論 文 の 内 容 の 要 旨

論文題目 Development of Charge-Conversional Polyion Complex Micelles
for Intracellular Delivery of Proteins
（細胞内へのタンパク質デリバリーを指向した電荷転換ポリイオンコンプレックスミセルの開発）

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1. Introduction

Proteins, which are abundant in the body, are responsible for most of the biological processes, exerting highly complex functions at their biologically programmed destinations. Therefore, use of proteins as therapeutics is the most decisive treatment for many target diseases. However, these protein drugs have limitation for therapeutic use because of unspecific interaction among other proteins in tissues, and it is difficult to deliver it into specific target cells. Thus, the establishment of promising intracellular protein delivery system is highly desired to fully use the unexplored potential of protein drugs. Although many reports on the strategies for intracellular protein delivery have been published over the last decade, most have focused on enhancing the cellular uptake efficiency of proteins, however, other major issues, such as obstacles after administration and escape from the lysosomal degradation pathway, have not been appropriately resolved to date.

To this end, the Kataoka group at The University of Tokyo has developed a charge-converted, protein-loaded polymeric micelle. The fundamental concept underlying this micelle is based on a transient protein modification, which converts primary amino groups in lysine residues to amides containing carboxylate such that electrostatic interaction between the modified protein derivative and synthetic polycatiomers induces the spontaneous formation of a core-shell structured polyion complex (PIC) micelle. A unique characteristic of this system is its pH-sensitive cleavability, which leads to the recovery of the original lysine residues as well as biological activity of the protein in the acidic cellular compartments. Based on this principle, my doctoral study aims to (1) identify an appropriate modification degree that determines how many lysine residues in a proteins should be conjugated by charge-conversional moieties, (2) enhance the shielding of the PIC micelle using combinatorial mixtures containing both block and homo catiomers, and (3) stabilize the PIC micelle by the disulfide crosslinking of the polymeric shell.

2. Charge-conversional modification of proteins

Among various charge-conversional moieties, citraconic anhydride (-Cit) was determined to provide the most effective amide linkage because of its moderately stable nature in weak acid. Other moieties such as succinic anhydride (-Suc) and carboxydimethyl maleate (-CDM) generated highly stable and unstable amide bonds, respectively. This limited their use for intracellular protein delivery that requires late endosomal and lysosomal pH to work as a trigger signal for recovering original proteins and releasing them from the PIC micelles. Cis-aconitic anhydride (-Aco) generated moderately stable amide bonds similar to -Cit; however, the Aco modification was determined to induce distortion of secondary structures, including α-helix of the native proteins, leading to possible denaturation and permanent activity loss of proteins.

The degree of Cit modification was precisely controlled between 25% and 95% and generated four anti-nuclear pore complex (NPC) immunoglobulin G (IgG) antibody derivatives, namely, IgG-25, -50, -75, and -95. Acid-triggered cleavage of these derivatives was confirmed by monitoring the residual amine concentrations when incubated at pH 5.5, which demonstrated that lowly charge-converted IgG derivatives (IgG-25 and -50) can recover original amines within 4 h, whereas highly charge-converted IgG derivatives (IgG-75 and -95) cannot recover original amines even after 12 h. This tendency was also observed in terms of the biological activity recovery at pH 5.5, wherein IgG-25 and -50 could recover a fully competent immunoaffinity toward an antigen within 4 h, whereas the IgG-75 and -95 showed recovery of up to 70% of the original immunoaffinity toward an antigen at 4 h. These results suggest possible limitation of charge-conversional modification, particularly for highly charge-converted protein derivatives.

3. Preparation of protein-loaded PIC micelles

IgG derivatives mentioned in the previous chapter (IgG-25, -50, -75 and -95) were mixed with a synthetic polycatiomer, poly(ethylene glycol) (PEG)-{N-[N'-(2-aminoethyl)-2-aminoethyl]aspartamide} [PEG-PAsp(DET)), to generate antibody-loaded PIC micelles with different modification degrees of charge-conversion (IgG-25/m, -50/m, -75/m, and -95/m). Micelle size was strictly dependent on the modification degree of the IgG derivatives, with IgG-25/m demonstrating the largest (123 nm) and IgG-95/m demonstrating the smallest (50 nm) hydrodynamic diameters. These results suggest that the strong charge of the protein derivatives brings the polymers closer to themselves, generating a more condensed structure by enhanced electrostatic interactions.

Micelle stability was determined using fluorescence correlation spectroscopy (FCS), which suggested that modification degree plays a major role in maintaining micellar structure. Briefly, IgG-75/m and -95/m showed almost no change in their diffusion coefficient when incubating the micelle in a medium containing 150 mM NaCl and 10% fetal bovine serum (FBS). However, IgG-25/m showed substantial dissociation and IgG-50/m showed moderate stability under the same conditions. This may might be the result of a controlled shielding effect of the micelles because the association numbers increased according to the modification degree and reached a plateau with IgG-95/m, where 1.7 polymer molecules could be associated to one antibody molecule. Moreover, in all the formulations, polymer shielding of the micelles could be maximized at a specific molar mixing ratio between the polymer and antibody to 4. At this ratio, the association numbers of the IgG-loaded PIC micelle were proportional to the modification degree of charge-conversion, indicating that polymer shielding is mainly driven by charge-charge interactions.

In vitro delivery efficiency of the protein-loaded PIC micelles was confirmed by fluorescent imaging cytometry analysis of HeLa cells after the cells were treated using Cy5-labeled cytochrome-loaded PIC micelles (Cyt-25/m, -50/m, -75/m, and -95/m). Among these formulations, Cyt-95/m showed the most efficient cellular uptake efficiency with 24-fold higher uptake efficiency than that of native cytochrome. Moreover, the biological activity of protein-loaded PIC micelles was confirmed using real-time confocal laser scanning microscopy (CLSM) of the mouse colon carcinoma (C26) cells during incubation with Alexa Fluor 647-labeled anti-NPC IgG antibody-loaded PIC micelles (IgG-25/m, -50/m, -75/m, and 95). It is noteworthy that only IgG-50/m could successfully recognize the antigen. For IgG-50/m, delivered antibodies were readily localized to the nuclear membrane of cells, and the fraction of these cells increased up to 24 h. However, in case of IgG-25/m, detectable amounts of antibodies were rarely internalized to the cells, without any sign of subcellular antigen recognition. Conversely, for IgG-75/m and -95/m, although substantial fluorescent signal intensity in the cells was observed, neither micelle demonstrated any sign of subcellular antigen recognition. These results can be explained by the low stability of IgG-25/m; thus, most of these derivatives in the medium are supposed to be dissociated before

their cellular uptake. For IgG-75/m and -95/m, majority of the micelles are supposed to be internalized to the cells; however, their highly stable amide bonds may limit complete cleavage when entrapped in acidic cellular compartments; therefore, they are presumably released to the cytoplasm without having proper immunoaffinity toward the antigen.

4. Biological evaluation of anti-nuclear pore complex IgG antibody incorporated block copolymer and homo polymer combined PIC micelles

The use of PEG-PAsp(DET) has been motivated by its pH-sensitively induced di-protonation state of side chains, which has efficient membrane destabilization activity in acidic cellular compartments. However, excess PEG coating not only leads to reduced affinity against cellular membranes, but also affords limited association to the PIC micelle because of the strong steric repulsion between adjacent PEG chains. Thus, systemic engineering for the manipulation of the PEG fraction in the micelle is highly desirable obtain enhanced biological performance of protein-loaded PIC micelles.

To this end, a homopolymer without PEG segment, homo-PAsp(DET), was integrated into the system, such that certain fractions of PEG-PAsp(DET) (B) could be substituted by homo-PAsp(DET) (H). Through this manipulation, the H integrated PIC micelles demonstrated a controlled size with narrow polydispersity index (PDI), where IgG-50/m showed the smallest hydrodynamic diameter of 98 nm and gradually increased as the fraction of homo polymer increased up to 75% (IgG-50/$m_{+Chomo\ 75\%}$, 188 nm). The subscript $C_{homo\ 75\%}$ here indicates that the B and H polymer mixture comprises 25% PEG-PAsp(DET) and 75% homo-PAsp(DET), based on their concentration of amino groups in the mixture. The stability of the H integrated PIC micelles was fundamentally dependent on C_{homo}, because IgG-50/$m_{+Chomo\ 75\%}$ showed almost no change but other formulations showed a gradual increase in diffusion coefficient according to the FCS measurement. This is the direct consequence of the highly effective shielding of IgG-50/$m_{+Chomo\ 75\%}$, as evidenced by its association efficiency, which increased to 90%, whereas that of IgG-50/m was 21%. This means 90% and 21%, respectively, of added polymers in the antibody/polymer mixture could actually be associated to the antibody.

The *in vitro* delivery efficiency of the micelles were investigated using fluorescent imaging cytometry and clearly demonstrated that the IgG-50/$m_{+Chomo\ 75\%}$ outperformed other formulations, with enhanced cellular uptake and antigen recognition efficiencies. These results indicate that the integration of H could successfully induce enhanced shielding, stability, and biological performance of IgG-loaded PIC micelles.

5. Installation of disulfide crosslinking to the polymeric shell

The systemic administration, primarily by intravenous injection, is the most decisive and efficient but relatively non-invasive method of *in vivo* administration. For the successful systemic administration of protein-loaded micelles, an appropriate blood circulation profile is required. However, the preliminary screening of the PIC micelles revealed that both IgG-50/m and -50/$m_{+Chomo\ 75\%}$ demonstrated extremely short half-lives in the blood stream after intravenous injection, indicating limited *in vivo* application. As a solution to this dilemma, disulfide crosslinking between polycatiomer sidechains are expected to enhance the stability of the PIC micelle. To install activated free thiol groups to the polymer, sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido) hexanoate (sulfo-LC-SPDP) was reacted with the PEG-PAsp(DET); subsequently, it was treated with dithiothreitol (DTT), such that a PEG-based block copolymer containing both sulfhydryl and cationic segments [PEG-PAsp(DET/DET-SH)] could be prepared. This polymer was then mixed with a charge-converted IgG derivative (IgG-50) to initiate the formation of the PIC micelle. The formation of the PIC micelle is an instant process, but the mixture was incubated in the mild oxidant for 96 h to induce disulfide formation between the sulfhydryl groups.

Prepared crosslinked IgG-loaded micelles (IgG-Cit CL/m) showed the highest stability against elevated

salt concentrations (1 M) and medium (150 mM NaCl and 10% FBS), indicating that crosslinking is effective for protecting the micelles from counterion exchange. Moreover, significantly enhanced cellular uptake and intracellular antigen recognition efficiency of IgG-Cit CL/m was confirmed using fluorescent imaging cytometry, supporting promising biological feasibility of crosslinked IgG micelles.

Intratumoral administration of IgG-Cit CL/m also showed significantly enhanced tumor penetration and retention ability of crosslinked micelles, as evidenced by CLSM observation of cryostat sections of tumors 24 h post-injection. Furthermore, half-life of the IgG-Cit CL/m in blood stream was confirmed to be longer than 2 h, suggesting the feasibility of crosslinked micelles for clinical applications.

6. Summary and outlook

In an effort to improve the charge-conversional protein-loaded PIC micelles, I have achieved three major advancements that provide a detailed insight and enhance the biological performance of the system. First, a favorable modification degree of charge-conversion was identified. Second, homo-PAsp(DET) integration into the system afforded an optimized structure that provided both highly efficient cellular uptake and intracellular antigen recognition in biological evaluations *in vitro*. Third, the disulfide crosslinking of the polymeric shell facilitated the formation of highly stable micelles with incomparably extended half-lives in the bloodstream. In summary, an advanced systemic configuration of protein-loaded PIC micelles for intracellular delivery was successfully discovered, and this will strongly impact the protein-based biomedical field. Based on these results, further research into the clinical application of this system is warranted and could proceed through the use of therapeutic antibodies, such as anti-IDO and anti-aurora kinase antibodies to obtain anticancer effects through the inhibition of cancer overexpressing enzymes.