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Detection of Noncovalent Complexes in Biological Samples by Intensity Fading and High-Mass Detection MALDI-TOF Mass Spectrometry

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Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has not yet contributed widely to the study of intact noncovalent biomolecular complexes, because MALDI is known to cause dissociation of the interaction partners and induce formation of nonspecific aggregates. Here, we present a new strategy to circumvent this problem. It is based on intensity fading (in the low *m*/*z* range) and high-mass detection MALDI mass spectrometry (MS), using a cryodetector (in the high *m*/*z* range), with and without chemical cross-linking of the interaction partners. The study focuses on noncovalent interactions between the human enzyme carboxypeptidase A (hCPA) and three protease inhibitors (PCI, TCI, and LCI) present in heterogeneous mixtures of other nonbinding molecules derived from a biological source, an extract from leech (*Hirudo medicinalis*). Another example involves an extract of the sea anemone *Stichodactyla helianthus*, which is used without previous fractionation to detect the specific complex between the enzyme trypsin and the endogenous SphI-1 inhibitor. The results give insight into the mechanism of intensity fading MS and demonstrate that the specificity of binding is greatly favored when the overall concentrations of the analytes (nonbinding molecules, protease inhibitor and target enzyme) present in a biological sample of interest are kept at low concentrations, in the submicromolar range. Higher concentrations may lead to unspecific interactions and the formation of aggregates both during the MALDI process and during reaction with the cross-linking reagents. This strategy is expected to advance the field of high-throughput affinity-based approaches, by taking advantage of a new generation of high mass detectors for MALDI-TOF instruments.

Keywords: mass spectrometry • MALDI-TOF • high-mass detection • cryodetection • microchannel plates • intensity fading • noncovalent complexes • cross-linking

Introduction

One of the next frontiers in proteome research is the study of protein−ligand interactions, protein−peptide and protein−protein complexes, and entire protein interaction networks by high-throughput methods. Sensitive, mass spectrometric high-throughput methods, for example matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, appear to be well positioned to make significant contributions to this area. The wide variety of experimental conditions and instrumental parameters allow one, in principle, to detect strong (specific) noncovalent interactions by MALDI MS. However, the study of intact noncovalent biomolecular complexes by MALDI MS has found limited use, because it generally causes dissociation of the interaction partners and formation of nonspecific aggregates (false positives). Since the initial studies of Karas and Hillenkamp,[1−3] relatively few cases have been reported where specific noncovalent complexes,[4−7] rather than dissociated partners or nonspecific aggregates,[8,9] were successfully observed with MALDI MS. An excellent review article in this field has recently been published by Bolbach.[10] One possible strategy to avoid the dissociation during the MALDI process is the stabilization of the complexes by chemical cross-linking. Chemical cross-linking in combination with mass spectrometry[11,12] has been mainly applied for mapping 3D structures of proteins and protein complexes in vitro[13] and to analyze multiprotein complexes in vivo.[14,15] In these cases, mass spectrometry was used in the final step, for identifying peptides after digesting the cross-linked complexes.

Only a few examples have been published showing the capacity of chemical cross-linking and MALDI MS to analyze specifically stabilized intact complexes.[16−19] One of the major

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difficulties when analyzing full-length protein complexes by MALDI MS is the limitation of standard instruments to detect them in the high-mass range. Such analyses, however, would be of great interest for the direct determination of the stoichiometry and architecture of biologically active multiprotein assemblies and insights into the affinity of the interacting partners.

Within the context of the formation of protein−ligand complexes, the intensity fading MS approach has recently been established.[20] It is based on the observation of a reduction in the relative signal of a small MW ligand when its receptor protein is added to the MALDI sample. We proposed that intensity fading MS may be an advantageous strategy to detect noncovalent interactions when direct detection of intact complexes in solution is difficult.[20,21] Although the underlying mechanism is not completely understood, we have suggested that the signal of the ligand molecule fades because the intact noncovalent complex is formed and partially survives the MALDI desorption/ionization process in samples prepared by the dried-droplet method. Analyte suppression effects that are sometimes observed in MALDI experiments may contribute to the ligand fading,[22] although it is very unlikely that they would operate so specifically. However, neither the specific intact complexes nor the receptor protein alone (after putative partial dissociation of the complex) could ever be detected during such intensity fading assays. We hypothesized[22] that ion detection is compromised because of the usual drop-off of the detection efficiency with increasing mass, due the decreasing impact velocity on conventional ion-to-electron conversion detectors (e.g., microchannel plates, MCPs).

Cryodetection is an ion detection technology that shows no decrease in sensitivity with increasing mass. The cryodetector technology[23,24] has the capacity to analyze the protein content of highly heterogeneous protein mixtures with the same sensitivity, independent of their mass.[25] The benefits of cryodetector technology combined with chemical cross-linking for protein complexomics have recently been demonstrated by Nazabal et al.[26]

The goals of the present study were 2-fold: first, to test the combination of the intensity fading and cryodetection MALDI strategy with real-world, complex biological mixtures, and second, to gain insight into the underlying mechanism of intensity fading MS, profiting from the capabilities of the cryodetector technology for TOF MS. For this purpose, we studied the intact noncovalent complexes formed between recombinant human carboxypeptidase A (hCPA) and three inhibitors, all of them well characterized: potato carboxypeptidase inhibitor (PCI),[27] tick carboxypeptidase inhibitor (TCI),[28] and leech carboxypeptidase inhibitor (LCI).[29] Human carboxypeptidase A is an interesting biomedical target enzyme as it is involved in prostate cancer,[30] and also because it is one of the "canonical" forms of a large family of metalloenzymes of great biotechnological and biomedical interest.[31] Standard intensity fading assays of these inhibitors within highly heterogeneous biological mixtures, using a conventional MCP detector for MALDI-TOF MS were first carried out. We then used chemical cross-linking and cryodetection MALDI-TOF mass spectrometry to confirm these results and to detect the complexes formed in the high mass range. The results expand this new strategy as a more general high throughput binding assay to detect noncovalent interactions in complex biological samples.

Experimental Section

Materials. The MALDI matrices sinapic acid (SA) and 2,6-dihydroxyacetophenone (DHAP) were purchased from Sigma-Aldrich (Buchs, Switzerland) and were used without further purification. Ammonium citrate was purchased from Fluka (Buchs, Switzerland). Acetonitrile (ACN) was LiChrosolv grade (Merck, Darmstadt, Germany). Trypsin (modified/sequencing grade) was purchased from Promega (Madison, WI). Human carboxypeptidase A (hCPA), human kallikrein, potato carboxypeptidase inhibitor (PCI), tick carboxypeptidase inhibitor (TCI), and leech carboxypeptidase inhibitor (LCI) were obtained as previously described.[27−29,32,33] All aqueous solutions were prepared using Milli-Q water filtered with a 0.2 μm membrane filter (Millipore, Bedford, MA). Extract from *Hirudo medicinalis* was supplied by the group of Profs. H. Fritz and C. Sommerhoff (Chirurgischen Klinik Innenstadt, Ludwig-Maximilians-Universitat, Munich, Germany). An extract from *Stichodactyla helianthus* was prepared as previously reported[34] and supplied by the group of Prof. M. Angeles Chavez (Department of Biochemistry, Universidad de La Habana, Cuba).

Biomolecule Interaction Experiments. (a) Biological Sample Preparation. Lyophilized samples of LCI, PCI, TCI, and hCPA were dissolved in 20mM ammonium acetate at the desired concentration. Trypsin was dissolved in Milli-Q water at the desired concentration. Lyophilized leech extract (~2 mg) was dissolved and subjected to a reversed-phase HPLC on a Vydac C_{18} column, using a linear gradient from 10 to 50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min for 60 min. RP-HPLC fractions enriched with proteins in the mass range of the human carboxypeptidase inhibitors were collected, lyophilized, and dissolved in Milli-Q water. Possible human carboxypeptidase A inhibitory activity of the selected fractions was checked by measuring the inhibition of the hydrolysis of the chromogenic substrate *N*-(4-methoxyphenylazoformyl)-Phe-OH by human carboxypeptidase type A at 350 nm. Lyophilized extract of *Stichodactyla helianthus* was directly dissolved in deionized water at a concentration of 10 mg/mL without further fractionation.

(b) Binding Experiments in Solution. Nonbinding control molecules (RP-HPLC fractions of *Hirudo medicinalis*) were diluted to the desired concentration, which gave MALDI MS ion signals intensities resembling those of the human carboxypeptidase A inhibitors assayed in parallel. Although MALDI is not a quantitative technique, we assume the relative concentration of each of the observed nonbinding molecules in the mass spectra to be in the same range as that of the inhibitors (~0.2−1 pmol/μL). The reacting partners (1 μL of each) (protease inhibitor, nonbinding molecules, and protease or ammonium acetate) were mixed and incubated for 3 min at room temperature. As the inhibitor was endogenous in the case of the SphI-1 inhibitor from *Stichodactylus helianthus*, only 1 μL of both the complex biological mixture and the protease were incubated for 3 min at room temperature. The sample was mixed with a matrix solution (1:2 v/v) of sinapic acid (10 mg/mL) containing 30% acetonitrile (v/v) diluted in deionized water (pH ~3), or 2,6-dihydroxyacetophenone (10 mg/mL) containing 30% acetonitrile and 20 mM ammonium citrate (v/v) diluted in deionized water (pH ~ 5). After mixing, 0.5 μL of the mixture were deposited on the MALDI target using the dried-droplet method.[35]

(c) Binding Experiments with Chemical Cross-linking. The cross-linking reactions were carried out using a commercial kit (K100 Kit, CovalX, Zürich, Switzerland) containing different

cross-linkers, suberic acid bis(3-sulfo-*N*-hydroxysuccinimide ester), suberic acid bis(*N*-hydroxysuccinimide ester), disuccinimidyl tartrate, and dithio-bis(succinimidyl) propionate, with affinity for accessible ⟨-amine groups present on the N-termini of proteins and ∑-amines on lysine residues. The cross-linking chemistry and its specificity has been studied and described in detail in ref 26. The interacting partners forming noncovalent complexes were present in the 0.5−1.0 μM concentration range, in a volume of 10 μL. After addition of the cross-linking reagent, the sample was incubated for 4 h to achieve complete reaction. After cross-linking, all samples were dried using a SpeedVac to remove the solvent. The sample was redissolved in 10 μL of deionized water, and 1 μL of the sample containing the stabilized complex was mixed with 2 μL of the matrix solution of sinapic acid (10 mg/mL) containing 70% acetonitrile, 30% deionized water, and 0.1% TFA. After mixing, 0.5 μL of the mixture were deposited on the MALDI target using the dried-droplet method.

MALDI-TOF Mass Spectrometry with MCP Detector. MALDI mass spectra were obtained using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser, a gridless ion source, delayed-extraction (DE), and a 2-GHz digitizer. The instrument was operated in linear mode by applying an accelerating voltage of 20 kV. Delayed extraction was used, and the delay time was set according to the molecular weight of the analytes (protease inhibitor or complex) to optimize resolution of its molecular ions. Mass spectra were acquired by averaging 300−600 shots (3 different locations on one sample spot and 100−200 shots per location). The laser pulse energy was adjusted to slightly above threshold for ion production for each MALDI matrix. All subsequent mass spectra acquisition were performed applying the same laser fluence, including the control spectra and after addition of the target protein.

Cryodetection MALDI-TOF MS. All the high mass measurements were performed on the macromizer instrument (Comet AG, Flamatt, Switzerland). The macromizer is a special MALDI-TOF mass spectrometer designed to optimize ion transmission onto the small area of the cryodetector array. Every part of the instrument including all necessary ion optics, lasers and electronics were designed to enhance detection of high mass ions. A 337 nm nitrogen laser (VSL 337ND-S; Spectra-Physics; Mountain View, CA) capable of producing energies up to 90 mJ with 4 ns pulse widths at 30 Hz repetition rate (although the data adquisition itself is limited to 10 Hz) is focused to a spot size of approximately 0.1 mm (measured using burn paper) for desorption/ionization. The repeller/sample plate is maintained at voltages from 0 to 20 kV. The extraction plate is operated using delayed extraction times from 200 ns to 25 μs, lowering the voltage applied to it by 0−3000 V below the source plate. For all work described in this paper, a 15 kV sample plate voltage, a 3 kV extraction voltage, a 8 kV Einzel lens voltage and a 4 μs extraction delay time were used. The detector used in the current macromizer instrumentation is a superconducting tunnel junction (STJ) array (VTT Technical Research Centre of Finland; Espoo, Finland). The entire detector is housed inside a magnetic field coil which is present to suppress Cooper pair tunneling and other effects such as Fiske resonances. Because the STJ detector measures thermal currents and because the materials rely upon superconducting principles to operate, it is necessary to cool the detector to temperatures much below the material's superconducting temperature gap. The detector is attached to a coldfinger and is maintained at a temperature of approximately 350 mK.

Results

Intensity Fading MALDI-TOF MS Assays. We studied the specific binding of the hCPA to three different protein inhibitors of this enzyme: PCI (4298 Da), LCI (7395 Da), and TCI (7935 Da) when added to heterogeneous mixtures of 30−50 unknown nonbinding molecules derived from a natural extract of the leech *Hirudo medicinalis*, rich in peptides and small proteins.[21] A key piece of information available about the compounds present in this extract was the absence of any hCPA inhibitory activity as measured by classical spectrophotometric assays (data not shown). This sample represents a real-world experimental problem in which specific ligands are not yet purified from the biological extract. Figures 1, 2, and 3a,b display the MALDI mass spectra before and after the addition of hCPA (approximately 1:1 molar ratio with respect to the specific inhibitors). A clear fading of the PCI (0.6 pmol/μL), TCI (0.6 pmol/μL), and LCI (0.9 pmol/μL) signals was observed. Figure 1c shows the mass spectra for increasing amounts of hCPA added to the heterogeneous mixture. Gradual fading of the relative intensity of PCI is observed. The signal disappears almost completely at a 1:1 molar ratio of hCPA:PCI. The other signals in the sample are virtually unaffected by the addition of the target protein, confirming that analyte suppression effects due to the MALDI process are unimportant at the concentrations used. Note that none of the specific complexes formed with the inhibitors (neither free hCPA) could be detected in the high mass range of the spectra recorded with the MCP detector (Figure 1b, inset). As a negative control, a protease of alternative binding specificity, human kallikrein, was added to the heterogeneous mixtures containing the hCPA inhibitors (1:1 molar ratio in the sub-micromolar range). Relative intensities, including those of the hCPA inhibitors, remained virtually unaffected by the presence of the human kallikrein (∼0.8 pmol/μL) (data not shown). This further supports the specificity of the intensity fading approach.

A similar analysis of specific binding using a naturally occurring trypsin inhibitor (SphI-1) and trypsin, followed by the intensity fading approach, was also applied to a biological extract of the sea anemone *Stichodactyla helianthus*, as previously described.[20] These organisms have been found to be an important source of bioactive molecules. Figure 4a,b shows the MALDI mass spectrum of a whole body extract of the sea anemone before and after the addition of trypsin. One signal in the mass spectrum, at $m/z = 6110$, that faded upon the addition of trypsin (0.8 pmol/μL), has been assigned as the SphI-1 Kunitz protease inhibitor by Delfin et al.[34] In this case, as SphI-1 is an endogenous inhibitor, the relative concentration of this analyte in the extract was unknown. Accordingly, increasing amounts of trypsin were added to the heterogeneous mixture to reach an almost complete fading of the signal corresponding to the protease inhibitor (data not shown). As in the above cases, the intact specific complex formed with the inhibitor could not be detected in the mass spectrum recorded with the MCP detector (data not shown).

Detection of Intact of Noncovalent Complexes by Cryodetection MALDI-TOF MS. In the following, we present results from the first commercially available MALDI-TOF mass spectrometer specifically designed for the sensitive detection of very high mass ions. Reproducing the experimental conditions (analyte concentration, MALDI matrix, laser shots, etc.) em-

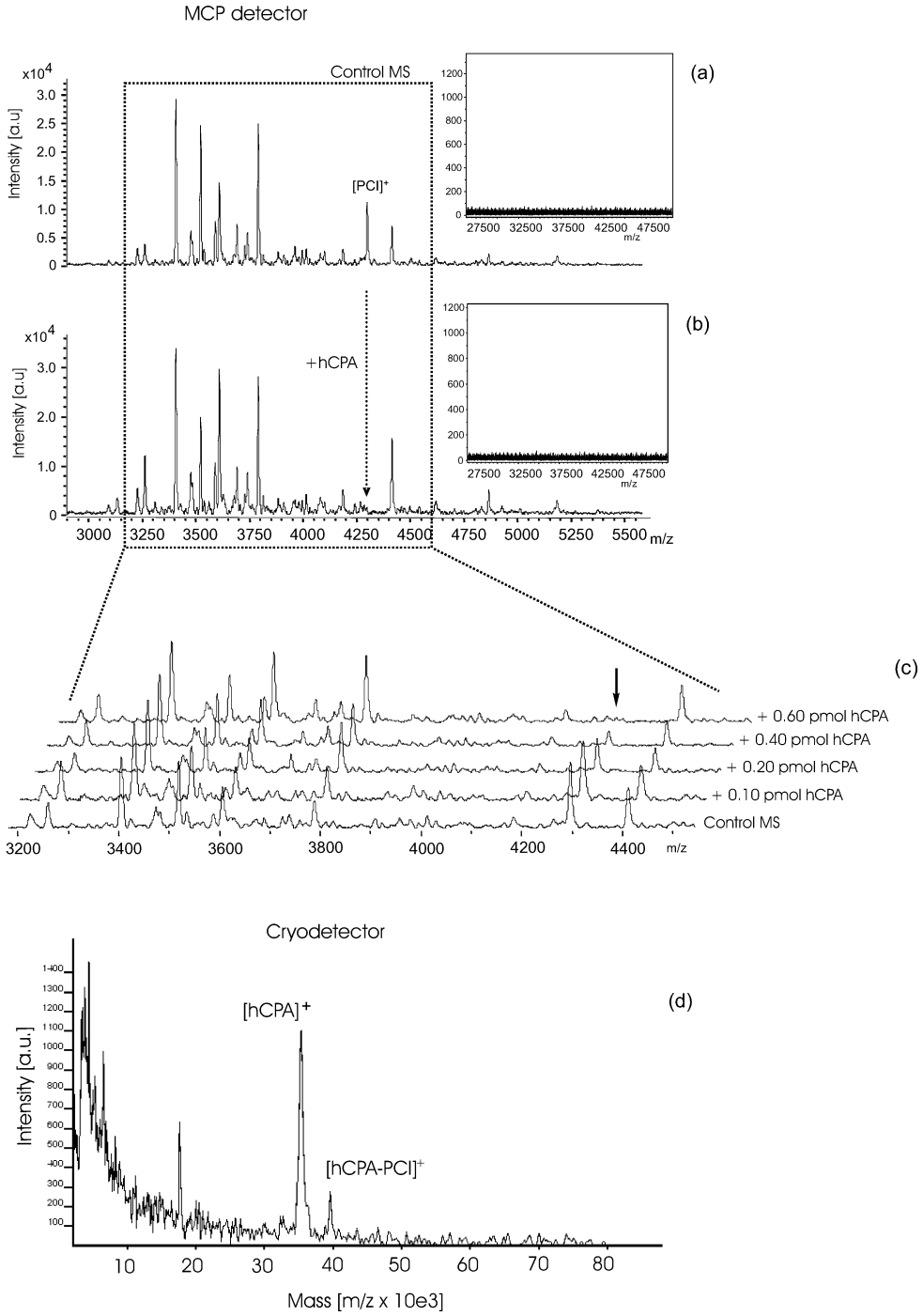

Figure 1. MALDI-TOF mass spectra of a complex mixture containing more than 50 nonbinding molecules plus potato carboxypeptidase inhibitor (PCI) (0.6 pmol/μL) (a) before (control MS) and (b) after the addition of human carboxypeptidase A (hCPA) (0.6 pmol/μL). The PCI peak is highlighted by a dotted arrow. Spectra covering only a limited mass range are displayed (3.0−5.5 kDa) for ease of visualization, however data was acquired over a much larger mass range showing the true complexity of nonbinding molecules. (Insets) Spectra covering the high mass range (27−50 kDa) before and after the addition of hCPA. Sinapic acid was used as matrix and dried-droplet as sample preparation. (c) MALDI-TOF mass spectra of the heterogeneous mixture containing nonbinding peptides and 0.6 pmol of potato carboxypeptidase inhibitor (PCI) in the presence of increasing amounts of human carboxypeptidase A (hCPA) (0.1, 0.2, 0.4, and 0.6 pmol). The PCI peak, indicated by an arrow, is observed to fade gradually until at an equimolar concentration of PCI and hCPA, the signal vanishes. (d) Cryodetection MALDI-TOF mass spectrum of the same mixture of nonbinding molecules analyzed in (a) in the presence of PCI (0.9 pmol/μL), after the addition of hCPA (0.5 pmol/μL). Sinapic acid was used as matrix and dried-droplet as sample preparation.

ployed for the intensity fading MS assays with the standard MALDI mass spectrometer, we used the cryodetector instrument to analyze the high m/z range of the samples described in the previous section. Figures 1d, 2c, and 3c show the mass spectra after the addition of the hCPA (0.5, ..., 0.9 pmol/μL) to the heterogeneous mixtures. The mass spectra clearly display the signal of the free hCPA, as well as the specific noncovalent complex formed with their natural inhibitors. The

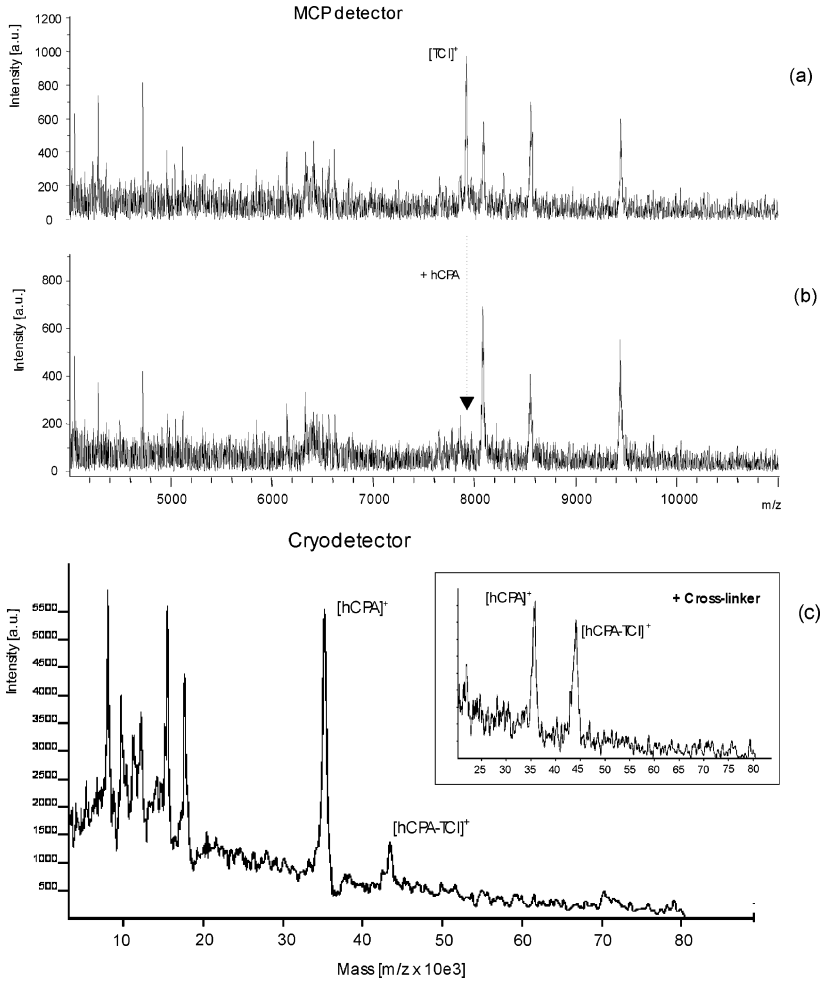

Figure 2. MALDI-TOF mass spectra of a complex mixture containing more than 30 nonbinding molecules plus tick carboxypeptidase inhibitor (TCI; 0.6 pmol/μL) (a) before (control MS) and (b) after the addition of human carboxypeptidase A (hCPA) (0.8 pmol/μL). The TCI ion peak is highlighted by a dotted arrow. Only a limited mass range is displayed (4.0−11.0 kDa) for ease of visualization. DHAP/ammonium citrate was used as matrix and dried-droplet as sample preparation. (c) Cryodetection MALDI-TOF mass spectrum of a complex mixture of nonbinding molecules and TCI (0.5 pmol/μL) after the addition of hCPA (0.9 pmol/μL). DHAP/ammonium citrate was used as matrix and dried-droplet as sample preparation. (Inset) Cryodetection MALDI-TOF mass spectrum of the same sample analyzed in (c) after the addition of hCPA (0.6 pmol/μL) in the presence of cross-linker reagents (4 h of reaction).

specificity of binding in this assay is confirmed by the absence of any kind of other complexes or aggregates (cluster ions). All three mass spectra show that the relative intensity of the specific complex is far less abundant than free hCPA. The low resolution, which is particularly evident for the low mass components (<10 kDa) of the complex mixture in the mass spectra, is an inherent characteristic of the macromizer instrument.

On the other hand, when the concentration of the hCPA was increased ~5-fold (4.75 pmol/μL) in the binding reaction, nonspecific aggregates of higher relative intensity than the specific complex appear in the mass spectra. This is exemplified in Figure 3d, where a hCPA dimer is detected. Considering the capacity of the cryodetector technology for intact protein analysis of highly complex mixtures, with the same sensitivity, independent of their mass, it becomes obvious that the amount of the hCPA dimer is more abundant than that observed for the hCPA-LCI complex. Therefore, in this particular experiment, the specific binding of LCI in the heterogeneous sample to the added hCPA cannot be confirmed, because aggregates most probably form in the plume, during the desorption/ionization process.[6,22]

Combination of Chemical Cross-Linking and Cryodetection MALDI-TOF. Chemical cross-linking stabilizes noncovalent complexes, but at the same time, it may induce unspecific aggregation of proteins under some experimental conditions. The same binding assays described in the previous sections were performed using similar complex mixtures of nonbinding molecules in combination with chemical cross-linking reagents. Figures 2c and 3c (insets) show the mass spectra after 4 h of reaction. In the entire mixture, and using the cryodetector, it is exclusively the noncovalent complexes hCPA-TCI (0.6 pmol/μL of hCPA and 0.5 pmol/μL of TCI) and hCPA-LCI (0.5 pmol/μL of hCPA and LCI) that became stabilized. The signal intensities corresponding to these complexes are far greater than those observed without the addition of the cross-linking reagents, reaching approximately the same relative intensity than that of free hCPA. No other binding to hCPA was observed, confirming the specificity of the assay. Again, when the concentration of the hCPA, the hCPA inhibitors, as well as the noninteracting compounds in the heterogeneous mixture was significantly larger (≈ 20 pmol/μL and above), we observed the formation of nonspecific complexes, together with the specific one, in the mass spectra (data not shown). On the other hand,

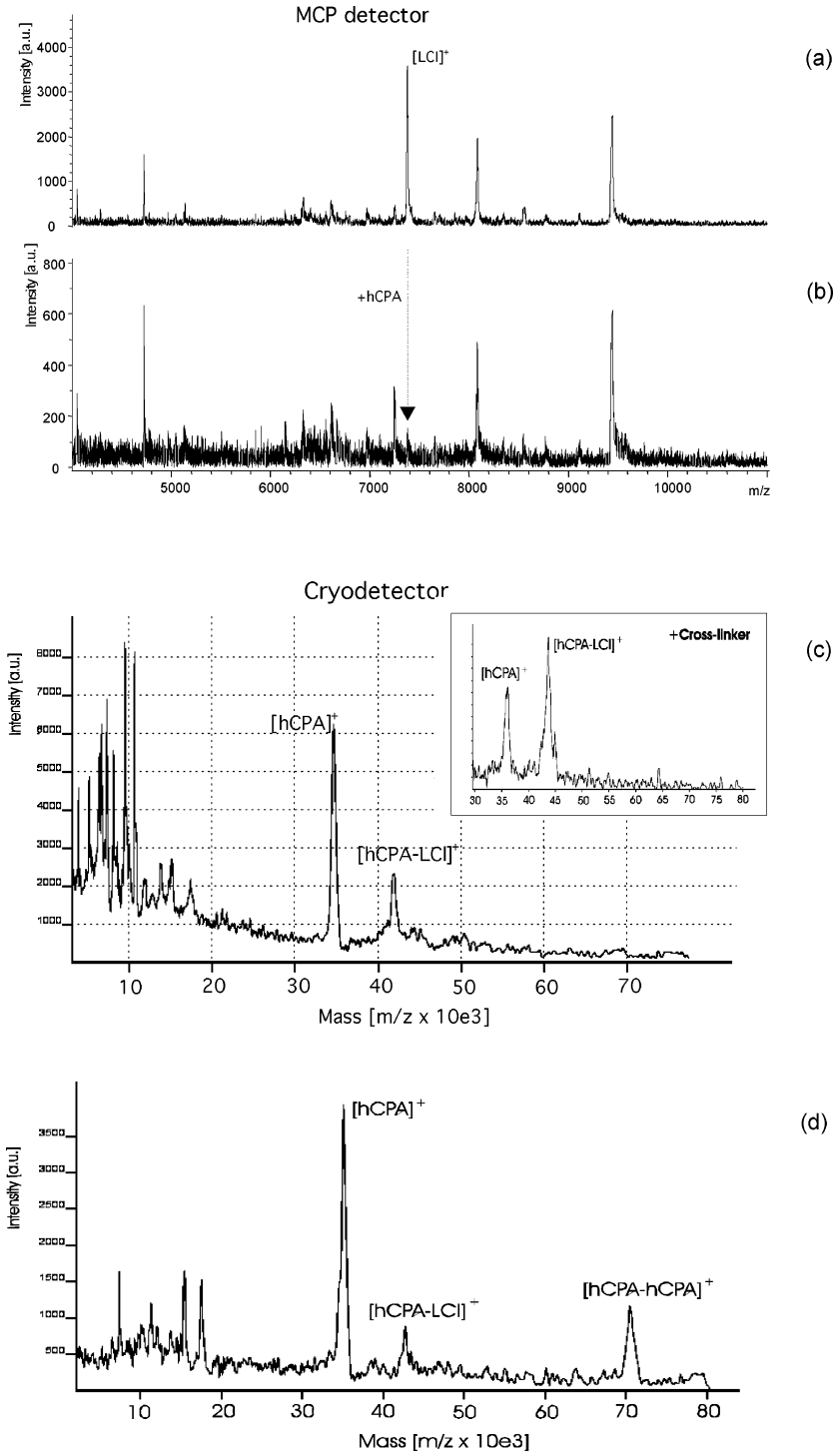

Figure 3. MALDI-TOF mass spectra of a complex mixture containing more than 30 nonbinding molecules plus leech carboxypeptidase inhibitor (LCI; 0.9 pmol/μL) (a) before (control MS) and (b) after the addition of human carboxypeptidase A (hCPA) (0.9 pmol/μL). LCI ion peak is highlighted by a dotted arrow. Only a limited mass range is displayed (4.0−11.0 kDa) for ease of visualization. DHAP/ammonium citrate was used as matrix and dried-droplet as sample preparation. (c) Cryodetection MALDI-TOF mass spectrum of a complex mixture of nonbinding molecules and LCI (0.9 pmol/μL) after the addition of hCPA (0.9 pmol/μL). DHAP/ammonium citrate was used as matrix and dried-droplet as sample preparation. (Inset) Cryodetection MALDI-TOF mass spectrum of the same sample analyzed in Figure 3c and LCI (0.5 pmol/μL) after the addition of hCPA (0.5 pmol/μL) in the presence of cross-linker reagents (4 h of reaction). (d) Cryodetection MALDI-TOF mass spectrum of a complex mixture of nonbinding molecules plus LCI (0.9 pmol/μL) after the addition of hCPA (4.75 pmol/μL). DHAP/ammonium citrate was used as matrix and dried-droplet as sample preparation.

when an extract from *Stichodactyla helianthus* was analyzed, the mass spectrum in Figure 4c indicated that the trypsin-SpHI1 inhibitor complex (0.8 pmol/μL of trypsin) was stabilized by the cross-linking reagents. No evidence at all of nonspecific complexes or aggregates with trypsin was found with this binding assay.

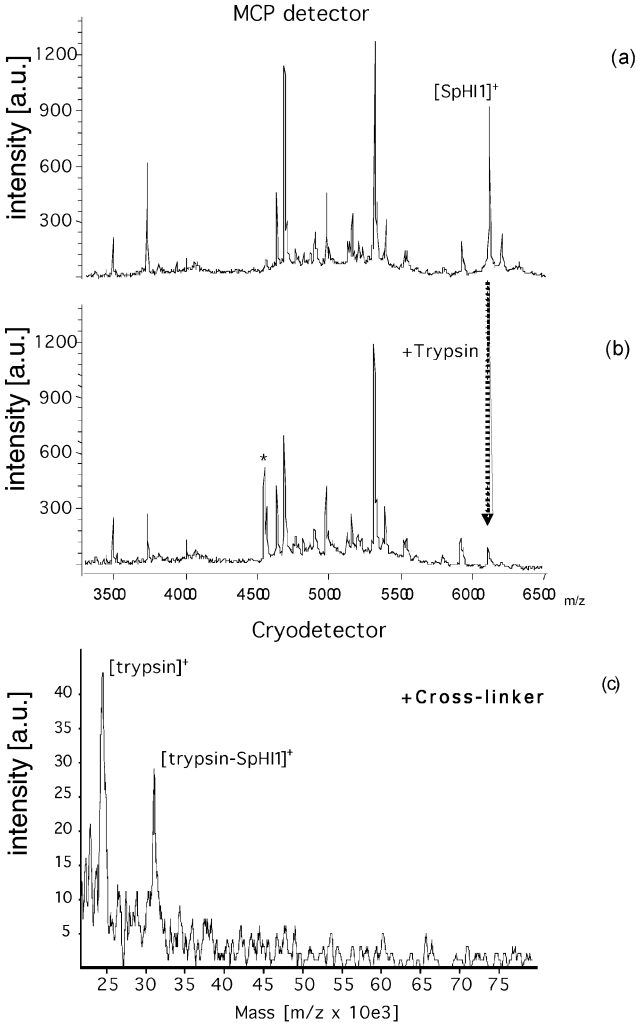

Figure 4. MALDI-TOF mass spectra of the sea anemone *Stichodactyla helianthus* (a) before (control MS) and (b) after the addition of trypsin (0.8 pmol/μL). The SpHI1 ion peak is highlighted by a dotted arrow. Sinapic acid was used as matrix and dried-droplet as sample preparation. The asterisk indicates an ion corresponding to a product of autolysis of trypsin. (c) Cryodetection MALDI-TOF mass spectrum of the same sample analyzed in Figure 4a after the addition of trypsin (0.8 pmol/μL) in the presence of cross-linker reagents (4 h of reaction).

Discussion

The results presented in this work answer one of the previously unsolved questions about the "intensity fading" mechanism in MALDI-TOF MS, i.e., the absence of a signal corresponding to the intact specific complex formed between the "faded" small protein ligand and the target protein added to the sample. On the basis of the results presented here, we can safely conclude that detection technology based on microchannel plates (MCPs), which is the standard detector for most commercial MALDI instruments, is the main factor that prevents detection of these noncovalent complexes. MCPs work by ejection of electrons following the impact of high velocity ions, initiating an electron cascade. The ion-to-electron conversion is extremely inefficient for large, low velocity ions; in fact, an exponential decrease in efficiency can be observed at higher masses. Additionally, MCP channels have a very slow refresh time, on the order of several milliseconds. This means that when an ion hits a channel, it is turned off for the duration of the analysis (microseconds). When measuring complex samples that contain ions over a broad mass range, the lower mass ions will saturate channels of the detector preventing the analysis of the higher mass ions, again leading to an additional decrease in sensitivity for the latter.[23,24] This is different for cryodetectors,[25] which are single ion counting devices, and therefore it becomes easy to detect high-mass macromolecules without the saturation effect observed for MCP detectors. With or without chemical cross-linking, our results show how the cryodetection technology allows the direct analysis of specific noncovalent protein complexes.

From our results, it becomes obvious that a significant degree of dissociation of the noncovalent complexes during the MALDI process with the present methodologies is inevitable when they are not stabilized covalently by chemical cross-linking. This is due to MALDI technology, which is not as "soft" as electrospray. Disruption of noncovalent complexes by MALDI seems to be enhanced by the nonphysiological conditions used for sample preparation (e.g., organic solvent, acidic pH, interaction of the noncovalent complex with the matrix molecules in solution and after crystallization, etc). Our experiments were performed with sinapic acid and 2,6-dyhidroxyacetophenone as MALDI matrices, using the dried-droplet sample preparation method. Further experiments should be carried out with other interacting partners and experimental parameters (i.e., first shot phenomenon) to investigate to what extent the differences in detection technology are responsible for the visualization of noncovalent complexes by MALDI-TOF mass spectrometry. In the present study, we took advantage of the intrinsic properties of a high-mass detector based on the cryodetection technology, which allowed us to work with complex mixtures of different proteins in the sub-micromolar concentration range. These experimental conditions avoid the formation of unspecific aggregates, which is an inherent and perhaps inevitable property of MALDI at high analyte concentrations. Therefore, the study of binding specificities by MALDI MS is improved because possible secondary reactions and collisions in the plume are significantly reduced. These results are in agreement with a recent systematic study of the intensity fading phenomenon,[22] in which protein concentration was shown to be an important factor for the success of the intensity fading experiments.

It is worth mentioning that cryodetection MALDI-TOF MS only avoids signal suppression effects at the level of detection. Signal suppression can still occur during desorption/ionization, which is another intrinsic property of MALDI caused by the limited number of useful desorption sites in the matrix[36] and by competition of the analytes for a limited number of charges (protons and cations).[37−39] In this sense, we have observed that when the overall concentrations of the nonbinding molecules and of the small ligand were significantly increased in the sample mixture compared to hCPA, or alternatively, the concentration of the hCPA was decreased, the relative intensity of the ion corresponding to the intact noncovalent complex (and to the free hCPA) was dramatically reduced or even missing in the mass spectra (data not shown). This clearly indicates that competition of the analytes for a limited number of charges can take place during the desorption/ionization processes. The extent of this will be determined by the physicochemical properties. However, by proper choice of the absolute and relative concentrations, MALDI signal suppression effects can be minimized.

Even using long incubation times and a variety of experimental conditions, it was not possible to observe exclusively the stabilized form of the complexes in the mass spectra. For instance, approximately 50% of the hCPA and trypsin remain in unbound form when analyzed by our approach. A similar phenomenon was observed in previous reports,[16−19] in which a significant part of monomers remained in the free state after the reaction with cross-linkers. This may be due to the limited reaction efficiency of cross-linker with primary amino groups of the proteins, the primary targets of the cross-linking agents used.[26] Also, in our case, some key amino acids of the active center of the enzymes can be chemically modified, affecting the equilibrium of forming the complex and the affinity between the interacting partners.

Nazabal et al.[26] have recently highlighted the power of chemical cross-linking combined with high-mass detection MALDI-TOF MS to detect intact noncovalent complexes in biological samples. This work confirms these findings with very different interacting partners, and shows its applicability for high-throughput screening assays. Only a few studies have been reported about the use of cross-linking and subsequent analysis of the intact stabilized complexes by MALDI-TOF mass spectrometry.[16−19] However, these studies were basically designed for the detection of hetero- or homooligomers (i.e., lysozyme, carbonic anhydrase, pyruvate kinase, hemoglobin) formed from simple mixtures composed exclusively of purified monomers. In addition, the concentration of the monomers was 20−50 μM, leading also to the formation of nonspecific aggregates in some cases. In our study, we found sub-μM concentrations to be the optimum range to avoid the formation of nonspecific complexes and, therefore, to increase the specificity of the binding assay.

Conclusions

The implementation of MALDI MS technology in reliable high-throughput binding assays is of great interest for the emerging field of interactomics and for ligand discovery in the both basic and applied research (i.e., in the pharmaceutical industry). In the present work, we have applied a new MALDI-TOF high-mass detector instrument equipped with the cryodetection technology (macromizer) to the detection on noncovalent complexes in complex biological mixtures, and compared the performance with current MCP detectors. The characteristics of the present high-mass detector technology, notably its ability to analyze highly heterogeneous protein mixtures with uniform sensitivity and in a mass-independent fashion, permitted us to carry out binding assays in the submicromolar concentration range. This was not previously possible in the high m/z range on standard MALDI-TOF instruments based on MCP detectors. In combination with chemical cross-linking, specifically bound complexes can be maintained throughout the MALDI analysis, whereas the formation of nonspecific complexes or aggregates during the desorption/ionization process or by unspecific reaction with the chemical cross-linkers is avoided. In the past, these have been the main shortcomings associated with the study of noncovalent interactions by MALDI MS, which may explain its rather limited contributions to this field. One way to partially circumvent this has been through indirect approaches, such as the observation of the decrease in the intensity of spectral signals belonging to protein ligands when target proteins are added to the sample, which we called intensity fading MS.[20−21] In this work, we were able to further support the proposed mechanism of intensity fading through direct observation of the complexes in the high mass range.

We strongly believe that new developments in ion detection, such as cryodetection for MALDI mass spectrometry, open up new prospects to implement reliable high-throughput affinity-based methods to detect biomolecular interactions in biological samples. Although the combination of cross-linking of the interacting partners and cryodetection MS have shown to be advantageous, we firmly believe that cryodetection MS (or equivalent technologies) has great potential beyond the strategy presented in the present work. The capacity to analyze the protein content of highly heterogeneous mixtures, with the same sensitivity, independent of their mass, may be exploited with "top-down" proteomic strategies to detect and characterize high molecular weight complexes, perhaps in combination with FTICR mass spectrometry. Cryodetection MS can be also coupled to classical "bottom-up" proteomic strategies such as protein tagging and affinity purification, surface plasmon resonance, immunoprecipitation, protein arrays, etc., as an alternative to 1D gel electrophoresis before enzymatic digestion. Cryodetection MS would improve sensitivity and speed, generate more informative and accurate data, and add the possibility to obtain stoichiometric information directly. These are some of the new challenges ahead of us.

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