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The importance of valency in enhancing the import and cell routing potential of protein transduction domain-containing molecules

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Abstract

Protein transduction domains (PTDs) are peptides that afford the internalization of cargo macromolecules (including plasmid DNA, proteins, liposomes, and nanoparticles). In the case of polycationic peptides, the efficiency of PTDs to promote cellular uptake is directly related to their molecular mass or their polyvalent presentation. Similarly, the efficiency of routing to the nucleus increases with the number of nuclear localization signals (NLS) associated with a cargo. The quantitative enhancement, however, depends on the identity of the PTD sequence as well as the targeted cell type. Thus the choice and multivalent presentation of PTD and NLS sequences are important criteria guiding the design of macromolecules intended for specific intracellular localization. This review outlines synthetic and recombinant strategies whereby PTDs and signal sequences can be assembled into multivalent peptide dendrimers and promote the uptake and routing of their cargoes. In particular, the tetramerization domain of the tumour suppressor p53 ($p53^{tet}$) is emerging as a useful scaffold to present multiple routing and targeting moieties. Short cationic peptides fused to the 31-residue long $p53^{tet}$ sequence resulted in tetramers displaying a significant enhancement (up to 1000 fold) in terms of their ability to be imported into cells and delivered to the cell nucleus in relation to their monomeric analogues. The design of future polycationic peptide dendrimers as effective delivering vehicles will need to incorporate selective cell targeting functions and provide solutions to the issue of endosomal entrapment.

1. The concept of multivalency and its applications

Multivalency (or polyvalency) refers to the presence of multiple copies of a functional element within a single molecule. In terms of receptor–ligand interactions, a multivalent ligand may interact with a multivalent receptor or a cluster of monovalent receptors [1]. Multivalent interactions are commonly observed in biological systems; examples include viral and bacterial adhesion to host cell surfaces, host cell–cell interactions, and many aspects of the humoral immune response. A major advantage conferred by multivalent interactions is the often dramatic enhancement in avidity of a polyvalent ligand for its receptor over the affinity of a monovalent counterpart [2,3]. More specifically, the display of several spacially proximal ligands within a molecular scaffold results in a greatly increased local concentration of recognition elements near a targeted receptor, a phenomenon known as the chelate effect [1,4,5].

One of the earliest examples of multivalent interactions is the oligomerization or duplication of a protein ligand such as an Ig domain (exemplified by the structure of IgG and IgM) or a lectin-binding motif (such as the binding subunits of cholera toxin and Shiga toxin) [6]. This concept has recently been exploited for the design of single-chain antibodies (scF*v*) oligomers. Specifically, scF*v*'s are genetically engineered antibody fragments built by linking the V_H and V_L chains of a known antibody through a flexible linker to produce a single polypeptide chain mimic of the original Fab domain [7]. Because of their small size (~27 kDa) and the practical fact that they are amenable to expression as recombinant proteins, scF*v*'s have become popular reagents for antibody-based therapies [8]. However, their reduced mass and monovalency also shorten their in vivo half-lives due to rapid renal clearance. By fusing an oligomerization motif to the scF*v* domain, non-covalent dimer (diabodies), trimers (triabodies) and tetramers

Abbreviations: Antp *Drosophila*, antennapedia homeodomain; NLS, nuclear localization sequence; Np, nucleoplasmin; $p53^{mono}$, monomeric form of human p53 tetramerization domain (residues 325–355) harbouring a L334P mutation; $p53^{tet}$, human p53 tetramerization domain (residues 325–355); PTD, protein transduction domain; scF*v*, single-chain antibody; Tat, HIV-1 transactivator of transcription

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(tetrabodies) of scF*v*'s have been developed which exhibit increases in both in vivo half-lives, tumour uptake and residence time in tumours [9–13]. The use of oligomerization domains to create multivalent scF*v*'s is essentially reminiscent of the multivalent architecture of IgG and IgM molecules.

Polyvalent scF*v*'s represent but one example of a more general phenomenon in which the multivalent display of a targeting function on a macromolecule can lead to a quantitative increase in localization of such constructs to cells. A number of publications have described the relationship between valency and avidity, mostly as it pertains to the design of synthetic multivalent ligands for use as inhibitors and activators [1,3,14]. Protein transduction domains (PTDs) and in particular polycationic peptides have also been used to shuttle macromolecules such as DNA, proteins and other cargoes across cellular membranes. This review will highlight the importance of valency effects in designing effective PTD-containing macromolecules and its impact in terms of cellular import with a focus on polycationic cell-penetrating peptides. Examples of covalent and non-covalent strategies for displaying multiple copies of PTD peptides onto macromolecules will be discussed in the context of enhancing the uptake of such conjugates by cells. In this regard, short self-assembling peptides derived from known oligomerization domains of proteins are emerging as excellent scaffolds for generating defined protein-like constructs displaying multiple PTD domains. More precisely, we report our recent progress in using the human p53 tetramerization domain, in promoting the cellular uptake and nuclear routing of cationic peptides.

1.1. A role for multivalency in transduction and transfection mediated by polycationic PTDs

Polycationic peptide sequences such as polylysine, polyarginine and polyornithine are known for their cell penetrating properties [15–17]. From a historical perspective, Ryser had demonstrated in 1965 that homopolymers of cationic amino acids were able to facilitate the import of albumin into adherent cell monolayers as well as cell suspensions [18,19]. More importantly, the level of albumin import correlated directly with the molecular size of the polycations (Fig. 1A) [20]. The correlation further suggested that a minimal peptide mass of 500 to 900 Da, corresponding to 4 to 8 amino acids in length, was necessary to effect albumin import [20]. This projection was subsequently proven with the discovery that short arginine- and lysine-rich peptides derived from the HIV-1 transactivator of transcription domain (Tat peptide), the third helix of the *Drosophila* Antennapedia homeodomain (Antp peptide), and herpesvirus VP22 could facilitate protein transduction, DNA transfection and the shuttling of other macromolecular cargoes into eukaryotic cells in the context of in vitro and in vivo experiments [21–23]. Although these peptides are at the low end of peptide masses proposed by Ryser to cause cellular uptake [18,20], they are surprisingly efficient at importing a wide range of cargoes into cells. Multivalency may provide the rationale for the observed effectiveness of PTD peptides. A role for multivalency in enhancing the internalization property of

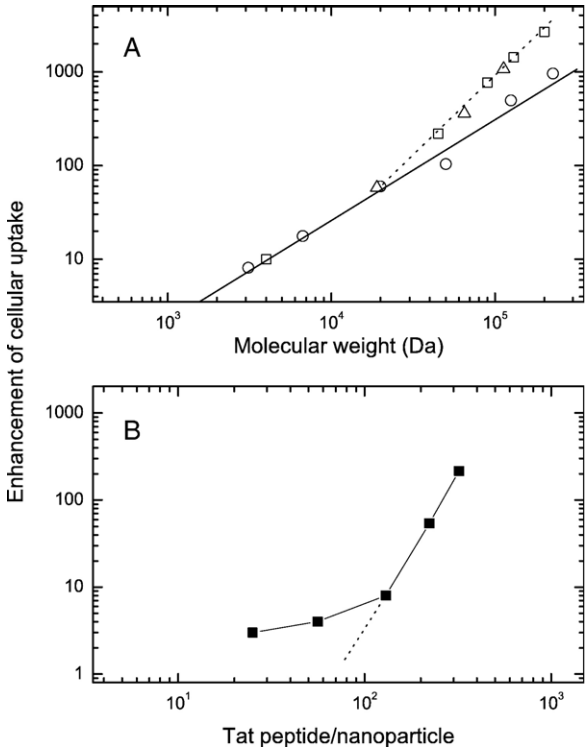

Fig. 1. The importance of cationic peptide mass and PTD-to-cargo ratio in enhancing the cellular uptake of macromolecules. (A) Linear relationship linking an increase in molecular mass of poly-L-lysine (○), poly-D-lysine (△) and poly-L-ornithine (□) conjugated to radiolabelled human serum albumin to the enhancement in cellular uptake of the resulting complex relative to radiolabelled albumin alone (adapted from [20]). (B) Tat peptides were attached to the surface of radiolabelled superparamagnetic iron oxide nanoparticles at various ratios and its uptake expressed relative to the nanoparticle with the lowest Tat peptide/nanoparticle ratio (redrawn from [29]).

short cationic peptides was first demonstrated by Gariépy and coworkers who showed that the assembly of pentalysine sequences into a multivalent scaffold can mimic the enhancement of cellular uptake observed by Ryser for much longer cationic peptide polymers [24–28]. More recently, the level of conjugation of HIV-1 Tat peptides to superparamagnetic nanoparticles was shown to influence their cellular uptake by mouse lymphocytes (Fig. 1B) [29]. Specifically, the coupling of up to 15 Tat peptides linked per nanoparticle generated a 100-fold increase in relative uptake. Both Fig. 1A and B indicate, in two distinct experiments, that a threshold value must be reached before the level of cellular uptake becomes linearly related to the mass of a polycationic peptide or to the PTD-to-cargo ratio. The biphasic nature of the curve in Fig. 1B suggests that one mechanism of cellular uptake becomes dominant when a threshold of PTD-to-particle (valency number) is reached.

1.2. Oligomerization of a cargo results in a multivalent display of PTDs

An analysis of the published literature to date on the use of short cationic PTD peptides provides many examples linking

Table 1
A representative list of multivalent PTD-based systems in the literature

Oligomerization state	Cargoes	PTDs	References
Dimer	β-galactosidase	Tat	[30–32]
	Superoxide dismutase	Tat	[33,34]
		Oligolysine (9)	[35]
	Caspase-3	Tat	[36]
	Thymidine kinase	Tat	[37]
		HSV-1 VP22	[38,39]
Tetramer	Ovalbumin	Tat	[40–42]
	p53	HSV-1 VP22	[22]
		Oligoarginine (11)	[43,44]
	Avidin/Streptavidin	Tat	[45]
		Antp	[46,47]
	Catalase	Tat	[48,49]
	Beta-glucuronidase	Tat	[50]
Hexamer	Glutamate dehydrogenase	Tat	[51]
Octamer	Rhodamine	Loligomers	[52]
	CTL antigen	Loligomers	[26]
Oligomer multivalent complex	p16(INK4a)	Tat	[53,54]
		Antp	[55]
	Human Serum Albumin	poly-L-lysine	[18,20]
	Liposome/lipid envelope	Tat	[56,57]
		Antp	[58,59]
		Oligoarginine (8)	[60]
	Nanoparticles	Tat	[61–63]
	DNA/plasmids	Tat	[64–66]
		Antp	[67]
		Poly-L-lysine	[68–70]
		Oligolysines	[71]
		Loligomers	[27]
	Heparan Sulfate	Tat	[72]
Aggregated Complex *	Grb10 SH2 domain	Antp	[73]
	BH4 domain of Bcl-XL	Tat	[74]
	FNK, a Bcl-XL derivative	Tat	[75]
	CRAC domain of benzodiazepine receptor	Tat	[76]
	Influenza matrix protein epitope	Tat	[77]

* Peptide sequences evaluated using TANGO algorithm [78,79] to determine the likelihood of α-helix and β-sheet driven aggregation.

valency effects and the internalization efficiency of such short peptides (Table 1). In many instances, it is the cargo itself that affords the multivalent assembly of PTDs, when the cargo naturally exists in an oligomeric state. For example, several types of PTD sequences (Antp, VP22 and oligoarginine) have been fused to human p53, a tumour suppressor containing a tetramerization domain (Table 1). Proteases such as Cu,Zn-superoxide dismutase, which form a highly stable dimer [80–82], and β-galactosidase, a highly thermostable dimer at alkaline pH [83–85], retain their quaternary structure even when fused as cargoes to PTDs [83,84,86,87]. Avidin and streptavidin can present either four copies of PTDs when both entities are engineered as fusion proteins [88,89] or up to four copies of PTDs per avidin/streptavidin when these proteins are mixed with biotinylated PTD peptides [90].

1.3. Self-aggregation, complexation and covalent attachment lead to the macromolecular display of multiple PTD peptides

Aggregation, complexation, and coupling mechanisms have either been observed or exploited to pair multiple PTD peptides with a cargo such as plasmid DNA, liposomes or nanoparticles [57,59,62,66]. Torchilin and Levchenko's groups have reported the preparation of liposomes incorporating the PEGylated lipid *p*-nitrophenylcarbonyl-PEG-phosphatidylethanolamine (pNP-PEG-PE) as a strategy to attach Tat peptides through the *p*-nitrophenylcarbonyl moieties on the liposome surface [57]. On average, over 100 Tat peptides were presented on the surface of each liposome [57]. In addition, these authors reported the transfection of mouse fibroblasts as well as Lewis lung carcinoma tumour cells in the mouse using Tat peptide-attached liposome preparations containing only a small quantity (≤10 mol%) of cationic lipids (to further promote DNA condensation) [91,92]. In their study, Tseng et al. demonstrated that both Tat and Antp peptides can enhance the efficiency of translocation of liposomes in proportion to the number of peptides attached to the liposomal surface [59]. Their work also showed that the observed enhancement in cellular uptake was dependant on the PTD peptide used and the cell line being transfected. Specifically, the addition of five Tat peptides/liposome produced almost a 100-fold increase in liposome translocation, while 50 Antp peptides/liposome were necessary to observe a significant translocation enhancement in comparison to non-conjugated liposomes. The dextran coat covering magnetic nanoparticles has also be modified with Tat peptides to achieve a high valency of approximately 20 peptides per nanoparticle [29,93]. Such particles have been used to track hematopoietic stem cells and antigen-specific T lymphocytes [62,94]. As in the case of liposomes, the internalization efficiency of such particles was proportional to the number of PTD peptides conjugated to their surface [29].

Branched peptides containing eight PTD peptides have also been useful in condensing DNA for cell transduction purposes [27]. Specifically, plasmid DNA needed to be condensed and its negative charges neutralized with cationic peptide dendrimers in order to create DNA–peptide complexes capable of being internalized by cells. For instance, Singh et al. showed that a minimal weight ratio of 0.5 of a nucleus-directed polycationic peptide dendrimer termed loligomer 4 was needed to abolish the electrophoretic mobility of a condensed peptide/plasmid complex [27]. For optimal transfection efficiency, the loligomer 4:plasmid ratio needed to be even higher (about 3) [27].

Finally, the multivalent presentation of PTD peptides may also occur as a result of an aggregation event with their cargo molecules. For instance, fusion constructs of Antp and Tat PTDs with peptide/protein cargoes such as the Grb10 SH2 domain or the BH4 domain of Bcl-XL are predicted to form molecular aggregates in solution by virtue of a stretch of 5 or more amino acids in the cargo peptide sequences which displays a greater than 50% probability of undergoing β-strand

driven aggregation, as predicted using the algorithm TANGO. This algorithm calculates the propensity of peptide sequence in forming secondary structure-based aggregates [78,79] (Table 1).

In summary, the physical features of a cargo as well as the methods of introducing PTD peptides onto a cargo can readily lead to the assembly of conjugates or complexes presenting multiple copies of a PTD peptide.

1.4. The rate of nuclear import is also dependent on the number of nuclear localization sequences associated with a cargo

Intracellular routing signals, in particular the SV40 large T-antigen nuclear localization sequence (NLS), have been extensively used to further relocate a fraction of imported molecules to the nucleus of targeted cells in an effort to deliver agents such as photosensitizers near nucleic acids [95].

Laskey and coworkers were the first investigators to demonstrate the existence of a nuclear localization domain while studying the protein nucleoplasmin (Np), an acidic protein abundantly expressed in the *Xenopus* oocyte nucleus. Np is a pentamer in which a bipartite NLS is incorporated in the C-terminal tail domain of each Np monomer. Laskey's group showed that microinjecting Np pentamers containing a single NLS tail (instead of five) per unit into the cytoplasm of oocytes was sufficient for transport of the protein into the oocyte nucleus [96]. More importantly, the rate of accumulation of Np in the cell nucleus depended on the number of NLS present per pentamer [96]. Dworetzky et al. subsequently showed that the relative nuclear uptake of gold nanoparticles coated with Np or BSA conjugated with the SV40 large T-antigen NLS increases with the number of NLS associated with such particles [97]. In summary, the import and the routing of macromolecules to the cell nucleus are events that can be enhanced through valency effects. Incidentally, since NLS sequences are by their very nature arginine- and lysine-rich peptides, they may also by themselves serve as PTD domains.

1.5. Incorporating multivalency into the design of PTD-containing vehicles

Both covalent and non-covalent approaches can be used to design constructs or cargoes harbouring multiple copies of cell-penetrating and intracellular routing peptides. The simplest but least controlled approach is the covalent integration of PTD and NLS sequences into protein or macromolecular scaffolds using chemical crosslinkers to produce complexes with high peptide-to-cargo ratios (high valency). Polylysine has been randomly coupled to larger macromolecules (serum albumin) to create macroscopic drug carriers [98].

Alternatively, polylysine chains have been assembled in a systematic way onto branched lysine core peptides called loligomers [26,27]. The term loligomer is derived from the Latin root *loligo* referring to members of the squid family, thereby emphasizing the branched or "squid-like" nature of these peptides. Loligomers have been designed to contain eight SV40 large T-antigen NLS sequences as well as eight pentalysine stretches acting as PTD sequences which allow such peptide dendrimers to penetrate cells and relocate to their nucleus [52]. Loligomers also include a C-terminal arm to introduce reporter groups into their structures [28]. These peptide dendrimers typically outperform their linear, monomeric homologues (one arm of the dendrimer incorporating the PTD and NLS sequences) in terms of cellular uptake and nuclear localization into CHO cells [52]. Loligomers harbouring antigenic epitopes have also been shown to generate cytotoxic T-cell responses [26]. Although the loligomer design is a significant improvement from the linear presentation of cationic PTD sequences, their assembly by solid-phase peptide synthesis places limits on the size and homogeneity of the final constructs.

Recombinant approaches offer a defined, systematic approach to incorporate PTD and NLS peptides into fusion constructs. For example, protein constructs can be engineered to integrate a linear series of routing peptides. As the size of such peptides increases, the resulting constructs are expected to adopt a tertiary structure (tertiary fold) that may interfere with the presentation of individual domains. By introducing an oligomerization domain within linear peptide constructs, peptide dendrimers can instead be created. This display strategy would guide the folding on such peptides into known quaternary structures (as dictated by the oligomerization domain) and permit the equivalent exposure of multiple routing domains via several N- and C-termini. As a prerequisite, these self-assembling domains must be encoded by relatively short peptide sequences and must create stable quaternary structures. Coil-coiled sequences (leucine zipper; multiple of heptad sequences of 28 to 35 amino acids in length) [99] and the p53 tetramerization domain ($p53^{tet}$; 31 amino acids) represent established examples of oligomerizing sequences. The $p53^{tet}$ domain offers the advantage of forming tetrameric peptide dendrimers that approximate the 8-branch scaffold of loligomers (tetramers with 4 N- and 4 C-terminal sites for introducing functional domains). In summary, the incorporation of a self-assembling domain into a linear peptide sequence permits the use of standard recombinant approaches to string together routing and functional domains in a modular fashion. This modular approach to designing peptide dendrimers as delivery vehicles represents a powerful approach for exploiting the concept of valency in enhancing the delivery of cargoes into cells and tissues.

1.6. Peptide dendrimers based on the non-covalent assembly of peptides incorporating the p53 tetramerization domain

Peptide-based delivery vehicles can be assembled into dendrimers by inserting the minimal tetramerization domain of human tumour suppressor p53 within their sequence. This tetramerization domain is a 31-amino acid long peptide representing residues 325 to 355 of human p53. It self-assembles into a stable tetramer with a melting temperature near 80 °C under physiological conditions [100]. The crystal and NMR structures of $p53^{tet}$ indicate that residues 325–255 forms a "dimer of dimers" in which each monomer adopts a β strand-turn-α helix fold conformation [101] (Fig. 2A). In order to

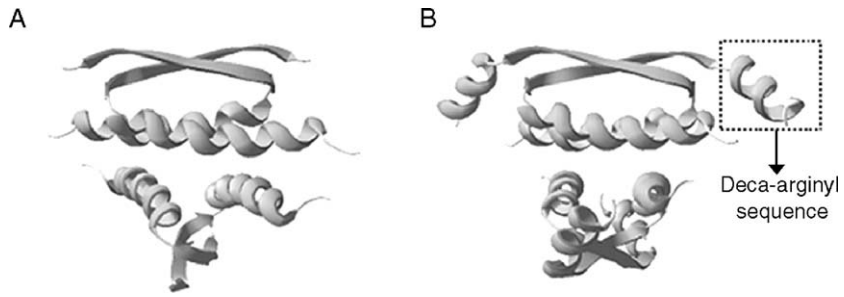

Fig. 2. Ribbon representations of (A) the minimal p53 tetramerization domain (325–355) from coordinates derived by NMR spectroscopy (Protein Data Bank code: 1PES [101]) and (B) a deca-arginyl-p53^{tet} fusion construct (10R-p53^{tet}) where a sequence of 10 arginine residues was fused to the N-terminus of the minimal p53 tetramerization domain. This model was constructed from the Swiss PDB Viewer Molecule Builder and Energy Minimization functions.

design peptide dendrimers that would exploit the valency effects previously observed for loligomers, we have recently constructed peptides (by synthetic or recombinant approaches) in which PTDs as well as other functional motifs were directly fused to either the C- or N-terminus of the p53 tetramerization sequence. The resulting constructs were shown to self-assemble into peptide dendrimers (tetramers). More specifically, we have fused a deca-arginyl (10R), deca-lysyl (10K) or the HIV-1 Tat sequence (GRKKRRQRRRAP; residues 48–60) to the N-terminus of the p53^{tet} domain. A ribbon model of the structure of the deca-arginyl p53^{tet} peptide is shown in Fig. 2B. Peptides containing both the PTD sequences as well as the NLS from the SV40 large T-antigen were also constructed. Monomeric versions (p53^{mono}) of these tetrameric peptide vehicles were generated by inserting a L334P mutation within the p53 tetrameric domain [102]. Routing functions such as the cellular uptake and nuclear localization of the tetrameric constructs were shown to be dramatically enhanced with the resulting tetravalent peptides typically displaying between 10- and 100-fold enhancement in cellular import and intracellular routing properties in relation to their monomeric analogues (Fig. 3A, first 6 h) [103]. These constructs were not toxic to cells. Flow cytometry results and transfection assays indicated that tetravalent deca-arginyl peptides (10R-p53^{tet} and NLS-10R-p53^{tet}) were the most efficiently routed constructs into cells.

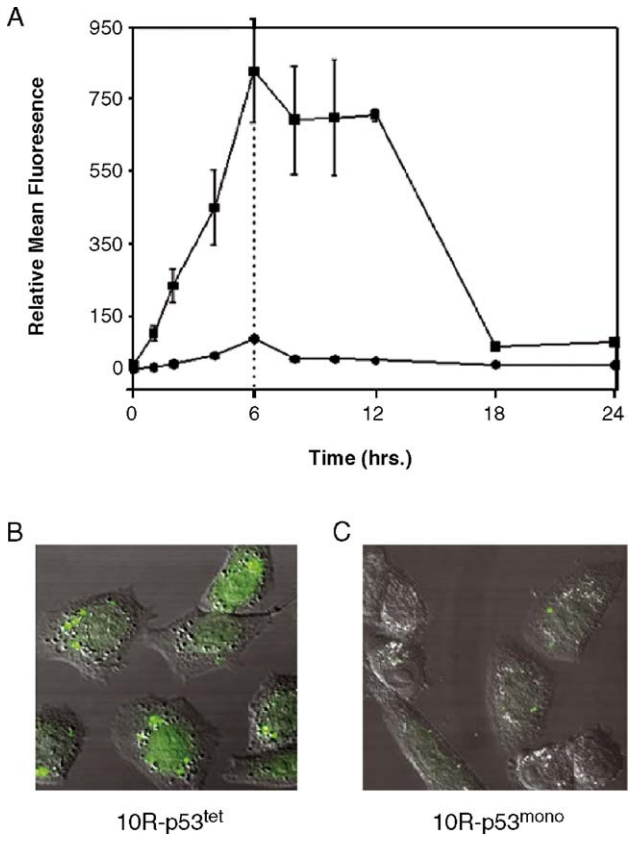

Fig. 3. Specific retention of fluorescein-labelled 10R-p53^{tet} peptides localized to the nucleus of CHO cells following the removal of peptides from the cell medium. (A) Retention of either tetrameric (10R-p53^{tet}, ■) or monomeric forms (10R-p53^{mono}; L334P mutation [102], ●) of fluorescein-labelled constructs peptides in CHO cells as measured by flow cytometry following the removal of the peptides from the medium 6 h post incubation. Cells were originally exposed to a 0.5-μM solution of one of the peptides in medium for 6 h and were subsequently incubated in peptide-free medium for an additional 18 h. (B and C) Superimposed phase-contrast and confocal images of viable CHO cells taken 18 h post incubation (12 h after the removal of peptides from the medium) suggest that most of the remaining fluorescein-labelled peptides (green colour) have localized to the cell nucleus. 10R-p53^{tet} and 10R-p53^{mono} peptides were not toxic to CHO cells even at doses up to 25 μM.

1.7. p53^{tet}-based peptide dendrimers containing cationic PTDs enter cells in a manner similar to PTDs linked to other cargoes

Mechanistic studies confirmed that deca-arginyl molecules fused to a p53^{tet} domain (10R-p53^{tet} peptides) initially bind to heparan sulfate carbohydrates on the cell surface and are internalized into eukaryotic cells via a clathrin-coated pit mediated process [103]. Most of the imported peptides accumulate into endosome-like vesicles. As shown in Fig. 3, when such peptides were removed from the cell medium, most of the fluorescein-labelled 10R-p53^{tet} peptides eventually disappeared from the cytosol with the exception of a significant fraction that had relocated to the cell nucleus (Fig. 3B and C). Flow cytometry data (Fig. 3A), however, indicate that the majority of the 10R-p53^{tet} peptides imported into cells were either degraded or routed back to the extracellular space (exocytosis) without ever reaching the cytosol. There is thus a need to develop strategies to facilitate peptide escape from such vesicular compartments. pH responsive peptides such as GALA have been shown to display endosomolytic properties in vitro [104]. Polyhistidine sequences, in view of their imidazole groups (pK_a ~6), may in theory buffer the endosomal microenvironment in which the pH

drops to 5. This buffering capacity would act as a "proton sponge" that eventually may rupture the endosomal membrane and allow peptide escape [105–108].

Other targeting functions must also be embedded into these peptide dendrimers to increase their therapeutic potential. As an example, delivery vehicles are needed to selectively target cancer cells. Receptors for short peptide hormones such as LHRH and somatostatin (octreotide) analogues are overexpressed on certain cancer cells [109–111] and as such, these peptides could be fused to $p53^{tet}$ containing peptides. It is, however, hoped that short peptides or small protein domains derived from combinatorial peptide or protein library searches may provide ligands with more selectivity for cancer cells [112–114].

2. Summary

The multivalent presentation of PTDs and other routing signals represents a powerful method for enhancing the cellular uptake and nuclear localization of many cargo macromolecules. Multivalency can be achieved using the oligomeric potential of the cargo macromolecule or by designing highly functionalized peptide dendrimers using synthetic or recombinant methods. The tetramerization domain of human p53 is a useful scaffold that is amenable to recombinant manipulation to present multiple equivalents of routing and targeting moieties. $p53^{tet}$-based constructs harbouring tetravalent oligocationic signals have been shown to efficiently enter cells and localize to their nucleus relative to their monomeric homologues. Future development of these delivery vehicles will involve incorporating other functional moieties that limit endosomal trapping and afford selectivity for molecular targets on cancer cells.

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