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Peptides for Specifically Targeting Nanoparticles to Cellular Organelles: *Quo Vadis*?

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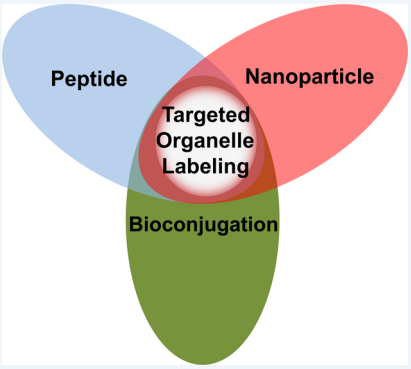
Supporting Information

CONSPECTUS: The interfacing of nanomaterials and especially nanoparticles within all aspects of biological research continues to grow at a nearly unabated pace with projected applications focusing on powerful new tools for cellular labeling, imaging, and sensing, theranostic materials, and drug delivery. At the most fundamental level, many of these nanoparticles are meant to target not only very specific cell-types, regardless of whether they are in a culture, tissue, an animal model, or ultimately a patient, but also in many cases a specific subcellular organelle. During this process, these materials will undergo a complex journey that must first find the target cell of interest, then be taken up by those cells across the extracellular membrane, and ultimately localize to a desired subcellular organelle, which may include the nucleus, plasma membrane, endolysosomal system, mitochondria, cytosol, or endoplasmic reticulum. To accomplish these complex tasks in the correct sequence, researchers are increasingly interested in selecting for and exploiting targeting peptides that can impart the requisite capabilities to a given nanoparticle construct. There are also a number of related criteria that need careful consideration for this undertaking centering on the nature and properties of the peptide vector itself, the peptide−nanoparticle conjugate characteristics, and the target cell.



Here, we highlight some important issues and key research areas related to this burgeoning field. We begin by providing a brief overview of some criteria for optimal attachment of peptides to nanoparticles, the predominant methods by which nanoparticles enter cells, and some of the peptide sequences that have been utilized to facilitate nanoparticle delivery to cells focusing on those that engender the initial targeting and uptake. Because almost all materials delivered to cells by peptides utilize the endosomal system of vesicular transport and in many cases remain sequestered within the vesicles, we critically evaluate the issue of endosomal escape in the context of some recently reported successes in this regard. Following from this, peptides that have been reported to deliver nanoparticles to specific subcellular compartments are examined with a focus on what they delivered and the putative mechanisms by which they were able to accomplish this. The last section focuses on two areas that are critical to realizing this overall approach in the long term. The first is how to select for peptidyl sequences capable of improved or more specific cellular or subcellular targeting based upon principles commonly associated with drug discovery. The second looks at what has been done to create modular peptides that incorporate multiple desirable functionalities within a single, contiguous sequence. This provides a viable alternative to either the almost insurmountable challenge of finding one sequence capable of all functions or, alternatively, attaching different peptides with different functionalities to the same nanoparticle in different ratios when trying to orchestrate their net effects. Finally, we conclude with a brief perspective on the future evolution and broader impact of this growing area of bionanoscience.

I. INTRODUCTION

Interfacing nanoparticles (NPs), which can typically range from "hard" inorganic metallic nanocrystals such as gold nanoparticles or luminescent semiconductor quantum dots (QDs) to "soft" dendrimers and recombinantly expressed viral capsids, with living cells has undergone decades of research, and efforts have progressed from use as mere cellular labels to elegant demonstrations of multifunctional constructs capable of simultaneously labeling, tracking, and delivering a drug or sensor cargo or both to cells.[1,2] Progress on these materials has been facilitated by cumulative improvements in the synthesis of high quality NPs, the solubilizing ligands that provide the NPs with colloidal stability, NP bioconjugation chemistries, and the cellular delivery modalities utilized.[3] The delivery regimes currently available for providing cellular uptake of NPs can be generally subdivided into three primary classes: (i) passive, where the NP is just exposed to the cells for some time period, (ii) active, where a

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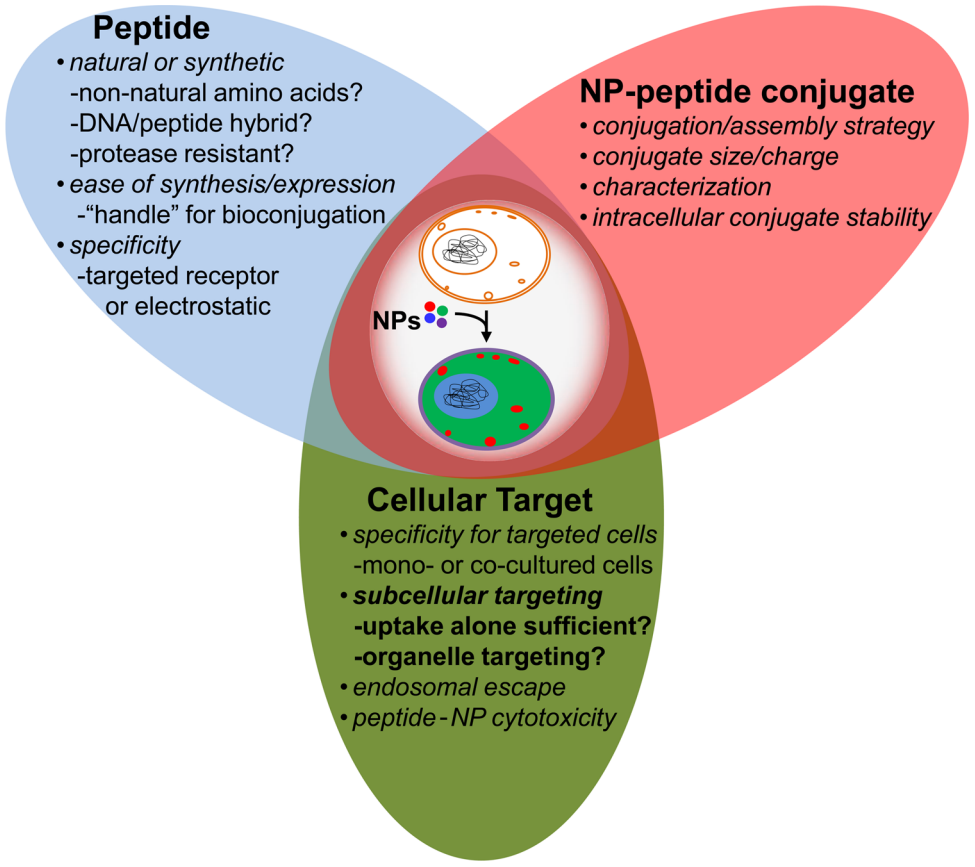

Figure 1. Generalized considerations regarding peptide-mediated nanoparticle cellular delivery. Multiple factors are involved in selecting peptides for NP cellular delivery including initial bioconjugation chemistry, choice of peptide, mechanism of NP uptake/intracellular NP fate, and target specificity.

physicochemical technique such as electroporation is utilized, and (iii) facilitated, which can depend on a transfection reagent (e.g., Lipofectamine) or the appending of a biological (e.g., the protein transferrin) or biologically active agent (e.g., charged polymers, drugs) to the NP to mediate cellular uptake (see ref 4 for a comprehensive review of NP cellular delivery modalities). Of the latter facilitated methods, appending biologicals to the NP often strikes the best balance between specificity and cellular viability, and here peptides have emerged as the preferred biological agent for facilitating NP cellular uptake due primarily to their desirable features. These include: (i) compactness potentially minimizing the resulting NP−conjugate hydrodynamic diameter, (ii) high avidity from presenting multiple copies simultaneously around the NP, (iii) relatively facile synthesis unless the peptide is highly modified, (iv) ability to incorporate functional chemical groups or "handles" for bioconjugation, (v) access to a plethora of non-natural residues, which significantly increases chemical diversity; ,and (vi) potentially reduced immunogenicity, which improves biocompatibility.

The next challenge for multifunctional NPs goes beyond just entering the cell to now targeting specific subcellular organelles in cell culture, tissue, animal models, or even a patient, and here we examine what peptides have to offer for this challenge by discussing key research areas and related issues (Figure 1). We utilize selected examples to highlight important concepts within this theme and apologize for any omissions or related work we overlook.

II. NANOPARTICLE−PEPTIDE BIOCONJUGATION

Critical to efficient use of cell-targeted NP−peptide constructs is the underlying bioconjugation chemistry used to assemble them and this important starting point is still often overlooked. Ideally, attachment chemistry should allow any peptide to homogeneously attach to any NP with control over the resulting display orientation, ratio, separation distance, and affinity.[5−7] Unfortunately, the commonly utilized bioconjugation chemistries cannot yet even provide a plurality of these. Three general attachment strategies are currently utilized, namely, electrostatic assembly, select noncovalent binding interactions (e.g., biotin−avidin), and covalent chemistries.[5,6] Each of these approaches has its own set of benefits, such as simplicity or access to affordable commercial reagents, and, conversely, liabilities which can include cross-linking during bioconjugation, purification requirements, and heterogeneous attachment/orientation of the biological on the NP;[8] the latter being the most important and hardest to control. See refs 5 and 6 for an in-depth discussion of NP bioconjugation.

Development of new chemoselective chemistries such as hydrazone ligation and the family of "click" reactions are slowly providing a growing library of new bioconjugation alternatives.[5,6] However, these almost invariably still require requisite reactive groups to be displayed on both peptide and NP, and these are, in turn, often introduced using current labeling chemistry, which reintroduces many of the original issues. Hexahistidine (His_6)-driven metal affinity coordination is a prime example epitomizing what chemoselective and related chemistries can potentially offer to overcome many of the problems associated with current approaches. In practice, we have found it to provide many of the ideal bioconjugation characteristics while still being very simple to implement and highly reliable ($K_D \approx 10^{-9}$ M). His_6 are commonly engineered into expressed proteins using commercially available plasmids for their subsequent purification over nitrilotriacetic

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Table 1. Peptides Facilitating Nanoparticle Endosomal Escape[a]

nanoparticle material	size (nm)	peptide sequence	peptide origin	refs
gold	25	CGGFSTSLRARKA and analogs	adenoviral NLS, others	47
	~20	MVKSKIGSWILVLFVAMWSDVGLCKKRPKP	bovine prion protein-derived peptide	26
	100−180	K_8/R_8	lysine/arginine-rich domain	48
	120	GLFEAIEGFIENGWEGMIDGWYGC	influenza-derived fusogenic peptide (INF-7)	25
multifunctional envelope-type lipid nanodevice (MEND)	160−180	WEAALAEALAEALAEHLAEALAEALEALAA	GALA	49
	131	stearylated-R_8/GALA	GALA	24
PEI/DNA	120−140	LLGRRGWEVLKYWWNLLQYWSQELC	HIV gp41	50
	200−400	PEG-KALA	arginine-rich domain	51
peptide/siRNA	>100	LIRLWSHLIHIWFQNRRLKWKKK	helix-producing vector	52
quantum dot	10−15	WGDDap[a](*Pal*)VKIKKP$_9$GGH$_6$	peptide JB577 (modular peptide)	27, 43
superparamagnetic iron oxide	45	GRKKRRQRRRGYKC	HIV-1 TAT protein	53

[a]Dap(*Pal*) = palmitoyl attached to peptide via a diaminopropionic group; INF = influenza; NLS = nuclear localization signal; PEI = polyethylenimine. More examples can be found in Table S2, Supporting Information.

acid (NTA)-media.[5,6,9,10] For conjugating peptides to QDs in particular, the peptides can be site-specifically synthesized with a His_6 (i.e., either internal or terminal) to facilitate subsequent coordination to ZnS-overcoated QDs.[9,10] This ratiometric self-assembly chemistry usually only requires simple mixing with no purification needed, unless the peptide is in vast excess over QD concentration,[11] while providing for control over peptide ratio, separation, and orientation on the QD. Equally important, it has been exhaustively demonstrated.[9] Commercial availability of reactive-NTA ligands means that almost any NP can be easily functionalized or its surface chemically interconverted to display this ligand and coordinate His_6-appended peptides in a similar manner. Ultimately, no single chemistry will be appropriate for all needs with the choice of chemistry utilized depending on the final application and, of course, practical experimental requirements. A related and complex issue is that of characterizing the number of peptides attached to the NPs.[12] Nevertheless, optimizing NP−peptide display will result in less material being required, improved functionality, and providing room for other "cargo" on the NP.

III. NANOPARTICLE UPTAKE INTO CELLS

Peptides have facilitated the cellular uptake of a wide array of NPs into cells, see refs 1 and 4 and Table S1, Supporting Information for an overview, which highlights the diversity of sequences and nanoparticulate materials. More importantly, several generalized themes have already emerged concerning peptide-mediated delivery of NPs. First, except for a few limited hydrophobic cargos that directly translocate across the plasma membrane, the vast majority of NPs and NP−peptide complexes enter the cell via endocytosis.[13−15] This invariably sequesters the NPs within the endolysosomal system and its vesicular complex where they cannot access subcellular locations without an escape mechanism. Second, NP delivery peptides exhibit a range of cell-type specificity. Positively charged peptides and especially the ubiquitous HIV-1 derived trans-activating transcriptional activator or TAT peptide (i.e., polyarginine) typically use non-specific electrostatic interactions with heparan sulfate proteoglycans on the ubiquitously expressed glycocalyx to initiate uptake,[14] while other peptides (e.g., RGD) rely on receptor−ligand interactions with $\alpha_v\beta_3$ integrin receptors to target primarily endothelial cells. Along with other sequences such as penetratin[16] or transportan,[17] these two classes of motifs tend to form the bulk of what is currently used for generalized cellular uptake of NPs.[13,14] As the name indicates, this class of peptides is usually referred to by the general term—cell penetrating peptides (CPPs). It is also important to be cognizant that the size, aspect ratio, and charge of the NP−peptide conjugate can impact cellular uptake efficiency and distribution kinetics in a manner that is still not fully elucidated.[15,18] If the goal is merely labeling cells for imaging or visualization and subsequent tracking, then endocytosed NPs may be sufficient. However, if the goal is targeting a specific subcellular organelle, then more elegant delivery and targeting schemes are required.

IV. PEPTIDES MEDIATING NANOPARTICLE ENDOSOMAL ESCAPE

Strategies to achieve NP release from the endocytic system to the cytosol initially relied on "proton sponges" to promote vesicle swelling/rupture or, alternatively, direct perturbation of the membrane by a peptide to render the vesicles leaky.[19] The sponge approach relies on delivering NPs to cells in the presence of proton absorbing polymers, such as polyethylenimine (PEI), and these are either mixed with the NPs in excess for delivery or attached to their surface.[20] Subsequent proton "sponging" or absorption in the endocytic vesicles leads to osmotic pressure across the membrane, which drives vesicular swelling and ultimately rupture and the release of contents. The major drawback is the significant resulting cytotoxicity, which commonly occurs as a "side-effect" of this strategy, that, for all intents and purposes, functions by gross disruption of intracellular vesicles. This can, however, be sometimes mitigated by modulating the PEI size and content.[21] Fusogenic, membrane-perturbing peptides have also been demonstrated for endosomal escape of NPs, see Table 1 for examples. Given their inherent membrane insertion/disrupting capabilities, the majority of these sequences are derived from viral coat proteins and typically display a balance between positively charged residues (for initial cell binding) and aliphatic residues (to enhance insertion−vesicle disruption). Amphipathic peptides originating from the fusogenic *Haemophilus influenza* hemagglutinin (HA) proteins and related viruses tend to provide the starting points for designing such sequences.[22] In one representative example highlighting their utility, Futaki used the pH-sensitive GALA fusogenic peptide to accelerate the endosomal escape of plasmid/liposome complexes to the cytosol.[23] This glutamate−alanine−leucine repeat rich peptide, originally designed as a synthetic mimic of a viral fusion protein, transitions from a random coil to an α helix when pH drops from ~7 to 5, forming a transmembrane pore that induces leakage in the acidifying endocytic vesicles. Harashima similarly exploited the

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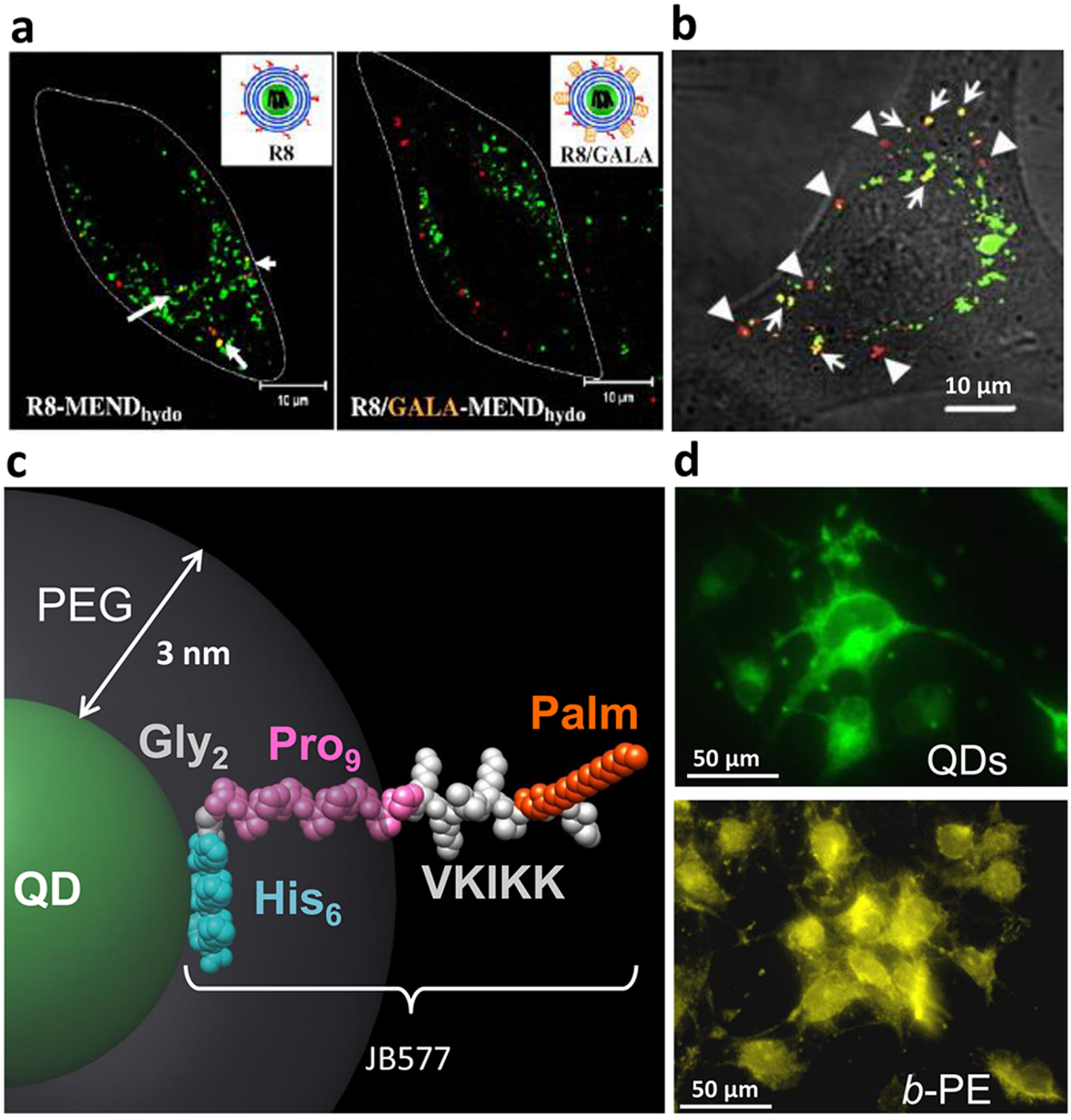

Figure 2. Endosomal escape. (a) GALA peptide-enhanced endosomal release of multilayer enveloped lipid NP device (MEND) NPs for siRNA delivery. R_8 peptide−siRNA complexes in the lipid envelope of MENDs without (left) and with GALA peptides (right). Enhanced NP release was evidenced by the increased red fluorescence. Reprinted with permission from ref 24. Copyright 2011 Elsevier. (b) Stearylated INF-7 peptide (from influenza) facilitates escape of rhodamine-labeled plasmid DNA (red) from endosomes (green). Arrow head, escaped DNA; arrow, plasmid colocalized in endosomes. Reprinted with permission from ref 59. Copyright 2009 Elsevier. (c) JB577 peptide schematic as assembled on the QD surface showing its multiple functional modules and how it presents the VKIKK/palmitoyl (Palm) domains beyond the PEG layer. (d) JB577-facilitated endosomal escape of 550 nm emitting QDs and 250 kDa *b*-phycoerythrin light harvesting complexes (*b*-PE) in COS-1 cells. Uniform staining excluding the nucleus reflects cytosolic delivery. Reprinted with permission from ref 27. Copyright 2013 American Chemical Society.

GALA motif to enhance siRNA endosomal release from multilayer enveloped lipid NP devices (MENDs) realizing a 10-fold increase in gene silencing compared with controls as demonstrated by the two-color micrographs in Figure 2a.[24] Another stearylated IFN7 peptide derived from the N-terminus of HA2 facilitated endosomal release of both large gold-NPs (AuNPs, ∼120 nm)[25] and MENDs as again reflected by the red fluorescence in Figure 2b.[22] Interestingly, the search for improved NP−fusogenic peptides has even extended to bovine prion proteins.[26]

In contrast to relying on viral mimicry, we described a modular or multidomain multifunctional peptide (referred to as JB577) capable of delivering NPs to the cytosol in a process that peaked 48 h following endocytic uptake.[27] Here, the multiple domains that comprise this modular peptide include: (1) His_6 for QD for QD assembly, (ii) a polyproline helical linker to extend away from the QD surface, (iii) a VKIKK domain to mediate membrane binding and endocytosis, and (iv) an aliphatic palmitoyl moiety for membrane insertion (Figure 2c). The latter two modules originate from a CVKIKK sequence in the C-terminus of the K-Ras4A signaling protein, where the C is palmitoylated in the endoplasmic reticulum (ER) allowing it to subsequently insert into the membrane for assembly of the Ras signaling cascade. Confirming the efficacy of this modular approach, NPs and other materials delivered to the cytosol with this peptide included QDs, AuNPs, dendrimers, and various proteins such as a 250 kDa light harvesting complex (Figure 2d). Despite all these examples, we do

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Table 2. Peptides for Targeting Nanoparticles to Subcellular Locations[a]

NP Material	size (nm)	peptide sequence	peptide origin	refs
		Membrane		
QD	11.5	WGDDab[a](*Pal*)VKIKKP$_9$GGH$_6$	JB858 (variant of peptide JB577)	27, 43
liposomes	>100	ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT	bacteriorhodopsin helix C	29
		Endosomal/Lysosomal		
gold	1.5	WGRRVRRRIRRPPPPPPPPPP	HIV-1 TAT	54
	13	YQRLC (fusion to TAT/penetratin peptide)	lysosomal targeting peptide	16
polystyrene	100	NNQKIVNLKEKVAQLEA	fibrinogen-derived ICAM-1 binding sequence	30
peptide/siRNA	70−100	AGYLLGKINLALAALAKKIL	transportan 10	17
		Mitochondria		
iron oxide	76	MALLRGVFIVAAKRTPFGAYGC	mitochondrial 3-oxoacyl-coenzyme-A	55
	80−100	[KLAKLAK]$_2$CGKRK	hybrid tumor homing/proapoptotic peptide	32
QD	11.5	KLWHIKARPVPLRRASGTLGRLLLPTLVSM-*Hyd*-GL(Aib)AAGGH$_6$	chemoselectively ligated cytochrome-C oxidase peptide	43
		Nuclear Localization		
gold	2.4	WGRRVRRRIRRPPPPPPPPPP	HIV-1 TAT	36
liposomes	120	GLFEAIEGFIENGWEGMIDGWYGC	influenza derived fusogenic peptide	25
silica	25−105	GRKKRRQRRRPQ	HIV-1 TAT protein NLS	56
		Endoplasmic Reticulum (ER)		
poly(γ-glutamic acid) NPs	>100	MRYMILGLLALAAVCSA	ER-insertion signal sequence	33
gold	20	KDEL	ligand for ER receptor KDELR	34
PLGA	350	AAKKKAA	ER targeting moiety	57
		Cytosolic		
QD	<15	(DS)$_x$	dentin phosphophoryn	58
	<15	HGLASTLTRWAHYNALIRAFGGG	herpes simplex type 1 virus	37

[a]Dab(*Pal*) = palmitoyl attached to peptide via diaminobutyric group; (Aib) = α-amino isobutyric acid; *Hyd* = hydrazone bond; PLGA = poly(lactic-*co*-glycolic acid). More examples can be found in Table S3, Supporting Information.

note a general trend where endosomal escape peptides that appear to function well for one material−cell-type configuration do not readily translate to another.[28] Clearly, there is far more to learn about how these NP−peptide conjugates function across the spectrum of targeted cellular applications.

V. PEPTIDES FOR NANOPARTICLE TARGETING TO SUBCELLULAR LOCATIONS AND ORGANELLES

Several peptides capable of localizing NPs to specific subcellular locations or organelles have already been demonstrated; for some examples, see Table 2. These peptides are laying the groundwork to potentially create a "plug and play" library where almost any organelle can be targeted by any NP through the choice of peptide displayed on its surface. The plasma membrane is the first point of contact for NP conjugates with cells and NP probes that can localize onto this lipid bilayer are quite valuable for understanding these first critical interactions. However, the constitutive nature of endocytosis has made identifying such peptides quite challenging. To address this problem, Andreev developed a pH-sensitive, membrane-localizing peptide (pHLIP) from bacteriorhodopsin that binds the membrane as a disordered peptide at neutral pH and inserts across the bilayer as an alanine/leucine helix at lower pH where they can remained localized for several hours.[29] In performing a structure−activity relationship (SAR) study on the above-described JB577, modification of the palmitoyl-peptide attachment moiety by addition of one carbon from a diaminopropyl (Dap/C$_3$) to a diaminobutyric (Dab/C$_4$) unexpectedly altered the QD delivery activity from endosomal uptake and cytosolic release to localization of the QDs to the plasma membrane where they resided for days in culture, see Figure 3a.[27]

Moving inward from the membrane, the vesicles of the endo-lysosomal system are another desirable target for NP delivery especially since some disease states are associated with deficient enzymes in this pathway (e.g., Fabry disease). To target these compartments, Garnacho used a lysine-rich peptide from fibrinogen's intracellular adhesion molecule-1 (ICAM-1)-binding sequence for lysosomal uptake of polystyrene nanocarriers (Figure 3b).[30] As mentioned earlier, endosomal uptake and endosomal sequestration is the mechanism and ultimate fate by which most NPs enter and reside in cells; thus use of generalized CPPs can in many cases be sufficient to target these vesicles. It has also been shown that fusion of a lysosomal sorting motif such as YQRLC to the CPP can help ensure a final delivery of the target NP to that organelle.[16] Peptide targeting of NPs to mitochondria has also been demonstrated. In one of the earlier confirmations of this possibility, Hoshino used a 30-mer peptide derived from the cytochrome-C oxidase enzyme to deliver QDs to mitochondria in Vero cells.[31] In another example of a multifunctional and modular peptidic sequence, Ruoslahti identified a CGKRK sequence with specificity for tumor cells and conjugated this to an amphipathic proapoptotic [KLAKLAK]$_2$ peptide meant to destabilize mitochondrial membranes. Demonstrating theranostic (i.e., therapy and diagnostic) type properties, the hybrid peptide was capable of localizing iron oxide NPs to mitochondria for simultaneous imaging and killing of tumor endothelial cells (Figure 3c).[32] Peptides can also direct NPs to the endoplasmic reticulum (ER) of the secretory pathway by exploiting specific ER signaling and retention motifs. For example, Matsuo used ER-insertion signals to deliver poly(γ-glutamic acid) NPs displaying antigenic peptide vaccines for processing through the secretory pathway before being displayed on the cell surface. This induced cellular immune responses, including cytotoxic T lymphocyte activity in mice.[33] Switching to a KDEL an ER retention signal (KDEL) similarly allowed for rapid delivery of AuNPs (15 min) to the ER.[34]

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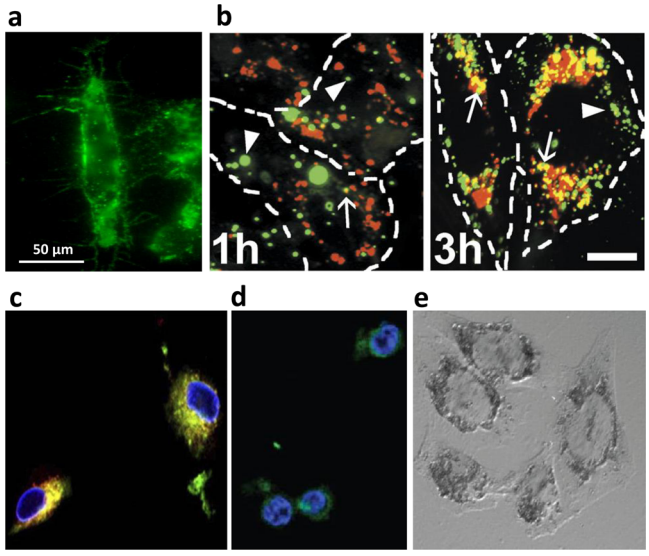

Figure 3. Targeted nanoparticle delivery to subcellular compartments. (a) QD membrane labeling by a modified JB577 (see Figure 2c) that increased the palmitoyl linker by one carbon bond. Note the labeling of cellular processes in this PC12 pheochromocytoma cell. Reprinted with permission from ref 27. Copyright 2013 American Chemical Society. (b) Lysosomes of endothelial cells labeled with polystyrene NPs (green) displaying fibrinogen-derived peptides colocalized (yellow) with dextran (red) at 1 and 3 h following delivery showing increased colocalization with time. Reprinted with permission from ref 30. Copyright 2012 American Society for Pharmacology and Experimental Therapeutics. (c) Iron oxide NPs localized to mitochondria of endothelial cells with strong fluorescence reflecting the large number of mitochondria typically found in cells. Nuclei (blue), NPs (green), MitoTracker (red), colocalized NPs and Mitotracker (yellow). Reprinted with permission from ref 32. Copyright 2011 National Academy of Sciences. (d) PEGylated gold NPs bearing SV40 NLS peptides (green) show accumulation at the nuclear envelope (DAPI, nucleus), and (e) nuclear accumulation (silver staining). Reprinted with permission from ref 35. Copyright 2012 Dove Medical Press.

As the site of genetic storage and initial gene transcription, nuclei are one of the primary organelles for NP targeting, and delivery here commonly exploits the Simian virus large T antigen nuclear localizing sequence (SV40 NLS).[4] Moving beyond attaining nuclear delivery, Tsai elegantly demonstrated that a combination of this peptide and NPs could actually block transcripts from exiting the nucleus. PEGylated 13 nm diameter AuNPs appended with SV40 NLS demonstrated a specific accumulation at the cytoplasmic side of the nuclear membrane in HeLa cells resulting in blockage of nucleocytoplasmic transport and prevention of RNA export and nuclear shuttling of signaling proteins (Figure 3d,e).[35] This, however, was not observed in SiHa cells, indicating cell-line dependent responses to this conjugate and suggesting the exciting possibility of a new type of NP-based tumor specific therapy. It is also highly probable that there is a NP size dependency to the process for attaining nuclear entry or blockage as noted before.[36]

Some peptides have been reported to provide for membrane translocation of both themselves and appended NP cargo directly to the cytosol. This is a very contentious issue in this field with strong proponents both for and against this process. It is now somewhat accepted that this direct translocation mechanism does occur; however, as stated before the vast majority of NP materials enter cells by some form of endocytosis.[4,13,14] Thus, in order to assert this as the primary mechanism for NP delivery to the cytosol, several critical criteria must be rigorously met: demonstration that cytosolic delivery is energy-independent (i.e., not endocytosis) and stringent imaging with multiple well-discriminated cellular markers. In limited instances strong evidence confirming these criteria have been met. For example, Galdiero described a gH625 amphipathic peptide that delivered QDs across the membrane of HeLa cells. Counterstaining and control experiments in the absence of endocytosis confirmed membrane translocation of the conjugates.[37] In another such example, Liu utilized histidine/arginine-rich peptides that noncovalently bound to QDs to achieve cytosolic delivery in 4 min where they colocalized with F-actin in the cytosol.[38]

VI. SELECTING IMPROVED PEPTIDYL MOTIFS AND MODULAR MULTIFUNCTIONAL PEPTIDES

The growing interest in organelle-specific NP delivery is, in turn, driving research into identifying new peptidyl motifs that can impart novel functionalities to the NP−peptide conjugate. Identifying these novel peptides typically involves either directed *in vitro* evolution and library selection or structure/activity relationship (SAR) analyses based on an initial rational design. In a demonstrative example of the former approach, Ting used phage display screening combined with biotin ligase substrate selectivity to develop a strategy allowing for two-color labeling of cell membranes with different colors of QDs.[39] Cells were grown to express *Escherichia coli* and yeast biotin ligase, and this was confirmed by fusing these proteins to different fluorescent proteins. Acceptor peptides selected for either the *E. coli* or yeast biotin ligase displayed on two different colors of QDs provided the specificity to bind to their cognate enzymatic partners on the target cells (Figure 4a).[39,40] The ability to site-specifically label cell membrane targets with two different NPs will clearly provide an important tool for probing the complex interplay, turnover, and signaling that continuously takes place here. In another study that sought to identify new CPP sequences, Banta used plasmid display to screen a randomized 14-mer peptide library in PC12 cells.[41] The library was fused to a DNA-binding protein so that once expressed, the CPP was noncovalently associated with its encoding plasmid, which facilitated its selection. This produced peptide SG3 (RLSGMNEVLSFRWL), which differs considerably from other CPPs in that it is not rich in cationic amino acids. In an example of utilizing SAR to improve peptide function, Brock investigated the effects of conformational constraint on increasing CPP activity.[42] Studying bicyclic peptides where the number/position of arginines was varied yielded a general correlation between uptake efficiency and the number of arginines (Figure 4b,c). Linear stretches of arginines coupled outside the bicycle were as effective in promoting cellular uptake as a substitution inside the bicyclic structure.

In addition to new CPPs, there is strong interest in developing modular/multifunctional peptides, that is, those incorporating multiple desirable targeting and delivery functionalities within a single, contiguous sequence. These contrast with monofunctional peptides or motifs (e.g., TAT) where a single domain imparts a single function such as cellular uptake. Such modular peptides would provide a viable alternative to either the almost insurmountable challenge of finding one sequence capable of all desired functions or, alternatively, attaching different peptides with different functionalities to the same NP, in what would most likely be different ratios, when trying to orchestrate their net effects. The aforementioned JB577 (see Figure 2c,d) epitomizes the concept of modularity because it contains four domains each of which provides a different functionality.[27,28] Another

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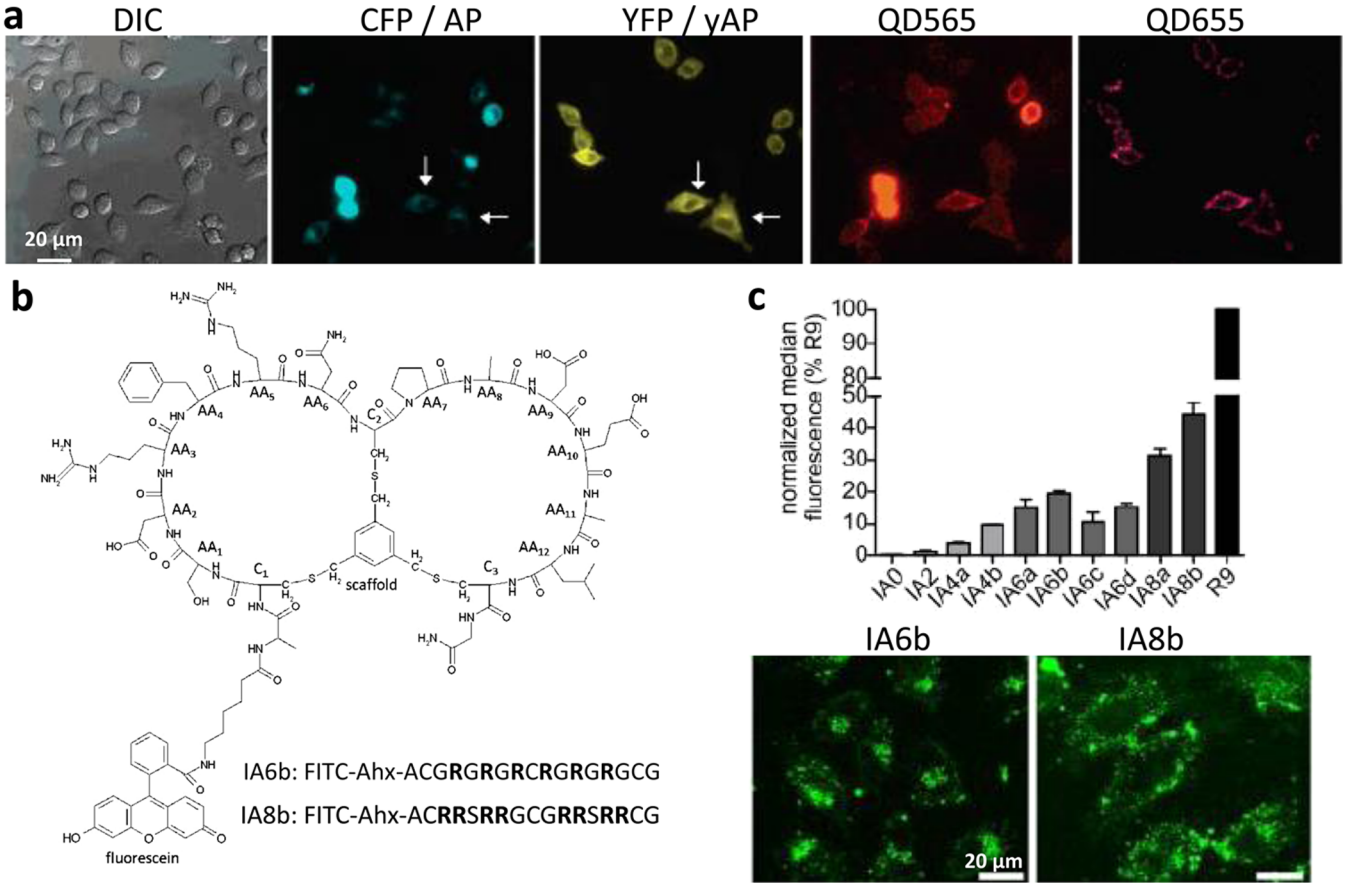

Figure 4. Selecting improved peptidyl motifs for cellular nanoparticle delivery. (a) Two-color QD labeling of HeLa cells coexpressing cyanine fluorescent protein (CFP) and *E. coli* biotin ligase acceptor peptide (AP) labeled with 565 nm-emitting QDs and cells coexpressing yellow fluorescent protein (YFP) and a yeast biotin ligase acceptor peptide (yAP) probed with 655 nm QDs. Arrows indicate cells coexpressing AP and yAP acceptor peptides and labeled with both QDs. Reprinted with permission from ref 39. Copyright 2007 American Chemical Society. (b) Bicyclic peptide structure used to interrogate effects of arginine presentation on cellular uptake (left). Inset shows two representative peptide sequences. (c) Flow cytometry (top) and confocal imaging (bottom) showing positive correlation between arginine number and display position compared with a control CPP (R9). Reprinted with permission from ref 42. Copyright 2014 American Chemical Society.

prominent example of such a sequence is Pep-1, which displays three domains: a hydrophobic domain (KETWWETWWTEW) mediating interactions with the membrane and noncovalent assembly to other peptide/proteins, a hydrophilic/charged region (KKKRKV) for solubility, and a flexible linker domain (SQP) joining the two (see Figure 5a). Commercially available as Chariot, Pep-1 has already delivered both peptide[17] and QD cargos[43] to a variety of cells. In the example shown in Figure 5a, not only does Pep-1 deliver a dye cargo to the cellular cytosol, it continues on to the nucleus on its own confirming that beyond modular, it may also be multifunctional.

In an alternative and somewhat ingenious approach to modularity, Tagalakis incorporated Y and ME27 targeting/uptake peptides into pegylated liposomal siRNA carriers to demonstrate the efficient inhibition of GAPDH expression in Neuro-2A cells (Figure 5b).[44] Both peptides contain domains for cell targeting/internalization, and here they work in tandem to facilitate cell targeting/siRNA-mediated gene knockdown. Lastly, Andaloussi et al. developed a multifunctional peptide vector for delivery of siRNA NPs that, similar to the palmitate on JB577, incorporates a non-amino acid moiety.[17] Termed PepFect6, the peptide is derived from the canonical transportan-10 peptide (Table 2), which contains a portion of the neuropeptide galanin at the N-terminus and a stearyl group at the C-terminus (for lipophilic interactions). Further modification with proton-accepting trifluoromethylquinoline facilitated endosomal escape of siRNA by promoting osmotic swelling. These constructs efficiently delivered siRNA directed against the Oct6 mouse embryonic stem cell pluripotency regulator, Figure 5d.

VII. PERSPECTIVE

In looking to the future, we draw from some recent results to see what more may be possible. Of course, far more work is still required to optimize both the assembly of the NP−peptide conjugates themselves and their subsequent bioanalytical characterization.[5−7,12] Both these issues, and especially the latter, will be critical if and when regulatory approval is ever sought for such nanomaterials. A recent example where two chemoselective chemistries, namely, (His_6)-peptide self-assembly with on-QD aniline catalyzed hydrazone ligation, were coupled to yield controlled NP biofunctionalization highlights what potentially is possible with designer chemistries and NPs.[10] Here almost all the "ideal" criteria desired from such NP chemistry were met. Given the complexity of the cellular environment, undertaking an efficient, homogeneous, and controlled NP−bioconjugate assembly will help to ensure optimal performance within the cell while requiring a minimal amount of material. Reducing the amount of NP materials to which a cell is exposed will also

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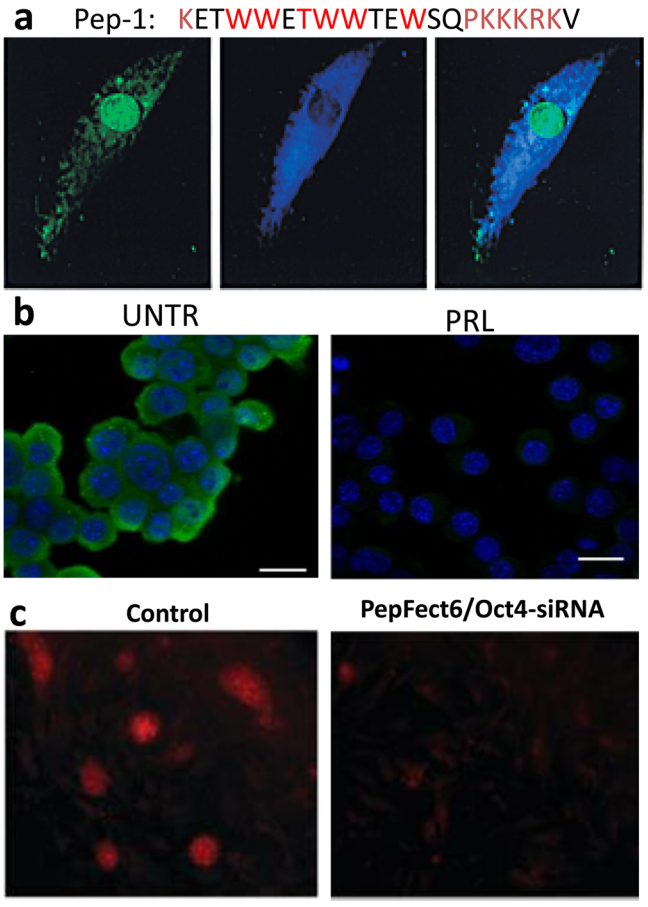

Figure 5. Modular, multifunctional peptides. (a) Pep-1 peptide (green, left) delivers a coumarin-labeled peptide cargo (blue, middle) to the cytosol while the Pep-1 carrier localizes to the nucleus of human fibroblasts after dissociation from the cargo (right). Reprinted with permission from ref 60. Copyright 2001 Nature Publishing Group. (b) Pegylated RNA–liposome (PRL) NPs comprised of cationic peptides Y and ME27 (P), siRNA (R), and liposomes (L) delivered to Neuro-2A cells for knockdown of GAPDH expression. Fluorescent staining of GAPDH expression in green. Untreated (UNTR) or nonpegylated lipid carriers show no knockdown of gene expression, while pegylated lipid formulations (PRL) showed significant inhibition of GAPDH expression. Reprinted with permission from ref 44. Copyright 2014 Elsevier. (c) PepFect6 delivery of Oct6 (pluripotency regulator) siRNA results in >50% knockdown of expression (red) in mouse embryonic stem cells. Reprinted with permission from ref 17. Copyright 2011 Oxford University Press.

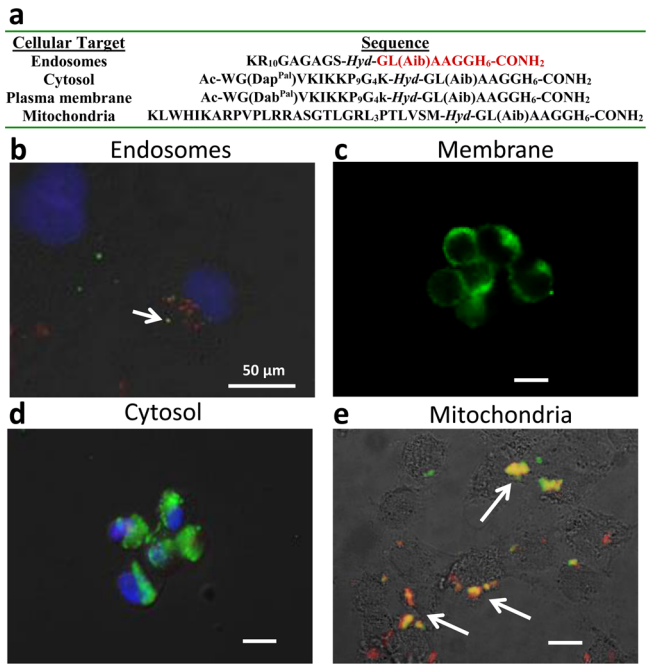

Figure 6. "Plug-and-play" modular QD delivery to discrete subcellular targets in COS-1 cells. (a) Sequences of each peptide and their cellular targets. Red highlights the common His_6 starter peptide to which the targeting sequences are ligated. *Hyd*, hydrazone bond formed during ligation; Aib, α-amino isobutyric acid; $CONH_2$, amide/Ac-acetyl; Dap, diaminopropionic acid; Dab, diaminobutyric acid; Pal, palmitoyl group. (b) Peptide-mediated QD delivery to endosomes. Arrow indicates region of colocalization of CPP-delivered QDs with the AF647-Tf marker within endosomes. (c) Membrane labeling peptide localizes QDs to the plasma membrane. (d) Palmitoylated peptide provides for initial endocytosis and subsequent release of QDs to the cytosol. (e) Mitochondrial targeting peptide directs QD delivery. Marker shows morphology of mitochondria colabeled with Mitotracker Red. Arrows indicate QD–mitochondria colocalization. For all images, QDs in green, nuclei in blue, and markers in red. Reprinted with permission from ref 43. Copyright 2013 Royal Society of Chemistry.

minimize any toxicity while maintaining the expected cellular function.[45]

The ultimate goal for organelle targeting peptides and NPs would be the creation of a "plug-and-play" library where choice of the delivery peptide conjugated to any desired NP material would determine its intracellular fate. These peptides should also be very efficient allowing for a minimal number to be displayed on the NP, thus leaving room for exogenous sensor and drug cargo as part of the NP conjugate. Some recent work suggests that this may indeed be attainable. To initially assemble the peptides used in this demonstration, aniline catalyzed hydrazone ligation was again utilized.[43] Identical His_6 "starter" peptides were coupled to four distinct targeting sequences (Figure 6a), which were then, in turn, self-assembled to QDs by metal affinity providing each conjugate with the ability to undergo specific delivery to either the plasma membrane, endosomes, mitochondria, or the cytosol, see micrographs in Figure 6b–e.[43] Following from this, it is not unreasonable to expect an expansion of the peptide library with the near-term description of other peptidyl motifs targeting far more subcellular locations. This will continuously expand the list of available cellular targets and their combinatorial use in more complex NP-labeling and delivery schemes should soon thereafter also start to appear.

Performing a SAR-type study on the JB577 endosomal escape peptide by discretely modifying the sequences of each of its modules led to selection of far more potent peptides along with one that unexpectedly localized QDs to the plasma membrane.[27] These findings have important long-term implications for the overall current effort. It implies that more complex selection strategies using peptide display on NPs can be implemented in the context of cellular delivery and targeting in an assay format reminiscent of that used in drug discovery. Such selection schemes in conjunction with the power of peptide combinatorial chemistry will surely provide new sequences capable of imbuing NPs with a host of desirable functionalities.[46] Further, incorporating non-natural residues displaying chemical functionalities not typically found on amino acids or even in biological environments into this mix can potentially provide access to almost unlimited chemical diversity. As we witnessed with the membrane localization, unexpected findings can sometimes be

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the more exciting. Such systems will also provide fundamental insight into mechanisms directing membrane translocation and escape of NPs from within endosomes. Understanding these complex processes can also allow targeted studies into important related issues such as NP toxicity and effects from long-term cellular persistence of constituent materials. Ultimately, the ability to deliver designer theranostic NPs to the subcellular structures of targeted cells in a patient's body will draw heavily from the initial lessons learned with these peptides.

■ ASSOCIATED CONTENT

Supporting Information

Lists of representative peptides used for cellular uptake of NPs along with more examples of peptides from Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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