Cell-Penetrating Compounds

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A Straightforward Approach for Cellular-Uptake Quantification**

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Cell-penetrating compounds are able to cross biological membranes and deliver bioactive cargo into cell compartments (cytoplasm, nucleus).[1] Different methods of detection have been employed to study their cellular uptake. Fluorescent dyes and radioactive labels are commonly used. However, direct quantification of internalized compounds is a lot more difficult, and different studies have led to different results. The pioneering study of Burlina et al.[2] constituted a real breakthrough in proposing a highly reproducible quantification method based on MALDI-TOF MS to measure the concentration of the internalized peptides. After cell lysis, this method requires the capture of the biotin-labeled cell-penetrating peptides (CPPs). This step is particularly critical for the accuracy of the quantification. Indeed, the lysate may contain molecules that may hamper the CPP capture by streptavidin-coated magnetic beads. However, the attractiveness of such an MS-based methodology for accurate CPP quantification from complex biological media could be greatly enhanced by avoiding affinity labeling and subsequent purification.

We report herein a sensitive general method for the quantification of internalized compounds into cells by MALDI-TOF mass spectrometry that combines existing analytical tools for highly sensitive peptide detection[3] and very accurate protein/peptide quantitation.[4] We previously reported an original approach in which peptides derivatized by α-cyano-4-hydroxycinnamic acid (HCCA) were readily identified by selective enhancement and discrimination of the MALDI MS signals in a neutral matrix, such as α-cyano-4-hydroxycinnamic methyl ester (HCCE).[3] This combination (HCCA tag and HCCE matrix) enabled us to discriminate signals induced by peptides of interest that were present in low concentrations from those of unlabeled more abundant peptides. Reliable accurate measurement of protein expression was demonstrated in quantitative proteomics by Oda et al.,[4] who used a stable-isotope-labeling MS-based strategy. We therefore decided to prepare a heavy (D_4) analogue of the UV-light-absorbing label HCCA for the quantification of CPP cellular uptake.

We synthesized CPPs coupled with light (D_0) or heavy (D_4) HCCA through an aminohexanoic acid (Ahx) spacer (Figure 1 A). Ahx is commonly used as a spacer between cargo and CPPs to prepare N-terminally tagged conjugates.[5,6] The ability of these compounds to penetrate cells was readily determined by comparison of the MS signals induced by tagged compounds with those of the overrepresented untagged materials. Thus, no separation procedure was required. The material that penetrated cells was quantified by comparison of the signals due to the light tag with the corresponding signals corresponding to deuterated heavy HCCA. The methodology (described in Figure 1 B) was validated by using four different compounds: the two widely used CPPs penetratin and nonaarginine $(Arg)_9$,[7,8] the benzothiazepine-derived oligomer $(DBT)_4$, which we previously identified as a potent cell-penetrating nonpeptide (CPNP),[9] and a tripeptide (FAK) as a negative control (Table 1). All compounds were prepared by microwave-assisted solid-phase synthesis.[10] Figure 1 A summarizes the synthetic and analytic workflows. The key step of the synthesis was a Knoevenagel condensation with commercially available deuterated *p*-hydroxybenzaldehyde.[11]

Before performing internalization experiments, we checked that HCCA-tagged peptides could be detected in a crude cell lysate by MALDI-TOF MS up to a 10^{-10} M concentration (see Figure S1 in the Supporting information), which corresponds to the possible concentration of internalized compound in the sample after cell lysis.[2] To highlight the HCCE/HCCA matrix-discrimination effect, N-terminal acetylated peptides were prepared and mixed at different concentrations along with HCCA-tagged peptides in a crude cell lysate. Equimolar mixtures of HCCA-CPPs and Ac-CCPs diluted in water/acetonitrile were mixed in a cell lysate to afford a 5×10^{-6} to 5×10^{-11} M concentration of each peptide species. Samples were prepared either in an HCCA matrix or in a neutral HCCE matrix to assess the discrimination effect (see Figure S1 in the Supporting Information). The MALDI-TOF spectra were quite clean, and very few signals were observed for the cell lysate or the buffer. HCCA-tagged peptides were still readily detected at 5×10^{-9} M in the HCCA matrix and 5×10^{-10} M in the HCCE matrix. Ac-CPPs were not detected at a concentration of 5×10^{-8} M. These

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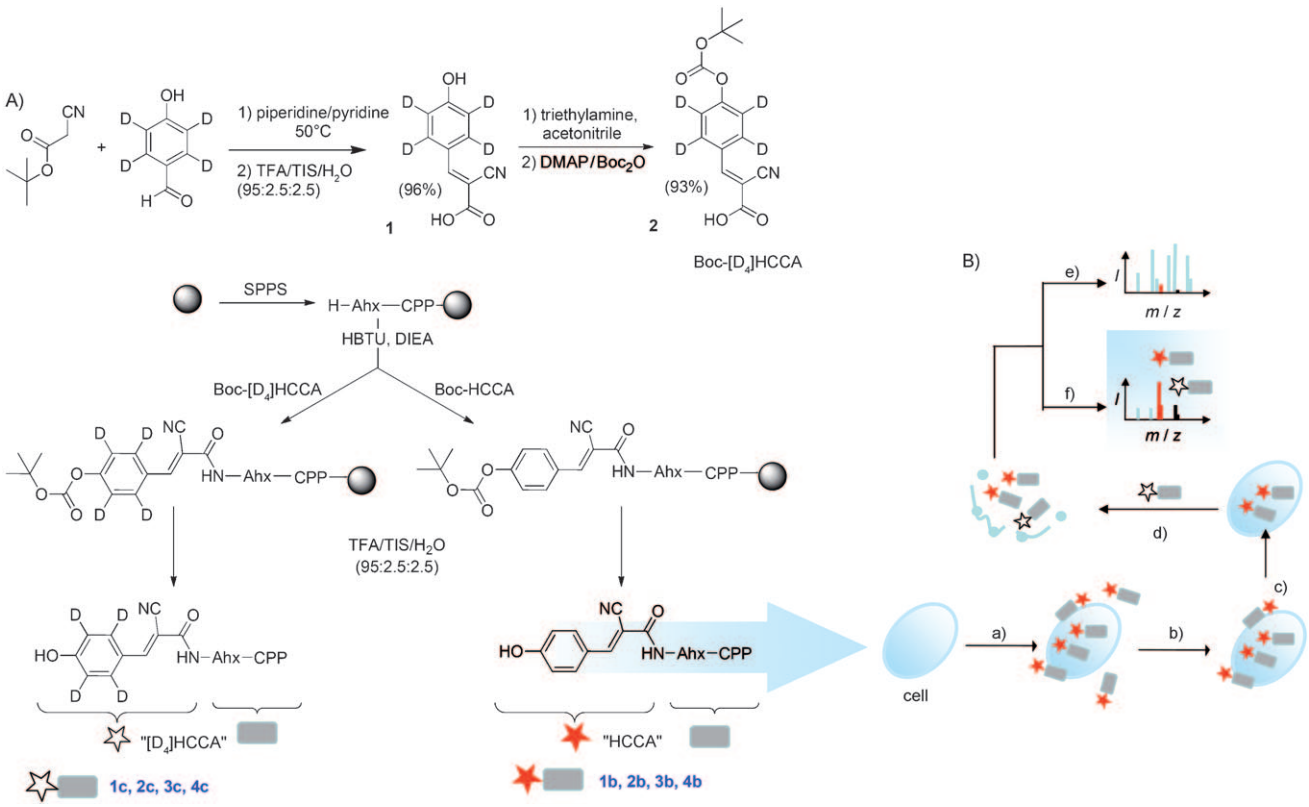
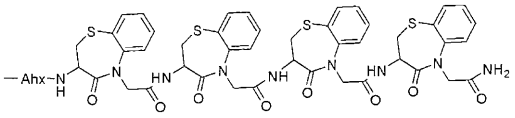


Figure 1. A) Synthesis of light (D_0) and heavy (D_4) HCCA-tagged CPPs. B) Strategy for the direct quantification of the cellular uptake of CPPs (and a CPNP) by MALDI-TOF MS by using the HCCA/HCCE discrimination effect: a) incubation of cells with the HCCA-tagged CPP; b) washing step; c) enzymatic stripping of the cell membrane; d) lysis of cells, followed by the addition of a precise amount of the deuterated-HCCA-tagged CPP as an internal standard; e) MALDI-TOF analysis of the whole cell lysate in the HCCA matrix; f) MALDI-TOF analysis of the whole cell lysate in the "neutral" HCCE matrix. The HCCA/HCCE signal-discrimination effect enables the enhancement of the CPP signal in a complex mixture. The internalized CPP can be quantified on the basis of the ratio between the $[M+H]^+$ peaks of the deuterated and nondeuterated HCCA-tagged CPP. Boc = *tert*-butoxycarbonyl, DIEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, SPPS = solid-phase peptide synthesis, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

Table 1: Intracellular uptake of peptides, as determined by mass spectrometry.

Compound		N terminus	Sequence	*m/z* $[M+H]^+$	*m/z* $[M+Na]^+$	Intracellular uptake [μM]	Intracellular uptake [μM][a]
penetratin	**1a**	Ac-	-Ahx-RQIKIWFQNRRMKWKK-NH_2	2400.4	2422.4	1.21	3.5
	1b	HCCA-		2529.4	2555.4		
	1c	[D_4]HCCA		2533.4	2559.4		
Arg_9	**2a**	Ac-	-Ahx-RRRRRRRRR-NH_2	1578.0	1600.0	1.08	4.5
	2b	HCCA-		1707.0	1729.0		
	2c	[D_4]HCCA		1711.0	1733.0		
DBT_4	**3a**	Ac-		1109.3	1131.3		
	3b	HCCA-		1238.3	1260.3	8.7	–
	3c	[D_4]HCCA		1242.3	1264.3		
FAK (negative control)	**4a**	Ac-	-Ahx-FAK-OH	520.3	542.3	none	–
	4b	HCCA-		649.3	671.3		
	4c	[D_4]HCCA		653.3	675.3		

[a] Intracellular uptake concentration reported by Burlina et al.,[2] who used biotin-labeled analogues.

results are in accordance with our observations during the analysis of tagged peptides from cytochrome *c*.[12]

Generally, the HCCA-discriminating effect increased when the concentration of the analyte (HCCA-CPP or Ac-CPP) decreased. This phenomenon makes HCCA tagging a method of choice for the detection and quantification of low-abundance peptides in a complex mixture. Furthermore, the baseline of spectra obtained in the HCCE matrix was flatter than that of spectra obtained in the HCCA matrix (see Figure S1 in the Supporting Information). This flat baseline is particularly favorable for the purpose of peak integration and quantification. The expected discrimination effect was less

significant with the $(DBT)_4$ sequence: the intensity of the Ac-Ahx-$(DBT)_4$-NH_2 $[M+Na]^+$ peak at m/z 1018.3 was only two times lower than that of the HCCA-Ahx-$(DBT)_4$-NH_2 $[M+Na]^+$ peak at m/z 1260.4 in the HCCE matrix (see Figure S1 in the Supporting Information).

Internalization experiments were performed on the MDA-MB-231 cell line according to the modified protocol described by Burlina et al.[2] (Figure 1B). The HCCA-tagged compounds **1b**, **2b**, **3b**, and **4b** were incubated at a concentration of 10 μM with 2×10^5 MDA-MB-231 breast cancer cells for 3 hours at 37 °C. The biological samples were then split into two pools to be submitted or not to trypsin stripping of cell-membrane-bound CPPs.[13] Cells were lysed for 20 minutes with lysis buffer containing 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) and ethylenediaminetetraacetic acid (EDTA), and membrane fragments were removed by centrifugation. The supernatant was collected and added to known amounts of the corresponding deuterated CPP. In this way, both internalized CPPs and internal standards ([D_4]HCCA-labeled CPPs) were exposed to degradation by proteases remaining in the medium.

Samples were deposited directly on the MALDI probe, mixed with the HCCA or neutral HCCE matrix, and analyzed. Spectra were averaged for statistical sampling from a hundred laser shots recorded on different spots of the deposit. The areas of all isotope peaks of the light and heavy compounds corresponding to monoprotonated compounds $[M+H]^+$ were used for quantification. In the case of DBT derivatives (Table 1, **3b** and **3c**), the isotope peaks of the sodiated ions $[M+Na]^+$ were chosen for quantification, as they are more intense than those of the monoprotonated ions. The mass increment of 4 Da between the compounds tagged with light HCCA and heavy, isotopically labeled [D_4]HCCA gave two isotopic distributions that partially overlapped at the edges. For example, the ion at m/z 2533.6 in Figure 2 was produced by the cumulated contributions of the light-HCCA-tagged molecule containing four naturally occurring ^{13}C atoms (very tiny abundance) and of the heavy-[D_4]HCCA-tagged counterpart (major intensity). The ratio of these two isotopic contributions was estimated and taken into account in all results after calculation and integration of the isotopic patterns by using the algorithm SNAP (Sophisticated Numerical Annotation Procedure) from the software FlexAnalysis. This algorithm helped us to determine and separate the exact isotopic pattern of molecules tagged with light and heavy HCCA, even when overlapping occurred (Figure 2).

Absolute quantification was more precise when light and heavy compounds displayed similar intensities and thus similar concentrations in the sample. A logarithmic range of concentrations of the deuterated standards was used to determine accurate concentration values. Each CPP concentration was calculated by using the more accurately known concentration values of the [D_4]HCCA-tagged compound (Figure 2; see also Figure S4 in the Supporting Information). Quantification was performed with ratios varying from 0.1:1 to 4:1. The analysis of several deposits of the same cell lysate yielded highly reproducible results (a maximum of 10% variation between samples). The analysis of different deposits of various biological samples (cell lysates with a variable number of MDA-MB-231 cells) also showed good reproducibility.

By using this protocol, we calculated the intracellular concentration of the four compounds (Table 1). A concentration of 1.21 μM for the penetratin derivative **1b** was found, and 1.08 μM for the Arg_9 derivative **2b.** These two values are in full accordance with the previously reported MS quantification of N-terminally biotinylated CPPs.[2] As expected, the negative-control peptide **4b** was not detected in the cell lysate; the internal standard **4c** was detected at a concentration of 10^{-8} M. The highest intracellular concentration (8.70 μM) was found for the labeled DBT_4 CPNP **3b**. This value corresponds to an absolute amount of 13×10^{-18} mol in a single cell.

Experiments were repeated with the pool of trypsin-untreated cells. A comparison of the two series reflected the proportion of the CPP (or CPNP) that remained bound to the membrane. Depending on the CPP (or CPNP), the amount of

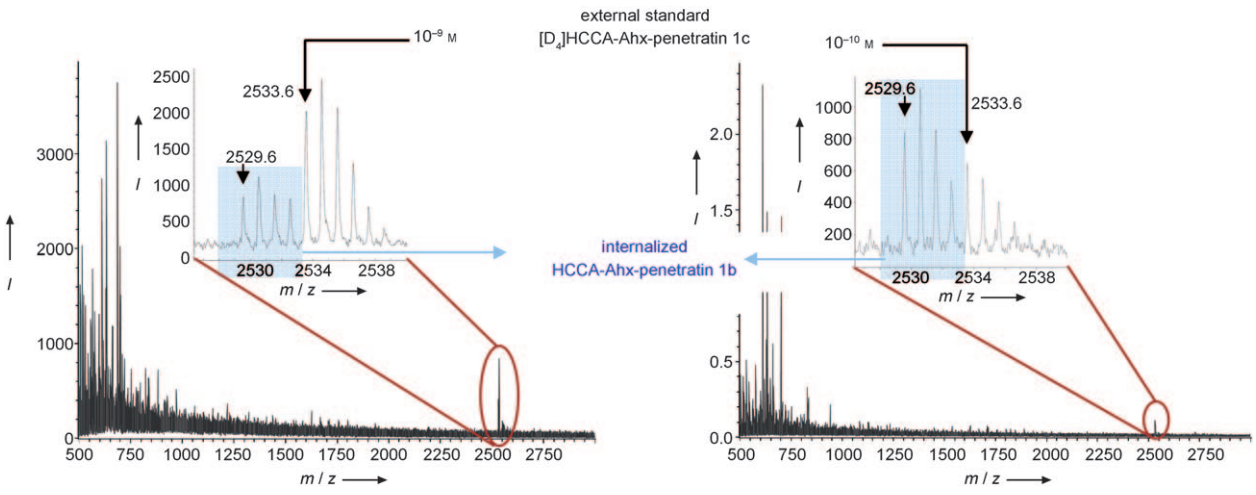


Figure 2. Quantification of HCCA-Ahx-penetratin (**1b**) through the addition of its deuterated counterpart at a concentration of 10^{-9} or 10^{-10} M (samples were prepared in an HCCE matrix).

internalized compounds was 10–100 times lower after trypsin treatment (see Figure S4 in the Supporting Information).

In conclusion, we have described a straightforward and efficient method for the quantification of cell-penetrating compounds. The originality of the methodology relies first on the use of the combination of HCCA as a UV-light-absorbing tag and the neutral HCCE matrix, which enabled the very sensitive detection of the compounds of interest by mass spectrometry even in complex biological samples, and second on the stable isotope labeling of such compounds for their reliable quantification. The procedure does not require any purification step, including biotin-based capture. This methodology was successfully applied to the quantification of two different types of cell-penetrating compounds, the CPP family and the CPNP DBT_4. DBT_4 was internalized about eight times more efficiently than Arg_9 or penetratin in MDA-MB-231 breast cancer cells. These results are in agreement with fluorescence-based assays and confirm the significance of noncharged, nonpeptide oligomers as a new class of cell-penetrating compounds.

Experimental Section

The [D_4]HCCA-tagged CPP solubilized in a mixture of acetonitrile/water/trifluoroacetic acid (50:50:0.1 v/v/v; 5 μL) at different concentrations was added to the cell sample (5 μL). After mixing and centrifugation, the mixture was analyzed by MALDI-TOF MS. To improve the signal resolution when the concentration of the internalized compound was very low, the mixture was concentrated by lyophilization and dissolved in acetonitrile/water/trifluoroacetic acid (50:50:0.1 v/v/v).

Each sample was analyzed with both the HCCA and the HCCE matrix twice. When the HCCA matrix was used, 0.5 μL of the sample was mixed on the MALDI probe with 0.5 μL of the matrix solution (half-saturated in acetonitrile/water/trifluoroacetic acid 50:50:0.1 v/v/v). When the HCCE matrix was used, 0.5 μL of the HCCE matrix (saturated in acetone) was deposited and dried on the MALDI probe, and then the peptide solution (0.5 μL) was deposited.

The isotopic group of peaks of each cell-penetrating compound was integrated with the FlexAnalysis software (version 2.2, Bruker Daltonics). The SNAP algorithm was used to determine the area of each isotopic pattern of light and heavy molecules by calculating their isotopic distribution.

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