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Highly parallel nano-synthesis of cleavable peptide-dye conjugates on cellulose membranes

Kai Licha,[a] Sarah Bhargava,[b] Christiane Rheinländer,[a] Andreas Becker,[a]
Jens Schneider-Mergener [b] and Rudolf Volkmer-Engert [b,*]

[a]*Institut für Diagnostikforschung an der Freien Universität Berlin, Spandauer Damm 130, D-14050 Berlin, Germany*
[b]*Institut für Medizinische Immunologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Schumannstr. 20-21, D-10117 Berlin, Germany*

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Abstract

Treatment of mercapto-functionalized cellulose membranes with preformed Fmoc-amino acid 3-bromopropyl esters yielded membrane-bound amino acids connected via a stable thioether and a cleavable ester bond. This synthesis strategy allows the highly parallel preparation of peptides that can be solubilized from the solid support. We apply this approach to the synthesis of novel peptide–cyanine dye conjugates which are potentially useful as fluorescent contrast agents targeted to tumor-specific receptors. © 2000 Elsevier Science Ltd. All rights reserved.

Recently, we described a strategy for the spot synthesis[1] of cleavable peptides and organic compounds on amino-functionalized cellulose membrane[2] (CAPE-membrane[3]), using a stably attached HMB-linker. This approach was successfully applied to the identification of peptidic HIV-1 antagonists[4] or PDZ protein domain binding peptides.[5] However, this synthesis strategy is critical because the yields of the amino acid esterifications employing a benzylic hydroxyl function at the cellulose-bound HMB linker are generally low and also strongly depend on the type of amino acid used. Therefore, a more robust method for the automatic synthesis of cleavable peptides on cellulose membranes was developed using preformed Fmoc-amino acid 3-bromopropyl esters and mercapto-functionalized cellulose membranes.

Peptides with high affinities for tumor-specific receptors can serve as carrier molecules for diagnostic effectors, such as radiolabels or fluorescent dyes, to achieve improved tumor detectability in diagnostic processes.[6] Accordingly, we extended our approach to the preparation of complete conjugates consisting of a peptide and a fluorescent cyanine dye, and to the characterization of these conjugates in cell-based screening assays after cleavage from the cellulose membrane. Cyanine dyes have proved to be promising contrast agents for the in vivo fluorescence demarcation of tumors.[7,8] The synthesized peptides are structurally derived from vasoactive intestinal peptide (VIP), the natural ligand of VIP receptors, that are described as promising tumor markers.[9]

* Corresponding author.

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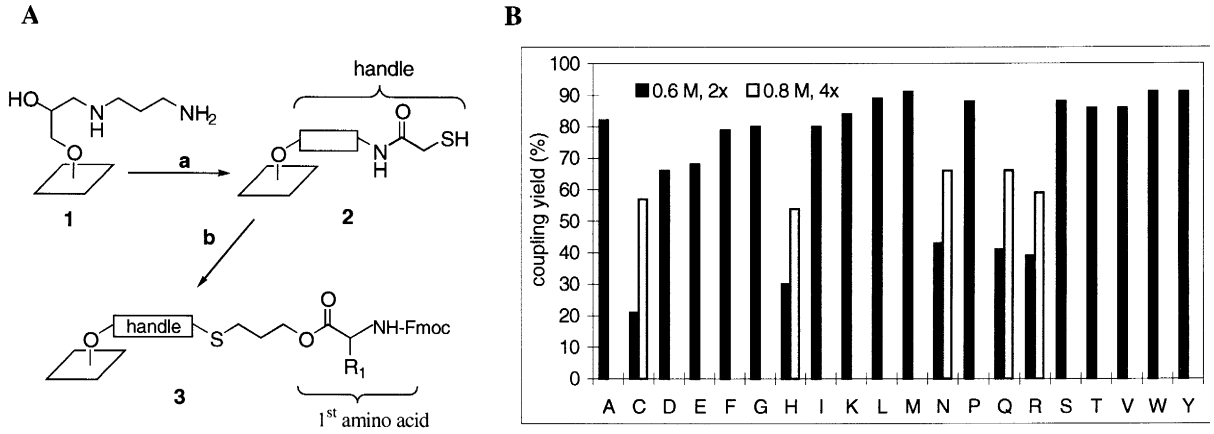

Fig. 1. **A**: Reaction scheme for the preparation of mercapto-functionalized cellulose membranes and attachment of the amino acids. **a**: (i) Br–CH_2–COONSu, (ii) Trt-SH/DIEA, (iii) TFA; **b**: (i) Cs_2CO_3, (ii) Fmoc-aa 3-bromopropyl ester. **B**: Coupling efficiency of the amino acid 3-bromo-propyl esters to the mercapto-functionalized membrane. One letter code for the amino acids is used. Sterical hindrance of the bulky protection groups (Trt, Pmc) of C, H, N, Q and R most likely accounts for the different coupling efficiencies. Applying a 0.8 M solution of the critical Fmoc-amino acid-3-bromopropyl esters and repeated coupling (4×) significantly reduced the differences in coupling yields

The steps towards the synthesis of cleavable peptides and peptide-dye conjugates are shown in Fig. 1. First, the *N*-modified CAPE-membrane **1** was prepared.[10] In contrast to the previously described method,[2] the present approach leads to significantly higher loadings. *N*-Acylation of **1** in a spot-synthetic manner using bromoacetic acid *N*-hydroxysuccinimide ester[11] (1 M solution of BrAcOSu in DMF, 1 µl for each spot, double coupling, 15 min each) followed by treatment with triphenylmethylmercaptan (1 M solution of Trt-SH in DMF containing 1 equiv. DIEA, 2×0.5 h each) and subsequent deprotection of the mercapto-function[12] results in the mercapto-functionalized membrane **2** (89% yield[12]). *S*-Alkylation of **2** with Fmoc-amino acid 3-bromopropyl esters leads to the membrane-bound amino acids **3** (see experimental note[13]). Coupling efficiency is shown in Fig. 1. The Fmoc-amino acid 3-bromopropyl esters (except for arginine) were prepared by *O*-acylation of 1-bromo-3-propanol with Fmoc-amino acid fluorides[14] in excellent yields.[15]

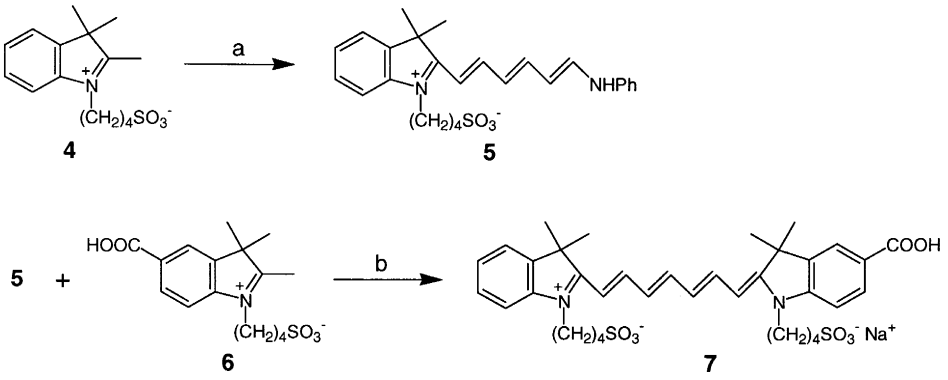

Fig. 2. Reaction scheme for the synthesis of 1,1′-bis-(4-sulfobutyl)indotricarbocyanine-5-carboxylic acid, sodium salt **7**. **a**: glutaconic aldehyde dianilide hydrochloride, Ac_2O, 30 min at 120°C; **b**: Ac_2O, AcOH, NaOAc, 30 min at 120°C

After attachment of the first amino acid (Fig. 1), several model peptides were completed by the standard spot synthesis protocol and the N-termini were dye-labeled by *N*-acylation with the indotricar-

bocyanine **7** (Fig. 2) (TBTU activation: 0.3 M solution of **7** in DMF containing 1 equiv. TBTU and 2 equiv. DIEA, 1 µl for each spot, double coupling, 20 min each).

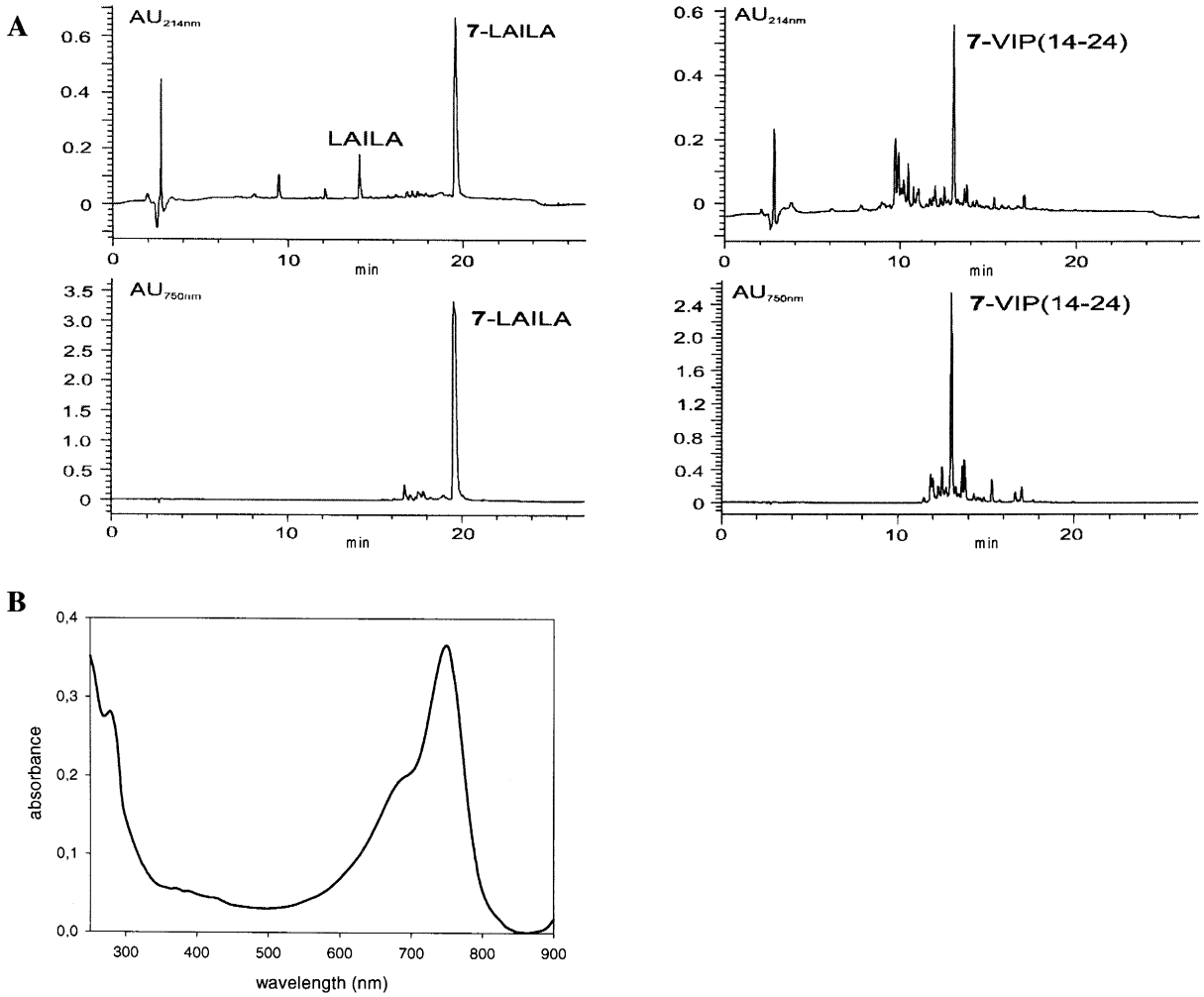

Fig. 3. **A**: RP-HPLC chromatograms of dye-peptide conjugates synthesized and cellulose. Model peptide LAILA (left) and 11 metric VIP-derivative VIP (14–24) (right) with N-terminal conjugated cyanine dye **7**. Detection at 214 nm (upper part) and 750 nm (lower part) are shown for both peptides. A linear gradient of 6–40% acetonitrile/water (0.05% TFA) was used for 20 min at 1.2 ml flow rate on a RP-C_{18} column. **B**: Absorbance spectrum of **7**-VIP (14–24) showing the peptide absorption at 280 nm and the dye absorption at 750 nm

Indotricarbocyanines were chosen as potential fluorescent contrast agents because of their high molar extinction coefficients and sufficient fluorescence quantum yields in the near-infrared spectral range (700–900 nm),[7] thus providing optical detectability within the living tissue.[16] The hydrophilic, carboxy-functionalized derivative **7** was synthesized for peptide conjugation (Fig. 2). The synthesis was performed by the reaction of 4-sulfobutyl-2,3,3-trimethyl-3*H*-indolenin[17] with 1 equiv. glutaconic aldehyde dianilide in acetic anhydride leading to the intermediate **5**. Subsequent addition of 1.1 equiv. 5-carboxy-4-sulfobutyl-2,3,3-trimethyl-3*H*-indolenin[18] followed by acetic acid and sodium acetate (3.5 equiv.) to the reaction mixture finally produced **7** in 60% yield after precipitation with diethyl ether and

purification by reversed phase chromatography (Europrep C-18, 30 μ, Knauer) using water/methanol as eluants.

Side-chain deprotection of the membrane-bound peptide-dye conjugates was achieved by treatment of the cellulose membrane with a mixture of: (i) 95% TFA, 1% phenol, 3% TIBS, 2% water for 0.5 h and (ii) 50% TFA, 1% phenol, 3% TIBS, 2% water in DCM for 3 h. The peptide-dye conjugates were finally released as peptide-carboxamides or free acids and analyzed by RP-HPLC, mass spectrometry and UV spectroscopy. Ammonolysis of the membrane-bound peptide-conjugates with ammonia gas as described[2] leads to peptide-carboxamides. However, substantial decomposition of the indotricarbocyanine part of the peptide was apparent in the UV spectra (data not shown). Therefore, the peptide-dye conjugates were released as free acids by: (i) punching out the spots followed by (ii) treatment of each spot with 200 μl of an aqueous methanolic solution (1:1) containing 25 μmol NaOH for 30 min[19] and (iii) neutralization of the mixture with dilute hydrochloric acid. In Fig. 3 two RP-HPLC chromatograms of released peptide-dye conjugates are shown by way of example. The calculated mass was verified by MALDI-TOF mass spectrometry (data not shown) for all the conjugates. The UV spectrum of the peptide-dye conjugate (Fig. 3) revealed the intact dye structure with its typical absorption peak at 750 nm.

The described method is applied in the simultaneous automatic preparation of different dye-labeled VIP derived peptides, e.g. for a mutation analysis of the VIP peptide. Internalization efficiency of the conjugates is currently being determined in transfected RIN38 tumor cells expressing the VIP receptor VPAC1.

In conclusion, this approach allows a highly parallel automatic nano-synthesis of peptides (ca. 560 compounds per 18×28 cm membrane affording up to 100 nmol compound per spot at a spot size of 0.25 cm^2) that permit N-terminal conjugation with fluorescent dyes and the cleavage of complete dye-peptide conjugates for screening assays.

Acknowledgements

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3. Abbreviations: CAPE: cellulose-amino-hydroxypropyl ether; DBF: dibenzofulvene; DIEA: diisopropylethylamine; DCM: dichloromethane; HMB: 4-hydroxymethylbenzoic acid; NMI: *N*-methylimidazole; NTB: 2-nitro-5-thiobenzoate; TFA: trifluoroacetic acid; TBTU: *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-tetrafluoroborate; TIBS: tri-isobutylsilane.
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10. The cellulose sheet (18×28 cm) (Whatmann 50, Maidstone, UK) was washed (5 min) with 50 ml of methanol containing 1 ml of perchloric acid (60% in water). After drying it was treated with a solution of 4 ml of epibromohydrine and 400 μl

of perchloric acid (60% in water) in 36 ml of dioxane for 1–3 hours, washed with 50 ml of ethanol (15 min) and incubated with a 50% solution of 1,3-diaminopropane in DMF (60 ml) overnight. Washing with DMF (3×), ethanol (2×), water (2×), ethanol (2×), 5 M sodium methanolate/methanol, methanol and water yielded the amino-functionalized membrane **1** (400–1000 nmol/cm^2). Loading was determined by incubation of a membrane spot with a 0.6 M solution of Fmoc-β-alanine-Opfp in DMF and measuring the absorbance of the released Fmoc-group (UV-absorbance of the DBF-piperidine adduct: ε_{302}=8100).

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13. Different conditions for the formation of the thioether bond were tested, revealing a clear preference for the caesium-thiolate. Therefore, the modified cellulose membranes **2** were incubated with a 20% aqueous solution of Cs_2CO_3 (2 min), followed by washing steps including water, ethanol and ether. *S*-Alkylation with the Fmoc-amino acid 3-bromopropyl esters (1.2 µl of a 0.6 M solution in DMF per spot, double coupling, 15 min each). Coupling yields were determined by measuring the absorbance of the released Fmoc-group.
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15. The Fmoc-amino acid 3-bromopropyl esters are solid except for cysteine and proline. Synthetic procedure: For good results Fmoc-amino acid fluorides (Carpino, L. A.; Sadat-Aalaee, D.; Chao, H. G.; DeSelms, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 9651) were freshly prepared and used without further characterization and purification. To a stirred solution of the Fmoc-amino acid fluoride (10 mmol) in DCM (100 ml) were added 1-bromo-3-propanol (874 µl, 10 mmol) and NMI (795 µl, 10 mmol). After 0.5 h the reaction mixture was washed with 3% citric acid and brine. The organic phase was dried (Na_2SO_4) and evaporated in vacuo. Yields of the 3-bromopropyl esters were in the range of 89–95% as determined by RP-HPLC. The Fmoc-Arg(Pmc) 3-bromo-propylester was prepared by esterification of Fmoc-Arg(Pmc)-OH using 1 equiv. 1-bromo-3-propanol, 1.1 equiv. DCC, 2 equiv. NMI and DCM as solvent. The resulting ester was isolated after 12 h in 79% yield.
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19. Before punching out the spots, the membrane was washed with phosphate buffer (0.1 M, pH 7.5), water, ethanol and dried. A single spot was incubated with a mixture containing 50 µl of methanol, 100 µl of water and 50 µl of a 0.5 M methanolic solution of sodium methanolate.