Efficient and High-Speed Transduction of an Antibody into Living Cells Using a Multifunctional Nanocarrier System to Control Intracellular Trafficking

YUMA YAMADA,[1] SANDRA MILENA VERGARA PEREZ,[1] MAI TABATA,[1] JIRO ABE,[1,2] YUKARI YASUZAKI,[1] HIDEYOSHI HARASHIMA[1]

[1]Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan
[2]Department of Pediatrics, Hokkaido University Hospital, Sapporo 060-8638, Japan

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ABSTRACT: The transduction of antibodies into living cells would represent a major contribution to both basic and applied biomedical fields, as currently available methods suffer from limitations such as low-uptake efficiency and endosomal entrapment. In this study, a liposome-based carrier was designed to overcome these issues. Liposomes were modified with octaarginine (R8), a cell penetrating peptide and GALA, a pH-sensitive fusogenic peptide. The presence of R8 enhanced the cellular uptake of antibodies, whereas GALA reduced endosomal entrapment, resulting in antibodies being released into the cytosol within 30 min. Moreover, compared with commercially available reagents for delivering antibodies, our system was superior in both cellular uptake and endosomal escape. In addition, specific antibodies delivered by R8-GALA liposomes were found to be associated with their epitope, confirming the preservation of functionality. This system for the efficient and high-speed cytosolic delivery of an antibody provides a valuable tool that can be useful in basic and applied research for analyzing the expression and function of intracellular molecules. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2845–2854, 2015

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INTRODUCTION

The high specificity of antibodies explains their extensive use in both basic and applied research for analyzing the expression and function of proteins. However, their use for studying intracellular phenomena in living cells requires a delivery system that can overcome the cell membrane barrier and release functional antibodies into the cytosol. To date, several attempts to transduce antibodies into intact cells have been reported, including mechanical approaches such as electroporation[1] and microinjection,[2] or the use of cellular uptake mediators such as cationic liposomes,[3,4] amphiphilic peptides,[5–7] polymeric carriers,[8,9] and recombinant viruses.[10] Nevertheless, these strategies remain problematic, and show low-transduction efficiencies, long periods for the effective dissociation and release of the antibody from the carrier, or time-consuming preparation methods.

Octaarginine (R8), a cell-penetrating peptide that is similar to other arginine-rich peptides such as HIV-1 TAT[11] and oligoarginines,[12] has been shown to be translocated across the cytoplasmic membrane. In addition, these arginine-rich peptides are able to mediate the membrane translocation of macromolecules such as peptides, proteins, and oligonucleotides.[13–15] In a previous study, R8 was incorporated into liposomes through a stearyl moiety that firmly anchors the cell-penetrating peptide to the liposomal surface while allowing the polyarginine component to interact with the cytoplasmic membrane.[16] The inclusion of R8 on the surface of the liposomes promoted the cellular uptake of carriers. Moreover, high contents of R8 resulted in stimulation of macropinocytosis compared with clathrin-mediated endocytosis promoted by low-density R8 modification.[16] So far, liposomes composed of fusogenic lipids and modified with R8 have been successfully used for the delivery of several macromolecules including pDNA,[17] oligoDNA,[18] siRNA,[19] the green fluorescence protein,[20] superoxide dismutase,[21] and DNase I protein.[22]

On the contrary, a common problem associated with the efficient cellular delivery of macromolecules using non-viral delivery systems is that the carriers accumulate in the endosomes/lysosomes.[23–25] Endosomal escape of the macromolecules is a critical step for obtaining the desired biological function. Therefore, the inclusion of an endosomal release device is useful in the design of delivery systems. GALA (WEAALAEALAEALAEHLAEALAEALEALAA) is a synthetic peptide that at neutral pH displays a random coil but under acidic conditions adopts an amphipathic α-helical structure.[26] At pH values lower than 6.0, GALA has a high affinity for neutral and negatively charged membranes, inducing fusion and fragmentation of model membranes via pore formation.[27,28] Therefore, the GALA peptide has been extensively used as an endosomal disrupting peptide in a number of delivery systems.[29,30] Previously, GALA was successfully integrated to liposomes via N-terminal modification with cholesteryl moiety.[31] The inclusion of GALA in the surface of liposomes promoted the destabilization of both the endosomal and liposomal membranes producing the cytosolic delivery of the encapsulated molecules. Moreover, the synergistic

Correspondence to: Hideyoshi Harashima (Telephone: +81-11-706-3919; Fax: +81-11-706-4879; E-mail: harasima@pharm.hokudai.ac.jp)

Yuma Yamada and Sandra Milena Vergara Perez contributed equally to this work.

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combination of R8 and GALA in liposomes was showed to be effective for siRNA delivery.[32]

In this study, we report on an alternate method for antibody delivery based on liposomes that contain functional devices that permit the major barriers to antibody delivery to be overcome. We prepared liposomes modified with R8, a cell-penetrating peptide, and GALA, a pH-sensitive fusogenic peptide. The cellular uptake of the antibody by the carrier was evaluated by flow cytometry. We also observed intracellular trafficking of the antibody using confocal laser scanning microscopy (CLSM). Finally, the R8-GALA liposomes were used to deliver specific antibodies in order to confirm the integrity of the antibodies released into the cytosol.

EXPERIMENTAL

Materials

Cholesteryl hemisuccinate (CHEMS), amiloride, and Fillipin III were purchased from Sigma (St. Louis, Missouri). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and rhodamine–DOPE were purchased from Avanti Polar Lipids Inc. (Alabaster, Alaska). Stearyl octaarginine[12,33] and cholesteryl–GALA[29,31] were obtained from Kurabo Industries Ltd. (Osaka, Japan). Goat IgG was purchased from Rockland (Gilbertsville, Pennsylvania). HeLa human cervix carcinoma cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle medium (D'MEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (Carlsbad, California). All other chemicals were commercially available reagent-grade products.

Preparation of Carriers

In a typical preparation, DOPE and CHEMS were dispersed in the bottom of a glass tube, with or without cholesteryl–GALA [DOPE/CHEMS/(+/−)cholesteryl–GALA, in a molar ratio of 9/2/0.22]. The solvent was removed by vacuum treatment and the lipid film was hydrated with a solution of Goat IgG or Goat $IgG^{Alexa488}$ at 0.125 mg/mL in 10 mM HEPES buffer (pH 7.4). $IgG^{Alexa488}$ was prepared with an AlexaFluor®488 Protein Labeling Kit (Invitrogen Corporation). After a 10-min incubation at room temperature, the lipid film was sonicated for 2 min in a bath-type sonicator (Aiwa, Tokyo, Japan) to give Lip(IgG) or GALA-Lip(IgG) (final composition 550 μM lipid, 0.125 mg/mL IgG). Subsequent modification with R8, to give R8-Lip(IgG) or R8-GALA-Lip(IgG), involved incubating liposomes in the presence of stearyl-R8, for 30 min at room temperature (11 μM final concentration). To prepare lipoplex-style carriers (R8-Lip—IgG and R8-GALA-Lip—IgG), the lipid film was hydrated with HEPES buffer, modified with stearyl-R8 and then incubated with an antibody solution for 15 min at room temperature, in a volume ratio of empty liposomes to antibody solution of 1:1 (final composition 275 μM lipid, 0.0625 mg/mL IgG). For the observation of the lipid phase of liposomes, 1% rhodamine-DOPE was introduced in the lipid film. The average diameter and the ζ-potential of liposomes were determined through dynamic light scattering and Laser Doppler Electrophoresis (Zetasizer Nano ZS ZEN3600; Malvern Instruments, Worcestershire, UK).

Cellular Uptake Assay

HeLa cells (2×10^5 cells) were seeded in six-well plates 24 h before the experiment. Cells were incubated for 1 h with carriers [27.5 μM lipid, 6.25 μg/mL IgG (15% $IgG^{Alexa488}$), D'MEM, FBS(−)], or 10, 15, 30, 45, 60, or 120 min in the experiment for cellular uptake as a function of incubation time. The cells were then washed twice with a heparin solution (20 U/mL) and trypsinized. After adding complete medium [D'MEM, FBS(+)], the cells were precipitated and the pellet suspended in phosphate-buffer saline [0.5% bovine serum albumin (BSA), 0.1% sodium azide]. The fluorescence of samples was registered in a FACScan instrument and analyzed with the CellQuest software (Becton Dickinson, Franklin Lakes, New Jersey). A total of 10,000 cells were analyzed in each sample.

To investigate the mechanism responsible for the cellular uptake of carriers, the cells were incubated with rhodamine-DOPE-labeled R8-GALA-Lip-IgG, R8-GALA liposomes and R8-liposomes. The cells were treated with inhibitors (5 mM amiloride, 0.5 M sucrose, or 1 μg/mL Fillipin III) for 30 min before adding the carriers to the cells. The relative cellular uptake when the cells were treated with inhibitors was calculated as follows:

Relative cellular uptake value (%) $= U_P/U_A \times 100$ where U_P and U_A represent the cellular uptake of the rhodamine-DOPE-labeled carriers when cells were treated with carriers in the presence and absence of inhibitors, respectively.

CLSM Observations

HeLa cells (5×10^4 cells) were seeded in 35-mm glass-bottom dishes 24 h before the experiment. Cells were incubated in the presence of carriers [13.75 μM lipid, 3.125 μg/mL $IgG^{Alexa488}$, D'MEM, FBS(−)] for 1 h. After washing with heparin (40 U/mL), complete medium [D'MEM, FBS(+)] was added and the solution incubated for 1 h. For the time course analysis of antibody delivery, the cells were incubated in the presence of carriers for 15, 30, 60, or 120 min. After washing (heparin 40 U/mL), complete medium was added and CLSM observations were performed immediately. Nuclei were stained with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). For the analysis of colocalization between antibodies and endosomes, acidic compartments were stained with LysoTracker Red DND-99 (Invitrogen Corporation). CLSM images were obtained using a Nikon A1 confocal imaging system (Nikon, Tokyo, Japan) equipped with the objective lens Plan Apo 60x/1.20 PFS WI and Plan Apo 20x/0.75 PFS dry, and a first dichroic mirror (405/488/561/640).

Comparison of R8-GALA Liposome with Commercially Available Reagents

Complexation of antibodies with the delivery reagents Chariot™ (Active Motif, Carlsbad, California) and Pro-Ject™ (Thermo Scientific, Waltham, Massachusetts) was carried out according to manufacturer's recommendations (see Supporting Information for details). The cellular uptake of antibodies was determined as described above with HeLa cells incubated in the presence of carriers corresponding to 2 μg of IgG (40% $IgG^{Alexa488}$). In the case of CLSM observations, cells were incubated with carriers corresponding to 3 μg/mL $IgG^{Alexa488}$ and treated as described above.

Delivery of Specific Antibodies

HeLa cells (5×10^4 cells) were seeded in 35-mm glass-bottom dishes (precoated with gelatin) 48 ± 4 h before the experiment. Cells were incubated in the presence of free antinuclear