CelluSpots Arrays as an Alternative to Peptide Arrays on Membrane Supports

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Abstract: Peptide arrays are useful tools to characterize antibodies, to determine sequence specificities of enzymes, or to find interaction partners to given peptide sequences. One widely-used format for such arrays is a cellulose sheet with hundreds of synthetic peptides bound to it. These SPOT arrays have been used successfully in a broad range of applications since their invention at the beginning of the nineties. The simplicity and robustness of this method along with the fully automated synthesis to generate custom arrays with high peptide densities made the SPOT method popular. A drawback of the SPOT method is the use of large reagent volumes and the limited throughput with only one copy of the library. CelluSpots represent a new method that retains the advantages of the SPOT method but allows the production of hundreds of identical copies on microscope slides for parallel screenings with low sample volumes.

Keywords: Antibody profiling, CelluSpots, kinase substrates, peptide arrays, peptide conjugates, protein interaction.

I. INTRODUCTION

Peptide arrays have become widely applied tools for screening approaches in life science research. A variety of biomolecular binding events or enzymatic modifications can be investigated by using peptide arrays including epitope mapping and characterization of antibodies [1-3], protein-protein interactions [4-9], protein-DNA interactions [10], peptide-cell interactions [11, 12] or kinase- [13-15] and protease-substrates [16]. In particular, *in situ* synthesis of peptides on cellulose membranes (SPOT) has been intensively used since its introduction in 1990 [17]. Results from several hundred publications underline the importance of this technique. Some of the main reasons for the popularity of the SPOT method are its simplicity and robustness. The SPOT-technology can be easily adapted even in laboratories that are not experienced in peptide chemistry. Parallel peptide synthesizers allow the fully automated synthesis of hundreds of peptides onto derivatized cellulose membranes. The resulting peptide arrays can be used to determine antibody epitopes, protein binding domains, phosphorylation sites and other aspects of molecular recognition [18, 19]. Additional favorable factors of the method were the biocompatibility of cellulose with high peptide densities in the range of several hundred nmol/mm^2 as compared to conventional monolayer arrays on glass substrates, fmol/mm^2. These high spot densities enabled the detection of even low affinity binders with a K_d in the μM range, almost up to 1 mM. SPOT arrays do, however, have limitations when reducing the spot size below 1 mm and they become costly and tedious when large numbers of identical arrays are required for parallel screening experiments. To overcome these limitations a new method was introduced [20, 21], that enables the production of hundreds of identical peptide arrays on coated microscope slides from a single synthesis run.

Here, we present the manufacturing process of CelluSpots peptide arrays and their major advantages compared to SPOT arrays, especially for screening applications. Furthermore, we describe four examples using this novel array format in the field of kinase screening, protein-protein interaction and antibody profiling with human sera incubations.

II. PREPARATION OF PEPTIDE-CELLULOSE CONJUGATES

The production of CelluSpots arrays starts with the Fmoc based synthesis approach of peptides on individual chemically modified cellulose discs (INTAVIS, Cologne, Germany), which are arranged in 384-well synthesis frames placed in a MultiPep RS synthesizer (INTAVIS, Cologne, Germany). This approach allowed the synthesis of peptides with a variety of modifications such as phosphorylation, acetylation or methylation sites. For synthesis, HOBt ester building blocks of the amino acids can be automatically generated by the synthesizer or pre-activated OPfp or HOBt esters can be used and directly distributed to the appropriate positions during the subsequent synthesis cycles. After the completion of the reaction, the side-chain protection groups were removed by a treatment in a cleavage solution (80% (v/v) TFA + 5% (v/v) dist. water + 3% (v/v) TIPS + 12% (v/v) DCM) followed by an elongated treatment of the discs in an acidic cleavage cocktail (88.5% (v/v) TFA + 4% (v/v) TFMSA + 5% (v/v) dist. water + 2.5% (v/v) TIPS) in order to solubilize the polymeric support. The chemical bond between the peptides and the cellulose polymers is stable under these conditions. The resulting peptide-cellulose conjugates were precipitated in ether to remove the acids. The peptide conjugates were then dissolved in DMSO. The polymer stock solutions were spotted as 1:2 dilutions in a mixture of DMSO and SSC buffer (2 parts DMSO and one part 1x SSC buffer; 20x SSC buffer: 3 M NaCl (175 g/L), 0.3 M $Na_3C_6H_5O_7 \cdot 2H_2O$ (88 g/L), adjust pH to 7.0 with 1 M HCl) by a slide spotting robot (INTAVIS, Cologne, Germany) and adsorbed onto standard microscope slides with white surface coating (INTAVIS, Cologne, Germany). Mini-arrays were generated by an application of 50-100 nl per spot with robust pipetting robots leading to spot diameters in the range of 500 μm. A more compact array can be generated if smaller volumes are distributed with a microarray spotter, frequently used for the production of DNA microarrays. These instruments are equipped with split pins, ring-and-pin or piezoelectric nozzles. After evaporation of solvent, the peptide-cellulose conjugates form three-dimensional structures that cannot be dissolved in aqueous buffers. The production process of the CelluSpots arrays is shown in Fig. (**1**).

The white surface coating of the microscope slides enables detection by standard chromogenic methods used for Western Blots. On mini-arrays with spot diameters of 300 μm or more the colored spots were visible by eye and were analyzed by conventional scanners. Therefore, expensive laboratory equipment like a fluorescence or chemiluminescence scanner is not necessarily needed. However, the detection of biomolecules that were bound to CelluSpots slides is not limited to the use of colorimetric substrates. If low affinity binding partners are investigated the use of more sensitive detection methods like chemiluminescence will prove advantageous.

III. EXAMPLES OF CELLUSPOTS PEPTIDE ARRAYS

a) Sera Screening to Discover Immunodominant Epitopes

Parallel incubations of human sera on peptide arrays enable the fast and economic detection of immunodominant epitopes. In a typical experimental set-up, potential antigen-spanning overlapping peptides were spotted onto coated microscope slides. Identical ar-

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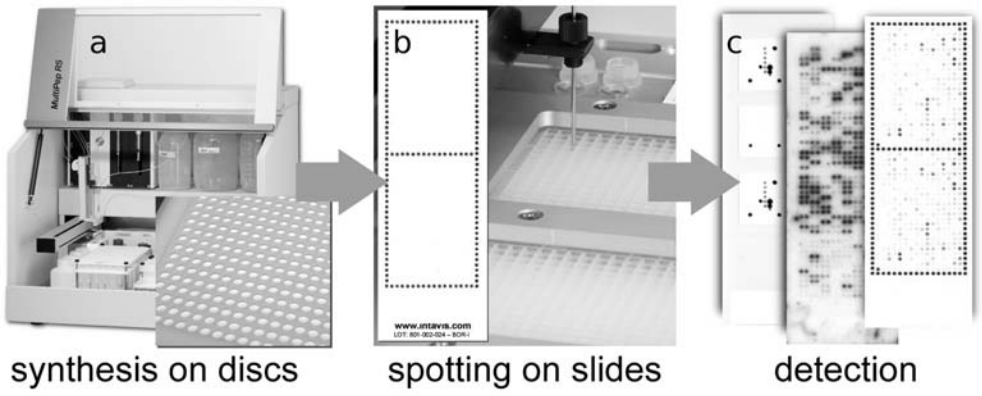

Fig. (1). Production procedure of CelluSpots peptide arrays. Peptides are synthesized on individual discs arranged in 384-well synthesis plates placed in a MultiPep RS peptide synthesizer (**a**). After solubilization, the peptide-cellulose conjugates are spotted onto coated microscope slides (**b**) that can be used for incubations (**c**) followed by detection (chemiluminescence, colorimetric substrates, autoradiography or fluorescence).

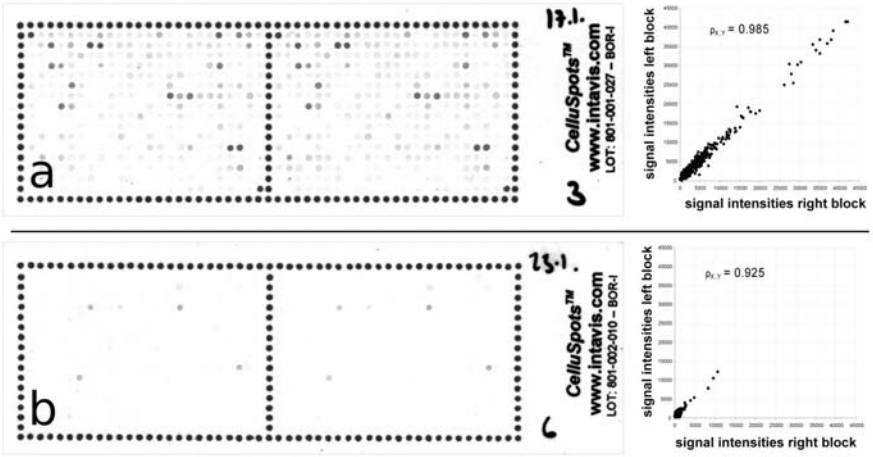

Fig. (2). CelluSpots peptide arrays containing overlapping peptides of several *Borrelia* antigens spotted in duplicate, incubated with a dilution of human sera. Serum of an infected person (**a**); negative control serum (**b**). The correlation coefficient of signal intensities for the two duplicate blocks is 0.985 for the positive and 0.925 for the negative serum incubation.

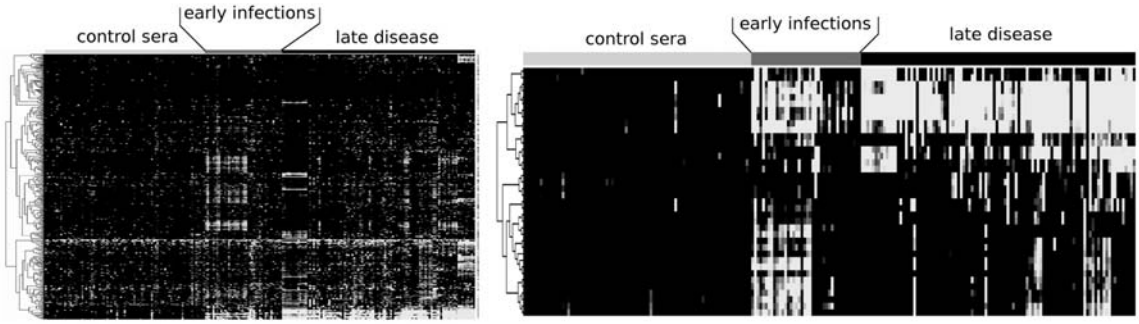

Fig. (3). Left: Cluster analysis of all 211 serum samples (columns) and 768 peptide spots (rows). Right: Cluster of all 211 serum samples (columns) and 38 chosen dominant epitopes (rows).

rays were then used to incubate positive and negative control sera in parallel. After detection and read-out of signal intensities, clustering algorithms were helpful to visualize and compare individual groups of serum samples (representing different disease states for example). This approach has the capability to discover new major immunodominant epitopes that play important roles in microbial or viral infections and allergic or autoimmune diseases. The discovery of these sequences could be of interest for the development of diagnostic tests and vaccines.

To identify immunodominant epitopes of *Borrelia* antigens hundreds of CelluSpots arrays representing overlapping 20mer peptides of *Borrelia* surface proteins were incubated with characterized human serum samples. More than 200 human serum samples were incubated on peptide arrays in total. The arrays consisted of two identical blocks, each with 384 peptide conjugates surrounded by red orientation marks. After incubation with diluted human serum, bound IgG antibodies were detected by anti-IgG-peroxidase conjugates with DAB solution as the substrate. Hierarchical cluster analysis was used to select the most relevant epitopes [22]. Results of this screening approach are shown in Figs. (**2**) and (**3**).

The cluster of 38 selected major immunodominant epitopes (Fig. (**3**), right) shows the significant difference between negative

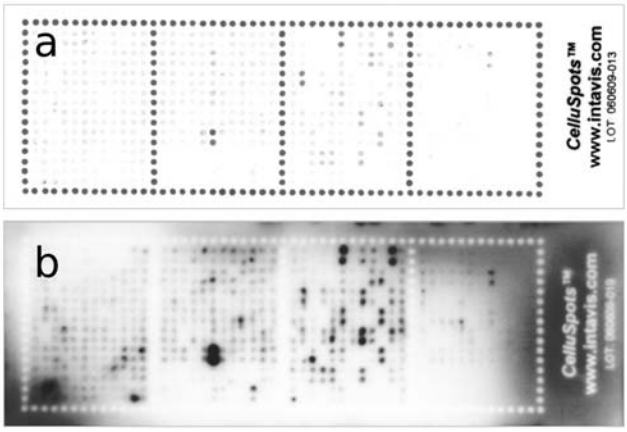

Fig. (4). CelluSpots peptide arrays with a phospho-serine containing peptide library, incubated with a phospho-specific antibody and analyzed by enzymatic color precipitation (**a**) and chemiluminescence (**b**). Each of the 4 blocks of the peptide arrays contains 192 spots (96 spots in duplicates), surrounded by colored spots as orientation marks.

control sera (columns marked in light grey, left side), early infections (columns marked in grey, middle) and late disease states (columns marked in black, right side). The patterns of signal intensities shown in each row, representing the individual peptides, allow discrimination between samples of infected and non-infected people. Some of the peptides in this cluster originated from the VlsE antigen, which contains the immunodominant IR6 invariable region and upon which the C6 ELISA is based [23-25].

b) Characterization and Profiling of Antibodies

Phosphorylation of tyrosine, serine and threonine residues by protein kinases play a major role in the regulation of cellular pathways, especially those involved in signal transduction, cell growth and development. It is suggested that the human genome contains more than 500 protein kinase genes [26]. One research tool used to detect the phosphorylation status of proteins are phospho-specific antibodies. Thousands of mono- and polyclonal antibodies are on the market that bind specifically to certain target sequences. Both, the sequence specificity and cross-reactivity were critical factors to interpret scientific data generated based on these antibodies [27-29]. In order to prevent cross-reactivity, a detailed characterization of the antibodies with respect to sequence variations of the epitope that can be recognized with similar affinity, was essential.

Peptide arrays helped to characterize the specificity and potential cross-reactivity of antibodies. As one example, peptide arrays were generated that contain possible variations of phosphorylated sequences with common motifs of Akt/PKB kinase substrates (R-X-R-X-X-S) [30]. Akt is involved in signal transduction pathways of cell proliferation, angiogenesis and apoptosis. The peptide array shown in Fig. (**4**) was designed as a replacement study: each position (1 to 12) of a 12 amino acid long sequence X-X-R-X-R-X-X-pS-X-X-X-X was replaced by either one of the 20 common amino acids, a mixture of amino acids (X), pS, pT or pY. This resulted in an array of 12 x 24 peptide variations spotted in duplicates. The arrays were incubated with an antibody directed against the phosphorylated epitope sequence M-S-G-R-P-R-T-T-pS-F-A-E-S-C-K-P (pS = phosphorylated serine at position 9) within GSK-3 beta. For the first array (Fig. (**4**), **a**) the detection was performed with a secondary antibody labeled with alkaline phosphatase and NBT/BCIP as colorimetric substrate. For the second array a horseradish peroxidase conjugated antibody was used for chemiluminescence detection (Fig. (**4**), **b**). The analysis of signal intensities showed that the phosphospecific antibody has the following sequence specificity: -**T**-**pS**-**F**/L-**A**-H/pS. The letters in bold were identical to the original sequence of the phosphorylated GSK-3 beta sequence.

c) Identification and Characterization of Kinase Substrates

Kinase-mediated posttranslational phosphorylation of serine, threonine and tyrosine residues often leads to a functional change of the modified substrate protein by changing the enzyme activity, binding affinity to other proteins or its cellular localization. Even though the substrate specificity of kinases was one important key to understand the impact of these enzymes on the cellular level, only a fraction of kinases has been fully characterized so far. One method to investigate the kinase substrate specificity was the use of peptide arrays. The CelluSpots peptide array technology enables a fast and versatile tool to determine the specificity of kinases towards specific peptide substrate motifs. Therefore, compilations of consensus sequences and known kinase substrates from literature and databases have been synthesized and spotted onto microscope slides which resulted in ready-to-screen kinase substrate arrays.

One example, shown in Fig. (**5**), is the characterization of two different but structurally related tyrosine kinases fms-like tyrosine kinase 3 (FLT3) and platelet-derived growth factor receptor (PDFGR) on an array representing a set of tyrosine kinase substrates. Arrays with 384 peptide conjugates spotted in duplicate

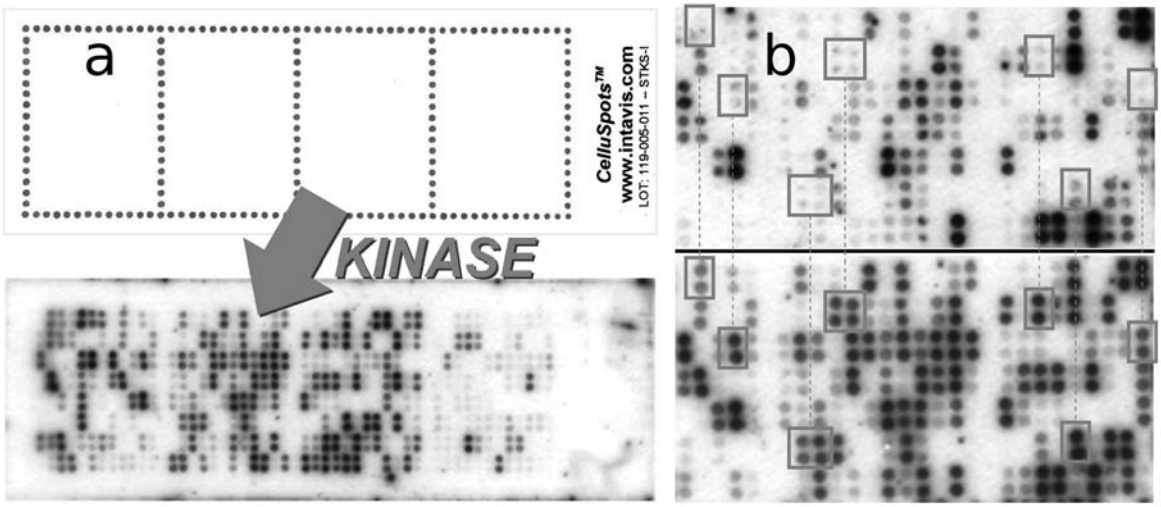

Fig. (5). (**a**) CelluSpots array representing 384 kinase substrates spotted in duplicate before and after incubation and autoradiographic detection. (**b**) Comparison of substrate specificity for two different kinases (top: FLT3, bottom: PDFGR). Some major differences are marked. Peptide conjugates are spotted in duplicates on all arrays. (Frank-D. Bohmer, Jena University Hospital).

were incubated with the two kinases of interest with labelled ATP (ATP-gamma ^{32}P) as a substrate. After several washing steps the dry slides were exposed to X-ray films. Data analysis revealed a set of peptide motifs that are substrates for both kinases whereas some of the substrates were phosphorylated exclusively by PDFGR. Additionally, a new substrate for FLT3 kinase was found that can be used to detect the FLT3 activity as well as to screen and characterize new FLT3 inhibitors [31].

In a second application example, Olaussen *et al.* [32] studied the effect of two inhibitors (pazobanib and lapatinib) on the kinome of A549 cells. The cells were incubated either with one inhibitor or a mixture of both. After 12 h the cell lysates were incubated on CelluSpots tyrosine kinase arrays followed by the detection of phosphorylated peptides with an anti-phospho-tyrosine antibody. The results showed differences in the phosphorylation profile of both substances. A combination of both inhibitors caused a significant inhibition of some kinases that were not inhibited by either one alone.

Both examples demonstrated that peptide arrays representing kinsase substrates can be valuable tools for inhibition studies, to characterize the substrate specificity of kinases and to explore new kinase substrates.

d) Protein Interaction Studies

Protein-protein interactions are involved in numerous important biological processes in living cells like cell adhesion, enzyme substrate interaction and growth factor recognition. One of the most important functions is signal transduction that is mediated by two or more interacting proteins which form cascades and complex networks. Defects in this cellular information processing are the cause for various diseases such as diabetes, cancer, and autoimmunity.

A large number of protein binding domains like SH2 [8], SH3 [33], PTB [9] or PDZ [34] are already known. Many of these protein interaction sites are represented by linear binding motifs. This enables binding assays of proteins towards peptide arrays containing hundreds potential interaction motifs [35]. Recently Katz *et al.* studied the molecular interaction between Bcl-2 and ASPP2, which are key proteins in the apoptotic pathway. Based on CelluSpots peptide arrays and quantitative fluorescence spectroscopy studies the authors showed that Bcl-2 binds sequences from three loops (ASPP2 945-961, 988-1002 and 1065-1082) of the C-terminal part of ASPP2 [36] with nanomolar affinities.

CONCLUSION

The CelluSpots peptide arrays are a versatile tool for the detailed analysis of antibody epitopes, proteins-protein interactions, and the substrate specificity of enzymes such as kinases. They have the potential to facilitate the evaluation of antibody and enzyme specificity and to characterize protein binding domains. Arrays containing sequence variations of common recognition motifs can be produced easily in numerous identical copies, giving the opportunity to screen many different antibodies, proteins or other biomolecules simultaneously against the same set of peptides. The biggest advantage of the CelluSpots arrays compared to the SPOT membranes is the ability to generate hundreds of identical arrays from one initial synthesis. This allows economical parallel screening approaches and repeatability of experiments. The smaller format of the arrays reduces the sample consumption for incubations and might therefore enable experiments that have not been possible before with peptide arrays on membranes.

ABBREVIATIONS

ASPP2	=	apoptosis-stimulating protein of p53 2
ATP	=	adenosine-5'-triphosphate
BCIP	=	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
Bcl-2	=	B-cell lymphoma 2
DAB	=	3,3'-diaminobenzidine
DCM	=	dichloromethane
DMSO	=	dimethyl sulphoxide
DNA	=	deoxyribonucleic acid
ELISA	=	enzyme-linked immunosorbent assays
FLT3	=	fms-like tyrosine kinase 3
Fmoc	=	fluorenylmethoxycarbonyl
GSK-3 beta	=	glycogen synthase kinase 3 beta
HOBt	=	hydroxybenzotriazole
IgG	=	immunoglobulin G
K_d	=	dissociation constant
$Na_3C_6H_5O_7 \cdot 2H_2O$	=	tribasic sodium citrate dihydrate
NaCl	=	sodium chloride
NBT	=	nitro-blue tetrazolium chloride
OPfp	=	pentafluorophenyl
PDFGR	=	platelet-derived growth factor receptor
PKB	=	protein kinase B
pS	=	phosphoserine
PTB	=	phosphotyrosine-binding domain
pT	=	phosphothreonine
pY	=	phosphotyrosine
SH2	=	Src homology 2
SH3	=	Src-homology 3
TIPS	=	triisopropylsilane
TFA	=	trifluoroacetic acid
TFMSA	=	trifluoromethanesulfonic acid
VlsE	=	variable major protein-like sequence, expressed

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