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An integrated cross-linking-MS approach to investigate cell penetrating peptides interacting partners

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ABSTRACT

Cell penetrating peptides (CPPs) are attracting attention because of their ability to deliver biologically active molecules into cells. On their way they can interact with membrane and intracellular proteins. To fully understand and improve CPP efficiency as drug delivery tools, their partners need to be identified. To investigate CPP-protein complexes, chemical cross-linking coupled to mass spectrometry is a relevant method. With this aim, we developed an original approach based on two parallel strategies, an intact complex analysis and a bottom-up one, to have a global characterization of the cross-linked complexes composition as well as a detailed mapping of the interaction zones.

Biological significance: The robust and efficient cross-linking-MS workflow presented here can easily be adapted to any CPP-protein interacting system and could thus contribute to a better understanding of CPPs activity as cell-specific drug delivery tools. We validated the relevancy of this cross-linking-MS approach with two biologically active CPPs, $(R/W)_9$ and $(R/W)_{16}$, and two interacting protein partners, actin and albumin, previously reported using isothermal titration calorimetry (ITC) and NMR. Cross-linking-MS results obtained on these previous studies allowed us to go further by providing a detailed mapping of the interaction zones. The identified interaction zones between actin and CPPs $(R/W)_9$ and $(R/W)_{16}$ are biologically meaningful. Two cross-linked zones [46–57] and [202–210] of actin are indeed involved in the modulation of its dynamics. Moreover, [46–57] domain has also been described as one interaction domain for thymosin β4 whose actin binding can be displaced by competition with $(R/W)_{16}$ (NMR experiments).

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1. Introduction

Twenty years ago, the discovery of peptides able to ubiquitously cross cellular membranes commonly named cell penetrating peptides (CPPs), with very limited toxicity, launched a novel field in molecular delivery based on these non-invasive vectors. Most CPPs are positively charged peptides though the presence of few anionic or hydrophobic CPPs was also demonstrated. After a decade of debate on the trafficking routes of CPPs to the heart of cells, it is now more or less accepted that these peptides use concomitantly different internalization pathways, including pinocytosis and direct membrane translocation processes [1]. CPPs are generally considered as biologically inert intracellular delivery tools. However, some CPPs have intrinsically biological activity and are part of a recently described class of CPPs baptized bioportide [2].

For instance, previous studies showed, that only the two CPPs $(R/W)_9$ and $(R/W)_{16}$ (RRWWRRWRR and RRWRRWWRRWWRRWRR respectively) among other CPPs tested, are able to remodel the actin cytoskeleton in oncogen transformed NIH3T3/EWS-Fli cells once these CPPs had crossed the plasmamembrane [3]. In order to explain the actin-remodeling activity of the two CPPs, the hypothesis of a direct interaction with actin was tested. $(R/W)_9$ and $(R/W)_{16}$ peptides were actually found to directly interact *in vitro* with G-actin by NMR and ITC experiments [3] ($Kd \approx 10\,\mu M$ and $Kd = 0.4\,\mu M$, respectively). In addition, competitive binding experiments by NMR showed that $(R/W)_{16}$ was able to displace the actin sequestering protein thymosin β4 from G-actin [3].

It was also recently reported that arginine-rich CPPs interact with serum proteins like albumin, modifying their ability to internalize in cells [4]. Therefore our aim in this study was to analyze further the interaction of $(R/W)_9$ and $(R/W)_{16}$ with actin and albumin.

For this purpose, chemical cross-linking was chosen. Cross-linking reactions are conventionally based on the use of a bifunctional cross-linker, which is a carbon chain spacer bearing reactive sites at both ends that can either be identical (homobifunctional) or different (heterobifunctional). Reactive sites are mainly activated esters targeting either lysine residues (*e.g.* N-hydroxysuccinimide (NHS) ester) or cysteine residues (*e.g.* maleimide ester) although side reactions with tyrosine, threonine and serine have been reported [5].

By creating a covalent bond between two or more interacting partners, chemical cross-linking gives a snapshot of a molecule's environment and, combined with MS, constitutes a powerful tool to map protein-protein or peptide–protein interactions (distance constraints and interacting domains). However chemical cross-linking is often characterized by low reaction yields. In addition, a wide variety of cross-linked products are usually created. Therefore, cross-linkers bearing an affinity tag [6,7] allowing selective enrichment of the sample in cross-linked species have been developed. Cleavable cross-linkers [8,9], isotope labeled cross-linkers [10] or cross-linkers bearing a CHCA matrix moiety for MALDI analysis [11] improve detection and identification/characterization of the cross-linked species. Affinity purification using a tagged cross-linker or interacting partners [12] and SDS-PAGE are the most commonly used off-line techniques but chromatographic methods like strong cation-exchange (SCX) [13,14] and size exclusion chromatography (SEC) [15] constitute promising approaches to enrich samples in cross-linked species.

Analysis of low abundant cross-linked peptides requires high sensitivity for their detection and high mass accuracy (mass error < 10 ppm) for their identification since the number of combination of two peptides is enormous. Moreover tandem MS of cross-linked peptides is required for the characterization of the interaction zones.

A number of tailored softwares have been developed to deal with the selective acquisition or interpretation of these MS/MS spectra. Among others, FINDX [16] has been designed to selectively fragment inter-protein cross-links by LC–MALDI-TOF/TOF using $^{14}N/^{15}N$ mixed isotope strategy, xQuest [13] is dedicated to the search of isotopically tagged cross-linked peptides and CrossWork [17] or Xlink-Identifier [18] support label-free analyses of chemical cross-linking samples.

In this study, we set-up a general *in vitro* analytical workflow coupling cross-linking and mass spectrometry (cross-linking-MS), involving enrichment steps as well as manual or automated MS and MS/MS data processing to test potential interacting partners of any CPP sequence.

To validate our cross-linking-MS workflow, we studied the systems described above: $(R/W)_9$ or $(R/W)_{16}$ interacting with actin or albumin. For this purpose, CPP analogs suitable for chemical cross-linking experiments, were synthesized with the following sequences: $Biot(O_2)$-G_4-K-RRWWRRWRR-NH_2 and $Biot(O_2)$-G_4-K-RRWRRWWRRWWRRWRR-NH_2, respectively. The K residue added at the N-terminus of the peptides sequences allowed the cross-linking reaction, the biotin tag ($Biot(O_2)$) was added for purification purpose and was separated from the biologically active motif by a four G residues spacer. This {$Biot(O)_2$-G_n-K-} group is easy to add at the N-terminus of peptides either manually or automatically during peptide synthesis whatever the peptide sequence.

The originality of our approach resides in the comprehensive study of the cross-linking reaction mixture from two angles: a global view of the interacting system with an intact complex analysis combined to the precise characterization of interacting zones by a bottom-up analysis. The intact complex analysis is based on the MALDI-TOF analysis in linear mode of the cross-linking reaction mixtures and on the modeling of the spectra obtained using the in-house SIMUL-XL program. The bottom-up analysis consists in the tryptic digestion of the cross-linking reaction mixtures followed by the affinity purification (biotin/streptavidin) of the biotinylated cross-linked peptides and their analysis by tandem MS (MALDI-TOF/TOF and/or nanoLC–ESI-Orbitrap). The MS/MS data are either manually interpreted with the help of GPMAW software [19] (MALDI-TOF/TOF spectra) or automatically searched using Xlink-Identifier software [18] (nanoLC–ESI-MS/MS spectra).

For practical reasons the intact complex analysis was developed only with $(R/W)_9$ and both actin and albumin proteins. In contrast, the bottom-up analysis was performed using both $(R/W)_9$ and $(R/W)_{16}$ and both actin and albumin proteins.