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Article

Potentiating the membrane interaction of an attenuated cationic amphiphilic lytic peptide for intracellular protein delivery by anchoring with pyrene moiety

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Abstract

We previously reported an approach for intracellular protein delivery by attenuating membrane-lytic activity of cationic amphiphilic peptides on cell surfaces. HAad is one such peptides that cytosolically delivers proteins of interest, including antibodies, by stimulating their endosomal escape. Additionally, HAad elicits ruffling of cell membrane, accompanied by transient membrane permeabilization, allowing for the efficient cytosolic translocation of proteins. In this study, we prepared a conjugate of HAad with pyrenebutyric acid as a membrane-anchoring unit (pBu-HAad). pBu-HAad demonstrated protein delivery into cells with only 1/20 concentration of HAad. However, the conjugates with cholesteryl hemisuccinate and aliphatic fatty acids (C=3, 6, and 10) did not yield such marked effects. The results of time-course and inhibitor studies suggest that the membrane anchoring of HAad by a pyrene moiety leads to enhanced peptide-membrane interaction and to loosen lipid packing, thus facilitating cytosolic translocation through membranes.

Keywords

cytosolic protein delivery; amphiphilic cationic lytic peptide; pyrenebutyrate; lipid packing; transient permeabilization of cellular membrane

Introduction

There is a considerable amount of research dedicated to the delivery of macromolecules including antibodies to cell interiors, which is useful not only for chemistry-driven biology but also in therapeutics.[1-3] To achieve this objective, numerous approaches have been reported based on membrane-active peptides, polymers, lipids, and virus infection systems.[4-12] These approaches generally employ endocytosis as the delivery route. For their successful delivery, the macromolecules must be efficiently encapsulated into endosomes and then released into the cytosol. Failure in endosomal escape should lead to the degradation of the macromolecules in lysosomes without obtaining the expected bioactivity. Thus, formulation of macromolecules with delivery vehicles is often necessary, and there is currently a substantial room for establishing more simple and efficient intracellular delivery systems.

To achieve endosomal escape, the selective destabilization of the endosomal membrane is expected, wherein the perturbation of other membranes, such as cell membrane, may yield disorder of cell functions. To achieve selectivity, differences in extracellular pH (i.e., neutral) from intra-endosomal pH (~5) are generally exploited. A variety of pH-sensitive peptides and polymers for the preferential destabilization of endosomal membranes have therefore been developed.[4-11] A valid approach involves the use of glutamic acid (Glu) in membrane destabilizing peptides as a safety-catch;[8,10]

charged Glu inhibits hydrophobic interaction with the cell membrane at neutral pH, and protonation of Glu at acidic pH enables the interaction to endosomal membrane.

Our laboratory has developed a strategy for the cytosolic delivery using attenuated cationic amphiphilic lytic (ACAL) peptides.[13-17] L17E (IWLTALKFLGKHAAKHEAKQQLSKL-amide) is a peptide derived from a spider venom, M-lycotoxin,[18] which has a potential cationic amphiphilic structure and strong membrane lytic activity (Figure 1A). To attenuate the lytic activity against cell membrane, Leu at position 17 in potential hydrophobic face of M-lycotoxin was replaced with Glu while retaining net positive charges of the peptide (L17E peptide). Since cell surface is rich in negatively charged molecules (e.g., membrane-associated proteoglycans and sialic acid), L17E may be effectively adsorbed on cell surfaces and taken up into endosomes without notable cytotoxicity. The successful cytosolic delivery of various proteins including immunoglobulin G (IgG) was achieved by a simple coincubation with L17E. Initially, the endosomal membranes were considered the main site of action of L17E. However, a later study elucidated that L17E induces ruffling of cell membrane, wherein the transient permeabilization of the ruffled membranes at a very early stage of endocytosis should be the main site of action of attaining the cytosolic translocation of the molecules of interest.[19] Loosening of lipid packing accompanied by membrane

ruffling is proposed to enhance the membrane interaction of L17E to permeabilizing membranes.

Considering this mode of action, we developed an advanced form of L17E, HAad, with mutations of His-to-Ala and Glu/Gln-to-aminoadipic acid (Aad) (IWLTALKFLGKAAAKAXAKQXLSKL-amide, X; L-2-aminoadipic acid (Aad)).[17] Through these mutations, HAad acquired significant endosomolytic activity, in addition to the ability to allow cytosolic access of proteins of interest through ruffled membranes at a very early stage of endocytosis. Using the same concentration with L17E, the delivery of IgG to 1.5-fold higher numbers of cells was attained or HAad showed a comparable efficacy of delivery with a half concentration of L17E. However, HAad still requires a concentration of 20-40 μM to achieve a satisfactory level of delivery. Thus, the aim of this study was to reduce the concentration of HAad by enhancing membrane interaction using a membrane-anchoring moiety while retaining the efficacy in cytosolic delivery of bioactive proteins (Figure 1B).

It has been reported by us and others that the attachment of hydrophobic moieties (including fatty acids and cholesterol) to membrane-interacting peptides leads to the enhancement of the interaction.[20-23] With the expectation that the anchoring of HAad with the cell membrane and the enhancement in the membrane interaction may result in the

reduction of the required amount of HAad for intracellular delivery, the effect of various membrane-anchoring moieties was studied. A marked improvement in delivery efficacy was observed for HAad by conjugation with pyrenebutyryl (pBu) group, which achieved a cellular distribution of Dex10-Alexa using 1/20 concentration (*i.e.*, 2 μM) of HAad. Loosening of lipid packing accompanied this treatment. The result of time-course and inhibitor studies suggested that the pBu conjugate of HAad may facilitate transient membrane permeabilization at very early stages in endocytosis. As a result, the unique feature of the pBu moiety in the membrane anchoring was demonstrated.

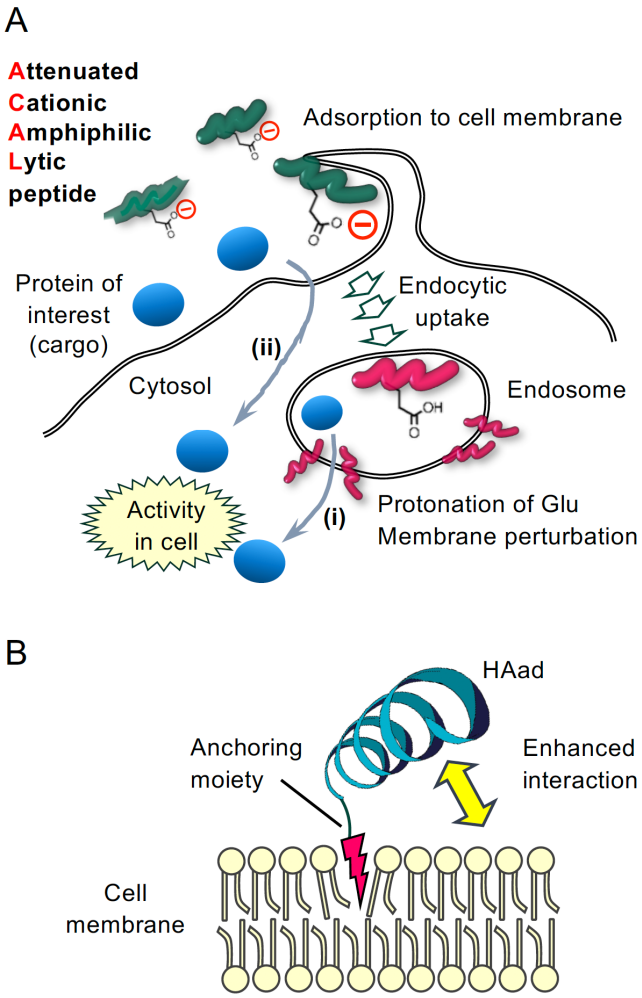


Figure 1. (A) Mode of action of attenuated cationic amphiphilic lytic (ACAL) peptides. Amino acid replacement in hydrophobic face of a cationic amphiphilic peptide to negatively charged amino acid (e.g., Glu) attenuates its lytic activity. Endocytic uptake and endosomal acidification leads to a decrease in the negative charge (protonation of Glu), the recovery of its lytic activity, and the cytosolic release of the cargo protein (route **(i)** in original design). Later study indicated that the ACAL peptide may also induce membrane ruffling, allowing for the transient perturbation of the cell membrane and cargo translocation into cells at a very early stages of endocytosis (route **(ii)**). Translocation using route **(ii)** was not observed under energy deficient condition (e.g., 4°C), and was therefore different from the canonical method of pore formation in the membrane. (B) Concept of this study: attachment of a membrane anchoring moiety to HAad (a representative ACAL peptide) may potentiate the membrane interaction of the peptide and delivery activity.

Results and Discussion

Anchoring of HAad with fatty acids

Acyl moieties are used to enhance the membrane interaction of bioactive peptides and may thus be used to reduce the concentration of HAad to attain the cytosolic delivery of bioactive proteins.[20-23] In this context, propionyl (C3), hexanoyl (C6), and decanoyl (C10) moieties were employed for anchoring HAad to membranes (Figure 2A). A tetraglycine (GGGG) sequence was employed as a linker, which was placed either on the N- or C-termini of HAad. A lysine residue was placed on the other side of the GGGG linker against the HAad sequence and the corresponding fatty acid was conjugated with the side chain amino group of lysine (Figure S1). The peptide chain of each peptide was constructed using Fmoc-solid-phase peptide synthesis on a Rink amide resin (Figure S1). The Mtt (=methyltrityl) group was employed for the side chain protecting group of lysine to be modified with acyl moieties and *t*-butyloxycarbonyl (Boc) group for other lysine residues, respectively.[24] After the construction of each peptide chain, the N-terminus amino group was protected with Boc group. The side chain Mtt group was selectively removed on the resin using hexafluoroisopropanol/dichloromethane (1:4) treatment. The liberated epsilon amino moiety of the lysine side chain was acylated using each fatty acid using 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate, 1-

hydroxybenzotriazole and *N,N*-diisopropylethylamine as a coupling system. The peptide resin was treated with a mixture of trifluoroacetic acid/1,2-ethanedithiol (95:5) and the resulting peptide was purified using high-performance liquid chromatography (HPLC).

Prior to evaluating the delivery activity of each peptide, cytotoxicity was analyzed using WST-8 assay (cell viability assay based on the activity of mitochondrial succinic dehydrogenase) (Figure S2).[25] This indicated the tendency of N-terminally acylated peptides to have a higher cytotoxicity than C-terminally acylated peptides. Peptides modified with longer acyl chains also showed a higher cytotoxicity. The delivery efficacy of the peptides was thus analyzed using the following peptide concentration to maximize delivery activity without yielding notable cytotoxicity: C3-HAad, 40 μM; C6-HAad, 20 μM; C10-HAad, 2 μM; HAad-C3, 40 μM; HAad-C6, 30 μM; HAad-C10, 10 μM.

The ability of these HAad derivatives to deliver macromolecules into cells was evaluated using Dex10-Alexa as a model. HeLa cells were treated with Dex10-Alexa in the presence of these peptides for 1 h. The cellular distribution of Dex10-Alexa signals was analyzed by confocal laser scanning microscopy (CLSM) (Figure 2B). When cells were treated with Dex10-Alexa in the absence of peptides, punctate, dot-like signals were observed (Figure 2B, no peptide). This suggests that Dex10-Alexa was taken up by the

cells by endocytosis, where Dex10-Alexa was engulfed into cells while encapsulated into vesicular compartments (i.e., endosomes). This also suggests that the majority of Dex10-Alexa was still trapped in endosomes and was not significantly released into the cytosol. When the cells were treated with Dex10-Alexa in the presence of 40 μM HAad, cells with Dex10-Alexa signals in the whole cellular area were predominantly observed (Figure 2B, HAad 40 μM). This suggests that Dex10-Alexa reaches the cytosol by escaping from endosomes or by other methods. Enhancing this feature is expected to result in intracellular delivery. The CLSM images suggested that around 75% of cells showed diffuse cytosolic Dex10-Alexa signals by the treatment with 40 μM HAad (Figure 2C).

Compared with HAad, N-terminal acylated HAad showed poor delivery activity (Figure 2B, C3-HAad, C6-HAad, and C10-HAad). The percentage of cells with diffuse cytosolic Dex10-Alexa was less than 40% (Figure 2C). On the other hand, C-terminal acylated derivatives yielded 60-70% of cells having cytosolic Dex10-Alexa signals (Figure 2B, HAad-C3, HAad-C6, HAad-C10). Typically, HAad-C10 delivered Dex10-Alexa into 67% of cells at 10 μM. This result implies that anchoring HAad with decanoic acid may reduce the concentration needed to obtain Dex10-Alexa delivery to ~70% of cells to 1/4 of HAad.

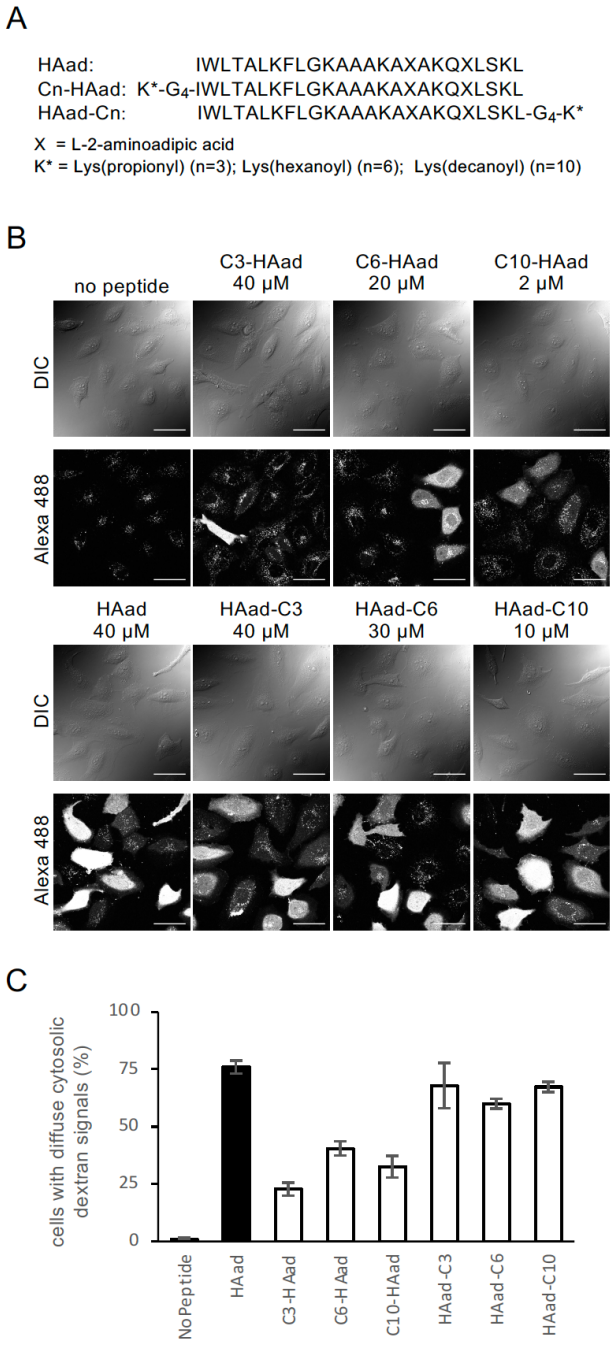


Figure 2. Anchoring HAad using linear aliphatic acyl groups. (A) Sequence of peptides. C-termini of all the peptides are amidated. (B) Cytosolic appearance of Dex10-Alexa treated with HAad with and without anchoring moieties for 1 h in serum-free α-MEM. Scale bar, 50 μm. (C) Percentage of cells accompanied by diffuse cytosolic dextran signals in (B). Results are shown as mean ± standard error (SE) (n = 3).

Anchoring of HAad with cholesterol

The above-mentioned results suggest that fatty acids bearing a longer aliphatic chain (e.g., stearic acid) may recruit HAad to the membrane to enhance the delivery effect. However, the possible increase in cytotoxicity along with the extension of aliphatic chains (Figure S2) motivated us to consider other types of anchoring. Choresterol is a major component of the cell membrane and has been used to anchor the moiety of membrane-interacting peptides.[22] Therefore, HAad derivatives modified with cholesteryl hemisuccinate, Chol-HAad and HAad-Chol, were similarly prepared (Figure 3A, Figure S1). The results from the WST-8 assay suggest that these peptides can be used at 5 μM without notable cell death. Disappointedly, treatment with 5 μM of Chol-HAad and HAad-Chol led to a weak delivery of Dex10-Alexa into the cytosol (Figure 3B, C), implying that cholesterol is not suitable for anchoring moiety of HAad.

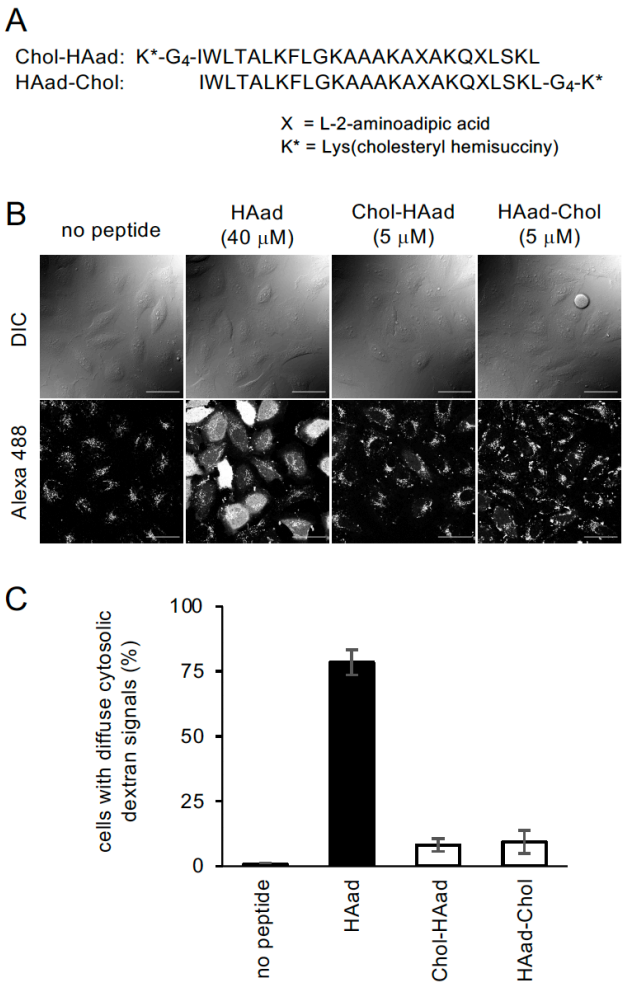


Figure 3. Anchoring HAad using cholesteryl hemisuccinicyl (Chol) moiety. (A) Sequence of peptides. C-termini of the peptides are amidated. (B) Cytosolic appearance of Dex10-Alexa treated with HAad with and without anchoring moieties for 1 h. Scale bar, 50 μm. (C) Percentage of cells accompanied by diffuse cytosolic dextran signals in (B). Results are shown as mean ± SE (n = 3).

Improved cytosolic delivery by conjugation of HAad with pBu

As described above, in our previous study, we demonstrated that L17E induces ruffling of cell membranes. The mode of action of L17E is deduced that interaction of L17E with the ruffled membrane, which leads to transient permeabilization and the resulting cellular internalization of biomacromolecules. This is accompanied by loosening of lipid packing of the cell membrane.[19] On the other hand, arginine-rich peptides including HIV-1 TAT peptide and oligoarginines are among the representative cell-penetrating peptides (CPPs), which have an ability to deliver bioactive molecules into cells. We observed a marked facilitation of cell-membrane translocation of arginine-rich CPPs in the presence of pyrenebutyric acid (pBu-OH).[26] pBu-OH was also found to have a salient effect on loosening lipid packing, providing a compelling reason for facilitating the translocation by pBu-OH.[27] Given that lipid packing loosening plays a role in intracellular delivery using L17E, the use of the pyrenebutyryl (pBu) moiety for membrane anchoring of HAad may promote the cytosolic delivery.

pBu-HAad and HAad-pBu were prepared similarly as described above (Figure 4A, Figure S1). The WST-8 assay indicated that 2 μM was the maximum concentration for both peptides to yield no notable cell death as in the use of 40 μM HAad (Figure S4A,B). The treatment of cells with 2 μM pBu-HAad and HAad-pBu yielded a marked

cytosolic labeling with Dex10-Alexa, as in the treatment with 40 μM HAad (Figure 4B), indicating the potential of both peptides for the cytosolic delivery of biomacromolecules. The Dex10-Alexa treatment of cells with 2 μM pBu-OH or HAad, or with a mixture of these, only yielded punctate Dex10-Alexa signals (Figure 4B), indicating a lack of the respective components of pBu-HAad and HAad-pBu for notable cytosolic Dex10-Alexa delivery activity. The CLSM images suggested that 75% of cells showed diffuse cytosolic Dex10-Alexa signals by the treatment with 2 μM pBu-HAad or HAad-pBu (Figure 4C), which was comparable with that obtained by treatment with 40 μM HAad. When the total cellular uptake of Dex10-Alexa by each treatment was compared using flow cytometry analysis, 2 μM pBu-HAad yielded a comparable Dex10-Alexa uptake to 40 μM HAad and a higher uptake than 2 μM HAad-pBu (Figure 4D).

Although a mixture of 2 μM HAad and pBu-OH did not markedly promote intracellular Dex10-Alexa delivery (Figure 4B,C), the use of higher concentrations of HAad and pBu-OH did have this effect (Figure S5A). Cells were treated with pBu-OH (25 μM, the same concentration used in the previous report[27]) for 30 min prior to incubation with Dex10-Alexa in the presence of HAad (20 or 40 μM) and pBu-OH (25 μM) for 1 h. In the presence of pBu-OH, there was a significant increase in the number of cells with diffuse cytosolic Dex10-Alexa signals (Figure S5B). Increases in cytosolic

Dex10-Alexa distribution of 50-to-75% (use of 20 μM HAad) and 75-to-80% (use of 40 μM HAad) were observed by the addition of 25 μM pBu-OH. On the other hand, there was no significant cytosolic Dex10-Alexa distribution when cells were treated only with 25 μM pBu-OH in the absence of HAad (Figure S5C).

Serum is often reported to diminish intracellular delivery efficacy. However, this effect was not very obvious in the case of HAad. In fact, cellular treatment with Dex10-Alexa in the presence of 40 μM HAad in serum-containing medium yielded >60% cells with cytosolic Dex10-Alexa signals as reported previously[17] (Figure S6A). A reduction in the delivery efficacy was observed for pBu-HAad and HAad-pBu. However, serum also reduced the cytotoxicity of these peptides, and no significant cytotoxicity was observed in serum-containing medium for these peptides up to 10 μM (Figure S4C). The use of 10 μM pBu-HAad yielded a similar extent of cytosolic Dex10-Alexa distribution to that obtained by 40 μM HAad (Figure S6A,B). Compared with pBu-HAad, a lesser extent of cytosolic distribution of Dex10-Alexa was observed in the presence of 10 μM HAad-pBu in serum-containing medium.

Regardless of the presence of serum, pBu-HAad yielded better delivery results (Figures 4 and S6). Therefore, pBu-HAad was used in further studies. However, the reasons underlying the better results obtained using the N-terminal conjugation with the

pBu moiety were not clear. One possible explanation could be that HAad-pBu may have a higher interaction with serum proteins compared with pBu-HAad, resulting in a lesser extent of membrane interaction and cellular internalization. However, of course, further study is needed to elucidate the reasons for this difference.

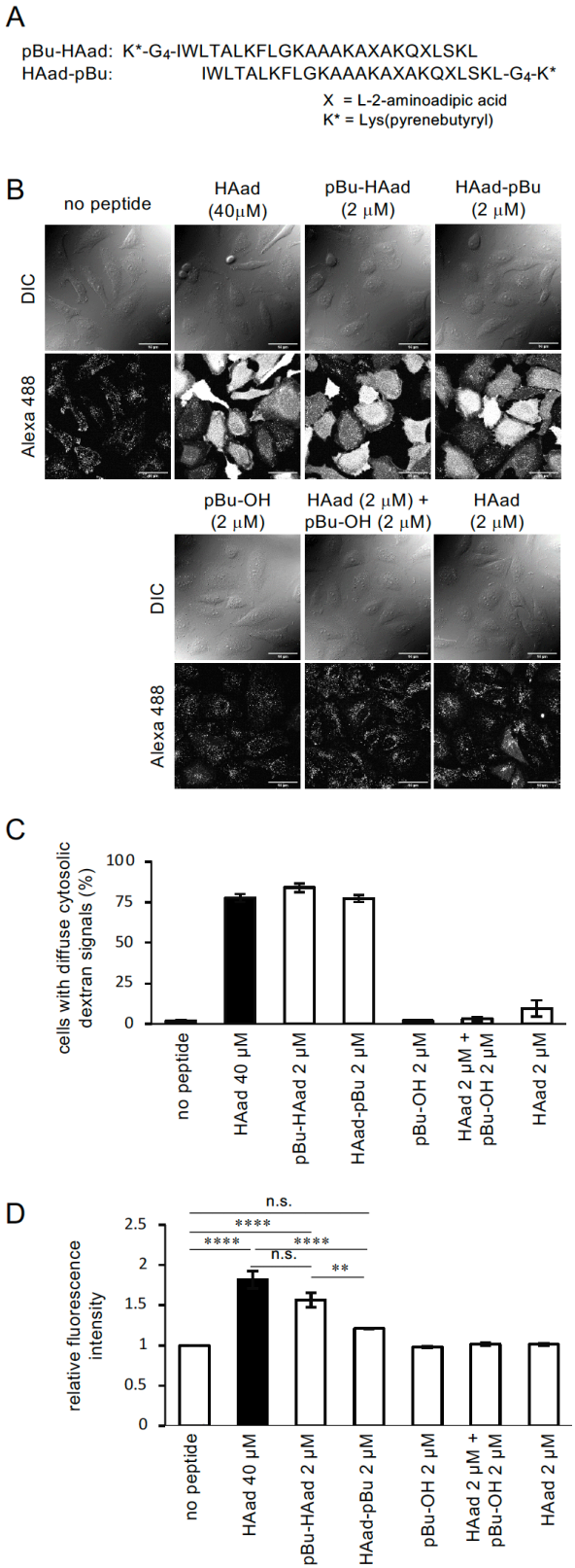


Figure 4. Anchoring HAad using pyrenebutyryl (pBu) moiety. (A) Sequence of peptides. C-termini of the peptides are amidated. (B) Cytosolic appearance of Dex10-Alexa treated with HAad with and without anchoring moieties for 1 h. Scale bar, 50 μm. (C) Percentage of cells accompanied by diffuse cytosolic dextran signals in (B). Results are shown as mean ± SE (n = 3). (D) Flow cytometry analysis of total cellular uptake of Dex10-Alexa. Results are shown as mean ± SE (n = 3). **; $p < 0.01$, ****; $p < 0.0001$, n.s.; no significant difference (one-way ANOVA with Tukey-Kramer's honestly significant difference (HSD) test).

Effective delivery of biomacromolecules into cytosol by pBu-HAad

Next, the applicability of pBu-HAad-mediated cytosolic protein delivery was evaluated (Figure 5). HAad is capable of delivering human immunoglobulin G (IgG, ~150 kDa) into cells.[17] pBu-HAad (2 μM) was also able to deliver IgG. However, the efficacy was slightly lower than that obtained by HAad 40 μM (Figure 5A, B). The treatment of cells with Alexa Fluor 488-labeled IgG (IgG-Alexa) in the presence of 2 μM pBu-HAad yielded diffuse cytosolic IgG-Alexa signals in ~1/3 of cells, whereas half of the cells had diffuse cytosolic IgG-Alexa signals when treated with HAad (40 μM).

For proteins of smaller molecular weight, pBu-HAad (2 μM) seemed effective, similar to HAad (40 μM). As the Cre-loxP system is widely used for the spatiotemporal activation of gene of interest,[29] a loxP-DsRed-loxP-EGFP plasmid was transiently transfected into HeLa cells. The cells were then treated with Cre recombinase (~38 kDa, 10 μM) in the presence of HAad (40 μM) and pBu-HAad (2 μM), respectively (Figure 5C, D). In the absence of these peptides, DsRed-to-EGFP recombination occurred only at a marginal level. In the presence of HAad (40 μM) and pBu-HAad (2 μM), about half of the transfected cells showed EGFP signals, indicating the successful cytosolic delivery of vital Cre recombinase in either case and a comparable delivery efficacy using either system.

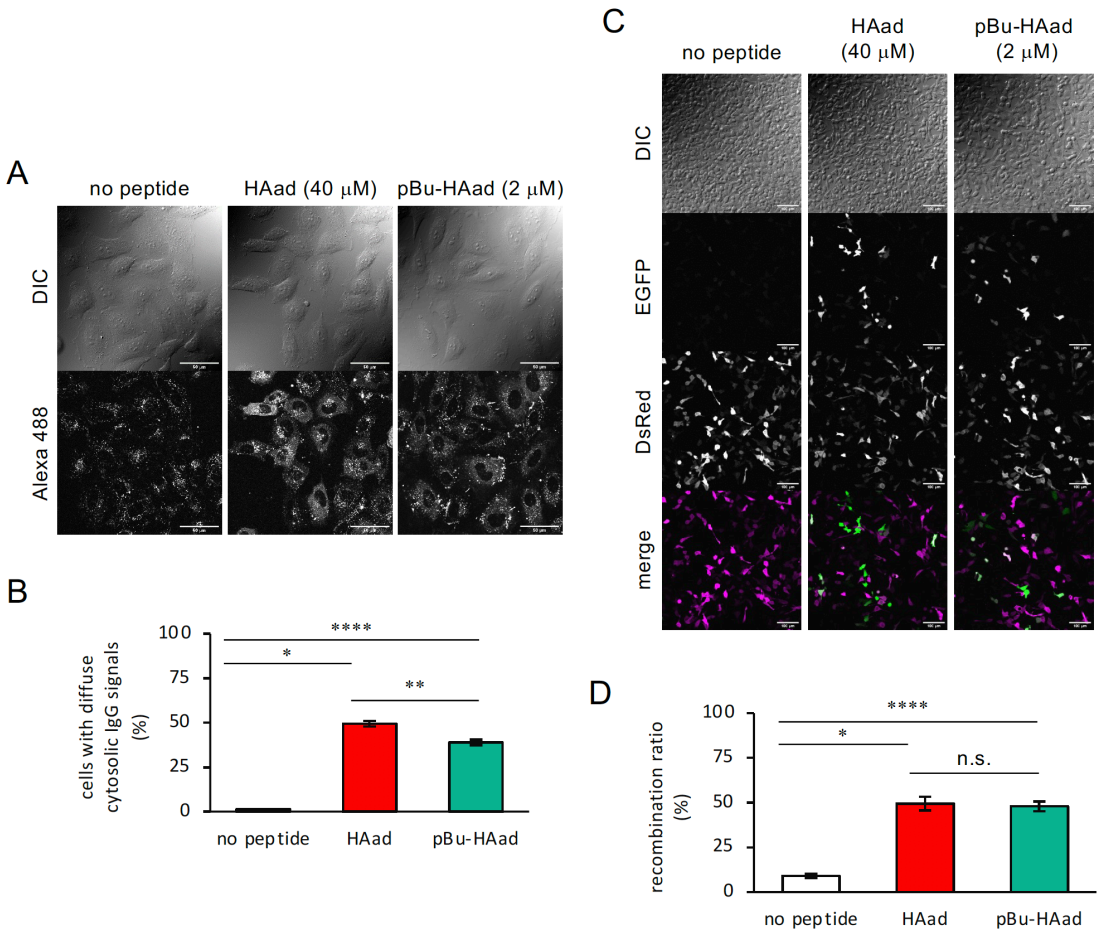


Figure 5. Cytosolic protein delivery using pBu-HAad. (A) Cytosolic delivery of IgG-Alexa by peptides. Scale bar, 50 μm. (B) Percentage of cells showing diffuse cytosolic IgG signals in (A). Results are shown as mean ± SE (n = 3). *; $p < 0.05$, **; $p < 0.01$, ****; $p < 0.0001$ (one-way ANOVA with Tukey-Kramer's HSD test). (C) Cre recombinase-mediated recombination in cells expressing loxP-DsRed-STOP-loxP-EGFP plasmid treated by peptides and Cre recombinase. Scale bar, 100 μm. (D) Recombination ratio of cells treated by peptides and Cre recombinase. Results are shown as mean ± SE (n = 3). *; $p < 0.05$, ****; $p < 0.0001$, n.s.; no significant difference (one-way ANOVA with Tukey-Kramer's HSD test).

Mode of cellular uptake of pBu-HAad

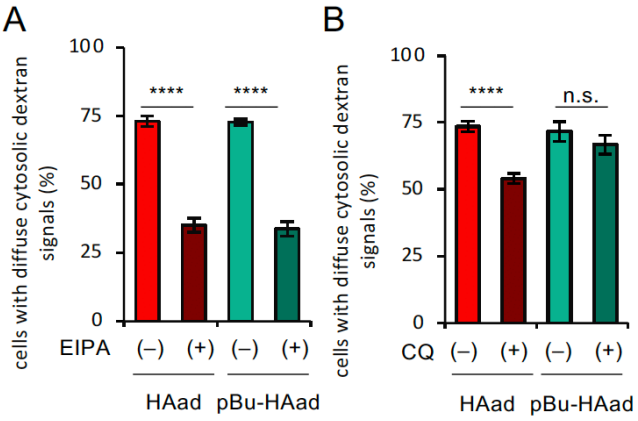
It has been reported that the cellular uptake of Dex10-Alexa in the presence of L17E is inhibited by the treatment with 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), an inhibitor of the Na^+/H^+ exchanger and membrane ruffling.[13,30] Since HAad is thought to share similar uptake methods with L17E, the effect of EIPA on the HAad-promoted cytosolic translocation of Dex10-Alexa was analyzed (Figure 6A). As expected, a significant decrease in the cytosolic localization of Dex10-Alexa was observed by the treatment with EIPA. A similar effect of EIPA was observed for the pBu-HAad-mediated Dex10-Alexa delivery to the cytosol, indicating the importance of membrane ruffling for the cytosolic delivery of macromolecules using these peptides.

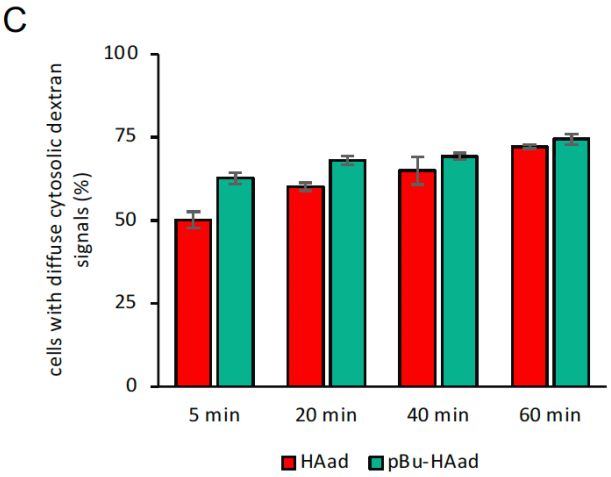
Chloroquine (CQ) is an inhibitor of endosomal acidification.[31] HAad is considered to allow the cytosolic translocation of Dex10-Alexa both at a very early stage of endocytosis and after endosomal maturation accompanied by endosomal acidification. The significant suppression of cytosolic Dex10-Alexa translocation by the CQ treatment suggests that the cytosolic release of Dex10-Alexa release from mature endosomes is involved in HAad-mediated cytosolic delivery (Figure 6B). On the other hand, no significant effect of CQ was observed for pBu-HAad-mediated Dex10-Alexa delivery to the cytosol, suggesting a difference in the mode of delivery using HAad and pBu-HAad.

A time-course study to assess the number of cells bearing diffuse Dex10-Alexa signals was conducted. Cells were treated with Dex10-Alexa in the presence of 40 μM HAad or 2 μM pBu-HAad for 5, 20, 40, and 60 min (Figure 6C). As reported previously,[17] Dex10-Alexa was cytosolically delivered into 50% of the cells in the presence of HAad in the first 5 min. Then, a gradual increase in the number of cells with cytosolic Dex10-Alexa signalwas observed, reaching 72% in 60 min. On the other hand, when treated with pBu-HAad, the percentage of cells with cytosolic Dex10-Alexa signals in the first 5 min was about 60%, 10% higher than that obtained by the use of HAad. However, the cell percentages attaining the cytosolic Dex10-Alexa signal in 60 min were comparable (74%) with that obtained using HAad. This suggests that, although 40 μM HAad and 2 μM pBu-HAad yield similar efficacy to cytosolic Dex10-Alexa delivery, pBu-HAad may attain cytosolic Dex10-Alexa translocation at the earlier stages in the endocytic process than HAad.

We previously reported that the treatment of cells with L17E leads to membrane ruffling, accompanied by loosening of lipid packing in cell membranes.[19] Thus, we speculated that the transient interaction of L17E with membranes with loosened lipid packing may facilitate the cytosolic translocation of biomacromolecules in a very early stages of endocytosis. The loosening of lipid packing can be evaluated using the general

polarization (GP) value of cell membranes treated with the environment-sensitive fluorescent dye di-4-ANEPPDHQ.[32] A decrease in the GP value implies loosening of lipid packing. Cells were stained with di-4-ANEPPDHQ and the decrease in GP value was evaluated by comparing cellular images 5 min before and after peptide treatment. Similar to L17E, the treatment of cells with 40 μM of HAad led to decrease in the GP value of the cell membranes (Figure 6D). Notably, the decrease in GP value obtained by treatment with 2 μM pBu-HAad was even lower than that obtained by 40 μM HAad (20-fold higher concentration). Additionally, treatment with 2 μM HAad alone yielded substantively no decrease in GP value, although 2 μM pBu-OH alone yielded a larger decrease in GP value than 2 μM pBu-HAad. Therefore, modification with the pBu moiety enhanced the membrane interaction of HAad and facilitated the cytosolic translocation of biomacromolecules.





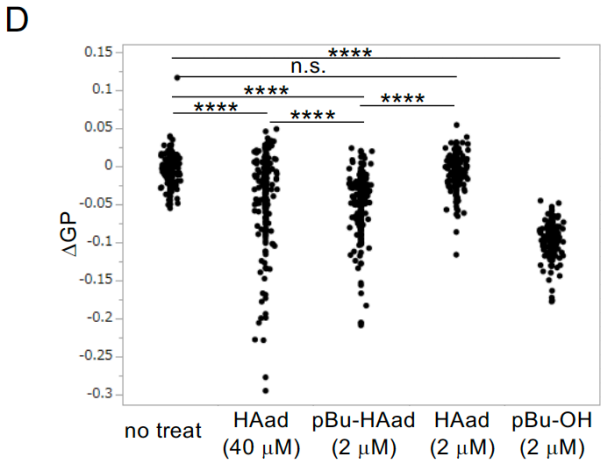


Figure 6. Mode of action of pBu-HAad. (A, B) Effect of EIPA (A) and chloroquine (CQ) (B) on the activities in the cytosolic Dex10-Alexa delivery of HAad or pBu-HAad. (C) Time course study of cytosolic delivery of Dex10-Alexa by HAad or pBu-HAad. Results are shown as mean ± SE (n = 3). ****; $p < 0.0001$, n.s.; no significant difference (one-way ANOVA with Tukey-Kramer's HSD test). (D) Changes in GP values (DGPs) at cell membrane treated by HAad (2 or 40 μM), pBu-HAad (2 μM) or pBu-OH (2 μM). DGP was calculated as $GP_{after} - GP_{before}$, where GP_{after} is GP at 5 min after treatment and GP_{before} is GP at 5 min before treatment. Results are shown as dot plot (n = 240). ****; $p <$ 0.0001, n.s.; no significant difference (Kruskal-Wallis' test followed by Steel-Dwass' post hoc test).

Conclusion

In this study, the effect of membrane anchoring of ACAL peptides on their mode of membrane interaction was evaluated using HAad as a model. The side chain of lysine residues, which was placed at the N- or C-termini of HAad via a tetraglycine linker, was acylated with propionic, hexanoic, and decanoic acids. The decanoyl moiety tethered at the C-terminal side of HAad exerted the most prominent effect, yielding a comparable delivery efficacy with 1/4 concentration of 40 μM HAad. Unexpectedly, cholesterol modification, which enhances the hydrophobic property to the peptide, yielded a poor delivery activity. On the other hand, a marked improvement in delivery efficacy was observed for HAad conjugated with pyrenebutyryl (pBu) group. A comparable efficacy of cellular distribution of Dex10-Alexa and Cre recombinase was successfully achieved by pBu-HAad using 1/20 concentration of HAad. Additionally, a marked cytosolic translocation of Dex10-Alexa (up to ~80% cells) was attained by incubation with 40 μM HAad together with 25 μM 1-pyrenebutyric acid (unconjugated with HAad). The results of the study of the mechanism of action of pBu-HAad suggest that this peptide accelerates the cytosolic translocation of biomacromolecules in the initial stages of endocytosis compared with HAad, where lipid packing loosening is likely to play a role. Further studies are needed to analyze the mechanism in more detail, together with the

applicability to other types of cells. Nevertheless, this study demonstrates the effect of the membrane anchoring of ACAL peptides and the unique features of the pyrene moiety in membrane interactions.

Acknowledgements

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Supporting Information

The following Supporting Information is available free of charge at http://pubs.acs.org.

Materials and Methods, Table S1, and Figures S1-S6 (PDF).

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