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Short Peptide Motifs for Long-Lasting Anchoring to the Cell Surface

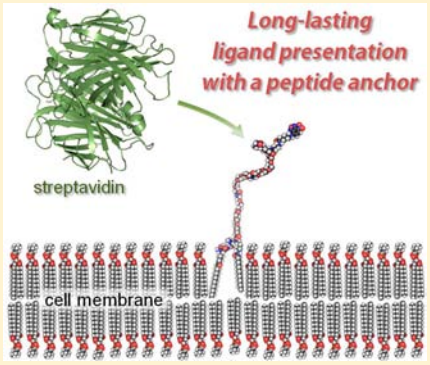
Masayoshi Matsuda,[†] Wataru Hatanaka,[†] Masafumi Takeo,[†] Chan Woo Kim,[†,‡,§] Takuro Niidome,[†,‡,§] Tatsuhiro Yamamoto,[§] Akihiro Kishimura,[†,‡,§,∥] Takeshi Mori,*[,†,‡,§] and Yoshiki Katayama*[,†,‡,§,∥,⊥]

[†]Graduate School of Systems Life Science, [‡]Department of Applied Chemistry, [§]Center for Future Chemistry, and [∥]Center for Molecular Systems, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan

[⊥]Center for Advanced Medical Innovation, Kyushu University, 3-1-3 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Supporting Information

ABSTRACT: A rational design strategy has been developed for the construction of stable peptide-based anchors for the efficient modification of cell surfaces. Six types of peptide composed of five residues with divalent hydrophobic groups have been designed using this new strategy. Among them, a peptide with a sequence of NBD-Lys-Lys(X)-Lys-Lys-Lys(X)-NH_2 (NBD: fluorophore, Lys(X): *N*-ε-palmitoyl-L-lysine) was found to show the highest modification efficacy and longevity in culture medium. The good performance of this peptide was attributed to (1) its high aqueous solubility, which allowed it to partition from the medium to the cell surface, and (2) the high binding affinity of the saturated palmitoyl groups to the cell membrane. We found that the distribution of the peptide was affected by recycling endosome, which enabled the representation of the peptide following its endocytotic disappearance from the cell membrane. Biotin was also presented on the cell surface using this peptide-based anchor to examine its recognition by streptavidin. The efficacy of the recognition process increased as the length of the oligoethylene glycol spacer increased, indicating that it was necessary for the biotin tag to move away from the membrane glycoproteins on the cell surface to facilitate its efficient recognition by streptavidin.



INTRODUCTION

The cell surface is a dynamic outer environment that is filled with a variety of different proteins, including receptors, ligand proteins, and adhesive proteins, which allow the cell to interact with a range of molecules as well as neighboring cells.[1] This cellular response can be modified by the artificial presentation of bioactive molecules on the cell surface according to a process known as cell surface engineering.[2−4] Cell surface engineering has recently been applied to the development of cell delivery systems capable of targeting specific tissues through ligand modification[5,6] and to the endocytotic uptake of desired molecules through receptor modification.[7−9]

Several techniques have been reported for presentation of bioactive molecules on the surfaces of cells, including covalent attachment to membrane proteins,[10−12] modification of cell surface glycoproteins thorough oligosaccharide biosynthesis,[13,14] and hydrophobic anchoring to the cell membrane.[15−17] The latter of these techniques has several advantages over the others, such as lower cytotoxicity, rapid modification, and its ability to be applied to a wide variety of different molecules and cells. Practically, the process of hydrophobic anchoring simply involves mixing the hydrophobic anchors with cells, which allows for the spontaneous transfer of the anchors from the solution phase to the outer leaflet of the plasma membrane. It has been reported that bioactive molecules such as proteins,[5,6,18] peptides,[19] carbohydrates,[7,8] and DNA[20,21] were successfully presented via hydrophobic anchors.

Despite the superiority of the hydrophobic anchoring technique, its application has been limited by the relatively short duration of the anchors on the cell membrane because of dissociation processes or endocytotic disappearance from the cell membrane. Two different approaches have been reported to address these limitations, including (1) increasing the number of hydrophobic anchoring groups, and (2) extending the acyl chain length of the anchoring groups. For the first approach, Nagamune et al.[22] reported that lipid-based divalent acyl groups were more stable than monovalent acyl anchors. We previously reported a series of polymer-based anchors bearing multiple hydrophobic anchoring units along the main hydrophilic polymer, which prolonged the longevity of the anchor on the cell surface.[9] With regard to the second approach, Iwata et al.[23] clearly showed that the use of phospholipid-based anchors with longer acyl chains led to an increase in the retention time of the anchor on the cell surface.[23] This effect has been confirmed by other groups based on their extensive studies.[24,25]

Most of the anchoring molecules reported to date for cell surface presentation have been based on phospholipids.[21−24] Another promising molecular design strategy for the development of stable anchoring systems involves the use of peptide-based hydrophobic anchors. Membrane-associating proteins are

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Table 1. Characterization of the Peptides

name	peptide sequence (1 2 3 4 5)	F.I. ($\times 10^{-4}$ a.u.)[a]	solubility (%)[b]	$t_{1/2}$ (h)[c]
XX1	NBD-Lys-Lys(X)-Lys-Lys(X)-Lys-NH_2	1.2	0	-[d]
XX2	NBD-Lys-Lys(X)-Lys-Lys - Lys(X)-NH_2	4.0	72	14
ZZ	NBD-Lys-Lys(Z)-Lys-Lys(Z)-Lys-NH_2	3.7	58	6.7
XZ	NBD-Lys-Lys(X)-Lys-Lys(Z)-Lys-NH_2	1.5	0	-[d]
XB	NBD-Lys-Lys(X)-Lys-Lys(B)-Lys-NH_2	1.2	74	-[d]
BB	NBD-Lys-Lys(B)-Lys-Lys(B)-Lys-NH_2	1.1	82	-[d]

[a]Fluorescence intensities (F.I.) of the cells measured by fluorescence cytometry immediately after their modification with each peptide. [b]Defined as remaining amount of peptide in the filtrate fraction after filtration through a 0.22-μm filter. [c]Half-life of fluorescence intensity. [d]Not determined due to the low modification efficacy.

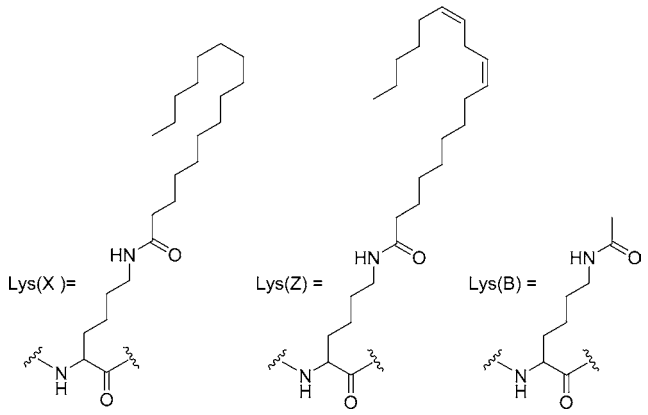
known to use peptide-based hydrophobic anchors for stable anchoring on the inner leaflet of the cell membrane.[26] For example, Src family protein kinases and G-proteins contain typically divalent hydrophobic anchors on their *N*- or *C*-termini that they use for stable anchoring.[26] Silvius et al.[15] collected short peptides from the termini of these proteins and found that divalent acyl-modified peptides have more extended duration on the liposome membranes than monovalent ones.

Peptide-based anchors have several critical advantages over lipid-based anchors, in that they allow for the precise design of diverse peptide sequences and facilitate the conjugation of bioactive molecules such as peptides through sequential synthesis on a solid phase. However, to the best of our knowledge, there have so far been no reports in the literature pertaining to the rational design of peptide-based anchors for the cell surface modification. Herein, we report the rational design of a series of peptide-based anchors capable of presenting bioactive molecules on the cell surface. From a practical perspective, anchors should be able to satisfy two contradictory conditions, in that they should possess sufficient aqueous solubility to allow them to partition efficiently into the cell membrane during the modification step, and they should exhibit good stability on the cell surface once anchored. We have succeeded in developing peptides with these properties and have applied them to the presentation of a bioactive ligand, biotin, on the cell surface to evaluate its recognition by a complementary protein, streptavidin.

RESULTS AND DISCUSSION

Design of the Peptides. Six peptides consisting of five amino acid residues were rationally designed as anchoring motifs for the cell surface modification (Table 1). The peptides consisted of three lysine residues and no more than two hydrophobic anchors. Three different types of hydrophobically modified L-lysine residues were used in the current study (Chart 1), including L-lysine residues modified with saturated palmitoyl group (X), unsaturated linoleoyl (Z), and acetyl (B) groups. The B group was used to evaluate the impact of the hydrophobic groups on the cell surface modification. It was envisaged that the three lysine residues in the six peptides designed in the current study would enhance the solubility of the peptides as well their stability in terms of their ability to anchor to the cell surface through electrostatic interactions with the negatively charged cell membrane.[27] Hydrophobic L-lysine derivatives were introduced at the second and the fourth positions of these peptides except for XX2, which included an X group at the second and the fifth positions. The *N*-termini of the peptides were labeled with NBD as a fluorophore.

Chart 1. Structures of the Hydrophobic Lysine Derivatives



Modification Efficacy of Peptides. Peptides modified with hydrophobic anchors showed poor solubility properties in DPBS, and the cell surface modification of the peptides was therefore performed in an isotonic 300 mM mannitol solution buffered with 10 mM HEPES (pH 7.4). Figure 1a,b shows the fluorescence images and results of the fluorescence cytometry experiments with K562 cells modified with each peptide for 5 min at 37 °C, respectively. Table 1 provides a summary of the median fluorescence intensities of the peptide-modified cells, which were determined by fluorescence cytometry. Relatively bright fluorescence signals were observed for the NBD both on the cell surface and within the cells for XX2, ZZ, and XZ, which all contained two hydrophobic lysine derivatives (Figure 1a). The fluorescence signals observed within the cells were co-localized with LysoTracker Red, which indicated that the peptides were rapidly internalized through endocytosis (this result will be discussed in greater detail later). XB and BB, which included one and no hydrophobic lysine derivatives, respectively, showed weak and no fluorescence, which indicated that the hydrophobic lysine derivative was essential to the success of the cell surface modification process.

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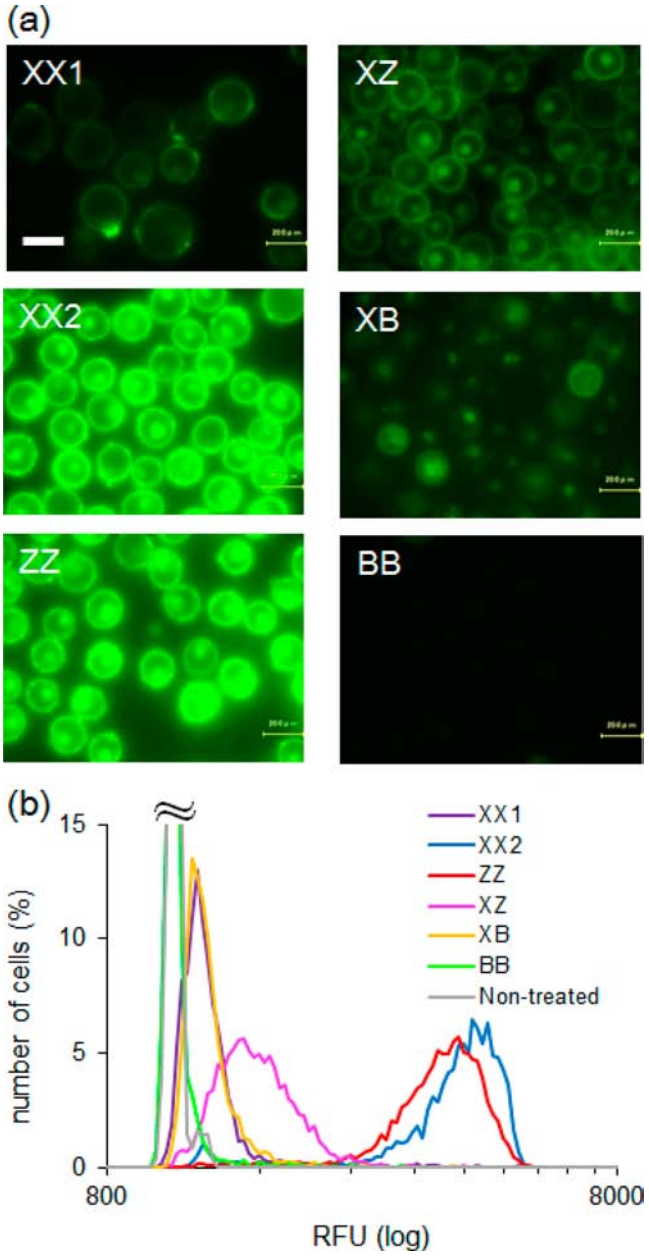

Figure 1. (a) Fluorescence microscopy images of K562 cells immediately after their modification with the peptides (6.0 μM) for 5 min at 37 °C. Scale bar: 20 μm. (b) Fluorescence cytometry analysis of the peptide-modified K562 cells.

The marked difference in the modification efficacy of the four different types of peptide (i.e., XX1, XX2, ZZ, and XZ) was attributed to the solubility properties of the peptides in the isotonic aqueous medium used in the cell surface modification process. Figure 2 shows the absorption spectra of XX1 and XX2 in DMSO (solid line) and the isotonic aqueous medium (broken line), respectively. Hypochromism and the blue shift of NBD were observed in the absorption spectra of these peptides in the isotonic buffer, which suggested that H-aggregation was occurring between the NBD groups of these peptides. To examine the solubility of the peptides in the isotonic aqueous medium, the peptide dispersions were filtered through a 0.22 μm filter and the absorption spectra of the resulting filtrates were measured to quantify the amount of peptide remaining in solution (dotted line in Figure 2). Almost all of the XX1 was

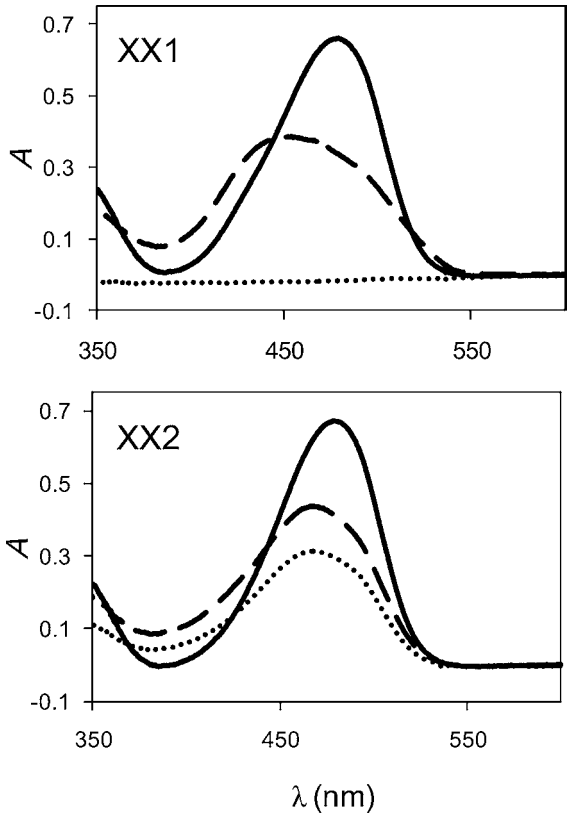

Figure 2. Absorption spectra of 6.0 μM solutions of the XX1 and XX2 peptides in DMSO (solid line), and in 10 mM HEPES buffer containing 300 mM mannitol before (dashed line) and after filtration (dotted line).

removed by filtration, whereas 72% of the XX2 peptide remained in the filtrate. The remaining absorption in the filtrate was defined as the solubility and the solubility of each peptide is summarized in Table 1. The solubilities of the four different types of peptide were found to be proportional to their cellular fluorescence intensity. Thus, the solubility of the peptide was determined to play a critical role in the effective partitioning of the peptide from the aqueous phase to the cell membrane. Notably, the solubilities of XX1 and XX2 were found to be quite different, even though they contained the same two hydrophobic lysine derivatives. The poor solubility of XX1 was attributed to the periodicity of the X residues in its peptide sequence (n and n+2 positions), which was the same as that of a β-sheet.[28] For this reason, XX1 tended to form β-sheets, which resulted in the aggregation of the peptide. Despite possessing a similar β-sheet periodicity, the ZZ peptide, which contained two unsaturated linoleoyl groups, showed relatively high solubility that led to the efficient modification on the cell surface. The high solubility of ZZ was attributed to the poor packing capability of the linoleoyl group. Similar levels of solubility dependence with regard to the anchoring efficacy have been reported in lipid-based anchors, where a dioleoyl lipid including one unsaturated bond was more soluble than a distearoyl lipid, which led to a higher modification ratio.[24]

The longevities of the peptides on the cell surface were then determined at 37 °C in RPMI-1640 medium containing 10% FBS. Figure 3 shows the fluorescence intensities of the cells modified with XX2 and ZZ plotted against incubation time. The results in this figure revealed that the fluorescence intensity decreased with time, which indicated that the peptides were

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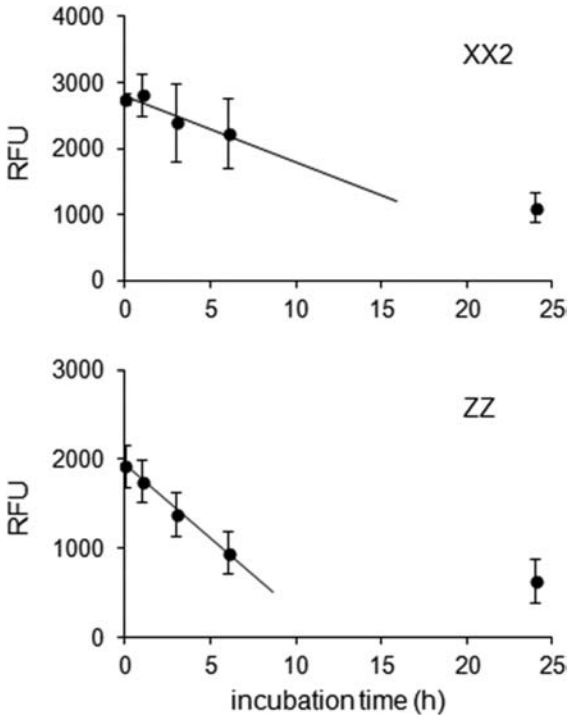

Figure 3. Decay in the fluorescence intensity signals of the XX2 and ZZ peptides in cells over time. The median relative fluorescence unit (RFU) values of the peptide modified cells were subtracted from those of the unmodified cells and plotted against the incubation time at 37 °C. Each curve represents the best fitted result of initial 6 h with a linear function.

dissociating from the cells. The initial decay in cellular fluorescence intensity was fitted with a linear function and the half-lives of the peptides used for the cellular modification process were determined from the function to be 14 and 6.8 h for XX2 and ZZ, respectively. We also examined the longevities of these peptides on another cell line, Jurkat (Supporting Information Figure S1). The half-lives of these peptides were determined to be 37 and 23 h for XX2 and ZZ, respectively. Thus, in the both cell lines, XX2 was found to be the best peptide-based anchor of the different peptides tested in this study because of the highest level of modification and the longest duration following the cellular modification process. It is noteworthy that the half-life of XX2 on the both cell lines (14 and 37 h) was longer than that of the corresponding lipid-based anchor bearing unsaturated hydrocarbon groups (8 h).[22] The longer half-life of XX2 than ZZ should reflect the higher affinity of the saturated alkyl group to the cell membrane than the unsaturated one probably due to the more hydrophobic characteristics of the palmitoyl modified XX2, which was evidenced by its lower solubility (Table 1).

If the peptides are hydrolyzed by the cellular protease, the half-life of the peptides determined here should include the effect of the hydrolysis. The effect of the peptide hydrolysis will be examined by replacing the amino acids with D-type ones, which will be done in our future work. The modification of the acyl groups on peptides is known to increase the resistance against the protease.[29]

We examined the effect of the kind of fluorophore modified to XX2 on the efficacy of the cell surface modification. Cy3 was modified on the peptide terminus instead of NBD (Supporting Information Figure S2a). The Cy3-modified XX2 showed fluorescence on cell surface similarly to NBD-modified XX2, while showed strange fluorescence intensity change with time; first increased then decreased with time (Supporting Information Figure S2b,c). This behavior cannot be explained at present, but the aggregating property of Cy3 may contribute to the fluorescence quenching in the initial stage.

According to the knowledge we obtained here, we designed another peptide by improving XX2; NBD-Lys-Lys(X)-Lys-Lys-Lys(X)-Lys-Lys-Lys(X)-NH_2. This peptide possesses three palmitoyl groups at the second, fifth, and eighth positions, while avoiding the periodicity of the β-sheet. We expected that the peptide retains adequate solubility but shows higher stability on the cell membrane due to the three palmitoyl groups. As a result, the fluorescence was observed on the cell surface, indicating successful modification (Supporting Information Figure S3). However, unfortunately, the peptide is quite less soluble and the amount of the modified peptide was too small to determine the half-life (Supporting Information Figure S3). Thus, it was concluded that two palmitoyl groups is the maximum number to be modified on one peptide to afford the enough solubility.

Characteristics of XX2 Peptide Anchor. We examined the peptide density on the surface of the K562 cells by varying the concentration of the peptide during the modification process. The amount of modified peptide on the surface was determined from the fluorescence of the NBD. As shown in Figure 4a, the peptide density increased in a linear manner with peptide concentration. The modification efficacy of the XX2 peptide was found to be high level regardless of the concentration (60−70% of the amount of the added peptide). The density of the peptide was calculated to be 11×10^8 molecules/cell at a concentration of 10 μM. The modification ratio of XX2 was 3.6-fold higher than that of the commercially available NBD-labeled lipid (Chart 2) when it was modified at a concentration of 6.0 μM (Figure 4a). The high modification efficacy of XX2 was attributed to its high solubility.

The cytotoxicity of XX2 was examined at two time points after the modification; just after and 48 h after modification. Figure 4b shows the results of 48 h after modification. No cytotoxicity was observed up to 2.0 μM of XX2, and almost the same results were obtained for just after modification (Supporting Information Figure S4). Thus, the cytotoxicity of the peptide is found to be brought by the initial binding of the peptide with the cell membrane which may disrupt the membrane structure. It was also found that the modified peptide does not show detectable effect on the cell growth at least for 48 h. The relatively inert characteristics of XX2 toward the cell provided certain advantages in terms of its application as an anchoring molecule. Ishiwata et al. also reported negligible cytotoxicity for cholesterol-modified PEG after modification on the cell membrane by the hydrophobic anchoring.[30] They found that the cellular modification of the cholesterol-modified PEG with relatively high molecular weight specifically inhibits the clathrin-dependent endocytosis.[30,31] Our peptides may also affect the cellular functions such as endocytosis. The effect of our peptides on such cellular functions should be the focus of our future work. We then proceeded to examine the intracellular localization of XX2 using CLSM by comparing its properties with those of the commercially available cell staining reagent, CellBrite Green (Chart 2). As shown in Figure 5, CellBrite Green localized in all of the membranes, including the plasma, lysosomal, endosomal, and nuclear membranes. In contrast, XX2 was localized

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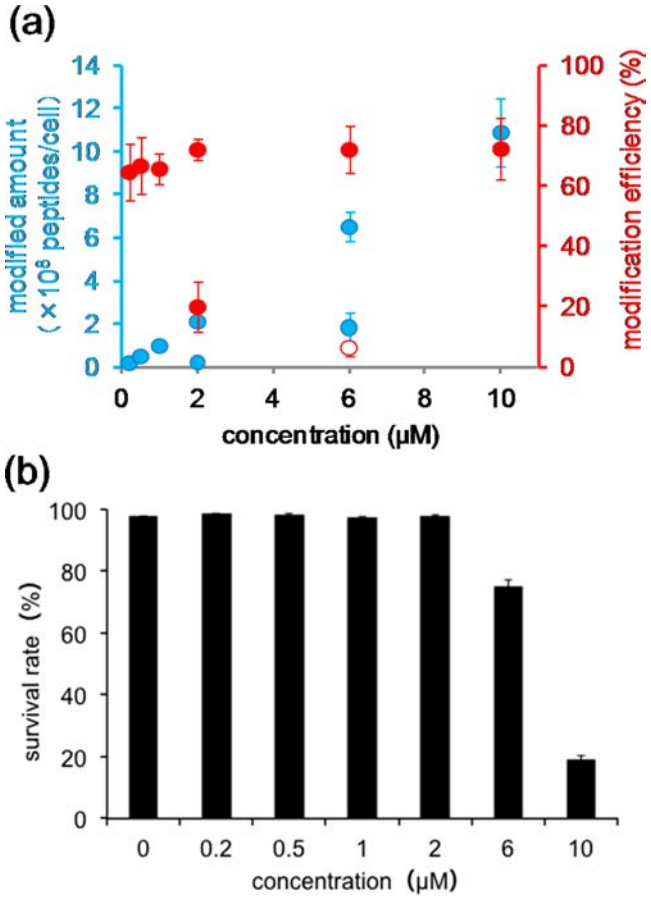
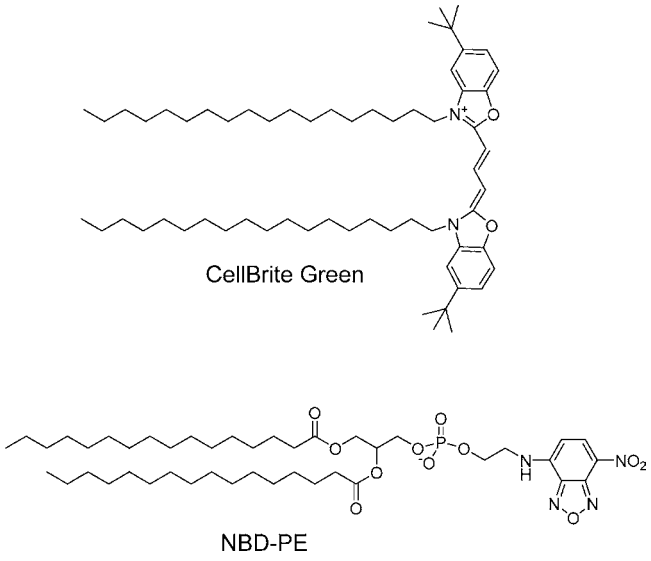


Figure 4. (a) Concentration dependence of the modified amount (blue symbols) and modification efficacy (red symbols) of the XX2 peptide (closed circle) and NBD-PE (open circle) modified on the cell surface. (b) Cytotoxicity of the XX2 peptide toward K562 cells after 48 h from the modification.

Chart 2. Chemical Structures of NBD-PE and CellBrite Green



specifically in the plasma, lysosomal, and endosomal membranes. The nonspecific localization of CellBrite Green in all of the membranes was attributed to its hydrophobic structure, which resulted in its high membrane permeability. The specific localization of XX2 indicated that its poor membrane permeability would lead to it being anchored to the outer leaflet of the plasma membrane followed by its distribution within the inner leaflet of the lysosomal and endosomal membranes through endocytosis. The poor membrane permeability of XX2 was attributed to its amphiphilic characteristics.

Recycling of XX2. We found that XX2 was represented on the cell surface though the recycling endosome following its endocytotic disappearance from the cell surface. Similar levels of representation through the recycling endosome have been reported for endogenous receptors such as transferrin and G protein-coupled receptors,[32−36] as well as a cholesterol-containing artificial amphiphile.[1] To prove the recycling of XX2, the fluorescent NBD group of XX2 on the outer leaflet of the plasma membrane was selectively quenched by sodium dithionite[5] and the subsequent representation of XX2 through the recycling endosome was monitored by fluorescence microscopy (Figure 6). K562 cells were initially modified with 6.0 μM of XX2 and incubated at 37 °C for 30 min to allow for endocytotic uptake, and the cells were then cooled to 4 °C to reduce the rate of the recycling process (Figure 6a). The fluorescence signal of the NBD group on the XX2 peptide in the outer leaflet of the plasma membrane was then quenched by treatment with ice-cold sodium dithionite (Figure 6b), and the resulting cells were maintained at 4 or 37 °C for 30 min (Figure 6c and d, respectively). A significant recovery in the fluorescence signal was observed on the cell surface at 37 °C, which indicated that XX2 was being represented on the cell surface through an energy-dependent recycling process. This recycling capability of XX2 would be beneficial for the long-lasting presentation of specific molecules on the cell surface.

Ligand Modification of the Cell Surface Using Peptide Anchor XX2. We investigated the presentation of the bioactive ligand biotin on the cell surface using XX2 as peptide-based anchor, and went on to evaluate the recognition of this material using streptavidin. In this particular case, we synthesized three different types of biotinylated XX2, which were functionalized with flexible oligoethylene glycol linkers of different lengths (Chart 3, Table 2) to evaluate the effect of the linker length on the recognition process. These biotinylated XX2 peptides were added to K562 cells at a concentration of 2.0 μM, and the cells were then added 0.2 μM of Cy3-labeled streptavidin. Figure 7a shows the fluorescence images of the cells modified with XX2−3 (linker's repeating unit n = 3). The red fluorescence of streptavidin and the green fluorescence of XX2−3 were co-localized on the cell surface, which revealed that the biotin had been successfully presented through the peptide anchor XX2 to be recognized by streptavidin.

Figure 7b shows the results of the fluorescence cytometry analyses of the cells modified with the three different types of peptide. The amounts of the modified peptide on the cell surface tended to decrease with increasing spacer length. This tendency may be attributed to an increase in the solubility of the peptides with increasing spacer length, which resulted in a reduction in the partitioning of the peptides to the cell membrane. The half-lives of these peptides are determined similarly to Figure 3 (Supporting Information Figure S5) and are summarized in Table 2. XX2−3 shows the shortest half-life, which probably reflects the high solubility of this peptide due to the longest hydrophilic spacer. Figure 7c shows the fluorescence cytometry results for the bound streptavidin. It is noteworthy that the amount of bound streptavidin was the

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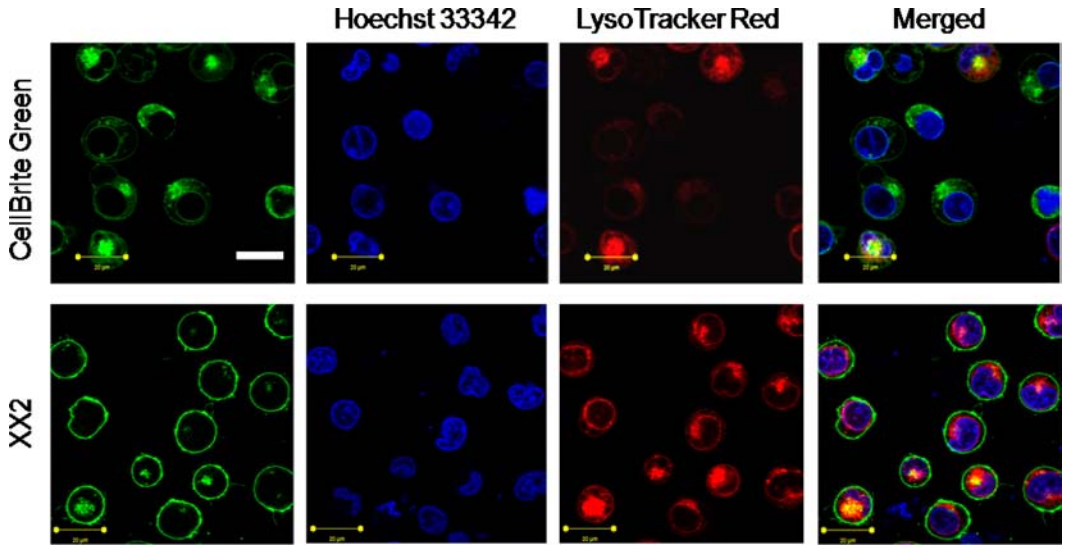

Figure 5. Confocal fluorescence laser scanning microscopy images after treatment with XX2 (6.0 μM) or CellBrite Green for 20 min at 37 °C. The cell nuclei and lysosome/endosome were stained with Hoechst 33342 and LysoTracker Red, respectively. Scale bar: 20 μm.

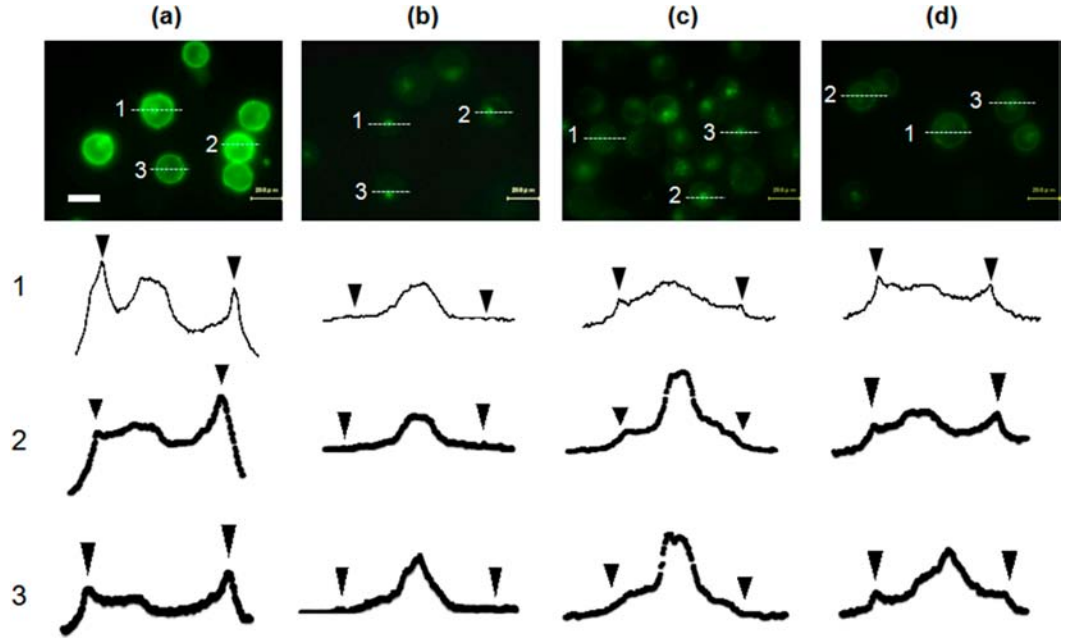

Figure 6. Fluorescent microscopic monitoring for the representation of the XX2 peptide through the recycling endosome (upper panels) and line profiles of the position of the dashed line (lower panels). K562 cells were treated with 6.0 μM of XX2 (a) followed by the bleaching of the cell surface NBD (b). The cells were maintained at 4 (c) or 37 °C (d) for 30 min. Scale bar in the upper panel: 20 μm. The arrows in the lower panels indicate the positions of the cell membranes.

highest in the longest spacer peptide, XX2−3 in spite of the least amount of modification of this peptide. In comparison with XX2−3, the relatively small effect of XX2−1 and XX2−2 on improvement of the streptavidin binding indicates the presence of a critical value in the spacer length on the promotion of the streptavidin binding. The binding efficacy of streptavidin to the surface biotin increased by 30% on going from XX2−1 to XX2−3. This result is entirely plausible because the cell surface is filled with membrane proteins and some of them contain dense polysaccharide chains.[1] The surface-presented streptavidin could be used as a cross-linker to present biotinylated molecules on the cell surface.[4]

CONCLUSION

We have developed peptide-based anchoring motifs for the presentation of specific molecules on the cell surface over long periods of time. The XX2 peptide provided the best performance of the six peptides designed and synthesized in the current study, and was modified on the cell surface with high efficiency (60−70%) because of its relatively high solubility in the aqueous medium, which led to its rapid partitioning to the cell membrane. The periodicity of the hydrophobically modified amino acid residues along the peptide sequence was found to have a critical impact on the solubility characteristics of the peptides. Once XX2 had been anchored to the cell membrane, its presentation lasted for a

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Chart 3. Chemical Structure of the Biotinylated Peptide Anchors (n = 1, 2, and 3)

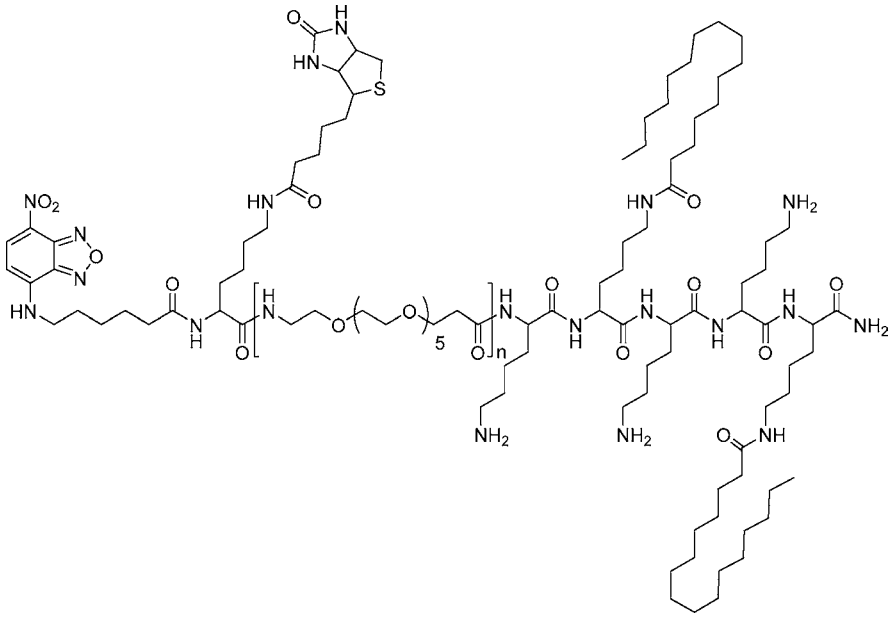

Table 2. Characteristics of the Biotin-Modified Peptide Anchors

name	n of spacer	extended spacer length (nm)	normalized F.I. of peptide[a]	normalized F.I. ratio of SA/peptide[b]	$t_{1/2}$ (h)
XX2	-	-	1.00	-	14
XX2−1	1	2.7	0.84	1.0	9.8
XX2−2	2	5.4	0.84	0.94	11
XX2−3	3	8.1	0.74	1.3	7.0

[a]Determined by fluorescent cytometry. [b]Normalized based on the F.I. of XX2−1.

long time with a half-life of 14 h. The long half-life of XX2 was attributed to the high affinity of its saturated palmitoyl groups for the cell membrane. XX2 was found to be recycled back to the cell membrane through endocytotic uptake, which made a significant contribution to its observed long-lasting presentation. Biotin was also attached to the cell surface using XX2 as a peptide anchor, and the efficacy of the surface binding of biotin was examined based on its recognition by streptavidin. The use of a long spacer between biotin and the peptide anchor enabled efficient binding with streptavidin on the cell surface likely because of a reduction in steric hindrance from the cell surface, which is crowded with membrane proteins. The peptide motif described in this paper could therefore be used to present bioactive molecules such as peptides for cell delivery and could be used to control cell−cell and cell−material adhesion. Studies toward these applications are currently underway in our laboratory and will be reported in due course.

EXPERIMENTAL PROCEDURES

Materials. NovaSyn TGR resin and Fmoc amino acids were purchased from Novabiochem (Darmstadt, Germany). 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), piperidine, trifluoroacetic acid (TFA), *N*,*N*′-diisopropylethylamine (DIEA), and *N*-methylpyrrolidone (NMP) were purchased from Watanabe Chemical (Hiroshima, Japan). HPLC grade acetonitrile and sodium dithionite were purchased from Nacalai Tesque (Kyoto, Japan). The *N*,*N*-dimethylformamide (DMF) and diethyl ether used for peptide synthesis were purchased from Kanto Chemical (Tokyo, Japan). Dimethyl sulfoxide (DMSO) for biochemistry was purchased from Wako Pure Chemical (Osaka, Japan). Triisopropylsilane and D-glucose were purchased from Tokyo Chemical Industry (Tokyo, Japan). 6-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoic acid (NBD-hexanoic acid), Hoechst 33342, and Lysotracker Red DND-99 were purchased from Invitrogen. D-Mannitol was purchased from Sigma. CellBrite Green Cytoplasmic Membrane-Labeling Kit was purchased from Biotium. FluoroLinkCy3-labeled streptavidin (Cy3-SA) was purchased from Amersham Biosciences. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (16:0 NBD PE) was purchased from Avanti Polar Lipids.

Synthesis of Peptides. All of the peptide sequences were synthesized on the 0.025 mmol scale using from the corresponding Fmoc-amino acids using Fmoc-based chemistry on a NovaSyn TGR resin. Fmoc-*N*-ε-Biotinyl-L-lysine was used for the biotin modification experiments. Fmoc-*N*-ε-Palmitoyl-L-lysine was used for the synthesis of the palmitate-modified peptides, whereas the linoleate-modified peptides were synthesized using Fmoc-Lys(Mtt)-OH as follows to allow for the modification of the linoleic acid residues. Following the modification of the *N*-termini peptides of NBD-X, the Mtt groups of the Lys(Mtt) residues were removed via the treatment of the peptide with 1% (v/v) TFA in dichloromethane for 30 min at room temperature. Linoleic acid was then introduced to the ε-amino groups of the unprotected lysine residues. The peptides were then cleaved from the resin by treatment with TFA containing 2.5% (v/v) triisopropylsilane and 2.5% (v/v) water, and purified by reversed-phase high-performance liquid chromatography (HPLC) (Hitachi LaChrom Elite, Tokyo, Japan) on a Protein-R column (Nacalai Tesque; 10 × 150 mm) using a mobile phase consisting of acetonitrile/0.1% TFA (20% v/v to 100% v/v). The obtained peptides were identified by a matrix assisted laser desorption ionization time-of-flight mass spectrometer. The peptides were

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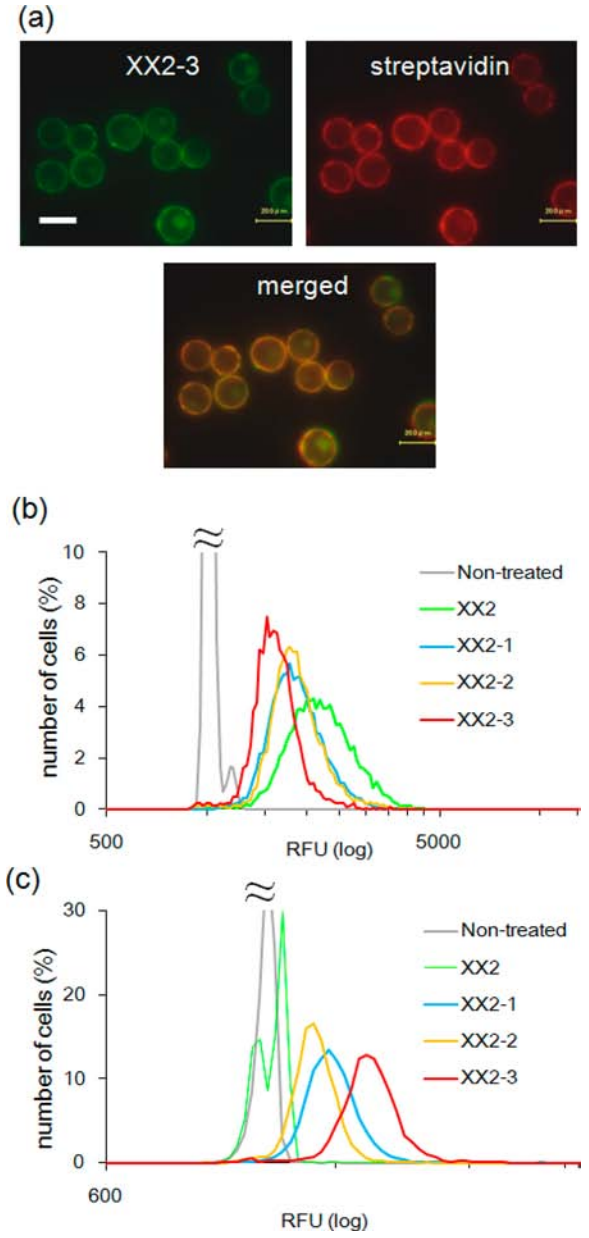

Figure 7. (a) Fluorescence microscopic images of the XX2−3 modified K562 cells treated with Cy3-labeled streptavidin. Scale bar: 20 μm. (b,c) Fluorescence cytometry results for K562 cells modified with three different types of biotin-modified XX2 followed by streptavidin addition. The cells were monitored by the fluorescence signals of the biotin-modified XX2 (b) and streptavidin (c), respectively.

dissolved in DMSO at a concentration of 1.0 mM, and the resulting solutions were stored at 5 °C prior to their analysis. The concentration of the peptide was determined from the absorption of a solution of NBD in DMSO at 478 nm (molar absorption coefficient = 33 186/M/cm).[37]

Evaluation of the Solubilities of the Peptides. A portion (20 μL) of a 1.0 mM DMSO solution of peptide was mixed with 980 μL of 10 mM HEPES buffer containing 300 mM mannitol (pH 7.4) (final peptide conc. 20 μM), and the resulting dispersion was vortexed to ensure complete dispersion. The peptide solution was then held for 30 min at room temperature before being passed through a 0.22 μm Millex-GV filter (Millipore). The maximum absorption of the solution was then measured at 478 nm using a UV-2550 ultraviolet/visible spectrophotometer (Shimadzu), and the result compared with that determined prior to filtration to determine the solubility of the peptide, which was defined as (A_{478} after filtration)/(A_{478} before filtration).

Cell Culture. K562 cells were cultured in RPMI-1640 medium (Wako) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY, USA). Cells were harvested in a humidified atmosphere containing 5% CO_2 at 37 °C.

Modification of Peptides on Cells. K562 cells (2×10^6) were collected by centrifugation. After removal of the supernatant, the cells were washed twice with medium without serum. The cells were then resuspended in 250 μL of 10 mM HEPES buffer containing 300 mM mannitol. Peptide solutions (total 250 μL, final conc. of 12 μM) were then prepared by diluting 3.0 μL of 1.0 mM DMSO peptide solutions with 247 μL of 10 mM HEPES buffer containing 300 mM mannitol (pH 7.4). Immediately after the preparation of the peptide solution, the material was mixed with a cell suspension, and the cells were then incubated for 5 min at 37 °C to allow for the peptide modification process to occur. The cells were then washed with serum-containing medium (2 × 2 mL) to allow for the removal of any unmodified peptide. Thee fluorescence intensities of the peptides on the cell surface were estimated using a BZ-8000 fluorescence microscope (Keyence) and a Tali Image-Based Cytometer (Life Technologies). The retention times of the peptide modifications on the cells were determined by Tali Image-Based Cytometer.

Quantification of the Amount of Modified Peptides on the Cells. K562 cells were modified with peptides according to the procedures described above. The cells were then collected by centrifugation and the supernatant was collected. A 100 μL aliquot of the supernatant was diluted with 900 μL of DMSO and the peptide absorbance derived from the NBD was measured at 478 nm using a 96-well microplate reader (WALLAC 1420 Multilabel Counter, PerkinElmer). The concentrations of peptide in the supernatant were determined using a calibration curve.

Evaluation of the Cytotoxicity of the Peptide. K562 cells were modified with peptides according to the procedure described above. After the modification process, 100 μL of the resulting cell suspension was transferred to a 96-well microplate and incubated for 48 h at 37 °C. Then the suspension was treated with 1.0 μL of Tali Dead Cell Red reagent (containing propiodium iodide). The resulting mixture was gently agitated for 3 min and the dead and living cells were then counted by Tali Image-Based Cytometer.

Confocal Laser Scanning Microscopy (CLSM) Observation. K562 cells were modified with peptides according to the procedure described above. The cells were then stained with LysoTracker Red (final concentration of dye = 1 μM) and Hoechst 33342 (final concentration of dye = 8.1 μM) and held for 30 min at 37 °C. The cells were observed using a LSM700 confocal laser scanning microscope (Carl Zeiss).

Analysis Following the Recycling of the Peptide. K562 cells were modified with peptides according to the procedure described above. The cells were then placed on ice for 2 min to inhibit plasma membrane recycling. The cells were then treated with 1.0 mL of ice-cold medium containing 30 mM sodium dithionite and held at 4 °C for 5 min to quench the cell surface NBD. The cells were then washed with Dulbecco's phosphate-

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buffered saline (DPBS) containing 2 g/L of glucose, and resuspended in 2 mL of the same buffer, before being equally partitioned into two 1.5 mL test tubes. The test tubes were then held at 4 or 37 °C for 30 min before being transferred to a glass-bottomed 96-well microplate for observation with a BZ-8000 fluorescence microscope.

Biotin Presentation on Cells and Its Recognition with Streptavidin. K562 cells were modified with 2.0 μM of biotinylated peptides according to the procedures described above. Cy3-SA (0.2 μM) was then added to a dispersion of the cells in FBS containing RPMI-1640 medium, and the resulting mixture was gently shaken for 10 min at room temperature. The cells were then washed twice with FBS-containing RPMI-1640 medium before being observed with a BZ-8000 fluorescence microscope and analyzed using a Tali Image-Based Cytometer.

■ ASSOCIATED CONTENT

Supporting Information

Additional graphics. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: mori.takeshi.880@m.kyushu-u.ac.jp. Tel: +81-92-802-2849. Fax: +81-92-802-2849.

*E-mail: ykatatcm@mail.cstm.kyushu-u.ac.jp. Tel: +81-92-802-2849. Fax: +81-92-802-2849.

Author Contributions

Masayoshi Matsuda and Wataru Hatanaka contributed equally to this work.

Notes

The authors declare no competing financial interest.

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