus (BDV)	Peptide scan (b)	Billich *et al*., 2002
Polyclonal antibodies, bovine mycoplasmosis, surface lipoprotein (Vsps), immunogenic antigens, *Mycoplasma bovis*	Peptide scan (b)	Sachse *et al*., 2000
Polyclonal antibodies, cadherins, protocadherin 12, endothel, trophoblast, mesangial cells, mice knockout	Peptide scan (b)	Rampon *et al*., 2005
Polyclonal antibodies, *E. coli*, isoforms, *Chlamydomonas reinhardtii*, 14-3-3-ß-galactosidase (ß-Gal)	Peptide scan (b)	Voigt *et al*., 2001
Polyclonal antibodies, herpes simplex virus type 1 and 2, glycoprotein C	Peptide scan (b)	Ackermann *et al*., 1998
Polyclonal antibodies, human rotavirus (HRV), VP8, Wa strain, virus neutralization	Peptide scan (b) Substitution analysis (b) Length analysis (b)	Kovacs-Nolan *et al*., 2003
Polyclonal antibodies, IgG, blood clotting factor VIII, inhibiting antibodies, PEGylation, therapeutic substances	Substitution analysis (b)	Kopecky *et al*., 2006a
Polyclonal antibodies, malaria, symptomatic and asymptomatic groups, *Plasmodium falciparum*, merozoite surface glycoprotein-1 (MSP1)	Peptide scan (b)	Fu *et al*., 2000
Polyclonal antibodies, monoclonal antibody, acute myocardial infarction, human cardiac troponin I, diagnosis	Peptide scan (b)	Ferrieres *et al*., 1998
Polyclonal antibodies, monoclonal antibody, *Neisseria meningitidis*, vaccine candidates, lipoprotein GNA1870, bactericidal antibodies	Peptide scan (b)	Giuliani *et al*., 2005
Polyclonal antibodies, monoclonal antibody, psychiatric disorders, Borna Disease Virus (BDV)	Peptide scan (b)	Schwemmle and Billich, 2004
Polyclonal antibodies, monoclonal antibody, serotype, rice yellow mottle virus (RYMV), *Sobemovirus*	Peptide scan (b)	Fargette *et al*., 2002
Polyclonal antibodies, peptide vaccine, lipoprotein (AvgC), immunogenic antigens, *Mycoplasma agalactiae*	Peptide scan (b)	Santona *et al*., 2002

Table 6. Contd.

Keywords	Techniques*	Reference
Polyclonal antibodies, vaccine, immunogenicity, HIV-1, V-3 region,	Alanine scan (b) Length analysis (b)	Cruz *et al.*, 2004b
Polyclonal antibody, antibody purification, bacteriophage ø29, connector protein	Peptide scan (b)	Valle *et al.*, 1999
Polyclonal antibody, autoantigen, polymyositis-scleroderma overlap syndrome, heterochromatin protein p25ß	Peptide scan (b) Glycine scan (b) Length analyses (b) Substitution analysis (b)	Blüthner *et al.*, 2000
Polyclonal antibody, CMV-26, method description	Peptide scan (b) Length analysis (b)	Frank, 1992
Polyclonal antibody, food allergy, wheat, conformational epitopes, IgE-binding epitopes, wheat-dependent exercise-induced anaphylaxis (WDEIA)	Peptide scan (b)	Battais *et al*., 2005
Tobacco mosaic virus protein, Fab57P, plasmon resonance, key amino acids	Peptide scan (b) Substitution analysis (b)	Choulier *et al.*, 2001
Vaccine, HIV-1, V-3 region, cross reactivity, antibody response, consensus sequence, mixotope	Peptide scan (b)	Cruz *et al*., 2004a
Vaccine, humoral immune response, B-cell epitopes, *Anaplasma marginale*, major surface protein (MSP)	Peptide scan (b)	Garcia-Garcia, 2004
Ligand-receptor interaction		
Acetylcholine receptor, muscle nicotinic acetylcholine receptor, receptor binding site, mimotopes, a-bungarotoxin	Combinatorial library (b) Substitution analysis(b)	Bracci *et al.*, 2001
Angiogenesis, VEGF, VEGFR-2, endothelial cells, D,L-peptides	Substitution analysis (b)	Piossek *et al*., 2003
Angiogenesis, VEGF, VEGFR-2, endothelial cells, dimerized peptides,	Peptide scan (b) Substitution analysis (b)	Piossek *et al*., 1999a

Table 6. Contd.

Keywords	Techniques*	Reference
Autoimmunity, T helper (Th) lymphocytes, cross-reactivity, T-cell epitope	Subsitution analysis (s)	Kamradt and Volkmer-Engert, 2004
Cytokines, cytokine cross-talk, interleukin 10 (IL-10), IL-22, receptor binding site	Peptide scan (b)	Wolk *et al.*, 2005
Fibrinogen, fibrinogen receptor, fibrinogen-binding motif prediction, group B Streptococcus	Peptide scan (b) Alanine scan (b)	Schubert *et al.*, 2002
Human polymeric Ig receptor (pIgR), ectodomains 3 and 4, SpsA, *Streptococcus pneumoniae*	Peptide scan (b)	Elm *et al.*, 2004
Import receptor, mitochondria, *Saccharomyces cerevisiae*, Tom70, preprotein	Peptide scan (b)	Brix *et al.*, 2000
Immune response, receptor, human interleukin-6, IL-6 receptor	Peptide scan (b) Subsitution analysis (b)	Weiergräber *et al.*, 1996
Inflammation, innate immunity, non-redundant functions, soluble pattern recognition receptor, fibroblast growth factor-2 (FGF2), long-pentraxin 3 (PTX3)	Peptide scan (b)	Camozzi *et al.*, 2006
Interleukin-10 receptor (IL-10R), interleukin-10, discontinuous epitope, polyvinylene difluoride membranes, electrotransfer	Peptide scan (b)	Reineke *et al.*, 1998
In situ stimulation, T helper cell hybridoma, antigen processing, mass spectrometry, rabies virus nucleoprotein	Peptide scan (b)	Otvos Jr. *et al.*, 2000
Phage display, site-directed C3a receptor antibodies	Peptide scan (b)	Hawlisch *et al.*, 1998
Preproteins, mitochondria, preprotein translocase, import receptors, cytosolic domains, Tom20, Tom22, Tom70, critical determinant of internal targeting	Peptide scan (b)	Brix *et al.*, 1999
Signalling, signal transduction, antiviral properties, human immunodeficiency virus 1 (HIV-1), SDF-1-derived small peptides, SDF-1 chemokine, chemokine receptor CXCR4	Substitution analysis (b) Peptide scan (b) Deletion scan (s)	Heveker er al., 1998
Tumor imaging, *in vivo* imaging, vasoactive intestinal peptide (VIP), VIP-dye conjugates	Subsitution analysis (s)	Bhargava *et al.*, 2002

Table 6. Contd.

Keywords	Techniques*	Reference
Peptide-microbe interaction		
Antimicrobial peptides, Bac2A, *P. aeruginosa*, Gram positive, Gram negative, yeast, MIC, scrambled peptides, structure/function relation	Scrambled peptide library (s) Substitution analysis (s)	Hilpert *et al.*, 2006
Antimicrobial peptides, bactenecin, Bac2A, *P. aeruginosa*, Gram positive, Gram negative, yeast, MIC	Substitution analysis (s)	Hilpert *et al.*, 2005a
Protein mimicking / protein design / modeling		
CB4-1 recognition, binding specificity, anti-p24 (HIV-1) monoclonal antibody, X-ray crystallography	Selected peptide library (b)	Hoffmüller *et al.*, 1999b
CB4-1, binding specificity, antibody supertopes, monoclonal antibodies, cross reactivity, polyspecificity	Substitution analysis (b)	Kramer and Schneider-Mergener, 1998a
De novo design, copper centers, synthetic four-helix-bundle proteins, TASP, UV-vis spectra, resonance Raman, EPR spectra intermediate	Combinatorial library (b)	Schnepf *et al.*, 2001
De novo design, genetic algorithm, computer-assisted drug discovery, thrombin inhibitor screening	Random peptide library (s) Selected peptide library (s) Substitution analysis (s)	Kamphausen *et al.*, 2002
Molecular recognition, antibody complementarity-determining region (CDR), porphyrin-binding peptides, ^{1}H NMR spectroscopy	Subtitution analysis (b) Length analysis (b) Alanine scan (b)	Takahashi *et al.*, 2000
Monoclonal antibody, IL-10, TNF-α, discontinuous epitope, mini protein, linker optimization	Peptide scan (b) Substitution analysis (b) Duo-cystin-scan (b)	Reineke *et al.*, 1999e
Spectroscopic identification, TASP, copper centers, synthetic four-helix bundle proteins, UV-vis absorption, resonance Raman, electron paramagnetic resonance, magnetic circular dichroism spectroscopic	Combinatorial library (b)	Schnepf *et al.*, 2004
TASP, four-helix-bundle hemoproteins, cofactor properties, redox potential	Combinatorial library (b)	Rau *et al.*, 2000

Table 6. Contd.

Keywords	Techniques*	Reference
Protein-protein interaction / peptide-protein interaction		
Adaptor protein, tight junction, SH3 and GuK domains, occludin, zona occludens protein 1 (ZO-1)	Peptide scan (b)	Schmidt *et al.*, 2004
Affinity chromatography, monolith, blood coagulation factor VIII, von Willebrand factor	Substitution analysis (b)	Pflegerl *et al.*, 2002
Alzheimer's disease, diabetes, hemodialysis, glycation/fructation, advanced glycation end products (AGEs)	Combinatorial library (b)	Münch *et al.*, 1999
Alzheimer's disease, plaques, tangles, phosphorylation, tau, amyloid-ß protein (Aß)	Peptide scan (b)	Guo *et al.*, 2006
Assembly, multiprotein complexes, proline-rich sequences, dynamics simulations, GYF (glycine-tyrosine-phenylalanine)	Subsitution analysis (b)	Gu el al., 2005
α_2-macroglobulin, α_2-macroglobulin-binding protein (GRAB), virulence factor, *Streptococci*	Length analysis (b) Substitution analysis (b)	Godehardt *et al.*, 2004
Calmodulin, calcium, potassium channel, fluorescence correlation spectroscopy, patch clamp, surface plasmon resonance spectroscopy, human *ether à go-go* potassium channels (hEAG1)	Peptide scan (b)	Ziechner *et al*., 2006
Calmodulin, mastoparan variants, protein design, phage display, affinity enrichment, two dimensional screening	Length analyses (b)	Hultschig and Frank, 2004a
cAMP-phosphodiesterase, cAMP dependent protein kinase, barrestin, phosphorylation, RACK1, PKA, $ß_2$-adrenergic receptor	Peptide scan (b) Length analyses (b) Substitution analysis (b)	Bolger *et al.*, 2006
Carbon regulation, phosphorylation, *Escherichia coli, Salmonella typhimurium*, phosphoenolpyruvate-glucose phosphotransferase system, signal-transducing $EIIA^{Glc}$	Peptide scan (b) Substitution analysis (b)	Blüschke *et al.*, 2006
Cell adhesion, Ca^{2+} independent, calmodulin, phosphorilation, protein kinase C (PKC)	Peptide scan (b)	Edlund *et al.*, 1998
Cell adhesion, immunoglobulin supergene family, C-CAM/calmodulin interaction	Peptide scan (b)	Edlund *et al.*, 1996
Chaperone, small heat shock protein, αA-crystallin, αB-crystallin, mutations	Peptide scan (b)	Sreelakshmi *et al.*, 2004
Clathrin, membrane transport, vesicles, auxilin, hsc70	Peptide scan (b)	Scheele *et al.*, 2003

Table 6. Contd.

Keywords	Techniques*	Reference
Crystal structure, site-directed mutagenesis, cyanobacterial light-harvesting complexes, proteolytically degraded, bleaching, phycobilisomes, chlorosis	Peptide scan (b)	Bienert *et al.*, 2006
Crystal structure, rearrangement, vinculin-binding site, talin rod	Selected substitution analysis (b)	Papagrigoriou *et al.*, 2004
C-termini, PDZ-protein domain	Combinatorial library (b) Substitution analysis (b)	Hoffmüller *et al.*, 1999a
Cytoskeleton, cytokinesis, morphogenesis, motility, IQGAP1, scaffolding protein, Rho GTPase, Cdc42	Peptide scan (b)	Mataraza *et al.*, 2003
Cytoskeleton, talin, vinculin, four helix bundle	Peptide scan (b)	Gingras *et al.*, 2006
Drug development, delineation, nuclear accumulation, T-cell factor (Tcf) family, ß-catenin, adenomatous polyposis coli, Tcf4, E-cadherin	Alanine scan (b) Length analysis (b) Peptide scan (b)	Gail *et al.*, 2005
Epitope-targeted proteome analysis, cDNA library, DNA microarray, experimental concept	Peptide library (b)	Frank *et al.*, 2002
EVH1 domain, ActA, *Listeria monocytogeneses*, actin remodelling	Peptide scan (b) Substitution analysis (b)	Niebuhr *et al.*, 1997
EVH1 domain, peptoid, NMR, cytoskeleton, Ena/VASP protein	Substitution analysis (b)	Zimmermann *et al.*, 2003
FBP28 WW domain variants, binding behavior	Substitution analysis (b)	Przezdziak *et al.*, 2002
Human cardiac troponin I, binding to cardiac troponin C, BIACORE, N-terminal cardio-specific extension of hcTnI, Ca^{2+}-dependent cTnC binding domains	Peptide scan (b)	Ferrieres *et al.*, 2000a
Human immunodeficiency virus type 1(HIV-1), capsid formation, dimerization, p24,	Peptide scan (b)	Hilpert *et al.*, 1999
Human immunodeficiency virus type 1 (HIV-1), discontinuous epitope, V3-directed immune response, gp120 V3 domain	Peptide scan (b)	Schreiber *et al.*, 1997
hYAP WW domain, structure-function relationship, NMR	Substitution analysis (b)	Toepert *et al.*, 2001

Table 6. Contd.

Keywords	Techniques*	Reference
hYAP65 WW domain, structural stabilization, CD measurement, double mutants	Double-substitution analysis (b)	Kretzschmar *et al*., 2005
Hypoxia, metabolic adaptation, signal transduction, hypoxia inducible hactor 1 (HIF-1), PI3K/Akt	Peptide scan (b)	Zhou *et al*., 2004
Immune response, sera of convalescent cases, SARS corona virus	Peptide scan (b)	Guo *et al*., 2004
Inflammation, inflammatory response, thrombosis, multiligand receptor, leukocyte integrin $\alpha_M ß_2$ (Mac-1)	Peptide scan (b) Substitution analysis (b)	Lishko *et al*., 2004
Inflammatory response, phagocytes, fibrinogen, integrin $\alpha_M ß_2$	Peptide scan (b)	Ugarova *et al*., 2003
Intracellular Ca^{2+} signalling, S100 protein family, I-S100C, annexin I	Peptide scan (b)	Seemann *et al*., 1996
In vivo studies, drosophila models, arginine/lysine-rich motifs, AAA-ATPases, mammalian valosin-containing protein VCP, polyglutamine protein ataxin-3 (Atx-3)	Peptide scan (b) Substitution analysis (b)	Boeddrich *et al*., 2006
Kinase, signal transduction, PAK/ERK2 association, cell proliferation, motility	Peptide scan (b)	Sundberg-Smith *et al*., 2005
Kinase-anchoring proteins (AKAP's) cAMP dependent protein kinases (PKA), regulatory subunits, isofrom specific peptide disrupters	Length analyses (b) Substitution analysis (b)	Burns-Hamuro *et al*., 2002
Leucine zipper GCN4, heterospecific coiled coil interactions, neighbourhood effects	Single and double Substitution analysis (b)	Portwich *et al*., 2002
Lipoprotein metabolism, human hepatic lipase, heparin, modelling	Peptide scan (b) Substitution analysis (b)	Yu and Hill, 2006
Microtubule assembly, tubulin binding sites, γ-tubulin	Peptide scan (b)	Llanos *et al*., 1999
Microtubule, STOP proteins, Ca^{2+}-calmodulin, cytoskeleton	Peptide scan (b)	Bosc *et al*., 2001
Minimal binding epitope, NMR structure, polyproline motif, YAP65 WW Domain	Substitution analysis (b) Length analysis (b)	Pires *et al*., 2001

Table 6. Contd.

Keywords	Techniques*	Reference
Mitochondria, preprotein, signal sequences, translocase, TOM, cytochrome c oxidase, phosphate carrier, charge	Peptide scan (b)	Brix *et al*., 1999
Mitochondria, Tim9p-Tim10p complex, ADP/ATP carrier, protein import, *Saccharomyces cerevisiae*	Peptide scan (b)	Curran *et al*., 2002
Monoclonal antibody, anticholera toxin, peptide epitope analogues, soft docking, flexible docking, ICM	Substitution analysis (b)	Stigler *et al*., 1999
Myosin, dynein intermediate chain, neuronal NO synthase, proapoptotic protein Bim, DAP1a, gephyrin, transcription factor NRF-1, PIN (protein inhibitor of neuronal NO synthase)	Peptide scan (b) Alanine scan (b) Glycine scan (b) Subsitution analysis (b)	Lajoix *et al*., 2004
Neuronal positioning, disabled (dab) protein family, phosphotyrosine binding domain, PTB, APP, internalization signals	Peptide scan (b) Alanine scan (b) Substitution analysis (b)	Howell *et al*., 1999
NMR, PDZ domain, AF6, acute myeloid leukemia, EPB2, consensus motif, binding specificity	Substitution analysis (b)	Boisguerin *et al*., 2003
NMR, phage display, consensus motif, cyclophilin A (CypA), peptidyl-prolyl *cis/tras*-isomerase	Substitution analysis (b) Peptide library (b)	Piotukh *et al*., 2005
PDZ domain, AF6, acute myeloid leukemia, EphB2-RTK, NMR, specificity, receptor-ligand	Substitution analysis (b)	Boisguerin *et al*., 2002
Peroxisomal protein import, Pex13p, Pex14p, SH3 domain, *Saccharomyces cerevisiae*, NMR, three-dimensional structure	Peptide scan (b) Length analysis (b) Substitution analysis (b)	Pires *et al*., 2003
Phosphorylation, rolipram, cAMP phoshodiesterase, PDE4, RACK1, PKA, cAMP dependent protein kinase, $ß_2$-adrenergic receptor	Peptide scan (b) Alanine scan (b) Length analysis (b)	Bolger *et al*., 2006
Plasmin(ogen)-binding motif, α-enolase, *Streptococcus pneumoniae*, intranasal infection, mouse model	Peptide scan (b)	Bergmann *et al*., 2003

Table 6. Contd.

Keywords	Techniques*	Reference
Platelet receptor, thrombosis, integrin $\alpha_{IIb}ß_3$, glycoprotein Iib/Iia, fibrinogen	Peptide scan (b) Substitution analysis (b)	Podolnikova *et al*., 2005
Porcine pleuropneumonia, *Actinobacillus pleuropneumonia*, tranferrin-binding protein, TfbA, iron source	Peptide scan (b)	Strutzberg *et al*., 1995
Protein complex, ligand specificity, dual epitope, VASP EVH1 domain, ActA, zyxin	Substitution analysis (b)	Ball *et al*., 2000
Protein domain, C-termini, dissociation constant prediction, NMR, ANOVA models, AF6, ERBIN, SNA1 (α-1-syntrophin) PDZ domains	Substitution analysis (b)	Wiedemann *et al*., 2004
Protein domain, DNA microarrays, phage display, proteomics, epitope-targeted proteome analysis	Peptide library (b)	Bialek *et al*., 2003
Protein phosphatase 1 (PP1), PP1α, Ras-activated Bad phosphatase, Bcl-2	Peptide scan (b)	Ayllon *et al*., 2001
Protein-protein interaction prediction, sequence-activity relationships, WW domain, ligand recognition propensities	Peptide library (b) Substitution analysis (b)	Otte *et al*., 2003
Sequence requirements, CD2 target sequence, GYF domain, spliceosomal function of CD2BP2	Substitution analysis (b) Peptide scan (b)	Kofler *et al*., 2004
SH2 domain, Nck1 and 2, infection strategy, crystal structures	Peptide scan (b) Substitution analysis (b)	Frese *et al*., 2006
SH3 domain, binding partners, phage display, consensus sequences, *Saccharomyces cerevisiae*	Selected peptide library (s)	Landgraf *et al*., 2002
SH3 domain, specificity, endophilin, amphiphysin, synaptojanin1, presynaptic compartment	Peptide scan (b) Substitution analysis (b)	Cestra *et al*., 1999
Sigma factor, chaperone, DnaK, DnaJ, rpoH, eubacteria	Peptide scan (b)	McCarty *et al*., 1996
Signal recogniation partical, chloroplast, cpSRP, substrate binding region	Peptide scan (b)	Groves *et al*., 2001
Small heat shock protein, sHSP Hsp18.1, Hsp17.7, substrate, HSP70, mode of action, plants	Peptide scan (b)	Wagner *et al*., 2005
Streptokinase, streptokinase therapy, human serum, antigenic regions, anti-streptokinase antibody	Peptide scan (b)	Torrens *et al*., 1999

Table 6. Contd.

Keywords	Techniques*	Reference
Structure-activity relationship, calmodulin	Length scan (b) Substitution analysis (b)	Hultschig *et al*., 2004b
Synergistic components, weak interaction, paired peptides, SPOT DS technique, protein-tyrosine phosphatase (PTP) 1B, extracellular signal-regulated kinase ERK-2, phosphorylation sites	Peptide scan (b)	Espanel *et al*., 2003
Syntrophin PDZ domain, ERBIN PDZ domain, cell proliferation, oncogenic transformation, C-terminus	Peptide library (b) Substitution analysis (b)	Boisguerin *et al*., 2004
T cell activation, Zap70 tyrosine kinase, CrkII adaptor protein, phosphorylation induced binding	Peptide scan (b)	Zhou *et al*., 2004
T cell binding promiscuity, autoimmune diseases, *Borrelia burgdorferi*, OspA, T cell hybridomas	Selected peptide library (s)	Kramer *et al*., 1999c
T cell receptor (TCR)-mediated signaling, SH2 and SH3 domain, binding specificity	Peptide scan (b) Alanine scan (b) Substitution analysis (b)	Berry *et al*., 2002
Translation assembly, cooperative, eIF4E, eIF4G, regulation, yeast gene expression	Peptide scan (b)	Ptushkina *et al*., 1998
Tumor necrosis factor-receptor (TNF-R55), neutral shingomyelinase (N-Smase), inflammatory and immunoregulatory responses	Peptide scan (b)	Adam-Klages *et al*., 1996
Transcription factor, STAT5, cytokine receptors, mitogenic and antiapoptotic signaling	Peptide scan (b)	Bittorf *et al*., 2000
Vaccination, intranasal route, ganglioside GM-1, mucosal adjutants, phage display	Peptide scan (b)	Montaner *et al*., 2006
WISE (whole interactome scanning experiment), SH3 domain, yeast, phage display, proteomic tool	Combinatorial library (b)	Landgraf *et al*. 200
WW domain, binding specificity, phosphorylated amino acid, native peptide ligation	Substitution analysis (b)	Toepert *et al*., 2003
WW domain ligands, binding specificity, essential recognition motifs	Substitution analysis (b)	Otte *et al*., 2002
Yes accociate protein (YAP), p53-binding protein-2, WW domain, SH3 domain, yeast two hybrid screening	Peptide scan (b) Alanine scan (b)	Espanel and Sudol, 2001

Table 6. Contd.

Keywords	Techniques*	Reference
Screen for antibody epitope		
Autoantibodies, glutamic acid decarboxylase (GAD), insulin-dependent diabetes mellitus (IDDM)	Alanine scan (b)	Rharbaoui *et al*., 1998
Autoantibodies, mRNA, neurological disease, Sjogren's syndrome, GW bodies, GW 182, cytoplasmic autoantigen GW182	Peptide scan (b)	Eystathioy *et al*., 2003
Autoantigen, Epstein-Barr virus, anti-nuclear antibodies, shared epitopes, cryptic epitopes	Substitution analysis (b) Peptide library (b)	Mahler *et al*., 2001
Autoimmune thyroid diseases, human anti-thyroperoxidase autoantibodies, discontinuous immunodominant region	Peptide scan (b) Alanine scan (b)	Bresson *et al*., 2003
Filamentous bacteriophage, phage display, major coat protein, major coat protein g8p	Glycine scan (b) Glycine scan (progressive) (b)	Kneissel *et al*., 1999
Innate imunity, inflammation, antigen presentation(TAP) deficiency, Gram- bacterial infections	Peptide scan (b)	Schultz *et al*., 2003
Monoclonal antibody, antigen-antibody complex, antibody paratope	Peptide scan (b) Alanine scan (b)	Laune *et al*., 2002
Monoclonal antibody, antibody polyspecificity, phage display, structure model, autoimmunity	Length analysis (b) Substitution analysis (b)	Otte *et al*., 2006
Monoclonal antibody, cyclic peptides, TGFα, antibody cross reactivity, isothermal titration calorimetry	Combinatorial library (b) Substitution analysis (b)	Hahn *et al*., 2001
Monoclonal antibody, CB4-1, ligand specificity, molecular evolution, transition pathways	Substitution analysis (b)	Hoffmüller *et al*., 2000
Monoclonal antibody, p24, HIV-1, cholera toxin transformation, D-peptide analogs	Substitution analysis (b)	Kramer *et al*., 1998
Monoclonal antibody, p24, HIV-1, positional scanning library, autoimmunity, paratope, polyspecificity, cross-reactivity	Substitution analysis (b) Combinatorial library (b)	Kramer *et al*., 1997
Monoclonal antibody, paratope, site-directed mutagenesis, HIV, ELISA, CD measurements, anti-CD4 antibody 13B8.2, BIACORE, flow cytometry	Peptide scan (b) Alanine scan (b)	Bes *et al*., 2003

Table 6. Contd.

Keywords	Techniques*	Reference
Monoclonal antibody, phage display libraries, polyspecificity, autoimmunity, anti-cholera toxin peptide 3 (CTP3)	Substitution analysis(b) Length analysis (b)	Otte *et al*., 2006
Monoclonal antibody, prions, PrPSc, transmissible spongiform encephalopathites (TSEs)	Peptide scan (b)	Korth *et al*.,1997
Multiple epitopes, primary Sjögren´s syndrome (SS), systemic lupus erythematosus (SLE), La(SS-B) autoantigen, serum antibodies, autoantibodies	Peptide scan (b)	Haaheim *et al*., 1996
Phage display, comparative epitope, delineation, papillomavirus type 16, E6 oncoprotein	Peptide scan (b) Substitution analysis (b)	Choulier *et al*., 2002
Polyclonal antibodies, IgG, blood clotting factor VIII, inhibiting antibodies	Combinatorial library (b)	Kopecky *et al*., 2005
Polyclonal antibodies, phage display, coeliac disease, gliadin antibodies	Peptide scan (b)	Osman *et al*., 2000
Screen for enzyme-inhibitors		
Anti-inflammatory effect, polyunsaturated fatty acids, ß-casein, lipoxygenases (LOXs)	Peptide scan (b)	Schurink *et al*., 2006
ß-Lactamase, broad-spectrum peptide inhibitors, phage diplay, antibiotics	Substitution analysis (b)	Huang *et al*., 2003
ß-Secretase 1 (BACE1), Alzheimer´s disease, aspartic protease, optimization, unique binding mode, crystallography, bis-statine	Substitution analysis (b) Length analysis (b)	Bridges *et al*., 2006
Carboxypeptidase inhibitor, potato carboxypeptidase inhibitor, rational structure-based design	Peptide scan (b)	Lazoura *et al*., 2002
cGMP dependent protein kinase (PKG), protein kinase inhibition, membrane permeant peptide blockers	Peptide library (b)	Taylor *et al*., 2005
Kinase, cGMP dependent protein kinase type Iα Iß, cGPK, vascular contractility, protein kinase inhibition, membrane translocation sequence	Combinatorial library (b)	Dostmann *et al*., 2000
Kinase, cGPK, vascular contractility, smooth muscle	Combinatorial library (b)	Dostmann *et al*., 2002
Phosphotransferase system, bacterial, phosphohistidine, active-site inhibitor, enzyme I inhibiting peptides, Phosphor-pyruvate dependant sugar- phosphotransferase system	Combinatorial library (b)	Mukhija *et al*., 1998

Table 6. Contd.

Keywords	Techniques*	Reference
Protease, serine protease, OMTKY3, porcine pancreatic elastase, optimizing specificity, D-amino acids	Substitution analysis (b) Substitution analysis (s) Length analysis (b) Cystine scan (s)	Hilpert *et al*., 2000
Protease, serine protease, porcine pancreatic elastase, proteinase K, trypsin, D-amino acids, sunflower inhibitor, specificity, scaffold	Substitution analysis (b)	Hilpert *et al*., 2005b
Protease, serine protease, porcine pancreatic elastase, squash inhibitor, hybrid inhibitor, isothermal titration calorimetry, D-amino acids, sunflower inhibitor, specificity	Substitution analysis (b) Substitution analysis (s) Random library (b)	Höhne and Hilpert, 2005
Protein kinase inhibitors, cAMP-dependent protein kinase, selective cyclic GMP-dependent protein, kinase Iα substrate	Combinatorial library (b)	Dostmann *et al*., 1999
Small functional peptides, human α-thrombin, vitro selection, mRNA display	Peptide scan (b) Substitution analysis (b) Length analysis (b)	Raffler *et al*., 2003
Screen for and characterization of metal binding		
Transforming growth factor-beta (TGFß1), monoclonal antibody, nickel ions, technetium-99m, peptide mixtures	Combinatorial library (b)	Kramer *et al*., 1994
Technetium-99m, radioimmunodetection (RAID), peptide mixtures, single chain Fv fragment	Combinatorial library (b)	Malin *et al*., 1995a
Transforming growth factor-alpha (TGFα), monoclonal antibody, silver ions, DNA (15mer), peptide mixtures	Combinatorial library (b) Random library (b)	Kramer *et al*., 1993
Cadmium, metal tolerance, phytochelatin, *Schizosaccharomyces*	Peptide scan (b)	Maier *et al*., 2003

Table 6. Contd.

Keywords	Techniques*	Reference
Substrate characterization		
cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), serine/threonine kinases, protein phosphorylation, recognition motif, specificity	Combinatorial library (b)	Tegge *et al*., 1995a and b
Chaperone, DnaJ, DnaK, *E. coli*, substrate specificity, stereo-specificity, mode of action	Peptide scan (b)	Rüdiger *et al*., 2001
Chaperone, DnaK mutants, *E. coli*, hydrophobic arch, modification of substrate recognition pattern	Peptide scan (b) Combinatorial library (b)	Rüdiger *et al*., 2000
Chaperone, DnaK, *E. coli*, substrate recognition pattern, ATP-dependent, energy contribution, substrate prediction, structural discrimination	Peptide scan (b) Combinatorial library (b)	Rüdiger *et al*., 1997
Chaperone, DnaK, *E. coli*, substrate specificity, IL-10 superfamily, interferon-γ, dimeric folding interface	Peptide scan (b)	Vandenbroeck *et al*., 2002
Chaperone, HscA, substrate recognition pattern, Hsca-mutants, structural discrimination	Substitution analysis (b)	Tapley *et al*., 2006
Chaperone, HscC, Hsp70. structure-function analysis, *Escherichia coli*	Peptide scan (b)	Kluck *et al*., 2002
Chaperone, outer membrane, porins, periplasmic chaperone, peptidyl-prolyl isomerase (PPIase) SurA	Peptide scan (b)	Hennecke *et al*., 2005
Chaperone, SecB, substrate recognition pattern, protein translocation, substrate prediction	Peptide scan (b)	Knoblauch *et al*., 1999
Down syndrome, substrate recognition, specificity determinants, protein kinase DYRKIA	Substitution analysis (b)	Himpel *et al*., 2000
Phosphorylation, activity of NF-κB, transactivating p65 (ReIA) subunit, basal activity	Peptide scan (b) Alanine scan (b)	Buss *et al*., 2004
Protease, ADAM protease, candidate substrates, ectodomain shedding, type I transmembrane protein, extracellular metalloprotease domain	Peptide scan (b) (in microtiter plates)	Naus *et al*., 2006
Protease, chaperone, *E. coli*, substrate recognition pattern, DegP protease, serine protease, PapA pilin	Peptide scan (b) (in microtiter plates)	Jones *et al*., 2002
Protease, human transferrin receptor (TfR), ectodomain shedding, inhibitor profile, serine protease, neutrophil elastase, cathepsin G	Peptide scan (b) (in microtiter plates)	Kaup *et al*., 2002

Table 6. Contd.

Keywords	Techniques*	Reference
Protease, prostate cancer, human glandular kallikrein 2 (hK2), proteolytic activation of cytotoxic prodrugs	Peptide scan (b) (in microtiter plates)	Janssen *et al*., 2004
Protein degradation, *E. coli*, N-end rule pathway, ClpS, charge, unstructured regions	Peptide scan (b)	Erbse *et al*., 2006
Protein kinase C, PKC, CK1, CK2, selectivity, phosphorylation	Peptide scan (b)	Toomik and Ek, 1997
Protein tyrosine phosphatases (PTPs), dephosphorylation, SPOT-DS, protocols	Combinatorial library (b)	Espanel and van Huijsduijnen, 2005
Protein-tyrosine phosphatase (PTPs), phage display library, substrate specificity, substrate-trapping, GST-PTP fusion proteins	Peptide scan (b)	Wälchli *et al*., 2004
Protein tyrosine phosphatases (PTPs), substrate specificity, dephosphorylation, signaling pathway	Combinatorial library (b) Peptide scan (b) Substitution analysis (b) Ala reverse scanning library(b)	Espanel *et al*., 2002
Signal transduction, androgen receptor, steroids, nuclear protein kinase, ANPK	Peptide scan (b)	Moilanen *et al*., 1998
Sucrose metabolism, maize seedlings, Ca^{2+} dependent protein kinase, CDPK-1, sucrose synthase type II	Peptide scan (b)	Loog *et al*., 2000
Twin-arginine translocation (Tat) systems, *Bacillus subtilis*	Peptide scan (b)	Schreiber *et al*., 2006
Twin-arginine translocation (Tat) systems, *Bacillus subtilis*, *pre-proteins*, $TatA_d$	Peptide scan (b) Substitution analysis (b)	Pop *et al*., 2003
Tyrosine phosphorylation sites, high affinity receptor for IgE, rat basophilic leukemia cells, PKCδ	Peptide library (b)	Szallasi *et al*., 1995
Zap70 protein tyrosine kinase, T cell development, phosphorylation, CT10 regulator of kinase II (CrkII) adapter protein	Peptide scan (b)	Gelkop *et al*., 2005

Table 6. Contd.

Keywords	Techniques*	Reference
T-cell epitope mapping		
CD4 and CD8 T cell epitopes, *Listeria monocytogenes*, enzyme-linked immunospot (ELISPOT) assay	Peptide scan (b)	Geginat *et al.*, 2001
$CD4^+$ human T cell clones, MHC class II, DQ dependent epitopes	Peptide scan (b) Length analysis (b)	Adler *et al*., 1994
CD8 T cell epitope mapping, *Listeria monocytogenes*, P815 cells, antigenicity	Selected peptides (s)	Wenschuh *et al*., 2002
Cross-reactivity, autoimmune disease, Borrelia burgdorferi, molecular mimicry, outer surface protein A (OspA),	Substitution analysis (s)	Maier *et al*., 2000
Cross-reactivity, autoimmune disease, mice model, encephalomyelitis, myelin basic protein	Substitution analysis (s)	Grogan *et al*., 1999
Intercellular pathogen, *Listeria monocytogenes*, secreted p60 protein, CD4 T cells, protection	Peptide scan (b)	Geginat *et al*., 1998

[#] The updated table will be available at www.kaihilpert.de/~spotapplications

* (b) indicates that the peptides are synthesized on cellulose and are still bound on the cellulose during the assay
(s) indicates that the peptides are synthesized on cellulose, cleaved from the cellulose support and used as soluble peptides during the assay

In addition to linear, or so-called continuous, antibody epitopes, there are also discontinuous or structural antibody epitopes. A discontinuous epitope comprises different parts of a protein, that are not neighboring in the primary sequence, but come close together due to the protein folding into its proper three-dimensional structure. A peptide scan of such an epitope may detect the different peptides that combine to form the structural epitope, if the binding strength of each single small linear peptide towards the antibody is within the range of detection of this method. Peptide antibody interactions with a dissociation constant of around 10^{-3} - 10^{-4} M remain detectable (Reineke *et al*., 1996 and 1999e; Kramer *et al*., 1999a). To detect true epitopes and exclude paratopes or other non-epitope interactions, it is necessary to have a very sensitive detection method on the cellulose membranes in combination with the use of soluble peptides in a competitive ELISA, or other methods using the native antigen as a competitor. This method was successfully used to detect discontinuous epitopes (e.g. Reineke *et al*., 1995; Gao and Esnouf, 1996). For discontinuous epitopes where no binding or weak binding of the individual linear peptides is observed to hamper investigations of the interaction, a so-called "duotope scan" was developed (*Figure 3b*). The discontinuous epitope of an antibody against hen egg-white lysozyme was determined using a "duotope scan", since a peptide scan showed no signals. The detected epitope represented the same region as detected by X-ray crystallography (Reineke *et al*, 2002b).

Reineke and co-workers used an antibody that could neutralize the biological action of IL-10, and the corresponding discontinuous epitopes, to mimic the biological function of IL-10. The optimization process for mimicking IL-10 involved identifying linear peptide fragments of the antibody epitope (peptide scan), optimizing the binding of each peptide separately towards the antibody (substitution analysis), combining both peptides via a linker, optimizing the resulting peptide, including the linker sequence ("duotope" scan and substitution analysis of a 32-mer peptide) and finally a "cysteine-scan", where all combinations of two cysteines in the molecule were tested to discover the most active cyclic peptide (Reineke *et al*., 1999e).

If the immune system mistakes self-tissues for non-self and mounts an inappropriate attack, the result is an autoimmune disease. Many different autoimmune diseases are known which can effect different parts of the body: for example, the digestive tract (Crohn's disease), blood and blood vessels (pernicious anemia), eyes (uveitis), glands (type 1 diabetes mellitus), heart (myocarditis), joints (rheumatoid arthritis), kidneys (glomerulonephritis), lungs (sarcoidosis), muscles (polymyositis), nerves and brain (multiple sclerosis) and skin (scleroderma). SPOT technology can support research in this area, since it provides a rapid and comprehensive approach to mapping epitopes and understanding cross-activity and polyspecificity. Using an epitope scan, Härkönen and co-workers (2002) were able to demonstrate that enterovirus infection in humans may occasionally induce a humoral response that cross-reacts with tyrosine phosphatases (IA-2/IAR), representing a major target autoantigen in type 1 diabetes. Using the same method, Esposito *et al* (1999) could demonstrate that autoantibodies from patients with multiple sclerosis bound to epitopes of human transaldolase, and at the same time showed cross-reactivity to peptides derived from the capsid of Epstein-Barr and Herpes Simplex Virus type 1. These data suggest that cross-reactivity caused by molecular mimicry may play a critical role in autoimmune diseases. Similar investigations with T cell epitopes were performed, demonstrating that T-cell cross-

reactivity is a common phenomenon, and therefore the existence of cross-reactive epitopes alone does not trigger autoimmunity (Grogan *et al*., 1999; Maier *et al*., 2000). T cell recognition is highly degenerate, and structural criteria rather than sequence homology are important for T cell cross-activity (Kamradt and Volkmer-Engert, 2004).

In some cases, antibodies can bind and inhibit the human blood clotting factor VIII and cause bleeding disorders. An iterative deconvolution of a linear combinatorial library of 10-mer peptides was used to screen for peptides that can neutralize these antibodies. It was shown for the first time that short peptides can be used to compete for polyclonal inhibitory IgG from various patients (Kopecky *et al*., 2005). These peptides were used to study PEGylation with a view toward using them as therapeutic substances (Kopecky *et al*., 2006a)

Peptide scan technology was also used to identify the B-cell epitopes of EEA1 (early endosome antigen1). Analyses of patient serum using these epitopes suggest that it is possible to discriminate non-neuronal and neurological autoimmune diseases. This information can then be used for new diagnostic tests (Selak *et al*., 2003).

PEPTIDE-RECEPTOR INTERACTIONS

For any biological systems it is important to react according to environmental conditions. Recognizing these conditions is often achieved through receptors that can interact with a narrow or broad range of ligands. Communication and regulation within multi-cellular organisms is also often mediated by ligand-receptor interactions. Therefore, studying such interactions is a major focus for understanding many different biological processes. This information can also lead to the development of new drugs against many different diseases. For receptors that bind peptides or proteins, SPOT synthesis can be a useful tool to study such interactions.

Vascular endothelial growth factor (VEGF) directly stimulates endothelial cell proliferation and migration through tyrosine kinase receptors. Inhibition of vascular growth is thought to be one of the most promising approaches in cancer therapy. The potential binding site of VEGF with its receptor was identified using SPOT technology (peptide scan). A substitution analysis of VEGF-derived peptides was used to study this interaction (Piossek *et al*., 1999a). The linear peptide identified was further investigated by several substitution analyses using D-amino acids (Piossek *et al*., 1999b). This provided a good starting point for developing anti-angiogenic peptide drugs.

Mapping of the interleukin-10/interleukin-10 receptor binding site by SPOT technology was described by Reineke *et al*. (1998b). In addition, two potential receptor binding sites for human granulocyte macrophage colony stimulating factor (hGM-CSF) were identified by these methods (Eberhardt *et al*., 2003)

PEPTIDE-ENZYME INTERACTIONS

Signal transduction, metabolism, cell proliferation/viability, differentiation and apoptosis are examples of biological processes in which kinases play a critical role. To understand their role in these processes it is important to understand the interactions

with their substrates. Analyses of the human genome revealed more than 500 kinases and increased the demand for tools to study their substrate specificities (Manning *et al*., 2002). Peptide arrays for kinase profiling are an ideal tool for addressing this demand. The peptides can be synthesized directly on a surface (cellulose, glass, polymers) or presynthesized and subsequently immobilized onto a surface. Kinase assays can be performed directly on the cellulose sheet (e.g., Moilanen *et al*., 1998, Loog *et al*., 2000) or alternatively, the peptide can be synthesized, cleaved from the membrane and spotted onto a glass microarray (e.g., Rychlewski *et al*., 2004; Panse *et al*., 2004; Schutkowski *et al*., 2004). When the natural kinase substrate is known, a peptide scan can be performed and the phospho-acceptor residue determined. Substitution analysis can be used to study kinase specificity or to optimize the substrate for a selected kinase (e.g. Toomik and Ek, 1997; Loog *et al*., 2000). In case no information about the substrate is known, randomized or combinatorial libraries can be used to map substrate specificity. A very efficient peptide substrate for protein kinase G (PKG) was designed by an iterative deconvolution of combinatorial libraries (Tegge *et al*., 1995b). Combinatorial libraries were also used to develop inhibitors of cyclic GMP-dependent protein kinase Iα (cGPK). One of these inhibitors was fused to two different cell membrane translocation sequences and the effect of this inhibitor studied *in vivo* (Dostmann *et al*., 2002). In addition, peptide libraries with fragments of human peptide sequences can be used to identify the kinase target. For this approach micro-peptide arrays are more suitable than cellulose membranes, since microarrays have a larger number of peptides per cm^2. Peptide phosphorylation can be detected by an anti-phosphorylated-amino acid antibody, or a phosphorylated-amino acid chelator or by mixing the catalysed ATP with labeled [γ^{32} or ^{33}P] ATP.

Conservative estimates of the total numer of distinct proteases in the human genome are between 700 and 2000 (Pessi, 2002). In addition, many pathogens produce their own proteases, which play a critical role in pathogenesis. Therefore, we can anticipate a constant supply of proteases that may become targets for drug discovery as well as being investigated to understand their functions. The successful development of HIV protease inhibitors illustrates the importance of studying these enzymes (Temesgen *et al*., 2006). Two types of investigations, assessing protease/substrate and protease/inhibitor interactions, have been carried out using SPOT technology. Membrane-bound peptides were used to map the substrate specificity of proteases at the P1 and P'1 sites (Schechter and Berger, 1967) surrounding the cleavage site, whereby membrane discs (polyaminoethylmethacrylamide) containing the dipeptide substrate and a chromophore were dipped into the wells of a microtitre plate containing the protease and its required reaction buffer. Subsequently, the absorbance of the liberated chromophore was measured in a time dependent manner (Duan and Laursen, 1994). This method was adapted to cellulose membranes using a fluorophore near the membrane-proximal C-terminus, with the protease cleavage site (P1 and P'1) in the middle of the peptide and a quencher molecule at the N-terminus. Cleaved peptides are detected at the membrane by the increase in fluorescence when the fluorescence quenching molecule is removed (Reineke *et al*., 1999c). Alternatively, the peptides can be tagged with a sequence, recognized by an antibody and a biotin molecule. After protease contact the cleaved peptides will be blotted on a streptavidin membrane and detected using an antibody (Kramer *et al*, 1999b). Both assays can be performed directly on the membrane and therefore many thousands of peptide variants can be

screened. The great advantage is that the affects of variations at all other subsites and not just P1 and P'1 can be investigated. An assay was also developed where the spots (large size) were punched out and transferred into microtitre plates. These peptides are tagged with a fluorescent moiety at the N-terminus. Measuring aliquots of a protease-containing solution in the wells of the microtitre plate permits the detection of the cleaved peptides via the N-terminal fluorescence tag (Reineke *et al*., 2000). Using this method, the *Escherichia coli* protease DegP (Jones *et al*., 2002), human glandular kallikrein 2 (Janssen *et al*., 2004) and human ADAM protease (Naus *et al*., 2006) were investigated with respect to their interaction with their substrates. In addition, Kaup *et al*. (2002) were able to determine the proteases responsible for the cleavage of human transferrin receptor.

Protease-inhibitor interactions are of great interest in drug development. A method for investigating these interactions was developed using SPOT technology. A series of peptide inhibitor variants were investigated for both binding to, and inhibition of, a serine protease (Hilpert *et al*., 2000 and 2005b; Höhne and Hilpert 2005). Binding was studied directly on the cellulose membrane using horseradish peroxidase (HRP) labeled proteases, while inhibition was measured by an assay in microtiter plates with punched out peptide spots and a chromogenic substrate. Both methods led to similar results, indicating that the binding assay, which is faster and easier to perform and also provides the opportunity to use thousands of peptide spots, is satisfactory for studying these interactions. Combining substitution analysis and length analysis, the designed new peptides inhibited the protease more strongly than the original peptide, with a high specificity towards the target protease. Substitution analyses using D-amino acids led to inhibitory peptides that were protected against exopeptidase attack. Hybrid peptide inhibitors were then investigated with respect to their thermodynamic parameters, as well as by X-ray crystallography in complex with a protease (Hilpert *et al*., 2002 and 2003; Ay *et al*. 2003). Selecting a small scaffold (14-mer) it was also possible to study both the binding to the protease and the influence of the scaffold on binding in one single experiment (Hilpert *et al*., 2005). The interaction of α_2-macroglobulin (a broad spectrum protease inhibitor) with Protein G related α_2-macroglobulin-binding protein (GRAB), a virulence factor of group A streptococci, was studied using length and substitution analysis. The interaction site was mapped, and mutation of the key amino acid involved in the interaction confirmed the results (Godehardt *et al*., 2004).

PROTEIN-PROTEIN INTERACTIONS

Since the DNA sequences for many organisms are now available, a resulting challenge is to understand gene regulation and the function of encoded proteins. Identifying the function of a protein and understanding the mechanism of action requires considerable effort. In addition, a protein's function is in most cases interlinked with a network of other proteins, which creates an even more complex situation in understanding the dynamics of biological systems. SPOT technology can be used to map the interaction sites of proteins, thus providing additional information to understand the network of protein-protein interactions for a single process, within a cell or within a whole organism.

Chaperone/heat shock proteins are important for a broad range of biological activities, e.g. protein-folding and responses to stress. SPOT synthesis was successfully used to discover the interactions of these proteins with themselves and with their substrates. Amongst those investigated were DnaK, SecB, Hsp18.1 and 17.7, GroEL, α-crystallin, DnaJ and HscA (Rüdiger *et al*., 1997, 2000 and 2001; Knoblauch *et al*., 1999; Wagner *et al*., 2005; Tapley *et al*., 2006; Sreelakshmi *et al*., 2004).

Peptide scan technology was also used to characterize the formation of the chloroplast signal recognition particle (cpSRP) complex and to map the substrate binding region of this complex (Groves *et al*., 2001). The same method was used to study the mitochondrial import of proteins (Brix *et al*., 1999; Ptushkina *et al*., 1998) and the interplay of microtubules with STOP proteins and Ca^{2+}-calmodulin (Bosc *et al*., 2001). Mapping of the dimerization sites of the capsid protein of HIV-1 (p24) was performed using SPOT technology to investigate this protein-protein interaction. Site directed mutagenesis and ultra-centrifugation were used to confirm these data (Hilpert *et al*., 1999).

Proteins often interact via distinct domains, for example SH3-, WW- and PDZ-domains, and this can be studied using SPOT technology. Besides peptide scan and substitution analyses to study known interaction partners, random libraries or iterative deconvolution of combinatorial peptide libraries can be used to find new binding peptides. The Src homology 3 (SH3) domain is a small protein domain of about 60 amino acids, often found associated with functions concerning the cytoskeleton, Ras protein and Src kinase. Using SPOT technology and phage display technology, the investigated SH3 domains of endophilin and amphiphysin showed different preferred binding sites, indicating that the binding repertoire of SH3 domains are more complex than originally predicted (Cestra *et al*. 1999). In contrast, peptide scan and substitution analysis were also used to investigate the binding specificities of the SH2 domains of Nck1 and Nck2, that had been predicted to be different, but indicated that they were essentially indistinguishable (Frese *et al*., 2006). In an excellent approach called WISE (whole interactome scanning experiment), all of the peptides in the yeast proteome with the potential to bind to eight SH3 domains were discovered (Landgraf *et al*., 2004; Santonico *et al*., 2005). In combination with NMR spectroscopy, an intensive study of a WW domain comprising of 44 amino acid was reported by Toepert and co-workers. All together, 12,696 peptide variants of the WW domain were synthesized on cellulose and investigated for peptide binding (Toepert *et al*., 2001 and 2003).

PEPTIDE-MICROBE INTERACTIONS

The rapid increase in antibiotic resistance has resulted in the diminished effectiveness of antibiotics, especially against hospital infections. For example, the rate of resistance to methicillin of coagulase-negative *Staphylococci* (CNS) reached 89.1%, while that of *Staphylococcus aureus* (known as MRSA) increased to 59%. Recent candidates for novel antimicrobials are host defense peptides, but the mode of action and sequence requirements are still poorly understood. Substitution analyses (Hilpert *et al*., 2005a), scrambled peptides (Hilpert *et al*., 2006) and random peptide libraries (unpublished results) have been used to discover novel antimicrobial peptides with superior broad spectrum activity, and to investigate the sequence requirements for antimicrobial activity in general. For this investigation peptides were cleaved from spots, and the

soluble peptides used in a microtitre plate assay. This assay was developed for screening peptides with killing activities against the Gram-negative bacteria *Pseudomonas aeruginosa*, but could be easily adapted to any other microbe.

PEPTIDE-METAL ION INTERACTIONS

Since many proteins are in complexes with metal ions, it is an interesting task to study the interaction of peptides with metal ions. In addition, some peptide-metal ion interactions are used in purification and protein detection, for example his-tag technology. In some cases the fusion of six histidines to a protein may influence folding, solubility, function and crystallization. Therefore, alternative binding sequences would be of general interest. For *in vivo* diagnostics, the metastable technetium isotope ^{99m}Tc is widely used, due to its physical properties, low cost and availability. Cellulose bound combinatorial libraries were successfully used to find specific binding sequences for ^{99m}Tc, as well as nickel, silver, iron, calcium, molybdenum, uranium, lead, gold, zinc and manganese (Kramer *et al*., 1994; Malin *et al*., 1995a and 1995b; Schneider-Mergner *et al*., 1996). Similarly the metal binding site in phytochelatin synthase was discovered using the peptide scan method with cellulose-bound peptides (Maier *et al*., 2003).

PEPTIDE-DNA INTERACTIONS

Many proteins can bind and interact with DNA or RNA, including polymerases, gyrases, helicases, ribosomes, transcriptional factors and restriction endonucleases. There is a great potential to study peptide-DNA or peptide-RNA interactions using the SPOT technology to increase our understanding of these proteins' functions. Combinatorial peptide libraries on cellulose membranes were successfully used to study peptide-DNA interactions (Kramer *et al*., 1993). Similarly the region of the endonuclease *Eco*RII involved in DNA recognition was identified using the peptide scan method, with the peptides synthesized on cellulose (Reuter *et al*., 1999; Reuter and Möncke-Buchner, 2002).

Conclusions

SPOT synthesis technology represents a well-established screening tool for biologically active peptides. From simple binding assays to more sophisticated enzyme assays and studies with living microbes or cells, a multitude of investigations are possible. We anticipate that SPOT synthesis will be adapted and used in new fields in the next few years. Peptide variants can be studied with relatively low cost and time investment. The technology has an advantage over biological screening technologies since all chemically available building blocks can be used in peptide synthesis. Besides being a screening tool, SPOT synthesis also provides array strategies for investigating interactions at a higher level and can therefore result in new discoveries as well as novel understanding of interactions. The technology (manual or automated) can be easily integrated into a laboratory, since the equipment is commercially available and does not require special conditions. The small amount of peptide per spot does not

easily allow purification or quantification, a potential drawback of this method. All results gained must be confirmed by selected peptides synthesized on resin. Nevertheless, the method is an excellent tool for gaining information and supporting traditional methods.

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