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Complete List of Authors:	Morimoto, Jumpei; The University of Tokyo, Chemistry and Biotechnology Amano, Rei; The University of Tokyo, Department of Chemistry and Biotechnology, Graduate School of Engineering Ono, Takahiro; The University of Tokyo, Department of Chemistry and Biotechnology, Graduate School of Engineering Sando, Shinsuke; The University of Tokyo, Department of Chemistry and Biotechnology, Graduate School of Engineering

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A Parallel Permeability Assay of Peptides across Artificial Membrane and Cell Monolayers Using a Fluorogenic Reaction

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Jumpei Morimoto,*[a] Rei Amano,[a] Takahiro Ono[a] and Shinsuke Sando*[ab]

Here, we report a facile permeability assay to quantitatively evaluate membrane permeability of multiple peptides in parralel. With a fluorogenic click reaction between azidocoumarin and a terminal alkyne tag introduced on a peptide, the peptide that crossed an artificial membrane or a cell monolayer was quantitatively detected. The method allows a rapid measurement of permeability of multiple compounds on a plate reader even in the presence of a complex mixture of biological molecules.

Peptide is a promising class of compounds that bind and manipulate proteins with high affinity and selectivity. However, most peptides are poorly membrane permeable and they are not absorbed in intestine and not penetrate into cells, which is limiting the pharmacological utility of peptides. Therefore, guiding principles need to be established for designing peptides that are likely to cross biological membranes. Recent efforts of permeability measurements involving artificial membranes and monolayers of cells, such as Caco-2 and MDCK-II cells, have revealed some essential determinants for high membrane permeability of peptide. For example, molecular weight[1], lipophilicity of side chains[2], the number of solvent-exposed amide protons[3–8] are suggested to be important determinants for membrane permeability of peptides. The permeability assays such as parallel artificial membrane permeability assay (PAMPA), Caco-2, MDCK-II assays are performed in a multi-well format and potentially useful for evaluating numbers of peptides at one time. However, the detection of peptides across membrane involves LC-MS analysis that requires one by one sample preparation and chromatographic separation, which significantly slows down the speed of the assay. Especially, when various biomolecules such as serum albumin are included in the assay to better mimic in vivo conditions[9,10], careful preprocessing will be necessary to remove such additives before the samples are applied to LC. Therefore, a more facile detection method of peptides is desired to elucidate relationship of peptide structures and membrane permeability.

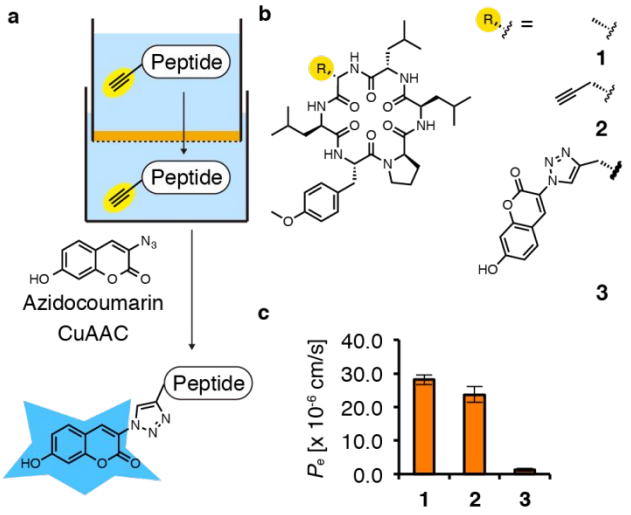

Fig. 1. (**a**) A schematic illustration of the membrane permeability assay utilizing a fluorogenic reaction. A terminal alkyne tag is highlighted in yellow. The fluorescent coumarin moiety is highlighted in blue. The layer shown in orange between the two chambers indicates artificial membrane (PAMPA) or cell monolayer (Caco-2 or MDCK-II assay). **(b)** Structures of model peptides that have no reporter group (**1**), a terminal alkyne group (**2**), or a fluorescent reporter group (**3**). The variable structure is highlighted with yellow circles. **(c)** Permeability of peptides **1**–**3** measured by PAMPA with HPLC-based quantification. Error bars represent standard deviations of triplicate.

Labeling of peptides with a fluorescent reporter group is an attractive method to achieve facile detection of peptides. However, large fluorescent groups greatly affect the abovementioned important physicochemical properties of peptides, such as molecular weight and lipophilicity, leading to largely altered membrane permeability of peptides. We assumed that introduction of a terminal alkyne tag instead of a fluorescent reporter group is a promising alternative strategy because the tag minimally affects membrane permeability of peptides. In addition, peptides with the tag can be selectively

[a] *Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.*
[b] *Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.*

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and quantitatively detected by reacting with an azide-containing fluorogenic dye under a copper catalyzed alkyne-azide cycloaddition (CuAAC) reaction conditions (**Fig. 1a**).[11] Particular azide-functionalized fluorogenic dyes are known to increase their fluorescence upon reacting with a terminal alkyne group, allowing lighting up the alkyne-tagged molecule with no need of removing unreacted dyes.[12] 3-Azido-7-hydroxycoumarin (azidocoumarin), first developed by Wang and co-workers, is a fluorogenic dye of high OFF/ON ratio at 478 nm emission.[13,14] The dye has been utilized for various applications such as selective labeling of newly synthesized alkyne containing proteins in bacterial and mammalian cells.[15–17] Utilizing the advantages of a minimal alkyne tag and a fluorogenic dye, we here developed a rapid assay for quantitatively evaluate permeability of peptides that cross artificial membrane or cell monolayers.

First, we evaluated influence of a terminal alkyne tag on membrane permeability of peptides. Introduction of a terminal alkyne is not supposed to significantly alter size or lipophilicity of the peptide, that are important determinants of membrane permeability. To experimentally confirm the assumption, permeability of peptides with and without a terminal alkyne tag across artificial membrane was measured using parallel artificial membrane permeability assay (PAMPA). As model peptides, we synthesized peptides **1**–**3** (**Fig. 1b and Fig. S1**). **1** is a derivative of peptides that was previously reported to be membrane permeable[18] and, therefore, expected to exhibit high membrane permeability. As a peptide with a terminal alkyne group, we synthesized peptide **2** that is a mutant of peptide **1** bearing a propargylglycine residue at alanine residue of peptide **1**. We also synthesized a derivative of peptide **3** that is another mutant of peptide **1** bearing a fluorescent group, hydroxycoumarin, at alanine residue of peptide **1** to investigate the influence of fluorescent labeling on permeability. Permeability of the peptides **1–3** across membrane was assessed by PAMPA (1% lecithin in dodecane as an artificial membrane) with typical HPLC-based quantifications and effective permeability coefficients (P_e) were calculated. As expected, peptide **1** and **2** exhibited similar membrane permeabilities while peptide **3** that bears a fluorescent group exhibited significantly lower P_e value than the other two peptides (**Fig. 1c and Fig. S2**). Based on the result, we concluded that introduction of a small alkyne tag does not significantly alter membrane permeability of peptides.

Next, we tested whether an alkyne-containing peptide can be quantitatively detected using a fluorogenic reaction. As shown in **Fig. 2a and b**, fluorescence of azidocoumarin increased upon CuAAC reaction with a model peptide **4**. When a series of concentrations of peptide **4** (0.05–20 μM) was reacted with azidocoumarin under the optimized CuAAC reaction conditions (100 μM azidocoumarin, 200 μM $CuSO_4$, 5 mM sodium ascorbate, 100 μM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) in phosphate buffer containing 30% DMSO), the fluorescence intensity after 1 h from the beginning of the measurement was proportional to the peptide concentration, validating that the peptide

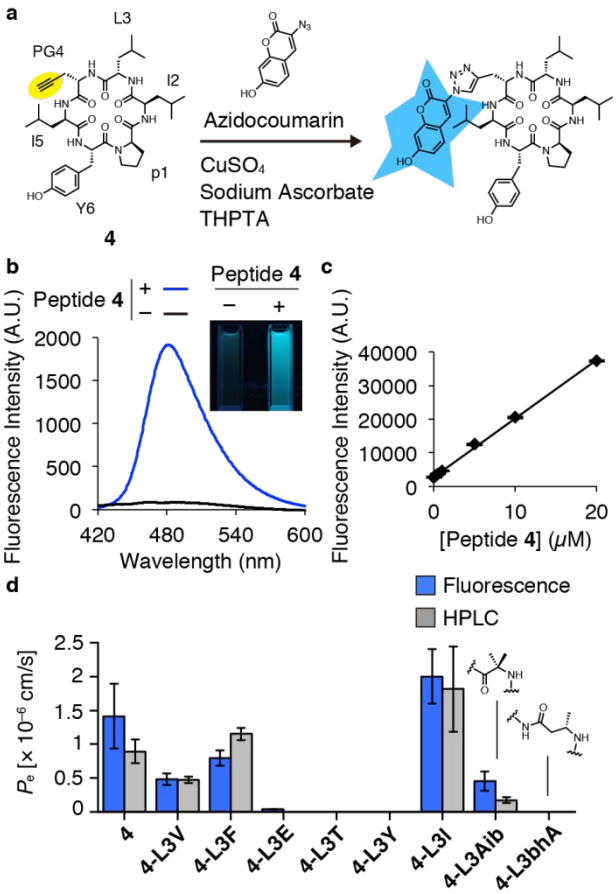

Fig. 2. Validation of membrane permeability assay of peptides using a fluorogenic reaction. **(a)** A scheme of the fluorogenic reaction between peptide **4** and azidocoumarin. **(b)** Fluorescence spectra of 1 μM azidocoumarin solution after 1 h incubation under the CuAAC reaction conditions (200 μM $CuSO_4$, 5 mM sodium ascorbate, 100 μM THPTA in phosphate buffer (pH 8.0) containing 30% DMSO) with 5 μM peptide **4** (blue) or without peptide **4** (black). The solution was 10-fold diluted before measurement. The spectra were measured with an excitation wavelength of 400 nm. Shown in the inset is a picture of azidocoumarin (left) and azidocoumarin-peptide conjugate (right) under a UV light. The solutions after the abovementioned CuAAC reaction were used without dilution. **(c)** Fluorescence intensities after 1 h reaction using 0.05–20 μM of peptide **4** and 100 μM azidocoumarin. Other reaction conditions are the same with **b**. Error bars represent standard deviations of triplicate. **(d)** P_e of the peptides evaluated by PAMPA. Values obtained by the fluorogenic reaction and HPLC are shown as blue bars and gray bars, respectively. Error bars represent standard deviations of triplicates.

concentration can be determined from the intensity of the generated fluorescence (**Fig. 2c**).

To test the validity of the proposed assay for evaluating membrane permeability of peptides using the fluorogenic reaction, PAMPA assay of a series of cyclic peptides was conducted and the peptide in a donor and an acceptor well was quantitated using the fluorogenic reaction (**Fig. 2d, blue bars**). To see how the natures of amino acid residues affect to membrane permeability of peptides, derivatives of peptide **4** that has a mutated residue from the original sequence were synthesized (**Fig. S3**) and tested. The peptides were first incubated in donor wells of a PAMPA plate for 18.5 h. The solution of peptides in the donor and acceptor wells after the incubation were transferred to a 96-well black plate and

reacted with azidocoumarin under the CuAAC conditions. The fluorescence intensities of donor and acceptor wells after 1 h were measured on a plate reader. To enable quantification, three different concentrations of each peptide (0.65–20 μM) were also reacted with azidocoumarin under the same conditions and the obtained values were used to generate a standard curve for each peptide (**Fig. S4**). Using the standard curve, concentration of each peptide in donor and acceptor well was determined and the value was used to calculate the P_e of the peptide. The P_e of the same peptides were also measured by HPLC to evaluate the accuracy of quantification by the fluorogenic reaction (**Fig. 2d, gray bars**). As a result, the permeability values of the peptides determined from the fluorogenic reaction and HPLC were consistent with each other, validating the utility of the fluorogenic reaction for quantitative measurement of membrane permeability of multiple peptides in parallel.

The obtained results from the permeability assay gave us a small set of structure-permeability relationship, which is well correlated with previous reports.[2,18] First, permeability of peptides with proteinogenic residues is governed by the lipophilicity of the side chain functionality. Derivatives having a mutation from Leu to Val or Phe (**4**-L3V or **4**-L3F) exhibited modest permeability while derivatives having a mutation from Leu to Glu, Thr or Tyr (**4**-L3E, **4**-L3T, **4**-L3Y) exhibited very low permeabilities. Substitution of the Leu residue with nonproteinogenic amino acid residues led to various results. Substitution with D-Leu (**4-L3l**) resulted in higher permeability than the original peptide. On the other hand, substitution with an α,α-disubstituted amino acid residue, i.e. aminoisobutyric acid, (**4-L3Aib**) resulted in modest decrease of permeability and substitution with β-homoalanine residue (**4-L3bhA**) completely diminished permeability of the peptide. These differences are probably produced from the difference in the number of solvent-exposed hydrogen bond donors due to the altered backbone conformation.

We next conducted MDCK-II assay[19] and quantitated the permeability values of peptides across the monolayer of MDCK-II cells using the fluorogenic reaction (**Fig. 3a**). The same set of peptides used for PAMPA (**4** and derivatives) was incubated in apical chambers of which the bottom is covered with a monolayer MDCK-II cells. After 2 h, peptides in the basolateral chambers were transferred to a 96-well black plate and reacted with azidocoumarin under the abovementioned CuAAC conditions. Prior to the assay, we confirmed that fluorescence intensity after the fluorogenic reaction in the buffer used for MDCK-II assay is again directly proportional to the peptide concentration between 0.05–20 μM (**Fig. S5**). Therefore, after MDCK-II assay, single concentration (20 μM) of each peptide was reacted with azidocoumarin under the CuAAC reaction conditions and the fluorescent intensity was used as a reference to determine the concentration of each peptide in the basolateral chamber. The determined concentration of each peptide was used to calculate P_e (**Fig. 3b, blue bars**). Because the peptide concentration in basolateral chamber was less than 10% of that in apical chamber, P_e was calculated assuming the steady-state flux

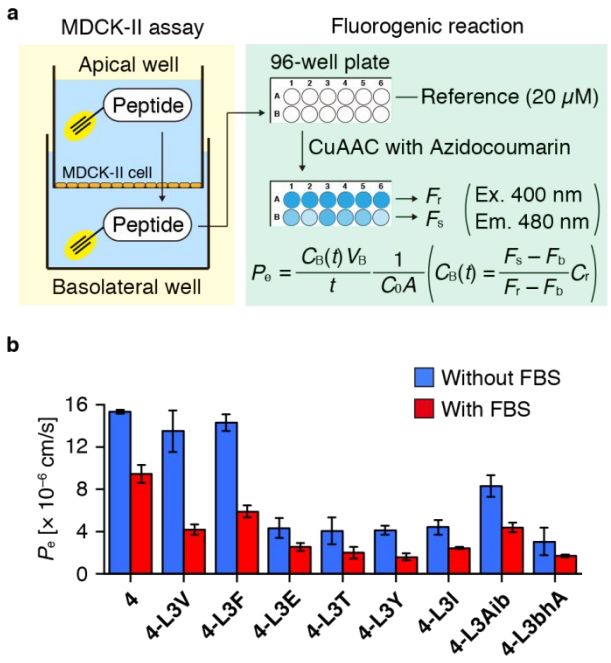

Fig. 3. MDCK-II assay using fluorogenic reaction. **(a)** A schematic illustration of MDCK-II assay followed by quantification using the fluorogenic reaction. From the intensity of the fluorescence generated from the CuAAC reaction between a peptide and azidocoumarin, P_e of the peptide was calculated using the equation shown in the figure. (See pages S8–9 of Supplementary Information for details.) $C_B(t)$: Concentration of peptide in basolateral chamber at time t. V_B: Volume of basolateral chamber. t: Incubation time. C_0: Initial concentration of peptide in apical chamber. A: filter area. F_s: Fluorescence intensity from peptide in basolateral chamber. F_r: Fluorescence intensity from reference peptide. F_b: Fluorescence intensity from a blank sample. C_r: Peptide concentration of reference. **(b)** Permeability values of peptide **4** and its derivatives are shown. Blue and red bars indicate permeability values obtained from the assay in the absence and presence of FBS, respectively. Error bars represent standard deviations of triplicate.

from apical to basolateral (the equation in **Fig. 3a**).[20] The P_e values of the tested peptides were also quantitated by HPLC to validate the method for quantitative evaluation of P_e values using the fluorogenic dye (**Fig. S6**). The values obtained from the two quantification methods were consistent, demonstrating that the fluorogenic reaction allows quantitative measurement of permeability across cell monolayers. The trend of the structure-permeability relationship obtained here was mostly similar to the PAMPA result.

Because a variety of proteins and other biomolecules exists in vivo that often affects to membrane permeability of peptides[21], the permeability values measured in the simple buffers may not be proper measures assessing the actual membrane permeabilities of the tested peptides. To make the tested environment more relevant to the actual biological environment, we conducted MDCK-II assay in cell culture medium containing 10% fetal bovine serum (FBS). When we tested the fluorogenic reaction of peptide **4** of predetermined concentrations with azidocoumarin using the same reaction conditions used for the serum-free assays, the reaction did not proceed efficiently presumably because copper was reacted with thiols existing in the biomolecules. However, simply increasing the copper concentration from 200 μM to 600 μM

solved the problem and the fluorescence intensity generated under the reaction conditions was confirmed to be directly proportional to the concentration of peptide **4** (**Fig. S7**). This warranted the utility of our system for quantitatively measuring the permeability even in the presence of a complex mixture of biological molecules. We conducted the MDCK-II assay of the same set of peptides (**4** and derivatives) using the FBS-containing medium. P_e values were determined in the same way with the MDCK-II assay without FBS described above. As a result, all the peptides exhibited lower permeability values than on the same assay in a serum-free buffer probably because the peptides were absorbed on serum proteins (**Fig. 3b**, red bars). Especially, peptides with hydrophobic residues such as leucine, valine and phenylalanine at the mutated residue exhibited much lower values in the presence of FBS.

In summary, we here demonstrated that implementing a fluorogenic reaction to in vitro permeability assays facilitates studies of structure-permeability relationship of peptides. The small size of the terminal alkyne group allowed rapid measurements of permeability of multiple peptides with little effects on their permeabilities. Our detection method based on a fluorogenic reaction does not require laborious preprocessing of samples to remove biomolecules such as proteins and small molecules that are generally required when peptides are quantitated by a LC-based method. This allowed rapid evaluation of the permeability of peptides even in the presence of a complex mixture of biomolecules. A large data set of membrane permeability of peptides obtained with the presented method together with previously reported cell-based permeability assays[22,23] will be useful resource for designing peptides that efficiently internalize into the cells and manipulate intracellular protein functions.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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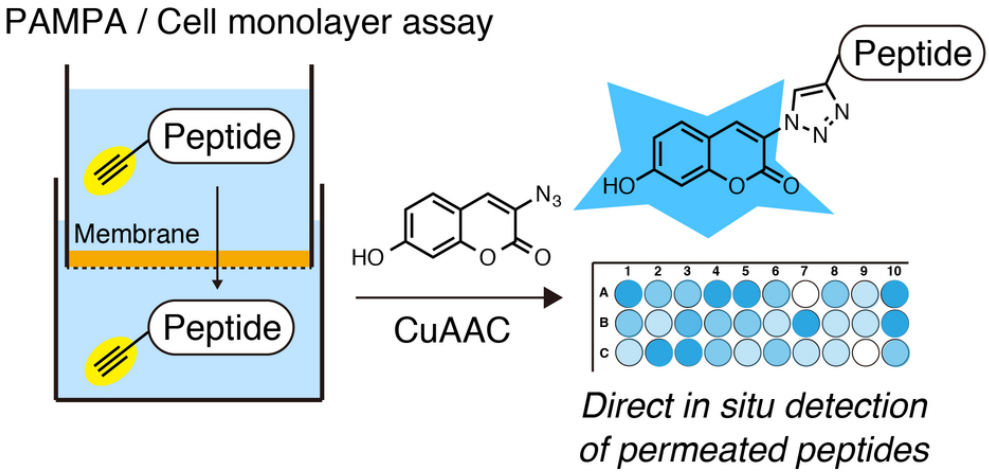

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