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Editorial

Preorganized cationic pillararene as efficient carriers for intracellularly delivering native proteins

As a novel clinical treatment technology, intracellular protein delivery has attracted great attention because of its ability to specifically manipulate cellular functions and accurately treat diseases by acting on intracellular targets. Although therapeutic proteins have shown great promise in the pharmaceutical industry, most of them have membrane impermeability and mainly act on extracellular targets, which limits the clinical application of therapeutic proteins to a certain extent. Therefore, in order to promote the clinical application of therapeutic proteins and protein-based biotechnology, the invention of excellent intracellular protein delivery technologies and carriers has become one of the current research hotpots. With the development of supramolecular chemistry, utilizing macrocyclic molecules to mimic cell-penetrating peptide has gradually attracted great attentions.

Pillararenes, a new generation of macrocyclic compounds which perform the advantages of easy modification and high selectivity on guest molecules, have shown excellent potential in adsorption separation [1], electronic devices [2], biomedicine [3] and so on. In a recent work, Ruibing Wang from University of Macau, Leyong Wang from Nanjing University and co-workers reported a guanidinium-perfunctionalized multifunctional micromolecular carrier using pillar[5]arene (GP5) to efficiently deliver the native proteins intracellularly (Fig. 1) [4]. After preorganized the guanidinium moieties on both sides of the pillar[5]arene skeleton, the GP5 gains the ability to not only assemble several proteins together and form the nano-aggregates *via* electrostatic interaction, but also pull them to the cell membrane, thereby facilitating the uptake of proteins.

The self-assembly behaviors of proteins with GP5 were investigated by using bovine serum albumin (BSA) as the model protein. With the [GP5]/[BSA] molar ratio increasing, the zeta potential of protein complex gradually changed from negative to positive, indicating that the guanidinium groups were distributed on the surface of protein complexes, where the electrostatic attraction between ionic surface of protein and cationic guanidinium groups on pillararene skeleton play an influential role. Transmission electron microscopic (TEM) images and dynamic light scattering (DLS) analysis showed that the GP5@BSA complex at [GP5]/[BSA] = 5/1 presented the spherical morphology with the average diameter of 60 nm, which is much larger than the size of a single protein's as 7.5 nm. Circular dichroism (CD) could also indicate that BSA remained secondary structure after the complexation with GP5. Furthermore, fluorescein isothiocyanate (FITC) labeled BSA was selected as model to evaluate the efficiency of intracellular protein delivery, demonstrating that the GP5@BSA complex could be efficiently transduced into Hela cell and other cell lines. After internalizing into the cells, GP5@BSA aggregates escaped endosomes after incubation for 4 h, and nano-aggregates would release the proteins due to the competitive binding between GP5 and various polyanionic species in the cell.

The protein delivery capacity of GP5 also showed universality. For example, both the negatively charged proteins (including yellow fluorescent protein (YFP), green fluorescent protein (GFP), and β-galactosidase (β-Gal)) and the positively charged proteins (including trypsin, cytochrome C (Cyt C), and ribonuclease A (RNase A)) were able to be transduced into HeLa cells, and the proteins activity was well maintained after cytosolic delivery using GP5, which was verified by horseradish peroxidase (HRP) and (β-Gal) activity experiments. After intracellular delivery of RNase A, trypsin, Cyt C, and anti-phospho-Akt (Ser473) antibody, all of these therapeutic proteins showed obvious cytotoxicity towards HeLa cells and the apoptosis rate of cells is higher than the rate of the untreated cells or the cells treated with Cyt C alone, which indicating the excellent proteins delivery ability of GP5.

The mechanism of efficient intracellular delivery of proteins through GP5 was also studied. A series of control molecules, including primary ammonium functionalized pillar[5]arene PAP5, quaternary ammonium functionalized pillar[5]arene QAP5, synthetic monomer of GP5 (Gu), guanidinium-modified calix[4,5]arene (GC4A and GC5A), and a guanidino groups modified linear cell-penetrating peptide (R8) were selected as comparing materials. Interestingly, all of these comparing materials were able to form the nano-aggregates with BSA *via* the electrostatic interactions, but none of the complexes formed by protein and comparing materials showed the intracellular protein delivery capability into HeLa cells, when the cell was incubated with GC4A@FITC-BSA or GC5A@FITC-BSA and the proteins could be absorbed on cell membrane. A possible reason may be that it is crucial for intracellular protein delivery to pre-organize the guanidinium groups on the pillararene framework. The pre-organized guanidiniums facilitate GP5 not only to act as a "molecular glue" to compound and glue several proteins together by salt bridges [5], but also to be partially distributed on the periphery of protein aggregates. The pre-organized multiple guanidinium groups could also be tightly bound with oxyanions on the cell membrane to induce following protein endocytosis (Fig. 2). This work provides a new solution for the design of artificial cell-penetrating system for protein delivery. Consequently, it showed an advanced perspective for the diseases treatment through pro-

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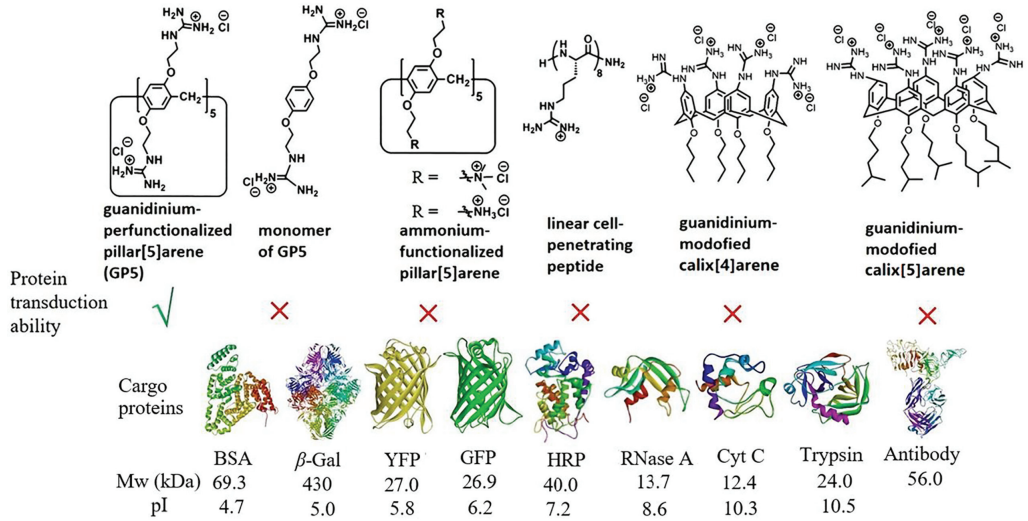

Fig. 1. Capability of GP5 and control molecules for intracellularly delivering native proteins with different isoelectric points and sizes. Reproduced with permission [4]. Copyright 2022, Elsevier.

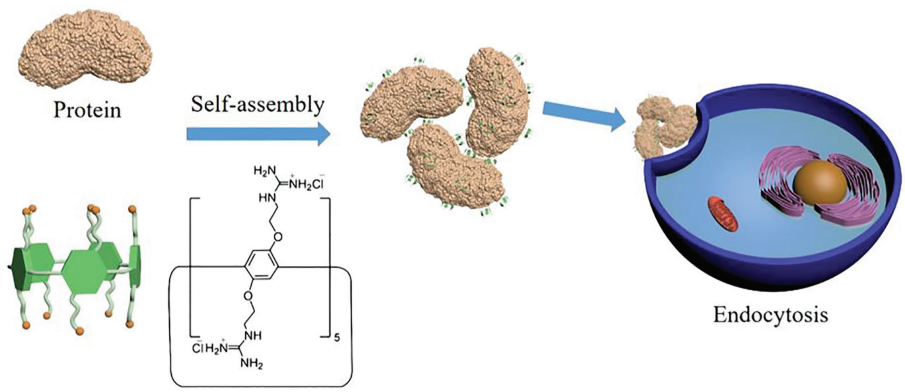

Fig. 2. The formation of the GP5@proteins nano-aggregates and the subsequent endocytosis of proteins.

teins delivery and we were convinced that the work indicated the potential utility of supramolecular chemistry in clinical medicine application.

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