Sensing Immune Responses with Customized Peptide Microarrays

Christopher Schirwitz, Felix F. Loeffler, Thomas Felgenhauer, Volker Stadler, Frank Breitling, and F. Ralf Bischoff

---

Sensing Immune Responses with Customized Peptide Microarrays

**Christopher Schirwitz · Felix F. Loeffler ·
Thomas Felgenhauer · Volker Stadler ·
Frank Breitling · F. Ralf Bischoff**

Abstract The intent to solve biological and biomedical questions in high-throughput led to an immense interest in microarray technologies. Nowadays, DNA microarrays are routinely used to screen for oligonucleotide interactions within a large variety of potential interaction partners. To study interactions on the protein level with the same efficiency, protein and peptide microarrays offer similar advantages, but their production is more demanding. A new technology to produce peptide microarrays with a laser printer provides access to affordable and highly complex peptide microarrays. Such a peptide microarray can contain up to 775 peptide spots per cm^2, whereby the position of each peptide spot and, thus, the amino acid sequence of the corresponding peptide, is exactly known. Compared to other techniques, such as the SPOT synthesis, more features per cm^2 at lower costs can be synthesized which paves the way for laser printed peptide microarrays to take on roles as efficient and affordable biomedical sensors. Here, we describe the laser printer-based synthesis of peptide microarrays and focus on an application involving the blood sera of *tetanus* immunized individuals, indicating the potential of peptide arrays to sense immune responses.

This article is part of the Topical Collection "In Focus: Future of Biosensors".

C. Schirwitz · F. F. Loeffler (✉) · F. R. Bischoff
Department Chip-based Peptide Libraries, German Cancer
Research Center (DKFZ), Im Neuenheimer Feld 280,
69120 Heidelberg, Germany
e-mail: f.loeffler@dkfz.de

C. Schirwitz
e-mail: c.schirwitz@dkfz.de

F. R. Bischoff
e-mail: r.bischoff@dkfz.de

T. Felgenhauer · V. Stadler
PEPperPRINT GmbH, Rischerstrasse 12,
69123 Heidelberg, Germany
e-mail: thomas.felgenhauer@pepperprint.com

V. Stadler
e-mail: volker.stadler@pepperprint.com

F. Breitling
Department Peptide Arrays and Antibody Libraries,
Karlsruhe Institute of Technology (KIT),
Hermann-von-Helmholtz-Platz 1,
76344 Eggenstein-Leopoldshafen, Germany
e-mail: frank.breitling@kit.edu

1 Introduction

Understanding the various interactions on the molecular level in a living system is of central interest in modern biomedical research. To study as many interactions as possible with minimum consumption of analyte/compound and within a short time frame, immense effort has been put on the development of high-throughput approaches. In the field of proteomics, cell-based (e.g. Phage display) [1–3], bead-based (e.g. "one-bead-one-compound" method) [4, 5], as well as array-based [6–8] techniques are applied to screen entire libraries of proteins or peptides for interaction partners. Since synthetic high-density DNA microarrays [9–11] have revolutionized the field of genomics, a similar tool is requested for proteomic research. However, the chemical synthesis of proteins and peptides is more demanding than the synthesis of DNA libraries because a larger variety of building blocks is needed. The first strategy to combinatorially synthesize peptide microarrays was suggested by Fodor et al. in 1991 [12]. Using photochemical protecting groups and "activating" desired synthesis locations with a light source, the lithographic method is, in principle, capable of producing highly

Springer

resolved arrays. However, the lithographic peptide synthesis requires a sequential application of monomer solutions, which is acceptable, when a rather limited number of monomers is needed (as in DNA microarray synthesis) [9]. Yet, this results in a high number of repetitive coupling cycles in the synthesis of peptide microarrays, where at least 20 different amino acids are used as building blocks. Thus, the first "fully" combinatorial approach to the synthesis of peptide microarrays by Ronald FRANK published in 1992 was a milestone in the development of this field. His SPOT synthesis is a spatially resolved spotting technique which, in general, uses the same principles as MERRIFIELD's solid phase peptide synthesis [13, 14]. Droplets containing the pre-activated monomers are applied to a modified cellulose sheet in a distinct pattern, whereby all 20 proteinogenic amino acids can be addressed in a single run. In the first step, the amino acids readily couple to functional groups linked to the cellulose. In the following steps the peptides are subsequently elongated [15, 16]. Orthogonal protection of the amino acids ensures that only the free amino terminus reacts with the next building block, while the side chains remain protected until the final deblocking step. However, these peptide arrays are limited in peptide spot density due to spreading and evaporation of the solvent during the synthesis. The direct SPOT synthesis thus reaches only a maximum resolution of 25 peptides per cm^2 (with up to 15 amino acid residues). To achieve a higher spot density on the array, labor-intensive steps, including peptide cleavage from the synthesis support and re-spotting, are required [17, 18]. Numerous applications of peptide arrays synthesized with the SPOT technique can be found in the literature [8, 17]. Still, commercial peptide arrays produced in situ by the SPOT synthesis are offered at a price of 7–14 € per peptide, which is quite inferior to the economic efficiency of DNA arrays [8].

A new approach developed in our research group has solved the problems in synthesizing affordable high density peptide microarrays by using solid amino acid microparticles. The microparticles comprise the Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) and orthopentafluorophenyl (Opfp) pre-activated amino acid building blocks and can be selectively addressed onto a solid support with a custom-built laser printer [19]. The microparticle-based synthesis reaches much higher resolutions than the SPOT synthesis because it circumvents the use of solvents and the described disadvantages [19, 20]. When a combinatorial layer consisting of a specific microparticle deposition pattern is completed, i.e. when all types of monomer particles are successfully addressed, the peptide synthesis is initiated by melting the polymeric microparticle matrix (see Fig. 1a, b). Highly viscous reaction spheres are formed in which the amino acid building blocks can diffuse to the surface and couple to the functional groups (see Fig. 1b). Standard washing and deblocking steps complete a synthesis cycle (see Fig. 1c, d).

Since all types of particles can be addressed in a single laser printer run, only 20 coupling cycles are, for example, required for 20meric peptides, whereby the resulting peptide quality is equivalent to standard synthesis from solution [19]. Strictly following MERRIFIELD's principle of orthogonal synthesis, up to 281,000 individual peptides can be arrayed on a single solid support (active area: 19 cm × 19 cm) with the latest laser printer generation (see Fig. 2) [21].

To synthesize peptides on a solid support, the substrate (which is standard microscopy glass), is routinely coated with a copolymer of poly(ethylene glycol) methacrylate (PEGMA) and polymethylmethacrylate (PMMA) (see Fig. 1i–ii) [22]. These covalently anchored graft polymer coatings can be functionalized with Fmoc-β-alanine to yield amino groups for the peptide synthesis (see Fig. 1iii) [23]. Furthermore, the composition of the polymer determines the protein repelling properties of the surface which can, thus, be fine tuned for the desired assay [22].

Here, we describe the synthesis and analysis of a peptide array, featuring the *tetanus toxin* protein (1315 amino acids), mapped onto an array of overlapping 15meric peptides. The bacterium *clostridium tetani* which elicits the *tetanus* disease is a very common pathogen. The pathogen produces a toxic protein, which is called *tetanus toxin* or *tetanospasmin*. In infected patients, this toxin blocks inhibitory neuron action, which leads to chronic muscle contraction and untreated eventually to death [24]. Fortunately, with respect to this disease, vaccination is available. Vaccination against the *tetanus toxin* is a routine procedure and almost every person in Western countries has had such a vaccination at least once in his or her life. Thus, *tetanus* seems to be an appropriate proof-of-principle target in sensing human immune responses. The human adaptive immune system reacts to a specific pathogen by evolving antibodies directed against it. Meanwhile, it is well known that immunodominant epitopes are prevalent in many pathogens. Those cause the adaptive immune system of different individuals to react in similar ways, evolving antibodies, which are directed against the same immunodominant epitope, e.g. in viruses [25, 26]. The question arises, whether it is possible to find immunodominant regions of a specific pathogen with our peptide-based approach. Therefore, a proof-of-principle peptide microarray experiment was devised.

2 Methods

2.1 Surface Preparation

2.1.1 Silanization

22 cm × 21 cm glass slides were cleaned and activated by overnight treatment with 1 M KOH in 2-propanol. The
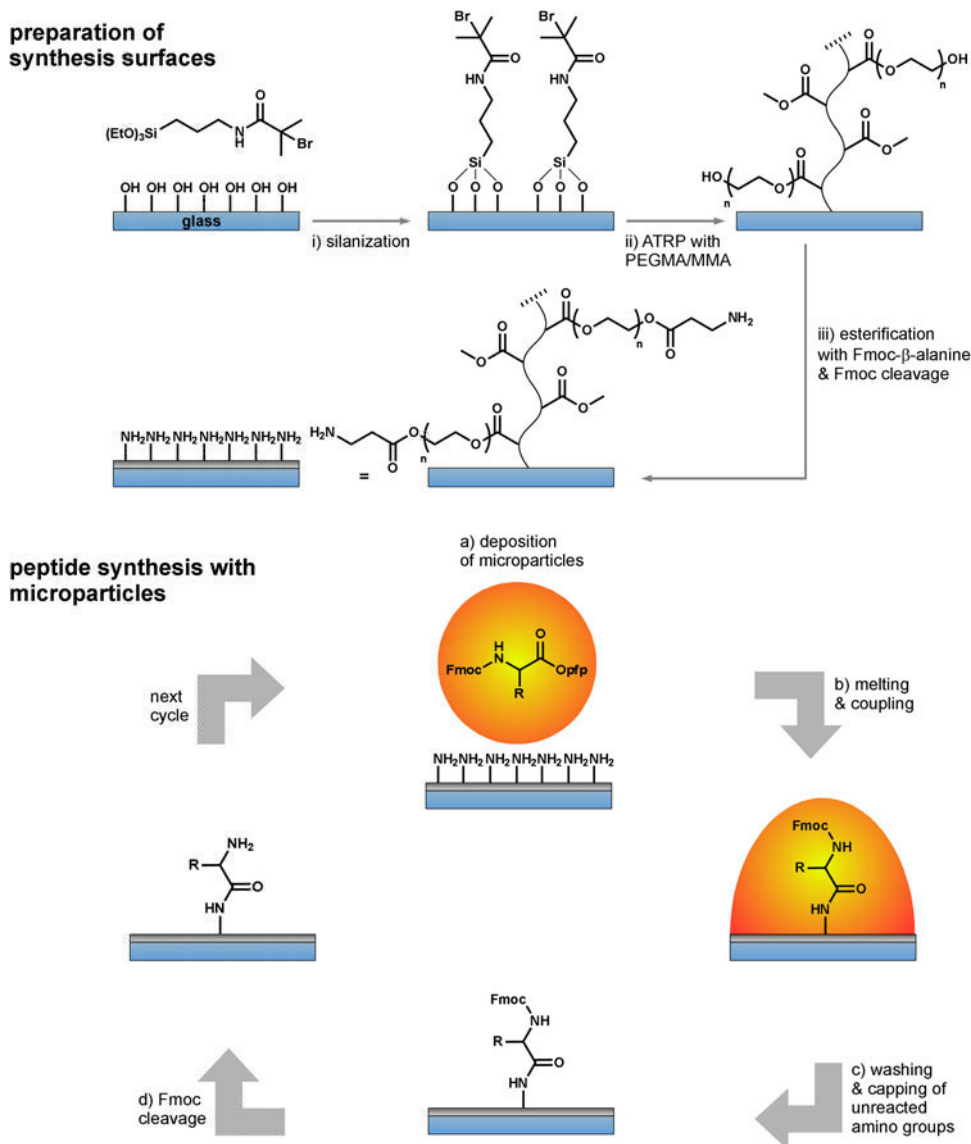
Fig. 1 Schematic of the preparation of synthesis surfaces for the laser printer-based peptide microarray synthesis and schematic of a synthesis cycle in the combinatorial synthesis. *i)* Cleaned and activated glass substrates are silanized with 2-bromo-*N*-(3-triethoxysilyl)propyl iso-butyramide to provide an initiator for the atom transfer radical polymerization (ATRP). *ii)* The ATRP with PEGMA and MMA yields a graft polymer coating which is covalently linked to the glass substrate (silane and MMA backbone are depicted as a sidled line). *iii)* The hydroxyl groups in the PEG side chains are esterified with Fmoc-*β*-alanine to provide amino groups for the peptide synthesis. Synthesis cycle: *a)* Microparticles containing the N-terminally Fmoc protected and C-terminally Opfp activated amino acids are deposited on the synthesis support. *b)* Heating of the slide leads to melting of the polymeric particle matrix and, thus, gives viscous reaction spheres in which the amino acids can couple to the amino groups on the slide. *c)* Routine washing removes microparticle residues such as matrix material and unreacted amino acids. Capping of unreacted amino groups on the surface helps to avoid formation of incorrect peptide sequences. *d)* Removal of the N-terminal Fmoc protecting groups renders new amino groups for the next synthesis cycle

slides were intensively rinsed with water followed by acetone, dried in a stream of air and then baked in an oven at 110 °C for 30 min. A 2-bromo-*N*-(3-triethoxysilyl)pro-pyl isobutyramide self assembled monolayer (SAM), serving as atom transfer polymerization (ATRP) initiator, was introduced according to the following protocol: A solution of 2 mM 2-bromo-*N*-(3-triethoxysilyl)propyl iso-butyramide (synthesized) and 8 mM propyltriethoxysilane (PTES, Sigma Aldrich, Germany) in anhydrous dichloro-methane (DCM) was prepared and directly added to the activated dry glass slides. The slides were left to react overnight under inert gas atmosphere. The DCM was then rinsed off the surfaces with an excess of absolute ethanol. The slides were washed three times for 5 min each with absolute ethanol, two times for 2 min each with acetone, dried in a stream of nitrogen, and then baked in a

Fig. 2 Pictures of the latest laser printer generation: The 24 linearly aligned printing cartridges (*right*) in the printing track form the core of the 5.5 m long device (*left*)

pre-heated oven at 110 °C for 2 h. After cooling to room temperature the slides were directly reacted in the polymerization step. 2-bromo-*N*-(3-triethoxysilyl)propyl isobutyramide is not commercially available and had to be synthesized according to [27] prior to silanization.

2.1.2 Atom Transfer Radical Polymerization (ATRP)

A 50–60 nm polymeric film consisting of 10 % PEGMA and 90 % PMMA was grafted to the silanized surfaces of the slides according to the following protocol: The silanized slides were placed in a suited reaction vessel which was adjusted in a desiccator and brought to inert gas atmosphere. Per glass slide, 43.75 mmol (14.4 mL) poly(ethylene glycol) methacrylate (PEGMA, $M_w \approx 360$ g/mol, $n \approx 5$, Sigma Aldrich, Germany), 393.75 mmol (41.9 mL) MMA (Merck, Germany), 2.20 mmol (455 μL) 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA, Merck, Germany) and 19.8 mmol (3.1 mL) tri(ethylene glycol) monomethyl ether (TEGMME, Merck, Germany) were dissolved in 185 mL dimethylsulfoxide (DMSO) in a nitrogen flask. The solution was degassed by evacuating the flask and flooding it with inert gas three times. 2.20 mmol (220 mg) CuCl (Sigma Aldrich, Germany) were added in inert gas counter stream. The solution was stirred until the copper salt was completely dissolved. The solution was then quickly filled into the reaction vessel inside the desiccator. The desiccator was again thoroughly evacuated and flooded with inert gas three times. The polymerization was left to react for 20 h at room temperature. Subsequently, the glass slides were washed five times for 5 min each with DMSO, two times for 5 min each with methanol, and two times for 10 min each with water. After rinsing with acetone, the surfaces were dried in a stream of nitrogen. The resulting polymer layer thickness can be verified via ellipsometry if a piece of silanized Si(100) wafer is processed in the same reaction vessel [22, 28].

2.1.3 Amino-modification of the Polymer Coating

A solution of 0.1 M Fmoc-*β*-alanine (Iris Biotech, Germany) in anhydrous *N,N*-dimethylformamide (DMF) was prepared in a nitrogen flask. 0.12 M *N,N*'-diisopropylcarbodiimide (DIC, Sigma Aldrich, Germany) were added and the solution was stirred for 5 min. Subsequently, 0.2 M *N*-methylimidazole (NMI, Sigma Aldrich) were added. The solution was directly added to the PEGMA/PMMA-modified glass slides which were placed in a suited reaction vessel inside a desiccator. The desiccator was then brought to inert gas atmosphere. The surfaces were left to react overnight. Afterwards, the slides were washed three times for 5 min each with DMF. To cap residual hydroxyl groups, the slides were directly incubated in a solution of 10 % (v/v) acetic anhydride (Ac$_2$O, Roth, Germany), 20 % (v/v) *N,N*-diisopropylethylamine (DIPEA, Merck, Germany), and 70 % (v/v) DMF overnight. After washing five times for 5 min each with DMF and two times for 2 min each with methanol the surfaces were dried in a stream of inert gas. To cleave the Fmoc protecting groups the slides were incubated in a solution of 20 % (v/v) piperidine (Sigma Aldrich, Germany) in DMF for 20 min. The Fmoc cleavage was followed by washing three times for 5 min each with DMF and two times for 3 min each with MeOH. For the peptide synthesis, the whole procedure was repeated two times to sequentially couple three *β*-alanine residues to the polymer coating [23].

2.2 Peptide Array Synthesis

2.2.1 Routine Peptide Synthesis with the Laser Printer

The current laser printer generation is equipped with 24 printing units which are assembled on a linear stage. Twenty cartridges are filled with Fmoc-amino acid particles (for particle synthesis and composition refer to [19]), whereas the remaining cartridges can be used to print

non-standard amino acids. The printing process is completely automated. A derivatized glass slide is inserted and the different amino acid toners are applied in micrometer resolution in a single run.

After printing the pattern, i.e. one complete combinatorial layer, the following routine coupling and washing steps were performed: The glass slides were transferred into a pre-heated oven and allowed to react at 90 °C for 90 min under inert gas atmosphere. After cooling to room temperature, unreacted amino groups were directly capped with 10 % (v/v) Ac_2O, 20 % (v/v) DIPEA, and 70 % (v/v) DMF by shaking the slides in an excess of this mixture for 30 min. Subsequently, the slides were washed two times for 5 min each with DMF and 5 min with acetone. The slides were either stored at 4 °C under argon atmosphere or directly deprotected for the next coupling cycle. To cleave the Fmoc protecting group, the slides were rocked in a solution of 20 % (v/v) piperidine in DMF for 20 min. Afterwards, the slides were washed three times for 5 min each with DMF, two times for 3 min each with MeOH, and then dried in a stream of inert gas.

2.2.2 Peptide Side-Chain Deprotection

To remove the side-chain protecting groups after the completed peptide synthesis, the array was rocked in a solution of 51 % (v/v) trifluoro acetic acid (Biosolve, The Netherlands), 44 % (v/v) DCM, 3 % (v/v) triisobutylsilane (TIBS, Sigma Aldrich, Germany), and 2 % (v/v) H_2O for 90 min. Subsequently, the slides were washed five times for 5 min each with DCM, two times for 2 min each with methanol, then rinsed with acetone, and dried in a stream of inert gas.

2.3 Immuno-Assay

2.3.1 Preparation of the Standard Incubation Buffer (Phosphate Buffer Saline)

Phosphate buffer saline (PBS, pH 7.4, 0.15 M) was prepared dissolving 137 mmol (8.0 g) NaCl, 2.7 mmol (0.2 g) KCl, 8.1 mmol (1.44 g) $Na_2HPO_4 \cdot 2 H_2O$, and 1.5 mmol (0.2 g) KH_2PO_4 in 1 L MilliQ (Millipore, US) water. 500 μL (0.05 % (v/v)) Tween20 (Sigma Aldrich, Germany) was added to prepare PBS-T.

2.3.2 Serum Acquisition

Blood samples were acquired from three adult individuals, between 8 weeks and 2 years after *tetanus* vaccination. While storing the samples in serum tubes at 4 °C overnight, the agglutination of the blood proceeds. Subsequently, the

serum was separated by centrifugation and then aliquoted and stored at −20 °C.

2.3.3 Array Processing and Readout

After peptide synthesis and side chain deprotection, the peptide arrays were incubated in PBS-T for 30 min, and then blocked in Rockland Blocking Buffer MB-070 (Rockland, US) for 1 h at room temperature. Blocking was followed by a short washing step (10 s) with PBS-T. Then, the arrays were directly incubated with the serum samples for 16 h (diluted 1:100 in PBS-T + 1:10 blocking buffer). After two short washing steps with PBS-T, binding was detected with the corresponding secondary antibodies F(ab')$_2$ goat-Anti-human conjugated with the fluorescent dye DyLight 680 (Thermo Scientific, US). After 30 min of incubation at room temperature, arrays were shortly washed with PBS-T and rinsed with MilliQ water to remove residual buffer salts prior to scanning.

Control HA epitope (YPYDVPDYA) and Flag epitope (DYKDDDDK) spots were stained with monoclonal mouse-Anti-Flag M2 IgG1 (Sigma Aldrich, US) conjugated with FluoProbes 752 (Lightning-Link, Innova Biosciences, UK) and monoclonal mouse-Anti-HA 12CA5 IgG antibodies (provided by Dr. G. Moldenhauer, DKFZ) conjugated with Atto680 (Lightning-Link, Innova Biosciences, UK). Both antibodies were diluted 1:1000 in PBS-T + 1:10 blocking buffer. Staining was performed for 30 min at room temperature followed by washing as described above.

2.3.4 Stripping Buffer and Protein Stripping

The stripping buffer was prepared dissolving 1 % (w/v) (1.0 g) sodium dodecyl sulfate (SDS) and 20 mmol (1.5 g) glycine in 100 mL MiliQ water and, afterwards adjusting the buffer with HCl to pH 2.0. The array was stripped from all antibodies and proteins by sonicating it for 30 min in the stripping buffer at 70 °C. Then, the array was sonicated in MiliQ water for additional 45 min at 70 °C, to remove residues of the stripping buffer.

2.3.5 Fluorescence Scanner

Fluorescence images were obtained with the Odyssey *Infrared Imager* (LI-COR, US). The scanner is equipped with two lasers (excitation wavelengths 685 and 785 nm) and filters optimized for the emission wavelengths 700 and 800 nm. Scanner sensitivities were set to 7.0 for the 700 and 800 nm channels, the focal plane was set to +0.8 mm. Note: In the acquired images, the 700 nm channel is displayed in red color and the 800 nm channel in green color (false colors).

3 Results

3.1 Peptide Array Synthesis

The task of finding a dominant immunogenic region was addressed by mapping the *tetanus toxin* on a peptide microarray. The amino acid sequence of the *tetanus toxin* (1315 amino acids) was taken from the literature ([29]) and was extended with 9 amino acids as initial and final linker (shown in Table 1). This sequence was divided in 1319 overlapping 15mer sequences and arranged in 20 × 66—1 spot duplicates, with an overlap of 14 amino acids in each following spot. The array was surrounded by alternating Flag and HA wild type epitopes serving as staining controls. Two replicas of the *tetanus toxin* array were printed on a glass slide coated with an approximately 50–60 nm thick graft polymer film (as determined by ellipsometry on a Si(100) reference, for details see [22]) composed of 10 % PEGMA and 90 % PMMA. Surface coating and peptide array synthesis were administered in the company PEP-perPRINT GmbH (Heidelberg, Germany) as described in the methods section.

3.2 Tetanus Toxin Mapping Using Laser Printed Arrays

The obtained arrays were incubated with the blood sera of three different *tetanus* vaccinated individuals to demonstrate the potential of peptide arrays in the readout of specific immune responses. The three sera were acquired from individuals with a *tetanus* vaccination within the last 2 years, but at least 8 weeks ago to assure that the immune system has had enough time for a response. In addition, one serum (see Fig. 3a) was positively tested for a sufficient titer of anti-*tetanus* antibodies, which indicates a sufficient immunity. Although an approach with only three sera is rather subjective, this first screen can pinpoint to those candidate peptides, which are often targeted by the immune system. Therefore, this screen was expected to yield candidates for smaller and more specialized peptide microarrays which should, in turn, allow for screens with even less serum consumption.

Figure 3 shows the results of the *tetanus toxin* mapping with three different sera on two different arrays (for economical reasons, one array was reused after antibody
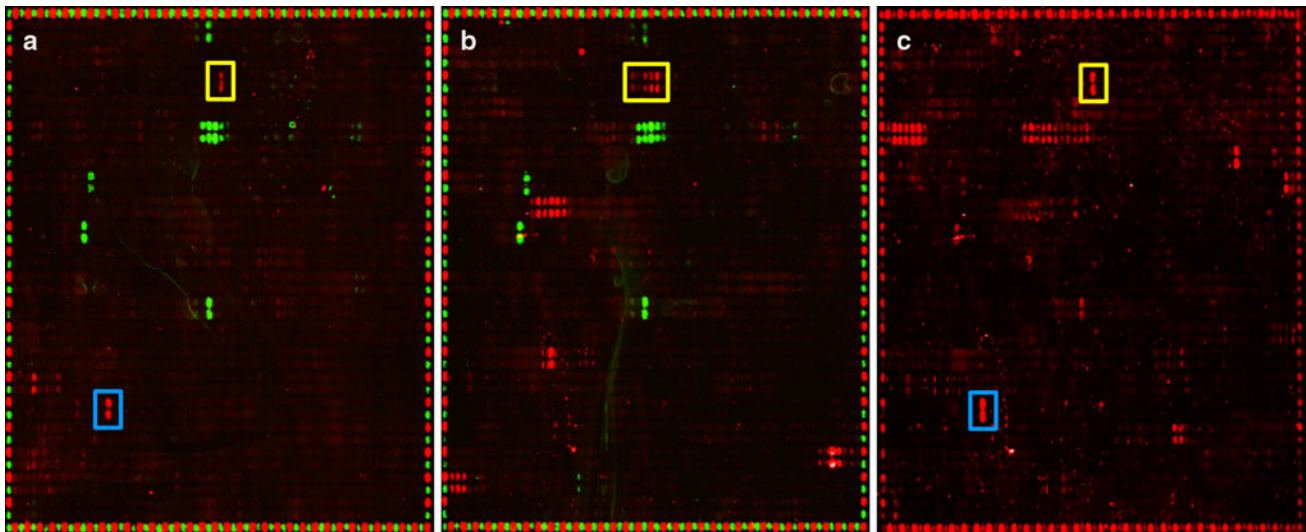
Table 1 Amino acid sequence of the *tetanus toxin* (1315 amino acids) amended with an initial and final linker of nine amino acids each (GSGSGSGSG). The light chain is located between the amino acids 2–457, the heavy chain begins at the amino acid number 458

```
GSGSGSGSG
MPITINNFRYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFN      60
PPSSLIEGASEYYDPNYLRTDSDKDRFLQTMVKLFNRIKNNVAGEALLDKIINAIPYLGN     120
SYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGPGPVLNKNEVRGIVLRVDN     180
KNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENITSLTIGKSKYFQDPALLLMHELIHVLH     240
GLYGMQVSSHEIIPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLNDYK     300
AIANKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSNGQYIVNEDKFQILYNSIMYGFTE     360
IELGKKFNIKTRLSYFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMRVNT     420
NAFRNVDGSGLVSKLIGLCKKIIPPTNIRENLYNRTASLTDLGGELCIKIKNEDLTFIAE     480
KNSFSEEPFQDEIVSYNTKNKPLNFNYSLDKIIVDYNLQSKITLPNDRTTPVTKGIPYAP     540
EYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIYSYFPSVI     600
SKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGN     660
FIGALETTGVVLLLEYIPEITLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYK     720
LVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKIYSGPDKEQIADEINNLKN     780
KLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLLEFDTQSKNILMQYIKANSKFIG     840
ITELKKLESKINKVFSTPIPFSYSKNLDCWVDNEEDIDVILKKSTILNLDINNDIISDIS     900
GFNSSVITYPDAQLVPGINGKAIHLVNNESSEVIVHKAMDIEYNDMFNNFTVSFWLRVPK     960
VSASHLEQYGTNEYSIISSMKKHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLP    1020
DKFNAYLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNITLKLDRCNNN    1080
NQYVSIDKFRIFCKALNPKEIEKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSSKDV    1140
QLKNITDYMYLTNAPSYTNGKLNIYYRRLYNGLKFIIKRYTPNNEIDSFVKSGDFIKLYV    1200
SYNNNEHIVGYPKDGNAFNNLDRILRVGYNAPGIPLYKKMEAVKLRDLKTYSVQLKLYDD    1260
KNASLGLVGTHNGQIGNDPNRDILIASNWYFNHLKDKILGCDWYFVPTDEGWTND        1315
GSGSGSGSG
```

Fig. 3 Epitope mapping of the *tetanus toxin*. The array content was fabricated as spot duplicates (center-to-center spot distance 254 μm × 508 μm) and the HA and Flag controls in the surrounding frame as single spots (*red/green*). Two arrays with identical peptide composition (1319 spot duplicates of 15meric peptides) were stained with two sera from *tetanus* vaccinated individuals (**a**, **b**). The array shown in (**b**) was then stripped from all antibodies using a *stripping buffer* and afterwards incubated with the serum taken from a third immunized individual (**c**). The array frames (controls) in (**a**, **b**) were stained with anti-Flag (shown in *green*) and anti-HA antibodies (shown in *red*). In array (**c**), the HA control staining was persistent to the stripping buffer, which is why a repeated control staining was omitted. Coinciding peptides in the *tetanus toxin* mapping are marked in yellow and blue boxes (also see "Discussion")

stripping). First, each array was incubated with the secondary goat-anti-human antibodies, to distinguish the binding events arising from binding of the secondary antibodies (data not shown). The results showed no evidence for significant binding of secondary antibodies. In a subsequent step, each array was incubated with the serum and, afterwards, binding events were detected with the secondary antibodies (fluorescence signals from the Atto 680 dye are displayed in red). After a first readout (not shown), the single spot control peptides (HA and Flag epitope frame) were stained with anti-Flag (displayed in green) and anti-HA antibodies (displayed in red) and the arrays were scanned again (Fig. 3a, b). In contrast to other microarray techniques, where circular or quadratic features are standard, the shape of the peptide spots resembles a rectangle which is an effect of the laser printing technique. The array content was fabricated as spot duplicates (two neighboring spots each) in order to rule out artifacts, with a single spot center-to-center distance of 254 μm × 508 μm. The immunostaining features low background noise and a homogenous control spot staining. Regarding the control staining of Flag epitopes, an observation was that anti-Flag antibodies also specifically bind to sequence variants of the Flag epitope within the array (additional green spots in Fig. 3a, b). In contrast, the anti-HA antibodies did not show such side-effects and binding was strictly limited to the HA epitopes in the control frame.

Figure 3c shows array (b) after antibody stripping (30 min in stripping buffer), incubation with the serum taken from a third individual, and detection of binding events with the secondary antibodies. Although the stripping procedure obviously did not remove the HA control peptide staining (dye conjugated antibodies or residual fluorescent molecules), efficient removal of the serum antibodies was checked by pre-staining with secondary anti-human antibodies as described before (data not shown).

Comparing the results of the three serum incubations, peptide spots with coinciding amino acid sequences were stained, which are highlighted in Fig. 3 (yellow and blue boxes). In particular, all three arrays exhibit a staining of the peptide with the sequence LIIFGPGPVLNKNEV (yellow boxes). A second peptide, which is only found on the arrays (a) and (c), has the amino acid sequence GNNLIWTLKDSAGEV (blue boxes).

4 Discussion

The *tetanus toxin* is composed of two domains, a heavy and a light chain. The heavy chain is required for cell binding and subsequent internalization into the cell, but only the light chain causes the intracellular toxic effect in neurons. For vaccination, the toxic light chain is inactivated by protein denaturation, yielding the so called *tetanus toxoid*, which has no neurotoxic effect. If injected, the body can create antibodies directed against this protein, making the human organism "immune" to the (active) tetanus toxin.

With respect to the results of the protein mapping, all three sera seem to contain an antibody species directed against the peptide LIIFGPGPVLNKNEV (Fig. 3, upper yellow box). This sequence is part of the light chain and is located between the 156th and 171st amino acid in the *tetanus toxin* sequence. In addition, the sera in (a) and (c) seem to contain antibodies against the peptide GNNLIWTLKDSAGEV which is located on the heavy chain (996–1011).

A remarkable result is that the coinciding spots in arrays (a) and (c) seem to exhibit no stained neighboring spots. This might indicate that the length of the binding epitope is exactly 15 amino acids. The epitope LIIFGPGPVLNKNEV is unique in the array and in the mapped protein. However, an intrinsic side-effect of protein mapping with surface-bound peptides can be steric hinderance by the surface coating which could have inhibited the binding of antibodies to adjacent peptides with partially identical sequence motifs. Further experiments with longer peptides (e.g. 17meric) might help to explore this phenomenon. Interestingly, in array (b), a shorter epitope seems to be recognized, which is indicated by stained neighboring spots. This might imply that the corresponding antibodies in serum (b) are directed against a shorter, approximately 10meric, binding epitope (approximately 5 neighboring double spots are stained; see Fig. 3b, yellow box).

5 Conclusions

Concluding from these results, the dominating antibody species formed upon *tetanus* vaccination might be directed against the amino acid sequence LIIFGPGPVLNKNEV. However, due to the limited approach involving only 3 sera, further experiments with additional sera from a representative number of individuals need to be performed to confirm this result. However, the experiment proves that peptide arrays are highly suited to readout immune responses, to map binding epitopes, and, thus, to develop highly specialized biosensors for diagnostics. Based on the described preliminary findings, smaller and more specialized arrays for the *tetanus toxin* mapping with only a few selected peptides will be produced to approach the next screenings with more array replicas per synthesis slide and less serum consumption per screen. In summary, the experiment underlines that customized peptide arrays are a versatile tool to sense immune-responses and, thus, to further develop biosensors for numerous biologically relevant peptide-protein interactions.

References

1. Smith G (1985) Science 228(4705):1315–1317. doi:10.1126/science.4001944
2. Dubel S, Breitling F, Fuchs P, Braunagel M, Klewinghaus I, Little M (1993) Gene 128(1):97–101. doi:10.1016/0378-1119(93)90159-z
3. Jostock T, Dubel S (2005) Comb Chem High Throughput Screen 8(2):127–133. doi:10.2174/1386207053258479
4. Ohlmeyer MH, Swanson RN, Dillard LW, Reader JC, Asouline G, Kobayashi R, Wigler M, Still WC (1993) Proc Nat Acad Sci 90(23):10922–10926
5. Liu RW, Enstrom AM, Lam KS (2003) Exp Hematol 31(1):11–30. doi:10.1016/s0301-472x(02)01008-1
6. Ekins RP (1989) J Pharm Biomed Anal 7(2):155–168. doi:10.1016/0731-7085(89)80079-2
7. MacBeath G, Schreiber SL (2000) Science 289(5485):1760–1763
8. Breitling F, Nesterov A, Stadler V, Felgenhauer T, Bischoff FR (2009) Mol BioSyst 5(3):224–234
9. Lipshutz RJ, Fodor SPA, Gingeras TR, Lockhart DJ (1999) Nat Genet 21:20–24. doi:10.1038/4447
10. Southern E, Mir K, Shchepinov M (1999) Nat Genet 21 (1 SUPPL.):5–9. doi:10.1038/4429
11. Egeland RD, Southern EM (2005) Nucleic acids res 33(14):e125
12. Fodor SPA, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Science 251(4995):767–773
13. Frank R (1992) Tetrahedron 48(42):9217–9232. doi:10.1016/s0040-4020(01)85612-x
14. Merrifield RB (1965) Science 150(3693):178–184
15. Frank R (2002) J Immunol Methods 267(1):13–26
16. Frank R (2002) Comb Chem High Throughput Screen 5(6):429–440
17. Volkmer R (2009) ChemBioChem 10(9):1431–1442. doi:10.1002/cbic.200900078
18. Winkler DF, Hilpert K, Brandt O, Hancock RE (2009) Methods Mol Biol (Clifton, NJ) 570:157–174
19. Stadler V, Felgenhauer T, Beyer M, Fernandez S, Leibe K, Güttler S, Gröning M, König K, Torralba G, Hausmann M, Lindenstruth V, Nesterov A, Block I, Pipkorn R, Poustka A, Bischoff FR, Breitling F (2008) Angewandte Chemie Int Edn 47(37):7132–7135
20. Breitling F, Felgenhauer T, Nesterov A, Lindenstruth V, Stadler V, Bischoff FR (2009) ChemBioChem 10(5):803–808
21. http://www.pepperprint.com/
22. Stadler V, Kirmse R, Beyer M, Breitling F, Ludwig T, Bischoff FR (2008) Langmuir 24(15):8151–8157
23. Beyer M, Felgenhauer T, Bischoff FR, Breitling F, Stadler V (2006) Biomaterials 27(18):3505–3514. doi:10.1016/j.biomaterials.2006.01.046
24. Janeway C (2005) Immunobiology: the immune system in health and disease, 6th edn. Garland Science, New York
25. Ferguson NM, Galvani AP, Bush RM (2003) Nature 422(6930):428–433. doi:10.1038/Nature01509

26. Tobin GJ, Trujillo JD, Bushnell RV, Lin G, Chaudhuri AR, Long JX, Barrerad J, Pena L, Grubman MJ, Nara PL (2008) Vaccine 26(49):6189–6199. doi:10.1016/j.vaccine.2008.09.080

27. Schirwitz C, Block I, König K, Nesterov A, Fernandez S, Felgenhauer T, Leibe K, Torralba G, Hausmann M, Lindenstruth V, Stadler V, Breitling F, Bischoff FR (2009) Curr Protoc Protein Sci (SUPPL. 57) Chapter 18:18.12.11–18.12.13

28. Stadler V, Beyer M, König K, Nesterov A, Torralba G, Lindenstruth V, Hausmann M, Bischoff FR, Breitling F (2007) J Proteome Res 6(8):3197–3202

29. Fairweather NF, Lyness VA (1986) Nucleic Acids Res 14(19): 7809–7812