Research Article

Journal of PeptideScience

Received: 8 December 2009 Revised: 18 February 2010 Accepted: 10 March 2010 Published online in Wiley Interscience: 7 May 2010

(www.interscience.com) DOI 10.1002/psc.1237

A study to assess the cross-reactivity of cellulose membrane-bound peptides with detection systems: an analysis at the amino acid level

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The growing demand for binding assays to study protein–protein interaction can be addressed by peptide array-based methods. The SPOT technique is a widespread peptide-array technology, which is able to distinguish semi-quantitatively the binding affinities of peptides to defined protein targets within one array. The quality of an assay system used for probing peptide arrays depends on the well-balanced combination of screening and read-out methods. The former address the steady-state of analyte capture, whereas the latter provide the means to detect captured analyte. In all cases, however, false-positive results can occur when challenging a peptide array with analyte or detecting captured analyte with label conjugates. Little is known about the cross-reactivity of peptides with the detection agents. Here, we describe at the amino acid level the potential of (i) 5-(and 6)-carboxytetramethylrhodamine (5(6)-TAMRA), (ii) fluoresceinisothiocyanate in form of the peptide-bound fluorescein-substituted thiourea derivative (FITC), and (iii) biotin/streptavidin-POD to cross-react with individual amino acids in a peptide sequence. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: SPOT synthesis; peptide array; cross-reactivity; TAMRA; FITC; biotin; streptavidin

Introduction

There are several commonly used methods to measure protein–protein interactions and binding affinities, such as enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance (SPR). In contrast to most of these methods, protein and peptide arrays on planar surfaces [1–4] allow high-throughput measurement because they provide a higher density of probes, so a multitude of molecular interactions can be measured in parallel. Array experiments have demonstrated their value for bimolecular binding assays [5,6], especially in the case of protein–protein interactions [4,7–9].

Synthetic peptide arrays [1,9] have several advantages: (i) peptide synthesis is faster and cheaper than expression-related techniques, (ii) peptide probes are stable moieties, and (iii) peptide synthesis allows incorporation of non-gene-encoded residues. A drawback of applying peptides instead of whole proteins as probes is information loss due to the missing structural context. This can be compensated for by adapting the task of the peptide-array experiment, for example, by focusing on modular binding events or by resolving immunorecognition to the epitope level. Peptide arrays are usually prepared in a micro- or macro-array format [10]. The latter kind of array is generated according to the SPOT synthesis approach [11], which is accessible even for non-specialized laboratories. The SPOT technology and many of its applications have been reviewed extensively [1,8,9]. In principle, signal intensities (SIs) – the output of this technique – can be used to roughly distinguish between different affinities [12]. The most important application of the SPOT technique, however, is to differentiate qualitatively between binding affinities of peptides to defined protein targets within one array using fluorescent or chemiluminescent read-out systems.

The quality of an assay system used for probing peptide arrays depends on the well-balanced combination of screening and read-out methods. The former address the steady-state of analyte capture, whereas the latter provide the means to detect captured analyte. Usually, both screening and read-out are carried out directly on the peptide array and are often performed as separate procedures. The visualization of peptides binding the interaction partner is done in an additional step, where the probed peptide array is subsequently immersed in a solution containing a label conjugate with high binding affinity to the analyte. Besides antibody-based immunoblotting techniques [13,14], the biotin/streptavidin-peroxidasis (-POD) system has recently been reported as a convenient combination of a non-interfering screening strategy (biotin-conjugated analytes) with a specific affinity-based read-out strategy (streptavidin-conjugated reporter) for peptide arrays [15,16]. More advantageously though, screening and read-out can be achieved simultaneously through direct labeling of the analyte with a detectable moiety, for instance with fluorescent dyes. These dyes can be incorporated synthetically [17,18] or via methods used in activity-based protein profiling [19]. In all cases, however, false-positive results can occur when challenging a peptide array with

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analyte or detecting captured analyte with label conjugates. This is due to the diversity of mechanisms through which peptides may directly interact with any of the detection agents. Control incubations using only the detection agents for the read-out procedure are always required and standard in good laboratory practice.

In spite of being a general problem, little is known about the cross-reactivity of peptides with the detection agents. Here, we describe the potential of (i) 5-(and 6)-carboxytetramethylrhodamine (5(6)-TAMRA), (ii) fluoresceinisothiocyanate in form of the peptide-bound fluorescein-substituted thiourea derivative (FITC), and (iii) biotin/streptavidin-POD to cross-react with individual amino acids in a peptide sequence.

Experimental Section

SPOT Synthesis

Cellulose-bound peptide arrays were prepared according to standard SPOT synthesis protocols using a SPOT synthesizer as described in detail in the literature [20]. The peptides were synthesized on amino-functionalized cellulose membranes of the ester type prepared by modifying a cellulose paper with Fmoc-β-alanine as the first spacer residue. In the second coupling step, the anchor position Fmoc-β-alanine-OPfp in dimethylsulfoxide (DMSO) was used. Residual amino functions between the spots were capped by acetylation. The Fmoc group was cleaved using 20% piperidine in dimethylformamide (DMF). The cellulose-bound peptide arrays were assembled on these membranes by using 0.3 M solutions of Fmoc-amino acid-OPfp in *N*-Methylpyrrolidone (NMP). Side-chain protection of the used Fmoc-amino acids was as follows: Glu, Asp (OtBu); Ser, Thr, Tyr (tBu); His, Lys, Trp (Boc); Asn, Gln, Cys (Trt); Arg (Pbf). After the last coupling step, the acid-labile protection groups of the amino acid side chains were cleaved using 90% trifluoro-acetic acid (TFA) for 30 min and 60% TFA for 3 h. Peptides were cleaved from the membrane using the standard protocol as described in detail in the literature [20] and dissolved in water (using 10% acetonitrile to increase solubility if necessary). HPLC analysis was performed using a linear solvent gradient (A: 0.05% TFA in water; B: 0.05% TFA in acetonitrile; gradient: 5–60% B over 30 min; UV detector at 214 nm; RP-18 column). α-Cyanocinnamic acid was used as a matrix for MALDI–TOF–MS analysis.

Peptide Synthesis on Resin

Soluble peptides – repeats of glycine – were synthesized (50 µmol scale) as amides on a multiple synthesizer according to the standard Fmoc machine protocol using TentaGel S RAM resin (Rapp Polymere) and PyBOP activation. Each peptide was *N*-terminally modified with 5(6)-TAMRA, fluoresceinisothiocyanate, or biotin using PyBOP activation. All peptides were analyzed by reversed-phase HPLC and MALDI–TOF. HPLC purification and analysis were performed using a linear solvent gradient (A: 0.05% TFA in water; B: 0.05% TFA in acetonitrile; gradient: 5–60% B over 30 min; UV detector at 214 nm; RP-18 column).

Binding Studies on Cellulose Membrane-Bound Peptides

All incubation and washing steps were carried out under gentle shaking and at room temperature. After washing, the membrane with ethanol once (for 10 min) and three times for 10 min with Tris-buffered saline [TBS: 50 mM Tris-(hydroxymethyl)-aminomethane, 137 mM NaCl, 2.7 mM KCl, adjusted to pH 8 with HCl/0.05%], the membrane-bound peptide arrays were blocked (3 h) with blocking buffer [casein-based blocking buffer concentrate (Sigma-Genosys, Cambridge, UK), 1 : 10 in T-TBS containing 5% (w/v) sucrose] and then washed with TBS (1 × 10 min). Subsequently, the peptide arrays were incubated with the labeled analytes ($c = 10$ µM) for 10 min in TBS-blocking buffer. After washing for 120 min with TBS, analysis and quantification of peptide-bound dyes/biotin/streptavidin-POD were carried out using a Lumi-Imager. In the case of biotin/streptavidin-POD, a chemiluminescent substrate was added beforehand. In case of densitometric analysis, the membranes were scanned and read-out directly by the Genespotter software. For further information, see Volkmer [9].

Measurement of Spot SIs

For each detection system, binding events were recorded by a cooled CCD-camera (TAMRA-fluorescence, FITC-fluorescence, and streptavidin-linked chemiluminescence) using a Lumi-Imager. In addition, TAMRA staining was also recorded by scanning in the visible light range, resulting in a digital image file (referred to as densitometric analysis). The SI of each spot was calculated by defining a spot radius that can be optimally applied to all spots in the image and taking the median value of the pixel intensity. The background signal was determined with a safety margin to each spot's circular region, and then the global background mean was subtracted from each individual spot signal. We refer to this parameter as SI. Grid-layer and SI were calculated using dedicated image analysis software (Genespotter, microdiscovery GmbH). Genespotter has a fully automatic grid-finding routine resulting in reproducible SIs. The median value of the intraspot distribution was sufficient to avoid saturation. Results are shown as the interspot global background corrected mean value over three replica spots for each sequence. TAMRA was measured at 645 nm, FITC at 520 nm, and streptavidin-POD via chemiluminescence. We chose to detect TAMRA at the aforementioned wavelength to lower background noise.

Results and Discussion

The rationale of our approach was to understand the potential cross-reactivity of common detection systems such as 5(6)-TAMRA, fluoresceinisothiocyanate in form of the peptide-bound FITC, biotin and streptavidin-POD with cellulose membrane-bound peptides at the amino acid level. To illuminate the potential interaction of these detection systems with individual amino acids, we designed 20 peptides of the sequence GGG$[B]_5$GGG. Herein, $[B]_5$ denotes five repeats of one of the 20 amino acids. Glycine was used to create non-reactive regions flanking the functional core at the *N*- and *C*-termini. This approach generates peptides of reasonable length for the homogeneous display of the defined cores. The peptides were prepared via SPOT synthesis, whereby each GGG$[B]_5$GGG sequence was repeated three times in columns on the peptide array (Figures 1–3, B and C as well as Scheme S1 in the supporting information).

In addition, we varied the core motif lengths from $[B]_5$ to $[B]_1$ and also the peptide-specific density in order to identify the effects on interaction. These additional arrays are shown in Figures S1–S6 (supporting information).

All membrane-bound peptides were analyzed by reversed-phase HPLC and MALDI–TOF. All masses except those of

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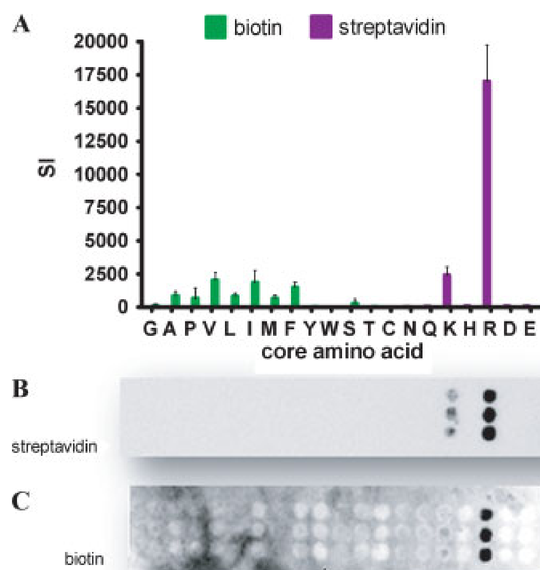
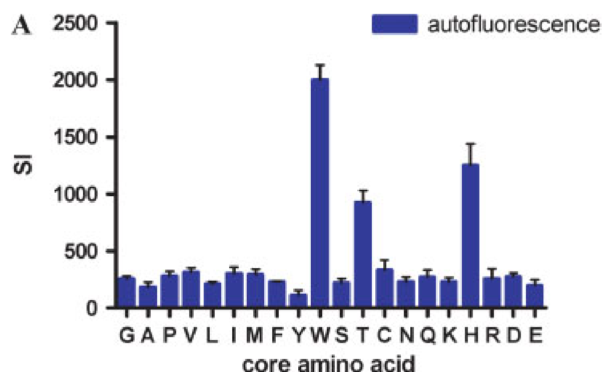
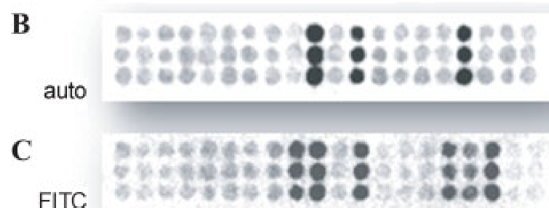


Figure 1. Streptavidin-POD (purple) and biotin (green) cross-reaction with membrane-bound peptides. (A) The spot signal measured through chemiluminiscence is calculated from a circular region around the spot center detected in the image. All signals below a SI of 1000 are at the background level and should therefore not be considered as interactions between the core and the detection system. Streptavidin-POD results were set as background for the calculation of biotin interactions. Owing to the direct interaction of streptavidin-POD with positively charged peptides, any further information about the cross-reactivity of biotin with Lys and Arg has been lost. (B) Each spot represents a cellulose membrane-bound peptide of the sequence GGG$[B]_5$GGG, where $[B]_5$ denotes five repeats of one of the 20 amino acids. Black spots denote interactions with streptavidin-POD and (C) with biotin-GGG/streptavidin-POD. The negative control without analyte shows no signal. Error bars represent the standard deviation of three spots.





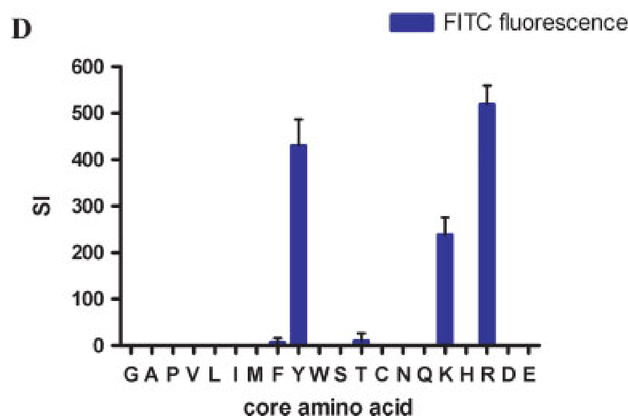


Figure 2. FITC cross-reaction. (A) Spot auto-fluorescence at 520 nm. The spot signal measured at 520 nm is calculated from a circular region around the spot center detected in the image shown in panel (B). (B) Each spot represents a cellulose membrane-bound peptide of the sequence GGG$[B]_5$GGG, where $[B]_5$ denotes five repeats of one of the 20 amino acids. (C) The same array incubated with FITC-GGG, black spots denote interaction – except for the Trp-, Thr-, and Arg-containing spots. (D) The spot signal measured at 520 nm is again calculated from a circular region around the spot center detected in the image. The SIs are background-subtracted (spot auto-fluorescence at 520 nm). Error bars represent the standard deviation of three spots.

cysteine-containing peptides were found, and the purity of the SPOT-synthesized peptides was determined (by HPLC) to be in the range of 25–85%, which is adequate for screening assays [20,21]. Because the masses of the cysteine-containing peptides were incorrect, the analytical results of these peptides were not taken into account.

As soluble interaction partners, peptides of the sequence Gly-Gly-Gly were synthesized, *N*-terminally modified with biotin, TAMRA, and FITC (label-GGG), and finally purified by HPLC. This tripeptide was used to better meet the assay conditions, because the aforementioned labels are usually chemically coupled to an analyte or a detection antibody.

Peptide arrays containing the core-motifs were incubated *in situ* with a label-GGG and evaluated via optical, fluorescent, and chemiluminescent methods. Strict conditions including short incubation periods and long-time washing procedures were applied to ensure stringency of binding. Binding experiments resulted in measurable spot SIs signifying directly or indirectly captured label conjugate. The tripeptide Gly-Gly-Gly itself does not contribute to the overall interaction of the conjugated construct (label-GGG) with membrane-bound peptides, as we found no evidence for experiment-spanning recurring signals that would indicate binding events of the Gly-Gly-Gly peptide (compare Figures 1–3). Therefore, we are able to specifically measure the direct influence of the detection system on the 20 core positions of the membrane-bound peptide probes.

Biotin-labeled samples were used to challenge our peptide arrays and were subsequently detected via the streptavidin-POD conjugate using chemiluminiscence. Streptavidin-POD was also tested directly on the membrane-bound peptides to differentiate between streptavidin-POD and biotin interactions. Figure 1 shows that streptavidin-POD is prone to cross-reaction with positively charged amino acids, such as lysine and arginine. However, the observed binding is most likely related to streptavidin, as it has previously been shown that peroxidase does not cross-react [8]. Overall, this set of interactions reveals a weak cross-reactive potential of biotin. The bulky aliphatic amino acids, such as valine and isoleucine, and the aromatic amino acid phenylalanine show a

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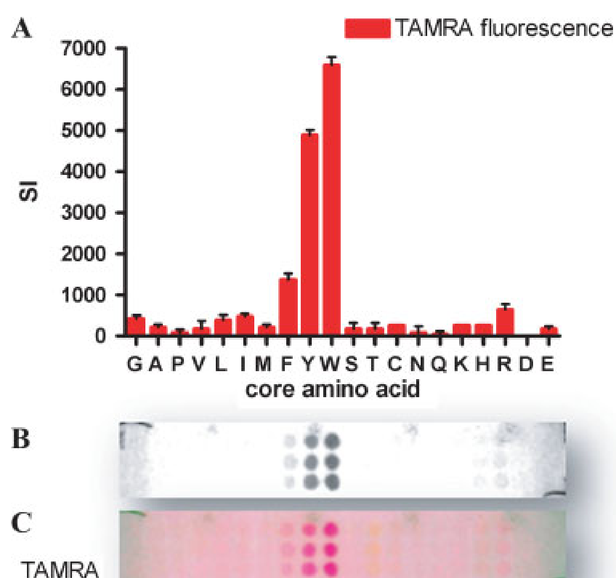

Figure 3. TAMRA cross-reaction. (A) Fluorescence emission of each corresponding spot measured at 645 nm is calculated from a circular region around the spot center detected in the image. All signals below a SI of 500 are at the background level and should therefore not be considered as interactions between the amino acid core and the detection system. (B) Fluorescent and (C) densitometric read-out. Each spot represents a cellulose membrane-bound peptide of the sequence $GGG[B]_5GGG$, where $[B]_5$ denotes five repeats of one of the 20 amino acids. Contrast is adjusted to ensure better visibility. The negative control without analyte shows no signal. Error bars represent the standard deviation of three spots.

signal slightly above the background. The smaller amino acids such as alanine, serine, proline, and leucine show insignificant signals scarcely visible against the background. Hence, it is reasonable to assume that the small biotin molecule cannot bind to a peptide probe when deeply buried inside the complex with streptavidin. According to the law of mass action, the interaction between biotin and streptavidin is favored ($K_D \sim 10^{-15}$), as the interaction of biotin with the peptides is not supposed to be covalent.

For fluorescent dye-labeled probes, it was necessary to consider any spot auto-fluorescence (membrane and/or peptides). The fluorescence emission of unchallenged peptide arrays in blocking buffer was measured prior to incubation at wavelengths corresponding to label emission (645 and 520 nm). As expected, no background signals were detected at 645 nm. As shown in Figure 2(A) and (B), common auto-fluorescence of the cellulose membrane can be observed at 520 nm. This is in accordance with the literature [22] and may result from membrane impurities bound during the processes of the synthesis cycles, e.g. Fmoc deprotection, side-chain deprotection, or coupling procedures. In addition, significant spot auto-fluorescence was measured at 520 nm for Trp-, His-, and, unexpectedly, also for Thr-containing peptides (Figure 2(A)). Although fluorescence of tryptophan and histidine can be explained by their aromatic ring systems containing more than six valence electrons, the observed fluorescence of the threonine core peptide still remains a challenge for interpretation.

Probing the peptide array with labeled GGG-peptides and comparing the recorded images with fluorescence records from the unchallenged arrays leads to additional signals. These signals are label-specific and indicate, in the context of this work, sorptive effects of the amino acid core composition. Besides the above-mentioned background effects, arrays challenged with FITC-GGG samples resulted in spot signals at 520 nm for Tyr-, Trp-, Thr-, Lys-, His-, and Arg-containing peptides (Figure 2(C)). After background correction for membrane auto-fluorescence, significant SIs remained for peptides containing Tyr, Lys, and Arg (Figure 2(D)). These amino acids are therefore interpreted as FITC cross-reactive moieties. Owing to the background correction of fluorescence signals at 520 nm, any further information about the cross-reactivity of Trp, His, or Thr was lost.

Our results draw a clear picture of the cross-reactivity of amino acid cores with the peptide TAMRA-GGG. As shown in Figure 3, significant spot SIs at 645 nm were observed for Phe, Tyr, and Trp cores. The strength of cross-reactivity between these amino acids and TAMRA follows the order Phe<Tyr<Trp. In addition, densitometry was used to read the capturing of TAMRA-GGG via staining (see experimental section). As shown in Figure 3(B) and (C), results are in accordance with the fluorescence read-out approach. The aromatic TAMRA moiety interacts exclusively with aromatic amino acid cores (Figure 3(A)). Therefore, aromatic stacking is most likely the common driving force for the amino acid–TAMRA interaction. Stacking is a widespread mechanism for stabilizing organic moieties. It is accomplished by the favorable interaction of π-electrons of aromatic systems [23]. In our case, the π-electron systems of TAMRA and the side group of Trp may interact in an energetically favorable manner via stacking interactions, which the smaller aromatic systems of Tyr and Phe possibly cannot provide to the same extent.

One open question is the influence of the peptide-specific density per spot on the binding of the label-GGG analytes. Therefore, peptide arrays with varied concentrations of $GGG[B]_5GGG$ were probed for binding with TAMRA-GGG, FITC-GGG, and Biotin-GGG. The amount of peptide per spot was adjusted as described in the literature [21]. The results are presented in the supporting information (Figures S1–S3). All detection methods show the reported behavior down to 6.25% of the initial concentration, where the signal breaks off due to the spot's low peptide density. The results suggest that the peptide-specific density influences the signal level while not being the cause of the interaction. Reducing the peptide density by a factor of 10 diminishes unwanted side effects. However, it may also result in general binder signal loss. An overall reduction of the peptide load of a membrane is therefore not advisable and has to be adapted to the object of research.

Probing the core reduction peptide arrays in which the core motif lengths vary from $[B]_5$ to $[B]_1$ reveals information about the critical length of the cross-reacting motif. As described above, the core reduction arrays were incubated with TAMRA-GGG, FITC-GGG, and Biotin-GGG. Figures S4–S6 (see supporting information) depict the results. A strong dependency on the quantity of aromatic amino acids can be observed for TAMRA. In the case of Trp, signals can be detected even when the core is reduced to one amino acid. The reduction library incubated with FITC-GGG shows signals above the background for all cross-reacting core reductions, but they also decrease with the length of the core. The analysis for biotin/streptavidin-POD reveals that interactions with Val, Leu, Ile, and Phe only occur if the cross-reacting amino acid is repeated more than four times. The interaction with positively charged amino acids can still be found at the critical length of two (Lys) or even just one (Arg) core position.

The approach using model peptides leads to conclusive results. However, these findings have to be verified in a more realistic setting. Therefore, we designed a 15-meric random peptide library of

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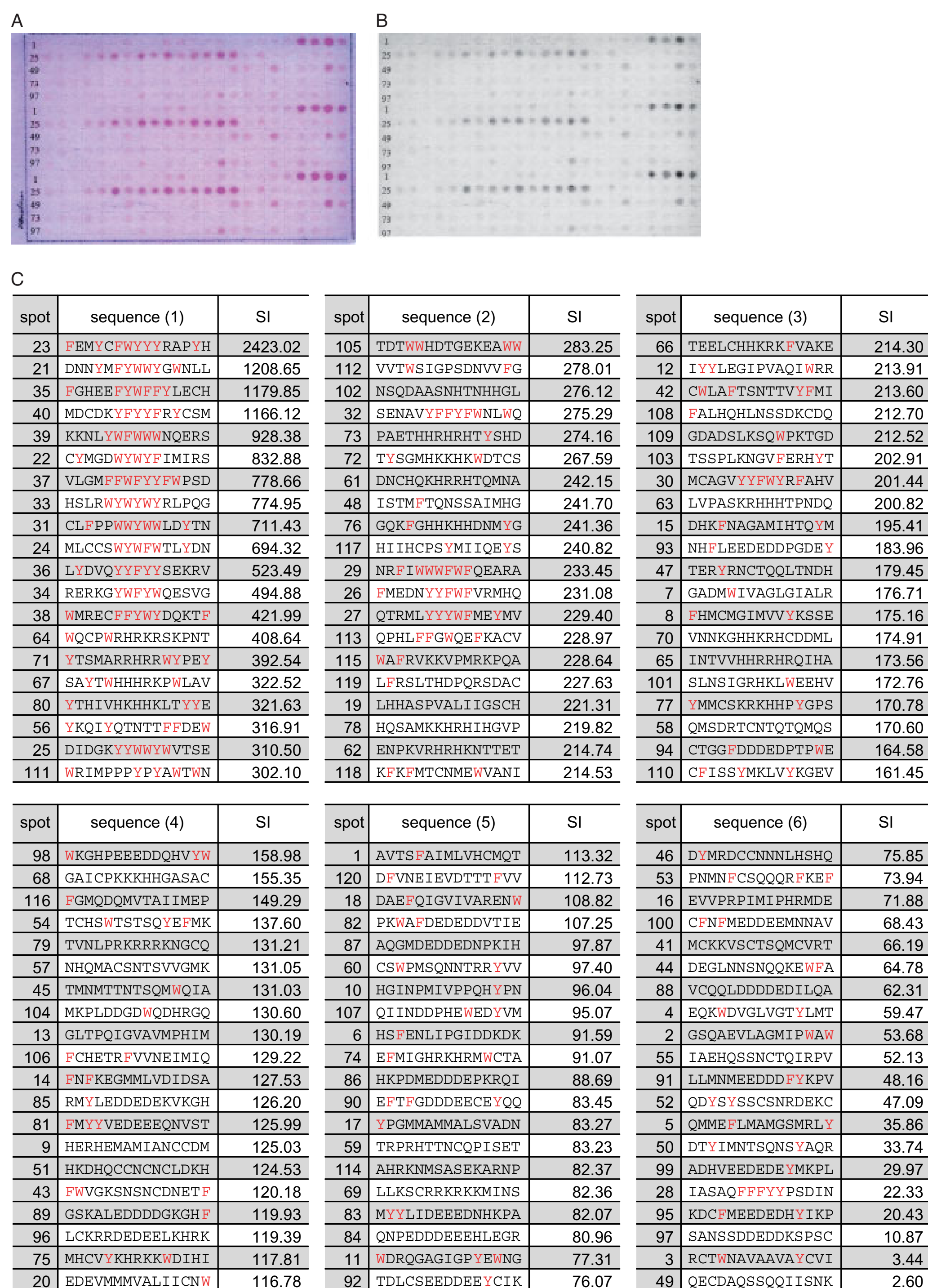

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spot	sequence (1)	SI
23	FEMYCFWYYYRAPYH	2423.02
21	DNNYMFYWWYGWNLL	1208.65
35	FGHEEFYWFFYLECH	1179.85
40	MDCDKYFYYFRYCSM	1166.12
39	KKNLYWFWWWNQERS	928.38
22	CYMGDWYWYFIMIRS	832.88
37	VLGMFFWFYYFWPSD	778.66
33	HSLRWYWYWYRLPQG	774.95
31	CLFPPWWYWWLDYTN	711.43
24	MLCCSWYWFWTLYDN	694.32
36	LYDVQYYFYYSEKRV	523.49
34	RERKGYWFYWQESVG	494.88
38	WMRECFFYWYDQKTF	421.99
64	WQCPWRHRKRSKPNT	408.64
71	YTSMARRHRRWYPEY	392.54
67	SAYTWHHHRKPWLAV	322.52
80	YTHIVHKHHKLTYYE	321.63
56	YKQIYQTNTTFFDEW	316.91
25	DIDGKYYWWYWVTSE	310.50
111	WRIMPPPYPYAWTWN	302.10

spot	sequence (2)	SI
105	TDTWWHDTGEKEAWW	283.25
112	VVTWSIGPSDNVVFG	278.01
102	NSQDAASNHTNHHGL	276.12
32	SENAVYFFYFWNLWQ	275.29
73	PAETHHRHRHTYSHD	274.16
72	TYSGMHKKHKWDTCS	267.59
61	DNCHQKHRRHTQMNA	242.15
48	ISTMFTQNSSAIMHG	241.70
76	GQKFGHHKHHDNMYG	241.36
117	HIIHCPSYMIIQEYS	240.82
29	NRFIWWWFWFQEARA	233.45
26	FMEDNYYFWFVRMHQ	231.08
27	QTRMLYYYWFMEYMV	229.40
113	QPHLFFGWQEFKACV	228.97
115	WAFRVKKVPMRKPQA	228.64
119	LFRSLTHDPQRSDAC	227.63
19	LHHASPVALIIGSCH	221.31
78	HQSAMKKHRHIHGVP	219.82
62	ENPKVRHRHKNTTET	214.74
118	KFKFMTCNMEWVANI	214.53

spot	sequence (3)	SI
66	TEELCHHKRKFVAKE	214.30
12	IYYLEGIPVAQIWRR	213.91
42	CWLAFTSNTTVYFMI	213.60
108	FALHQHLNSSDKCDQ	212.70
109	GDADSLKSQWPKTGD	212.52
103	TSSPLKNGVFERHYT	202.91
30	MCAGVYYFWYRFAHV	201.44
63	LVPASKRHHHTPNDQ	200.82
15	DHKFNAGAMIHTQYM	195.41
93	NHFLEEDEDDPGDEY	183.96
47	TERYRNCTQQLTNDH	179.45
7	GADMWIVAGLGIALR	176.71
8	FHMCMGIMVVYKSSE	175.16
70	VNNKGHHKRHCDDML	174.91
65	INTVVHHRRHRQIHA	173.56
101	SLNSIGRHKLWEEHV	172.76
77	YMMCSKRKHHPYGPS	170.78
58	QMSDRTCNTQTQMQS	170.60
94	CTGGFDDDEDPTPWE	164.58
110	CFISSYMKLVYKGEV	161.45

spot	sequence (4)	SI
98	WKGHPEEEDDQHVYW	158.98
68	GAICPKKKHHGASAC	155.35
116	FGMQDQMVTAIIMEP	149.29
54	TCHSWTSTSQYEFMK	137.60
79	TVNLPRKRRRKNGCQ	131.21
57	NHQMACSNTSVVGMK	131.05
45	TMNMTTNTSQMWQIA	131.03
104	MKPLDDGDWQDHRGQ	130.60
13	GLTPQIGVAVMPHIM	130.19
106	FCHETRFVVNEIMIQ	129.22
14	FNFKEGMMLVDIDSA	127.53
85	RMYLEDDEDEKVKGH	126.20
81	FMYYVEDEEEQNVST	125.99
9	HERHEMAMIANCCDM	125.03
51	HKDHQCCNCNCLDKH	124.53
43	FWVGKSNSNCDNETF	120.18
89	GSKALEDDDDGKGHF	119.93
96	LCKRRDEDEELKHRK	119.39
75	MHCVYKHRKKWDIHI	117.81
20	EDEVMMMVALIICNW	116.78

spot	sequence (5)	SI
1	AVTSFAIMLVHCMQT	113.32
120	DFVNEIEVDTTTFVV	112.73
18	DAEFQIGVIVARENW	108.82
82	PKWAFDEDEDDVTIE	107.25
87	AQGMDEDDEDNPKIH	97.87
60	CSWPMSQNNTRRYVV	97.40
10	HGINPMIVPPQHYPN	96.04
107	QIINDDPHEWEDYVM	95.07
6	HSFENLIPGIDDKDK	91.59
74	EFMIGHRKHRMWCTA	91.07
86	HKPDMEDDDEPKRQI	88.69
90	EFTFGDDDEECEYQQ	83.45
17	YPGMMAMMALSVADN	83.27
59	TRPRHTTNCQPISET	83.23
114	AHRKNMSASEKARNP	82.37
69	LLKSCRRKRKKMINS	82.36
83	MYYLIDEEEDNHKPA	82.07
84	QNPEDDDEEEHLEGR	80.96
11	WDRQGAGIGPYEWNG	77.31
92	TDLCSEEDDEEYCIK	76.07

spot	sequence (6)	SI
46	DYMRDCCNNNLHSHQ	75.85
53	PNMNFCSQQQRFKEF	73.94
16	EVVPRPIMIPHRMDE	71.88
100	CFNFMEDDEEMNNAV	68.43
41	MCKKVSCTSQMCVRT	66.19
44	DEGLNNSNQQKEWFA	64.78
88	VCQQLDDDDEDILQA	62.31
4	EQKWDVGLVGTYLMT	59.47
2	GSQAEVLAGMIPWAW	53.68
55	IAEHQSSNCTQIRPV	52.13
91	LLMNMEEDDDFYKPV	48.16
52	QDYSYSSCSNRDEKC	47.09
5	QMMEFLMAMGSMRLY	35.86
50	DTYIMNTSQNSYAQR	33.74
99	ADHVEEDEDEYMKPL	29.97
28	IASAQFFFYYPSDIN	22.33
95	KDCFMEEDEDHYIKP	20.43
97	SANSSDDEDDKSPSC	10.87
3	RCTWNAVAAVAYCVI	3.44
49	QECDAQSSQQIISNK	2.60

Figure 4. Random peptide library incubated with TAMRA-GGG. (A) Densitometric analysis and (B) fluorescence at 645 nm. The array (spots 1–120) is repeated three times resulting in three identical subarrays. Each of the 120 spots represents a cellulose membrane-bound 15-meric peptide of random sequence with weighted cores. Contrast is adjusted to ensure better visibility of the spots. (C) The spot signal measured at 645 nm is calculated from a circular region around the spot center detected in the image. Trp, Tyr, and Phe are highlighted in red. SI is the calculated mean of three spots.

120 peptides with physicochemically weighted cores and random flanking residues. These sequences were SPOT synthesized in triplication on a cellulose membrane and were probed, freshly prepared, for binding TAMRA-GGG (Figure 4), FITC-GGG, or Biotin-GGG (supporting information, Figures S7 and S8), respectively. Interestingly, in many cases with this set-up just two physicochemically similar cross-reactive amino acids close to each other suffice to observe the above-mentioned effects we revealed using model peptides. The density and frequency of the cross-reactive amino acids correlate with the intensity of the measured spot signals. A comparison shows that the SI of each of the 120 spots varies significantly depending on the detection method used.

In conclusion, we have demonstrated that several amino acids interact with TAMRA-, FITC-, or biotin-labeling agents as well

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as streptavidin-POD. We do not recommend FITC for read-out when probing peptide arrays on cellulose membranes for binding, and suggest considering these results for *in vivo* approaches. Besides the observed spot, auto-fluorescence of several amino acids and the cellulose membrane, label-specific cross-reactivity with positively charged amino acids was observed for FITC and streptavidin-POD. TAMRA, on the contrary, seems to be a more suitable screening/read-out system for probing peptide arrays on a cellulose membrane. However, the influence of aromatic amino acids, especially tryptophan, has to be taken into account. A critical view on peptide sequences is essential and a comparative approach using both TAMRA- and biotin-labeled analytes is recommended. Such an approach would compensate effects on label-specific cross-reactive amino acids.

One has to bear in mind that a method is always limited by the effectiveness and validity of the read-out system. To avoid unwanted side effects, the right choice of buffer solutions is advised. However, in the case of SPOT synthesis, even the optimal buffer composition [8,24] fails to prevent cross-reaction. Our approach identified several amino acids that interact with different detection systems. We highly recommend factoring in these results with the analysis of future measurements to prevent and identify false-positive results. Furthermore, as good experimental practice [25], we advocate testing the detection method of choice for its ability to cross-react before running the actual experiment. Taking these new results into consideration will strengthen the reliability of the analysis of SPOT synthesis generated data in the future.

Acknowledgements

CCM acknowledges financial support of the Manchot-Foundation and VT acknowledges financial support of the Deutsche Forschungsgemeinschaft SFB 449. We thank Ingrid Abfalter for stimulating discussions and proofreading. We thank Ines Kretzschmar for the continuous synthesis of high-quality peptides.

Supporting information

Supporting information may be found in the online version of this article.

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