A. Kramer
U. Reineke
L. Dong
B. Hoffmann
U. Hoffmüller
D. Winkler
R. Volkmer-Engert
J. Schneider-Mergener

Spot synthesis: observations and optimizations

Authors' affiliations:
A. Kramer, U. Reineke*, L. Dong, B. Hoffmann, U. Hoffmüller, D. Winkler, R. Volkmer-Engert and J. Schneider-Mergener,* Institut für Medizinische Immunologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, 10098 Berlin, Germany.
*Equal contributors.

Correspondence to:
Jens Schneider-Mergener
Institut für Medizinische Immunologie
Universitätsklinikum Charité
Humboldt-Universität Berlin
Schumannstraße 20–21
10098 Berlin, Germany
Tel: + 49 30 2802 6139
Fax: + 49 30 2802 6460
E-mail: jsm@charite.de

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Abstract: Positionally addressable syntheses of peptides on continuous cellulose membranes (spot synthesis) have often been reported in detail, but important questions dealing with synthesis quality, reproducibility and subsequent binding assays have largely been under-emphasized. In this report we have investigated some of these problems. The most important results were: (i) the signal intensity of ligate binding to cellulose-bound peptides and the affinity of the corresponding soluble peptides show good correlation, illustrated by three different ligate binding assays; (ii) reducing peptide density on the cellulose avoids the 'ring spot' effect, i.e. where less binding is observed in the spot-center compared to the rim. We recommend a peptide density of 10 nmol/cm^2 as a reasonable starting point for further optimization; (iii) statistical analysis of binding assay reproducibility with more than 15 000 peptides resulted in a mean standard signal deviation of 0.18; and (iv) optimization of side-chain deprotection revealed that a 30-min pretreatment of the cellulose with 90% trifluoroacetic acid followed by the standard deprotection protocol resulted in higher purity of the synthesized products.

Spot synthesis, the positionally addressable, parallel chemical synthesis of peptides on continuous cellulose membranes, was originally developed to facilitate mapping of antibody epitopes (1, 2). Since then this technique has been extended to the preparation of many different types of peptide libraries, including scanning and combinatorial libraries (3–7), amino acid cluster libraries (8), cyclic libraries (4, 9), positional scanning libraries (5, 10) as well as inverted libraries consisting of peptides with free carboxy termini (11,

12). These libraries have been used not only to identify peptides that bind to proteins (3, 4, 13–19), metals (3–5, 20) and nucleic acids (3, 21), but also for mapping the substrate specificity of enzymes such as proteases or kinases (22–26). The most common application so far has been the mapping of linear epitopes using overlapping peptides derived from the primary structure of a given protein (27–33), but also non-linear antibody epitopes and receptor–ligand interaction sites can be identified (13, 14, 17, 34, 35; for review see (36)). Evidence for different conformation states of proteins was also obtained by spot synthesis (37), while transformation of L-peptide epitopes into D-peptide analogs with comparable biological properties showed the potential of spot synthesis for using D- or unnatural amino acids (38). Furthermore, spot synthesis was also used for the parallel microsynthesis of soluble peptides (39, 40), allowing the identification of peptides which block HIV-1 entry in T-cell tropic strains via its CXCR4 coreceptor (41).

The technical protocols of spot synthesis have been described in detail (2, 4, 6, 7, 42). This paper aims to address several questions and problems primarily regarding binding studies on the cellulose membranes, but also concerning the quality of spot synthesis. Such questions include: do the results of the solid-phase binding assays correlate with dissociation constants obtained in subsequent ELISA experiments with the corresponding soluble peptides? How sensitive are binding assays on cellulose membranes? How reproducible are the signals? How can the 'ring spot' effect be avoided, in other words when the signal is more intense at the rim of the spot than in the center (43)? How pure are the peptides synthesized on cellulose membranes?

Materials and methods

Peptide synthesis on cellulose membranes – spot synthesis

Spot synthesis was performed on Whatman 50 paper (Whatman, Maidstone, UK) as described in detail previously (7). For the adjustment of peptide density see below.

Synthesis of N-acetyl-β-alanine-OPfp

To 0.1 mol *N*-acetyl-β-alanine-OH (purchased from Novabiochem, Läufelfingen, Switzerland) in DCM, 0.11 mol dicyclohexylcarbodiimide and 0.1 mol pentafluorophenol were added and stirred overnight. After filtration to remove dicyclohexylurea the reaction mixture was washed with water, with saturated $NaHCO_3$-solution, again with water and then dried over Na_2SO_4. The solvent was removed *in vacuo* yielding a white powder.

Deprotection experiments

Three different conditions for side-chain deprotection were used: (i) 50% TFA, 3% triisobutylsilane, 2% water, 1% phenol in DCM (deprotection solution A), for 2.5 h with shaking (standard condition). The membrane was then washed four times with DCM, three times with dimethylformamide (DMF) and twice with methanol (standard washing) and dried; (ii) 90% TFA, 3% triisobutylsilane, 2% water, 1% phenol in DCM (deprotection solution B), for 30 min without shaking, followed by standard washing and drying. The membrane was then treated for 150 min with deprotection solution A without shaking. After standard washing the membrane was dried; (iii) 90 min deprotection solution B (without shaking), followed by standard washing and drying and then for 90 min, again deprotection solution B (without shaking). After standard washing the membrane was dried.

Adjustment of peptide density

The cellulose membrane was functionalized with Fmoc-β-alanine as the first anchor residue as described (2, 4, 6, 7). At the second step for the coupling of the next anchor position, a mixture of different ratios of a 0.3-M solution of Fmoc-β-alanine-OPfp and a 0.3-M solution of *N*-acetyl-β-alanine-Opfp (see above) in dimethylsulfoxide was used (100%, 25%, 6.25%, 1.56%, 0.39% Fmoc-β-alanine-OPfp). For double-coupling each reaction was performed for 15 min.

Determination of peptide density

The peptide density on cellulose membranes was determined by measuring the released Fmoc-protecting group photometrically. Therefore, additional bigger spots ($\approx$ 0.3 cm^2) which can be punched out at each step of synthesis before removing the Fmoc-protecting group of the N-terminus were defined on the cellulose membrane. These spots were treated with 20% piperidine in DMF for 20 min to cleave the Fmoc-groups, and the Fmoc/piperidine solution was analyzed photometrically at the absorbance wavelength 302 nm. The peptide density was calculated using the extinction coefficient ε_{302} = 8100 L mol/cm.

Cleavage of peptides from the cellulose membrane

After cleavage of side-chain protecting groups, the cellulose membrane was washed three times for 1 h with 100 mM phosphate buffer, pH 7.5 (except for the peptides described in Fig. 4). The membrane was then dried and placed in a desiccator which was subsequently filled with ammonia. After 5 h single spots were punched out and cellulose-adsorbed peptides were dissolved in water.

Reversed phase HPLC

Analytical reversed-phase HPLC (RP-HPLC) of the cleaved peptides was performed on a Vydac C18 column (250 × 4.6 mm, Hesperia, CA, USA) using a linear gradient of 5–60% acetonitril vs. water (0.05% TFA) for 25 min at 1.2 mL/min flow rate. Absorbance was detected at 214 nm.

Mass spectrometry

Peptides cleaved from cellulose membranes were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a-cyano-hydroxy-cinnamic acid as a matrix (LaserTec FOCUSS mass spectrometer, GSG/VESTEC, Karlsruhe, Germany).

Antibody binding assays

Detection using a second peroxidase-conjugated antibody (Fig. 1A and Fig. 2)

The cellulose-bound peptides were treated with blocking buffer (Boehringer Mannheim, Germany) diluted 1 : 10 in T-TBS (Tris-buffered saline/0.05% Tween®20, pH 8.0) for 2 h and then incubated with 0.5 µg/mL CB4–1 (or 0.1–1 µg/mL for the other antibodies used in the 'ring spot' experiments of Fig. 2) in blocking buffer/T-TBS for 3 h. After washing three

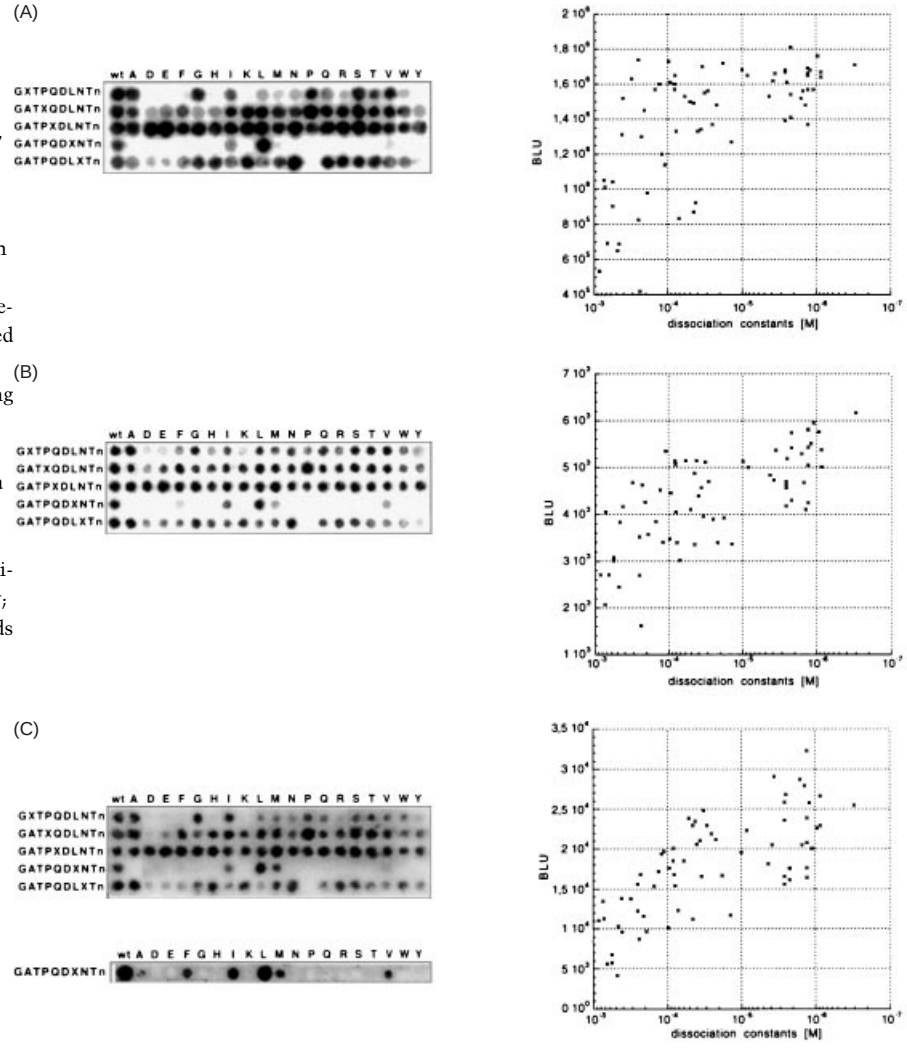

Figure 1. Binding of antibody CB4-1 (46) to epitope subtitution analogs detected by different methods. Five positions (X) of the epitope GATPQDLNTn (n = norleucine) were substituted by 19 L-amino acids (rows, cysteine omitted). The spots in the left column are identical and represent the starting peptide, other spots are single substitution analogs. (A) Two-step detection of peptide-bound CB4-1 using a peroxidase-conjugated antimouse IgG antibody. (B) One-step detection with CB4-1 directly conjugated to peroxidase. (C) Detection of CB4-1 after electrotransfer onto a PVDF membrane using a peroxidase-conjugated antimouse IgG antibody. On the lower left the leucine substitution row is shown separately after a 5-fold longer exposure. In all approaches visualization was carried out using a chemiluminescence detection system (Lumi-Imager™, Boehringer Mannheim, Germany; BLU = Boehringer light units). For all methods a signal intensity/dissociation constant correlation is shown using dissociation constants determined by ELISA with the corresponding soluble peptides (47).

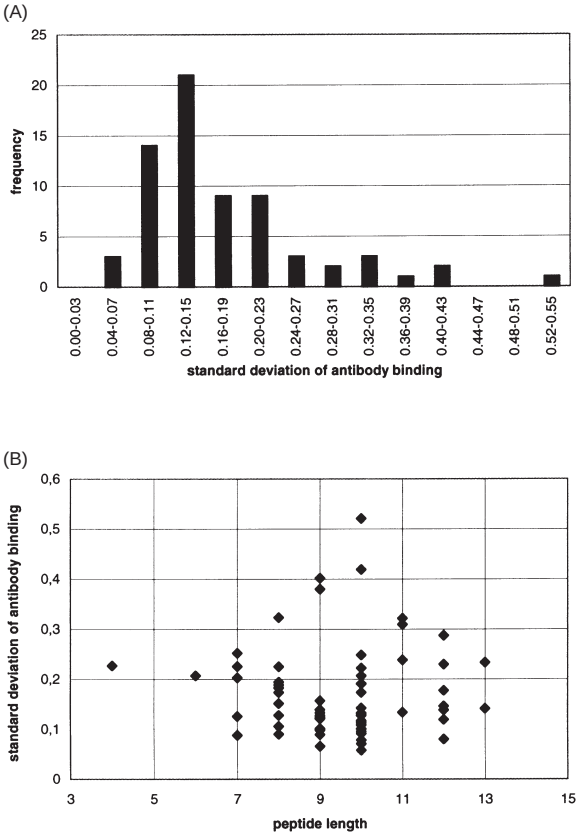

Figure 2. (A) Standard deviation frequency of binding signals of 68 different peptide/antibody interactions. Each peptide was synthesized on cellulose (at least 12 times), and binding was visualized and quantitated as described in Figure 1. (B) Correlation between standard deviation of antibody binding signals and the length of the respective epitope peptides.

times with T-TBS, 1 μg/mL anti-mouse IgG peroxidase-conjugated antibody (Sigma, Deisenhofen, Germany) in blocking buffer/T-TBS was added for 2 h. Following three washes for 10 min with T-TBS, detection was carried out with the SuperSignal™ chemiluminescence detection system (Pierce, Rockford, IL, USA) using the Lumi-Imager™ (Boehringer, Mannheim, Germany).

Detection using directly labeled CB4-1 (Fig. 1B)

The peptide membrane was blocked as described above and incubated with 0.5 μg/mL peroxidase-conjugated (44) CB4-1 in blocking buffer/T-TBS for 3 h. After washing three times with T-TBS, peptide-bound antibody was detected by chemiluminescence as described above.

Indirect detection following electrotransfer onto polyvinylene difluoride (PVDF) membranes (Fig. 1C)

After blocking with blocking buffer/T-TBS for 2 h the cellulose membrane was incubated with 2 μg/mL CB4-1 in blocking buffer/T-TBS for 3 h. The cellulose membrane was washed twice for 3 min with T-TBS, and to transfer peptide-bound antibody to PVDF membranes, electroblotted in a semi-dry blotter (Phase GmbH, Lübeck, Germany; constant power of 0.8 mA/cm² for 2 h; cathode buffer: 25 mM Tris base, 40 mM 6-aminohexane acid, 20% methanol; anode buffer I: 30 mM Tris base, 20% methanol; anode buffer II: 300 mM Tris base, 20% methanol) (16, 17). The PVDF membrane was blocked with blocking buffer/T-TBS and CB4-1 was detected using a peroxidase-conjugated antimouse IgG antibody in combination with the chemiluminescence system as described for the other detection methods.

Results and Discussion

Correlation between signal intensity and affinity

Two general questions arise concerning the signal intensity observed on the peptide membranes after binding assays and detection: (i) does the signal intensity serve as a relative measure for the affinity of the peptide–ligate interaction and (ii) how sensitive is the detection method? There are several factors which might influence the correlation between signal intensity and binding affinity: the synthesis itself can lead to different amounts of the correct full-length peptide in the spots, and the type of detection assay used is likely to be a critical step, while non-homogeneity of the cellulose membrane could additionally bias the results. Three general principles for the detection assays were compared, all of them having particular advantages and disadvantages and each being used to investigate different types of protein–protein interactions (13).

The most commonly used assay is based on detection directly on the peptide membrane following its incubation with the respective ligand. The signal is generated by specific antibodies against the ligate and a second enzyme-conjugated antibody specific for the constant region of the anti-ligate antibody, combined with a suitable substrate. In control experiments the interaction of both detection antibodies with the cellulose-bound peptides must be tested. This membrane assay may be accelerated if the ligate can be labeled directly with an enzyme, fluorescent dye or radioactive isotope since the incubation procedure is less time-consuming and avoids the problem of unspecific antibody interactions with the peptides. Only possible interactions of the peptides with the chosen enzyme label have to be checked as a control.

Indirect detection of peptide-bound ligands was introduced by Rüdiger *et al*. (16) using electrotransfer of bound ligates onto polyvinylene difluoride (PVDF) membranes prior to immunodetection steps. This procedure has particular advantages for investigating discontinuous protein–protein interactions. One broadly applied method for the mapping of protein–protein interactions is the use of protein-derived scans of overlapping peptides (45) which are probed with the respective binding partner. For the identification of linear binding epitopes the peptide–protein affinities are sufficiently high for standard incubation and detection procedures (17). However, the mapping of discontinuous binding sites using peptide scans is considerably more difficult due to the very low affinities of the individual parts of these epitopes with the respective binding partner (13, 17). During the steps involved in immunodetection directly on the membrane the binding equilibrium is shifted towards the non-complexed ligate. However, indirect detection on PVDF membranes captures the total bound protein, increasing the sensitivity. Furthermore, a major advantage of this method is that the detection antibodies are not in contact with the peptide membrane, an important aspect when mapping protein homodimerization sites because specific antibodies can also bind to the membrane-bound peptides derived from the same protein sequence.

In order to address the question of the correlation between signal intensity and affinity we compared the interaction of the monoclonal anti-p24 (HIV-1) antibody CB4-1 (46) with its peptide epitope using three detection methods. The dissociation constants of this antibody complexed with the wild-type peptide epitope and several amino acid substitution analogs were determined by competitive ELISA with soluble peptides (47, 48). Five positions of the wild-type epitope (GATPQDLNTn; n = norleucine) were substituted by all other genetically encoded amino acids (cysteine omitted) resulting in dissociation constants in the range of 3.0×10^{-7} M to not measurable ($> 10^{-3}$ M). The same peptides were spot-synthesized and probed with CB4-1 in the three different binding assays using (i) a second antibody, (ii) directly labeled CB4-1, or (iii) detection after electrotransfer onto PVDF membranes (see Materials and Methods). Figure 1 shows the binding patterns of the substitution analogs detected by the different methods and a correlation of the quantitated signal intensities with the dissociation constants with $K_D < 10^{-3}$ M. For all three methods the peptide membranes displayed almost the same spot pattern and a rough correlation between signal intensity and affinity. However, the absolute signal intensity values vary depending upon the exposure time used in the final detection step.

The leucine residue of the peptide epitope is the most critical for antibody binding and only the wild-type amino acid and the substitution to the physicochemically similar isoleucine led to measurable dissociation constants ($K_D < 10^{-3}$ M). Using the second antibody detection assay only these two peptides produced a signal in the leucine substitution row. In contrast, the methionine and valine substitution analogs were also detected with both the labeled CB4-1 (Fig. 1B) and the electrotransfer technique (Fig. 1C). For the latter method a 5-fold longer exposure of the leucine substitution row is displayed separately (Fig. 1C), showing additional signals for the phenylalanine and the alanine substitutions after 5-fold longer exposure time. This increase in sensitivity was not observed with longer exposures of the directly labeled CB4-1 assay (not shown). Therefore, the indirect electrotransfer method, as expected, was the most sensitive approach, probably because the washing time before transfer onto PVDF membranes is relatively short.

Statistics of antibody binding to peptides on cellulose membranes

To investigate the reproducibility of the signals obtained from binding assays with cellulose-bound peptides, we synthesized 68 different antibody epitopes of various lengths (6–13 residues), each between 12 and 26 times. After incubating the appropriate spots with their respective murine monoclonal IgG antibodies, and subsequent quantitation of second antibody binding using a chemiluminescence detection system (not shown), the standard deviations of measured binding intensities were calculated. Figure 2A shows the frequency of the standard deviations (mean standard deviation 0.18). About 70% of the 68 epitopes tested bound their respective antibodies with a standard deviation of chemiluminescence signals of less than 0.2. No correlation between standard deviation and the peptide length was obvious (Fig. 2B) suggesting, as expected, that in this range (6–13 amino acids) the quality of peptide synthesis (which should be the major source of variation) is affected more by the sequence rather than the overall length of the peptide.

'Ring-spot' effect in ligand binding experiments

Sometimes using cellulose-bound peptides for ligand binding experiments a 'ring-spot' effect is observed where the

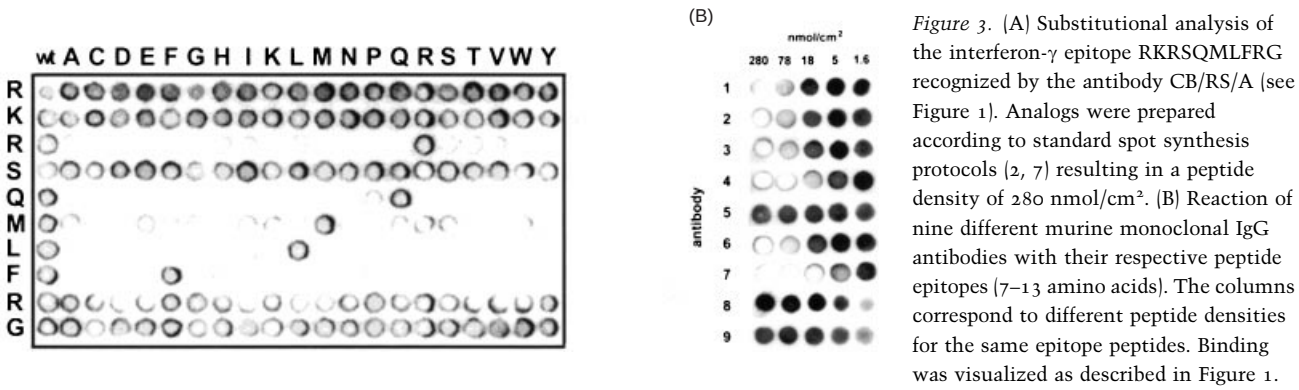

Figure 3. (A) Substitutional analysis of the interferon-γ epitope RKRSQMLFRG recognized by the antibody CB/RS/A (see Figure 1). Analogs were prepared according to standard spot synthesis protocols (2, 7) resulting in a peptide density of 280 nmol/cm². (B) Reaction of nine different murine monoclonal IgG antibodies with their respective peptide epitopes (7–13 amino acids). The columns correspond to different peptide densities for the same epitope peptides. Binding was visualized as described in Figure 1.

rim of a spot has a higher signal intensity compared to its center (45). Figure 3A shows a substitutional analysis of the epitope peptide RKRSQMLFRG recognized by the murine monoclonal anti-interferon-γ antibody CB/RS/A (R. Sabat, personal communication). Each position of the epitope was exchanged by all other 19 amino acids providing binding data for about 200 epitope variants. The irregular signals of the spots, however, did not allow the quantitation of antibody binding. In some cases it is even difficult to decide qualitatively between binding and non-binding (e.g. for the methionine variants). One explanation of this effect has been that a too-high concentration of enzyme in the center of the spot results in the exhaustion of substrate before the membrane can be placed in the Imager or the X-ray film cassette for detection (7). Extensive washing of the membrane followed by re-detection can solve this problem (not shown).

In some cases, however, the enzyme concentration cannot account for the 'ring-spot' effect, as it is also observed using chromogenic substrates. As another explanation, we speculated that the capillary effect of the cellulose causes a concentration gradient of amino acid solutions from the center to the edges of the spots during synthesis. The resulting differences in peptide density on the cellulose could influence the signal intensity such that a lower density of peptides (as at the rim of the spots) would provide a higher signal. In fact, decreasing the functionality of the cellulose membrane resulted in higher antibody-binding spot signals (Fig. 3B). The peptide density can be adjusted by spotting a mixture of Fmoc-β-alanine-OPfp and *N*-acetyl-β-alanine-OPfp in different ratios for coupling of the second anchor position. Referring to the starting membrane functionality (from the first derivatization step) the ratio of Fmoc-β-alanine to the total β-alanine (Fmoc-β-Ala + *N*-acetyl-β-Ala) in the applied mixtures corresponds exactly to the percentage of remaining functionality (not shown). By investigating several peptide–antibody-interactions (murine monoclonal IgGs) with respect to peptide density, the appearance of the 'ring-spot' effect was observed to be different for each system (Fig. 3B). In almost all cases the signals were higher with a lower peptide densitiy, leading to the conclusion that in order to obtain maximal and reliable signals the peptide density has to be adjusted for each peptide–ligand system. As a starting point a density of around 10 nmol/cm² is recommended, rather than a value of 280 nmol/cm² normally reached in the standard synthesis protocol (2, 7). Consequently, the amino acid concentration necessary for the subsequent coupling steps may be decreased by at least 5-fold without changing the ligate-binding properties of the synthesized peptides (data not shown), which reduces the costs of peptide synthesis considerably.

Analysis of peptide purity

Ammonolysis of the ester bond between peptide and linker molecules coupled to the cellulose was described by Volkmer-Engert *et al*. (39). The cleavage of cellulose-bound peptides without requiring a linker molecule was performed by treating the membrane for 5 h in ammonia atmosphere where the ester bond between the cellulose and the peptide (including the C-terminal β-alanine or glycine residues) was cleaved, but the peptides remained adsorbed to the cellulose. Subsequent punching out of the spots and elution of the peptides in water or buffer allows not only analysis of the synthesized products but also makes the peptides available for subsequent biological assays.

Analyzing the crude products of spot synthesis by RP-HPLC and mass spectrometry revealed, as might be expected, different results for different peptide sequences. Two examples are given. The RP-HPLC analysis of the peptide NH_2-GATPQDLNTMLββ-$CONH_2$ (β = β-alanine) showed about 70% pure product but also a deletion sequence, identified as a peptide lacking threonine

(Fig. 4A). However, the HPLC analysis of the IL-10-derived peptide NH_2-RKRSQMLFRGββ-$CONH_2$ showed the crude product to be very impure with signals at high retention times (Fig. 4B,i). These signals were identified by mass spectroscopy as being due to incomplete side-chain deprotection, especially of arginine and serine (data not shown). Signals at lower retention times (2–4 min) derived from the cellulose and could be reduced by extensive washing with water before ammonolysis (data not shown).

To improve side chain deprotection we tested different conditions with several model peptides. Treatment of the cellulose membrane using 90% TFA followed by standard deprotection (for exact protocols see Materials and Methods) resulted, in all cases, in a decrease in the signals at high retention times accompanied by an increase in the signals for the deprotected peptide. One example is given in Fig. 4B,ii. The deprotection efficiency could be further optimized by two treatments of the membrane for 90 min

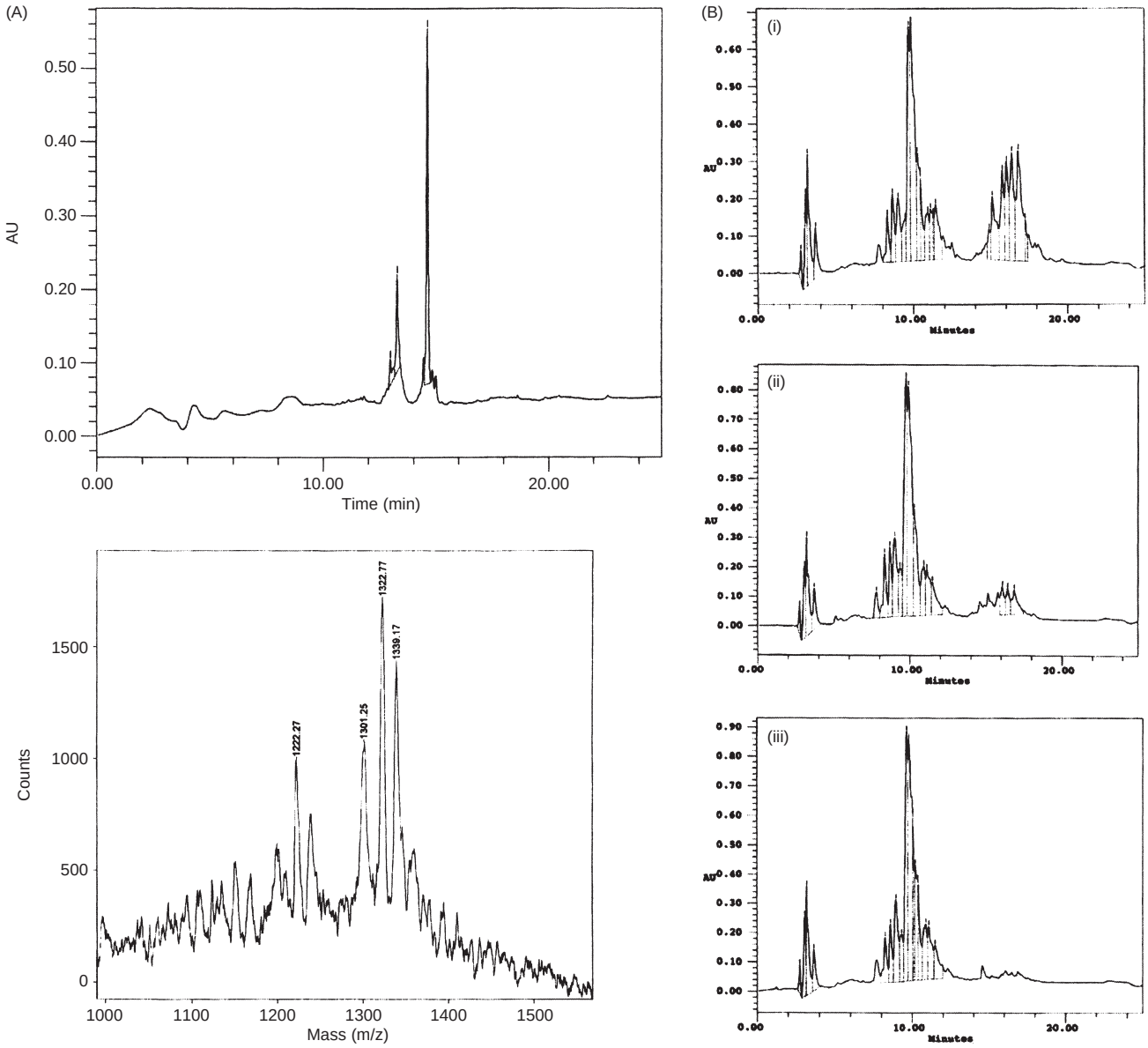

Figure 4. A: RP-HPLC and MALDI-TOF mass analyses of the 13-mer peptide NH_2-GATPQDLNTMLββ-$CONH_2$ (β = β-alanine) prepared by spot synthesis and obtained by cleaving the ester bond between the cellulose and the peptide by treatment with ammonia vapor. HPLC: Rt = 14.6 min: peptide; Rt = 13.3 min: deletion sequence (peptide lacking threonine). MS: m/z = 1301.25: M^+; 1322.77: $[M + Na]^+$; 1339.17: $[M + K]^+$; 1222.27: deletion sequence $[M$ – threonine + $Na]^+$. B: RP-HPLC analyses of the crude synthesis products of the 12-mer peptide NH_2-RKRSQMLFRGββ-$CONH_2$ (β = β-alanine) obtained after different side chain deprotection protocols and subsequent cleavage from the cellulose via ammonolysis (i). Standard side-chain deprotection protocol for spot synthesis (50% TFA, 2.5 h) (ii). Deprotection using 90% TFA for 30 min followed by the standard protocol (iii). Deprotection using two 90-min 90% TFA treatments.

with 90% TFA (Fig. 4B,iii). However, this led to mechanical instability of the cellulose. As a consequence, we recommend a new standard cleavage procedure based on 30-min pretreatment of the cellulose with 90% trifluoroacetic acid followed by the former standard deprotection protocol.

Nevertheless, even in those cases where the yield of cellulose-bound peptides analyzed by RP-HPLC and mass spectrometry was lower than 40%, solid-phase binding experiments with the respective monoclonal antibodies resulted in very reliable signals (not shown). Together with the knowledge that around 50-fold reductions in peptide density reveal better signals in most cases, this suggests that even very low amounts of the correct peptide in the spot can be detected in solid-phase binding experiments. This is of great importance for multiple peptide synthesis on cellulose membranes (up to 8000 peptides synthesized in parallel on a 20 × 30 cm sheet), where a complete analytical investigation of the synthesized peptides is obviously not practical.

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