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Introduction of 4-chloro-α-cyanocinnamic acid liquid matrices for high sensitivity UV-MALDI MS

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Running title: Liquid UV-MALDI MS using 4-chloro-α-cyanocinnamic acid

Nonstandard abbreviations: 3-AQ, 3-aminoquinoline; ABC, ammonium bicarbonate; AP, ammonium phosphate; CHCA, α-cyano-4-hydroxycinnamic acid; ClCCA, 4-chloro-α-cyanocinnamic acid; DHB, 2,5-dihydroxybenzoic acid; ILM, ionic liquid matrix; LSM, liquid support matrix; OGP, octyl β-D-glucopyranoside.

Keywords: ionic liquids, liquid matrices, MALDI, mass spectrometry

ABSTRACT

Matrix-assisted laser desorption/ionization (MALDI) is a key ionization technique in mass spectrometry (MS) for the analysis of labile macromolecules. An important area of study and improvements in relation to MALDI and its application in high-sensitivity MS is that of matrix design and sample preparation. Recently, 4-chloro-α-cyanocinnamic acid (ClCCA) has been introduced as a new rationally designed matrix and reported to provide an improved analytical performance as demonstrated by an increase in sequence coverage of protein digests obtained by peptide mass mapping (PMM) (Jaskolla, T. W *et al*. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 12200-12205). This new matrix shows the potential to be a superior alternative to the commonly used and highly successful α-cyano-4-hydroxycinnamic acid (CHCA). We have taken this design one step further by developing and optimizing an ionic liquid matrix (ILM) and liquid support matrix (LSM) using ClCCA as the principle chromophore and MALDI matrix compound. These new liquid matrices possess greater sample homogeneity and a simpler morphology. The data obtained from our studies show improved sequence coverage for BSA digests compared to the traditional CHCA crystalline matrix and for the ClCCA-containing ILM a similar performance to the ClCCA crystalline matrix down to 1 fmol of BSA digest prepared in a single MALDI sample droplet with current sensitivity levels in the attomole range. The LSMs show a high tolerance to contamination such as ammonium bicarbonate, a commonly used buffering agent.

INTRODUCTION

Matrix-assisted laser desorption/ionization[1] (MALDI) is one of the most commonly used ionization techniques for the analysis of (bio)molecules by mass spectrometry (MS) and has proven extremely useful in fields such as proteomics.[2-4] The choice of matrix can have a profound impact on peak intensities, relative abundance of analyte, fragmentation of the analyte and the types of analyte ions detectable. Considerable research has gone into trying to define what makes a suitable matrix for MALDI. In general, however, the only steadfast rule is that the matrix molecules should absorb the laser energy at the laser wavelength used without imparting excessive internal energy to the analyte, whilst promoting the desorption/ionization process (with ionization occurring by analyte protonation or deprotonation). Other desirable matrix qualities include vacuum-stability, chemical inertness, and being able to generate a homogeneous matrix/analyte mix. In practice, most matrices cannot meet all of the requirements so a compromise must be made based on the application. Whilst advances in the development of matrices in tandem with instrumental developments have allowed for an increase in sensitivity and general performance of MALDI MS, the gains made in terms of versatility have come predominately from the development of more effective matrices and optimisation of the sample preparation techniques.[5-8]

One development that seems particularly promising is the re-introduction of liquid matrices.[3, 9-13] Liquid matrices in the form of particle suspensions were first utilised by Tanaka *et al*[14] and have been reported for use in MALDI as long as MALDI itself. However, a lack of overall sensitivity meant that they lost favour in light of the advances made possible by the highly sensitive solid state matrices α-cyano-4-hydroxycinnamic acid (CHCA)[6] and 2,5-dihydroxybenzoic acid

(DHB)[5]. Recently, however, new liquid matrices have been introduced which promise to close this sensitivity gap. The liquid matrices essentially come in one of two forms, an ionic liquid matrix (ILM) or a liquid support matrix (LSM). Typically, ionic liquids (ILs) are salts that melt below 100°C, are non-explosive, non-flammable, stable below 250°C and possess negligible vapour pressure.[15] ILs suitable for MALDI were first introduced in 2001 by Armstrong *et al*.[16] and are typically created by the dissolution of an acidic MALDI matrix compound such as CHCA in the presence of a counter base such as 3-aminoquinoline[13] (3-AQ) or aniline[16] in a volatile solvent. Upon fast solvent evaporation a viscous vacuum-stable ILM remains. LSMs can be formed in a similar fashion to ILMs but with the addition of a liquid support such as glycerol.[3, 12] LSMs can also take the form of particle suspensions.[14, 17-19] However, the suspended particles in these matrices add only little if at all to the ionization process while the compounds suspended in the presented LSMs promote both desorption and ionization. Furthermore, laser energy deposition facilitated by suspended particles is markedly different to the energy absorption process provided by individual matrix molecules, i.e. in the case of UV-MALDI the electronic excitation of the matrix compound's chromophore.

Liquid matrices have a number of potential advantages over the commonly used crystalline matrices. They display superior sample homogeneity with a simpler morphology.[16, 20, 21] The liquid nature of these matrices allows for renewal and self-healing of the irradiated sample position enabling an extremely stable ion signal production and sample longevity.[22-24] Quantification can easily be achieved across a broad abundance range,[25, 26] and it has been shown that this can be performed without the need for an internal standard.[24] In addition it has also been shown that key properties of the matrix environment such as the pH can be tuned through the use of additives.[27] Liquid matrices have been used for the improved analysis of a variety of

molecules including peptides,[3, 28] proteins,[21] phosphopeptides,[29] glycopeptides,[30] ogliosaccarides,[30, 31] phospholipids,[32] and polymers.[16] Furthermore, liquid matrices have been reported to improve the results of peptide mass mapping (PMM), showing an increase in the Mascot-generated probability score for protein identification via database searching as well as improved protein sequence coverage when compared to crystalline CHCA.[3, 33]

Recently, 4-chloro-α-cyanocinnamic acid (ClCCA) has been introduced as a new rationally designed MALDI matrix compound.[34] As a crystalline matrix it has been shown to have great potential for the analysis of peptides by PMM showing an improvement in sequence coverage and sensitivity when compared to CHCA. This has been attributed to an increase in the number of small and/or acidic peptides which is hypothesised to be due to the lower proton affinity of the ClCCA matrix as compared to CHCA allowing for a more efficient proton transfer from the $[ClCCA+H]^+$ matrix ions to the analyte.[34]

We have taken this rational design one step further by developing and optimising two new liquid matrices, an ILM using ClCCA as the principle chromophore in combination with 3-AQ and an LSM with glycerol as the support. We have tested these two matrices in terms of their characteristics as liquid matrices and found them both to display a high degree of homogeneity as well as possessing similar signal stability in comparison to the two analogous matrices using CHCA as the principle chromophore. In addition the sequence coverage obtained using these matrices have been compared to their crystalline analogues using a bovine serum albumin tryptic digest as a model protein digest to determine as to whether the ClCCA-containing liquid matrices would provide a further increase in protein sequence coverage similar to that reported for CHCA-based liquid matrices over crystalline CHCA matrices.

EXPERIMENTAL

Materials

All solvents were purchased from Rathburn (Walkerburn, UK). The in-solution digest of bovine serum albumin (CAM-modified) was purchased from New England BioLabs (Hitchin, UK). All other reagents with the exception of ClCCA were purchased from Sigma (Poole, UK). ClCCA was synthesised by reacting malononitrile with 4-chlorobenzaldehyde in pyridine in the presence of piperidine (5 mol%). After 1 hour at 50°C the solvents were removed under reduced pressure. The remaining residue was acidified (1M hydrochloric acid) and then extracted with ethyl acetate. The ethyl acetate was dried (magnesium sulfate), filtered and then concentrated *in vacuo*. The resulting colourless solid was assessed by ^{1}H NMR and re-crystallised from hot ethanol until no signals that were not associated with the structure of the product were discernable giving a final impurity of less than 0.5%.

Instrumentation for MALDI-TOF MS

MALDI measurements were performed using an Ultraflex axial-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using FlexControl 3.0 acquisition software. Positive ion measurements were taken in reflectron mode using a nitrogen laser (337 nm) with an acceleration voltage of 25 kV and a delayed ion extraction setting of 40 ns. For all experiments, 384-well MTP 400μm-AnchorChip target plates were used (Bruker Daltonics).

MALDI sample preparation

Analyte preparation

Unless otherwise stated the analytes used were prepared in 50% MeOH / 0.05% TFA at a concentration to yield the final reported on-target amount after matrix and analyte premixing. The analyte composition of the individual analyte solutions used for each experiment can be found in the relevant subsections.

Solid state MALDI

Both the CHCA and ClCCA crystalline matrices for solid state MALDI were prepared at 3 mg/ml in acetone and diluted to a final concentration of 0.5 mg/ml in acetone:ethanol (1:4; v/v). In all cases the matrix solution was mixed with the analyte solution in a ratio of 2:1(v/v), and 1.5 µL of this mixture was deposited on the MALDI target. To increase MALDI sample homogeneity and concentration, the crystalline MALDI samples were re-crystallized on-target with 0.5 µL ethanol:acetone:0.1% trifluoroacetic acid (TFA) (6:3:1; v/v/v).

Liquid MALDI

The liquid matrices were prepared as follows. The CHCA-containing LSM was prepared by dissolving CHCA and 3-AQ in solution A (aqueous 10 mM ammonium phosphate (AP) 50% methanol solution) and glycerol in a ratio of approximately 1:3:5:5 (w/w/v/v) with 1 mg equating to 1 µL and then diluted by a factor of 30 with solution A. The ClCCA-containing LSM was prepared by dissolving ClCCA and 3-AQ in solution A and glycerol in a ratio of approximately 1:5:10:5 (w/w/v/v) and diluted by a factor of 20 with solution A. The CHCA-containing ILM was prepared by dissolving CHCA and 3-AQ in methanol in a ratio of 1:3:5 (w/w/v) and diluted by a factor of 30 with 50% methanol / 0.1% octyl β-D-glucopyranoside (OGP) with AP, which was adjusted to a final concentration of 5 mM. Finally, the ClCCA-containing ILM was prepared by dissolving ClCCA and 3-AQ in methanol in a ratio of 1:5:10 (w/w/v) and diluted by

a factor of 20 with 50% methanol with AP, which was adjusted to a final concentration of 5 mM. In all cases the initial stock solutions were sonicated until full dissolution occurred prior to the final dilution step. The stock solutions could be kept for several days at 4°C. For analysis the matrices were premixed 1:1 with analyte solution and a volume of 1 µL was deposited on the MALDI target.

MALDI-TOF analysis

MALDI imaging

The homogeneity of the MALDI sample preparation for each of the matrices was assessed by MALDI imaging. The on-target sample amounts were 100 fmol Bradykinin 1-7, 100 fmol Angiotensin I and 300 fmol ACTH clip 18-39. Custom geometry files (.xeo files) for FlexControl defining sampling positions were created using our in-house-written software and loaded by substituting the geometry file relating to the type of target plate used. The positions were sampled automatically using the AutoXecute function in FlexControl. An over-sampling technique was used by setting sample positions 25 µm apart (laser focus: ~50 µm). Spectra were acquired by data accumulation from a total of 10 laser pulses at each position. The resulting spectral files were combined and the peak intensity for a user defined mass (+/- 1Da-window) for each position was exported to Microsoft Excel™ using another in-house-written program.

Signal stability

To assess the signal stability and longevity of the liquid matrices 100 spectra were acquired from a central position of a liquid droplet containing 100 fmol of Angiotensin I. Each spectrum was

acquired as the sum of 100 single-shot spectra at 50 Hz with a gap of ~1 sec between each acquisition, resulting in a total of 10,000 individual exposures on a single position.

Quantitation

The ability of the ClCCA-containing LSM to perform quantitation without the need of an internal standard via the linear relationship between analyte amount present and signal response was assessed across a dynamic range of 5 – 1000 fmol (of each analyte) spotted on target using an equimolar mixture of 7 peptides. The mixture consisted of RFDS (m/z 524.25), Bradykinin (m/z 757.40), Angiotensin I (m/z 1296.68), Substance P (m/z 1347.74), Glu-Fibrinopeptide B (m/z 1570.68), ACTH (II) clip 18-39 (m/z 2465.20) and Somatostatin 28 (m/z 3147.47) (m/z values refer to monoisotopic protonated species). The peptide solutions were prepared in 50% MeOH / 0.05% TFA and premixed 1:1 with matrix with 1 µL spotted to give final on-target amounts of 5, 10, 50, 100, 250, 500 and 1000 fmols of each of the peptides. Four replicates of each dilution were spotted and 700 single-shot spectra were acquired from a single central position of which the last 500 were accumulated. The first 200 spectra were discarded to avoid any initial surface concentration effect as previously described.[24] The R^2 values were calculated from the average of the four spots.

PMM analysis

The BSA in-solution digest was dissolved in 50% methanol / 0.05% TFA for normal analysis or with 25mM ammonium bicarbonate (ABC) 50% MeOH / 0.05% TFA for an assessment of how the matrices respond to contamination with a commonly used buffering agent. The analyte was diluted for final on-target amounts of 100, 10 and 1 fmol with 1 µL of matrix/analyte mixture deposited for the liquid matrices and 1.5µL for the solid state matrices. A sum of five 100shot-

acquisitions was used for each analysis. Spectra were calibrated externally using a standard calibration mixture of nine peptides covering an m/z range of 524-3658. For the crystalline matrices, spectra were acquired by sampling multiple positions and selecting only those positions that gave a superior response in terms of the number of putative peptide signals, signal intensity, and resolution for the final summed spectra. In contrast, for the liquid matrices the spectra were acquired from a single central position only. Peak picking was performed with FlexAnalysis 2.4 using the SNAP (sophisticated numerical annotation procedure) algorithm. Peaks were picked between m/z 800-4000 with a maximum of 150 peaks and a signal-to-noise threshold of 4 (2 for 1fmol-samples). PMM analysis was performed using Mascot (Matrix Science Ltd., London, UK) against all entries in the Swiss-Prot database version 57.10 with a mass tolerance of 100 ppm, up to 3 missed cleavages, a fixed modification of cysteines by carbamidomethylation and a variable modification of methionine oxidation. In addition, a 250 attomol sample was analysed for the ClCCA-containing ILM. This was performed by diluting the BSA digest to 1 fmol/µL and then mixing it in a ratio of 1:2 with matrix solution prior to spotting out 0.75 µL resulting in 250 attomol of BSA on target. Spectra from a total of 1000 laser pulses were acquired for each of 4 replicates. Assignments were made using Biotools 3.1 (Bruker Daltonics) against the BSA sequence as listed in the Swiss-Prot database (P02769).

RESULTS AND DISCUSSION

Matrix Characteristics

The newly created ClCCA liquid matrices have very similar appearances to the previously reported CHCA-containing LSM.[3, 12] In the diluted form, the liquid matrices appear as a clear yellow liquid. After mixing with the analyte solution and deposition on the target, volatile solvent evaporation occurs. This results in the formation of a small, viscous, clear yellow, vacuum-stable matrix droplet, approximately 400-500 µm in diameter (Figure 1). The apparent viscosity of the droplet varies between the preparations, with the CHCA ILM appearing to show the highest degree of viscosity, followed by the ClCCA ILM preparation and then the two LSMs. The use of the 400µm-AnchorChip target was deemed to be preferable as the hydrophilic anchor aids in the localisation of the liquid matrix droplets as they shrink to cover the anchor. Spotting directly on to a hydrophobic portion of the target plate, which did not contain an anchor, results in the formation of a spot of similar size and dimensions. However, the final position of the spot is more variable. In general, at least for the matrices presented here, any smooth and/or hydrophobic surface can be utilised and for example, data has been published utilising polished stainless steel[30] and Teflon™ tape.[24] Attempts to use brushed stainless steel resulted in the formation of a thin film as the matrix droplet diameter did not reduce.

The CHCA-containing ILM has a tendency to form a thin film which appears to be less favourable for MS measurements as positions can easily become depleted of matrix forming a hole within the film rather than self-healing of the film. The addition of OGP was found to alleviate this problem promoting droplet formation. Increasing the level of OGP up to 0.1% within the diluted matrix resulted in a progressive alteration in the droplet appearance from thin

film to droplet formation (see Supplemental Data 1). Further increases of the level of OGP cause a disruption of the droplet's surface tension, which results in the formation of several smaller droplets of varying sizes. The exact nature of these effects has not been investigated.

AP can be added to all of the liquid matrices in order to avoid the presence of matrix clusters within the mass range of interest and reduce the formation of adduct peaks (mainly due to sodium and potassium)[3, 35, 36] For the CHCA-containing LSM without the addition of AP intense matrix cluster peaks are observed at m/z 855, 1060 and 1066. With the addition of AP at a concentration of 0.5 mM within the diluted matrix preparation these can be completely suppressed. This, however, is not sufficient to suppress adduct ion formation within the LSMs. It was found that the glycerol-based LSMs required an increased concentration of AP in comparison to the ILMs (10 mM versus 5 mM) in order to suppress adduct ion formation. The ILMs with the inclusion of AP have a very similar degree of adduct formation and matrix background in comparison to the solid state matrices. Unfortunately, the elevated low-mass matrix background signals below 650 Da seen within the LSMs are higher than those seen for the other matrices. However, this is below the mass range important for the performance of PMM experiments. It was also noted that further increasing the concentration of AP, resulted in a higher laser energy threshold for analyte ion detection for the glycerol-based LSMs but no significant further gains in terms of adduct suppression were observed. Without the addition of AP the laser energy thresholds for LSMs were similar to the ILMs. The apparent laser energy thresholds for analyte ion detection can be roughly summarized as follows: CHCA-solid state and CHCA-ILM < ClCCA-solid state and ClCCA-ILM < CHCA-LSM < ClCCA-LSM.

Initial stock solutions remained viable for up to one week if stored at 4°C. When deposited on the target the matrix droplets were useable without significant loss of sensitivity for at least 24 hours with or without being under vacuum, although it should be noted that when removed from the vacuum the ClCCA-containing liquid matrices became cloudy and solidified taking on a paste-like consistency within one hour. The CHCA-containing liquid matrices seemed to remain liquid for at least 5 days on the target after initial analysis but matrix performance degraded and the matrices turned to a dark brown colour.

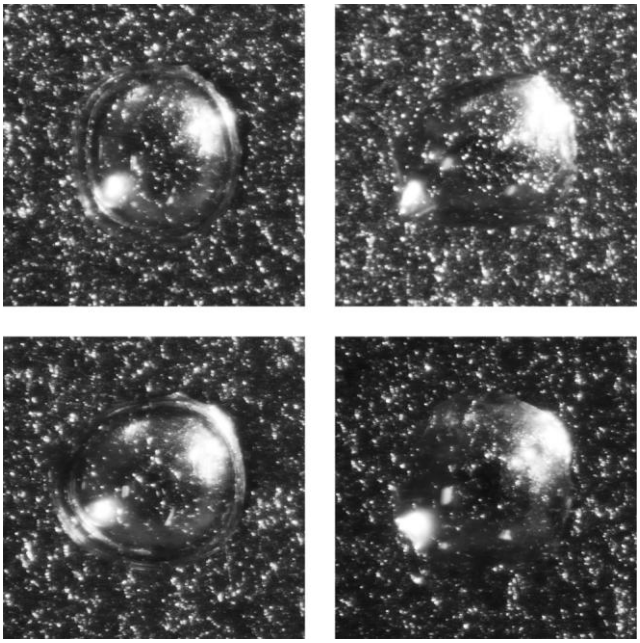

Figure 1. Liquid MALDI samples after evaporation of the volatile solvents. Top left, CHCA-containing glycerol LSM. Top right, CHCA-containing ILM. Bottom left, ClCCA-containing glycerol LSM. Bottom right, ClCCA-containing ILM. Sample appearance was unchanged after exposure to the vacuum. Each spot/droplet has a diameter of ~400 μm.

Homogeneity

Considering the tendency of crystalline matrices to form inhomogeneous analyte-matrix distributions, which can complicate analysis, the liquid matrices with their superior homogeneity can be highly desirable. In order to examine the analyte distributions various solid state and liquid matrices were analysed by MALDI imaging. Figure 2 shows the results of these analyses for the peptides Bradykinin 1-7, Angiotensin I, and ACTH clip 18-39. It can be seen that the liquid matrices, particularly the LSMs, have a much simpler analyte distribution in comparison to the crystalline matrices. The crystalline matrices (Figure 2a/b) have several pronounced "hot spots" displaying a complex distribution, which is different for each peptide. The MALDI images of the liquid matrices show less "hot spots" and a smoother analyte ion signal distribution (Figure 2c-f) somewhat reflecting the three-dimensional shape of the droplet and the incidence angle of the laser beam. The comparison of the distributions of the three analytes also shows that the liquid matrices enable a relatively similar analyte distribution in contrast to the differential, analyte-dependent ion signal distribution observable in crystalline matrices. However, it can be observed that the ILM samples (Figure 2d and f) show a slightly decreased homogeneity compared to the LSM samples. The reason for this is unclear but could be attributable to the process of solvent evaporation. It can be observed that as the ILM samples undergo the final stages of solvent evaporation two distinct domains form, an outer domain and an inner domain. The inner domain, which is slightly lighter in coloration, reduces in size until it is no longer apparent. It is possible that the slight decrease in homogeneity is due to a varying degree of concentration of some analytes within this inner domain. This is not observed for the LSM samples, possibly due to the glycerol support aiding in the homogeneity of the matrices by

allowing all components to remain evenly distributed during the volatile solvent evaporation process.

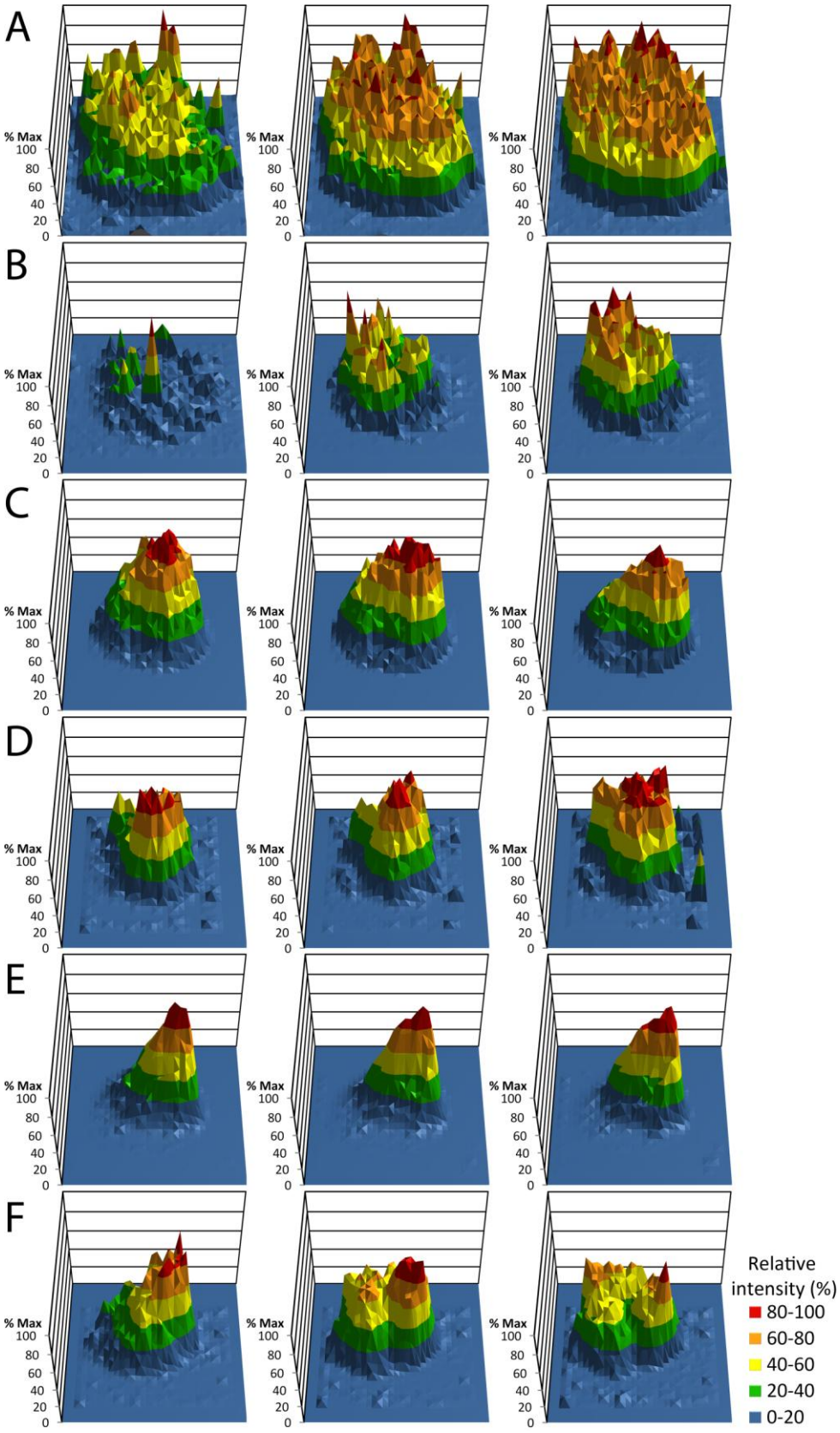
A
B
C
D
E
F
% Max
100
80
60
40
20
0
Relative intensity (%)
80-100
60-80
40-60
20-40
0-20

Figure 2. MALDI analyte ion signal distribution studied by MS imaging for the crystalline matrices CHCA (A) and ClCCA (B) and the liquid matrices CHCA-containing LSM (C), CHCA-containing ILM (D), ClCCA-containing LSM (E), and ClCCA-containing ILM (F). The distributions of the analytes Bradykinin 1-7 (left column; 100 fmol spotted), Angiotensin I (middle column; 100 fmol spotted) and ACTH 18-39 (right column; 300 fmol spotted) can be seen. The images were taken with a 25µm-raster (laser focus: ~50 µm). A total of 10 single-shot spectra at 50 Hz were recorded for each MS imaging point. The images are normalised against the maximum intensity obtained. Each image displays an irradiated area of 825 µm x 825 µm.

Continuous single position sampling

One key advantage of liquid MALDI is the ability to sample from a single position for extended periods of time or number of laser pulses without extensive ion signal fluctuation or loss of ion signal. This has advantages for techniques such as MS/MS or instrument tuning as a prolonged stable ion signal is desirable[24]. Figure 3 shows the stability of ion production for the peptide Angiotensin I (100 fmol spotted) over the course of 10,000 laser pulses recorded as a series of 100 spectra, each the summation of 100 single-shot spectra for each of the liquid matrices. The CHCA-containing liquid matrices (Figure 3a) appeared to possess greater stability in comparison to the ClCCA-containing liquid matrices (Figure 3b) with only a slight gradual decrease in signal intensity of ~25% over 10,000 laser pulses. Despite the ClCCA-containing LSM being less stable across the 10,000 laser pulses it maintained ion signal intensity. The ClCCA-containing ILM showed a less stable ion signal, decreasing by 61.7% after 5,000 laser pulses. However, the signal-to-noise ratio only fell by 35.3% to 493.3. This matrix may benefit from a lower laser pulse repetition rate as unlike the other liquid matrices, a localized distortion of the spot's hemi-spherical appearance was observed along with a minor darkening of the exposed area which could be due to inferior droplet renewal at the high laser pulse repetition rate of 50 Hz. In comparison, the ion signal was depleted within 1,000 laser pulses when continuously sampling a single position with either of the crystalline matrices. The ion signal could be extended for the crystalline matrices by rastering across the surface. However, the signal stability would be affected by the crystal morphology and the sample homogeneity at each position as has been previously shown[24] and can be seen in Figure 2a/b. Although thin-layer sample preparations can to some extent alleviate the problem of sample inhomogeneity, this is typically achieved at the expense of faster overall sample depletion, particularly when compared with liquid MALDI

samples.

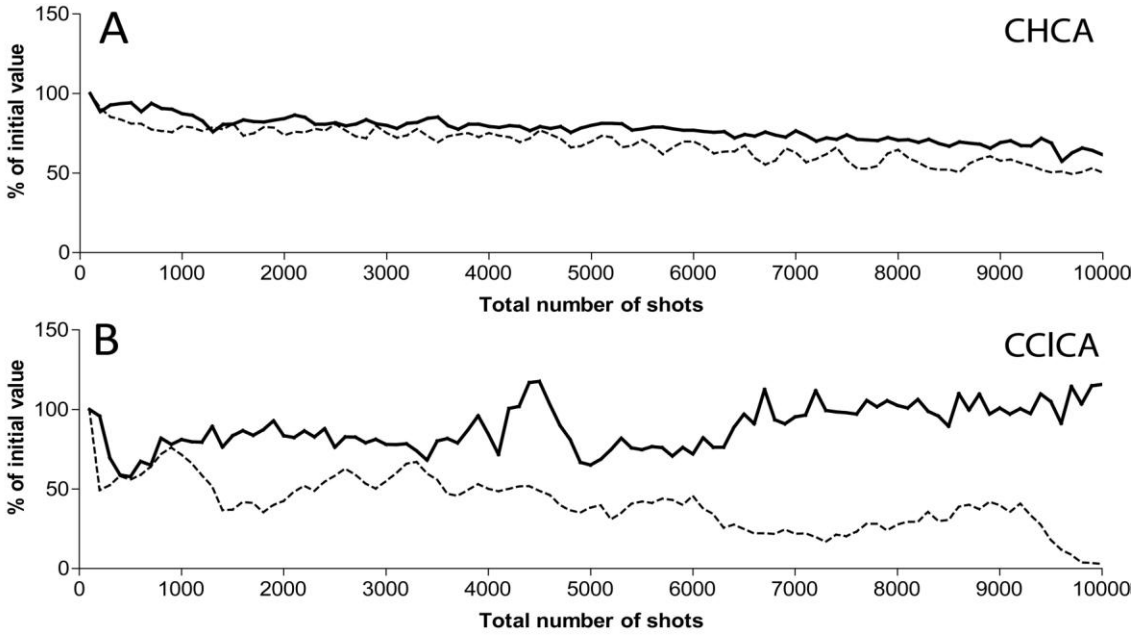

Figure 3. Ion signal intensity of the $[M+H]^+$ of Angiotensin I with an increasing number of laser pulses. Spectra were acquired as the sum of 100 single-shot spectra at 50 Hz from a single desorption position with ~1 sec between each accumulation, for a total of 10,000 laser pulses. Panel A, CHCA-containing LSM (solid line), CHCA-containing ILM (dotted line). Panel B, ClCCA-containing LSM (solid line), ClCCA-containing ILM (dotted line).

Quantitation

Stable ion flux also facilitates quantitative analysis. Quantitive analysis of peptides using liquid matrices has previously been demonstrated with a linear dynamic range of up to 3 orders of magnitude and extremely high R^2 value for the CHCA-containing LSM.[24] Consequently, the ability to perform quantitation was also assessed for the ClCCA-containing LSM using an equal mixture of 7 peptides (m/z 524-2465) with a dynamic range of 5 fmol – 1 pmol. The average R^2 value for the 7 peptides was 0.98 with three peptides having an R^2 value of greater than 0.99 (Figure 4).

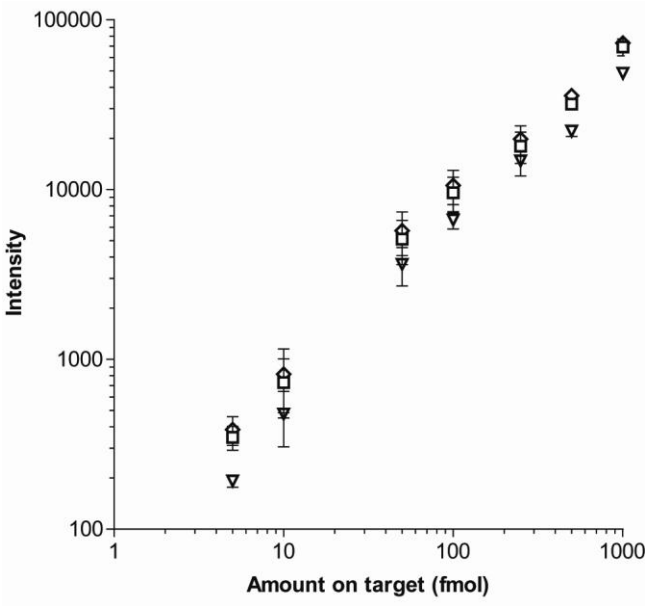

Figure 4. Ion signal intensity as a function of sample amount (5 – 1000 fmol spotted) for the ClCCA-containing LSM. Four spots were measured for each data point (500 laser pulses each). Data are shown for Angiotensin I (diamonds), Substance P (squares) and Glu-Fibrinopeptide B (circles). All R^2 values are greater than 0.99.

Peptide mass mapping

Previous analyses of tryptic BSA digests have shown that both ClCCA as crystalline matrix and the utilisation of liquid matrices lead to an increased sequence coverage.[3, 33, 37] We have investigated whether the same effect can be observed with the newly created ClCCA liquid matrices and whether the use of ClCCA will give a further increase over the CHCA-based liquid matrices. Table 1 shows the results of Mascot searches on spectra generated using 100, 10 and 1 fmol of a BSA in-solution digest for the crystalline and liquid matrices. Example spectra for each matrix at 100 fmol can be seen in Figure 5. The spectra shown in Figure 5 were selected as those that were closest to resembling the average displayed in Table 1 and are the result of the summation of 500 single-shot spectra. At an analyte level of 100 fmol all of the liquid matrices showed an increase in performance compared to the CHCA crystalline matrix. The ClCCA-containing ILM was relatively similar to the ClCCA crystalline matrix, with higher sequence coverage but a lower average number of matching peaks. At 10 fmol the performance of the ClCCA-containing ILM and ClCCA crystalline matrix was again similar while the other liquid matrices were roughly equal in performance to the CHCA crystalline matrix. With 1 fmol on target the ClCCA crystalline and ClCCA-containing ILM preparations outperformed the other matrices with the ClCCA-containing ILM showing a higher sequence coverage and higher number of matched peaks, as well as a higher MASCOT score when compared with its crystalline counterpart (Table 1).

The lower sequence coverage of the LSM samples compared to the ILM samples is possibly related to an effect of surface activity with some peptides being present at a lower concentration at the surface. Alternatively, the LSM samples may have a higher overall homogeneity with the peptides within the ILMs being concentrated to a higher degree at the surface, thereby increasing

the sensitivity. In either case the surface homogeneity, as investigated in Figure 2, is less affected and thus, further studies, for instance of the internal spatial distribution of the analyte and matrix components within the droplet, are necessary. However, it should be noted that in previous studies it has been found that for a liquid matrix comprising of diethanolamine and CHCA, surface partitioning is severely suppressed in comparison to aqueous systems.[38-40]

Table 1 – Results of Mascot searches for the analysis of 100, 10, and 1 fmol on-target amounts of BSA in-solution digest (averaged over 5 replicates for each matrix, 4 replicates for 1 fmol of analyte). Peaks were picked between m/z 800-4000 with a signal-to-noise ratio ≥ 4 (≥ 2 for 1 fmol).

Matrix	# of picked peaks	sequence coverage [%]	# of matched peaks ± SD	Mascot score (sig ≥ 70) ± SD
100 fmol				
ClCCA	142	79.4	58 ± 4	317.4 ± 32.8
ClCCA ILM	150	82.4	53 ± 1	269.4 ± 9.2
ClCCA LSM	150	75.4	49 ± 2	237.8 ± 14.8
CHCA	112	70.4	44 ± 5	241.2 ± 25.9
CHCA ILM	133	75.0	48 ± 5	256.4 ± 27.2
CHCA LSM	150	75.2	51 ± 2	248.0 ± 17.0
10 fmol				
ClCCA	105	74.8	49 ± 2	305.0 ± 15.9
ClCCA ILM	112	72.8	48 ± 3	288.6 ± 13.1
ClCCA LSM	87	54.8	35 ± 3	210.6 ± 26.3
CHCA	79	57.2	32 ± 3	181.0 ± 27.6
CHCA ILM	77	60.2	37 ± 5	251.6 ± 32.7
CHCA LSM	84	53.8	33 ± 2	182.8 ± 6.1
1 fmol				
ClCCA	70	41.3	24 ± 1	129.5 ± 21.0
ClCCA ILM	54	46.0	28 ± 2	199.0 ± 21.2
ClCCA LSM	28	26.3	13 ± 2	93.0 ± 21.2
CHCA	75	23.3	12 ± 3	32.5 ± 7.5
CHCA ILM	20	29.5	15 ± 5	148.3 ± 23.6
CHCA LSM	27	25.3	15 ± 1	115.0 ± 11.0

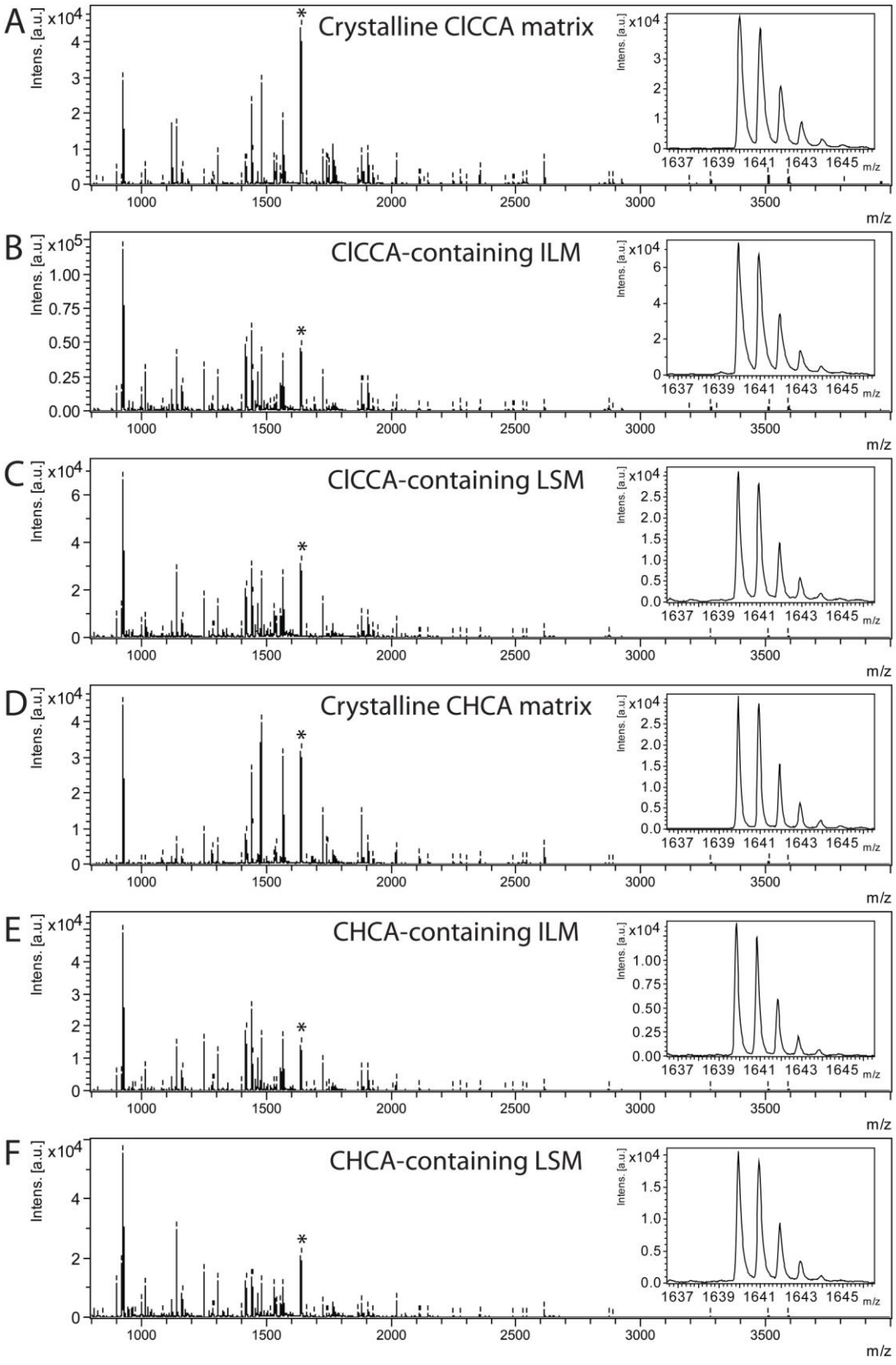
A
Crystalline ClCCA matrix
B
ClCCA-containing ILM
C
ClCCA-containing LSM
D
Crystalline CHCA matrix
E
CHCA-containing ILM
F
CHCA-containing LSM
Intens. [a.u.]
m/z
*

Figure 5. Example mass spectra of an in-solution BSA digest with 100 fmol spotted on target for (A) crystalline ClCCA matrix, (B) ClCCA-containing ILM, (C) ClCCA-containing LSM, (D) crystalline CHCA matrix, (E) CHCA-containing ILM and (F) CHCA-containing LSM. A total of 500 single-shot spectra were acquired per spectrum. Tick-marks denote peaks matching theoretical BSA tryptic digest masses. * denotes expanded peak.

It has previously been reported that the enhanced peptide recovery seen with solid state ClCCA-containing MALDI samples in comparison to solid state CHCA-containing samples is attributable to an increase in the observable number of small and/or acidic peptides.[34] In light of this the isoelectric point (IEP) values have been calculated for the tryptic BSA peptides identified in the various Mascot searches, as well as all those peptides which could have been theoretically observed based on the parameters used. This was performed using the ProtParam tool allowing for up to three missed cleavages and without consideration of post-translational modifications (http://www.expasy.org/tools/protparam.html). Interestingly, it was found that the average percentage of peptides matched with all matrices, which had an IEP < 7, was much higher than expected when compared to the percentage make up of all possibly matched peptides (~81-85% for the 100 fmol samples compared to 63% for the theoretical dataset). Similarly, the percentage of small peptides (<1500 Da) was also raised (~34-41% compared to 23%). Examining the average number of peaks matched with the BSA digest at a level of 100 fmol, it can be seen that all LMs and the solid state ClCCA-containing samples show an increase in the number of acidic peptides and small peptides when compared to the solid state CHCA-containing samples whilst the number of basic peptides and peptides above 1500 Da (with the exception of the solid state ClCCA-containing samples) do not increase significantly. The average number of peptides above 1500 Da is increased for the solid state ClCCA samples when compared to the solid state CHCA samples (38 ± 3.8 compared to 29 ± 2.5). At the 10 fmol level only the solid state ClCCA samples and ClCCA ILM samples show a significant increase in the average number of acidic peptides compared the solid state CHCA samples (40.6 ± 1.5 and 39.2 ± 2.2 compared to 27.2 ± 2.2) with all showing an increase in small peptides compared to solid state CHCA samples. At 1fmol however, the increases seen for the solid state ClCCA

samples and ClCCA ILM samples are approximately twofold for both large/small and acidic/basic peptides. It can be seen therefore that the data for the 100 and 10fmol-BSA digests concurs with the data which was previously reported by Jaskolla *et al.* [34] for the solid state ClCCA samples, and the same effect can be seen for the ClCCA ILM samples. However, the increases at the lower analyte level (1 fmol) appear to be of general nature. The lists of peptides matched in the various classes can be seen in Supplementary Data 2.

The CHCA-containing LSM has been reported to be superior in the analysis of biological samples of limited purity[3] and to have a high tolerance for contaminants and commonly used buffers such as ABC.[27] In a second set of experiments the PMM analysis with 100 fmol of BSA was repeated in the presence of ABC at 25 mM within the analyte solution. The results of this analysis can be seen in Table 2. In the presence of ABC, both crystalline matrices showed disturbed crystallisation even after re-crystallisation. Both ILMs showed evidence of an internalised cloudy sub-surface region occupying approximately one quarter of the surface area. Sampling of this region revealed that it was void of ion signal and so was avoided during data acquisition. In contrast the appearance of the LSMs did not alter. From the comparison of the data in Table 1 and 2 it can be seen that the presence of ABC had no negative effect on the performance of these matrices, which concurs with the previously reported data for the CHCA-containing LSM.[3, 27] Despite the overall lower performance of the LSMs compared to the ILMs seen in Table 1, the inclusion of glycerol can be seen to be beneficial in some cases by adding an increased level of robustness to the matrix preparations and ion signal stability.

Table 2 – Results of Mascot searches for the analysis of 100 fmol on-target amount of BSA in-solution digest in the presence of 25 mM ammonium bicarbonate (averaged over 5 replicates for each matrix). Peaks were picked between m/z 800-4000 with a signal-to-noise ratio ≥ 4.

Matrix	# of picked peaks	sequence coverage [%]	# of matched peaks ± SD	Mascot score (sig ≥ 70) ± SD
ClCCA	78	53.8	32 ± 9	190.2 ± 57.5
ClCCA ILM	150	68.3	43 ± 2	187.8 ± 24.1
ClCCA LSM	150	76.4	50 ± 3	230.6 ± 23.6
CHCA	147	42.7	24 ± 13	*(99.0/177.0)*[a]
CHCA ILM	135	45.0	31 ± 1	100.4 ± 5.5
CHCA LSM	149	72.4	48 ± 2	222.6 ± 13.6

[a] Only 2 of 5 search results reported significant hits.

Of the liquid matrices investigated here the ClCCA-containing ILM can be seen to have provided the highest levels of sequence coverage and peptide recovery at the lowest levels of analyte present. Recently, Jaskolla *et al.* have shown that ClCCA as crystalline matrix facilitates the analysis of attomol quantities of analyte present on the target.[41] Similar observations were made with the ClCCA-containing ILM. An average of 8 ± 1.4 matching peptides obtained from 4 replicates was observed, giving an average sequence coverage of 17.5 ± 3.6%. Despite the somewhat lower sequence coverage compared to the previously reported coverage for ClCCA as crystalline matrix, it represents a significant improvement in relation to both the CHCA LSM and solid state CHCA matrix. Due to the nature of the linear correlation between the concentration of analyte within a liquid matrix and the analyte response, it may be possible to improve this result further via a careful reduction in the matrix droplet size. Attempts to do this via greater matrix dilutions proved unsatisfactory, due to a concomitant higher concentration of contaminants within the shrunk droplet, leading to increased matrix adduct formation and general background noise. A method such as that described by Tu *et al* (2008)[42] for the precise deposition of a small quantity of diluted matrix solution may be preferable.

CONCLUSION

The data presented here shows that the novel ClCCA liquid matrices display similar characteristics to the previously reported CHCA liquid matrices. The glycerol-based LSMs show a particularly high homogeneity with a simple analyte distribution pattern, predominately independent of the peptide that is chosen as analyte, thus simplifying greatly the data acquisition. Although not quite as homogenous as the LSMs, the ILMs also show an improved analyte distribution and sample morphology in comparison to their crystalline counterparts. The investigated liquid matrices demonstrate a high degree of stability over a large number of measurements, allowing at least 10,000 laser pulses from a single position, and thus making them ideal for techniques such as MS/MS where a large number of spectra need to be acquired. This high degree of stability along with the high homogeneity enables quantitation to be performed without the need for an internal standard.

It can be seen from PMM analyses of a BSA digest that the investigated liquid matrices clearly outperform the crystalline CHCA matrix at the higher peptide concentrations (100 fmol) and at the very least show comparable results at the lower concentrations (10 and 1 fmol). The recently introduced ClCCA crystalline matrix again proves its superiority over the classical CHCA matrix. However, the novel ClCCA-containing ILM, as prepared and evaluated in this study, appears to be at least equally sensitive to its crystalline counterpart with somewhat better PMM performance at the 1fmol-level. Interestingly, the glycerol-based LSMs consistently outperformed all other matrices once the sample purity was deteriorated by adding 25 mM ABC.

From this data it can be concluded that the ClCCA-containing ILM represents a distinct improvement in terms of sensitivity compared to the other liquid matrices tested, and that it can

be seen as a viable alternative to its crystalline counterpart combining the desirable properties of a liquid matrix with the high sensitivity of the newly introduced ClCCA matrix compound.

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FIGURE LEGENDS

Figure 1. Liquid MALDI samples after evaporation of the volatile solvents. Top left, CHCA-containing glycerol LSM. Top right, CHCA-containing ILM. Bottom left, ClCCA-containing glycerol LSM. Bottom right, ClCCA-containing ILM. Sample appearance was unchanged after exposure to the vacuum. Each spot/droplet has a diameter of ~400 µm.

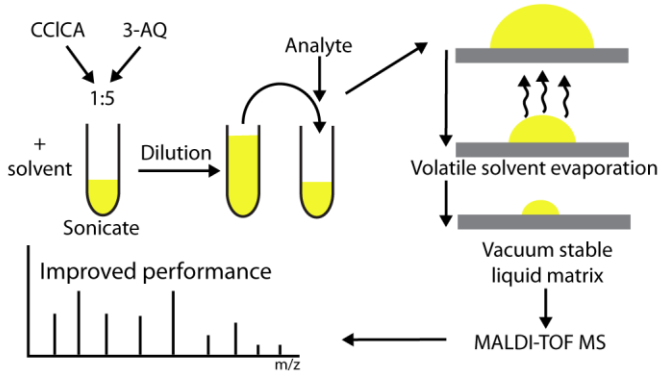
Figure 2. MALDI analyte ion signal distribution studied by MS imaging for the crystalline matrices CHCA (A) and ClCCA (B) and the liquid matrices CHCA-containing LSM (C), CHCA-containing ILM (D), ClCCA-containing LSM (E), and ClCCA-containing ILM (F). The distributions of the analytes Bradykinin 1-7 (left column; 100 fmol spotted), Angiotensin I (middle column; 100 fmol spotted) and ACTH 18-39 (right column; 300 fmol spotted) can be seen. The images were taken with a 25µm-raster (laser focus: ~50 µm). A total of 10 single-shot spectra at 50 Hz were recorded for each MS imaging point. The images are normalised against the maximum intensity obtained. Each image displays an irradiated area of 825 µm x 825 µm.

Figure 3. Ion signal intensity of the $[M+H]^+$ of Angiotensin I with an increasing number of laser pulses. Spectra were acquired as the sum of 100 single-shot spectra at 50 Hz from a single desorption position with ~1 sec between each accumulation, for a total of 10,000 laser pulses. Panel A, CHCA-containing LSM (solid line), CHCA-containing ILM (dotted line). Panel B, ClCCA-containing LSM (solid line), ClCCA-containing ILM (dotted line).

Figure 4. Ion signal intensity as a function of sample amount (5 – 1000 fmol spotted) for the ClCCA-containing LSM. Four spots were measured for each data point (500 laser pulses each). Data are shown for Angiotensin I (diamonds), Substance P (squares) and Glu-Fibrinopeptide B (circles). All R^2 values are greater than 0.99.

Figure 5. Example mass spectra of an in-solution BSA digest with 100 fmol spotted on target for (A) crystalline ClCCA matrix, (B) ClCCA-containing ILM, (C) ClCCA-containing LSM, (D) crystalline CHCA matrix, (E) CHCA-containing ILM and (F) CHCA-containing LSM. A total of 500 single-shot spectra were acquired per spectrum. Tick-marks denote peaks matching theoretical BSA tryptic digest masses. * denotes expanded peak.

TOC graphic and synopsis



The 4-chloro-α-cyanocinnamic acid (ClCCA) ionic liquid matrix (ILM) and liquid support matrix (LSM) show an improved sample morphology and homogeneity for UV-MALDI MS. In comparison to the crystalline ClCCA (and α-cyano-4-hydroxycinnamic acid), these matrices possess highly stable and durable analyte signal, allowing for quantitation over 3 orders of magnitude without an internal standard. In addition both ILM and LSM show an increase in performance and sensitivity for peptide mass mapping.