Nanotechnology for intracellular delivery and targeting of drugs, nanoreporters and nanoactuators

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The selective permeability of the plasma membrane prohibits most exogenous agents from gaining cellular access. A very effective class of transporters for the internalization of drugs, biosensors and imaging agents are cell penetrating peptides (CPPs), a group of short cationic sequences with a remarkable capacity for membrane translocation. Cell-penetrating peptides guarantee high delivery yield, low toxicity and the possibility to enter a wide range of target cells. Moreover they can act as molecular carriers for delivery of cargoes (e.g. proteins and nucleic acids) to relevant sub-cellular domains (mitochondria, endosomal vesicles and nucleus). This report describes recent advances in the use of Tat arginin-rich peptides and provides further information on the controversial mechanism of its nuclear entry. Nuclear localization is particularly significant, as it is one of the fundamental steps for gene-therapy approaches and can open the way to probe and modify cellular processes of utmost importance.

Peptide-mediated delivery of bioactive molecules appears to be a new biotechnology tool that in many aspects is superior to commonly used delivery agents (liposomes, microinjection, electroporation, viral systems). Cell penetrating peptides typically consist of short basic peptide sequences that could cross the plasma membrane and deliver their cargo directly into the cell. Among CPPs we focused our attention on the arginine-rich motif derived from the HIV transactivator of transcription protein Tat (residues 47-57 YGRKKRRQRRR of HIV-Tat protein full length). In order to elucidate the entrance pathway and to identify the intracellular trafficking properties of Tat-based vector, we engineered a recombinant fusion protein consisting of Tat sequence fused to the green fluorescent protein (GFP). The purified protein added to the culture medium first attaches to the cell membranes - presumably by ionic interactions - and then permeates cells by endocytosis. An array of endocytosis assays had demonstrated that Tat enters living HeLa cells using both clathrin-dependent pathway and macropinocytosis and progressively translocates into low-pH endosomes.

Once we had identified Tat protein as a vector system capable to cross cell membranes and elucidated its mechanism of internalization, we focused upon the investigation of the ability of Tat-derived peptides to transduce cargoes of different size in specific sub-cellular compartments. Cell penetrating properties depend upon the integrity of Tat arginine-rich motif, YGRKKRRQRRR, corresponding to a putative nuclear localization sequence (NLS) and the trans-activation responsive region-binding domain of the entire Tat protein.

To assess the nucleus/cytoplasm shuttling properties driven by Tat peptide we engineered green fluorescent protein (GFP)-based fusion proteins of different molecular size, both below and above the passive diffusion size limit through the nuclear pore (NP). These constructs represented a benchmark for intracellular passive diffusion behavior when expressed in cells and were also used as cargoes for prototypical active import by fusing them to the NLS (Nuclear Localization Sequence) of SV40. The behavior of these two classes of constructs was compared with the same GFP-cargoes fused to the Tat peptide. *In vivo* imaging allowed us to study Tat peptide-intracellular localization and dynamics (FRAP analysis). Our findings support a passive diffusion mechanism directing Tat movement across the nuclear envelope (NE) (Fig. 1).

The identification of this limitation of Tat peptide as a nuclear delivery vector prompted us to improve Tat functionality for the correct translocation of cargoes to the

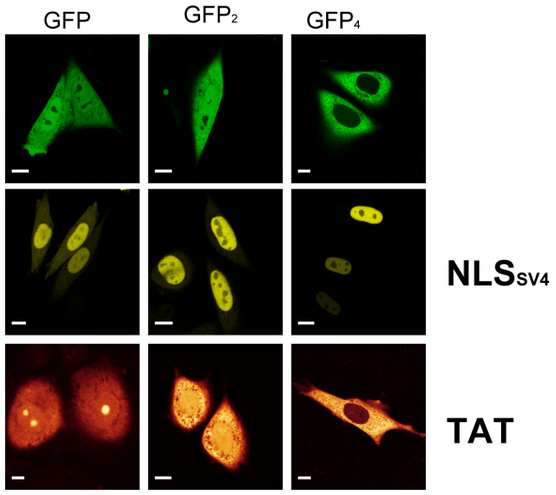


Fig.1
Cargo-dependent sub-cellular localization. As untagged constructs (above panel), Tat-GFP and Tat-GFP_2 are uniformly distributed between nucleus/cytoplasm compartments; Tat-GFP_4 is completely excluded from the nucleus (below panel). In contrast, NLS-tagged cargoes are predominantly localized in the nucleus of HeLa cells (middle panel).

nucleus. To accomplish this goal, we focused our study on the rational mutagenesis of the Tat peptide to afford variants with finely tuned intercompartmental dynamics and controllable nuclear transport mechanism. By mutagenesis of the carboxy-terminal stretch "RRR" of Tat sequence into "GGG" (not-charged residues), we engineered a novel variant of Tat peptide, Tat_{GGG}-EGFP with well-defined nuclear active transport properties. Remarkably, the Tat_{GGG} fused to GFP_4-cargo (110 Kda, exceeding the size-threshold value for passive diffusion through nuclear pore) was predominantly detected into the nucleus (Fig. 2 c): this indicates that the Tat_{GGG} sequence does determine active nuclear import of large cargoes. Conversely, the wild-type Tat peptide is not able to drive the active nuclear import of the same cargo, and is completely excluded from the nucleus (Fig 2 b), as untagged GFP_4 (Fig.2a).

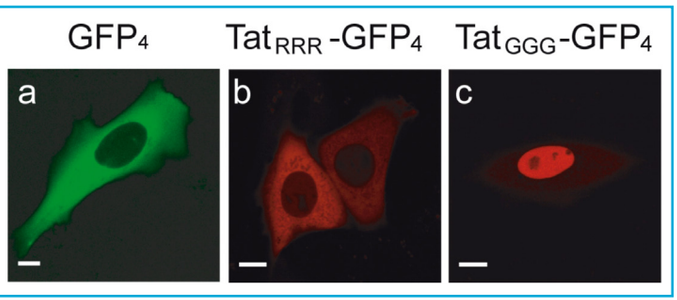
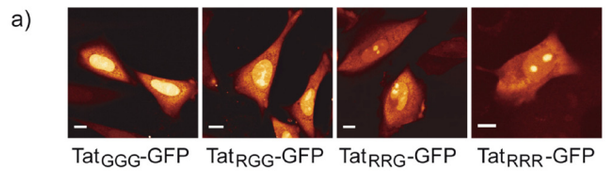


Fig. 2
Tat_{GGG}-mediated active nuclear accumulation of GFP_4 cargo, while Tat_{RRR} shown the same behavior of untagged GFP_4

Since the substitution of 'RRR' residues with 'GGG' allowed us to switch on and off the Tat-peptide nuclear-import properties, we explored the degree of tunability yielded by the variation of the number of positively charged C-terminal arginine residues. By *in vivo* imaging we found that the one-by-one replacement of glycine residues to wild-type Tat sequence gradually increases the effective nuclear accumulation of constructs in comparison with Tat_{RRR}-GFP (Fig.3a). Collected recovery curves by FRAP-analysis show that the addition of glycine residues can progressively accelerate the intercompartment mobility of Tat_{RRR}-derived mutants (Fig.3b) [2].

Our data on the intracellular properties of TAT_{GGG} confirmed our choice of this mutant as a major candidate for drug/nanoactuators delivery into the nuclear compartment. Indeed, analogously to the wild-type precursor, TAT_{GGG} fused



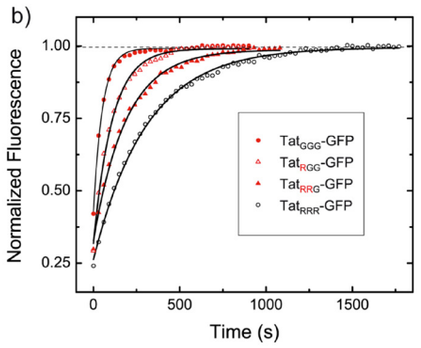


Fig. 3
Analysis of intracellular localization of Tat-derived mutants. Scale bar: 10 μm. (b) Kinetics of nucleoplasmic fluorescence recovery after photobleaching for Tat-derived mutants.

to cargoes molecules were shown to translocate through the cell membranes and internalize within endosomes.

As a first implementation of a Tat-driven nanoreporter we showed the intracellular delivery of a genetically encoded pH-reporter conjugated to Tat protein. E^{1}GFP is a pH-sensitive fluorescent protein with a pK_a close to 6.0 that is suitable for *in vivo* ratiometric emission pH-measurements. By fusing E^{1}GFP to Tat sequence we were able to monitor in real time the changes in vesicles pH starting from its initial cell surface value through the intracellular endocytic network. After 4 hours of internalization we observed that Tat-E^{1}GFP accumulated in two subpopulations of endosomes: the first accumulated in bright patches close to the plasma-membrane with a pH of 6.77± 0.13 and a second population represented by few clusters of vesicles embedded in the cytoplasm with a pH value of 5.78±0.08 (Fig.4)[3]

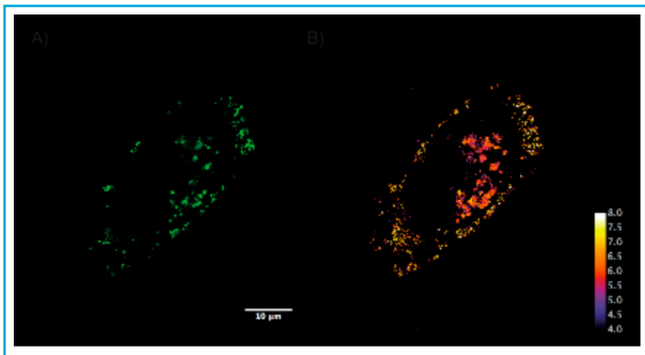


Fig.4
Confocal imaging of HeLa cells treated with Tat- E^{1}GFP (left panel). Ratiometric pH map of endosomal acidification during Tat-E^{1}GFP endocytosis (right panel). Image is color-encoded according to the look-up table on the right.

Based on these results a part of our recent research activity was devoted to engineering multifunctionalized nanoactuators endowed with cell-penetrating properties. PAMAM dendrimers represent an exciting new class of macromolecular architecture and they are very promising tools for therapeutic and diagnostic purposes. Unlike classical polymers, dendrimers have a high degree of molecular uniformity, narrow molecular weight distribution, specific size and shape characteristics, and a highly-functionalized terminal surface. The hyperbranched

architecture of these nanostructures allows us to include: 1) a biocompatible scaffold for functionalization; 2) a vector (i.e. TAT peptides derived thereof) for intracellular delivery and nuclear targeting of cargoes; 3) a fluorescence sensing-molecule that allows *in vivo* imaging.

References

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