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HIV TAT Protein Transduction Domain Mediated Cell Binding and Intracellular Delivery of Nanoparticles

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Abstract

Intracellular delivery of non-transported therapeutic agents has traditionally been thought possible only for low molecular weight (<500 DA) hydrophobic molecules. Higher molecular weight agents including oligonucleotides, proteins, DNA, liposomes, and nanoparticles do not readily enter the cytoplasm. However, the human immunodeficiency virus (HIV) trans-acting transcriptional activator (TAT) protein enters the cytosol by way of an 11 amino acid cationic peptide (TATp). When this cationic sequence is attached to a variety of small pharmacological agents, including paramagnetic ions[1,2] and proteins,[3–6] they are delivered into cells. Further, TATp modification of large cargo, such as proteins, polymers, and nanoparticles, may enable them to internalize into cells as well. The size limitations for cargo delivered by a single TATp are currently undetermined, but multiple TATp attached to polymers, nanoparticles, liposomes, and phage can definitely mediate their internalization. This process appears to follow an endocytotic or potocytic pathway and does not directly transfer the cargo into the cytoplasm of the cell. Here we review recent publications in which multiple TATp have been attached to and resulted in the successful intracellular delivery of nanoparticles.

Keywords

HIV; TAT; Human immunodeficiency virus; Trans-acting transcriptional activator; Liposome; Nanoparticle; Cationic; Peptide; Endocytosis; Potocytosis; Polymer; Heparan sulfate proteoglycan; CLIO; Cross-linked iron-oxide; Pharmacokinetic

INTRODUCTION

What Is Trans-Acting Transcriptional Activator?

Trans-acting transcriptional activator (TAT) is produced and secreted by HIV infected cells. TAT then enters the cytosol of adjacent cells, traffics to the nucleus, and directly participates in transcriptional regulation. The mechanism of TAT protein internalization was first investigated by Mann and Frankel[7] over a decade ago. They concluded that a cationic peptide sequence Trans-acting transcriptional activator peptide (TATp) with eight positively charged

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side chains (RKKRRQRRR) binds non-specifically to greater than 10^7 binding sites per cell and is required for transactivation activity. The fact that the peptide is short and highly charged suggests that it functions through an electrostatic mechanism and not via a specific protein–protein interface. The authors hypothesized that TATp fusion proteins could be tools for delivering active protein to the cytosol and nucleus. This discovery was not widely applied to enhance the uptake of other molecules (cargo) until the late 1990's. The upsurge in activity in this field is reviewed in a number of recent articles written on TATp and TAT-like sequences and their putative uses.[8–10] We focus here on reports in which multiple TATp sequences are attached to a nanoparticle (Table 1).

What Is “Trans-Acting Transcriptional Activator Peptide-like”

It is worth noting that the TAT peptide shares internalization characteristics with other short cationic polymers including polyarginine, the antennapedia peptide, penetratin, and the herpes virus peptide, VP22. These peptides are all cationic, and rich in arginine or lysine. In fact, polyarginine has been shown similarly effective at delivering cargo.[11–13] These peptides still function if their sequence is reversed or if the *D*-isomer amino acids are used. Wender and coworkers[12] report similar activity using a peptoid backbone instead of the natural peptide backbone. The peptides internalize by a common pathway that is reported to function at 4°C, where endosomal vesicles cannot fuse;[14,15] however, this may be an artifact of fixation.[16–19]

Why Is There Excitement over Trans-Acting Transcriptional Activator Peptide in the Drug Delivery Field?

Most importantly, these peptides can be used to ferry cargo across membranes into the cell and possibly into the cytosol. Cell penetrating peptides potentially offer a mechanism to bind and internalize large molecules and nanoparticles rapidly into cells *in vivo*. This would greatly benefit the fields of protein and gene delivery by allowing passage to targets within the cytoplasm and nucleus.[8–10] These peptides may also divert the cargo to an alternative trafficking pathway and permit the particle to avoid the lysosomal route; hence exposure to degrading enzymes.

MECHANISM

Despite the proliferation of new applications for these peptides, the mechanism by which they cross cell membrane remains uncertain. TATp, antennapedia, polyarginine, VP22, and similar cationic peptides convey similar internalization properties when attached to a cargo due to their high linear density of flexible, cationic side chains. The TAT protein interacts strongly with heparin oligosaccharides.[20,21] In fact heparan sulfated proteoglycans (HSPG) are critical for TATp internalization.[22–24] Negatively charged lipids[14] and sialic acids are another possible cell surface attachment site for TATp. Thus, TATp binds its cargo nonspecifically to negatively charged cell surface molecules via electrostatic forces. This interaction of TATp with negatively charged heparan is similar to what occurs when certain viruses bind to mammalian cells.[25] Cationic polymers and cationic liposomes also interact with negatively charged glycosaminoglycans.[26] Thus the mechanism of TATp internalization should be considered in the large context of the biology of glycosaminoglycans.

Three modes of internalization have been proposed for cationic peptide mediated internalization: endocytosis,[7] potocytosis,[27] and direct membrane destabilization.[14] Endocytosis and potocytosis would both lead to particle encapsulation within a cellular membrane, whereas membrane destabilization would allow direct access to the cytosol.

The initial mechanistic study of TAT protein internalization by Mann and Frankel[7] suggested adsorptive endocytosis. That is, TAT binds to the cell surface and is internalized into a clathrin-coated pit. Subsequent publications reported that TATp does not internalize by classical endocytotic mechanisms because it has the ability to internalize at 4°C or in the presence of inhibitors of endocytosis.[13,15] Recent reports indicate that data collected showing rapid uptake of TATp were obtained under fixation conditions that permeabilize the cell membrane sufficiently to allow nuclear localization of TATp and VP22.[16–19] Thus one cannot consider the rapid uptake of TATp at 4°C to be convincingly established.

In an effort to explain rapid TATp internalization at low temperatures, potocytosis was investigated.[27] Potocytosis is an alternative internalization method, similar to endocytosis. Unlike endocytosis, potocytosis internalizes surface bound cholesterol rich membrane into pits coated with caveolin proteins. Lending some credibility to this hypothesis, the addition of molecular blockers of potocytosis reduced the internalization of TATp coated phage by about 50%.[27]

Alternative explanations of the rapid antennapedia peptide internalization, suggested that the antennapedia peptide interacts directly with negatively charged lipids on the outer surface of the cell to form inverted micelles.[14] These inverted micelles would then open inward and allow the cationic peptide and cargo direct access to the cytosol. However, this mechanism does not easily explain the observation that TATp mediated internalization is saturable.[5] Additionally, evidence of contents leakage induced by TATp from model phospholipid vesicles has not been reported.

The size of the attached cargo has a very significant effect upon the mechanism of internalization. Silhol and coworkers[28] showed that TATp carrying a low-molecular weight fluorescent molecule is not dependent upon HSPG for internalization and uptake is not blocked at low temperatures. Uptake of the low molecular weight peptides are, however, easily saturated at about 1 μM. When TATp is attached to the green fluorescent protein (GFP) the results are strikingly different; GFP–TATp uptake exhibits strong temperature dependence and strong HSPG dependence. Without HSPG or at low temperatures, internalization is essentially abolished. Clearly the mechanism of internalization for the peptide may be very different than that for proteins and larger particles.

In light of recently reported fixation artifacts, it seems most likely that the mechanism of TATp mediated internalization of nanoparticulates is via adsorptive endocytosis. Individual TATp and similar cationic peptides have both the flexibility to penetrate into HSPG and a sufficient positive charge density to bind with moderate affinity. Multivalent TATp particles will therefore exhibit an even higher avidity for the cell surface. Surface binding becomes saturated as the HSPG are filled. Surface bound particles will then be internalized into a low pH compartment.[29] At this point, some undetermined mechanism may allow for escape to the cytosol. Thus the most recent evidence suggests that TATp modified particles bind to the cell surface through negatively charged cell surface molecules, principally heparan sulfate and possibly sialic acid. Subsequent to binding they are internalized either through endocytosis, macropinocytosis, or via caveolae.

APPLICATIONS

Polymers

Huang and coworkers[30] synthesized a 12 repeat block copolymer of PEG and lysine (MW = 26.9 kDa, 2.198 kDa/repeat). Lysine residues were incorporated as attachment points for peptides. Trans-acting transcriptional activator peptide (RKKRRQRRRK[Biotin] C-amide) were attached at the cysteine via a disulfide linkage to activated lysines on the PEG–lysine

copolymer. The carboxy terminal lysine sidechain was conjugated to biotin. The disulfide linkage was designed to break upon exposure to the reducing cytoplasmic environment. The resulting macromolecules had eight TATp branches per macromolecule. The macromolecule was stable under non-reducing conditions at physiological pH, but the addition of 3 mM glutathione released the TATp within minutes. Peptide delivery was assessed by inhibition of normal TAT transactivation as shown by the chloramphenicol acetyltransferase assay. In the CAT assay, cells are stably transfected with a CAT reporter gene under control of the HIV trans activator response (TAR) element. Subsequently, the cells are transfected with a plasmid that expresses a functional TAT protein, that upregulates TAR elements. The peptide, TATp, binds the TAR element, but does not induce transcription. Thus TATp competes with TAT protein and reduces CAT reporter expression. At similar peptide concentrations, both free TATp and the multivalent TATp macromolecule significantly reduced CAT activity. This indicates that when initially bound to the polymer backbone, TATp successfully internalizes, traffics to the nucleus, and reduces transactivation; however, it remains unclear whether TATp detached from the PEG-lysine copolymer before or after internalization.

Complexes

Because TATp is highly cationic, it can condense anionic polymers such as heparan sulfate and DNA into electrostatic stabilized complexes. Sandgren and coworkers[31] created such complexes by mixing polyanion with TATp (GRKKRRQRRRPPQC-amide) polycation using a 1: 1 w/w ratio. For heparan sulfate this corresponded to a 1.4: 1 positive to negative charge ratio. Both TATp and polyanion were labeled with fluorophores. The labeled complexes were incubated with cells and tracked by fluorescence microscopy. Both heparan sulfate-TATp and DNA-TATp complexes strongly bound on the cell surface in a heparan sulfate dependent manner. Binding and internalization were abolished in a proteoglycan deficient cell line. Chlorate treatment to prevent sulfation also abolished uptake. Treatment with chondroitin ABC lyase or heparitinase enzymes significantly reduced, but did not eliminate binding and internalization. By fluorescence microscopy, the heparan sulfate-TATp complex internalized to the cells and at 24 hours heparan sulfate could be seen in the nucleus; however, the fluorescence labeled TATp was retained in low pH vesicles that stained with lysotracker. At no time did the TATp escape from the low pH compartments and it was presumably degraded. Interestingly, this is in contrast to the observation that free TATp localizes to the nucleus. This suggests that free TATp and multivalent particulate TATp internalize by different mechanisms.

Morris and coworkers[32] synthesized a carboxy terminal cationic peptide with a nitrogen terminal hydrophobic end (acetyl-KETWWETWWTEWSQPKKKRKV-cysteamine). The cationic portion of this peptide was not derived from TAT, but instead from the cationic SV-40 nuclear localization sequence. The peptide has a hydrophobic sequence that associates with protein surfaces, presumably onto hydrophobic patches. The peptide was mixed with green fluorescent protein at a ratio of 30 to 1 and formed a complex consisting of 12–14 peptides per protein as assessed by gel filtration chromatography. The complexes delivered both fluorescence labeled peptide and GFP into up to 90% of the cells as indicated by fluorescence microscopy. Incubation in the presence of serum or at 4°C only slightly reduced the number of fluorescent cells. While this was not a TAT derived particle, it may share a similar mechanism of entry in to the cell. That is, the high multivalency of positive charge can induce similar particulate binding to cell surface proteoglycans and escape from endolysosomal degradation. The authors proposed formation of these hydrophobic complexes as a means to deliver otherwise unmodified proteins to the cell interior.

Shell Cross-Linked Nanoparticles

A stabilized micelle modified with TATp (GGGGYGRKKRRQRRR), attached by the nitrogen terminus, was prepared from shell cross-linked (SCK) nanoparticles.[33] These 13 nm particles

are formed around a micelle that is cross-linked at its surface. The majority (68%) of particles were 13 nm in diameter as measured by dynamic light scattering; however a significant fraction of large aggregates (329 nm) were formed (32%). It is not clear if the appearance of aggregates in the TATp modified nanoparticles is due to an intrinsic feature of TATp or due to the method used to attach the TATp to the particle surface. The fluorescent TATp nanoparticles were incubated with CHO cells and shown using confocal microscopy to bind to the cell whereas the unmodified nanoparticles did not associate with cells. However it was not determined if the 13 nm particles or the 329 nm particles were responsible for the cell association, so one cannot draw any conclusions concerning the number of TATp per particle required for cell association or internalization from this study.

Nanoparticles

Magnetic resonance imaging (MRI) can be used to track the location of cells loaded with contrast enhancing agents. One such agent is a nanoparticle designated a cross-linked-iron-oxide (CLIO) nanoparticle.[34] A CLIO particle has an iron oxide crystal core of about 5 nm in diameter. The iron core is covered with a biocompatible, cross-linked dextran coat. This forms a particle 41 nm in diameter that can be functionalized with different molecules and peptides at the surface. This type of cell label is of interest because in vitro labeled cells can be followed in vivo by magnetic resonance imaging. Josephson and coworkers[34] first reported preparing TATp labeled CLIO particles by attaching TATp (GGCGRKKRRQRRRGYK[FITC]-amide) at the nitrogen terminal cysteine via a disulfide bond. Particles were determined to have about seven peptides per particle, and this enhanced the cellular internalization of iron by greater than 100 fold compared to non-modified particles. Over a concentration range of 1 to 100 μg of iron per million cells the TATp CLIO particles did not show saturation. Microscopy was employed to document that the particles had internalized. Additionally, TATp CLIO labeled cells could be isolated by passage over a magnetic chromatography column.[34] This permits labeled cells to be recovered after injection into animals.

A different variation of the TATp modified CLIO particles were later prepared using a stable thioether linkage to attach a slightly different peptide (GRKKRRQRRRGYK[FITC]C-amide) at a cysteine located at the carboxy terminus.[35,36] These particles had 10 peptides per particle and were 65 nm in diameter. The direction of attachment had no effect upon cellular internalization. Dodd and coworkers[35] showed that up to 30,000 particles per cell were internalized into 97% of lymphocytes treated with the 8 $\mu\text{g}/\text{mL}$ TATp CLIO nanoparticles for 18 hours. TATp CLIO labeling of T cells also did not appear to affect cell behaviors including activation and activation induced cell death.[35] With importance regarding the in vivo use of TATp modified nanoparticles, Wunderbaldinger and coworkers[36] showed that the addition of 10 TATp per particle decreased the clearance half-life in the blood from 682 to 42 minutes compared to non-functionalized CLIO particles; however, biodistribution of the TATp functionalized particles did not differ significantly from the unmodified CLIO particles. This rapid elimination from the blood could be a troubling issue for the use of TATp modified nanoparticles to distribute to regions of the body with a small fraction of the blood flow. Most of the particles will be eliminated from the blood before a significant fraction of the dose circulates through a small target site such as a metastasis.

A third preparation of TATp modified CLIO particles were also examined[37,38] whereby the TATp sequence (GRKKRRQRRRGYK[FITC]C-amide) was linked to the cysteine at the carboxy terminus by a degradable disulfide bond. These particles were 45 nm in diameter. Again the direction of attachment for the peptide had did not prevent the TATp CLIO particles from binding and internalizing. The relationship between the number of TATp/particle and cell binding was examined by Zhao and coworkers,[38] who reported the effect of varying the

number of TATp per particle between 1 and 15. The uptake of particles increased 100 fold when the number of TATp per particle was increased from 1.2 to 15 TATp. Again this illustrates the cooperative nature of the binding observed with multivalent TATp particles.

Liposomes

Liposomes are synthetic vesicles consisting of bilayers of phospholipids similar to those found in mammalian cell membranes. Liposomes have been extensively investigated as platforms for drug, gene, and protein delivery. Neutrally charged liposomes interact poorly with cells, and these interactions are further reduced when a PEG polymer is attached to the surface. Such PEG shielded particles can circulate in the blood for several days. Recently, Torchilin and coworkers[39] have shown that TATp (YGRKKRRQRRR) attached via a PEG (MW = 3.0 kDa) polymer to a 200 nm diameter liposome results in improved binding and internalization. The chemistry of attachment formed an amide bond between the primary amines either at the nitrogen terminus or the lysine sidechains. With 100 to 500 peptides per liposome the particles were internalized into cytoplasmic vesicles within an hour. The attachment of 4.5% of 3000 MW PEG polymer to the surface of the liposome had little effect on binding and internalization, whereas the addition of 4.5% 5000 MW PEG polymer to the liposome surface blocked binding and internalization. In contrast to the TATp modified CLIO, the direct attachment of TATp to the liposome surface did not mediate association with cells. Torchilin[39] proposed a steric hindrance model to explain the lack of binding when TATp was attached directly to the liposome surface and to explain the ability of the 5000 MW PEG to interfere with cell association mediated by TATp coupled to the 3000 MW PEG.

In a short communication Torchilin and colleagues reported that in addition to delivering the polyanion dextran,[39] these TATp liposomes also deliver and express DNA.[40] Reportedly, TATp liposomes containing dextran show punctate staining within the cytoplasm within the first hour of incubation. From 2 to 4 hours the staining appears near the nucleus. Between 9 and 24 hours released dextran becomes visible throughout the cell. Torchilin also reported the transfection of cells with TATp liposomes.[40] Trans-acting transcriptional activator peptide labeled liposomes were prepared with less than 10% of the positively charged lipid DOTAP needed to form a complex with DNA. The liposomes and DNA were mixed together and placed on cells. Torchilin reports that TATp enabled this complex to transfect a high percentage of cells while TATp deficient complexes could not.[40] No obvious signs of cytotoxicity were reported.

Tseng and coworkers[29] have studied the kinetics of TATp modified liposomes with varied numbers of peptides on the particle surface. They conjugated the TATp sequence (CYGRKKRRQRRRPPQ) to a PEG (MW = 3.4 kDa) phosphatidylethanolamine. The chemistry of attachment was to the nitrogen terminal cysteine via a stable thioether bond. The TATp-PEG-lipid was inserted into preformed phosphatidylcholine: cholesterol (6: 4) 100 nm liposomes using a micelle insertion method. Greater than 90% insertion of TATp-PEG-lipid occurred in all preparations. Liposomes containing a pH sensitive dye, 8-hydroxypyrene-1,3,6-trisulfonic acid, were incubated with cells, and fluorescence was assayed using FACS. Tseng and coworkers[29] showed that increasing numbers of peptides per particle raised the fluorescence substantially. Internalization was evident above five peptides per particle. This increase in fluorescence began to saturate by 50 to 100 peptides per particle for HTB-9 cells but continued to increase linearly for A431 cells. The fact that a pH sensitive dye greatly increases in fluorescence upon binding strongly suggests that TATp labeled liposomes are entrapped separate from the cytoplasm, in a low pH compartment.

Additionally Tseng and coworkers[29] used doxorubicin (Dox) loaded TATp modified liposomes and measured cell proliferation and tumor killing. In vitro cell assays showed that TATp modified Dox loaded liposomes delivered 12 fold more Dox than unmodified Dox

loaded liposomes at 2 hours incubation with 1 μg Dox/mL. Still, the TATp modified Dox liposomes delivered about half as much Dox as that delivered by free drug. Cell proliferation assays revealed that TATp modified Dox loaded liposomes were only slightly more effective at reducing cell growth than the unmodified liposomes. In summary, free Dox had the most antiproliferative activity, while liposomal entrapped Dox reduced cellular exposure, with or without TATp modification.

Having shown a 12-fold improvement in delivery of Dox by TATp modification of the liposomes, Tseng and coworkers[29] present data from an *in vivo* test. They monitored tumor size as a function of time after treatment in a BALB/c mouse C26 hind limb tumor model. Both liposomal Dox formulations reduced tumor growth rate better than treatment with free drug; however, for the Dox loaded liposomes there was no significant difference between the groups treated with or without TATp modification. A likely explanation is that the presence of TATp on the liposome surface enhanced their elimination from circulation as was observed with the CLIO nanoparticles. This result illustrates a potential pitfall of TATp modified particles *in vivo*.

Phage

Trans-acting transcriptional activator peptide has also been used to modify biological viral particles for improved transfection. Eguchi and coworkers[27] created a variant of the lambda bacteriophage that expressed TATp on its capsid. They engineered a fusion protein of the TATp sequence (MLGISYGRKKRRQRRRPPPPQT) connected at the carboxy terminal residue to the D coat protein. A single Lambda phage, 55 nm in diameter, would express 420 TATp—D fusion proteins. Interestingly, these bacteriophage were able to transfect mammalian cells. Eguchi and coworkers[27] compared the TATp phage both with normal phage and phage modified with an integrin binding RGD sequence. The unmodified phage showed minimal cell binding, while both the RGD and TATp modified phage showed strong cellular binding.

In vitro, the TATp modified phage showed the most significant transfection with 30% of the cells transfected with 10^6 particles/cell.[27] Interestingly, the presence of serum in the media significantly accelerated the kinetics of transfection in COS-1 cells. Typically, serum proteins interfere with cationic transfection reagents, but this is not the case for TATp modified phage. The authors also investigated the possibility that potocytosis is involved with the uptake of TATp phage. COS-1 cells were incubated with TATp phage with or without the presence of filipin and nystatin, inhibitors of caveolae internalization. Both filipin and nystatin caused a 50% decrease in luciferase transfection compared to a control with no inhibitor. This result suggests that potocytosis may play a role in TATp mediated internalization of nanoparticles. *In vivo* TATp phage was able to mediate GFP transfection of mouse liver as observed by microscopy. The authors concluded that the binding and internalization seen in the TAT phage were dependent upon a low affinity absorption process and internalization may be partly due to potocytosis.

CONCLUSION

The HIV TAT peptide can effectively increase the binding and internalization to a wide array of cells both *in vivo* and *in vitro*. Nanoparticles between 13 and 200 nm in diameter with between 5 and 500 TAT peptides per particle show high avidity for polyanions on the cell surface such as heparan sulfate proteoglycans. As evidenced by pH sensitive dyes, TATp modified particles are targeted to low pH compartments, which may be independent from the classical lysosomal degradation pathway. This precludes the possibility that TATp nanoparticles have direct access to the cytosol, but instead must traffic through a vesicle such as a caveolae or endosome. The true mechanism of internalization has been difficult to determine, especially in light of recently described fixation artifacts; however, some of the

internalized cargo must reach the cytoplasm and nucleus because of the expression of proteins from delivered DNA. Lastly, the presence of unshielded TATp on a nanoparticle surface may greatly hinder its usefulness for intravenous administration. Without blocking TATp activity, injected nanoparticles may be rapidly cleared from the bloodstream. Regardless, the use of TATp for delivering therapeutic cargo is not restricted to small molecules and proteins, but extends to a variety of large polymers and nanoparticles.

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ABBREVIATIONS

CAT	chloramphenicol acetyltransferase
CLIO	cross-linked iron-oxide
Dox	doxorubicin
FACS	fluorescence activated cell sorting
GFP	green fluorescent protein
HIV	human immunodeficiency virus
HSPG	heparan sulfate proteoglycans
PEG	polyethylene glycol
TAR	trans activator response element
TAT	trans-acting transcriptional activator
TATp	TAT derived basic peptide
SCK	shell cross linked

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Table 1

Comparison of multivalent cationic peptide modifications of nanoparticles.

Peptide sequence ^a	Particle	Attachment	Size	Peptides/particle	Transfect	Ref.
Acetyl-KETWWETWWTEWSQPKKKRKV-cysteamine	Peptide-GFP	Hydrophobic complex	25–30 (kDA)	12–14	n.a.	32
RKKRRQRRRK[biotin]C-amide	PEG-lysine co-polymer	Disulfide	27 (kDA)	8	n.a.	30
GRKKRRQRRRPPQC-amide	Peptide-DNA	Electrostatic complex	?	n.a.	yes	31
G ^u GGGYGRKKRRQRRR	SCK	Amide	13 (nm)	?	n.a.	33
G ^u GGRKKRRQRRRGYK[FITC]-amide	CLIO	Disulfide	41 (nm)	7	n.a.	34
GRKKRRQRRRGYK[FITC]C-amide	CLIO	Disulfide	45 (nm)	1–15	n.a.	37, 38
GRKKRRQRRRGYK[FITC]C-amide	CLIO	Thio ether	65 (nm)	10	n.a.	35, 36
YGRKKRRQRRR	Liposome	Amide PEG (MW = 3.0 kDA)	200 (nm)	100–500	yes	39, 40
CYGRKKRRQRRRPPQ	Liposome	Thioether PEG (MW = 3.4 kDA)	100 (nm)	5–100	n.a.	29
MLGISYGRKKRRQRRRPPPPQT-D protein	λ Phage	Fusion coat protein D	55 (nm)	420	yes	27

Note: n.a., not applicable.

^aIf applicable, residue of covalent linkage to nanoparticle is underlined.