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REGULAR ARTICLE

Protein microarrays for antibody profiling: Specificity and affinity determination on a chip

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Protein microarray technology facilitates the detection and quantification of hundreds of binding reactions in one reaction from a minute amount of sample. Proof-of-concept studies have shown that the set-up of sensitive assay systems based on protein arrays is possible, however, the lack of specific capture reagents limits their use. Therefore, the generation and characterisation of capture molecules is one of the key topics for the development of protein array based systems. Recombinant antibody technologies, such as HuCAL® (human combinatorial antibody library; MorphoSys, Munich, Germany), allow the fast generation of highly specific binders to nearly any given target molecule. Although antibody libraries comprise billions of members, it is not the selection process, but the detailed characterisation of the pre-selected monoclonal antibodies that presents the bottleneck for the production of high numbers of specific binders. In order to obtain detailed information on the properties of such antibodies, a microarray-based method has been developed. We show that it is possible to define the specificity of recombinant Fab fragments by protein and peptide microarrays and that antibodies can be classified by binding patterns. Since the assay uses a miniaturised system for the detection of antibody-antigen interactions, the observed binding occurs under ambient analyte conditions as defined by Ekins (*J. Pharm. Biomed. Anal.* 1989, *7*, 155–168). This allows the determination of a relative affinity value for each binding event, and a ranking according to affinity is possible. The new microarray based approach has an extraordinary potential to speed up the screening process for the generation of recombinant antibodies with pre-defined selection criteria, since it is intrinsically a high-throughput technology.

Keywords:
Affinity / Antibody / Epitope mapping / HuCAL® / Peptide microarrays / Protein microarrays / Screening

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Abbreviations: aa, amino acid; **Ab**, antibody; **[Ab],** concentration of free antibody; **[Ab_0],** initial concentration of antibody; **AbAg complex**, antibody-antigen complex; **[AbAg]**, concentration of antibody-antigen complex; **Ag**, antigen; **[Ag]**, concentration of free antigen; **[Ag_0]**, initial concentration of antigen; **BBS**, borate-buffered saline solution; **HBS**, HEPES-buffered saline solution; **HuCAL**, human combinatorial antibody library; **SPR**, surface plasmon resonance

1 Introduction

Recent technological advances have resulted in the miniaturisation of ligand binding assays and highly sensitive systems have been developed that allow the quantification of analyte from a minute amount of sample [1, 2]. The possibility to parallelise a high number of assays has lead to the set-up of analytical systems that are capable of analysing thousands of parameters within a single experiment. Such global methods were initially employed to study gene expression, and the term DNA-chip or DNA-microarray was used to describe the technology [3]. While these experiments have proven the exceptional potential of micro-

arrays, their use is not limited to generate mRNA expression patterns. Over recent years, it could be demonstrated that a parallel analysis of proteins is feasible and systems using proteins as immobilised capture molecules have been developed [4–6]. The challenges are different when working with proteins, but nevertheless the underlying principle of a solid-phase binding assay is the same for DNA- or protein-based microarrays. A major difference has to be seen in the nature of the capture reagents that are used on the solid phase. The generation of complex DNA-microarrays became possible since the prediction and the *in vitro* synthesis of a nearly infinite number of highly specific capture molecules, namely, the complementary strand of the nucleic acid of interest is doable. For proteins the generation of capture reagents is a major issue since such binders are often not available and their generation can be time-consuming and expensive [7]. Today, mAbs produced by the classical hybridoma technique are the most common capture reagents for protein microarrays, but the more recently developed technologies to isolate binding molecules from large synthetic libraries allow a more focused approach.

As an *in vitro* technology, phage display based synthetic libraries allow the fast generation of mAbs against almost any given target molecule, including non-immunogenic and toxic ones. Such capture molecules are often required for the generation of biosensors used in environmental or medical screenings. Due to the selection process during phage display, such antibodies are *per se* monoclonal and usually a panel of highly specific binders is generated targeting various epitopes of the antigen. The diversity of such capture molecules on the 'chip' obviously increases its specificity for the target. The *in silico* design of such libraries furthermore allows the immobilisation of the capture antibody on the array *via* customised tags at pre-defined sites on the molecule. If required, even the antibody format can be switched between monovalent Fab and bivalent IgG constructs. Thus, the combinatorial antibody library HuCAL® [8, 9] that comprises all these features represents an ideal source for antibody array applications.

However, even if the generation of capture molecules has become a state-of-the-art procedure, it is the detailed characterisation of several hundreds to thousands of preselected binders from these libraries, which requires new innovative methods providing high-sensitivity and high-throughput at the same time. We have therefore developed a miniaturised and parallelised microarray system that is capable of providing such information. By using microarrays consisting of proteins and peptides, the concurrent analysis of multiple binding events is possible; this allows to define the epitope recognised by a given binder. On the same array, a relative affinity value for each binding event can be determined. The combination of both information can then be used to discriminate antibodies according to their affinities and by their specificity for the respective antigen.

2 Materials and methods

2.1 Antibody generation and affinity determination

Fab fragments were isolated from the HuCAL®-Fab 1 phage display antibody library [10] in a high-throughput procedure as described earlier [11]. The protein antigens were two recombinant proteins, one of which was derived from the cell surface glycoprotein CD11b (amino acid (aa) 133–337 of ITAM_HUMAN, purchased from the Institute of Bioanalytics, Goettingen, Germany); the peptide antigen (termed here M18) used is a synthetic 25mer peptide corresponding to amino acid 289–313. Expression of Fab fragments in *E. coli* TG-1 cells was carried out in shake flask cultures with 1 L of 2 × YT medium supplemented with 34 $\mu g \cdot mL^{-1}$ chloramphenicol. After induction with 0.5 mM IPTG, cells were grown at 22°C for 16 h. For preparation of periplasmic extracts, cell pellets were resuspended in BBS (200 mM boric acid containing 160 mM NaCl and 2 mM EDTA, pH 8.0) and centrifuged at 5000 × *g* for 30 min (4°C). Fab molecules isolated from the HuCAL®-Fab 1 library were purified by Streptactin® chromatography system as described by the manufacturer (IBA, Goettingen, Germany). The apparent molecular weights were determined by SEC with calibration standards. Concentrations were determined by UV-spectrophotometry. For microarray screening, periplasmic preparations of Fab fragments produced in 96-well microtitre plates were used.

Binding constants of monomeric fractions of purified Fab fragments were determined by surface plasmon resonance (SPR) using the BIAcore system (Uppsala, Sweden). All experiments were conducted in HBS buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) at a flow rate of 20 $\mu L \cdot min^{-1}$ at 25°C on a BIAcore3000™ instrument. Antigens in 100 mM sodium acetate pH 5.0 were coupled to a CM 5 sensor chip using standard EDC–NHS coupling chemistry. All sensograms were fitted globally using BIA evaluation software and a 1:1 binding model (Langmuir binding).

2.2 Peptide synthesis

Solid phase peptide synthesis [12] was performed on a multiple peptide synthesiser Syro II (MultiSynTech, Bochum, Germany) using Fmoc strategy [13]. Every coupling was followed by a capping of free amino functions with acetanhydride. Deprotecting and the separation of the peptides from the resin was done by incubation with 82.5% TFA/5% phenol/2.5% ethanedithiol/5% thioanisol (Fluka, Buchs, Switzerland). Quantification and quality control was done by HPLC (Gynkotek, Germering, Germany) and mass spectrometry (ESI-Q-Tof 1; Micromass, Manchester, UK). For epitope mapping, peptides were designed as 15mers with an overlap of 11 amino acids. Internal peptides were manufactured as amides. To allow the attachment of the peptides to carrier proteins, a spacer of two aminocaproic acid molecules and a cysteine was added to the *N*-terminus of the peptides.

2.3 Preparation of peptide bovine serum albumin conjugates

Peptide-BSA conjugates were prepared using pre-activated BSA with the bi-functional linker 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylic acid 3-sulpho-*N*-hydroxy-succinimideester (SMCC) (Merck Biosciences, Bad Soden, Germany). The peptides were reduced with one equivalent Tris-(2-carboxyethyl) phosphine (TCEP; nonthiol reducing agent, Fluka) at 500 μM prior conjugation to ensure thiol groups of the peptides were not oxidised. Peptides were mixed with the pre-activated BSA to give a 50-fold excess of peptide. Conjugation was performed in 20% DMF, 50 mM Na_2HPO_4, pH 7.2; 600 mM NaCl and 60 mM EDTA. Reaction mixtures were left for 3 h at room temperature under gentle mixing. Non-reacted peptides were separated from the conjugates by SEC using ready-made spin columns (Princeton Separations, Adelphia, USA) according to the manufacturer's protocol.

2.4 Array fabrication

Protein and peptide conjugate solutions were diluted in PBS (150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4) to a concentration of 100–400 $\mu g \cdot mL^{-1}$ and were spotted on CSS aldehyde slides (Telechem, Sunnyvale, CA, USA) using the Biochip Arrayer (PerkinElmer Life Sciences, Boston, MA, USA) or the GMS 417 micro-arrayer (Affymetrix, Santa Clara, CA, USA). The amount of liquid deposited in one spot was approximately 300 pL for the Biochip Arrayer and 150 pL for the GMS arrayer. Dot spacing was set to 375 μm. Protein microarrays consisting of 6 or 12 subarrays were generated on one slide. After the printing process, all slides were incubated overnight in a humid chamber to allow maximum binding of the protein or conjugates to the surface. To stabilise the arrays for storage, slides were incubated for 2 h in Blocking Solution (1.5% BSA (Carl Roth, Karlsruhe, Germany), 5% low fat milk powder (PAA Laboratories, Pasching, Austria) in PBS pH 7.4); the blocking solution was removed and slides were left to dry at room temperature for 2 h. The slides were stored at room temperature in the dark and were stable for at least 6 months (for detailed procedure see [14]).

2.5 Assay procedure

Before the assay, the subarrays on the slides were spatially seperated by disposable frames, and if required four slides were mounted into a slide holder (ProPlate™ Multiarray Slide System, Grace Bio-Labs, Bend, OR, USA). The system allows the printed protein microarrays to be integrated into automated liquid handling devices and up to several hundred subarrays can be processed in parallel. Stored slides were rehydrated for 10 min in washing buffer (0.1% Tween® 20 (Merck, Darmstadt, Germany) in PBS pH 7.4). After rehydration, the arrays were rinsed three times in washing buffer. Recombinant Fab fragments were diluted to a concentration of 1–10 $\mu g \cdot mL^{-1}$ in Assay Buffer (1.5% BSA, 2.5% low fat milk powder, 0.1% Tween® 20 in PBS pH 7.4) and 30 μL of the solution was added to the arrays. After 45 min the Fab fragment solution was removed and 1 $\mu g \cdot mL^{-1}$ CY5-conjugated goat-anti-human IgG (Dianova, Hamburg, Germany) in Assay Buffer was added. The arrays were incubated for another 45 min at room temperature; the incubation times can be varied (see below). After the assay, the slides were washed once with washing buffer, once with H_2O_{dd} and finally they were carefully blown dry in a stream of nitrogen (for detailed procedure see [14]).

2.6 Laser scanning and evaluation

Arrays were scanned using a confocal laser fluorescence scanner GMS 418 (Affymetrix) at maximum laser power. The photomultiplier gain was set to 60%. Fluorescence intensities of all spots were then quantified using ImaGene 4.0 software (Biodiscovery, El Segundo, CA, USA). For evaluation, the mean fluorescence of each spot was used.

3 Results and discussion

3.1 Affinity ranking using microarrays

The characterisation of an antibody-antigen binding reaction requires the determination of the kinetic constants of the binding. While classical methods (*e.g.* equilibrium dialysis) give accurate data, they are time-consuming and therefore not suited for high-throughput screening purposes. Alternative methods have been developed and current state-of-the-art technology for gaining kinetic binding data uses SPR as the readout system [15]. The method relies on a solid phase assay that allows an online monitoring of the binding of the analyte to an immobilised probe. Association and dissociation constants can be directly calculated. A major disadvantage of the method is its limitation in throughput using currently available instrumentation. Usually ELISA based methods that serve as pre-selection filter have to be included prior to detailed kinetic analyses by SPR.

The system described here combines the simplicity and speed of a standard ELISA procedure with the capability to determine relative affinity values. In a minimal configuration, the system allows the quantification and the determination of a relative affinity value of a binding molecule to its target in a single experiment. The assay principle relies on the fact that in a miniaturised solid phase assay system, the equilibrium constant that governs complex formation can be approximated as shown below.

The following reaction will be observed:

$$Ab + Ag \leftrightarrows AbAg\ complex \quad (1)$$

within the equilibrium:

$$K_A = \frac{[\mathrm{AbAg}]}{[\mathrm{Ab}_0 - \mathrm{AbAg}][\mathrm{Ag}_0 - \mathrm{AbAg}]} \quad (2)$$

In a miniaturised assay format (*e.g.* a protein microarray), it is possible to adjust the concentration of the immobilised antigen below $0.05/K_D$. This corresponds to the ambient analyte assay conditions that were first defined by Ekins [1]. Under these conditions, the amount of Ab in the [AbAg] complex relative to the amount of free Ab can be neglected and the mass action law can be written in good approximation as

$$K_A = \frac{[\mathrm{AbAg}]}{[\mathrm{Ab}_0][\mathrm{Ag}_0]} \quad (3)$$

In a system, where [Ag] is constant and [Ab] and [AbAg] can be measured, a relative affinity constant can be determined (4). Microarray based assay systems can meet the described conditions and, therefore, a system that allows the determination of such relative affinities was designed and tested.

$$\text{Relative affinity} = K_A \times [\mathrm{Ag}_0] = \frac{[\mathrm{AbAg}]}{[\mathrm{Ab}_0]} \quad (4)$$

As described above, the determination of a relative affinity value that is performed under 'ambient analyte conditions' requires the miniaturisation of the assay. Here the format of a printed protein microarray was chosen where the antigen was immobilised in a microspot. In our initial experiments the printing conditions for generating the microspots was adjusted accordingly and a maximum of 5 fmol of antigen was deposited in a microspot. Assuming that all the material stays in the spot and that all the interacting sites are accessible for the examined binding reaction, the maximal concentration of antigen in a 50 μL assay is not exceeding 100 pM. Therefore, ambient analyte conditions are met for a dissociation constant of an antibody antigen interaction in the low nanomolar range.

On an antigen microspot, the antigen-antibody interaction will occur and a measured signal on the microspot is directly dependent on (a) the affinity constant that governs the binding reaction and (b) the concentration of the antibody present in the sample (Fig. 1). Therefore, the introduction of a second microspot that permits the quantification of the antibody concentration is sufficient to set-up an assay system that can determine a relative affinity value. This second spot contains a capture reagent that recognises the constant regions of the antibody framework. Product formation is measured using an antibody specific detection reagent, here we have used a secondary antibody labelled with CY5 fluorophore. These two measurements were performed in the same experiment and the relative affinity value was determined under 'ambient analyte conditions'.

A screening system using an antigen microspot and an anti-Fab fragment specific antibody immobilised in a second microspot as the *'quantifier'* was set-up as described above and was used to rank a panel of 62 recombinant Fab

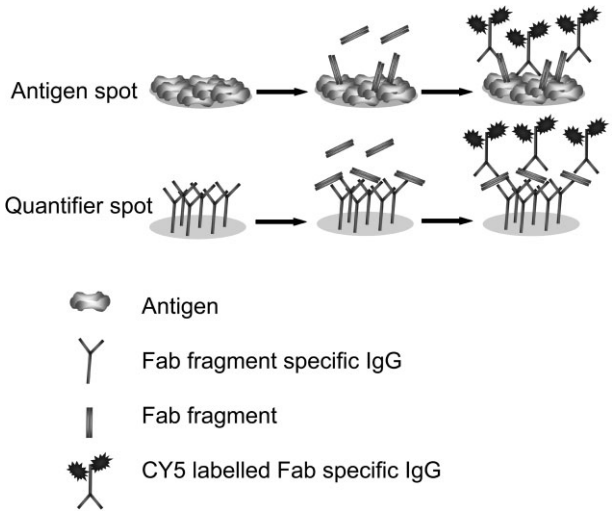

Figure 1. Principle of the two-spot affinity determination. For the determination of a relative affinity value, two microspots are required. One spot contains the antigen of interest, the other contains a capture molecule recognising the Fab fragment in its constant part. During the incubation of the Fab containing sample on the microspots, complex formation occurs on the spots and is detected by the addition of fluorescently labelled antibody. The signal intensities on the two spots are used to calculate an affinity value that combines the specific signal intensity on the antigen spot with the amount of antibody that is detected on the general Fab capture spot. To obtain a meaningful affinity value, the system has to be miniaturised in order to measure under ambient analyte conditions (see text).

fragments by their affinities. The samples used were crude periplasmic extracts prepared from *E. coli* and thus the screening process can be performed omitting laborious protein purification steps. In a parallel experiment, the same antibodies were purified and characterised by the SPR measurements. The resulting dissociation constants range from 1 nM to 1.2 μM. Relative affinity values were calculated from the obtained fluorescence intensities and were plotted against the known equilibrium constants as determined by SPR measurements (Table 1, Fig. 2).

The results prove that the method is capable of identifying binders possessing the highest affinity for their target. Most of the Fab fragments with affinity constants below 15 nM were identified. Only 3 out of 96 samples were identified as false positives and 4 high-affinity binders were not detected. However, these false negatives were present in duplicate samples in the microplate and therefore represent only two different antibodies.

While the presented set-up has been proven successful for the discrimination of high -and low-affinity binders, it is important to note that the data has not been collected under equilibrium conditions. As for most ELISA type assays, the procedure requires different incubation and washing steps, and only during the initial incubation of the antibody on the microarray, the formation of the antibody-antigen complex

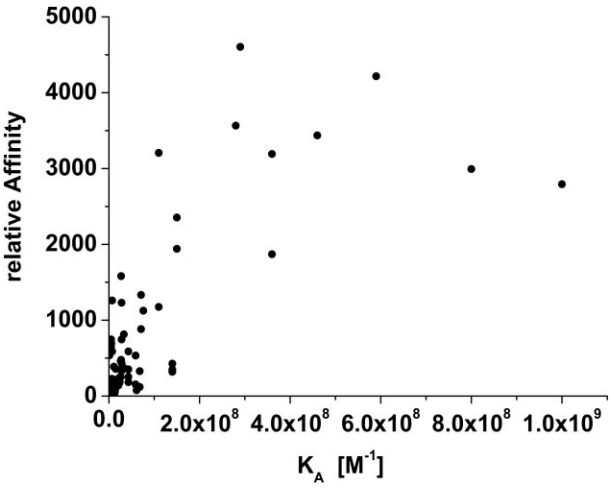

Figure 2. Affinity ranking of recombinant Fab fragments. Ninety-six crude bacterial lysates prepared from cultures expressing 62 different Fab fragments were examined using the described microarray based ranking method. Relative affinity values were calculated and plotted against the association constant K_A obtained by the SPR measurements with the corresponding purified Fab fragments. Clones expressing high affinity Fab fragments also show high-relative affinity values, thus clones with association constants of above 7×10^7 M^{-1} (corresponding to a dissociation constant of 15 nM) are identified easily (see text).

occurs. During the following incubation steps, the equilibrium will be changed since dissociation of the antibody-antigen complex will occur. The duration of the incubation and stringency of the washing steps therefore strongly influence the signals generated on the microarray. Long and extensive washing steps lead to affinity data sets that are biased towards the off kinetics of the binding process. While this presents a problem for the analysis of binding events with a fast off-kinetics, extended washing steps can be included to adapt the system for the identification of binders that do form a stable complex. Screening for highly affine antibodies is such a situation and here a bias towards the *k*off binding constant usually does not present a problem.

The microarray based assay system developed here shows several advantages. It allows the measurement under ambient analyte conditions and the miniaturisation results in low sample and material consumption. Nevertheless, the major benefit of array-based methods is the possibility to perform dozens to hundreds of measurements in parallel. Thus the expansion of the system allows the determination of affinity and binding specificity on the same array.

In an effort to combine both specificity determination and affinity estimation, a new method for peptide microarray generation was established.

Table 1. Affinity ranking of recombinant Fab fragments. Ninety-six crude bacterial lysates prepared from cultures expressing 62 different Fab fragments were examined using the described affinity ranking method and sorted by the obtained relative affinity values. The kinetic constants (k_{on}, k_{off}, K_A, K_D) that govern the binding reaction were determined by SPR are shown in the following columns. The results show that the method is capable of identifying binders possessing the highest affinity for their target

Relative Affinity	Kinetic Constants (BIAcore measurements)			
(Two spot assay)	k_{on} [s * M^{-1}]	k_{off} [s^{-1}]	K_A [M^{-1}]	K_D [M]
4604	8.9E+04	3.1E-04	2.9E+08	3.5E−09
4217	1.9E+05	3.2E−04	5.9E+08	1.7E−09
3565	1.0E+05	3.6E−04	2.8E+08	3.6E−09
3437	1.6E+05	3.5E−04	4.6E+08	2.2E−09
3206	2.8E+05	2.6E−03	1.1E+08	9.2E−09
3192	1.3E+05	3.6E−04	3.6E+08	2.8E−09
2993	1.2E+05	1.5E−04	8.0E+08	1.3E−09
2793	1.6E+05	1.6E−04	1.0E+09	1.0E−09
2354	7.9E+04	5.2E−04	1.5E+08	6.6E−09
1941	7.9E+04	5.2E−04	1.5E+08	6.6E−09
1870	1.4E+05	3.9E−04	3.6E+08	2.8E−09
1849	nd	nd	nd	nd
1792	nd	nd	nd	nd
1581	7.0E+04	2.6E−03	2.7E+07	3.7E−08
1446	nd	nd	nd	nd
1333	1.9E+05	2.6E−03	7.1E+07	1.4E−08
1260	9.0E+03	1.2E−03	7.2E+06	1.4E−07
1230	1.1E+05	4.1E−03	2.8E+07	3.6E−08
1175	2.8E+05	2.6E−03	1.1E+08	9.2E−09
1125	2.5E+05	3.3E−03	7.6E+07	1.3E−08
882	1.9E+05	2.6E−03	7.1E+07	1.4E−08
813	7.3E+04	2.2E−03	3.3E+07	3.0E−08
746	9.6E+04	3.4E−03	2.8E+07	3.6E−08
746	9.0E+03	1.9E−03	4.8E+06	2.1E−07

Table 1. Continued

Relative Affinity (Two spot assay)	Kinetic Constants (BIAcore measurements) k_{on} [s * M^{-1}]	k_{off} [s^{-1}]	K_A [M^{-1}]	K_D [M]
713	1.9E+05	4.0E−02	4.8E+06	2.1E−07
690	4.0E+04	7.6E−03	5.3E+06	1.9E−07
676	nd	nd	nd	nd
647	9.0E+03	1.9E−03	4.8E+06	2.1E−07
588	9.0E+03	1.2E−03	7.2E+06	1.4E−07
588	1.9E+05	4.4E−03	4.3E+07	2.3E−08
559	nd	nd	nd	nd
536	1.7E+05	1.5E−01	1.1E+06	8.8E−07
533	8.7E+04	1.5E−03	5.9E+07	1.7E−08
477	7.0E+04	2.6E−03	2.7E+07	3.7E−08
462	2.7E+04	1.0E−03	2.6E+07	3.9E−08
435	9.6E+04	3.4E−03	2.8E+07	3.6E−08
427	1.2E+05	8.7E−04	1.4E+08	7.3E−09
387	2.6E+04	2.4E−03	1.1E+07	9.2E−08
374	nd	nd	nd	nd
370	7.3E+04	2.2E−03	3.3E+07	3.0E−08
357	1.1E+05	7.1E−03	1.5E+07	6.8E−08
353	4.0E+04	1.4E−03	2.8E+07	3.5E−08
352	3.6E+05	8.4E−03	4.3E+07	2.3E−08
350	4.0E+04	2.9E−04	1.4E+08	7.2E−09
341	nd	nd	nd	nd
331	4.0E+04	1.4E−03	2.8E+07	3.5E−08
328	1.7E+05	2.5E−03	6.8E+07	1.5E−08
326	1.2E+05	8.7E−04	1.4E+08	7.3E−09
319	4.0E+04	2.9E−04	1.4E+08	7.2E−09
253	3.6E+05	8.4E−03	4.3E+07	2.3E−08
248	2.7E+04	1.0E−03	2.6E+07	3.9E−08
247	1.0E+05	4.3E−03	2.4E+07	4.2E−08
227	1.8E+05	2.7E−02	6.6E+06	1.5E−07
215	1.1E+05	7.1E−03	1.5E+07	6.8E−08
215	5.8E+04	3.0E−03	1.9E+07	5.1E−08
201	8.7E+04	1.5E−02	5.9E+06	1.7E−07
199	nd	nd	nd	nd
198	4.8E+05	8.2E−02	5.9E+06	1.7E−07
193	nd	nd	nd	nd
191	nd	nd	nd	nd
184	1.9E+05	4.4E−03	4.3E+07	2.3E−08
180	1.0E+05	4.3E−03	2.4E+07	4.2E−08
167	nd	nd	nd	nd
161	5.8E+04	3.0E−03	1.9E+07	5.1E−08
161	3.9E+05	1.0E−01	3.9E+06	2.6E−07
153	8.7E+04	1.5E−03	5.9E+07	1.7E−08
151	nd	nd	nd	nd
140	7.4E+05	3.5E−02	2.1E+07	4.8E−08
137	nd	nd	nd	nd
133	nd	nd	nd	nd
129	2.6E+04	2.4E−03	1.1E+07	9.2E−08
127	7.6E+03	1.6E−03	4.7E+06	2.1E−07
126	7.6E+03	1.6E−03	4.7E+06	2.1E−07
124	2.3E+05	2.2E−01	1.1E+06	9.4E−07
119	1.7E+05	2.5E−03	6.8E+07	1.5E−08
117	nd	nd	nd	nd
113	1.4E+04	5.8E−03	2.4E+06	4.2E−07
97	1.4E+04	5.8E−03	2.4E+06	4.2E−07
95	8.7E+04	1.5E−02	5.9E+06	1.7E−07
87	2.3E+05	2.2E−01	1.1E+06	9.4E−07

Table 1. Continued

Relative Affinity (Two spot assay)	Kinetic Constants (BIAcore measurements) k_{on} [s * M^{-1}]	k_{off} [s^{-1}]	K_A [M^{-1}]	K_D [M]
81	2.0E+05	2.2E−01	8.8E+05	1.1E−06
81	8.0E+04	5.6E−03	1.4E+07	7.0E−08
80	3.7E+05	6.2E−02	6.0E+06	1.7E−07
76	4.4E+05	7.3E−03	6.1E+07	1.7E−08
68	3.9E+05	1.0E−01	3.9E+06	2.6E−07
59	2.8E+05	9.1E−02	3.1E+06	3.3E−07
54	7.4E+04	2.6E−02	2.8E+06	3.5E−07
53	4.9E+04	5.3E−03	9.3E+06	1.1E−07
51	6.0E+04	4.8E−03	1.3E+07	8.0E−08
50	4.0E+04	7.6E−03	5.3E+06	1.9E−07
43	1.8E+05	2.7E−02	6.6E+06	1.5E−07
43	6.0E+04	4.8E−03	1.3E+07	8.0E−08
41	5.7E+05	5.7E−02	1.0E+07	1.0E−07
35	3.7E+05	6.2E−02	6.0E+06	1.7E−07
23	4.9E+04	5.3E−03	9.3E+06	1.1E−07
19	2.3E+05	2.5E−01	9.2E+05	1.1E−06

3.2 Generation of peptide microarrays

Two key parameters determine the quality of an antibody as an analytical tool. The affinity determines the sensitivity of an assay, which employs the antibody as a capture or detection reagent, whereas the specificity determines its use for a given application. A detailed knowledge on the antibody-antigen interaction includes the identification of the epitope recognised by the antibody. A variety of methods have been developed to define this epitope. Peptide-arrays have proven to be a powerful tool to fulfil this task, and different methods have been described that can be used to map the antibody-antigen interaction down to the level of the amino acid [17]. State-of-the-art systems use macroarrays of immobilised peptides that are manufactured by the direct synthesis [18, 19] on cellulose membranes. While this method allows hundreds of different peptides to be probed in one assay, it has a variety of drawbacks. Therefore, we focused on an approach that uses peptides conjugated to a carrier protein. This allows the generation of microarrays containing peptides and proteins. All peptides were made with conventional solid phase peptide synthesis [12, 13] and carry a sulfydryl-group at the *N*-terminus. Peptide-protein conjugates can easily be obtained by coupling cysteinyl-peptides to maleimide activated BSA. Microarrays consisting of peptide-conjugates are generated by using standard spotting technologies. There are several major advantages when comparing these peptide microarrays to the macroarrays produced by spot synthesis. Standard methods for microarray fabrication allow a spot density of 1000 spots *per* cm^2, a 500-fold higher density than can be obtained with spot synthesis; this reduces the amount of antibody required for one assay substantially, and a binding analysis can be performed with less then 100 ng of an antibody. The fabrication of microarrays by dedicated arraying robots allows the printing of hundreds of arrays from less then 1 mg of peptide in one production process; this allows the use of peptide microarrays as a screening tool. Library-based methods like the phage display technology employed in this study do require the analysis of hundreds to thousands of binders during the screening process. Macroarrays on cellulose membranes hardly fulfil this requirement because they are only reusable several times and have to be stripped in a time-consuming procedure. Alternatives like the light directed parallel on-chip synthesis of peptide microarrays became recently available, but their production costs are very high and therefore these are not yet economical [20]. The peptide-array fabrication shown here facilitates the analysis of hundreds of antibodies and an easy repetition of experiments. Furthermore, it allows the generation of arrays that not only contain peptides but also complete proteins within the same microarray. The approach chosen for the production of peptide microarrays also considers the problems that are known to occur during chemical synthesis of peptides. Truncated peptides that are often the result of sequence specific synthesis problems will not be coupled to the activated BSA-carrier since the sulfydryl-group required for the covalent coupling to the carrier is not present in these unwanted side-products of the synthesis. This is due to the capping step included in the peptide synthesis procedure, which prevents the further elongation of truncated peptides. Therefore, only full length, correctly synthesised peptides are coupled to the carrier protein resulting in a significant gain in quality of the peptide microarrays. This way of immobilising peptides and the fact that each of the used peptides has been characterised by HPLC and MS results in quality controlled peptides in the array, a feature which is not feasible using *in situ* peptide synthesis approaches.

3.3 Epitope mapping and affinity estimation using microarrays

A collection of Fab fragments isolated by two different panning strategies from the HuCAL library was chosen to prove the capabilities of the method. A microarray consisting of proteins and peptide-BSA conjugates was created. The molecules immobilised included the panning antigens and an antibody raised against the Fab portion of human IgG antibodies. The panning antigens were a recombinant fusion protein containing part of the extracellular domain (aa 162–330) of the CD11b protein and M18, a 25mer peptide derived from aa 289–313 of the CD11b protein. This part of the array was used to estimate the relative affinity of the examined binders to the two different types of antigen. The second part of the array consisted of a set of overlapping peptides derived from the sequence of the part of the extracellular domain (aa 162–330) of the CD11b protein that was used for the panning procedure.

The peptide part of the microarray consisted of 42 peptides. Each peptide was 15 aa long with an overlapping sequence of 11 aa to the subsequent peptide. Moreover, different peptides were used as negative controls (myc-tag peptide, FLAG-tag peptide). Using these mapping arrays, linear epitopes for three out of six Fab fragments were detected. These three Fab fragments showed interactions with a limited number of overlapping peptides and all epitopes could be mapped to a distinct region in the recombinant protein domain (Table 2). Interestingly, all the three epitope sequences are found in the same region of the protein. An antigenicity plot [21] of this region reveals a high-antigenic index for the recognised epitopes (data not shown). This demonstrates that this algorithm is not only suitable to find possible antigenic structures for a classical immunisation but might also give hints for directed panning strategies. The remaining three Fab fragments showed no signal above background on the peptide spots, but high signal on the recombinant protein domain, the CD11b fragment. The results indicate that these antibodies recognise structures that are likely to be discontinuous or conformational and therefore are not detectable using arrays of overlapping peptides.

Table 2. Linear peptide epitopes of protein binding Fab fragments. Epitopes were determined using peptide arrays containing 42 peptide-BSA conjugates, prepared from 15mer peptides with 13 aa overlap. Fab fragments were detected via fluorescently labelled goat anti-human IgG

Binder	Mapped epitope
Fab 6	no peptide binding
Fab 7	no peptide binding
Fab 8	no peptide binding
Fab 9	EDVIPEADREG
Fab 10	KFGDPLGYEDV
Fab 11	EDVIPEADREG

Five Fab fragments isolated against the peptide antigen M18 were characterised on the microarray, and as expected they showed reactivity towards their panning peptide; only one of the antibodies recognised the protein domain.

The relative affinity value that could be calculated using the antigen and the quantifier spot were used to rank the binders. The ranking order obtained using the relative affinity values from the microarray, correlates with the affinity constant determined by SPR. Therefore, combination of epitope mapping and an affinity ranking is possible using protein and peptide microarrays.

3.4 Detailed epitope characterisation

To get a complete view on the epitope requirements of the isolated Fab fragments, a new set of peptides was synthesised and immobilised as peptide-BSA conjugates. The peptides were derived from the region of the CD11b protein that was used to design the M18 peptide, a 25mer that had been used to generate peptide specific Fab fragments. To determine the minimal sequence requirement for Fab binding an array containing a series of 48 truncated peptides in triplicates, starting from both the *C*-terminus and the *N*-terminus of the M18 were designed. These allowed the definition of the minimal epitope required for binding of the different Fab fragments (Fig. 3, Table 3) to their antigen. By combining the information obtained in the overlapping peptide approach with arrays consisting of all possible truncation of the antigenic region of interest, the definition of a minimal binding motif of an antibody is possible.

In addition to the identification of the minimal epitope of an antibody, we also employed peptide arrays for the determination of the influence of each individual position in a peptide epitope on the binding of an anti-peptide antibody. The characterisation of Fab 4, a Fab fragment that recognises the recombinant protein domain at a linear epitope of 7 aa length is shown exemplary in Fig. 4. The approach chosen uses a positional scanning peptide library for the 25mer M18. Here, 25 different libraries were synthesised, where each individual amino acid position in the peptide sequence was randomised by allowing the presence of all 20 amino acids. Therefore, sequence specificity is lost at the randomised position and the requirement for a specific amino acid is indicated by a loss of signal on a peptide spot made from such a positional scan library. Figure 4 demonstrates the power of this type of peptide array; for positions important for binding, signal intensity is reduced dramatically, whereas positions that are less important do not lead to a substantial loss in binding activity. With this finding the influence of each residue of the epitope can be weighted. For Fab 4, a displacement of position 1, 2, 3 and 7 leads to a substantial loss of signal intensity; for position 4, 5 and 6, no decrease of signal was observed. This means that the first three and the seventh positions of the minimal binding sequence are the residues that are most important for the interaction

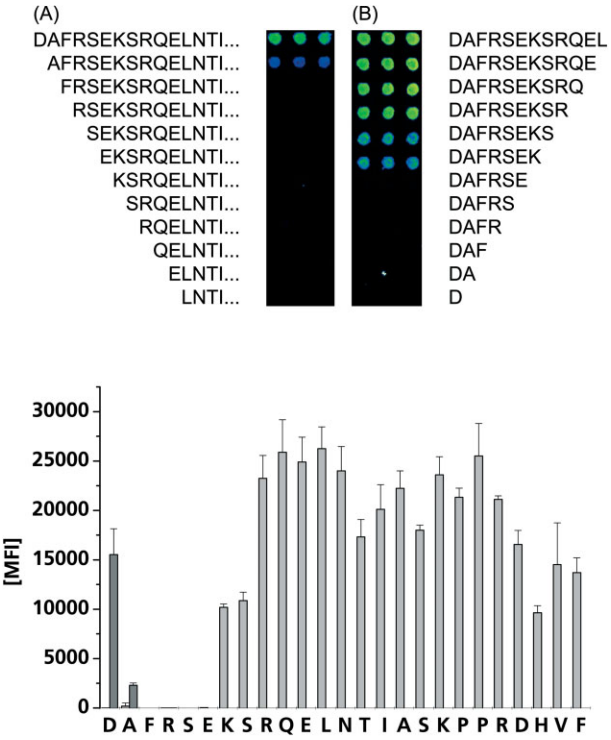

Figure 3. Epitope mapping using peptide microarrays. To define the minimal epitope requirements for the isolated Fab fragments, a strategy using a peptide array was employed. The array was created from a set of *N*-terminal (A) and *C*-terminal (B) truncations of the peptide of interest. Fab fragments were incubated on the array and visualised with a fluorescently labelled detection antibody. An image of an array incubated with Fab 4 is shown and the minimal binding sequence was determined to correspond to DAFRSEK. The bar chart shows the mean fluorescence of three spots plotted against the M18 25mer sequence. *C*-terminal truncated peptides are coloured in dark grey and *N*-terminal truncated peptides in light grey.

Table 3. Minimal binding sequences of the M18 peptide binding Fab fragments. Minimal epitopes were specified using peptide arrays containing 50 peptide-BSA conjugates. The peptide-BSA conjugates carried peptides truncated as well from the *N*-terminus as from the *C*-terminus. Bound Fab fragments were detected with a CY5 labelled goat anti-human IgG

Binder	Minimal binding sequence
Fab 1	KPPRDHVF
Fab 2	SKPPRDHVF
Fab 3	KPPRDHV
Fab 4	DAFRSEK
Fab 5	KPPRDHVF

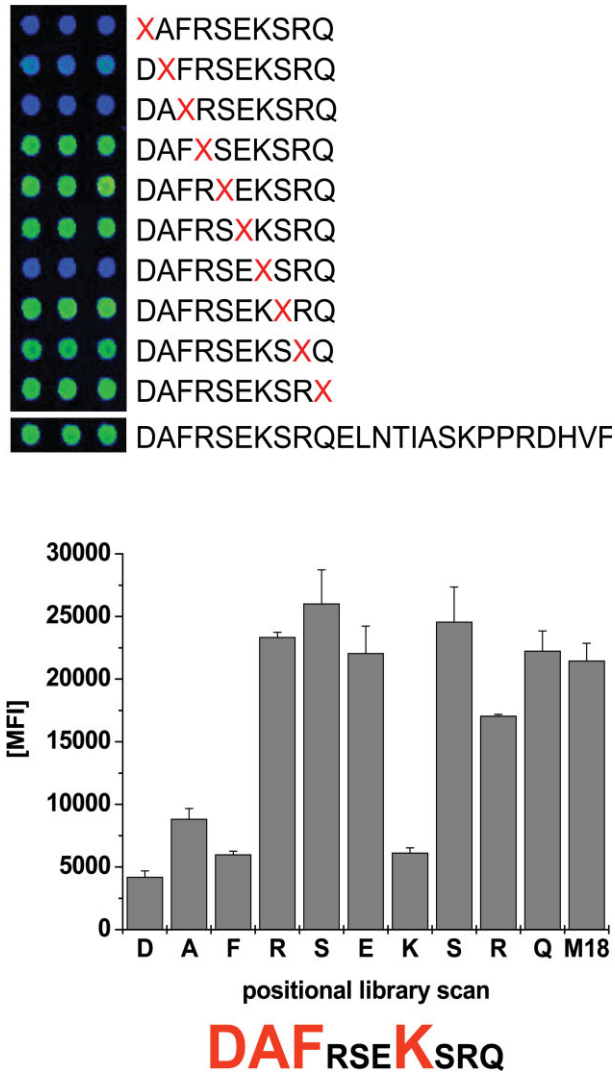

Figure 4. Epitope mapping using a positional X-library scan peptide microarray. A set of 25 different libraries was synthesised, in which each individual amino acid position in the sequence of the initial 25mer peptide was randomised by allowing the presence of all 20 amino acids. Arrays containing these libraries were generated, Fab fragments were incubated on the array and visualised with a fluorescently labelled detection antibody. The image shows an array incubated with Fab 4. The obtained signal intensity on the microspots reflects the influence of the single residues on the binding to the Fab fragment. The displacement of the 4th, 5th and the 6th positions by the entire set of amino acids of the epitope leads to no significant loss of the signal. However, the X-library at positions 1, 2, 3 and 7 results in a decreased signal on the microspot containing these peptides. Those four residues are responsible for the antibody-peptide interaction.

with the Fab fragment. This weighting allows the definition of functional binding motifs and it is useful for the identification of antibodies with similar properties. In addition, it is a valuable information identifying cross-reactivities. The functional binding motifs derived from the peptide array can be used in motif searches in genome or protein databases and might help to identify cross-reactivities at an early stage of antibody characterisation.

4 Concluding remarks

Array technology allows the investigation of a large number of interactions in distinct microspots in one single experiment. We have established a microarray-based system-containing proteins and peptide-BSA conjugates to screen large numbers of recombinant antibodies for affinity and specificity. A value that reflects the affinity of a binding reaction can be obtained easily by a two spot analysis, by measuring the antigen-antibody signal and the antibody concentration. Under 'ambient analyte assay conditions' the antibody concentration in the liquid phase changes only marginally and the amount of antibody-antigen complex is directly dependent on the concentration of the antibody. Therefore, the amount of binder captured on an immobilised antigen is directly dependent on the affinity constant that governs the binding reaction and the concentration of the binder present in the sample. The major advantage of this approach is its ease and speed, compared to current state-of-the-art methods. Furthermore, the new method facilitates affinity screening of antibodies from crude cell lysates within a single experiment.

In addition, this microarray-based system allows to include an efficient epitope mapping of recombinant Fab fragments. Epitope mapping of recombinant antibodies using overlapping peptides in combination with the full-length protein revealed for several binders discrete peptide epitopes, whereas other recombinant binders only recognised the full-length protein on the microarray. Peptide binders were further characterised according to their minimal binding sequence using *C*- and *N*-terminal truncated peptides. With a library scan, it was possible to test the sequence specificity of the examined binder.

Our approach facilitates the easy determination of relative affinity and the recognition of important amino acids in the binding event of antibodies. In principle, such a detailed screening procedure for affinity and specificity can be performed within one single experiment, and could be applied into the recombinant antibody generation at an early stage of the screening. In the proteomic era, there is a tremendous need for high-throughput technologies to analyse the function of the proteome. Within the last few years, microarray technology has expanded beyond DNA chips. A large variety of protein microarray-based approaches has already demonstrated that this technology is capable of filling the gap between genomics and proteomics [5, 6]. Highly specific and selective capture molecules are an absolute pre-requisite for the design and generation of protein and DNA microarrays. These requirements are met very easily for DNA microarrays due to the complementary nature of the DNA molecule. Proteins are much more complex and it is not possible to predict highly selective binders, just based on the amino acid sequence of the target molecule. Each binder has to be generated and characterised not only for its affinity but also for its recognition of the antigen. The microarray based screening method presented here is perfectly suited to characterise a large number of binding molecules simultaneously for sequence specificity, selectivity and affinity.

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