Supporting Information for

Anomalous Reflection of Gold Applicable for a Practical Protein-Detecting Chip Platform

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METHODS
General information
Absorption spectra were acquired on a Shimadzu UV-2550 spectrophotometer equipped with a thermoregulator using a quartz cell (10 mm pathlength). Fluorescence spectra were acquired on a Hitachi F-2500 fluorescence spectrophotometer equipped with a magnetic stirrer and a thermoregulator using a quartz cell (10 x 10 mm pathlength). All proteins (calmodulin, protein kinase A, β-galactosidase, and α-amylase) were purchased from Sigma-Aldrich Japan. All solvents and reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as received except otherwise noticed. Fmoc-amino acid derivatives and reagents used in peptide syntheses were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Acetonitrile (HPLC grade) was used for HPLC analysis and purification. 5-(and 6)-Carboxytetramethylrhodamine, succinimidyl ester (TAMRA-OSu) was purchased from Molecular Probes. Analytical HPLC and purification of peptides were performed on a Hitachi L7000 or a Shimadzu LC2010C system equipped with a Wacosil 5C18 or a YMC-pack ODS-A (4.6 x 150 mm) with a linear gradient of acetonitrile/0.1% TFA at a flow rate of 1.0 mL/min for analysis, and a YMC ODS A323 (10 x 250 mm) at a flow rate of 3.0 mL/min for preparative purification. Mass spectra were obtained via a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS, Kompact MALDI III, Shimadzu) with 3,5-dimethoxy-4-hydroxycinnamic acid (SA) as matrix.

Preparation of TAMRA-containing peptides
Peptides were synthesized by means of Fmoc chemistry on Rink amide MBHA resin (Novabiochem) with 2-((1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole monohydrate (HOBt) (HBTU–HOBt method) or O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU method) as coupling reagents (Fig. S1).1 Side chains of following amino acids were protected with acetamidomethyl (Acm) for Cys, t-butyloxycarbonyl (Boc) or 4-methyltrityl (Mtt) for Lys, and t-butyl ester (O-tBu) for Glu. Initially, Fmoc-Lys(Mtt)-Gly-resin (70.5 mol) was treated with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/dichloromethane (DCM) (1/5/94, v/v/v) at room temperature to remove the Mtt protecting group of the lysine residue, followed by acetylation with acetic anhydride (0.70 mmol) and diisopropylethylamine (DIEA) in N-methyl-2-pyrrolidone (NMP), affording Fmoc-Lys(Ac)-Gly-resin. Both Ac-Cys(Acm)-Gly-Lys(Mtt)-[(LK(Boc)K(Boc)LLK(Boc)L)_2 or (LE(O-tBu)E(O-tBu)LLK(Boc)L)_2] -Lys(Ac)-Gly-resins were prepared in 15 µmol scale. The obtained peptide-bound resin was treated with TFA/TIS/DCM (1/5/94, v/v/v) at room temperature to remove the Mtt protecting group of the lysine residue, followed by acetylation with acetic anhydride (0.70 mmol) and disopropylethylamine (DIEA) in N-methyl-2-pyrrolidone (NMP), affording Fmoc-Lys(Ac)-Gly-resin. Both Ac-Cys(Acm)-Gly-Lys(Mtt)-[(LK(Boc)K(Boc)LLK(Boc)L)_2 or (LE(O-tBu)E(O-tBu)LLK(Boc)L)_2] -Lys(Ac)-Gly-resins were prepared in 15 µmol scale. The obtained peptide-bound resin was treated with TFA/TIS/DCM (1/5/94, v/v/v) at room temperature to remove the Mtt protecting group of the lysine residue. The resulting peptide-bound resin was treated with TAMRA-OSu (1 eq) and DIEA (2 eq) in NMP at room temperature in the dark. After 5 h, DIEA (3 eq) was added again and the reaction mixture was shaken at room temperature in the dark. After
12 h, reaction mixture was filtered and washed with NMP then CHCl₃. All of the protecting groups except Acm were removed by the treatment of TFA/m-cresol/ethanedithiol (EDT)/thioanisole (TA) (40/1/3/3, v/v/v/v) for 1 h at room temperature. The crude peptide obtained was purified by HPLC, lyophilized and characterized by MALDI-TOFMS, affording a fluffy magenta powder: **Acm-S-L8K6-T** (16.8 mg, 41%): obsd, 2731.7 [(M + H)⁺]; calcd 2733.5, **Acm-S-L8K2E4-T** (21.1 mg, 51%): obsd, 2737.8 [(M + H)⁺]; calcd, 2737.3.

The Acm protecting group for cystein side chain protection in the peptides was cleaved according to the literature.² **Acm-S-L8K6-T** (2.0 mg, 0.73 µmol) was treated with silver tetrafluoroborate (2.9 mg, 15 µmol) and anisole (0.80 µL, 7.3 µmol) in TFA (600 µL) for 1 h at 0 °C. The solvent was evaporated with a stream of nitrogen. The resulting crude mixture was solidified with diethylether, centrifuged, and dried. The crude peptide was treated with dithiothreitol (DTT, 5.7 mg, 37 µmol) in 10% acetic acid (10 mL) for 3 h at 25 °C. The reaction mixture was purified by a gel filtration column chromatography (Sephadex® G-10/10% acetic acid, 0.5 x 10 cm), then HPLC, followed by lyophilization, afforing a magenta **L8K6-T** (1.6 mg, 82%). Likewise, **Acm-S-L8K2E4-T** (2.2 mg, 0.80 µmol) was treated with silver tetrafluoroborate (3.1 mg, 16 µmol) and anisole (0.87 µL, 8.0 µmol) in TFA (600 µL) for 1 h at 0 °C then with DTT in 10% acetic acid at 25 °C, affording a magenta **L8E4K2-T** (1.1 mg, 52%) after HPLC purification. The peptides were characterized by MALDI-TOFMS: **L8K6-T**, obsd, 2664.2 [(M + H)⁺]; calcd 2662.4, **L8K2E4-T**, obsd, 2668.1 [(M + H)⁺]; calcd, 2666.2.

**Detection of CaM-peptide interactions in solution**

TAMRA-containing peptides were dissolved in methanol as a peptide stock solution and stored at −20 °C. Concentrations of the stock solutions were calculated by using absorbance at 545 nm in methanol (ε₅₄₅ = 95,000 M⁻¹ cm⁻¹).³ Fluorescence spectra of **Acm-S-L8K6-T** and **Acm-S-L8K2E4-T** were recorded as a function of calmodulin concentration in 20 mM Tris HCl buffer, 150 mM NaCl, 100 µM CaCl₂, 20 mM PEG2000 (pH 7.4) at 25 °C ([peptide] = 0.1 µM, λₑₓ = 554 nm, λₑₘ = 585 nm). Plots were fitted with a single site binding equation (Fig. S2).⁴

**Fabrication of peptide-immobilized gold surfaces**

General procedure for preparation of peptide-immobilized gold surfaces and detection with the AR apparatus: A glass slide (26 mm x 15 mm, Matsunami) was washed with Extran® MA02 (15 min x 1, Merck), MilliQ, 2-propanol (15 min x 1) and acetone (15 min x 1) then dried in an oven at 70 °C for 30 min. The cleaned glass slide was mounted in a vapor deposition system VE-2030 (Vacuum Device) and coated with Cr (1 nm in thickness, Nilaco) then Au (300 nm in thickness, Tanaka Kikinzoku Kogyo) under monitoring the thickness of metal layers by QCM. The gold substrate was deposited in a mixture of PEG₃-OH (1) and PEG₆-NHS (2) (Toyobo) at a total thiol concentration of 1.0 mM in ethanol over 12 h at room temperature. After washing the SAM-modified gold substrate with ethanol, the substrate was dried with a stream of air. The reaction cell was constructed to create...
a 400 µL volume of the reaction pool as described in Fig. 1B, and treated with a solution of maleimide linker (3)\textsuperscript{5} at concentration of 10 mM in 10 mM HEPES buffer (pH 8.0) for 35 min at room temperature, washed with the same buffer (x 5), then dried under a stream of air. The substrate in the cell was also treated with a peptide solution at concentration of 57 µM in 10 mM HEPES buffer (pH 8.0) for 30 min at room temperature, washed with the buffer (x 5), followed by quenching the remaining maleimide functionalities on the surface with 10 mM 2-mercaptoethanol in 10 mM HEPES buffer (pH 8.0) for 35 min at room temperature. The substrate in the cell was washed with the buffer (x 5), MilliQ (x 5), and dried under a stream of air, and used in the experiments. The amounts of SAMs, maleimide linker, and peptides immobilized onto the gold surfaces were estimated by difference in reflectivity compared with a bare gold surface in air after dryness of the substrates with a stream of air.\textsuperscript{6}

**On-chip assay protocol**

A reaction cell comprised of the modified gold substrate, a Teflon\textsuperscript{®} ring (20 mm x 10 mm in inner size, 2 mm in thickness) and another glass slide to create a 400 µL volume of the reaction pool was mounted in a hand-made AR apparatus equipped with a MCPD-3000 spectromultichannel photodetector (Otsuka Electronics), a PHL-150 halogen lamp (Mejiro Precision) and an optical fiber (1.2 mm in diameter, Otsuka Electronics) in the dark. Assaying buffer (400 µL of MilliQ containing 100 µM CaCl\textsubscript{2}) was injected into the reaction cell via a microsyringe and the endface of the optical fiber was brought to the glass surface of the reaction cell on a stage. The endface of the fiber was positioned as close as possible to the glass surface of the reaction cell. The reflections from the cell were monitored for 10 min at room temperature before measurements started. A solution of a protein of interest in the assaying buffer (4.0 µL) was injected into the reaction cell via a microsyringe and changes in reflectivity were recorded as a function of time. The amount of proteins bound to the surface was estimated by changes in reflectivity in solution according to the literature.\textsuperscript{6}

**References**

Figure S1. Synthetic scheme of TAMRA-containing peptides. The peptides were prepared by the solid phase synthesis methodology with Fmoc chemistry and the TAMRA dye was introduced into the peptide on resin after a selective cleavage of the protecting group of lysine side chains. Deresination/deprotection of the peptide-bound resin followed by purification by HPLC afforded pure magenta peptides. The obtained peptides that still possessed an acetamidomethyl (Acm) group for the cystein side chain protection were characterized by MALDI-TOFMS.
**Figure S2.** Changes in fluorescence intensity upon addition of calmodulin to the solutions of **Acm-S-L8K6-T** (closed circles) and **Acm-S-L8K2E4-T** (open circles) in 20 mM Tris HCl buffer, 150 mM NaCl, 100 μM CaCl₂, 20 mM PEG2000 (pH 7.4) at 25 °C ([peptide] = 0.1 μM, λ<sub>ex</sub> = 554 nm, λ<sub>em</sub> = 584 nm). Plots were fitted with a single site binding equation."
Figure S3. Detection of CaM–peptide interactions on the surfaces modified with different SAM compositions. (A) Real time monitoring of binding processes of CaM to the L8K6-T-modified surfaces with different molar fractions of 2 in the mixture of 1 and 2 in the SAM components \( \chi_{\text{NHS}} = \frac{[2]}{([1] + [2])} \) by the AR technique. Arrows show the time points of CaM injection \( (t = 300 \text{ sec}) \) and data acquisition \( (t = 2100 \text{ sec}) \). (B) Changes in the amount of CaM bound to the gold surfaces modified with L8K6-T or 2-mercaptoethanol (control surface) as a function of the molar fractions of 2 in the SAM components. Experiments were performed by the addition of 4.0 µL of CaM solution (final CaM concentration = 300 nM) to the reaction cell at room temperature.