Correspondence

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Development of a novel cholesterol tag-based system for transmembrane transport of protein drugs

Pengfei Zhao[1,3,4,§], Shuo Song[5,§], Zhuojun He[1,2,3,§],Guiqin Dai[1,3], Deliang Liu[1,3], Jiayin Shen[1,3], Tetsuya Asakawa[1,*], Mingbin Zheng[1,2,3,5,*], Hongzhou Lu[1,3,*]

[1] Institute of Neurology, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, Shenzhen, Guangdong, China;
[2] Key Laboratory for Nanomedicine, Guangdong Medical University, Dongguan, Guangdong, China;
[3] National Clinical Research Center for Infectious Disease, Shenzhen Clinical Medical Research Center for Tuberculosis, Institute for Hepatology, the Third People's Hospital of Shenzhen, Shenzhen, Guangdong, China;
[4] College of Physics and Optoelectronic Engineering, Shenzhen Key Laboratory of Photonics and Biophotonics, Key Laboratory of Optoelectronic Devices and Systems of the Ministry of Education and Guangdong Province, Shenzhen University, Shenzhen, China;
[5] Shenzhen Samii Medical Center, Shenzhen, Guangdong, China.

SUMMARY The main technological difficulties of developing an intracellular (transmembrane) transport system for protein drugs lie in two points: *i*) overcoming the barriers in the cellular membrane, and *ii*) loading enough protein drugs, and particularly high-dose proteins, into particles. To address these two technological problems, we recently developed a novel cholesterol tag (C-Tag)-based transmembrane transport system. This pilot study found that the C-Tag dramatically improved the cellular uptake of Fab (902-fold, *vs*. Fab alone) into living cells, indicating that it successfully achieved transmembrane transport. Moreover, C-Tag-mediated membrane transport was verified using micron-scale large unilamellar vesicles (LUVs, approximately 1.5 μm)-based particles. The C-Tagged Fab was able to permeate the liposomal bilayer and it greatly enhanced (a 10.1-fold increase *vs*. Fab alone) internalization of proteins into the LUV-based particles, indicating that the C-Tag loaded enough proteins into particles for use of high-dose proteins. Accordingly, we established a novel C-Tag-based transport system that has overcome the known technological difficulties of protein transmembrane delivery, and this might be a useful technology for drug development in the future.

Keywords protein drug, cell membrane, transmembrane transport, cholesterol tag, intraparticle delivery

1. The main difficulties in delivery of therapeutic protein drugs into the cytoplasm

Expression and regulation of proteins in living cells play a crucial role in biological processes, involving almost all forms of cellular physiology and disease progression (*1-4)*. In terms of diagnosis and treatment of some refractory diseases, a crucial task is to explore the cytosolic targets associated with cell necrocytosis, energy metabolism, and protein expression (*5*). During the development of a novel therapy, we sometimes need to deliver therapeutic proteins or drugs from extracellular fluid to the cytoplasm (*6*). However, cytosolic delivery of therapeutic proteins is usually hampered by cell membrane obstruction, endocytic sequestration, and lysosomal breakdown, which present notable barriers for approaching those intracellular targets (*7-9*). Hence, delivering therapeutic proteins/drugs into the cytoplasm is a challenge during the development of novel strategies to fight against these refractory diseases (*10,11*). Thus far, several physical technologies have been developed to address this problem. Approaches like direct microinjection of biologics into cytosols using a microinjector are plausible, but these approaches suffer from quite low throughput (*12*). High-throughput approaches, like squeezing and electroporation, have to punch transient pores at the cell membrane in order to establish temporary channels for material exchange between the extracellular and intracellular environments. However, these approaches suffer from uncontrollable material exchange, along with potential cell toxicity (*13,14*). Other intrinsic cellular mechanisms such as endocytosis have also been considered. Many chemical agents, such as liposomes, polymers, and cell-penetrating peptides (CPPs), are commonly delivered mostly into cells *via* endocytosis. Nevertheless, such endocytotic action is fundamentally regarded as a cellular self-defense mechanism, the basic function

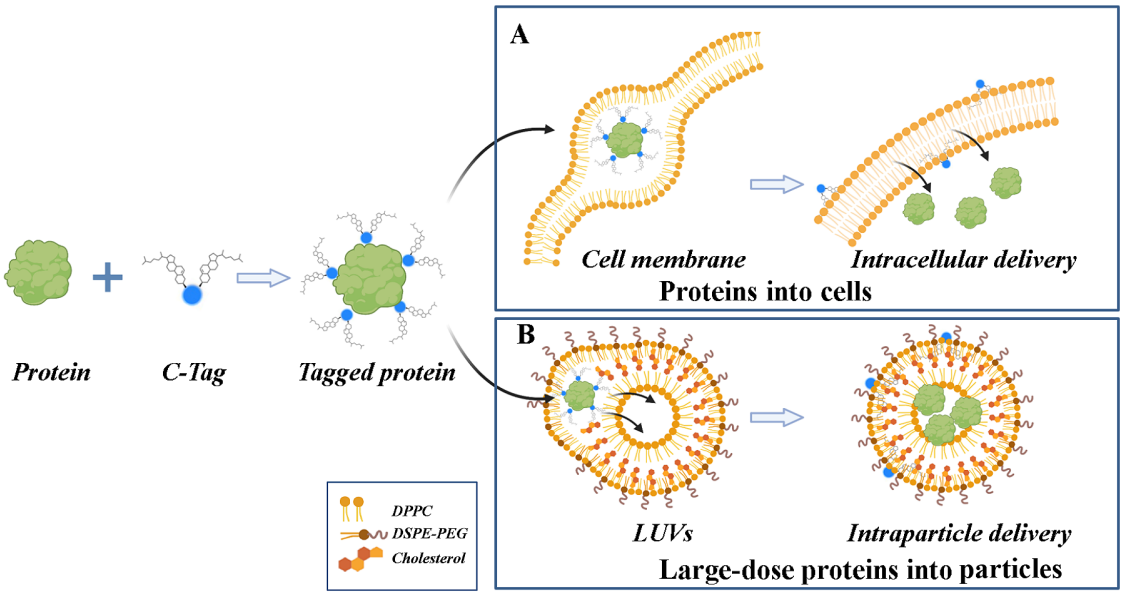

Figure 1. Schematic diagram of the novel C-Tag-based transmembrane transport system. The novel C-Tag-based transmembrane transport system addressed two technological difficulties: **(A)**. Transporting proteins into cells; **(B)**. Loading high-dose proteins into particles. The CB moiety of C-Tag non-covalently anchors onto the protein surface, and the cholesterol motif facilitates protein insertion into the hydrophobic lipid bilayers of cell membranes (or LUVs). Due to the noncovalent nature of C-Tag, the protein-tag complex embedded between the bilayers would separate, precluding the presence of the hydrophilic protein within the hydrophobic bilayer. Eventually, the proteins escape from the membrane and enter the cytoplasm (or LUV cavity), achieving protein transmembrane transport. Created with Biorender.com. CB: Coomassie blue; C-Tag: cholesterol tag; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPE-PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000], LUVs: large unilamellar vesicles.

of which is to prevent intact foreign biomolecules from entering the cytoplasm (*15,16)*. Thus, inducing such endocytotic action might not be able to maintain the biological activity of the delivered drugs. Thus, such "endocytotic action" should be avoided when developing novel delivery vehicles/systems/strategies to transport active therapeutic proteins into cytosolic targets because of their "inactivation". Lipid-based vehicles are the most common class of FDA-approved micro-nano particles, and they have a simple formulation and involve self-assembly, biocompatibility, and bioavailability (*15*). By incorporating a stimuli-response and cationic design, such lipid-based particles might also facilitate endosomal escape and fulfill potent protein internalization, which might be conveniently used in the scenario of disease treatment (*17*). Nevertheless, conventional lipid-based nanoparticles cannot sufficiently encapsulate cargo proteins due to uncontrollable particle assembly and an amphiphilic protein structure, hence limiting their further use in cytosolic protein delivery and personalized precision medicine (*18*). Accordingly, the aforementioned technologies have certain drawbacks and are far from satisfactory. Development of a novel delivery system is an urgent task for treating some refractory diseases (*15,19*), but the main technological difficulties lie in two points: *i*) The difficulty of achieving satisfactory intracellular (transmembrane) transport (the problem of "Transporting proteins into cells") and *ii*) The difficulty of filling the delivery vehicle with high-dose proteins (the problem of "Loading particles with high-dose proteins"). To address these two technological problems, we recently developed a novel cholesterol tag (C-Tag)-based transmembrane transport system (Figure 1).

2. Establishment of a novel C-Tag-based transmembrane transport system

A novel C-Tag-based transmembrane transport system was developed to achieve transmembrane delivery of protein drugs into cytoplasm with complete biological activity, along with ability to fill the delivery vehicle with high-dose proteins.

To overcome the first technological problem to achieve intracellular delivery, a cholesterol-based protein delivery tag was established by conjugating a Coomassie blue (CB) molecule with two copies of cholesterol (Figure 2A). Cholesterol is a natural component of eukaryote cell membranes, and CB can non-covalently bind to the protein surface. As shown as in Figure 1, the C-Tag can anchor onto the protein surface and facilitate permeation of the protein directly into cells without generating any transient pores or reducing drug activity (*19*). C-Tag therefore can serve as a promising tool to pull linked proteins into the cell bilayer and eventually transported proteins into the cytoplasm rather than *via* endocytosis (Figure 1A). Our previous study suggested that compact proteins are highly amenable to transmembrane delivery, whereas large proteins tend to enter cells *via* endocytosis (*19,20*). Hence, the compact Fab fragments (~55 kDa) of

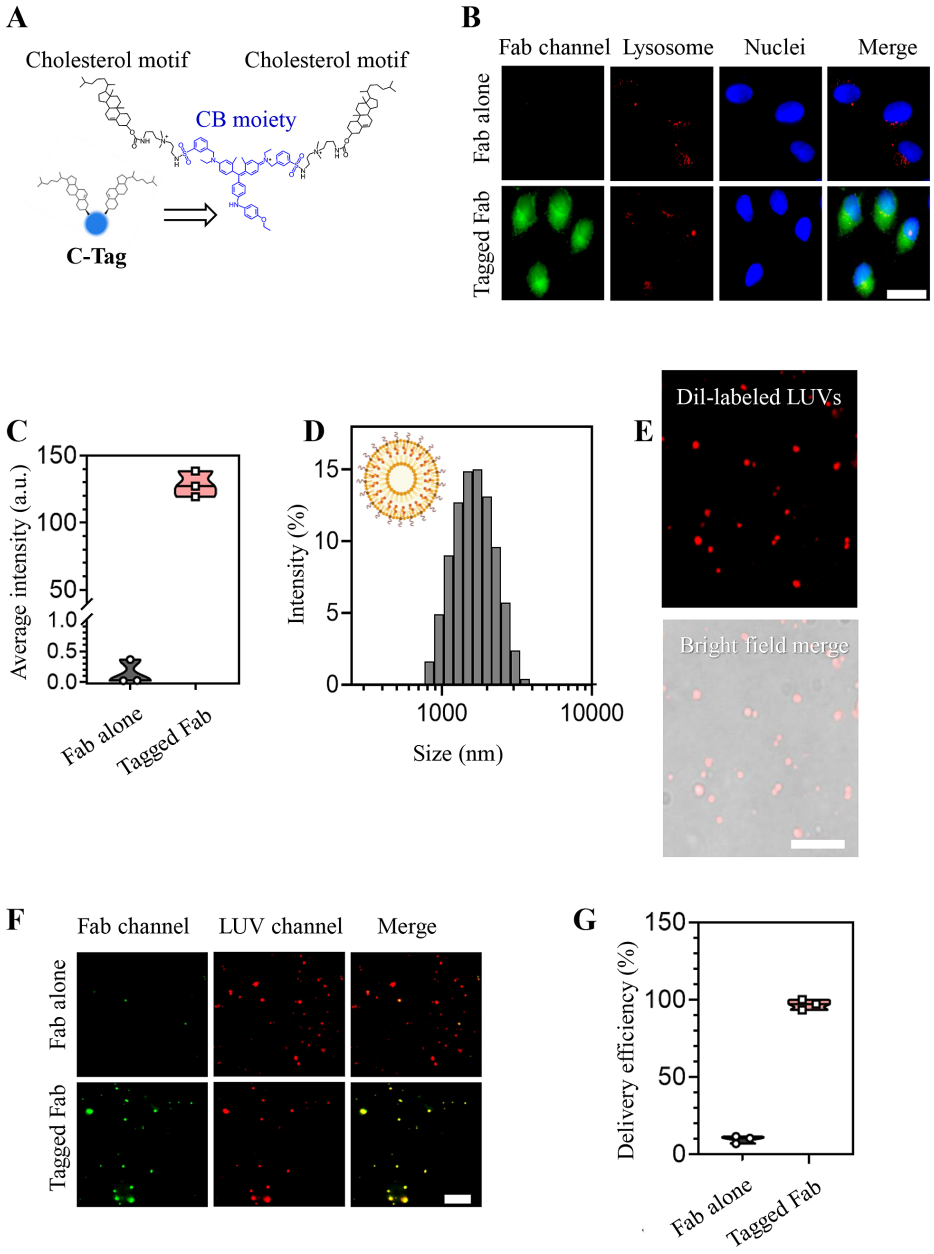

Figure 2. Verification of the C-Tag-based transmembrane transport system. (A). The structural formula of the noncovalent cholesterol tag (C-Tag). C-Tag consisted of a CB G250 and two copies of cholesterol molecules. **(B)** and **(C)**. Fluorescent images and quantitative analysis verified the efficiency of intracellular (transmembrane) transport of proteins in this system. HeLa cells (a human cervical carcinoma cell line) were used in experiments. Fab was labeled with Alexa Fluor488® (green), and lysosomes were stained with LysoTracker Red (red). Bar: 20 μm. **(D)**. Size and morphology of the micron-scale LUVs. **(E)**. The LUVs were stained with the lipophilic tracer Dil (red). Bar: 10 μm. **(F)** and **(G)**. Evaluating the efficiency of filling the LUVs with high-dose proteins. Fab was labeled with Alexa Fluor488® (green), and the LUVs were stained with Dil (red). Bar: 10 μm.

antibodies might be optimal candidates for the validation of C-Tag-mediated cytosolic delivery since Fab is known to engage in antigen recognition along with binding during the immune response. As shown in Figure 2B, the tagged Fab displayed bright and homogeneous intracellular fluorescence without apparent punctate bright spots or lysosome co-localization (manifestations of endocytosis, protein drugs may aggregate in the lysosome and be inactivated by enzymolysis). These results indicated a special pattern of Fab distribution within the cytoplasm *via* direct membrane permeation rather than endocytosis in the living cells. Importantly, the tagged Fab achieved a 902-fold increase in protein internalization (*vs*. Fab alone) and little Fab distribution in lysosomes, so endocytic sequestration and subsequent lysosomal hydrolysis were fundamentally avoided (Figure 2C). Thus, the C-Tag effectively avoids endocytosis and induces cytosolic delivery of proteins, thus providing a novel but useful pathway to achieve effective transmembrane transport of protein drugs while also, importantly, satisfactorily maintaining both the integrity and bioactivities of these protein drugs. In this

regard, C-Tag represents an advanced transmembrane technology to deliver protein drugs into cells for them to be efficacious by intervening in intracellular targets while avoiding endocytic sequestration.

The second technological problem is to achieve intraparticle delivery for high-dose proteins, and this can be addressed by designing a nano-particle that enables encapsulation of high-dose proteins to achieve satisfactory delivery efficiency and bioavailability (Figure 1B). Accordingly, micron-scale large unilamellar vesicles (LUVs) were prepared by thin film hydration followed by repeated extrusions. In brief, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG), and cholesterol were dissolved in a chloroform solution, and a thin film was formed using a rotary evaporator. The film was subsequently hydrated in a 4% ethanol aqueous solution and sonicated and repeatedly extruded with a mini extruder (pore diameter = 1.0 μm). The bilayer LUVs had a hydrodynamic size of 1.5 ± 0.2 μm (Figure 2D). Observed with fluorescent microscopy, the LUVs (stained with Dil) exhibited excellent spherical morphology, a uniform size, and uniform dispersion in the aqueous solution (Figure 2E), indicating that the LUVs might be a satisfactory candidate for protein drug encapsulation. To investigate whether LUVs can encapsulate proteins *via* C-Tag-mediated protein transportation, the efficiency with which a given amount of Fab was internalized into LUVs *via* cholesterol tagging was evaluated. As shown as in Figures 2F and G, Fab alone barely co-localized with LUVs, indicating poor internalization of Fab into the cavity of LUVs. However, C-tagged Fab greatly enhanced (above 10-fold) internalization of proteins into LUVs (Figures 2F and G). Thus, the intraparticle delivery of high-dose proteins was achieved.

3. Insights and future perspectives

Reported here is a novel C-Tag-based transmembrane transport system that has addressed two existing technological difficulties. When this noncovalent cholesterol tagging technique was used, preliminary data proved that this C-Tag-based transmembrane tool provided effective transport of protein drugs into both the cytoplasm and the LUVs (particularly for loading high-dose protein). To the extent known, this is the first lipid-based transmembrane tool that has addressed these two technological difficulties. *First*, this novel technology enables the transport of proteins directly into cytosols and it avoids endocytic sequestration. When C-Tag mixes with proteins, the CB moiety of C-Tag non-covalently anchors onto the protein surface and facilitates protein insertion into the lipid bilayers of cell membranes. This allows proteins to dissociate from the bilayers and enter the cytoplasm rather than *via* endocytosis (Figure 1A). Hence, this technology has three advantages in terms of transmembrane transport: *i*) avoiding cell endocytosis, *ii*) sustaining cell integrity (no transient poles are required), *iii*) importantly, maintaining the protein bioactivity. *Second*, LUVs were used to create particles to verify the efficiency with which C-Tag filled particles with high-dose proteins (Figure 1B). Thanks to the concept of C-Tag technology, all technological difficulties were overcome, and transmembrane transport even of high-dose proteins was achieved. Overcoming the barriers in the cellular membrane and loading enough protein drugs into nanoparticles is important. Thus, this technology might provide a novel and powerful platform for medicine design. Due to the "refractory" nature of some diseases, intervention in intracellular targets, and particularly cytosolic targets, might be an ultimate solution (*vs*. conventional treatments). In this context, the technology described in this study may kindle the flame of hope to change the clinical outcomes for these patients from "incurable" to "treatable".

The clinical use of this C-Tag-based transmembrane transport system is highly anticipated, but its safety, efficacy, and indications are still unknown. A future study will focus on further verification of this system *in vitro* and *in vivo*. Even though thus is a promising platform for designing novel lipid-based micro-nano medicines, exploring its indications is also crucial.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Tetsuya Asakawa and Mingbin Zheng, Institute of Neurology, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, 29 Bulan Road, Shenzhen, Guangdong 518112, China.
E-mail: asakawat1971@gmail.com (TA); mingbinzheng@126.com (MZ)

Hongzhou Lu, Department of Infectious Diseases, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, 29 Bulan Road, Shenzhen, Guangdong 518112, China.
E-mail: luhongzhou@fudan.edu.cn

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