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Recognition of a monoclonal antibody against a small molecular weight antigen by monitoring the antigen–antibody reaction using fluorescence labeled structured peptides

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ABSTRACT

Interaction between proteins (as analytes) and de novo designed structured peptides as capture molecules cause structural changes, which are reflected in fluorescent-intensity changes of labeled peptides in a dose dependent manner. In contrast to conventional detection methods our detection system does not involve the detection of specific molecules themselves in a 1:1 manner, but uses the principle of the differences in fluorescent intensity changes of capture peptides upon addition of analytes. Instead of the use of secondary antibodies we have attempted monitoring these structural changes by an array of de novo designed synthetic and structured peptides. In the present study we have focused on a recognition system, 5-fluorouracil, as a low molecular antigen and a monoclonal antibody against 5-FU. The fluorescent intensity changes of fluorescent labeled peptides have been measured after incubation with a monoclonal antibody and again after further incubation with the antigen, 5-FU. Unique intensity changes were found for several peptides in the fluorescent peptide library that allowed the visualization as a color-coded protein fingerprint. The peptide screen used in the present study offers a useful detection system as capture molecules for peptide-based microarrays.

Proteins consist of 20 different natural amino acids with post-translational modifications and exhibit structures containing α-helices, β-sheets and β-loops. Hence, protein–protein interactions can be mimicked by protein-peptides in which the peptides have the appropriate secondary structures. During the last decade considerable effort has been devoted to the development of a novel bio-detection system using labeled structured peptides, arrayed on a chip surface, since characterization of proteins by high-throughput technologies are in great demand. The structural changes of the protein as an analyte are reflected in the fluorescent-intensity changes of peptides as capture molecules in a dose dependent manner. The changes can be visualized as a pattern in a color bar-code which is designated a 'protein fingerprint'.[1–7] In these studies we have constructed peptide libraries. Our previous study indicated that a microarray system allowed minimization of amounts of both analytes (nano gram) and capture molecules (several femto mole).[5,7] In contrast to a conventional detection method, using a set of 'antigen–antibodies', our detection system does not involve the detection of specific molecules themselves (so called 'biomarker') in a 1:1 manner, but the principle of detection is the differences in fluorescent intensity change caused by the analytes. In the recognition of antigens by antibodies the structures of captured antibodies are changed and we have attempted monitoring these structural changes by de novo designed structured peptides instead of the detection by secondary antibodies conventionally used in ELISA. In the present study we have focused on a recognition system, 5-fluorouracil (5-FU) as a low molecular antigen and a monoclonal antibody against 5-FU. Since 5-FU is commonly used for therapeutics and its blood concentration is important for prognostic information. Hence conventionally 5-FU can be detected by HPLC and mass spectrometry (MS), and the use of an anti-5-FU antibody allows simplification of the detection of the concentration in the blood to prevent overdosing.[8] The structure of 5-FU is similar to uracil and is converted to F-dUMP. However the thymidylate synthetase in cells is inhibited and thymine is not produced because of the presence of the fluoride atom in 5-FU.[9]

The construction of the structured peptide libraries used in the present study[10] has been reported previously.[2] Briefly the assembly was performed using automated synthesizers, PSSM-8 (Shimadzu, Kyoto) and PetiSyzer®s (HiPep Laboratories, Kyoto) by the Fmoc solid-phase strategy and cleaved from resin. Hence, in consideration of cost efficiency as well as reproducibility,

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C-terminal labeling was carried out by the on-resin modification of the Lys residue using ε-Alloc-group to give ε-peptides on α-TAMRA-Lys (Fig. 1).[2a] Each peptide has been characterized by ion-trap LC–MS on HiPep-Cadenza (3.0 × 150 mm, HiPep Labs.) after purification on preparative scale columns (5 cm id × 25 cm, HiPep Labs.). Several peptides having difficult sequences could be obtained in higher yield by coupling at elevated temperature.[11] Thus we obtained more than 2000 different peptide derivatives by the improved solid-phase method and characterized these by on-line LC–MS.[2,12] Hence the spot synthesis,[13] where each synthetic peptide is neither identified nor characterized, cannot be applied as quality assurance is very important for quantitative analyses and reproducibility because the fluorescent intensity changes are significant but relatively small[1,2] as indicated in Tables 1–3. Altogether 288 de novo designed structured peptides (96 α-helix, 96 β-sheet and 96 β-loop peptides, respectively) have been selected with respect to their stability and solubility, and used as fluorescent labeled capture molecules (Supplemental Tables S1–3). All peptides were completely soluble under the conditions described below.

The fluorescent labeled peptides were now assayed against antibody and antigen.[14] The stock solution (1 μL) of 100 μM 288 labeled peptides in 80% DMSO were dispensed into the microtiter plates and diluted 100 μL with 10 mM sodium phosphate buffer (pH 7.0). After measuring the fluorescence intensity, these were incubated with the antibody (1 μg/mL) for 30 minutes at room temperature in the dark. After 30 min 5-FU (1 μg/mL), as antigen, was added and the fluorescence intensity was again measured. Figure 2 indicates the fluorescent intensity changes for each structured peptide, and their fluorescent changes are listed in Tables 1–3. Peptides showing significant changes, indicated in red, have been selected. Figure 3 shows the fluorescent intensity changes as a color-coded image, which was made by the Microsoft Excel 2008, hence fluorescent intensity obtained (I_0 is the initial intensity and I is the intensity obtained with the antibody). The changes in the upper panel I in Figure 3 are for α-helical peptides. The sequence A–E–E–A was seen to be a consensus sequence which was also found in other peptides, although their intensity changes were not significant. The peptide A0127 (Seq. α-TAMRA-G-LKKLLEILKKLLEI-KTYTE-GC(Acm)-NH_2), for example, which did not show a significant fluorescent intensity change, was a positively charged peptide. While other peptides having E–E replacement in the A–E–E–A sequence exhibited lower fluorescent

Table 1
The peptide sequence and fluorescent intensity ratios of the screened α-helical peptides which show significant fluorescence changes

#A	Sequence	$\Delta I/I_0$	
		Antigen (−)	Antigen (+)
A0076	Y1-**AEEA**ARAARRAAEA-Z1	−0.19	0.05
A0081	Y1-**AEEA**ARAAEEAARA-Z1	−0.17	0.01
A0315	Y1-**AEEA**AKIAEEAAKI-Z1	−0.11	0.10
A0392	Y1-**AEEA**FRFARRAFEF-Z1	−0.77	0.27
A0427	Y1-**AEEA**VKVAKKAVEV-Z1	−0.25	0.03

The consensus sequence is indicated in bold. Y1 = αTAMRA-G-, Z1 = -GC(Acm)-NH_2.

Table 2
The peptide sequences and fluorescent intensity ratios of the screened β-sheet peptides

#B	Sequence	$\Delta I/I_0$	
		Antigen (−)	Antigen (+)
B0010	Y2-RLRLRLRL-Z2	−0.41	−0.01
B0011	Y2-EAEAEAEA-Z2	−0.08	−0.02
B0036	Y2-KAKAEAEA-Z2	−0.17	0.006
B0042	Y2-KLEAEAEA-Z2	−0.2	0.007
B0051	Y2-RARAEAEA-Z2	−0.33	−0.005
B0266	Y2-KAEAEFEF-Z2	−0.17	0.22
B0276	Y2-RAEAEAEF-Z2	−0.19	0.1
B0325	Y2-KIKFRFRF-Z2	−0.27	0.01

Y2 = C(Acm)-G-, Z2 = -K-(αTAMRA)-G-NH_2.

Table 3
The peptide sequences and fluorescent intensity change value of screened 9 β-loop peptides

#L	Sequence	$\Delta I/I_0$	
		Antigen (−)	Antigen (+)
L0010	Y3-GDHE-Z3	−0.11	−0.007
L0017	Y3-YGDE-Z3	−0.09	−0.003
L0023	Y3-HDWE-Z3	−0.19	−0.022
L0028	Y3-EDSL-Z3	−0.28	0.03
L0037	Y3-SEQD-Z3	−0.13	0.006
L0043	Y3-WSDE-Z3	−0.15	0.014
L0086	Y3-GHSD-Z3	−0.17	−0.032
L0090	Y3-GDLQ-Z3	−0.09	−0.018
L0098	Y3-GPDW-Z3	−0.2	−0.02

Y3 = αTAMRA-KKITV-, Z3 = KTYTEGC(Acm)-NH_2.

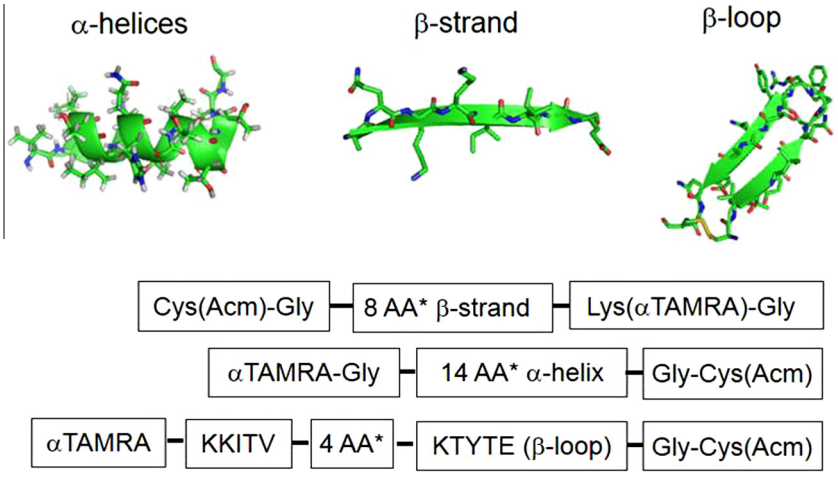

Figure 1. Architectures of the designed peptides as capture molecules. AA* indicates amino acid residues with diversity; TAMRA is tetramethylrhodamine used as fluorescent dye; Gly is used for the spacer; C-termini are amides.