Analytical Methods

Quantification of the Cellular Uptake of Cell-Penetrating Peptides by MALDI-TOF Mass Spectrometry**

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Cell-penetrating peptides (CPPs) are widely used to deliver biologically active molecules into cells.[1] We have successfully applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to the quantification of cellular uptake of CPPs. Penetratin, which corresponds to residues 43 to 58 of the *Antennapedia* homeodomain, was one of the first small CPPs to be discovered a decade ago.[2] Many others were identified soon after: Tat-derived peptides,[3] nuclear localization sequences,[4] transportan,[5] model amphipatic peptides,[6] polybasic peptides,[7,8] amphipathic proline-rich peptides,[9] and β peptides.[10] Many studies concerning the cellular uptake efficiencies of these CPPs have been reported, most of them based on the quantification of a radioactive or a fluorescent reporter group.[11] Only one example of direct CPP quantification has been reported, based on HPLC analysis of fluorescent CPPs.[12] The results strongly differ from one study to another. For example, the ratio between intracellular and extracellular concentrations of penetratin ranges from 0.6:1.0[13] to 95.0:1.0.[14] Discrepancies observed between studies can be explained by the use of different cell types and cell treatments (fixed or unfixed cells), different methods of detection, interference of the reporter group, or inaccurate distinction between internalized and membrane-associated peptides. In some of the studies the fraction of CPP bound to the external surface of the cells is removed by washing or by enzymatic digestion.[11] In other cases discrimination between internalized and membrane-bound peptide is achieved by chemical modification of the non-internalized peptide[12] or by the use of a reduction-sensitive fluorophore.[15] We report here a novel method based on MALDI-TOF MS to measure the concentration of internalized peptide and its application to the study of three widely used CPPs: penetratin, Tat_{48-59}, and $(Arg)_9$. The concept is outlined in Figure 1.

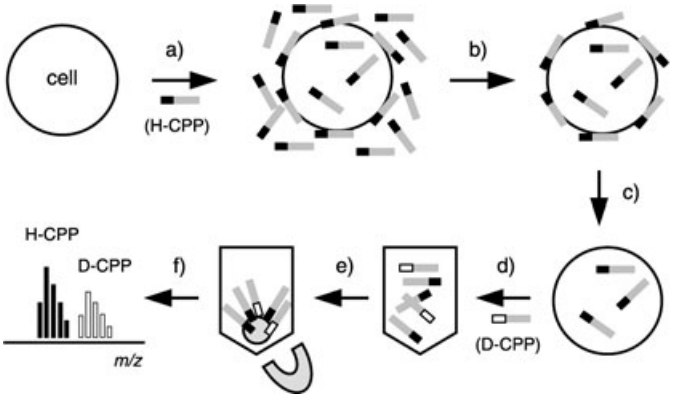

Figure 1. Quantification of CPP cellular uptake by MALDI-TOF MS. a) Cells are incubated with biotinylated non-deuterated CPP (H-CPP). b) Cells are washed. c) Membrane-bound CPP is digested by trypsin then trypsin inhibitor is added. d) A known and appropriate amount of internal standard (biotinylated deuterated CPP, D-CPP) is added, cell lysis is performed in 0.3% triton X100 and 1 M NaCl, and the sample is heated for 15 min at 100°C. e) Biotinylated peptides are captured by streptavidin-coated magnetic beads. f) Beads are washed and analyzed by MALDI-TOF MS. The ratio between the $[M+H]^+$ signal areas of H-CPP and D-CPP yields the absolute amount of internalized peptide.

Peptide quantification by MALDI-TOF MS can only be achieved by using the same peptide labeled with a stable isotope as an internal standard. Both peptides have identical chemical structure and exhibit the same efficiencies of desorption/ionization and detection. The relative intensity of the peptides $[M+H]^+$ signals therefore corresponds to the relative proportion of the peptides in the sample.[16,17] We

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[**] We thank Dr. Alain Prochiantz and Dr. Alain Joliot for fruitful discussions. We are also grateful to Dr. John Offer and Dr. Florian Toepert for their critical comments on the manuscript.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200500477

have previously reported a method to directly analyze by MALDI-TOF MS biotinylated peptides that were extracted from a complex mixture using streptavidin-coated magnetic beads.[18] We have now combined this technique with isotopic labeling to quantify cellular uptake of CPPs. The process of capturing the CPPs on beads enables concentration of the internalized CPP and its purification from cell lysate before MALDI-TOF MS analysis. This step is essential to facilitate detection of very small amounts of internalized CPP and also to allow distinction between the CPPs and endogenous peptides having the same molecular weight. The use of MALDI-TOF MS gives the advantage of direct detection of the peptide. Therefore, discrimination between internalized and membrane-bound peptide can be easily achieved by submitting the non-internalized peptide to a chemical or an enzymatic treatment that modifies its molecular weight. Intracellular degradation of the CPPs can also be studied. Finally, the simultaneous comparison of the cellular uptake efficiencies of several CPPs can be performed as long as the peptides have different molecular weights.

The CPPs used in this study were functionalized on their N-terminus with biotin and an isotopic tag composed either of four non-deuterated glycine residues (H-CPP, internalized) or four deuterated glycine residues (D-CPP, internal standard). The internalization experiments were performed following the protocol described in Figure 1 (see also the Supporting Information). The internal standard was submitted to the whole process of cell lysis and internalized CPP extraction to enable any degradation artefacts to be detected. Indeed this process allows the fast distinction between intracellular degradation of CPP (only H-CPP digests would be detected) and degradation of CPP occurring during sample preparation (the same H-CPP and D-CPP digests would be detected) by MALDI-TOF MS (see the Supporting Information). The conditions used for cell lysis and peptide capture were found to be particularly critical for the accuracy of the quantification. Indeed, the lysate may contain molecules that exhibit affinity for the CPPs and may thus hamper their capture by streptavidin-coated magnetic beads. This situation may lead to discrimination during capture between the internalized CPP and the internal standard since both peptides have not necessarily been exposed to the same interactions. Nucleic acids in particular were found to perturb the peptide extraction process. This problem could be overcome by treating the lysate with nucleases before peptide capture or by performing the capture in the presence of high salt concentrations. However, degradation of both the internalized peptide and the internal standard was then observed. This effect was possibly a result of the release of lysosomal enzymes during cell lysis. The addition of protease inhibitors during the whole process of peptide extraction was not sufficient to completely block degradation; this was only achieved by directly heating the cells in the lysis solution at 100 °C for 15 minutes and maintaining the samples at 4 °C for the rest of the extraction process (see the Supporting Information). In addition to protease denaturation, this heating step enables both the internal standard and the internalized CPP to associate to the same extent with lysate components and to be subsequently captured on streptavidin-coated magnetic beads with the same efficiency. After capture of the biotinylated peptides, lysate components were easily removed by using the protocol described previously for washing the beads.[18] The peptides associated with the beads were then mixed with the matrix α-cyano-4-hydroxycinnamic acid, deposited directly on the MALDI-TOF plate, and then analyzed. Spectra were averaged for statistical sampling from a few hundred laser shots recorded on different spots of the deposit. The acquisition of $[M+H]^+$ signals with the natural isotopic distribution for both H-CPP and D-CPP was the indication of a valid statistical sampling (Figure 2a and Supporting Information). The areas of the H-CPP and D-CPP $[M+H]^+$ signals, including all the isotopes, were used for the quantification. Quantification was more precise when H-CPP and D-CPP signals had similar intensities because the signal/noise ratio was then comparable for both peptides. In practice, the amount of internalized H-CPP was first evaluated by adding various known quantities of D-CPP to samples containing the internalized peptide. Quantification was then found to be feasible with our mass spectrometer for H-CPP/D-CPP signals ratios ranging from 0.2:1 to 5:1. The experiment was repeated, with the amount of D-CPP adjusted to get a signal ratio close to 1:1. Quantifications performed on different deposits of the same biological sample gave highly reproducible results (±5% variation).

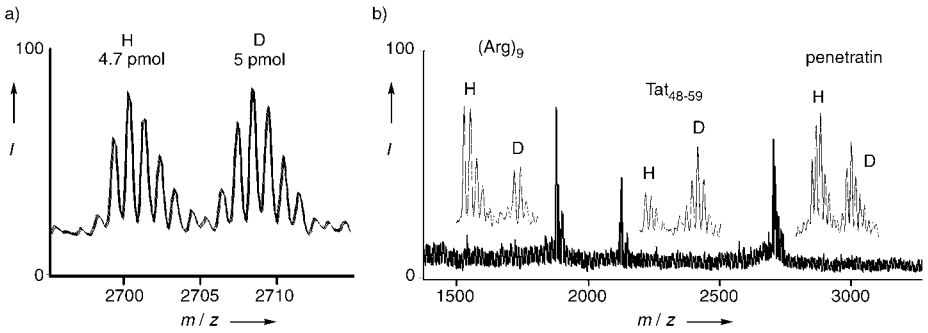

Figure 2. MALDI-TOF mass spectra obtained for internalization of a) penetratin and b) a mixture of penetratin (biotin-GGGG-RQIKIWFQNRRMKWKK-NH_2), Tat_{48-59} (biotin-GGGG-RKKRRQRRRPPQ-NH_2), and $(Arg)_9$ (biotin-GGGG-RRRRRRRRR-NH_2). H and D correspond to the $[M+H]^+$ peaks of the internalized CPPs and the internal standards, respectively.

By using this protocol we measured the intracellular concentrations of penetratin, Tat_{48-59}, and $(Arg)_9$. Each experiment was carried out by incubating 10^6 Chinese hamster ovary (CHO) cells (corresponding to a total intracellular volume of 1.5 μL) for 75 minutes at 37 °C with the CPP (7.5 μM). The highest intracellular concentration was found for $(Arg)_9$ corresponding to 4.5 ± 1 μM (average concentration ± standard deviation (± SD), $n = 10$) followed by penetratin (3.5 ± 0.8 μM, $n = 12$) and Tat_{48-59} (0.7 ± 0.2 μM, $n =$

10; Figures 2a and 3a). H-CPPs digests corresponding exclusively to cleavage at the C-terminus of basic residues were occasionally observed with low abundance. These species were likely formed by the trypsin treatment used to digest the membrane-bound H-CPP and not completely eliminated during the subsequent washing step (Figure 1). In contrast to other CPP uptake assays our mass spectrometry approach allowed the facile distinction between these false positives and the peptide fraction that was actually internalized. No other digestion products were detected (see the Supporting Information for full mass spectra). The trypsin treatment was omitted in another set of experiments aimed at evaluating the affinities of the CPPs for the cellular membrane. Depending on the CPP, the amount corresponding to the sum of internalized and membrane-bound peptide was 5 to 60 times higher than the amount of internalized peptide (Figure 3b). These results stress the importance of the trypsin digestion step combined with direct quantification of the CPP to accurately discriminate extracellular membrane-bound peptide from internalized peptide. They also show that the efficiency of CPP internalization is not directly linked to membrane affinity. Finally, the amount of internalized CPPs was measured when the cells were incubated with a mixture of the three CPPs (7.5 μM each; Figures 2b and 3a). No cell lysis during incubation was observed. The relative internalization efficiencies of the CPPs were found to be equivalent to those calculated from the single CPP incubation experiments, but lower amounts of internalized peptide were measured (Figure 3a). These data show that no competition occurs between the three CPPs and suggest that a limited number of internalization domains are available. Experiments using CPP mixtures are more applicable for screening than for absolute quantification of cellular uptake. Indeed, saturation phenomena can occur as observed with the CPP mixture used in this study. Other CPP mixtures may also lead to competition between individual peptides. Furthermore, low-intensity signals may be observed in the MALDI-TOF mass spectra for CPPs that are the most poorly internalized.

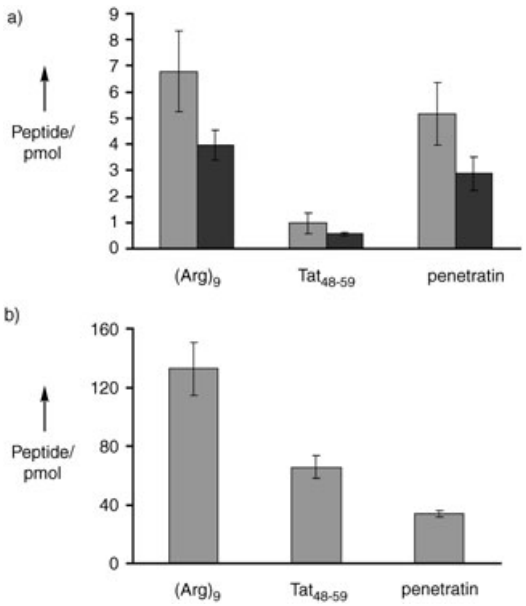

Figure 3. a) Amount of intact internalized CPP. Gray and black bars correspond to incubation of 10^6 CHO cells with each CPP independently or a mixture of the three CPPs, respectively. Each data is the average result of at least three independent experiments performed in duplicates or triplicates (± SD). Comparison of the mean differences between intracellular amounts of the three CPPs was performed with the Welch t test (populations with unequal SD) and was found significant ($p = 0.016$) between penetratin and $(Arg)_9$ and very significant ($p < 0.0001$) between penetratin or $(Arg)_9$ and Tat_{48-59}. b) Total amount of membrane-bound and internalized CPP. Data correspond to incubation of 10^6 CHO cells with each CPP independently.

In contrast to the majority of data reported in the literature for penetratin, Tat-derived peptides, and $(Arg)_9$, the intracellular CPP concentration was found in this study to be lower than the extracellular CPP concentration. Some of the high values of internalized CPP reported in the literature are likely explained by an incomplete elimination of the fraction of membrane-bound peptide. Results that closely match the ones determined in this study were obtained by fluorescence correlation microscopy, which allows unambiguous discrimination between extracellular and intracellular CPP.[13]

The detailed analysis of intracellular degradation of CPPs by using MALDI-TOF MS has been reported before.[19,20] However, compared to the method used, our procedure offers the advantage of allowing distinction between degradation occurring during sample preparation and actual intracellular degradation, with the internal standard (D-CPP) serving as a control (see the Supporting Information).

In conclusion, the method presented here enables direct quantification of CPP with unambiguous discrimination between membrane-bound and intracellular CPP. It is the first method described that allows direct comparison of internalization efficiencies using mixtures of CPPs. It is simple to use, fast, and yields highly reproducible results. The process of sample analysis on the MALDI-TOF plate lends itself to automation for the high-throughput screening of CPP libraries. The present method will provide a reliable and efficient tool for the design and optimization of new CPPs and for the study of their internalization mechanisms.

Received: February 8, 2005
Published online: June 1, 2005

Keywords: analytical methods · drug delivery · isotopic labeling · mass spectrometry · peptides

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