Original Article

Generation of GFP Native Protein for Detection of Its Intracellular Uptake by Cell-Penetrating Peptides

(GFP / prokaryotic expression systems / cell-penetrating peptides / Pep-1 / CADY-2)

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Abstract. Different types of lipid- and polymer-based vectors have been developed to deliver proteins into cells, but these methods showed relatively poor efficiency. Recently, a group of short, highly basic peptides known as cell-penetrating peptides (CPPs) were used to carry polypeptides and proteins into cells. In this study, expression and purification of GFP protein was performed using the prokaryotic pET expression system. We used two amphipathic CPPs (Pep-1 and CADY-2) as a novel delivery system to transfer the GFP protein into cells. The morphological features of the CPP/GFP complexes were studied by scanning electron microscopy (SEM), Zetasizer, and SDS-PAGE. The efficiency of GFP transfection using Pep-1 and CADY-2 peptides and TurboFect reagent was compared with FITC-antibody protein control delivered by these transfection vehicles in the HEK-293T cell line. SEM data confirmed formation of discrete nanoparticles with a diameter of below 300 nm. Moreover, formation of the complexes was detected using SDS-PAGE as two individual bands, indicating non-covalent interaction. The size and homogeneity of Pep-1/GFP and CADY-2/GFP complexes were dependent on the ratio of peptide/cargo formulations, and responsible for their biological efficiency. The cells transfected by Pep-1/GFP and CADY-2/GFP complexes at a molar ratio of 20 : 1 demonstrated spreading green regions using fluorescent microscopy. Flow cytometry results showed that the transfection efficiency of Pep-based nanoparticles was similar to CADY-based nanoparticles and comparable with TurboFect-protein complexes. These data open an efficient way for future therapeutic purposes.

Introduction

The plasma membrane is a major barrier for penetration of proteins into the cells, and different delivery systems were thus developed for protein transfection with high efficiency without any cytotoxicity (Kurzawa et al., 2010). To date, several non-viral carriers have been improved including liposomes, polymers and cell-penetrating peptides (CPPs) for the delivery of biomacromolecules *in vitro* and *in vivo*. CPPs have successfully represented one of the most effective systems for delivering large proteins and peptides into cells with a therapeutic potential in a variety of viral diseases and cancers (Morris et al., 2008; Kurzawa et al., 2010; Koren and Torchilin, 2012; Liou et al., 2012; Bechara and Sagan, 2013; Farkhani et al., 2014; Wang et al., 2014). CPPs are short cationic peptide sequences derived from natural resources (e.g., partial sequences from transcription factors, bacterial or viral surface proteins, toxins, amphipathic helix-forming peptides) or synthetically designed in laboratory. They can be classified based on their structural properties (Morris et al., 2008; Kurzawa et al., 2010; Koren and Torchilin, 2012; Liou et al., 2012; Bechara and Sagan, 2013; Farkhani et al., 2014; Wang et al., 2014).

The efficient delivery of cargo into cells depends on specific factors including the cell line, passage number, temperature, exposure time, and concentrations of the cargo-CPP complex (Mussbach et al., 2011; Munyendo et al., 2012). The CPPs can link to cargo covalently (as a fusion or conjugate) or form a non-covalent complex with the cargo. The chemical linkage or conjugation methods are limited due to altering the biological activ-

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Abbreviations: bp – base pair, BSA – bovine serum albumin, CPP – cell-penetrating peptide, FCS – foetal calf serum, GFP – green fluorescent protein, HA2 – influenza virus haemagglutinin-2, IPTG – isopropyl-β-D-thiogalactopyranoside, LB – Luria-Bertani, PBS – phosphate-buffered saline, RFP – red fluorescent protein, SDS-PAGE – sodium dodecyl sulphate-polyacrylamide electrophoresis, SEM – scanning electron microscopy, TRITC – tetramethyl rhodamine iso-thiocyanate.

ity of the cargoes (Morris et al., 2008; Kurzawa et al., 2010; Koren and Torchilin, 2012; Liou et al., 2012; Bechara and Sagan, 2013; Farkhani et al., 2014; Wang et al., 2014). Thus, several studies suggested formation of a complex between the cargo molecules with suitable CPPs. It was observed that a 10- to 20-fold excess of CPP (based on the molar ratio) is necessary for peptide or protein transduction (Mussbach et al., 2011; Munyendo et al., 2012). Recently, the short primary or secondary amphipathic peptide carriers named PEP (e.g., Pep-1, Pep-2 and Pep-3) and CADY (e.g., CADY-1 and CADY-2) families, respectively, developed stable nanoparticles with cargoes without the need for chemical modifications or covalent linkage (Gros et al., 2006; Kurzawa et al., 2010). The Pep-1 peptide (Ac-KETWWETWWTEWSQPKKKRKV-Cy) was designed from three components including a hydrophobic N-terminal domain (a tryptophan-rich motif) for interaction with proteins and efficient targeting to the cell membrane, a hydrophilic C-terminal domain (KKKRKV) for solubility and intracellular delivery, and a short linker (SQP) for improvement of the flexibility and integrity of both domains (Gros et al., 2006; Kurzawa et al., 2010). Several modifications of Pep-1 sequences at the N-/C-terminal regions such as addition of acetyl (Ac) and cysteamide (Cy) groups have also been proposed to stabilize the cargo-carrier complexes and its transduction mechanism (Gros et al., 2006; Morris et al., 2008). CADY2 (Ac-GLWWRLWWRLRSWFRLWFRA-Cya) is also a highly hydrophobic and positively charged peptide with modified terminals (Kurzawa et al., 2010).

In the present study, we first generated green fluorescent protein (GFP) as a native protein in a bacterial system. GFP is a protein composed of 238 amino acid residues (~ 27 kDa) that exhibits bright green fluorescence when exposed to light and has been usually used as a reporter to follow the gene expression in transiently transfected mammalian cells (Subramanian et al., 1996). GFP is more sensitive than other reporter markers, requires no special cofactors for detection, and can be assessed with a spectrofluorimeter (Stretton et al., 1998). Then, we investigated the efficiency of the cell-penetrating peptides (Pep-1 and CADY-2) for delivering GFP into mammalian cells as compared to a commercial protein transfection reagent (TurboFect). TurboFect is a powerful tool for detecting the protein function in the cell, and plays an important role in the fields of cell biology and drug discovery (Oba and Tanaka, 2012). In addition, we compared the ability of Pep-1 and CADY-2 to interact with and deliver the GFP protein into HEK-293T cells. It should be noted that mammalian cells can strongly differ in their protein, proteoglycan and lipid composition of the membranes and in their signal pathways (Mussbach et al., 2011; Munyendo et al., 2012). Here, we used a certain adhesion cell line (HEK-293T) to compare the delivery efficiency of two CPPs. The transfection efficiency of CPPs was evaluated using fluorescent microscopy, flow cytometry, and western blot analysis. The estimation of internalized cargo amounts is a prerequisite for *in vivo* tests and therapeutic applications by other proteins.

Material and Methods

Plasmids and bacterial strains

E. coli DH5α was used as the primary host for the cloning procedures. *E. coli Rosetta* containing the T7 RNA polymerase gene under the control of the *lac* promoter, and pET−28a plasmid of 5369 base pairs (bp) controlled by the T7 promoter and the *lac* operator in *E. coli* were used as expression host and vector, respectively. The BL21 strain was cultured in Luria-Bertani (LB) medium supplemented with 50 μg/ml kanamycin.

Cell culture

Human HEK-293T cells were grown in complete RPMI-1640 medium (Sigma, Germany) supplemented with 10% heat-inactivated foetal calf serum (FCS, Gibco, Germany) at 37 °C in an atmosphere containing 5% CO_2.

Peptides

The PEP-1 (Ac-KETWWETWWTEWSQPKKKRKV-Cya) and CADY-2 (Ac-GLWWRLWWRLRSWFRLWFRA-Cya) peptides were purchased from Biomatik Corporation (Cambridge, Canada).

Preparation of pET expression vector harbouring the GFP gene

For generation of the GFP-expressing plasmid [pET-GFP], GFP was subcloned from the pEGFP-N3 eukaryotic expression vector (Clontech, Mountain View, CA) into the *Bam*HI/*Not*I cloning sites of pET-28a bacterial expression vector. The positive DH5α colonies harbouring pET-GFP were grown in LB broth medium supplemented with kanamycin and plasmid extraction was performed using a mini-kit (Qiagen, Hilden, Germany) according to a standard mini-prep protocol. The presence of the inserted GFP fragment was confirmed by restriction enzyme digestion as detected using gel electrophoresis and the fidelity of this construct (pET-GFP) was also confirmed by DNA sequencing.

Expression and purification of the recombinant GFP protein

The *E. coli Rosetta* strain was transformed with pET-GFP prepared. Then, a single colony was cultured in 5 ml LB medium containing kanamycin and incubated in a shaking incubator with constant agitation (~ 150 rpm). After 16–18 h, 500 μl of the cultured materials was removed and inoculated in 50 ml Ty2x medium. The culture was grown in an OD_{600} of 0.7–0.8 with vigorous shaking (~ 200 rpm) at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for GFP expression in *E. coli*. The incubation

period continued for another three hours at 37 °C with shaking at 200 rpm. Then, the cell pellet was harvested and analysed by 12% sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE). To visualize the protein bands, gel was stained with Coomassie brilliant blue. The expressed protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column under native conditions according to manufacturer's instructions (Qiagen).

Briefly, the cell pellet was solved in lysis buffer A (10 mM imidazole, pH 8) and placed in ice for 30 min and then sonicated for 10 min. The supernatant was recovered after centrifuging the disrupted cell suspension in 4000 × *g* for 20 min at 4 °C. The lysate was applied to the Ni-NTA column. Following three washes by buffer B (30 mM imidazole, pH 8), purified protein was eluted by 300 mM imidazole elution buffer (pH 8). The purified protein was dialysed with PBS (pH 7.2) at 4 °C overnight. The quality and quantity of purified recombinant GFP was determined by 12% SDS-PAGE gel electrophoresis and NanoDrop spectrophotometry, respectively.

Western blot analysis

For Western blot analysis, protein bands were separated in 12.5% (w/v) polyacrylamide gel and transferred to nitrocellulose membrane (Millipore, USA). The anti-GFP-HRP polyclonal antibody (Acris, USA, 1 : 5000 v/v) was used to confirm GFP protein expression under standard procedures. The immunoreactive protein bands were visualized using peroxidase substrate 3, 3'-diaminobenzidine (DAB, Sigma).

Preparation of carrier-cargo complexes

PEP-1/GFP and CADY/GFP complexes with molar ratios of 5 : 1, 10 : 1, 15 : 1 and 20 : 1 (1 μg GFP protein) were formed in 100 μl phosphate-buffered saline (PBS) and incubated for 30 min at room temperature. TurboFect (Fermentas, Germany) was used as a protein transfection reagent. TurboFect/GFP complexes were prepared according to manufacturer's instructions (Pro-Ject™ Reagent, Germany). Briefly, 2.5 μl of TurboFect protein transfection reagent was used for preparation of complexes containing 1.0 μg of GFP. On the other hand, 0.5 μg of FITC-antibody control protein diluted in 25 μl of PBS was delivered by TurboFect, Pep-1 and CADY-2. The presence of the complexes was confirmed by SDS-PAGE electrophoresis.

Physicochemical characterization

The size and zeta-potential of the peptide/protein complexes (PEP-1/GFP; CADY/GFP) at a molar ratio of 20 : 1 were measured by a Zetasizer Nano ZS instrument (Malvern Instruments, UK) at room temperature. In addition, the size and morphology of complexes were analysed at the same ratio with a scanning electron microscope (SEM; KYKY-EM3200 model, China).

Transfection assay

The HEK-293T cells were seeded onto 24-well culture plates (Greiner, Germany) at a density of 1×10^5 cells/well and incubated overnight in RPMI containing 10% FCS. After growth of the HEK-293T cells to 80 % confluency, the medium was replaced by serum-free medium and then 100 μl of each complex (i.e., Pep-1/GFP, and CADY/GFP at a molar ratio of 20 : 1; TurboFect/GFP; TurboFect/FITC; Pep-1/FITC; CADY-2/FITC) was applied to each well. After 1 h incubation at 37 °C, the cells were supplemented with fresh RPMI, 5% FCS in a total volume of 200 μl medium without removal of the PEP-1/GFP, CADY/GFP, Pep-1 FITC, and CADY-2/FITC overlay, and were further incubated for 3 h and 24 h. It should be noted that the cells were overlaid with 200 μl complete medium after 3 h incubation at 37 °C with TurboFect/GFP and TurboFect/FITC complexes in the medium without FCS. Then, the cells were treated with trypsin-EDTA, harvested by centrifugation and resuspended in PBS 1X. The level of GFP delivery or the percentage of transfected cells was monitored by fluorescence microscopy (Envert Fluorescent Ceti, Korea) and also quantified by a FACS Calibur flow cytometer (Partec, Germany). For each individual sample, 10,000 cells were counted.

Statistical analysis

Statistical analysis (Student's *t*-test) was performed by Prism 5.0 software (GraphPad, San Diego, CA) to analyse the percentage of PEP-1/GFP and CADY/GFP transfection using flow cytometry. The value of $P < 0.05$ was considered statistically significant. Similar results were obtained in two independent experiments.

Results

Expression and purification of the recombinant GFP protein

The *GFP* gene was subcloned into the expression vector pET28a, which enables expression of a fusion protein with a 6xHis-tag at the N-terminus. The *GFP* gene migrated as a band of ~ 720 bp in agarose gel, using PCR and enzyme digestion with *Bam*HI/*Not*I (Fig. 1). Its accuracy was confirmed by sequencing. The recombinant GFP protein was expressed and purified in the *Rosetta* strain. GFP migrated as a clear band of ~ 27 kDa protein in SDS-PAGE that was detectable using anti-GFP antibody in Western blot analysis. Purification of GFP was done under native conditions using affinity chromatography as shown in Fig. 2. It was interesting that the GFP protein bound to Ni-NTA beads completely, because there is no GFP band in the flow-through or crude protein (Fig. 2). In addition, the protein was eluted completely from Ni-NTA beads using 300 mM imidazole buffer.

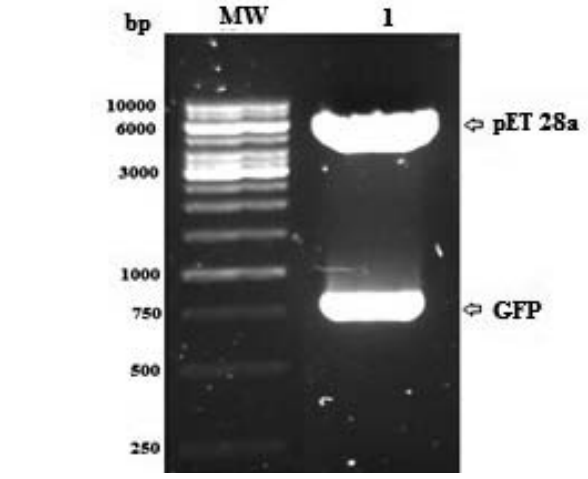

Fig. 1. Confirmation of the *GFP* gene cloned in the pET28a vector by restriction enzymes; MW is molecular weight marker (DNA ladder, 1 kb, Fermentas)

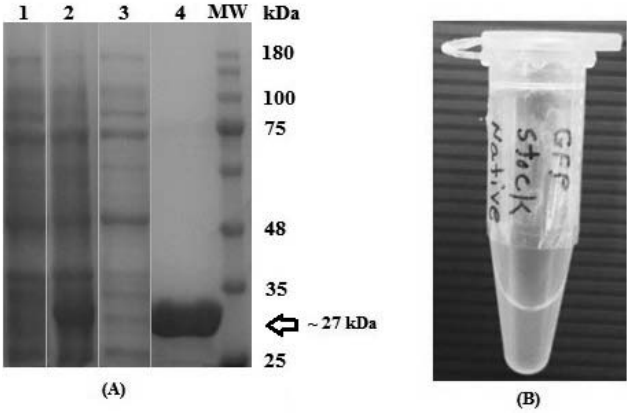

Fig. 2. **A**) Expression and purification of GFP protein in *E. coli* expression system. Lane **1**: before IPTG induction, lane **2**: after IPTG induction, lane **3**: flow-through (crude), lane **4**: GFP protein purified by affinity chromatography under native conditions. **MW** is molecular weight marker (prestained protein ladder, 10-170 kDa, Fermentas). **B**) The purified GFP solution

Confirmation of carrier-cargo complexes

The formation of carrier-cargo complexes (Pep-1/GFP or CADY/GFP) was confirmed by SDS-PAGE. As observed in Fig. 3A, chemical dissociation was detected as a dominant band of ~ 27 kDa related to the GFP protein along with the Pep-1 or CADY-2 peptide band in SDS-PAGE indicating formation of complexes over a range of molar ratios (5 : 1, 10 : 1, 15 : 1, and 20 : 1). In addition, the size and morphology of nanoparticles were analysed by SEM as shown in Fig. 3B. These complexes formed particles with an average diameter of 150–300 nm for Pep-1/GFP or CADY-2/GFP complexes. These data are in agreement with the particle hydrodynamic diameter and zeta potential analysis of Pep-1/GFP or CADY-2/GFP complexes at a molar ratio of 20 : 1 with dynamic light scattering.

Detection of GFP delivery by fluorescent microscopy and flow cytometry

The efficiency of GFP delivery in the HEK-293 T-cell line was investigated by CADY-2 and Pep-1 at a molar ratio of 20 : 1 for 4 and 24 h after transfection. The results were compared with the delivery efficiency of TurboFect transfection reagent. Incubation with GFP (control) showed no fluorescence at 4 and 24 h after transfecting cells. The transfection efficiency of the GFP and FITC-antibody control into the cells was indicated using fluorescent microscopy and flow cytometry in Fig. 4. The GFP delivery was detected in approximately 49.65 %, 57.58 %, and 58.70 % of HEK-293T cells treated with TurboFect, CADY-2 and Pep-1 at a molar ratio of 20 : 1, respectively. The cellular uptake of the FITC-antibody into the cells was 87.38 %, 78.27 %, and 83.04 % using TurboFect, CADY-2 and Pep-1, respectively. The cells treated with FITC-antibody without vehicle showed no fluorescence in this experiment. As observed in Fig. 4, the transfection efficiency of the Pep-based nanoparticles was similar to the CADY-based nanoparticles in the human cell line and comparable with TurboFect-protein complexes ($P > 0.05$). In addition, intracellular distribution of GFP and FITC-antibody

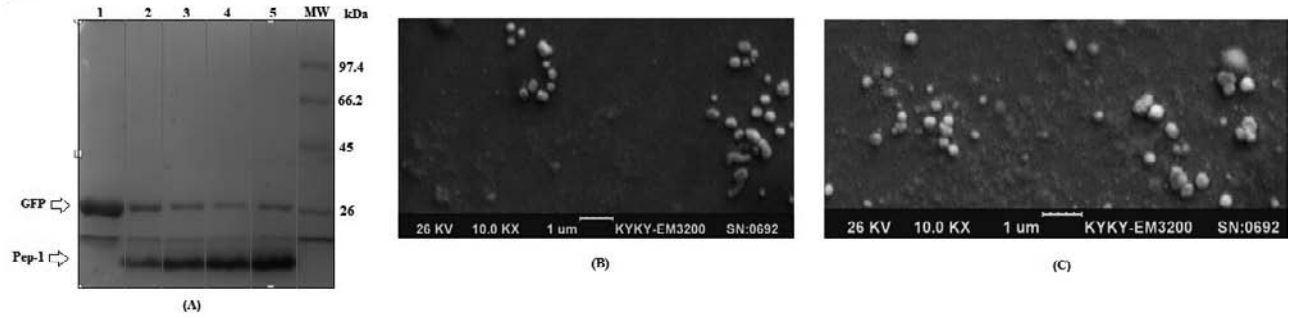

Fig. 3. **A**) Analysis of Pep-1/GFP complexes at different molar ratios. Lane **1:** purified GFP protein as a control, lane **2**: 5 : 1, lane **3**: 10 : 1, lane **4**: 15 : 1, lane **5**: 20 : 1. **B**) SEM electron microscopy of Pep-1/GFP complex. **C**) SEM electron microscopy of CADY-2/GFP complex. SEM micrograph of spherical nanoparticles formed at a molar ratio of 20 : 1 at 10,000 × magnification.

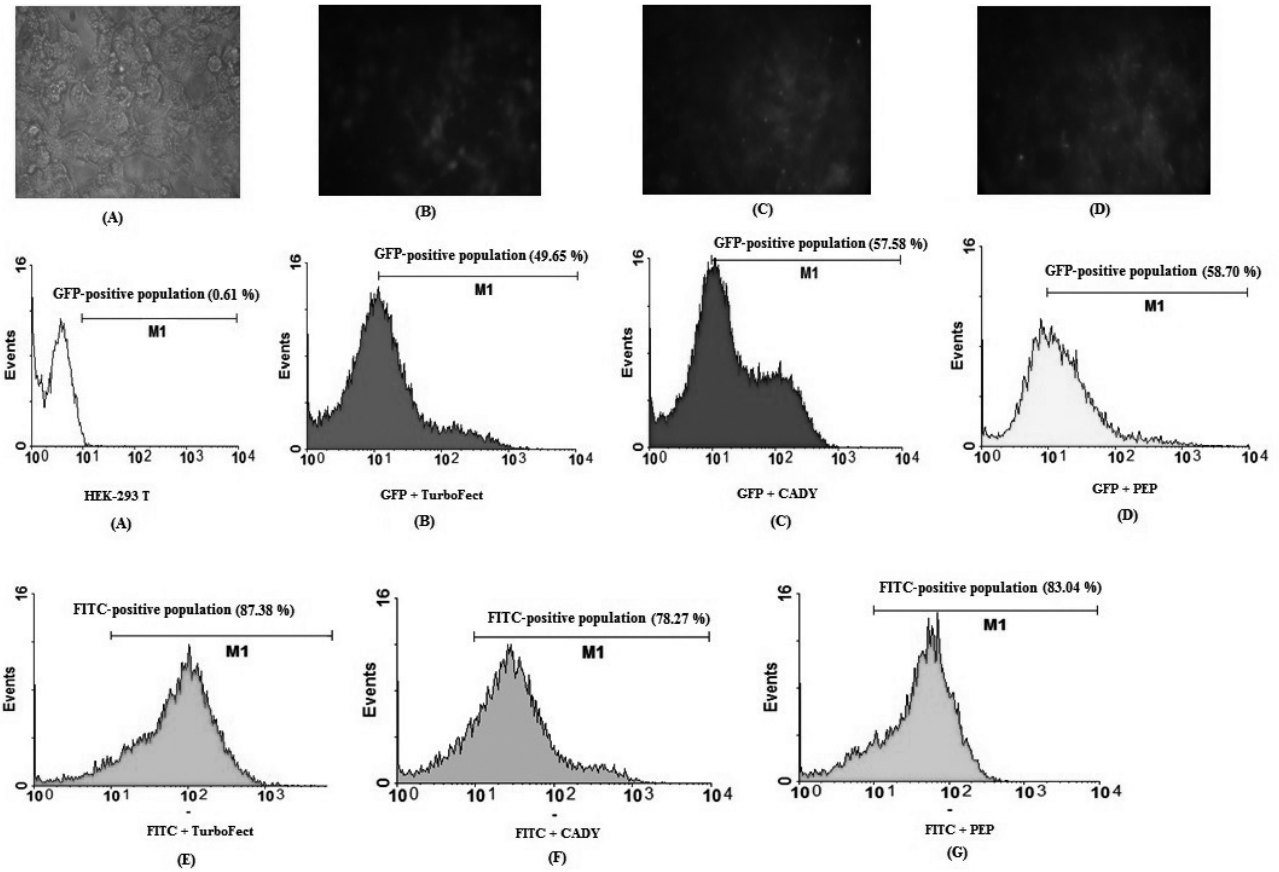

Fig. 4. Transfection efficiency of GFP using TurboFect (**B**), CADY-2 (**C**), and Pep-1 (**D**) at a molar ratio of 20 : 1 in HEK-293 T cells after 4 h. GFP delivery was monitored with epifluorescent microscopy and flow cytometry (**B-D**) as compared to the negative control (**A**).
Intracellular distribution of FITC-antibody complexes with TurboFect, CADY-2, and Pep-1 (**E-G**) was confirmed by flow cytometry (**E-G)** as a protein control.

was stable at 24 h post-transfection (data not shown). The cells transfected by the complexes showed spreading green regions as represented by fluorescent microscopy. In addition, our results indicated that both CPPs deliver FITC-antibody significantly more highly than GFP, indicating a different nature of the cargo-related cell uptake ($P < 0.05$).

Discussion

Several CPPs have been proposed for delivery of polypeptides and proteins into a wide variety of cells both *in vitro* and *in vivo* through either of two strategies: covalent, or complexed in a non-covalent approach. The studies showed that the delivery of therapeutic proteins (e.g., β-galactosidase: 120 kDa) into tissues and across the blood-brain barrier was restricted by the size and biochemical properties of the proteins. Intraperitoneal injection of the β-galactosidase protein fused to the HIV-1 Tat CPP indicated effective delivery of the fusion protein to the brain in mice (Munyendo et al., 2012). In addition, the researchers showed that among 11 different CPPs fused to the C-terminal of EGFP, Tat showed a potent ability to deliver the EGFP protein in macrophage J774 A.1 cells (Ma et al., 2014). On the other hand, a CPP tagged with an endosomolytic fusion peptide derived from the influenza virus haemagglutinin-2 (HA2) could significantly enhance the cytosolic delivery of red fluorescent proteins (RFPs) in human A549 cells without causing cytotoxicity (Liou et al., 2012). Other studies showed major differences in the uptake efficiency and cytotoxic effects using six different CPPs and six different adhesion and suspension cell lines (Mussbach et al., 2011). Most of the CPPs have been shown to be non-toxic. The mechanism of uptake of the CPP-cargo complexes across the plasma membrane remains unclear. It has been reported that both small and large proteins such as GFP (~ 27 kDa) and IgG (~ 150 kDa) have been efficiently delivered by the Tat peptide (Chugh et al., 2010). Several studies indicated that the delivery of proteins into cells by co-incubation with CPPs has several advantages such as deletion of the chemical coupling between the CPP and the cargo molecule (synthesis and purification procedures), and enhancement of the flexibility in CPP-cargo delivery. For example, fluoresceinyl YTA2 peptide was capable of delivering β-galactosidase and tetramethyl rhodamine iso-thiocyanate (TRITC)-labelled streptavidin into the Bowes cells by co-incubation (Myrberg et al., 2007).

In the present study, we used two CPPs known as Pep-1 and CADY-2, which allow formation of non-covalent complexes with the GFP protein due to the relatively easy handling procedure. The expression and purification of GFP protein was performed using the prokaryotic pET expression system. The *GFP* gene was cloned into the pET-28a vector and transformed into *Rosetta E. coli*. After induction of protein expression by 1 mM IPTG for 3 h, the His-tag-containing GFP protein was purified by affinity chromatography using Ni-NTA columns under native conditions. It is an attractive strategy to increase cellular internalization of an exogenous protein produced by a bacterial vector. The Pep-1/GFP and CADY/GFP complexes were formed at different molar ratios (5 : 1, 10 : 1, 15 : 1, 20 : 1) as observed in SDS-PAGE. We examined the size of Pep-1/GFP and CADY-2/GFP complexes at a molar ratio of 20 : 1 in water by light scattering and found that both complexes formed particles with a diameter of 150–300 nm. In order to further characterize the features of Pep-1/GFP and CADY-2/GFP particles, both complexes at a 20 : 1 molar ratio in water were spotted onto an intermetallic substrate and examined by SEM. Both Pep-1 and CADY-2 formed stable peptide-based nanostructures around GFP proteins with a symmetrical size distribution. In contrast, Pep-1 or CADY-2 alone did not form any particles.

Other studies also showed that the Pep-1/cargo interactions include the aromatic residues of the hydrophobic domain and also the helical structural organization of the Pep-1 carrier (Morris et al., 2008). In addition, both the size and homogeneity of the nanoparticles depend on the carrier/cargo ratio (Morris et al., 2008; Kurzawa et al., 2010). Indeed, an optimal delivery efficiency *in vivo* has been reported for a carrier/cargo ratio of ~ 10 : 1 to 20 : 1, dependent on the nature of the cargo. The use of greater ratios induces formation of larger particles due to aggregation and precipitation that hardly enter cells (Morris et al., 2008). In the next step of this study, the nanoparticles were overlaid onto cultured HEK 293T cells to evaluate their transduction efficiency. The protein uptake was estimated qualitatively by fluorescence microscopy and quantitatively by flow cytometry. Here, we compared the potency of Pep-1 and CADY-2 to interact with and internalize the GFP protein into HEK-293T cultured cells and provided evidence that CADY-2 can interact with GFP and deliver it efficiently into the cells similarly to the Pep-1 carrier. Furthermore, the FITC-antibody was delivered significantly more highly than GFP using Pep-1 and CADY-2 into the cells. This finding shows the correlation of physicochemical properties (e.g., size, charge) of cargoes with their cellular uptake by non-covalent CPPs.

The efficient carrier/cargo binding associated with the nature of the cargo will be important for the design of biomolecules for diagnostic or therapeutic purposes. One study has presented high affinity of CADY-2 and Pep-1 peptides for transferring two proteins (mRFP and GST-Cdk2) into mammalian cells (Kurzawa et al., 2010). Other studies have shown that the optimal Pep-1/cargo molar ratio for Pep-1-mediated delivery of p27Kip tumour suppressor (Pep-1/p27Kip complexes) into cells is 20 : 1, a ratio at which the cargo delivery is independent of the endosomal pathway, unlike delivery by most other CPPs (Munoz-Morris et al., 2007). In addition, Pep-1/protein complexes were delivered into the lungs of mice to generate alveolar wall apoptosis or to correct defects in the protein kinase A function (Heitz et al., 2009). However, few studies were conducted for assessment of Pep-1 and especially CADY-2 as a protein delivery system. On the other hand, three commercial protein transfection reagents including lipid-based ProDeliverIN, CPP-based Xfect, and cationic polymer-based TurboFect represented promising tools for delivering bovine serum albumin (BSA) in HeLa cells (Oba and Tanaka, 2012). These reagents can be used for *in vitro* experiments as controls. These products form non-covalent complexes with proteins, and can often be used in serum-free medium. Among them, TurboFect showed the highest protein transfection efficiency into the cells. As reported, the addition of serum to the cell culture medium significantly decreased the level of cellular protein uptake by TurboFect to less than 50 %. There is no cytotoxicity against HeLa cells using these reagents, indicating low cellular toxicity of each reagent (Oba and Tanaka, 2012).

In general, the data obtained from SEM experiments strongly suggest globular nanoparticle formation of Pep-1/GFP or CADY-2/GFP complexes for therapeutic purposes. Our results confirmed that both Pep1 and CADY2 are suitable carriers for GFP and can equally act to deliver GFP into HEK-293T cultured cells. This study will have important implications for evaluation of the efficiency of Pep-1/GFP and CADY-2/GFP complexes *in vivo*, e.g., their internalization into cells.

References

Bechara, C., Sagan, S. (2013) Cell-penetrating peptides: 20 years later, where do we stand? *FEBS Lett* **587,** 1693-1702.

Chugh, A., Eudes, F., Shim, Y. S. (2010) Cell-penetrating peptides: nanocarrier for macromolecule delivery in living cells. *IUBMB Life* **62,** 183-193.

Farkhani, S. M., Valizadeh, A., Karami, H., Mohammadi, S., Sohrabi, N., Badrzadeh, F. (2014) Cell penetrating peptides: efficient vectors for delivery of nanoparticles, nanocarriers, therapeutic and diagnostic molecules. *Peptides* **57,** 78-94.

Gros, E., Deshayes, S., Morris, M. C., Aldrian-Herrada, G., Depollier, J., Heitz, F., Divita G. (2006) A non-covalent peptide-based strategy for protein and peptide nucleic acid transduction. *Biochim. Biophys. Acta* **1758,** 384-393.

Heitz, F., Morris, M. C., Divita, G. (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br. J. Pharmacol.* **157,** 195-206.

Koren, E., Torchilin, V. P. (2012) Cell-penetrating peptides: breaking through to the other side. *Trends Mol. Med.* **18,** 385-393.

Kurzawa, L., Pellerano, M., Morris, M. C. (2010) PEP and CADY-mediated delivery of fluorescent peptides and proteins into living cells. *Biochim. Biophys. Acta* **1798,** 2274-2285.

Liou, J. S., Liu, B. R., Martin, A. L., Huang, Y. W., Chiang, H. J., Lee, H. J. (2012) Protein transduction in human cells is enhanced by cell-penetrating peptides fused with an endosomolytic HA2 sequence. *Peptides* **37,** 273-284.

Ma, J., Xu, J., Guan, L., Hu, T., Liu, Q., Xiao, J., Zhang, Y. (2014) Cell-penetrating peptides mediated protein cross-membrane delivery and its use in bacterial vector vaccine. *Fish Shellfish Immunol.* 39, 8-16.

Morris, M. C., Deshayes, S., Heitz, F., Divita, G. (2008) Cell-penetrating peptides: from molecular mechanisms to therapeutics. *Biol. Cell.* **100,** 201-217.

Munoz-Morris, M. A., Heitz, F., Divita, G., Morris, M. C. (2007) The peptide carrier Pep-1 forms biologically efficient nanoparticle complexes. *Biochem. Biophys. Res. Commun.* **355,** 877-882.

Munyendo, W. L., Lv, H., Benza-Ingoula, H., Baraza, L. D., Zhou J. (2012) Cell penetrating peptides in the delivery of biopharmaceuticals. *Biomolecules* **2**, 187-202.

Mussbach, F., Franke, M., Zoch, A., Schaefer, B., Reissmann, S. (2011) Transduction of peptides and proteins into live cells by cell penetrating peptides. *J. Cell. Biochem.* **112,** 3824-3833.

Myrberg, H., Lindgren, M., Langel, U. (2007) Protein delivery by the cell-penetrating peptide YTA2. *Bioconjug. Chem.* **18,** 170-174.

Oba, M., Tanaka, M. (2012) Intracellular internalization mechanism of protein transfection reagents. *Biol. Pharm. Bull.* **35,** 1064-1068.

Stretton, S., Techkarnjanaruk, S., Mclennan, A. M., Goodman, A. E. (1998) Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. *Appl. Environ. Microbiol.* **64,** 2554-2559.

Subramanian, S., Srienc, F. (1996) Quantitative analysis of transient gene expression in mammalian cells using the green fluorescent protein. *J. Biotechnol*. **49,** 137-151.

Wang, F., Wang, Y., Zhang, X., Zhang, W., Guo, S., Jin, F. (2014) Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery. *J. Control. Release* **174,** 126-136.