Supplementary Information for

A novel array format for monitoring cellular uptake using a photo-cleavable linker for peptide release

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Materials and Methods

General

All chemicals and solvents were of reagent or HPLC grade and were used without further purification. HPLC was performed on the Hitachi L7000 (Hitachi High-Technologies Co., Tokyo, Japan) or the Shimadzu LC2010C system (Shimadzu co., Kyoto, Japan) using a Wakosil 5C18 or a YMC-Pack ODS-A (4.6 x 150 mm; YMC Co., Ltd., Kyoto, Japan) for analysis, and a YMC ODS A323 (10 x 250 mm) for preparative purification with a linear gradient of acetonitrile / 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min for analyses and 3.0 ml/min for preparative separation, respectively. MALDI-TOF MS was measured on a Shimadzu KOMPACT MALDI III with 3,5-dimethoxy-4-hydroxycinnamic acid as matrix.

Synthesis of the model peptides

The TMR-KLA peptide and TMR-transportan peptide were synthesized on NovaSyn TGR resin (Merck Japan, Tokyo, Japan) by manual synthesis with Fmoc chemistry.[S1] Side chain protections were as follows: *t*-butyl (tBu) for Ser, Thr and Tyr; *t*-butyloxycarbonyl (Boc) for Lys, and Trp; trityl (Trt) for Asn and Cys residues. Initially, Fmoc-peptides were synthesized with Fmoc-AA-OH (3 eq.) by the HBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]–HOBt (1-hydroxy benzotriazole monohydrate) method. After assembly of the peptide sequences, the 5-(and-6)-carboxytetramethylrhodamine (TMR) moiety was introduced by using TMR (2 eq.) with HBTU (2 eq.), HOBt (2 eq.) and diisopropylethylamine (DIEA, 4 eq.) for 60 min. The peptides with fluorescent probes were cleaved from the resin and side chain protections were removed with TFA / *m*-cresol / ethandithiol / thioanisole (86/2/6/6, v/v) at room temperature for 1 h. The peptides were precipitated by addition of diethylether and collected by centrifugation. The peptide was purified by RP-HPLC and characterized by MALDI-TOF MS. The lyophilized peptide was dissolved every time before use. In order to estimate the concentration of the solution, TMR absorbance of a diluted solution of peptides was measured in 20 mM Tris-HCl containing 6 M guanidine hydrochloride (pH 7.0).

Synthesis of photo-cleavable linker (M-linker) peptide

Photo-cleavable (M-linker) peptide[S2] (Fig. S1a) was synthesized on 2-chlorotrityl chloride resin (Merck) by manual synthesis with Fmoc chemistry.[S1] Initially, Fmoc-Gly-OH (0.5 eq.) was coupled to the resin with DIEA (2.5 eq.) in dried dichloromethane (DCM) / *N*,*N*-dimethylformamide (DMF) (5/4, v/v) using a round bottom flask equipped with calcium chloride for 60 min. After the reaction, unreacted moieties on the resins were capped by methanol (ca. 5% volume of the reaction volume) for 15 min. Then, the resins were transferred to a peptide synthesis tube, and washed with DCM, methanol and DCM / DMF (1/1, v/v). Fmoc group was removed by 20% piperidine in DCM / DMF (1/1, v/v) for 15 min. After removal of Fmoc, the resins were washed with DCM / DMF (1/1, v/v) and diethylether. Fmoc-2-(2-aminoethoxy) ethoxy acetic acid, Fmoc-4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy]-butanoic acid and maleinimidopropionic acid were coupled by the HBTU-HOBt method as described in previous section. The peptide was cleaved from the resin with acetic acid / trifluoroethenol / dichloromethane (1/1/8, v/v) at room temperature for 2 h. After removal of the resins, adding hexane to the peptide solution and evaporating the solvent were repeated 3-5 times. Then, MilliQ was added to the evaporated sample and the sample was lyophilized.

Immobilisation of the peptides

Outline of the immobilisation was shown in Figure S1b. The C-terminal of M-linker (14 mM) was activated using N-hydroxysuccinimide (NHS) (10 eq.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC) (10 eq.) in 95 μL DMF (2 h). Then, 90 μL of the HEPES buffer (50 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 250 mM NaCl, pH 7.0) was added to the reaction solution. 5 μL of the activated M-linker solution was added to the 10 μL of poly-L-Lysine (PLK, MW. 150,000-300,000, final concentration 5 mg/ml) in the HEPES buffer and the solution was incubated for 30 min. After the coupling reaction, 100 μL of 2.2 mM peptide in the HEPES buffer was added to the reaction buffer, and the reaction solution was incubated for 30 min to give the peptide-M-linker-PLK conjugate. Finally, 200 μL of the HEPES buffer was added to the conjugate solution and 50 μL of the solution was dispensed to a

well of 96-well glass bottom plate (SF-G-P96, FPI, Kyoto, Japan) for immobilisation, incubated for 6, 30 and 48 h. After immobilisation, the well was washed with PBS buffer (Gibco, Tokyo, Japan) 200 μL and MilliQ water 200 μL (Both 10 times) and was dried with N_2 in a clean bench.

Peptide photo-release and determination of the released peptide concentration

100 μL of MilliQ was added to peptide-immobilised wells in a 96 well glass bottom plate, then UV light was irradiated from 20 cm above (irradiation area: 5 well x 5 well) using a UV-spot light source (Photocure 200, Hamamatsu Photonics Photocure 200) with filter (362±30 nm) and condenser lens (Photocure 200 series, E5147-06) for obtaining homogeneous UV-irradiation. After the irradiation, the peptide-released solution from the wells was collected. 100 μL of guanidine buffer (20 mM Tris-HCl containing 6 M guanidine thiocyanate (pH 7.0)) was added to the wells for washing out the rest and collected the peptide solution (final volume 200 μL). Fluorescence intensities of the final solution were measured by a Hitachi F2500 fluorescence spectrophotometer at 25°C (Ex. 560 nm, Em. 584 nm). The released peptide concentration was determined by comparing these fluorescence intensities with those of free peptide solution at well-defined concentrations.

Cell uptake assay with the peptide-immobilized plate using confocal microscopy

The Human cervical carcinoma (HeLa) cells were seeded $2x10^3$ cells in 100 μL media (DMEM supplemented with 1 % fetal bovine serum (FBS, BioWest, Nuaillé, France)) in per well of the peptide-immobilizing 96 well glass bottom plate. After 1-day culture, the media were removed and the cells were gently washed three times with the assay media (5 g DMEM powder (Sigma-Aldrich Japan) and 1.85g $NaHCO_3$ (Wako Pure Chemical Industries) in MilliQ 500 mL was filtered with 0.45 μm filter). Then, 100 U / mL penicillin (Sigma-Aldrich Japan) and 100 μg / mL streptomycin (Sigma-Aldrich Japan) were added to the filtered solution. After adding 100 μL of the assay media, UV light was irradiated from 20 cm above using the UV-spot light source with a filter (362±30 nm) and the condenser lens at 37 °C. After the light irradiation, incubation and removal of the media, 100 μL of 100 nM Mitotracker in the assay media was added to the wells and the plate was incubated for 30 min at 37 °C. Then, the cells were washed three times with the assay media.

Cells were observed under confocal microscopy IX70 with a FV300 system (Olympus, Tokyo, Japan). Image data were obtained with Fluoroview FV300 Ver. 4.3 (Olympus software). The TMR fluorescence was excited with the 543 nm HeNe(G) laser and was recorded using a filter (>570nm). The Mitotracker fluorescence was excited with the 488 nm Ar laser and was recorded using a filter (>510 nm).

References

S1 W. C. Chan and P. D. White, *Fmoc solid phase peptide synthesis: A practical approach*; Oxford University Press; New York; 2000.

S2 T. Kakiyama, K. Usui, K.-y. Tomizaki, M. Mie, E. Kobatake, and H. Mihara, *Polym. J.*, 2013, **45**, 535.

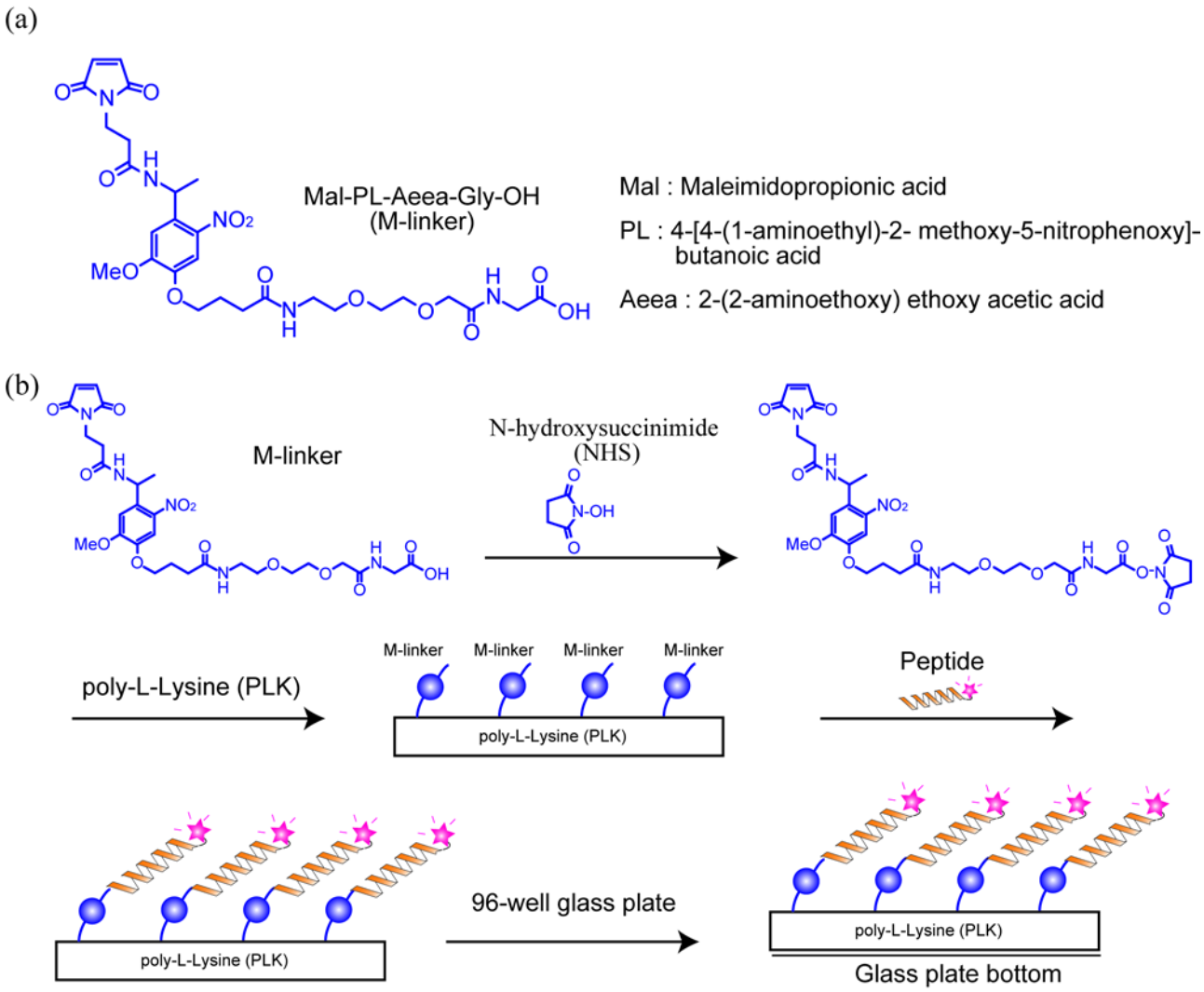

Fig. S1 (a) Structure of the photo-cleavable linker (M-linker).[S2] (b) Outline of the peptide immobilisation steps in this study.

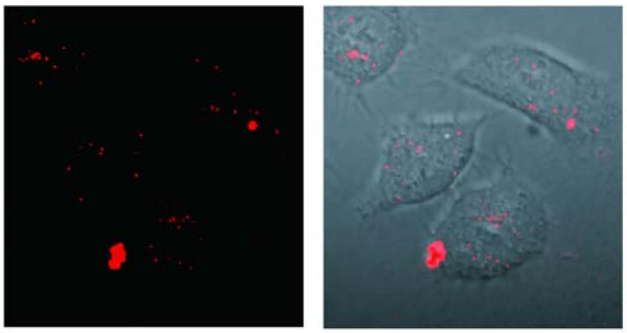

Fig. S2 Confocal micrographs of HeLa cells after HeLa cells were incubated for 1 h with TMR-KLA peptide solution at 1 μM.

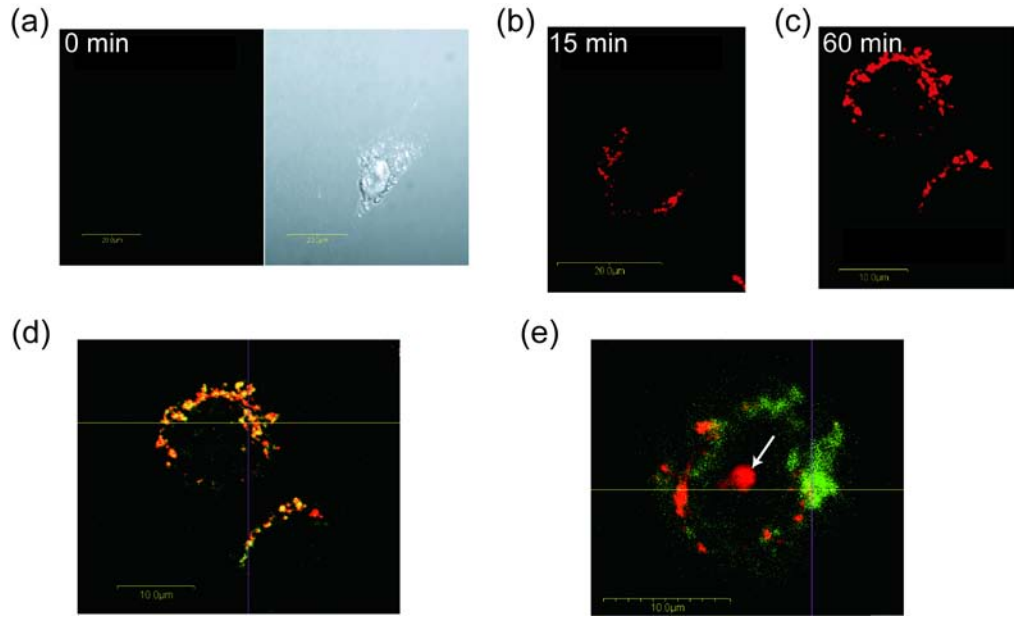

Fig. S3 Confocal micrographs of HeLa cells after UV light was irradiated to the TMR-KLA plate for (a) 0, (b) 15 and (c) 60 min with 48h-immobilised peptides and then HeLa cells were incubated for 1 h. Confocal micrographs of HeLa cells after UV light was irradiated to the plate for 60 min with Mitotracker (green) and immobilised peptides (red) of (d) TMR-KLA or (e) TMR-Transportan (A cellular nucleus was indicated by an arrow.).