Delivery of Nucleic Acids, Proteins, and Nanoparticles by Arginine-Rich Cell-Penetrating Peptides in Rotifers

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Abstract Cell-penetrating peptides (CPPs) are a group of short, membrane-permeable cationic peptides that represent a nonviral technology for delivering nanomaterials and macromolecules into live cells. In this study, two arginine-rich CPPs, HR9 and IR9, were found to be capable of entering rotifers. CPPs were able to efficiently deliver noncovalently associated with cargoes, including plasmid DNAs, red fluorescent proteins (RFPs), and semiconductor quantum dots, into rotifers. The functional reporter gene assay demonstrated that HR9-delivered plasmid DNAs containing the enhanced green fluorescent protein and *RFP* coding sequences could be actively expressed in rotifers. The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan assay further confirmed that CPP-mediated cargo delivery was not toxic to rotifers. Thus, these two CPPs hold a great potential for the delivery of exogenous genes, proteins, and nanoparticles in rotifers.

Abbreviations

CPPs	Cell-penetrating peptides
Cy3	Cyanine 3
DMSO	Dimethyl sulfoxide
dsRNA	Double-stranded RNA
EGFP	Enhanced green fluorescent protein
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HA2	Hemagglutinin-2
6His	Hexa-histidine
N/P	Nitrogen/phosphate
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
QDs	Quantum dots
R9	Nona-arginine
RFP	Red fluorescent protein
siRNA	Small interfering RNA

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Introduction

The efficient delivery of genetic material is a key challenge in transgenic biotechnology (Gama Sosa et al. 2010; Piedrahita and Olby 2011). Multiple gene delivery techniques have been developed. Physical insult (electroporation, microinjection, and particle bombardment), viral infection, and lipid fusion have been employed to overcome the impermeable cell membrane barrier that limits internalization of functional macromolecules (such as DNAs, RNAs, and proteins) (Jo and Tabata 2008). However, cell injury, cytotoxicity, immunogenicity, and low transgenic efficiency remain common problems in transgenesis (Jo and Tabata 2008). Thus, a highly efficient and biocompatible delivery system has been a critical pursuit for transgenesis, protein therapy, and delivery of biomarkers.

Nontoxic cell-penetrating peptides (CPPs) represent a nonviral technology capable of delivering a wide spectrum of biological macromolecules (such as DNAs, RNAs, and proteins) and nanomaterials into living cells (Gump and Dowdy 2007; Liu et al. 2011). CPPs (also known as protein

transduction domains) comprise a group of short, membrane-permeable cationic peptides derived from a number of natural proteins (Frankel and Pabo 1988; Green and Loewenstein 1988; Gump and Dowdy 2007). The process by which CPPs cross the cell membrane and deliver macromolecular cargoes is referred to as protein transduction (Wadia and Dowdy 2002; Gump and Dowdy 2007). Recently, we demonstrated that CPPs covalently conjugated with proteins (denoted as CPP–protein complexes) can enter animal and plant cells (Chang et al. 2005a; Liu et al. 2007; Li et al. 2010; Liou et al. 2012). Moreover, CPPs noncovalently conjugated with proteins (Wang et al. 2006; Chang et al. 2007; Hou et al. 2007; Liu et al. 2008; Hu et al. 2009; Lu et al. 2010; Liu et al. 2013a), DNAs (Chen et al. 2007; Lee et al. 2011; Dai et al. 2011; Chen et al. 2012; Liu et al. 2012, 2013b), small interfering RNAs (siRNAs) (Wang et al. 2007), or nanoparticles (Liu et al. 2010a, 2010b, 2011; Xu et al. 2010; Liu et al. 2011) (denoted as CPP/cargo complexes) are also efficiently delivered into cells.

The choice of a particular model organism or a gene delivery system depends on the purposes of the research. An ideal model organism would embody a low cost of cultivation, an ease of physical manipulation, and have available a plethora of genetic and molecular tools (Swanson et al. 2004). Rotifers are tiny zooplanktons (approximately<0.3 mm in length) that inhabit water (Oo et al. 2010; Dahms et al. 2011). They can reproduce sexually or asexually (parthenogenesis) and are key creatures at the base of aquatic food webs. Rotifers are one of the largest micro-invertebrate phyla in terms of biomass, number of species, and ecological importance (Snell and Hicks 2011). In some invertebrates, transfection of double-stranded RNA (dsRNA) can be achieved through ingestion by simply soaking the animals in dsRNA (Hannon 2002). SiRNA or dsRNA has been successfully transfected into rotifers by soaking, lipofection, and electroporation (Shearer and Snell 2007; Snell et al. 2009, 2011).

There have been a limited number of studies of gene delivery in invertebrates, including nematodes (*Caenorhabditis elegans*, May and Plasterk 2005), rotifers (Shearer and Snell 2007; Snell et al. 2011), and sponges (*Porifera*, Pfannkuchen and Brummer 2009). There have been no reports of CPP-mediated biomolecule delivery in rotifers. Development of CPP-mediated transfection and protein transduction tools for rotifers would open a new avenue of research in invertebrate research.

In this study, we adapted a novel CPP technology for delivery of DNAs, proteins, and quantum dots (QDs; colloidal semiconductor nanoparticles) into rotifers. The use of nanoparticles reflects recent interest in exploiting their unique optical properties for imaging and diagnosis procedures in vitro and in vivo (Liu et al. 2010b). Two arginine-rich CPPs, HR9 peptide previously employed by our group (Dai et al. 2011; Liu et al. 2011; Chen et al. 2012; Liu et al. 2012) and a novel IR9 peptide (manuscript submitted), were used. IR9 peptide is composed of nona-arginine (R9) and INF7, a glutamic acid-enriched hemagglutinin-2 (HA2) analog. INF7 has been identified as a potent endosome membrane-destabilizing peptide (Plank et al. 1994). We found that the fusogenic HA2 (INF7) peptide dramatically facilitates CPP-mediated protein entry as revealed by the release of endocytosed red fluorescent proteins (RFPs) into the cytoplasm (Liou et al. 2012).

Materials and Methods

Culture of Rotifers

Rotifers (*Brachionus calyciflorus*) (Bioprojects International Co., Kaohsiung, Taiwan) were cultured in freshwater supplemented with the Fresh Chlorella V-12 (Bioprojects). The culture system was air-pumped at a rate of 0.1–0.3 L/min according to the manufacturer's instructions. Rotifers were seeded at a density of 1×10^5 in each well of 24-well plates and incubated in a shaker incubator at 25–28 °C.

Plasmid, Peptide, and Protein Preparation

The mCherry plasmid has a coding sequence of hexa-histidine (6His)-tagged monomeric *RFPs* under the control of the T7 promoter (Shaner et al. 2004). The pR9-mCherry plasmid contains the coding sequences of 6His- and R9-tagged *RFPs* under the control of the T7 promoter (Lu et al. 2010). The pBlueScript-SK+plasmid is an empty vector (Agilent Technologies, Santa Clara, CA, USA). The pCS2+ EGFP and pCS2+ RFP (including pCS2+ DsRed and pCS2+ mCherry) plasmids contain the coding regions of enhanced green fluorescent protein (EGFP, GenBank accession number U76561) and *RFP* (DsRed1 and *mCherry* with GenBank accession numbers JF330266 and AY678264) under the control of the simian cytomegalovirus immediate–early enhancer/promoter sequence (GenBank accession number U38308) (Suhr et al. 2009; Kim et al. 2011). The pCS2+ mCherry plasmid was generated by the replacement of the *DsRed* coding sequence in the pCS2+ DsRed plasmid (Suhr et al. 2009) at *Eco*RI and *Bam*HI sites with a coding region of *mCherry* from the pR9-mCherry plasmid (Hu et al. 2009). The construct was confirmed by DNA sequencing. All plasmid DNAs were purified using a Nucleobond AX100 Kit (Machery-Nagel, Duren, Germany).

HR9 (CHHHHHRRRRRRRRRHHHHHC) peptide was synthesized as previously described (Liu et al. 2011). IR9

(GLFEAIEGFIENGWEGMIDGWYGRRRRRRRRR) peptide consisting of INF7 (Plank et al. 1994) and R9 in sequence was chemically synthesized (Genomics, Taipei, Taiwan). Green fluorescence-labeled HR9-FITC and IR9-FITC peptides containing fluorescein isothiocyanate (FITC) at their N-termini were synthesized (Genomics).

For protein expression, both mCherry and pR9-mCherry plasmids were transformed into *Escherichia coli* KRX strain (Promega, Madison, WI, USA) and induced, as previously described (Chang et al. 2005b; Lu et al. 2010). The expressed proteins (mCherry and R9-mCherry) were purified by one-step immobilized-metal chelating chromatography. The purified proteins were concentrated and subjected to dialysis using an Amicon Ultra-4 centrifugal filter device (Millipore, Billerica, MA, USA) as previously described (Lee et al. 2011). Proteins were quantified using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Entry of CPPs into Rotifers

To detect cellular uptake of CPP, rotifers were treated with 6 μM of HR9-FITC or IR9-FITC for 1 h at 28 °C as previously described (Dai et al. 2011; Liu et al. 2012). Live rotifers were washed and monitored without fixation. Fluorescent and bright-field images were recorded using a BD Pathway 435 System (BD Biosciences, Franklin Lakes, NJ, USA).

In Vitro Plasmid DNA Labeling

To prepare fluorescent DNAs, the pBlueScript-SK+plasmid DNA was in vitro labeled with the *Label*IT Cyanine 3 (Cy3) nucleic acid labeling kit (Mirus Bio, Madison, WI, USA) as previously described (Chen et al. 2007).

CPP-Mediated Cargo Delivery into Rotifers

To observe gene delivery mediated by CPPs, 3 μg of the Cy3-labeled pBlueScript-SK+plasmid DNA was incubated with a CPP (HR9, IR9, HR9-FITC, or IR9-FITC) at a molar nitrogen/phosphate (NH_3^+/PO_4^- or N/P) ratio of 3 in a final volume of 500 μl for 2 h at room temperature with agitation at 100 rpm as previously described (Dai et al. 2011; Liu et al. 2012). These CPP/Cy3-labeled DNA complexes were added to rotifers in 24-well plates, and the plates were incubated for 1 h at 28 °C. The rotifers were washed three times with freshwater using a Spectra/Mesh nylon filter of 41 μm in pore size (Spectrum Lab, Irving, TX, USA) to remove free CPP/Cy3-labeled DNA complexes. For comparison purposes, the Cy3-labeled plasmid DNA, pCS2+ EGFP, pCS2+ DsRed, or pCS2+ mCherry plasmid DNA was transfected using the jetPEI transfection reagent

(Polyplus-transfection, France) as previously described (Wang et al. 2007; Lee et al. 2011).

For the functional reporter gene assay, rotifers were treated with water, 3 μg of the pCS2+ EGFP plasmid DNA alone, or HR9 alone as controls, while rotifers were treated with HR9/pCS2+ EGFP plasmid DNA complexes prepared at an N/P ratio of 3 as an experimental group. Rotifers were incubated for 10 min at 28 °C. After that, the solution was removed, and rotifers were washed with freshwater thrice, followed by incubation at 28 °C for 1, 2, 3, or 24 h. Rotifers were then observed using the TCS SP5 and SP5 II confocal spectral microscope imaging systems (Leica, Wetzlar, Germany) or the Olympus BX51 inverted fluorescent microscope (Olympus, Center Valley, PA, USA).

To detect protein delivery mediated by CPPs, rotifers were treated with 30 μM of mCherry alone or mCherry noncovalently mixed with 30 μM of CPPs (HR9, IR9, HR9-FITC, or IR9-FITC) for 1 h at 25–28 °C as previously described (Hu et al. 2009). Rotifers were treated with 30 μM of R9-mCherry fusion protein as a positive control (Liou et al. 2012).

CdSe/ZnS QDs with the maximal emission peak wavelength of 625 nm (carboxyl-functionalized eFluor 625NC) were purchased from eBioscience (San Diego, CA, USA). The core of CdSe QD is 5 nm (Yu et al. 2003), and the functionalized CdSe/ZnS QD particles have a hydrodynamic size of about 25 nm in diameter (Xu et al. 2010). Six micromolars of each CPP (HR9, IR9, HR9-FITC, or IR9-FITC) was mixed with 100 nM of QDs at a molecular ratio of 60:1 for 2 h at room temperature as previously described (Liu et al. 2011). Rotifers were treated with QDs alone or CPP/QD complexes for 1 h at 25–28 °C.

Confocal and Fluorescent Microscopy

Green fluorescent protein (GFP), RFP, and bright-field images were recorded using a BD Pathway 435 System (BD Biosciences). The system includes both fluorescent and confocal microscopic sets. The parameters of microscopy were as follows: excitation at 482/35 nm and 543/22 nm for GFP and RFP, respectively, and emission at 536/40 and 593/40 nm for GFP and RFP, respectively. Bright-field images were used to determine rotifer morphology.

GFP, RFP, and bright-field images of gene expression were detected using the TCS SP5 confocal system (Leica), with excitation at 488 nm and emission at 520–568 nm for GFP and the SP5 II confocal system with excitation at 594 nm and emission at 582–680 nm for RFP, and the Olympus BX51 inverted fluorescent microscope (Olympus) with excitation at 460–490 nm and emission at 520 nm for GFP. The UN-SCAN-IT

software (Silk Scientific, Orem, UT, USA) was used to quantify fluorescent intensity that represented the relative efficiency of protein transduction (Chen et al. 2012).

Toxicity Measurement

Rotifers were treated with CPP-FITC, DNAs, QDs, CPP/DNA, CPP/QD complexes, R9-mCherry, or CPP/mCherry complexes for 24 h. Rotifers without treatment served as a negative control, while rotifers treated with 100 % dimethyl sulfoxide (DMSO) served as a positive control. Rotifer viability was determined using the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay (Dai et al. 2011). Rotifers were especially collected with a hand net (45-μm mesh corresponding to 300 holes per square inch) without centrifugation.

Statistical Analysis

Results are expressed as mean±standard deviation. Mean values and standard deviations were calculated from at least three independent experiments of triplicates per treatment group. Comparisons between the control and treated groups were performed by Student's t-test. Statistical significance was set at $P<0.05$ (*) and 0.01 (**).

Results

Entry of CPPs into Rotifers

To demonstrate that CPPs alone can enter live organisms, rotifers were treated with either HR9-FITC or IR9-FITC peptide. No signal was detected in the rotifers treated with water as a control using a fluorescent/confocal microscope (Fig. 1a). In contrast, green fluorescence was visualized in

the rotifers treated with either HR9-FITC (Fig. 1b) or IR9-FITC (Fig. 1c). This indicates that both HR9 and IR9 peptides can be internalized into rotifers.

CPP-Mediated Delivery of Genes

HR9 and IR9 are able to form stable noncovalent complexes with plasmid DNAs in vitro (Liu et al. 2011; manuscript submitted for publication). Rotifers were treated with Cy3-labeled DNAs alone, CPP alone, or CPP/Cy3-labeled DNA complexes. No signal was detected in the rotifers treated with water (Fig. 2a) or with Cy3-labeled DNAs alone (Fig. 2b) using a fluorescent/confocal microscope. Red fluorescent images were observed in the rotifers treated with jetPEI/Cy3-labeled DNA complexes as a positive control (Fig. 2c). We observed red fluorescent images in the rotifers treated with HR9/Cy3-labeled DNA complexes (Fig. 2d). Both green and red fluorescence were exhibited in the rotifers treated with HR9-FITC/Cy3-labeled DNA (Fig. 2e) or IR9-FITC/Cy3-labeled DNA (Fig. 2f) complexes. Overlaps between green fluorescent CPP-FITC and red fluorescent Cy3-labeled DNAs displayed a yellow color in the merged GFP and RFP images (Fig. 2e, f). These data indicated that HR9 and IR9 are effective to transport DNA into rotifers.

To determine whether cargo DNA can be functionally expressed after delivery by CPP in rotifers, *EGFP* and *RFP* reporter gene-containing plasmids were performed for the functional gene assay. Rotifers were treated with water, the pCS2+ EGFP plasmid DNA alone, HR9 alone, jetPEI/DNA, or HR9/DNA complexes. After 3 or 24 h of incubation, little signal was detected in the rotifers treated with water, DNA alone, or HR9 alone (Fig. 3a–c, g–i), due to chlorophyll autofluorescence of green algae, using a confocal microscope. In contrast, green fluorescence was displayed in the rotifers treated with jetPEI/DNA or HR9/DNA complexes

Fig. 1 Internalization of HR9 and IR9 peptides into rotifers. Rotifers were treated with **a** water (control), **b** HR9-FITC, or **c** IR9-FITC for 1 h. The GFP channel indicates the location of CPP-FITC. Images of bright-field, the RFP, and GFP channels are shown using a BD Pathway 435 System (BD Biosciences) at a magnification of ×200
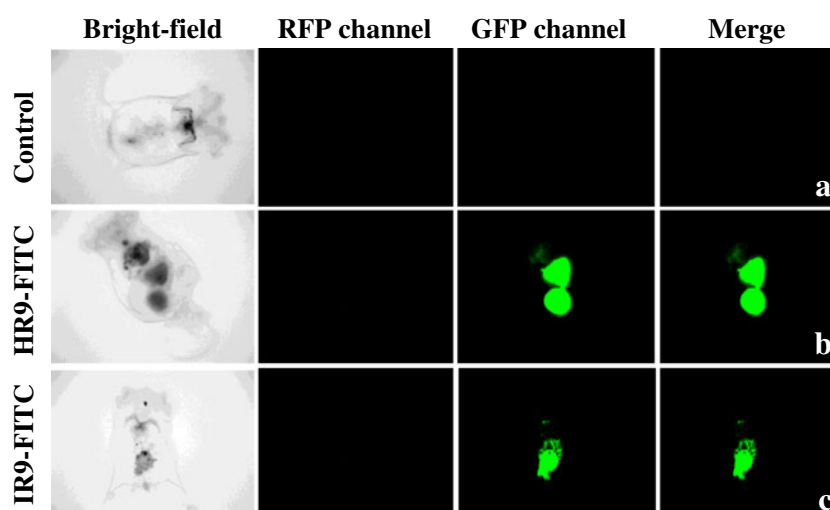
Fig. 2 Fluorescent microscopy of CPP-mediated delivery of Cy3-labeled DNAs into rotifers. Rotifers were treated with **a** water (control), **b** Cy3-labeled DNAs alone, **c** jetPEI/Cy3-labeled DNA, **d** HR9/Cy3-labeled DNA, **e** HR9-FITC/Cy3-labeled DNA, or **f** IR9-FITC/Cy3-labeled DNA complexes at an N/P ratio of 3. Images of bright-field, the RFP, and GFP channels are shown using a fluorescent microscope at a magnification of ×200. Overlap between green fluorescent CPP-FITC and red fluorescent Cy3-labeled DNA exhibits a yellow color in merged GFP and RFP images
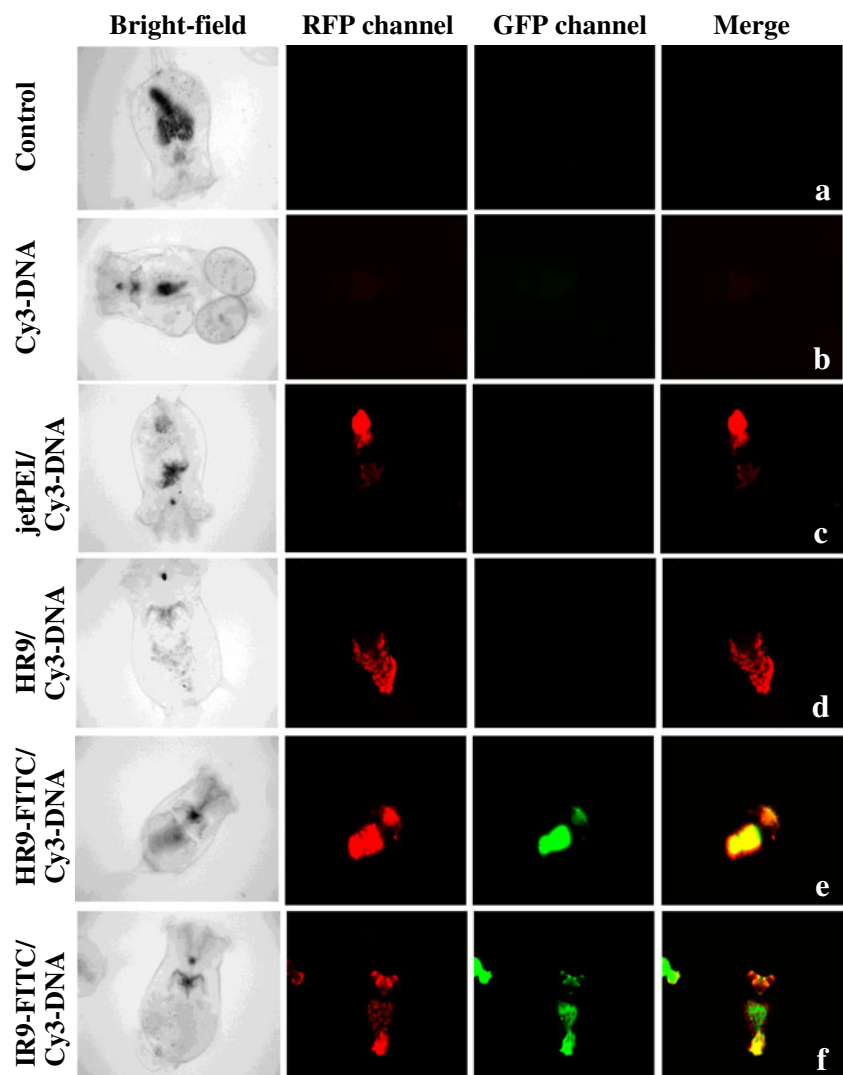
(Fig. 3d–f, j–l). After 1, 2, 3, or 24 h of incubation, similar results were observed in rotifers using a fluorescent microscope (Fig. S1 of the "Electronic supplementary material"). Rotifers were treated with water, the pCS2+ DsRed plasmid DNA alone, HR9 alone, jetPEI/DNA, or HR9/DNA complexes. After 3 or 24 h of incubation, no or little signal was detected in the rotifers treated with water, DNA alone, or HR9 alone (Fig. 3m–o, s–u). However, red fluorescence was detected in the rotifers treated with jetPEI/DNA or HR9/DNA complexes (Fig. 3p–r, v–x). Similar results were observed in the rotifers treated with pCS2+ mCherry plasmid (Fig. S2 of the "Electronic supplementary material"). These results indicate that HR9 is an effective transgenic carrier in Rotifera.

CPP-Mediated Delivery of Proteins

Rotifers were treated with mCherry alone, HR9/mCherry, HR9-FITC/mCherry, or IR9-FITC/mCherry complexes. Organisms treated with R9-mCherry fusion protein served as a positive control. No signal was detected in the rotifers treated with mCherry alone (Fig. 4a) using a fluorescent/confocal microscope. On the other hand, red fluorescence was detected when the rotifers were treated with either R9-mCherry or HR9/mCherry complexes (Fig. 4b, c). HR9-FITC/mCherry and IR9-FITC/mCherry complexes were internalized into rotifers as green and red fluorescent images, respectively (Fig. 4d, e). Overlaid images indicate colocalization of CPPs and mCherry. These data support the notion that arginine-rich HR9 and IR9 are effective protein carriers in rotifers.

CPP-Mediated Delivery of Nanoparticles

To study CPP-mediated delivery of nanoparticles, rotifers were treated with QDs alone, HR9-FITC/QD, or IR9-FITC/QD complexes. No fluorescent signal was observed in rotifers treated with water and QDs (Fig. 5a, b). In contrast,
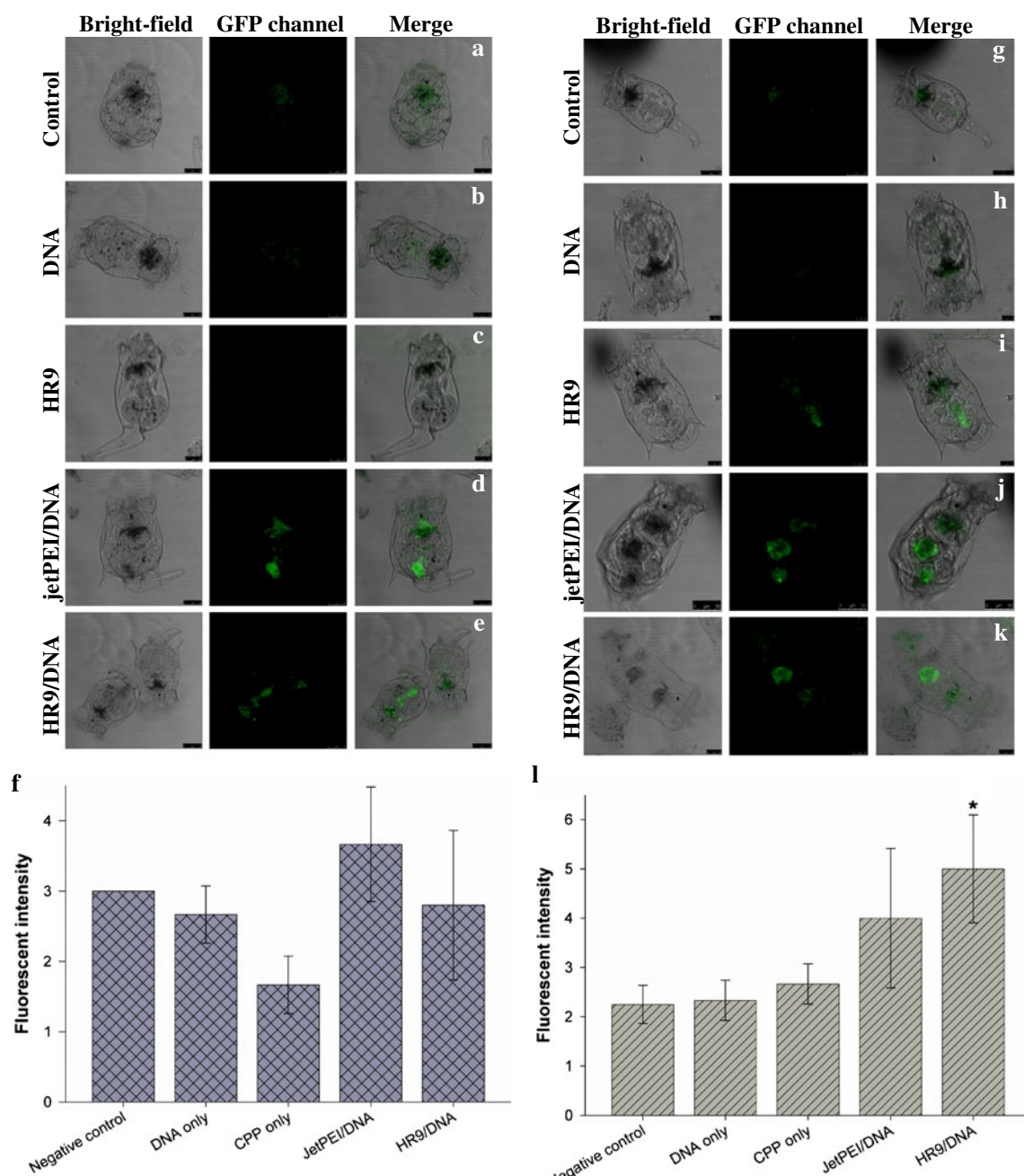
	Bright-field	GFP channel	Merge
Control			a
DNA			b
HR9			c
jetPEI/DNA			d
HR9/DNA			e

	Bright-field	GFP channel	Merge
Control			g
DNA			h
HR9			i
jetPEI/DNA			j
HR9/DNA			k

Fig. 3 Functional gene assay of CPP-delivered EGFP- and RFP-encoding plasmid DNAs in rotifers. Rotifers were treated with **a**, **g**, **m**, **s** water (negative control), **b**, **h** the pCS2+ EGFP plasmid DNA alone, **n**, **t** the pCS2+ DsRed plasmid DNA alone, **c**, **i**, **o**, **u** HR9 (CPP) alone, **d**, **j** jetPEI/pCS2+ EGFP, **p**, **v** jetPEI/pCS2+ DsRed, **e**, **k** HR9/pCS2+ EGFP, or **q**, **w** HR9/pCS2+ DsRed complexes, followed by incubation at 28 °C for 3 h (**a**–**f**, **m**–**r**) or 24 h (**g**–**l**, **s**–**x**). All images are shown using the TCS SP5 (*scale bars*, 25 μm for GFP) and SP5 II (*scale bars*, 100 μm for RFP) confocal systems (Leica) at a magnification of ×200. Gene expression intensity was determined from the digital image data from the functional gene assay and analyzed by the UN-SCAN-IT software. Significant differences at $P<0.05$ (*asterisk*) are indicated. Data were presented as mean±standard deviation from three independent experiments

rotifers internalized HR9-FITC/QD (Fig. 5c) and IR9-FITC/QD (Fig. 5d) complexes as shown by the green and red fluorescence. Superimposed images from GFP and RFP channels demonstrated colocalization of CPPs and QDs (Fig. 5c, d). These results indicate that arginine-rich HR9 and IR9 can deliver exogenous nanomaterials into rotifers.

Toxicity Assessment

To investigate whether treatments with CPPs, cargoes, and CPP/cargo complexes are toxic to rotifers, we first demonstrated a significant correlation ($R^2=0.9678$) between rotifer number and activity of MTT reduction (Fig. 6a). The MTT
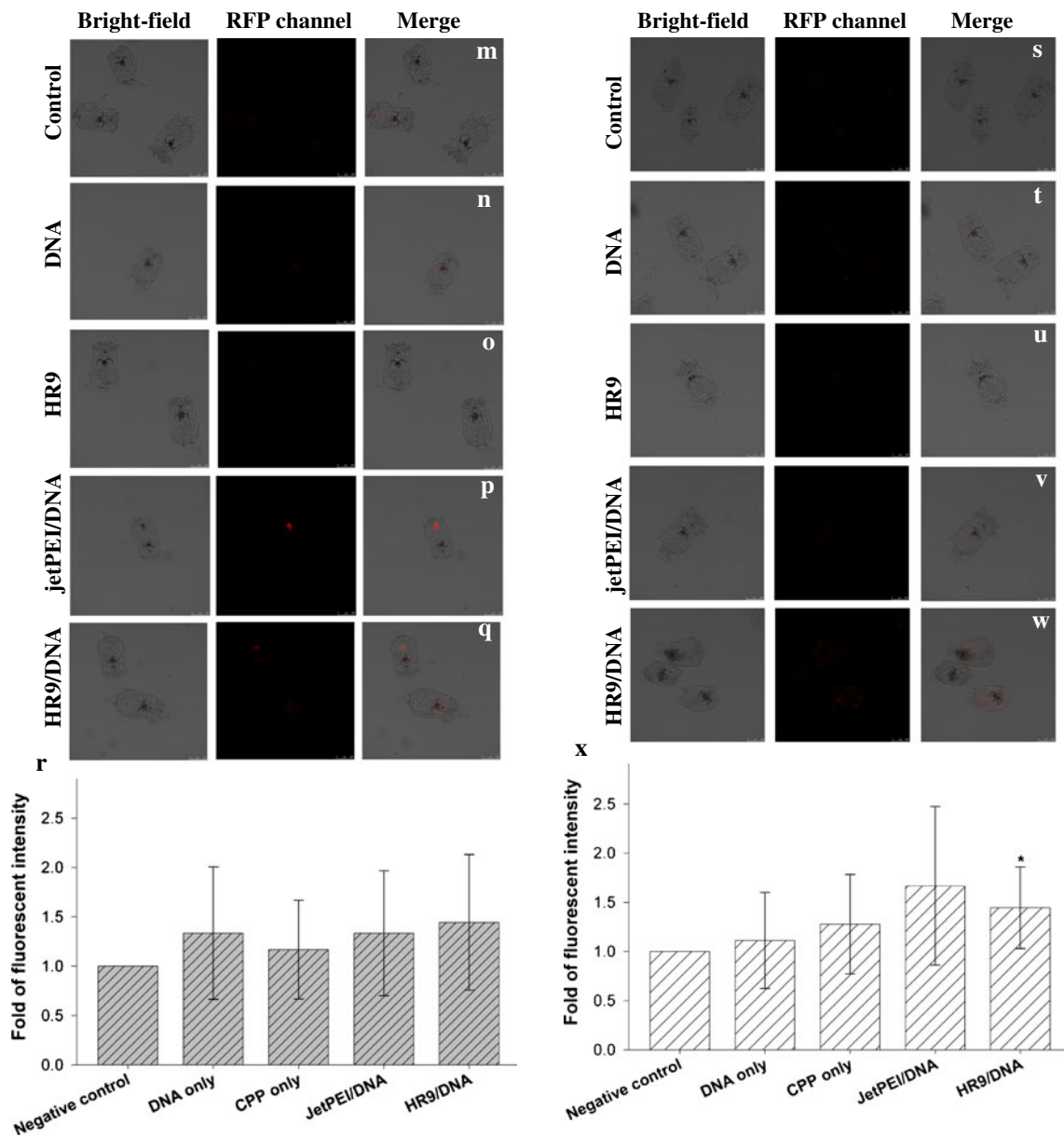
Fig. 3 (continued)

assay was then used to assess toxicity. Viability in treatment groups was not different from those in negative controls (Fig. 6b, c). This indicates that CPP-mediated cargo delivery is nontoxic to rotifers.

Relative Efficiency of Protein Transduction

Transduction efficiency in rotifers was determined by fluorescent intensity of CPP-delivered cargoes. HR9 and IR9 internalized rotifers at a high efficiency of 94 and 93 %, respectively (Fig. 7). The efficiency of CPP-mediated cargo delivery slightly differed among CPPs with the order of HR9/cargo (73 % averaged from

HR9/mCherry, HR9-FITC/mCherry, and HR9-FITC/QD complexes) >IR9/cargo (61 % averaged from IR9-FITC/mCherry and IR9-FITC/QD complexes) complexes.

Discussion

In recent years, CPPs have emerged as effective carriers for drug delivery, gene transfer, and DNA vaccination (Bolhassani et al. 2011). Our previous studies showed that the nontoxic R9 peptide is able to deliver noncovalently associated plasmid DNAs (Chen et al. 2007; Lee et al. 2011; Dai et al. 2011; Chen et al. 2012; Liu et al. 2012,

Fig. 4 Fluorescent microscopy of CPP-mediated delivery of proteins into rotifers. Rotifers were treated with **a** mCherry, **b** R9-mCherry, **c** HR9/mCherry, **d** HR9-FITC/mCherry, or **e** IR9-FITC/mCherry complexes. Images of bright-field, the RFP, and GFP channels are shown using a fluorescent microscope at a magnification of ×200. Overlap between green fluorescent CPP-FITC and red fluorescent mCherry exhibits a yellow color in merged GFP and RFP images
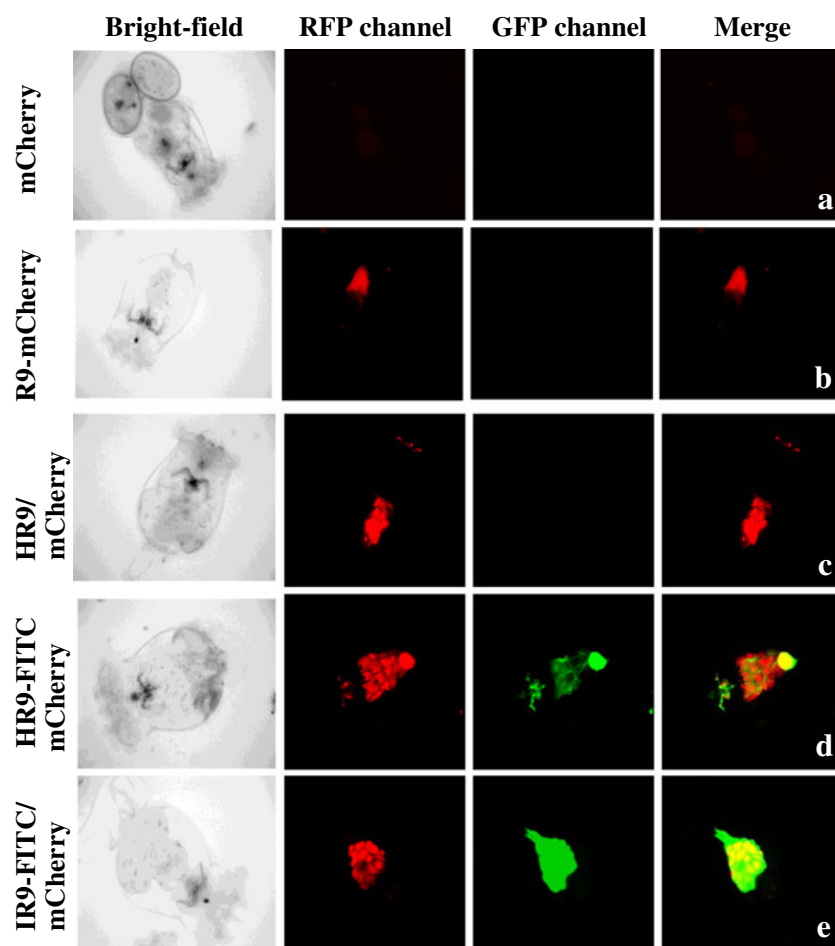

Fig. 5 Fluorescent microscopy of CPP-mediated delivery of nanoparticles into rotifers. Rotifers were treated with **a** water (control), **b** QDs, **c** HR9-FITC/QD, or **d** IR9-FITC/QD complexes. Images of bright-field, the RFP, and GFP channels are shown using a fluorescent microscope at a magnification of ×200. Overlap between CPP-FITC and QDs exhibits a yellow color in merged GFP and RFP images
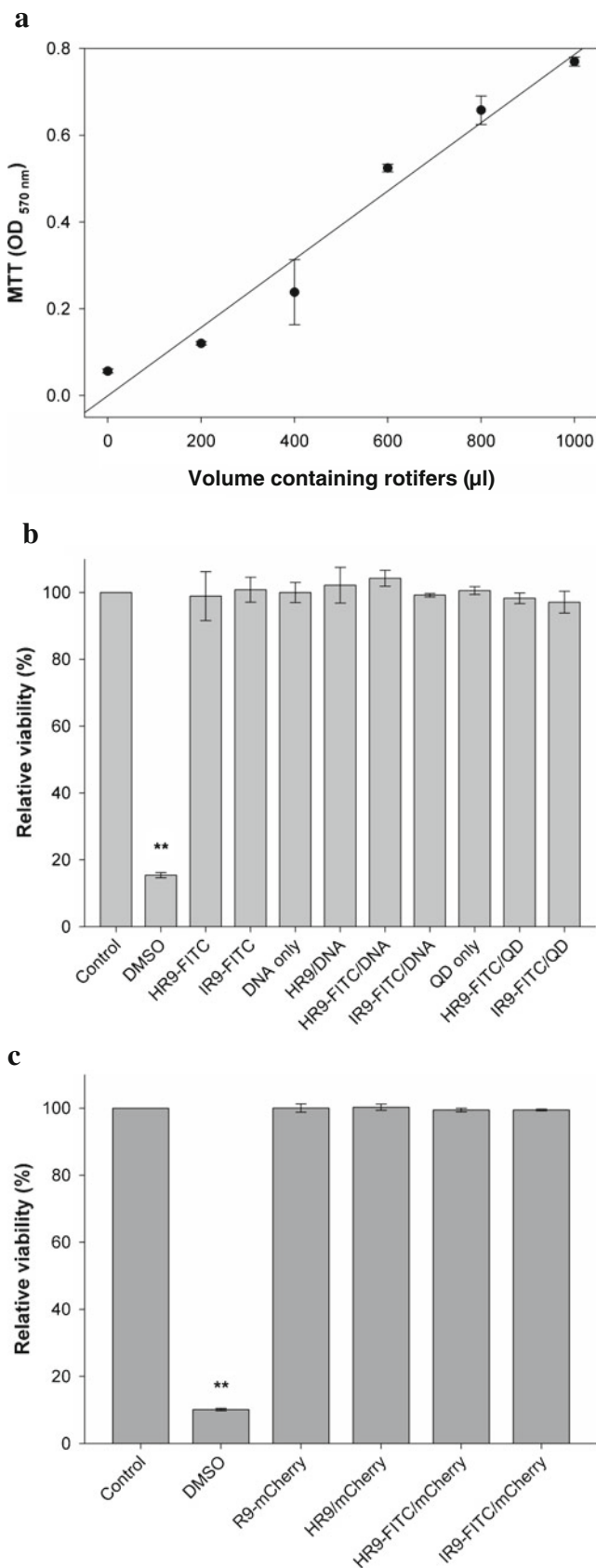
a

b

c
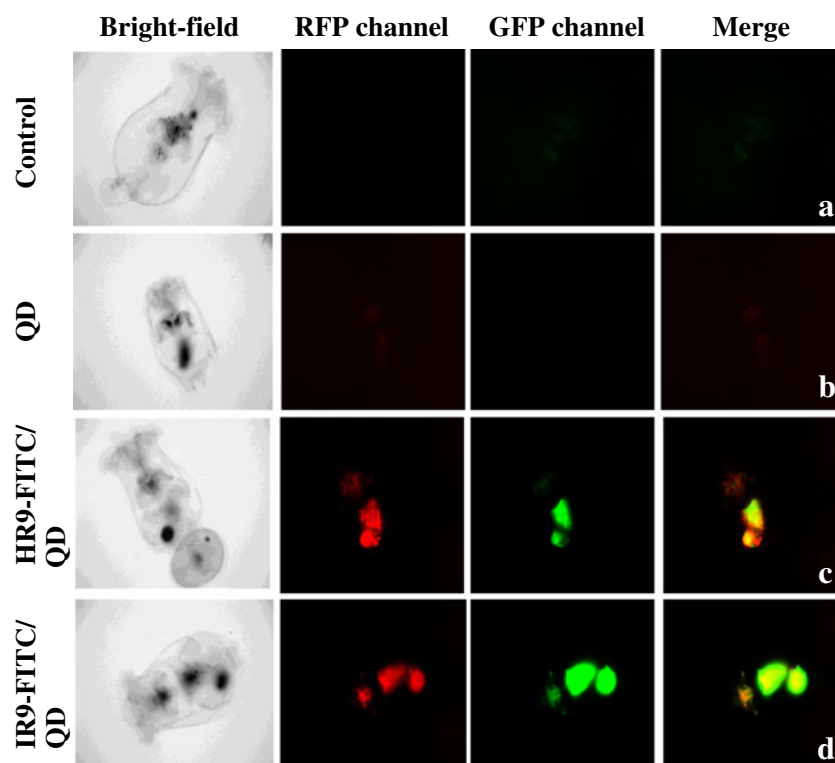
Fig. 6 Rotifer toxicity analysis using the MTT assay. **a** Linear regression graph of the results of rotifer viability as assessed by the MTT assay. The volume number containing rotifer was 1.32 ± 0.23 on average in each micro-liter. Influence of CPP-mediated DNA, QD (**b**), and protein (**c**) delivery on rotifer viability was analyzed by the MTT assay. Rotifers were treated with CPP-FITC, DNAs, QDs, CPP/DNA, CPP/QD complexes, R9-mCherry, or CPP/mCherry complexes. Rotifers treated with water (control) and 100 % DMSO served as negative and positive controls, respectively. Each treatment group was compared with the negative control. Significant differences at $P<0.01$ (*double asterisks*) are indicated. Data were presented as mean±standard deviation from three independent experiments

2013b) and siRNAs (Wang et al. 2007) into live cells. In the present study, we demonstrate that two arginine-rich CPPs (HR9 and IR9) are capable of entering rotifers. We then showed that these two CPPs could deliver noncovalently associated genes, proteins, and nanoparticles into rotifers.

Previous attempts to transfect plasmids containing the GFP reporter gene into rotifers have failed (Shearer and Snell 2007). Since rotifers are multicellular animals, the gene delivery systems shall be considered at an organism rather than a cellular level. As such, gene delivery into rotifers is probably more complex and variable than delivery to cultured cells. This may be the reason why reporter gene expression in rotifers has not been previously reported. Additionally, the timing of gene expression is critical in this aquatic micro-organism due to the high clearing rate in digestion (Snell and Hicks 2011). In the present study, we demonstrate that two arginine-rich CPPs can transport Cy3-labeled plasmid DNAs into rotifers following 1 h of exposure (Fig. 2). These results are consistent with those obtained with dsRNA delivered using lipofection transfection reagents (Snell et al. 2011). For gene expression assays, CPP-delivered plasmid DNAs encoding the *EGFP* and *RFP* reporter genes were successfully expressed in rotifers for the first time. These data are in agreement with our previous results obtained with CPP-mediated gene expression in plants (Chen et al. 2007) and paramecia (Dai et al. 2011). The success of gene expression in rotifers may rely heavily on high capacity of gene expression driven by the simian cytomegalovirus immediate–early transcription unit IE94 (Kim et al. 2011) at extremely low-level gene expression conditions.

The mechanism of cellular internalization of CPP/cargo complexes is still incompletely understood, although several mechanisms have been proposed (Deshayes et al. 2010). Recent studies have focused on two major mechanisms: direct membrane translocation and endocytic pathways (Nakase et al. 2010; Schmidt et al. 2010; Madani et al. 2011; van den Berg and Dowdy 2011; Mager et al. 2012). We observed that internalization of HR9/cargo complexes into human A549 cells is mediated by direct membrane translocation (Liu et al. 2011). Hence, we
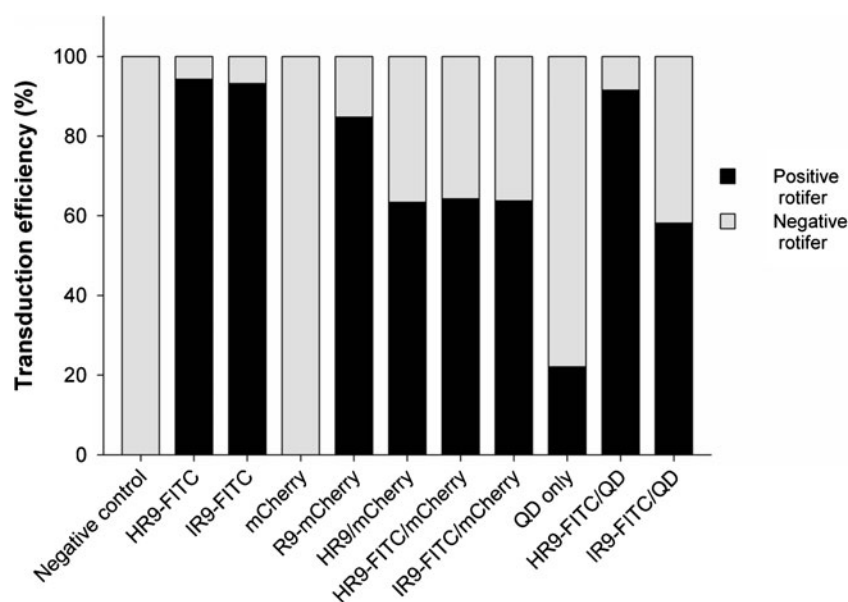
Fig. 7 Relative efficiency of protein transduction of various cargoes in rotifers. Rotifers were treated with those materials described in Figs. 1, 2, 3, 4, and 5. Relative intensities of fluorescent images after protein transduction were analyzed using UN-SCAN-IT software. Positive rotifers were defined as the rotifers with fluorescent signals observed using a fluorescent microscope, while negative rotifers were defined as the rotifers without any fluorescent signal. The total number of rotifers was combined from the number of both positive and negative rotifers. Data were presented as mean±standard deviation from three independent experiments

speculate that direct membrane translocation is probably a route used for the delivery of HR9/cargo complexes into rotifers in the present study. In our previous study, endocytosis was the major route for cellular uptake of R9-HA2-tagged RFP (Liou et al. 2012). The endosomolytic HA2 (INF7) peptide promoted the escape of RFPs from endosomes into the cytoplasm, ultimately increasing the cytosolic content of R9-HA2-tagged RFP (Liou et al. 2012). We predict that rotifers may utilize endocytosis as a major pathway to deliver IR9/cargo complexes.

QDs possess unique quantum properties that support a broad range of imaging applications (Zhang and Wang 2012). Advantages of QDs include photostability, narrow emission peak, high quantum yield, resistance to degradation, and broad size-dependent photoluminescence (Chen and Gerion 2004; Michalet et al. 2005). Thus, QDs are powerful imaging molecules and suitable for long-term and multiplexing biological imaging. Carboxylated and biotinylated QDs could be transferred to rotifers through dietary uptake of ciliated protozoans (Holbrook et al. 2008). QDs alone are not taken up by cells (Liu et al. 2010a; 2011; Xu et al. 2010); however, CPPs could form a stably noncovalent complex with QDs that leads to increase in uptake of QDs by cultured human cells (Liu et al. 2010a). In the present study, we found that QDs alone do not directly enter rotifers (Fig. 5c), but arginine-rich CPPs can effectively deliver QDs into rotifers. Our data are consistent with other studies that used QDs as biomarkers in various organisms (Feder et al. 2009; Son et al. 2009; Stylianou and Skourides 2009). QDs were successfully used in vertebrate model organisms, such as zebrafish (Son et al. 2009), amphibian model organisms such as *Xenopus* (Stylianou and Skourides 2009), and Protista–insect vector interactions such as *Trypanosoma cruzi–Rhodnius prolixus* (Feder et al. 2009).

Thus, we demonstrate that two arginine-rich CPPs can deliver nucleic acids, proteins, and nanoparticles into rotifers, important micrograzers at the base of the aquatic food web. Arginine-rich CPPs, especially HR9, appear to be a highly efficient and promising tool for gene transfer in rotifers. This nontoxic and efficient CPP-mediated protein transduction system may facilitate the study of gene and protein functions in invertebrates as well as the use of nanoparticles as markers for biomolecular trafficking both in vitro and in vivo.

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