Journal of Bioscience and Bioengineering
VOL. 120 No. 6, 630–636, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

Photosensitizer and polycationic peptide-labeled streptavidin as a nano-carrier for light-controlled protein transduction

Kosuke Minamihata,[1] Yasukazu Maeda,[1] Satoshi Yamaguchi,[1] Wataru Ishihara,[1] Akira Ishiwatari,[1] Satoshi Takamori,[1] Shinya Yamahira,[1] and Teruyuki Nagamune[1,2,*]

Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan[1] and Department of Bioengineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan[2]

Received 9 February 2015; accepted 2 April 2015
Available online 29 April 2015

Transductions of exogenous proteins into cells enable the precise study of the effect of the transduced proteins on cellular functions. Accordingly, the protein transduction technique, which can control the release of proteins into the cytosol with certainty and high-throughput, is highly desired in various research fields. In this study, streptavidin (SA) labeled with a photosensitizer and cell-permeable peptides (CPP) was proposed as a nano-carrier for light-controlled protein transduction. SA was modified with biotinylated oligo-arginine peptides (R_{pep}), which were functionalized with Alexa Fluor 546 (AF546), to achieve cell penetrating and endosomal escape functionalities. The SA-R_{pep} complex was efficiently internalized into living HeLa cells corresponding to the length and the modification number of R_{pep}. SA conjugated with more than three equimolar AF546-modified R_{pep} consisting of fifteen arginine residues was achieved to diffuse throughout the cytosol without cytotoxicity by irradiation of the excitation light for AF546. The optimized nano-carrier was confirmed to transduce a biotinylated model cargo protein, enhanced green fluorescent protein fused with thioredoxin (tEGFP) into the cytosol at the light-irradiated area. The results provided proof-of-principle that SA possessing multiple AF546-modified R_{pep} has the potential to be a versatile and facile carrier for light-controlled protein transduction into the cytosol of mammalian cells.

[**Key words:** Protein transduction; Photosensitizer; Cell-permeable peptides; Nano-carrier; Streptavidin]

Intracellular delivery of exogenous DNAs and proteins into target cells is a critical tool for modulating cellular functions in clinical (1) and laboratory research settings (2). Compared with protein expression through gene transfer, protein transduction is a more direct approach that avoids complicated transcriptional, translational, and post-translational processes from DNA to active protein (3). Accordingly, protein transduction can be a more rapid, easily controllable, and reliable method for modulating cellular functions. To effectively use this approach, mechanical direct protein transduction methods such as electroporation (4), sonoporation (5), and microinjection (6) are employed in applications. However, membrane poration methods often cause cell death, because some essential housekeeping molecules are released through the plasma membrane pores (7). Microinjection is the most direct method, and therefore, can most accurately control both the timing of transduction and the concentration of exogenous proteins. Nonetheless, although the device for microinjection has been improved, the extremely-low throughput of this method is a significant disadvantage in many therapeutic and research applications (8).

Cell-permeable carriers consisting of peptides (9), cationic polymers (10), liposomes (11), as well as other approaches (12,13), were actively studied as alternative methods because of their relatively-low cytotoxicity and high throughput. In particular, cell-permeable peptides (CPPs), such as oligo-arginine peptides (14) and TAT peptides (15) promote efficient protein transduction, and hence CPPs have been studied extensively over the past few decades (9,14–16). Because transduction using such cell-permeable carriers enter the endocytotic pathway (17), a release mechanism for the transduced proteins from endosomes is essential, otherwise these endosome located proteins are eventually degraded before functioning in the target cell. Therefore, cell-permeable carriers with an endosomal escape function have been developed (18). A major mechanism to facilitate endosomal release is the so-called proton sponge effect (19). For example, a proton-absorbing polymer, such as polyethyleneimine, absorbs protons in response to the low pH condition in endosomes, and induces proton flux into endosomes along with Cl^- ions and water molecules. This influx of molecules results in the rupture of the endosomes (19). However, in these approaches, the desired amount of target proteins often take several hours to be released into the cytosol, because of the gradual accumulation with functional carriers in the endosome and subsequent rupture. Accordingly, it is difficult to control the amount of the transduced protein in the cytosol at the desired time. Thus, the advantages of protein transduction over gene transduction are currently weakened because of the lack of a spatiotemporally controlled and high-throughput transduction method.

* Corresponding author at: Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. Tel.: +81 3 5841 7328; fax: +81 3 5452 5209.
E-mail address: nagamune@bio.t.u-tokyo.ac.jp (T. Nagamune).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.04.001

In endocytotic methods, an alternative way to achieve spatiotemporally-controlled endosomal release is to use photosensitizers that generate singlet oxygen upon light irradiation (20). In this approach, the generated singlet oxygen destabilizes the endosomal membrane by reacting with the membrane lipids and eventually leads to endosomal rupture. Photosensitizers used for generating singlet oxygen are fluorescent molecules such as fluorescein and Alexa Fluor 546 (AF546). There have been several reports in which proteins labeled with photosensitizers were first internalized into endosomes by using CPPs, and the subsequent light-induced excitation of the photosensitizer successfully mediated release of proteins from endosomes into cytosols (21,22). Furthermore, it was demonstrated that protein delivery into the cytosol was spatially controlled by limiting the irradiation area of light (21,22). However, the applications of CPPs and photosensitizer-mediated protein transduction are limited to only a few types of proteins, and the methods to modify the target protein with CPPs and photosensitizers are designed on a case-by-case basis. Therefore, it is important to construct a cell-permeable carrier equipped with photosensitizers as a versatile vehicle for spatiotemporally controlled protein delivery into the cytosol.

Here, we propose a streptavidin (SA)-based nano-carrier as a versatile vehicle for protein transduction. SA is a tetramer protein and each subunit can spontaneously bind to one biotin molecule with extremely high affinity: four biotinylated molecules are simultaneously modified on one SA molecule only by mixing in solution. Accordingly, light-controllable nano-carriers are simply formed by attaching photosensitizer-modified biotinylated CPPs to a few binding sites on SA, and furthermore, a biotinylated target protein is also conjugated with this carrier complex by using the remaining biotin binding site of SA. In this study, oligo-arginine peptides (hereafter abbreviated as R_{pep}), R9 and R15, were selected as CPPs, and AF546 was employed as a photosensitizer. N-terminal biotinylated R_{pep} was modified with an AF546, and subsequently conjugated to SA through biotin-SA binding. We initially evaluated the cell permeability, the cytotoxicity, and the light-induced endosomal escape function of the carrier complexes under various conditions, where the ratios of functionalized R_{pep} to SA and the length of R_{pep} were varied. Under the optimal conditions, a biotinylated target protein, a chimera of thioredoxin and an enhanced green fluorescent protein (EGFP) (hereafter abbreviated as tEGFP) were conjugated to SA together with the functionalized R_{pep}, and this SA-R_{pep}-tEGFP conjugate was then transduced into the cytosol of living cells by the CPPs and photosensitizer-mediated transduction system (Fig. 1).

MATERIALS AND METHODS

Materials and instruments Biotinylated oligo-arginine peptide, R9 (biotin-GGGYCGR_9), was synthesized by a conventional Fmoc solid-phase peptide synthesis method, and R15 (biotin-GGGR_{15}GYC) was purchased from Toray Research Center, Inc. (Tokyo, Japan). Streptavidin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Streptavidin labeled with Alexa Fluor 546 (AF546), AF546-C5-maleimide, and Opti-MEM I Reduced Serum Media were purchased from Life Technologies (Carlsbad, CA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). HeLa cells were provided by the Riken Cell Bank (Tsukuba, Japan). Flow cytometry analysis for quantification of cellular fluorescence intensity was measured by a FACSCalibur flow cytometer (Becton–Dickinson, Lexington, KY, USA). Fluorescence microscopic observations and light irradiation for photosensitizer-mediated endosomal release were conducted on a fluorescent microscope, IX81 (Olympus, Tokyo, Japan). Confocal laser scanning microscopy (CLSM) observations were carried out using a LSM510 (Carl Zeiss Co., Ltd., Jena, Germany).

Preparation of biotinylated-tEGFP The expression of a chimera protein of thioredoxin and EGFP (tEGFP) tagged with a peptide sequence of GGGC at its C-terminus was conducted in the *E. coli* Rosetta (DE3) pLysS strain. After transformation of the expression vector coding tEGFP (pET32b + EGFP-GGGC) and inoculating on LB agar medium containing chloramphenicol (Cm) (34 mg/L) and ampicillin (Amp) (100 mg/L), a single colony was selected and cultured in 5 mL of LB medium containing the same antibiotics. The cells were precultured O/N at 37°C with 220 rpm shaking and then added to 1 L of TB medium containing Cm and Amp as antibiotics. The main culture was grown at 37°C and 120 rpm and when the OD_{600} reached 0.7, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 1 mM to induce the expression of tEGFP. The culturing temperature was lowered to 16°C with shaking held at 120 rpm. The cells were grown O/N. The cells were pelleted by centrifugation at 6000 ×*g* for 20 min and kept frozen at −30°C until purification. The cells were dispersed with 100 mL of 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 1 mM dithiothreitol (DTT) and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and sonicated by 15 × (1 min sonication steps followed by 3 min cooling). The supernatant was obtained by centrifugation at 22,000 ×*g* for 30 min and injected onto four 5-mL HisTrap FF crude columns (GE Healthcare Life Sciences, Amersham, UK) equilibrated with HisTrap binding buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 20 mM imidazole). The four columns were washed with 5-column volumes (i.e., 1 column volume was 20 mL, therefore in total 100 mL was used) of the HisTrap binding buffer and proteins were eluted by a gradient of the HisTrap

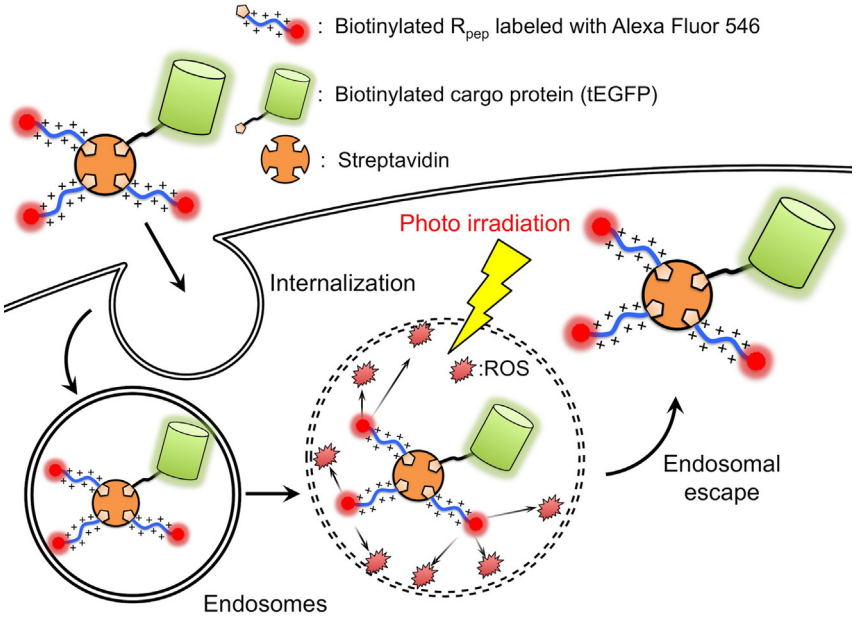

FIG. 1. Schematic illustration of protein transduction using the streptavidin based nano-carrier.