

Artificial nanocage formed via self-assembly of β -annulus peptide for delivering biofunctional proteins into cell interiors

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Abstract

Nanocarriers that deliver functional proteins to cell interiors are an attractive platform for the intracellular delivery of intact proteins without further modification, with *in vivo* compatibility. Development of efficient methods for cargo protein encapsulation and release in recipient cell cytosol is needed. Herein, we assess the feasibility of the above requirements using a protein nanocage (artificial nanocage) without compromising the structure and functions of the original protein and allowing design flexibility of the surfaces and interiors. The protein nanocage formed via the self-assembly of β -annulus peptide (24-amino acid peptide) in water was used as a model framework. The nitrilotriacetic acid moiety was displayed on nanocage lumen for effective encapsulation of hexahistidine-tagged proteins in the presence of Ni^{2+} , and the amphiphilic cationic lytic peptide HAad was displayed on nanocage surface to attain cell permeability. Successful intracellular delivery of cargo proteins and targeting of cytosolic proteins by a nanobody were achieved, indicating the validity of the approach employed in this study.

Keywords

drug delivery; amphiphilic cationic lytic peptide; β -annulus peptide; self-assembly; nanocage

Introduction

Although proteins including antibodies and small molecule antibodies exhibit considerable therapeutic potential, their inherently low membrane permeability is an obstacle for their targeted delivery in cells.¹⁻³ Conjugation with peptides and polymers can increase the membrane-permeability of cargo proteins;⁴⁻⁶ however, sophisticated methods of conjugation are often needed. Conjugation with heterogeneous molecules may lead to aggregation due to an imbalance in charges and hydrophobicity. Nanoparticles can be used to encapsulate biopharmaceuticals without any chemical modifications. The nanocarriers should effectively encapsulate the biopharmaceuticals and release them into cells.

Various nanoparticles are available including those based on natural/synthetic polymers, lipids, and organic/inorganic materials having their characteristic properties. Among these, protein nanocages have attractive features.^{7,8} Compared with other nanocarriers, protein nanocages exhibit a more precise design on their surfaces and interiors, which facilitates the efficient delivery of biopharmaceuticals.⁹⁻¹¹ The abilities of nanoparticles for targeting specific organs and cells enable their *in vivo* application. Designing the protein nanocages is also a challenge in chemical biology.

For the application of protein nanocages in intracellular delivery, effective encapsulation of cargo proteins and their release in recipient cells is needed. Electrostatic interactions can be used for protein encapsulation in nanocages, and are typically used for nucleic acid delivery.¹²⁻¹⁶ Harsh conditions (*e.g.*, low pH/organic solvents) are sometimes used for the encapsulation of nucleic acids in nanocarriers, but may cause protein denaturation.¹⁷ Additionally, all proteins are not highly charged. Therefore, more general approaches for protein encapsulation should be used. The approaches that precisely control the number of encapsulated cargos and their topologies in the particles are preferred.¹⁸ Previous studies have reported few approaches of cell permeation for the efficient delivery of cargo proteins in the cytosol of recipient cells using protein nanocages. One possible reason for the unsuccessful cargo protein delivery could be the inefficient endosomal release of cargo proteins. We aimed to study the applicability of the attenuated cationic amphiphilic peptide HAad, which was successfully used in our previous studies for the intracellular delivery of various proteins including immunoglobulin G in the cytosol with considerable efficacy.^{5, 19-21} We also evaluated the feasibility of protein delivery in cells using protein nanocages composed of β -annulus peptide²²⁻²⁴ as a model framework. For encapsulation of proteins into nanocages, the efficacy of the affinity tag was evaluated. The nitrilotriacetic acid (NTA) moiety was

displayed on nanocage lumen as a model tag for effective encapsulation of hexahistidine (His₆)-tagged proteins in the presence of Ni²⁺. HAad peptide was displayed on nanocage surface to attain cell permeability. The efficient cytosolic delivery of His₆-tagged enhanced green fluorescent protein (EGFP-His₆) and successful targeting of cytosolic protein by a nanobody (*i.e.*, a small antibody-like protein) fused with EGFP-His₆ was achieved by this system.

Results and discussion

Design of β -annulus-based artificial peptide nanocage decorated with HAad

The β -annulus peptide is a 24-amino acid peptide derived from the structural backbone of the dodecahedral capsid structure of the tomato bushy stunt virus (INHVGGTGGAIMAPVAVTRQLVGS) (Figure 1A). Matsuura *et al.* reported that this peptide self-assembles to form a peptide nanocage structure in water, having about 30–50 nm diameter with a hollow interior (Figure S1).²² Previous findings have shown that the N-terminus of the β -annulus peptide faces inward, whereas the C-terminus faces outward of the nanocage.²⁵ Therefore, the attachment of a suitable tag segment having affinity toward proteins to the N-terminal of the β -annulus peptide may allow efficient encapsulation of cargo proteins inside the nanocage.

Endocytosis is a major cellular pathway for the cellular uptake of biomacromolecules and materials trapped in the vesicular compartments. Since these vesicles finally fuse with lysosomes that digest the endocytosed macromolecules, encapsulated cargo proteins should be released from the nanocage and endosomes before being degraded in the lysosome. Therefore, we attached HAad peptide to the C-terminus of β -annulus peptide so that HAad peptide was displayed on nanocage surface. HAad stimulates endocytic uptake.^{5, 19-21} The pH inside the endosome decreases to about 5,

along with endosomal maturation. HAad increases membrane interactions to perturb the membrane, thus facilitating the endosomal escape of cargo proteins. The display of many HAad segments on nanocage surface should also increase the local concentration of peptides and membrane interactions, thus facilitating the local perturbation of membranes. Therefore, effective delivery of cargo proteins in the cytosol using HAad-decorated nanocage was expected.

The encapsulation efficacy of cargo proteins in nanocarriers is a critical factor in their formulation, thus lowering the loss of non-encapsulated proteins. The Ni-NTA affinity tag exhibits a high affinity toward His₆-tagged proteins.²⁶ The size of the His₆-tag is small enough to have no affect on the intrinsic protein function, hence this techniques is frequently employed in protein purification used for cell biological studies.²⁷ Feasibility of encapsulation of various functional proteins in β -annulus peptide and other protein-based nanocages using Ni-NTA has already been exemplified.^{15, 28} When delivered into cells, non-covalent methods for binding of His₆-tagged proteins with the capsid protein are favorably for their cytosolic liberation along with the membrane interaction of the HAad-decorated nanocages. Since there is no affinity tag to capture proteins regardless of physicochemical properties (e.g., sequence, charge, and size), the above feature of His₆-tag is preferable in the context of our study.

Direct conjugation of cargo proteins with the nanocage component is another option for encapsulating cargo proteins;²⁹⁻³² however, the attachment to extra segment (especially those having a high membrane interaction ability, like HAad) may affect the cellular localization and the function of the cargo proteins.³³ In this study, the NTA group was, therefore, attached to the N-terminus of β -annulus peptide derivatives to determine whether the disposition of an appropriate affinity tag in nanocage lumen promotes cargo encapsulation.

Previous studies have used virus-derived self-assembled proteins that yielded particle-like structures to deliver nucleic acids and proteins.²⁹⁻³² The sequences of these assembly proteins are often longer than those prepared via solid-phase peptide synthesis (SPPS) and must be prepared through recombinant methods, whereas β -annulus is a 24-amino acid residue peptide easily prepared via SPPS. The employment of β -annulus peptide should thus provide more flexibility in designing the delivery system, thereby allowing specific chemical modifications (e.g., NTA group)/cross-linking, and the use of peptides containing non-standard amino acids, such as Aad in HAad, as shown in this study. As described above, attachment of the HAad and the NTA group to the C- and N-terminal sides of β -annulus allows for their exposures on both capsid surface and interior.²⁸ The possible topological control of the β -annulus-attached segments is

advantageous in the functional design compared with other peptide-based nanoparticle structures.³⁴ Nucleic acid-based particles are another possible templates for the delivery of nanocages, which allow for topographically specific functionalization for surface modification and cargo encapsulation.³⁵⁻³⁷ However, cell surfaces are rich in negatively charged molecules, such as proteoglycans and sialic acids,³⁸ that may hinder cellular uptake. Additionally, cationic charges in HAad are important for its endosomolytic activity,^{19, 39} while attachment to negatively charged frameworks can diminish such activity. Therefore, we employed peptide-based nanocage in this study.

To assess the preferred mode of membrane interactions with HAad peptide, two types of HAad conjugation to the β -annulus peptide were studied, the N-terminus or C-terminus of HAad (NTA- β -annulus-N[HAad] (Figure 1B(ii)) and NTA- β -annulus-C[HAad] (Figure 1B(iii)), respectively). Additionally, the β -annulus peptide without conjugation with HAad peptide (NTA- β -annulus, Figure 1B(i)) was prepared as a control. In this study, oxidation-prone methionine (Met, position 12) in the original β -annulus sequence was substituted with non-oxidizable norleucine (Nle) for the ease of handling.

For the preparation of NTA- β -annulus-N[HAad] and NTA- β -annulus-C[HAad], cysteine and glycyl-lysine residues were attached to the N- and C-termini of the β -annulus segment. The NTA moieties were introduced by reacting N-[5-(3'-

maleimidopropylamido)-1-carboxypentyl] iminodiacetic acid (maleimido-C3-NTA)²⁸ with the N-terminal cysteine. For the conjugation of β -annulus and HAad segments, strain-promoted azide-alkyne cycloaddition was performed using dibenzocyclooctine (DBCO).⁴⁰ The azide group bearing a polyethylene glycol linker was attached to the C-terminus lysine of the β -annulus using azido-dPEG₁₂-NHS ester (NHS = *N*-hydroxysuccinimide). The DBCO group was introduced using the corresponding maleimide on the cysteine, placed either at the N- or C-terminus of the HAad segment *via* a tetraglycine linker to yield NTA- β -annulus-N[HAad] and NTA- β -annulus-C[HAad], respectively. The details of conjugate preparation are described in the Supporting Information and [Figures S2–S4](#).

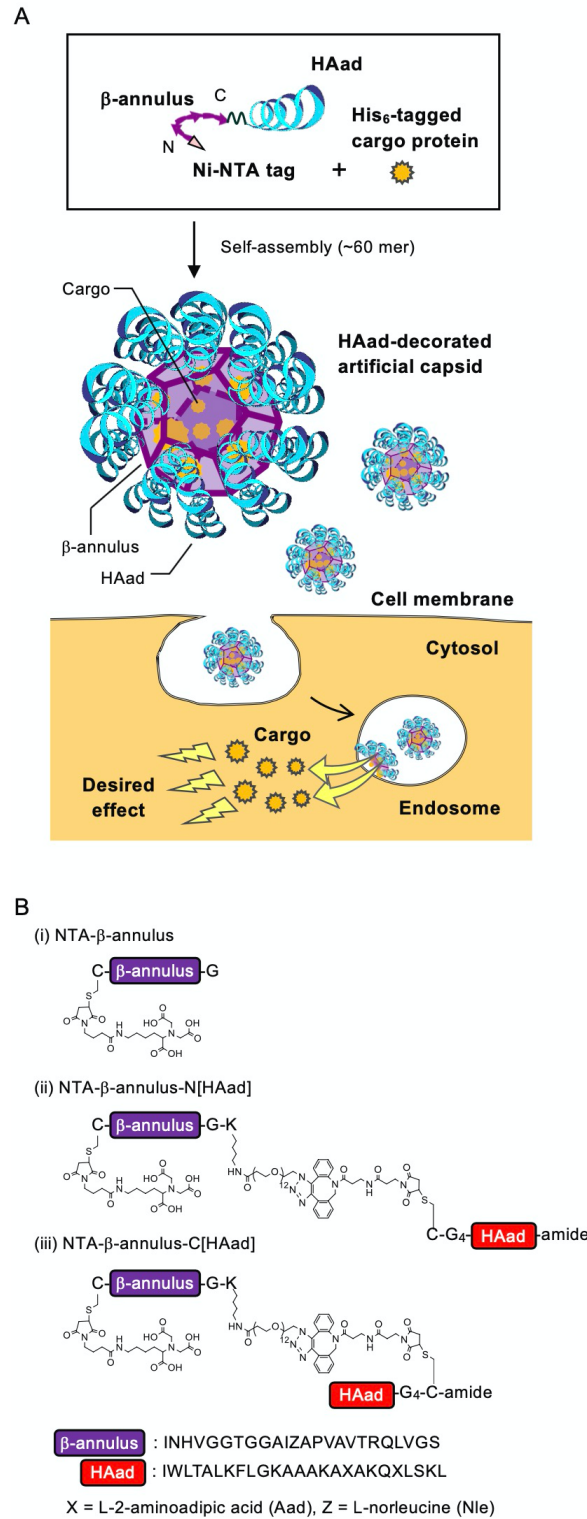


Figure 1. (A) Schematic illustration of the artificial peptide nanocage prepared via the assembly of β -annulus peptides, displaying the HAad intracellular delivery peptide on nanocage surface and encapsulating cargo proteins using the affinity tag (*i.e.*, Ni-NTA). N and C denote the N- and C-terminal sides, respectively. (B) Structures of β -annulus peptides bearing the NTA and HAad moieties.

Confirmation of the formation of artificial peptide nanocage decorated with HAad

NTA- β -annulus-N[HAad] and NTA- β -annulus-C[HAad] that were prepared spontaneously formed nanoparticle structures as expected, similar to those of the NTA- β -annulus. This was confirmed by dynamic light scattering (DLS) measurements after incubation of 40 μ M peptides in H₂O for 1 h at 25 °C (Figure 2A). The NTA- β -annulus was suggested to form particles of approximately 40 nm in diameter (Figure 2A(i)), which is consistent with a previous report (46.8 ± 10.5 nm).²⁸ The size of the particles formed by NTA- β -annulus-N[HAad] and NTA- β -annulus-C[HAad] was about 30 nm (Figure 2A(ii) and (iii)).

Transmission electron microscopic (TEM) analysis further confirmed the formation of spherical structures by these peptides with diameters of about 50 nm (Figure 2B). The peptides in H₂O (40 μ M) were incubated for 1 h at 25 °C. The solutions were then transferred to a TEM grid and then negative staining was performed.

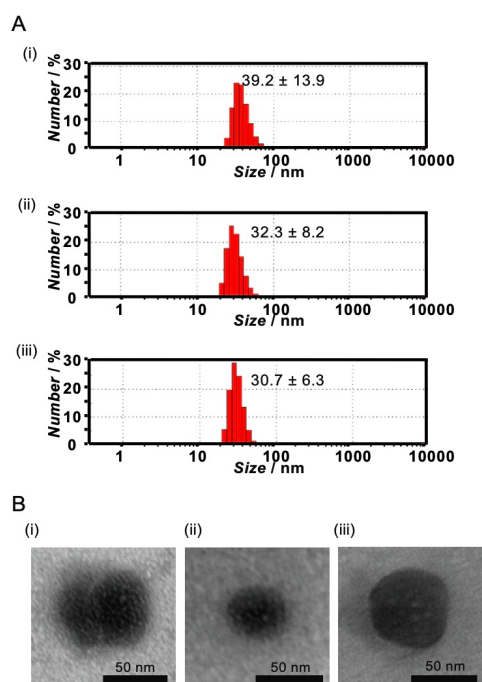


Figure 2. Formation of peptide nanocage structures via (i) NTA-β-annulus; (ii) NTA-β-annulus-N[HAad]; and (iii) NTA-β-annulus-C[HAad]. (A) Dynamic light scattering analysis of 40 μM peptide solutions in water. (B) Transmission electron microscopy images stained with EM stainer (purchased from Nisshin EM).

Cellular uptake and distribution of the artificial peptide nanocage decorated with HAad

We next determined whether the artificial nanocages decorated with HAad were internalized in the cells. To visualize the nanocages, the conjugates of β-annulus and HAad labeled with Alexa Fluor 488 were prepared (Figure 3A). Peptides were prepared similarly as in the case of the NTA-modified conjugates using Alexa Fluor 488-maleimide instead of maleimido-C3-NTA to yield Alexa488-β-annulus-N[HAad] and Alexa488-β-

annulus-C[HAad] (Figure 3A (ii) and (iii), respectively). Alexa488- β -annulus was also prepared as a control (Figure 3A (i)). These conjugates were dissolved in phosphate-buffered saline (PBS) containing CaCl_2 and MgCl_2 (PBS (+)) and incubated for 1 h at 25 °C (final peptide concentration, 40 μM), under the identical conditions described in the previous section to confirm the formation of nanocage structures (Figure 2A and B).

The confocal laser scanning microscopy (CLSM) analysis of HeLa cells treated with the above solution of Alexa488- β -annulus (Figure 3A (i)) for 1 h at 37 °C showed punctate and dot-like signals (Figure 3B (i)), suggesting that Alexa488- β -annulus taken up by the cells was predominantly retained in the endosomal vesicles. A similar tendency was observed for the cells treated with Alexa488- β -annulus-N[HAad] solution (Figure 3B (ii)). On the other hand, the cells treated with Alexa488- β -annulus-C[HAad] solution showed diffuse Alexa Fluor 488 signals in the cytosol in addition to punctate signals, suggesting cytosolic translocation of Alexa488- β -annulus-C[HAad] (Figure 3B (iii)). N-termini often play crucial roles in the interaction between amphiphilic helical peptides and membranes.⁴¹ The increased cytosolic translocation of Alexa488- β -annulus-C[HAad] may be explained by the fact that the HAad segment in Alexa488- β -annulus-C[HAad] had a free N-terminus but that in Alexa488- β -annulus-N[HAad] was used for crosslinking.

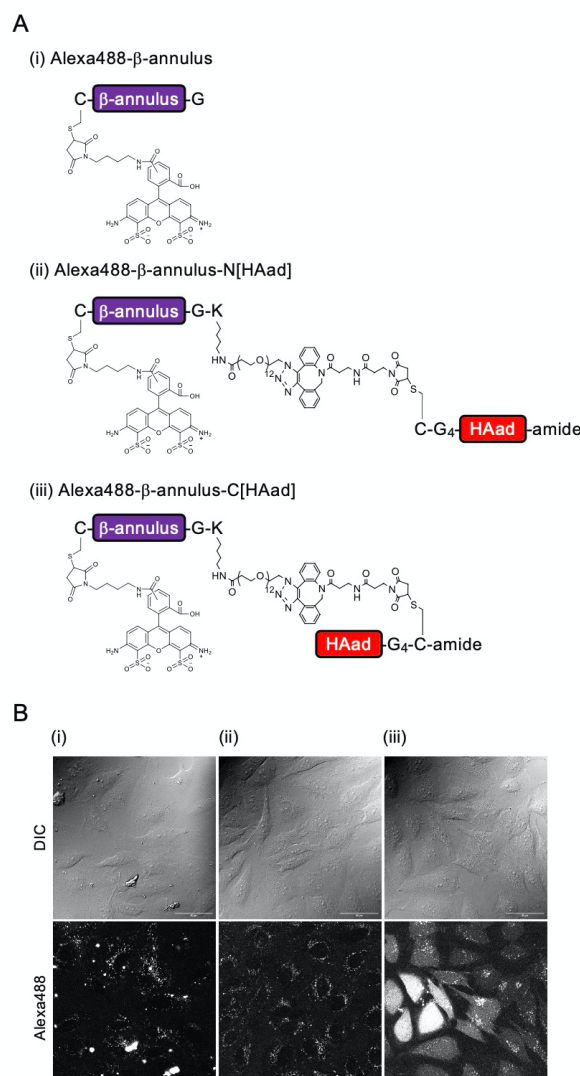


Figure 3. (A) Structures of Alexa Fluor 488-labeled peptides: (i) Alexa488- β -annulus; (ii) Alexa488- β -annulus-N[HAad]; and (iii) Alexa488- β -annulus-C[HAad]. (B) Cellular distribution of Alexa Fluor 488-labeled peptides. Each peptide was dissolved in PBS (+) (final concentration, 40 μ M) and incubated at 25 $^{\circ}$ C for 1 h to allow for nanocage formation. The solution was added to HeLa cells, and the cells were incubated at 37 $^{\circ}$ C for 1 h. Scale bars, 50 μ m.

Encapsulation of His-tagged protein in an artificial peptide nanocage

Although Alexa488- β -annulus-C[HAad] solution yielded cytosolic localization of the peptide (Figure 3B(iii)), this did not ensure the delivery of the nanocage formed by Alexa488- β -annulus-C[HAad]. We next examined whether NTA- β -annulus-C[HAad] forms a nanocage encapsulating EGFP-His₆ in the presence of Ni²⁺ by ultrafiltration using EGFP-His₆ as a model (Figure 4). EGFP-His₆ (~27 kDa) should permeate through the ultrafiltration filter having a molecular weight cutoff of 100 K (MWCO 100 K). Conversely, if 60 molecules of NTA- β -annulus-C[HAad] (~7 kDa) assemble to form a nanocage and encapsulate EGFP-His₆, the mass of Ni-NTA- β -annulus-C[HAad] containing an EGFP-His₆ molecule can be ~450 kDa, which is retained on the filter of MWCO 100-K filter. The encapsulation efficacy of EGFP-His₆ in the nanocage can be evaluated in terms of the amount of EGFP-His₆ in the filtrate versus that remaining on the filter.

NTA- β -annulus-C[HAad] (40 μ M) was incubated with 5 μ M EGFP-His₆ in the presence or absence of 40 μ M NiCl₂ for 1 h at 25 °C and then subject to ultrafiltration using the MWCO 100 K filter. The residue and the filtrate were diluted to the same volume (Figure 4A). The band intensity analysis of each fraction performed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 4B) showed no substantive

EGFP-His₆ remaining on the ultrafilter when EGFP-His₆ solution alone or a mixture of NTA- β -annulus-C[HAad] and EGFP-His₆ in the absence of Ni²⁺ (Figure 4C). However, when NTA- β -annulus-C[HAad] and EGFP-His₆ were incubated in the presence of Ni²⁺, EGFP-His₆ predominantly resided on the filter. The encapsulation efficiency evaluated from the band intensity was 93%. Although further topological investigations are warranted to assess the HAad and NTA moieties, if properly located on the surface and inside of the β -annulus-based nanocage, respectively, it is plausible that most of the EGFP-His₆ would be retained inside the nanocage; the dissociation constant of His₆-tagged proteins with Ni-NTA has been estimated within the micromolar range⁴² and, considering that the initial concentration of EGFP-His₆ in the preparation of the nanocage was 5 μ M, EGFP-His₆ may therefore be gradually released from the nanocage when exposed on the nanocage surface facing solvents without containing EGFP-His₆. At least 6 residues are typically needed for the interaction of poly-histidine with the NTA,²⁶ which is retained even in the presence of the relevant amino acids within the nanocage structure (e.g., His/Arg in β -annulus, and Trp/Lys in HAad).

The concentration of NTA- β -annulus-C[HAad] needed to form HAad-decorated artificial nanocages was also investigated via DLS (Figure S5). The peptide was dissolved in water at a final concentration of 40 μ M, and subsequently diluted to yield 20, 10, 5, 2,

1, 0.5, and 0.2 μM . Concentration-dependent, linear increase in the particle number was observed at NTA- β -annulus-C[HAad] concentrations higher than 5 μM , suggestive of the critical aggregation concentration (CAC) of NTA- β -annulus-C[HAad]. Particles with diameters of 20–30 nm were formed at peptide concentrations higher than the CAC.

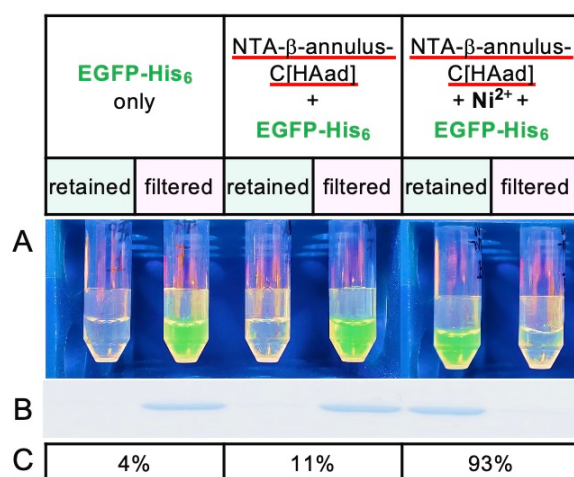


Figure 4. Evaluation of encapsulation ratios of His₆-tagged enhanced green fluorescent protein (EGFP-His₆) in the artificial nanocage formed by NTA- β -annulus-C[HAad] in the absence and presence of Ni²⁺. (A) Fluorescence images of EGFP-His₆ retained on the ultrafilter and filtrates. Each sample was diluted to yield an equal volume; (B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of each fraction; (C) Percentages of EGFP-His₆ retained on the ultrafilter (suggestive of encapsulated EGFP-His₆ in the nanocage) analyzed from the band intensities.

Cytosolic delivery of the His-tagged protein by HAad-decorated nanocage

HAad-decorated nanocages formed using NTA- β -annulus-C[HAad] successfully delivered the His-tagged proteins into the cytosol in the presence of Ni^{2+} (Figure 5). Following the procedures of verification of EGFP-His₆ encapsulation into the nanocage (Figure 4), 5 μM EGFP-His₆ was incubated with NTA- β -annulus-C[HAad] (40 μM) in the presence of Ni^{2+} (40 μM) in PBS (+) for 1 h at 25 °C. The samples were added to HeLa cells and incubated for 1 h at 37 °C. HeLa cells were treated with 5 μM EGFP-His₆ alone for 1 h at 37 °C as a control. CLSM analysis of the cells treated with EGFP-His₆ alone yielded punctate, dot-like EGFP-His₆ signals (Figure 5 (i)), suggesting that the EGFP-His₆ was taken up by the cells *via* endocytosis and predominantly captured in the endosomes without being released into the cytosol. In contrast, diffuse EGFP-His₆ signals distributed throughout the cells were observed in ~70% of the cells treated with EGFP-His₆ that was pre-incubated with NTA- β -annulus-C[HAad] in the presence of Ni^{2+} (Figure 5 (iv)), suggesting the cytosolic release of EGFP-His₆ from the nanocage. When the same experiment was performed in the absence of Ni^{2+} , where HAad-decorated nanocage was formed but EGFP-His₆ may not be encapsulated because of the lack of chelate formation with the nanocage, the cells with diffuse cytosolic EGFP-His₆ signals were about 50% (Figure 5 (v)), suggesting the importance of EGFP-His₆ encapsulation in the nanocage to

achieve cytosolic translocation with optimal efficacy. As controls, treatment of the cells with EGFP-His₆ incubated with NTA- β -annulus in the presence of Ni²⁺ yielded no marked diffuse cytosolic EGFP-His₆ signals, where EGFP-His₆ could be encapsulated in the nanocage, but the nanocage had no HAad modification (Figure 5 (ii)). The same experiment using NTA- β -annulus-N[HAad] yielded only about 25% of the cells with cytosolic EGFP-His₆ signals (Figure 5 (iii)). These results suggested the importance of nanocages displaying HAad with an appropriate orientation or topology and encapsulation of EGFP-His₆ using Ni-NTA to achieve effective cytosolic delivery by this nanocage-mediated approach. The percentage of cells with diffuse cytosolic EGFP-His₆ signals was almost the same in the presence of 10% bovine serum (BS) compared with that in its absence (Figure S6 (i) and (ii)), although some extent of decrease in the fluorescence intensities of cytosolic EGFP-His₆ signals was observed. Decreased efficacy of the intracellular delivery of biomacromolecules is often observed in the presence of serum proteins.^{43, 44} The retention in the delivery activity of HAad-decorated nanocages in the presence of serum suggested the possible expandability of this system for *in vivo* applications.

Although the β -annulus nanocage is membrane impermeable and the conjugation of HAad with the nanocage could reduce membrane-permeabilization

activity of HAad, this was not observed. No apparent difference in the efficacy of cytosolic delivery of EGFP-His₆ was observed when delivered using nanocage compared with delivered using HAad without tagged with the nanocage (Figure S6 (i) and (iii)).

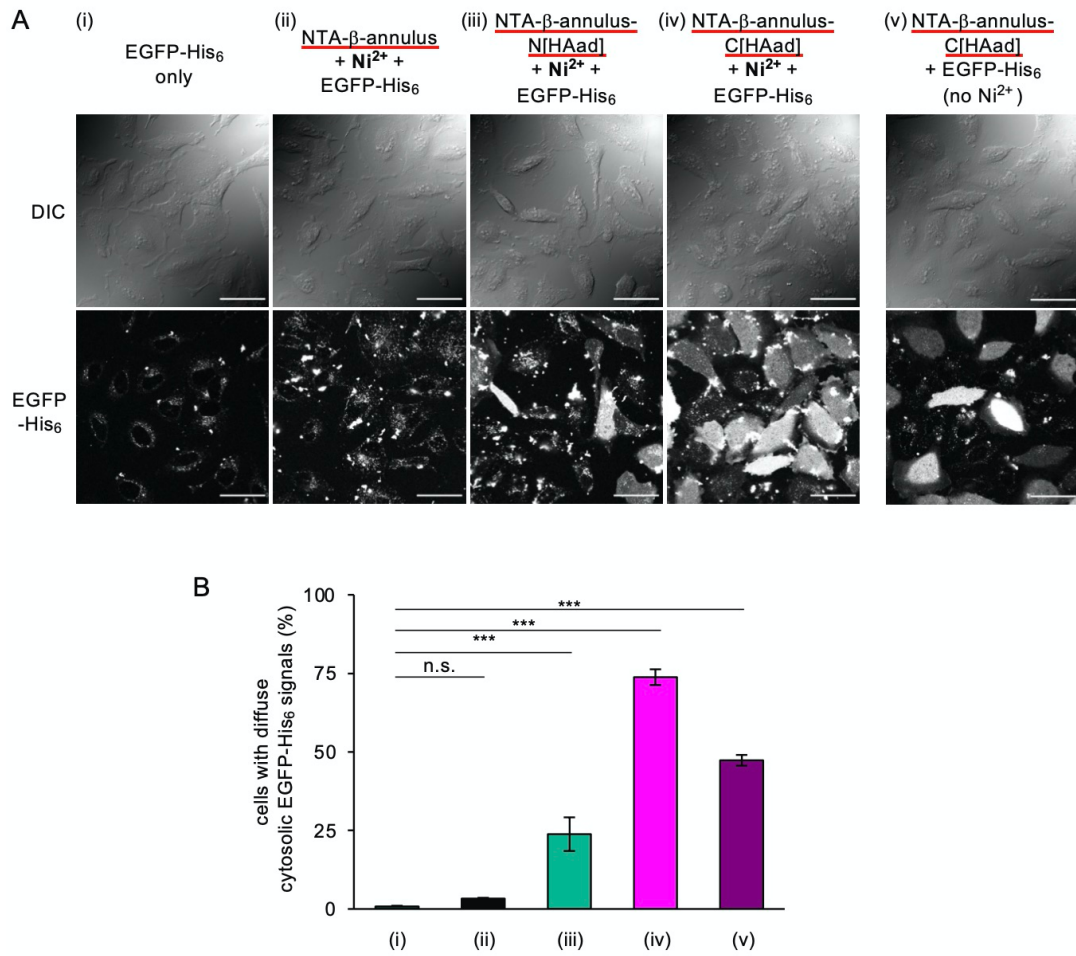


Figure 5. Cytosolic delivery of EGFP-His₆ by HAad-decorated nanocage. (A) Cellular distribution of EGFP-His₆. Scale bars, 50 μm. (B) Percentage of cells yielding diffuse cytosolic EGFP-His₆ signals. Results are presented as mean ± standard error of the mean (n = 3). ***p < 0.001; n.s., not significantly different vs. no peptide (one-way analysis of variance followed by Dunnett's post hoc test).

Targeting a cytosolic protein by a nanobody encapsulated in the HAad-decorated nanocage

Small antibody-like proteins including nanobodies are a promising class of biopharmaceuticals.⁴⁵ Considerable research has been undergone on the development of antibody-like proteins that modulate extracellular drug targets. Although there are several potential drug targets within the cells including disease-related protein-protein interactions, the difficulty in accessibility of these proteins into cell interiors reduces their applications. We demonstrated the potential applicability of the HAad-decorated nanocage for the delivery of nanobodies (variable domains of heavy chains of heavy chain antibodies from camelid, VHH) targeting intracellular proteins. Lifeact-mCherry was used as the model target within the cell. Lifeact is a peptide with 17 amino acids that binds to filamentous actin (F-actin) protein in living cells.⁴⁶ An anti-mCherry nanobody fused with EGFP-His₆ was designed as a cargo protein for visualizing the intracellular localization of the nanobody and for facilitating its encapsulation in the HAad-decorated nanocage *via* the interaction with His₆-tag (Figure 6A).

HeLa cells stably expressing Lifeact-mCherry were used to visualize the F-actin structure in the cells (Figure 6). Following the procedures to deliver encapsulated EGFP-His₆ to the cell interior using the HAad-decorated nanocage, an anti-mCherry nanobody

fused with EGFP-His₆ was pre-incubated in NTA- β -annulus-C[HAad] in PBS (+) containing Ni²⁺ and 10% BS for 1 h at 25 °C and then added to the HeLa cells. After incubation for 1 h at 37 °C, the cells were washed and incubated for 4 h. CLSM analysis of the cells yielded EGFP signals within the cells well colocalized with mCherry signals (Figure 6B, lower panels).

In contrast to the intracellular delivery of EGFP-His₆ (Figure 5A), the signals from anti-mCherry nanobody-EGFP-His₆ outlined cytosolic, filamentous structures, similar to the F-actin structure stained with Lifeact-mCherry. The generation of white filamentous structures in the merged image strongly suggested the colocalization of Lifeact-mCherry (magenta) and anti-mCherry nanobody-EGFP-His₆ (green) in the cells (yielding white signals), indicating the capability of the nanobody to recognize cellular proteins delivered by the HAad-decorated nanocage. Meanwhile, no significant colocalization of anti-mCherry nanobody-EGFP-His₆ signals with Lifeact-mCherry was observed when anti-mCherry nanobody-EGFP-His₆ was added to the cells without encapsulation in the nanocage of Ni²⁺/NTA- β -annulus-C[HAad] (Figure 6B, upper panels). Large dot-like EGFP signals observed in cells both in the absence and presence of Ni²⁺/NTA- β -annulus-C[HAad] (Figure 6B) represent the precipitates of anti-mCherry nanobody-EGFP-His₆.

Overall, although some extent of aggregation was observed extracellularly, the successful cytosolic delivery and recognition of the cellular target were achieved by anti-mCherry nanobody-EGFP-His₆ using the HAad-decorated nanocage.

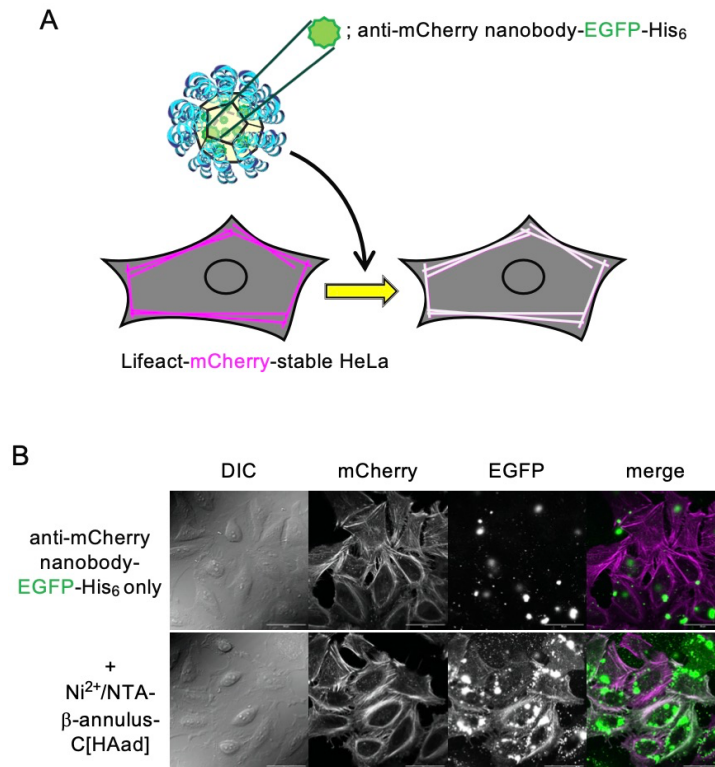


Figure 6. Recognition of the cytosolic protein by anti-mCherry nanobody-EGFP-His₆ intracellularly delivered by Ni²⁺/NTA-β-annulus-C[HAad]. (A) Outline of the experiment. (B) Cellular localization of Lifeact-mCherry and anti-mCherry nanobody-EGFP-His₆ delivered by Ni²⁺/NTA-β-annulus-C[HAad]. Scale bars, 50 μm.

Conclusions

In this study, we evaluated the feasibility of HAad-decorated peptide nanocages to efficiently deliver encapsulated proteins in the cytosol. The HAad-decorated nanocage was formed by the self-assembly of NTA- β -annulus-C[HAad] and the sphere-like structure was confirmed via TEM analysis. Encapsulation of cargo proteins in a nanocage is crucial for avoiding the loss of cargo proteins during formulation. The nanocage was designed to display the NTA group in the interior, facilitating the encapsulation of His₆-tagged proteins. Efficient cytosolic delivery of EGFP-His₆ and anti-mCherry nanobody-EGFP-His₆ fusion protein was achieved using the HAad-decorated nanocage. Notably, in the latter case, the targetability of anti-mCherry nanobody-EGFP-His₆ to Lifeact-mCherry was exemplified by co-staining the cytoskeletal F-actin structure.

For efficient cytosolic delivery, encapsulation of cargo proteins in nanocage structures and the effective release of encapsulated proteins into the cytosol are important. Therefore, a balance between encapsulation and release is required, which was attained in this study using NTA- β -annulus-C[HAad]. Although a remarkable extent of cytosolic appearance of the delivered proteins was observed in 75% of cells, a relatively high concentration of HAad-decorated nanocage (40 μ M as HAad concentration), ensuring the retention of the nanocage structures encapsulating His₆-tagged proteins, was used.

A more detailed study is needed on the design of nanocage structures to improve the delivery efficacy along with the methods of cellular uptake and cytosolic release of cargo proteins. In this study, we showed the potential use of artificial nanocarriers with appropriate membrane-permeabilizing peptides for the intracellular delivery of proteins with specific functions, which is an important first step in designing nanocarriers capable of delivering encapsulated proteins into specific cells or organs.

Experimental Section

Materials.

All chemicals, materials, and cell culture media were purchased from Peptide Institute, Thermo Scientific, and FUJIFILM Wako Pure Chemicals unless otherwise specified.

Solid-phase peptide synthesis.

All linear peptides were synthesized *via* Fmoc solid-phase peptide synthesis (SPPS) using a Rink amide resin (TGS-RAM; Shimadzu – for peptides having a C-terminal amide structure) or a Wang resin (TGS-PHB; Shimadzu – for peptides having C-terminal carboxyl group). The 3-[bis(dimethylamino)methyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate (HBTU)/hydroxybenzotriazole (HOBt) coupling system was employed for peptide chain elongation.⁴⁷ Deprotection of protecting groups and detachment of the peptides from the resin was conducted using trifluoroacetic acid (TFA)/ethanedithiol (EDT) (95:5 (v:v)) at room temperature (~23 °C) for 3 h. Peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column. Masses were confirmed by matrix-assisted laser desorption ionization – time-of-flight mass spectrometry (MALDI-TOF MS). Peptide sequences and the obtained masses are summarized in Table S1.

Preparation of conjugates

The methods of conjugate preparation are described in the Supporting Information and [Figures S2–S4](#).

Dynamic light scattering (DLS)

Each peptide (8 nmol) was dissolved in 200 μ L of ultrapure water filtered by 0.2 μ m filter to yield 40 μ M peptide solution and incubated for 1 h at 25 °C. The solution was then transferred to disposable cuvettes (ZEN0040, Malvern Panalytical) and DLS was measured using Zetasizer Nano S (Malvern Panalytical) under the following condition: temperature, 25 °C; measurement angle, 173°; measurement time, 10 sec for 10 times; number of measurements, 3 times. For investigation of critical aggregation concentration, 40 μ M peptide solution incubated for 1 h at 25°C were diluted stepwise to yield 20, 10, 5, 2, 1, 0.5, 0.1 μ M peptide solutions in the cuvette and DLS was measured.

Transmission electron microscopy (TEM)

Each peptide (4 nmol) was dissolved in 100 μ L of ultrapure water to yield 40 μ M peptide solution and incubated for 1 h at 25 °C. After incubation, 5 μ L of the solution was applied

to C-SMART Hydrophobic carbon film TEM grid (10 nm, ALLIANCE Biosystems) for 60 sec and were then removed. Grids were then negatively stained using EM stainer (Nisshin-EM) for 15 min and then the stain was removed. Grids were dried in vacuo and observed by TEM (JEOL JEM-1400 Plus) with an acceleration voltage of 80 kV.

Cell culture

HeLa cells (human epithelial carcinoma cell line) obtained from The European Collection of Authenticated Cell Cultures (ECACC) (93021013) were cultured in α -minimum essential medium (α -MEM) supplemented with 10% (v/v) heat-inactivated bovine serum (BS) (α -MEM(+)). Cells were maintained at 37 °C, in a humidified 5% CO₂ incubator and subculture was conducted every 3-4 days.

Confocal laser scanning microscope (CLSM) analysis of Alexa488-labeled peptides

Cells were seeded in 35 mm glass-bottom dishes (Iwaki) and were cultured at 37 °C, with 5% CO₂ for 24 h up to 80-90% confluence. Alexa488-labeled peptides (2 nmol) were dissolved in 200 μ L PBS(+) to yield 10 μ M peptide solutions and incubated for 1 h at 25 °C. Cells were washed twice with PBS(-) and were then incubated with the peptide solution for 1 h at 37 °C. After washing twice with PBS(-), α -MEM(+) was added on

dishes and cellular localization of Alexa488-labeled peptides was analyzed via live-cell imaging using FV3000 Olympus CLSM.

Establishing Lifeact-mCherry-expressing HeLa cells³⁶

HeLa cells were transfected with the Lifeact-mCherry plasmid (kindly provided by Prof. T. Itoh) with Lipofectamine 3000 (Invitrogen) according to manufacturer's protocol. After 24 h, the media was changed to fresh α -MEM(+). The cells were incubated for an additional 72 h before selection in the presence of 600 μ g/mL G418 (FUJIFILM Wako) for 1 week. Colonies expressing Lifeact-mCherry were picked and expanded.

Protein expression and purification

pET-EGFP-His₆ plasmid was constructed as described in the previous paper.¹⁹ The EGFP-His₆ protein was expressed in *E.coli* BL21 (DE3) cells by induction with 1 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) for 12 h at 18 °C. Induced cells were resuspended in lysis buffer (20 mM Tris, 300 mM NaCl, 10 mM Imidazole, pH 8.0), sonicated, and centrifuged at 18,000 rpm for 30 min. From the soluble fraction the EGFP-His₆ protein was purified using Ni-NTA-agarose (QIAGEN) and concentrated with

Amicon Ultra-4 (molecular weight cut off (MWCO) 10 kDa) centrifugal filter units (Merck Millipore) using PBS(-).

To construct anti-mCherry nanobody-EGFP-His₆, anti-mCherry nanobody was amplified from pGEX6P1-anti-mCherry nanobody⁴⁸ (kindly provided by Profs. K. Nakayama and Y. Kato) using the following primers: Forward, 5'-AGA AGG AGA TAT ACC ATG GCG CAA GTT-3'; Reverse, 5'-ACG TTA CTT GTA TCC ATG GAA ATG GCT TCT TTG CT-3' and inserted to NcoI site of pET-EGFP-His₆ to yield anti-mCherry nanobody-EGFP-His₆ plasmid. The orientation of insertion of anti-mCherry nanobody was confirmed by sequence analysis. The anti-mCherry nanobody-EGFP-His₆ protein was expressed in *E.coli* BL21 (DE3) cells by induction with 1 mM of IPTG for 12 h at 18 °C. Induced cells were resuspended in lysis buffer, sonicated, and centrifuged at 18,000 rpm for 30 min. From the soluble fraction the anti-mCherry nanobody-EGFP-His₆ protein was purified using Ni-NTA-agarose and concentrated with Amicon Ultra-4 (MWCO 10 kDa) centrifugal filter units using PBS(-).

Evaluation of encapsulation efficacy

Solutions of PBS(+) (500 µL) containing 40 µM peptide, 5 µM EGFP-His₆ and 40 µM NiCl₂ or those without containing NiCl₂ were incubated for 1 h at 25°C. The solutions

were transferred to Amicon Ultra 0.5 mL ultrafiltration unit (MWCO 100K, Merck Millipore) and centrifuged at $14,000\times g$ for 15 min. The sample remaining on the filter (20 μ L) and filtrate (480 μ L) were diluted with PBS(+) to 500 μ L and the pictures of the tubes were recorded by Samsung SCV45. The samples (15 μ L) were then mixed with 5 μ L of 4x SDS sample buffer containing 2-mercaptoethanol and boiled at 95 °C for 5 min. SDS-PAGE was conducted with 15% polyacrylamide gel at 100 V for 1 h. The gel was stained with rapid stain CBB (Nacalai Tesque) and the band intensity of the gel was quantified using ImageJ 1.53c (National Institutes of Health, USA).

CLSM analysis of cytosolic delivery of EGFP-His₆

Cells were seeded in 35 mm glass-bottom dishes and were cultured at 37 °C, with 5% CO₂ for 24 h up to 80-90% confluence. NTA-conjugated peptides (8 nmol) were dissolved in 200 μ L of PBS(+) containing 5 μ M EGFP-His₆ to yield 40 μ M peptide solutions and incubated in the presence and absence of 40 μ M NiCl₂ or 10% (v/v) BS for 1 h at 25°C. Cells were washed twice with PBS(−) and were then incubated with the incubated solution PBS(−) for 1 h at 37 °C. After washing twice with PBS(−), the nuclei were stained by 5 μ g/mL Hoechst 33342 (Invitrogen) and α -MEM(+) was added on dishes. Cellular localization of EGFP-His₆ was analyzed *via* live-cell imaging using FV3000

Olympus CLSM. The number of cells with diffuse cytosolic dextran signals in total > 200 cells were counted for each sample on ImageJ software.

CLSM analysis of cellular distribution of anti-mCherry nanobody-EGFP-His₆ protein

Lifect-mCherry-expressing HeLa cells were seeded in 35 mm glass bottom dishes and were cultured at 37 °C, with 5% CO₂ for 24 h up to 80-90% confluence. NTA-conjugated peptides (8 nmol) were dissolved in 200 µL of PBS(+) containing 5 µM anti-mCherry nanobody-EGFP-His₆ and 10% (v/v) BS to yield 40 µM peptide solutions and incubated in the presence of 40 µM NiCl₂ for 1 h at 25 °C. Cells were washed twice with PBS(-) and were then incubated with the incubated solution for 1 h at 37 °C. After washing twice with PBS(-), α-MEM(+) was added on dishes and cellular localization of anti-mCherry nanobody-EGFP-His₆ was analyzed *via* live-cell imaging using FV3000 Olympus CLSM.

Statistical Analyses.

All data were presented as mean ± standard error (SE) of three independent biological experiments (n = 3). Graphs were drawn and statistical analyses were performed using JMP Pro 14 (SAS Institute, Cary, NC, USA). For multiple comparison analyses, one-way

analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used. Differences were considered significant when the calculated p-value was < 0.05 .

Associated content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.

Preparation of conjugates, Table S1 and Figures S1-S6

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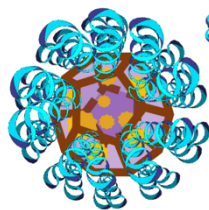
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Table of Contents Graphic

Artificial nanocage decorated
with the amphiphilic cationic
lytic peptide (HAad)



Attaining a marked
cytosolic protein delivery

