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review

Spot-Synthesis: Easy Multiple Preparation of Synthetic Peptides on Cellulose Membrane for the Analysis of Anti-Protein Antibodies

*Daniela Lucchesi and Paolo Rovero**

CNR – Istituto di Mutagenesi e Differenziamento,
Laboratorio di Sintesi Peptidica, Via Svezia 2A, 56124 Pisa, Italy

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Summary

The SPOT synthesis is a novel technique for the simultaneous multiple synthesis of peptides covalently linked to cellulose paper, using conventional solid-phase synthesis strategy. The sets of paper-bound peptides thus obtained can be directly used in solid-phase assays, including ELISA. The technique is a powerful tool for the study of protein-interactions and has been widely applied to antibody characterization and epitope mapping studies. The SPOT technique provides a simple, economic and rapid access to large numbers of short peptide sequences or peptide mixtures which can be tested concomitantly with a single serum and repeatedly with many sera on the same membrane. Recent applications of SPOT synthesis to epitope mapping are reviewed in this report.

Keywords: cellulose paper, epitope mapping, enzyme-linked immunosorbent assay (ELISA), multiple peptide synthesis, peptide libraries.

Introduction

»Ubiquitous in modern life, the stuff of ordinary cellulose appears as the ephemeral and the long lasting, the sacred and the mundane. Such is the magic of paper.« When writing this conclusion to an interesting article entitled »The Magic of Paper« *(1)*, National Geographic author J. R. Louma was probably not thinking about the increasing interest that cellulose paper is gaining in the field of immunochemical techniques. However, the disclosure of cellulose as a solid support suitable for peptide synthesis and, most importantly, the development of a straightforward strategy of multiple peptide synthesis on cellulose membranes which can be used directly for solid-phase immunoassays, opened very promising perspectives. As expected, a number of applications of this strategy have been recently published. This report reviews both the basic principles of the method and its published applications in the study of peptide-antibody interactions.

Over the past decade, peptides have become increasingly important in almost all areas of immunological research *(2,3)*. Indeed, the discovery *(4)* and the subsequent development *(5)* of the solid-phase method for the synthesis of peptides greatly contributed to the large diffusion of immunochemical methods based on synthetic peptides. Among these, enzyme-linked immunosorbent assay (ELISA) is the most commonly used technique. The methodology is based on peptides, either free or coupled to a carrier protein, that are attached to the plastic surface of ELISA plates, in order to allow detection of binding interaction between the peptide and an antibody. Thus, from a practical point of view, the peptide is first synthesized on one solid support and then removed from it and attached to a different one to facilitate antibody screening. Starting from this observation, Geysen *et al.* (6,7) proposed a method for the synthesis of peptides on plastic rods suitable for direct ELISA screening (PEPSCAN). The technology was developed to enable the simultaneous synthesis of a great number of peptides in a re-usable format; as a result, in many laboratories the availability of synthetic peptides no longer limitated the collection of immunological data. A further improvement in this direction followed the observation that cellulose paper can be conveniently used as a support for both the synthesis and the screening of peptides.

*Author to whom correspondance should be addressed at the above address; e-mail p.rovero@imd.pi.cnr.it

Membranes as solid support for peptide synthesis

The first reports on the use of cellulose paper as a support for solid-phase synthesis are due to Frank *et al.* who described the preparation of oligodeoxyribonucleotides *(8)* and peptides *(9,10)*. These authors stressed the suitability of this support for simultaneous multiple synthesis, following the »segmental support« approach *(9)*. The term »segmental support« refers to a mechanically and chemically stable, non-interchangeable entity of support material. This type of support allows one to perform in one pot all the common reaction cycles of the growing peptides and to separate them when necessary, without contamination. In this way, the number of reaction cycles necessary to assemble a set of different sequences can be reduced considerably, as compared to the number of individual synthetic steps, when performed separately *(9)*.

The validity of the method was confirmed by several authors. Thus, Eichler *et al. (11,12)* reported the synthesis of several sets of model peptides on filter paper, while alternative membrane supports were proposed: cotton *(13)*, polystyrene-grafted polyethylene films *(14)* and hydroxypropylacrylate-coated polypropylene membranes *(15)*. All these publications described only test syntheses; two of them also reported speculations on the suitability of this method for the assembly of membrane-bound peptides for direct use in the study of protein-protein interactions, including epitope mapping *(12,15)*.

Paper-bound peptides in immunological tests

The use of paper-bound peptides directly prepared on the membrane in immunological tests was described in at least three independent publications appeared in the early nineties. Krchnák *et al. (16)* described a »paper disc-ELISA« based on the synthesis of peptides on individual discs of filter paper (diameter 13 mm) which were subsequently cut in four parts and used as the solid phase in a conventional ELISA procedure. According to these authors, several peptides can be prepared simultaneously loading the paper discs into polypropylene flow reactors connected to a manually operated continuous-flow multiple synthesizer. Subsequently, the disc portions were incubated with sera in plastic syringes equipped with polypropylene sinters and the resulting colored solutions transfered to ELISA plates in order to measure the absorbance. As an example, four peptides (10–20 residues) known to detect antibodies against the respective viral proteins in ELISA were prepared and tested; all of them reacted strongly and specifically with relevant sera.

A similar approach was described by van't Hof *et al. (17,18)*, using the »tea-bag« strategy for the simultaneous synthesis of a large number of peptides on individual paper discs. Starting from the observation that the method of Krchnák *et al. (16)* is not practical for large numbers of peptides and sera, these authors used the »tea bag« method, sealing groups of about 100 paper discs (diameter 6 mm) into small polypropylene mesh bags which enabled an easy manual parallel multiple synthetic strategy, as described by Houghten *(19)*. The availability of multiple copies of the same paper disc-bound peptide enables one to test concomitantly a large number of sera, using a radioimmunoassay. The authors reported the preparation of 146 nonapeptides overlapping by eight residues, covering the sequence of the feline major allergen *Fel d I*; the binding sites found coincided with those detected by the PEPSCAN method and the signal-to-background ratio in the paper disc-RIA was found to be comparable to that in the PEPSCAN. The bound antibodies could be eluted from the paper disc-bound peptides, permitting regeneration and repeated use of the paper discs for immunological testing. According to the authors, the major advantage of this method is that large numbers of antibodies could be simultaneously tested with large numbers of peptides, due to the availability of many copies of each paper disc-bound peptide.

A fundamental further step toward the development of a really simple method for the simultaneous multiple synthesis of paper-bound peptides for immunological testing was done by Frank, with the disclosure of the SPOT-synthesis method *(20,21)*. The novelty of this procedure is that peptides are no longer prepared onto cut pieces of cellulose paper, which are treated as a conventional solid support for peptide synthesis and thus placed into the columns of a synthesizer or into the bags of the Houghten's procedure. In the Frank's method, each peptidic chain is assembled in a very small limited circular area of the cellulose membrane (»spot«), originated by the adsorption of a droplet of liquid.

The SPOT technique

Principle of the method

The principle on which the SPOT technique is based has been described by Frank and coworkers in several recent papers *(21–25)*. Briefly, a droplet of liquid dispensed onto a porous membrane such as cellulose paper is adosrbed and forms a circular spot. Using a solvent of low volatility containing appropriate reagents, the spot can be considered as a reactor where chemical reactions involving reactive functions anchored to the matrix can take place, as in batch-wise conventional solid-phase synthesis. A great number of distinct spots can be arranged on a membrane sheet and each one is individually addressable with a pipette for the delivery of the reactive. In practice, a large set of different peptides can be constructed dispensing individually on each spot a drop of a solution of the required activated amino acid. Subsequently, all the common steps of the synthetic process, i.e. washings, deprotections, etc., are carried out by immersing the whole sheet into the relevant solvent or reagent. The spotting procedure is easily amenable to automation, using a robot arm and a dispensing device.

Preparation of the membrane and assembly of the peptides

In the original method described by Frank *(21)*, arrays of peptides up to a length of 20 residues can be prepared manually using conventional mild Fmoc/t-Bu (Fmoc = fluorenyl-methyl-oxycarbonyl, t-Bu = tert-butyl) chemistry. In the first step, pure cellulose chromatogra-

phy paper is chemically derivatized through the uniform esterification of a N^{α}-Fmoc-protected βAla residue to available hydroxyl functions on the cellulose fibers of the whole membrane. The final substitution level (0.1–0.2 μmol/cm^2) is strongly dependent on several critical factors, including thickness of the paper and purity of the reagents. According to the author *(21, 25)* and to our own experience, this step is the most critical one. However, functionalized membranes are now commercially available. In the next step, the Fmoc group is cleaved and the desired array of spots is generated by spotting a second residue of Fmoc-βAla; at this stage, the spotted volume determines the final size of the spot and thus the scale of the synthesis. Finally, all residual amino functions between spots are capped by acetylation and the Fmoc group on the spots is cleaved. Following this procedure, a βAla-βAla dipeptide anchor is generated at each spot, in order to facilitate the presentation of immobilized peptides in the subsequent immunological tests. It is also possible to cleave the peptides from the cellulose support, introducing a cleavable linker in place of the βAla-βAla anchor. A simple option proposed by Frank *(21)* is the use of a Boc-Lys-Pro (Boc = butyl-oxycarbonyl) linker *(26)*, introduced using Fmoc-Pro and Boc-Lys (Fmoc) in the two steps described above. In this case the peptide is released in solution upon treatment with neutral phosphate buffer via diketopiperazine formation. More recently a set of special safety-catch linkers has been described *(27)*, which enables one to cleave *unmodified peptides acid or amide* directly in neutral aqueous buffers.

Once the linker has been constructed, it is possible to stain free amino functions on the spots using bromophenol blue (BPB) *(28)*. Monitoring of each step of the synthesis and particularly of the coupling efficiency can be visually followed. A complete coupling produces a change of the spot color from blue to yellow. The elongation of the peptide chains is achieved spotting aliquots of the appropriate activated Fmoc-amino acid; if the color change is not obtained within 15 min, a second and even a third aliquot could be added, until coupling is driven to completion. In the Frank's original paper *(21)* Fmoc-amino acid pentafluorophenyl (Pfp)-esters in N-methyl-2-pyrrolidinone (NMP) are recommended for normal coupling, while the more reactive N-carboxy anhydrides (NCAs) are preferred for the slow coupling residues Val, Ile and Thr. In more recently published protocols *(25)*, hydroxybenzotriazole (HOBt) esters of Fmoc amino acids are prepared 30 min before use, adding 0.25 M N,N'-diisopropylcarbodiimide (DIC) to a 0.2 M solution of the amino acid in NMP, containing 0.25 M HOBt. The greater efficiency of DIC/HOBt preactivation was confirmed also by other authors *(29)*. After each coupling, unreacted amino functions are capped by acetylation, the Fmoc group is cleaved by treatment with piperidine and the spots are stained again with BPB before starting the next cycle. At the end of the synthesis, side chain protecting groups are cleaved by treatment with trifluoroacetic acid (TFA) in the presence of appropriate scavengers.

Binding-assay

Following the above protocol a large variety of paper-bound peptide sets can be obtained for virtually any kind of immunological tests and in a broader sense for the study of protein-protein interactions. Thus, overlapping fragments derived from a protein sequence are useful for mapping linear antigenic determinants or binding sites. Stepwise N- or C-terminally truncated fragments are used for the elucidation of the minimal binding sequence. Moreover, the synthesis of substitution analogues helps in the evaluation of the contribution of individual residues to binding. An example of this technique is the so-called »alanine scan« which consists of the systematic replacement by Ala of each residue of a given sequence. Finally, the SPOT method has been largely exploited for the synthesis of peptide libraries, for the *a priori* delineation of peptide binding sequences *(vide infra)*.

The binding of a protein, e.g. an antibody, to paper-bound peptides has been most frequently identified by a conventional solid-phase ELISA procedure, although other labelling techniques based on radioactive tracers or fluorescent dyes have been also applied. According to Frank *(21)*, standard enzyme-conjugate/chromogen combinations which form water insoluble colored products can be used. As a result, colored spots appear at the position of the binding. Most importantly, if the detection system does not chemically modify the peptide spots, both the dyes and the bound proteins can be removed from the membrane by washing with appropriate organic solvents and detergents (stripping procedure) in order to regenerate the peptide arrays on the membrane, which can be reused many times. This is clearly another very important positive feature of the SPOT technique.

Applications

B cell epitope mapping

Following the above protocol it is possible to obtain a large variety of paper-bound peptide sets, which can be applied to the study of the binding of synthetic peptides to proteins, DNA and also metal ions *(39,40)*. Indeed, a number of applications have been developed in the field of immunology. A first example was reported by Frank *(21)*, who used a series o 49 paper-bound overlapping decapeptides with an offset of one residue, derived from the CMV26 sequence, an immunogenic region of the human cytomegalovirus 36/40 K protein recognized by sera of several patients seropositive to the whole virus. A polyclonal serum raised in rabbits against a β-galactosidase-CMV26 fusion protein was tested in an ELISA procedure: two series of peptides reacted most strongly, involving decapeptides 11 to 16 (common sequence motif SLSSL) and decapeptides 30 to 33 (common sequence motif LDNDLMN). These two dominant epitopes where previously identified by two independent methods, thus showing the validity of the SPOT technique for linear epitope mapping. The SLSSL epitope region was then analysed in more detail using paper-bound peptides of different length and alanine substitutions. The identity and homogeneity of the peptides was shown by repetition of the synthesis using the cleavable Boc-Lys-Pro linker: high-pressure liquid chromatography (HPLC), mass spectrometry (MS) and amino acid analyses gave the expected results.

Reusch *et al. (30)* used the SPOT synthesis to prepare a set of 120 overlapping paper-bound decapeptides (offset 1), spanning the whole sequence of interleukin (IL)-4. These peptides were used to analyze the binding to a monoclonal antibody 3B9, raised against recombinant human IL-4, using a conventional ELISA procedure developed with a chemiluminescent reagent. A series of seven consecutive positive spots (common sequence $H^{76}KQL^{79}$) revealed that the epitope recognized by the 3B9 monoclonal antibody is located within the N-terminal region of IL-4, in agreement with evidence obtained by the same authors using different techniques.

Martens *et al. (319* reported the analysis of six monoclonal antibodies raised against morbillivirus P protein (507 residues), using a series of 13-mer peptides (offset 3). Two antibody binding sites were delineated within the C-terminal and a third one was located on the N-terminal part of the protein. These results compare well with those obtained in Western blots and in an ELISA using detergent extracts from virus-infected cells, at least as far as linear determinants are involved.

Human monoclonal antibodies were characterized using the SPOT technique by Siemoneit *et al. (32)*. These authors prepared a set of 15 nonamers (offset one) for the fine epitope mapping of a conserved peptide fragment (EP3, 18 residues) of the envelope glycoprotein E1 of the hepatitis C virus. Anti-hepatitis C virus-positive blood donors were screened for anti-EP3 antibodies with a conventional ELISA; from three positive blood donors, four anti-EP3 producing heterohybridoma cell lines were established. The fine study of antibody reactivity, analysed using the paper-bound overlapping nonapeptides, suggested the existence of different antigenic determinants within the EP3 fragments. However, while two overlapping epitopes were clearly delineated for the two IgG antibodies, in the case of the two IgM antibodies the paper-bound peptide approach was not sufficient for determining antibody recognition site(s). Both antibodies reacted with peptides in a broad and/or heterogeneous pattern, which did not allow the description of a single amino acid binding motif. Nevertheless, the problem seems not be related to the cellulose paper support of the SPOT method, since similar observations for human IgG and IgM using the PEPSCAN technology were reported *(6,7)*.

An epitope analysis of human sera of clinical relevance was reported by Haaheim *et al. (33)*, who investigated the epitope recognition pattern of La(SS-B) autoantibodies in sera from patient with Sjögren's syndrome (SS) and systemic lupus erythematous (SLE) using an array of 80 decapeptides (offset 5) from the human La(SS-B) autoantigen (408 residues). Tests performed with 14 SS and 6 SLE sera showed that the immune response to the La(SS-B) peptides was restricted and unique for each individual, with no particular pattern for any of the two deseases. Among the advantages of the SPOT technique, the authors observed that the use of small peptides as antibody targets allowed the precise identification of contiguous reactive sites, a point easily missed when using longer stretches of recombinant sequences as antigens.

An interesting paper by Halimi *et al. (34)* deals with a comparison between the SPOT technique and a standard ELISA performed with free peptides for the localization of linear epitopes of the U1 snRNP-C autoantigen. The first method was used to prepare 75 paper-bound decapeptides (offset 2), to be compared with 15 overlapping peptides (16-30 residues), synthesized with conventional Fmoc chemistry, removed from the resin and tested by ELISA after direct coupling to polyvinyl plates. Both systems were evaluated with antibodies raised in rabbits against synthetic peptides of U1C and sera from patients with autoimmune diseases. Positive spots were revealed using the enhanced chemiluminescence (ECL) immunoblotting reagents. An overall very good agreement was observed between ELISA based on the use of conventional longer peptides and the paper-bound decapeptides. As reported above (33), an advantage of using the SPOT method was to identify short antigenic regions within the segment covered by peptides of larger size. In general, the method was found to be reproducible and more than 15 different sera were tested on a single membrane without any apparent loss of spot antigenic activity. Moreover, these authors found no background in the tests with sera from autoimune patients, a problem reported in some cases when using the PEPSCAN approach. However, a certain lack of sensitivity was reported, as compared to conventional ELISA. Other reported limitations are linked to the lack of analytical control on the paper-bound peptides synthesized according to the SPOT procedure and the absence of the free charged N- and C-termini of the peptides, which in some instances can be recognized by the antibodies. However, these problems are inherent with the concept of unique solid support for both the synthesis and the immunological test and are found also in the PEPSCAN method.

A recent paper by Gao and Esnouf *(35)*, based on a variation of the SPOT technique, reports on a method for the elucidation of discontinuous epitopes using membrane-bound linear peptides. These authors described a variant of Frank's method, based on the use of a polyvinylidene difluoride membrane as the solid support. Functionalization of the membrane is achieved by treatment with ethylene diamine in order to introduce membrane-bound amino functions, to which a Fmoc-βAla residue is linked through an amide bond; the subsequent peptide chain elongation on this single residue linker is performed substantially as described by Frank. Antibody binding to immobilized peptides is revealed by the ECL technique. According to this protocol, the authors prepared a set of 236 overlapping octapeptides (offset one) spanning the entire sequence of β-factor XIIa, a fragment of human blood coagulation factor XII. The immobilized peptides were used to test a panel of three monoclonal antibodies raised against this protein. These antibodies were not able to recognize carboxymethylated or trypsin-digested β-factor XIIa, suggesting that the recognized epitope may be discontinuous. However, the antibodies recognized several individual octapeptides from different regions of the primary sequence of β-factor XIIa. To determine which residues were essential for the reaction with the antibodies, the peptides were further characterized by alanine substitutions and by the synthesis of peptides of shorter sequence. In conclusion, according to the authors, the study shows that the discon-

tinuous epitopes of the three monoclonal antibodies can be identified by this sensitive technique, based on membrane-immobilized synthetic peptides. These epitopes are composed of several discrete short peptides five to eight residues long, involving between 20 and 31 amino acids.

Peptide epitope libraries

A different powerful application of the SPOT technique was developed for the identification of the residues in the sequence of a known epitope which are critical for binding. A cellulose-bound peptide epitope library is constructed, synthesizing all the individual peptides in which each residue of the epitope is replaced by all the amino acids. The analysis of the antibody binding to these peptides can give information on the relative importance of each residue of the epitope.

Stigler *et al. (36)* applied this technique to the Fab fragment of the human monoclonal antibody 3D6, raised against the transmembrane protein gp41 of human immunodeficiency virus (HIV)-1. The epitope recognized by 3D6 is known to be located within the conserved region 395–620 of gp41. Using a panel of free overlapping peptides the authors identified the minimal binding epitope in the region 605–617 (13 residues). This peptide served as a starting point to prepare a cellulose-bound peptide epitope library in which each residue of the epitope was substituted by all L- and D-amino acids. The resulting 494 epitope variants, were subsequently analyzed for binding to the antibody 3D6. The information thus obtained on the relative importance of each residue of the epitope was combined with computer-assisted molecular modeling techniques to construct a model of the peptide-antibody complex.

Similarly, Volkmer-Engert *et al. (37)* described a cellulose-bound epitope library of the HIV-1 p24 decapeptide epitope recognized by the murine monoclonal antibody CB 4-1. Antibody-binding analysis of the 190 peptides obtained by replacement of each residue of the epitope by all L-amino acids followed the detection of key residues in binding. Interestingly, these results were found to correlate satisfactorily with those obtained with soluble peptides obtained by conventional strategies.

T cell epitope analysis

Besides the several studies showing the application of the SPOT method to B cell epitope mapping, at least one paper has been published demonstrating that SPOT synthesis is equally convenient to prepare solution phase peptides for T cell epitope analysis. Accordingly, Adler *et al. (38)* reported on the simultaneous synthesis of 49 overlapping 14-mer peptides (offset 5), spanning the sequence of the matrix protein of influenza virus, onto a cellulose membrane functionalized with the cleavable Pro-Lys-Boc linker. Final products (ca. 20 nmol each) were analyzed by HPLC and MS and used in the functional T cell assay, which enabled the characterization of the epitopes recognized by several clones.

Combinatorial peptide libraries

A further very promising application of the SPOT technology was reported by Schneider-Mergener and coworkers *(39–42)* and by Frank *et al. (43)*, who described the synthesis of combinatorial peptide libraries on cellulose membrane. In recent years different types of chemically synthesized or biologically generated peptides libraries consisting of millions of distinct molecules have been successfully used for the identification of peptides that bind to protein, such as antibodies. The combination of the SPOT synthesis with the library technique enabled the development of cellulose-bound peptide combinatorial libraries that have been successfully used in various assays, including epitope mapping.

In a first example of epitope analysis using a cellulose-bound peptide library Kramer *et al. (39,40)* reported the characterization of the linear epitope recognized by the monoclonal antibody Tab2, raised against transforming growth factor-α (TGFα). It is known that the epitope recognized by this antibody is located within the N-terminus of TGFα and corresponds to the linear sequence HFND. This experiment was conceived mainly as a test of the possibility to create cellulose-bound conbinatorial libraries using the SPOT technique. The library X-X-B_1--B_2-X-X (where X is a randomized position obtained coupling an equimolar mixture of 19 amino acids, while B_1 and B_2 are defined position) was a collection of 400 sublibraries; each sublibrary (a single spot) represented a mixture of all the possible hexapeptides (19^4 = 130.321) containing the defined dipeptide B_1B_2 in the central position and the full library (400 spots on a single 19 x 19 cm sheet of paper) contained all the possible 400 defined dipeptides. When the library was incubated with the antibody and developed following the conventional ELISA procedure, seven positive spots were detected: X-X-H-F--X-X, X-X-F-N-X- X, X-X-N-D-X-X, X-X-Y-F-X-X, X-X-Y--N-X-X, X-X-H-Y-X-X, X-X-Y-Y-X-X. The first three contain the expected dipeptides HF, FN and ND which made up the HFND epitope, while the other clearly indicate that tyrosine was also tolerated in positions 1 and 2, replacing phenylalanine and histidine, respectively *(39)*. In order to further explore the adjacent positions, three additional libraries, X-B_1-H-F-B_2-X, X-B_1-F-N-B_2-X and X-B_1-N-D-B_2-X, were synthesized and screened with Tab2. The results gave important information about the sequence of the epitope and the structural requirements for its binding to the antibody. These experiments showed that small epitopes can be conveniently mapped by the use of cellulose-bound combinatorial peptide libraries *(40)*.

A recent publication from the same gorup *(42)* reported a more detailed analysis of the same Tab2 antibody, using a slightly different approach, based on a »combinatorial clustered amino acid peptide library«. The aim was to overcome the problem of »combinatorial explosion« in the synthesis of combinatorial libraries: a library containing three defined B positions (X-X-B_1-B_2--B_3-X-X) would consist of 20^3 = 8,000 peptide mixtures (*i.e.* spots) and thus the synthesis would be very difficult, if not impossible. An alternative is to use in each defined position clusters of physicochemically similar residues instead of every single amino acid. The rationale of this approach is based on the assumption that physico-chemically related amino acids contribute similarly to binding. Thus, grouping the amino acids into six clusters would lower the number of peptide mixtures in

a combinatorial library with four defined positions from 20^4 (160,000) to 6^4 (1,296). Accordingly, the cellulose-bound library X-O_1-O_2-O_3-O_4-X (O = one of the six amino acid clusters [APG], [DE], [HKR], [NQST], [FYW] and [ILVM], cysteine omitted; X = randomized position) was synthesized and screened with the Tab2 antibody. Four sports gave a positive signal; the role of each amino acid in the clusters of the positive mixtures was further studied preparing a second-step library X-B_1-B_2-B_3-B_4-X made up of all the possible combinations of single amino acids of the clusters identified in the first screening (432 peptide mixtures). This detailed analysis led to the identification of the wild-type epitope sequence, but also of a large number of other sequences with comparable affinity.

Similarly, Frank *et al. (43)* showed that the »mixed coupling« approach can be used to generate randomized positions in paper-bound peptides, thus obtaining arrays of defined peptide pools. Starting from previously reported paradigms for the deconvolution of individual sequences by activity screening of random pools, these authors devise three general strategies which can be used in the case of cellulose-bound hexapeptidic libraries.

a) Positional scanning with a single fixed position (six libraries of 20 sublibraries each): B-X-X-X-X-X, X-B-X-X-X-X, X-X-B-X-X-X, X-X-X-B-X-X, X-X-X-X-B-X, X-X-X-X-X-B (X and B as defined above).

b) Iterative search starting with one or more defined positions (in the case of a defined dipeptide, as in the example given above, each library is made up of 400 sublibraries): X-X-B_1-B_2-X-X (first screening), X-B_1-O_3-O_4-B_2-X (second screening; O is an unvaried position), B_1-O_2-O_3-O_4-O_5-B_2 (third screening).

c) Dual-positional scanning (five libraries of 400 sublibraries each): B_1-B_2-X-X-X-X, X-B_1-B_2-X-X-X, X-X-B_1-B_2-X-X, X-X-X-B_1-B_2-X, X-X-X-X-B_1-B_2.

The three strategies were tested with the mouse monoclonal antibody 1D3, directed against the tubulin tyrosine ligase from pig; the smallest epitope recognized is NYGKYE and a broad range of single substitutions is tolerated, with tyrosine in position 2 and glycine in position 3 being the most critical residues. An ELISA procedure was used, followed by rapid quantitative evaluation of signals on the membrane by utilizing a digital recording laser densitometer which reads the optical densities of dye distributed over the entire membrane. The positional scanning approach did not give any significant result with 1D3. The authors speculate that pools containing more than four randomized X positions may be too complex for this type of solid-phase binding assay. The iterative search gave one clear dominant signal when screening the library X-X-B_1-B_2-X-X: the motif -GK-, which in fact is found in the central portion of the epitope. Also the second iterative step gave results fitting with the known features of the natural epitope. Similarly, in the dual-positional scanning the strongest signals from the first four sets were NY, YG, GK and KY, which match to give the natural epitope sequence NYGKY. The fifth set gave no significant signals over background for the C-terminal dipeptide, indicating that these positions are quite degenerate. Taken together, these data have shown the potential applicability of the cellulose-bound combinatorial library approach to the *a priori* epitope mapping.

An octapeptide membrane-bound library was synthesized for an iterative epitope search approach by Gao and Esnouf *(44)*, using the SPOT technique on the polyvinylidene difluoride polymer described above *(35)*. The epitope of a monoclonal antibody 201/9, raised against β-factor XIIa, was identified starting with a membrane-bound octapeptide library with two amino acids defined at positions 2 and 4: X-B_2-X-B_4-X-X-X-X. In the first cycle, using a sensitive chemiluminescent method for the detection of antibody binding, the peptide mixture X-P-X-N-X-X-X-X showed the strongest signal. In the second cycle, six libraried were synthesized: B_1-P-X-N-X-X-X-X, X-P-B_3-N-X-X-X-X, X-P-X-N-B_5-X-X-X, X-P-X-N-X-B_6-X-X, X-P-X-N-X-X-B_7-X and X-P-X-N-X-X-X-B_8. After screening these peptide mixtures, the residues representing the sequence of the epitope of 201/9 were elucidated. The identified sequence correlated well with that previously identified in the scan of membrane-bound overlapping peptides spanning the sequence of β-factor XIIa *(35)*.

Conclusions

The SPOT technique for the easy and simultaneous multiple synthesis of cellulose membrane-bound peptides, developed by Frank *(21)* has been widely applied to the study of the binding of synthetic peptides to proteins, metal ions and DNA. Also enzymatic reactions, most notably peptide phosphorylation, can be studied using this method. The recent combination of the SPOT synthesis with the combinatorial library technology opened even broader perspectives. In fact, with respect to the synthetic step, the SPOT technique provides a simple, economic and rapid access to large numbers of short peptide sequences or peptide mixtures which can be tested concomitantly with a single serum and repeatedly with many sera on the same membrane.

The advantages of the SPOT techniques over conventional ELISA are clearly related to the possibility offered by this method to obtain easily and without special equipment large sets of synthetic peptides directly linked to a solid support, in a format suitable for repeated assays. Among the limitations of the method, the lack of a direct analytical control of the synthetic peptides should be taken into account. Moreover, a lower sensitivity of the SPOT method, as compared to conventional ELISA, has been reported *(34)*. Nevertheless the many interesting applications of the SPOT technique reiewed in the present report indicate that this method offers promising perspectives in the field of epitope mapping.

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SPOT-sinteza: jednostavna mnogostruka priprava sintetskih peptida na celuloznoj membrani pogodna za analizu anti-protein antitijela

Sažetak

SPOT-sinteza je novi postupak za simultanu mnogostruku sintezu peptida, kovalentno vezanih na celulozni papir, koji koristi uobičajeni postupak u čvrstoj fazi. Tako dobiveni niz na papir vezanih peptida može se izravno primjenjivati pri ispitivanju u čvrstoj fazi, uključujući ELISA. Novi je postupak značajno oruđe pri proučavanju interakcija protein-protein, a u velikoj se mjeri primjenjuje za karakterizaciju antitijela i pri studiju mapiranja epitopa. SPOT-sinteza omogućava jednostavno, ekonomično i brzo dobivanje velikog broja kratkih peptidnih sekvencija ili smjese peptida što se mogu istodobno testirati s određenim serumom ili ponovljeno s mnogim serumima na istoj membrani. U ovom je revijalnom prikazu dan pregled niza primjena SPOT-sinteze za mapiranje epitopa, provedenih posljednjih pet godina.