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Combinatorial Optimization of Multiple MALDI Matrices on a Single Tissue Sample Using Inkjet Printing

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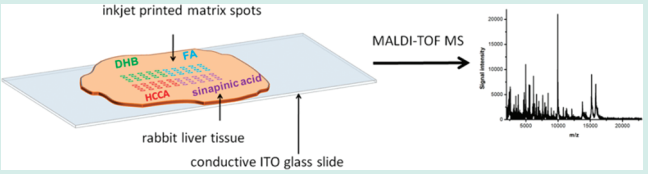
ABSTRACT: Taking advantage of the drop-on-demand capabilities of inkjet printing, the first example of a single tissue being used as a substrate for preparing combinatorial arrays of different matrix-assisted laser desorption/ionization (MALDI) matrices in multiple concentrations on a single chip is reported. By varying the number of droplets per spot that were printed, a gradient array of different amounts of matrix material could be printed on a single chip, while the selection of matrices could be adjusted by switching different matrix materials. The result was a two-dimensional array of multiple matrices on a single tissue slice, which could be analyzed microscopically and by MALDI to elucidate which combination of matrix and printing conditions offered the best resolution in terms of spot-to-spot distance and signal-to-noise ratios for proteins in the recorded MS spectra. This combinatorial approach enables the efficient optimization of possible matrices in an organized, side-by-side array format, which can particularly be useful for the detection of specific protein markers.



KEYWORDS: combinatorial chemistry, mass spectrometry, proteomics, surface analysis, MALDI-MSI, inkjet printing

INTRODUCTION

As a soft ionization tool for mass spectrometry, the applications of matrix-assisted laser desorption/ionization (MALDI) have continued to expand over the last quarter of a century, enabling the analysis of a wide range of nonvolatile components that would otherwise be impractical or inaccessible to characterize by other means.[1] One of the most interesting analytical applications of MALDI has been the characterization of intact samples, such as on a separation membrane or an electrophoresis gel.[2] Since laser optics offer the possibility of selective surface excitation, it allows for characterization of analyte spatial distribution. As a consequence molecular imaging of samples such as tissues is possible.[3] By mapping the distribution of different analytical signals in two dimensions, tissue samples may be visualized in entirely new MS dimensions, allowing for new possibilities to both identify new biomarkers, and opening new opportunities in life science disciplines such as pathology and histology.

However, there are some practical limitations to MALDI that make the fullest realization of these new possibilities more challenging. To wit, the signal intensity, and consequently the measurability, of most molecules is highly dependent on the selection of matrix material, the amount of matrix material added, the processing techniques used, and the use of additives for a better ionization efficiency. This sample preparation parameters, as well as instrument parameters, such as laser intensity, must be systematically and empirically investigated to identify the best combination for a given analyte. In many cases, the source of biological material is highly limited, making this problematic. In essence, this is an analytical combinatorial problem. While MALDI has been applied combinatorially for over a decade,[4] the approach has not yet been extended to the optimization of mapping studies.

While matrix spraying,[5] chemical vapor deposition,[6] and solvent-free crystal deposit techniques[7] are commonly used approaches to deposit matrix materials for MALDI mass spectrometric imaging (MSI) of tissue samples, one of the limitations is that these approaches generally allow for only one material to be deposited per sample, and only in one specific concentration. The use of multiple matrices on a single surface has been suggested,[8a] but to-date, has not been reported.

Inkjet printing has been widely used for the direct-writing of two-dimensional features. The accurate placement of predetermined quantities of material, which can be performed without the need for pre-patterning steps, makes inkjet printing very

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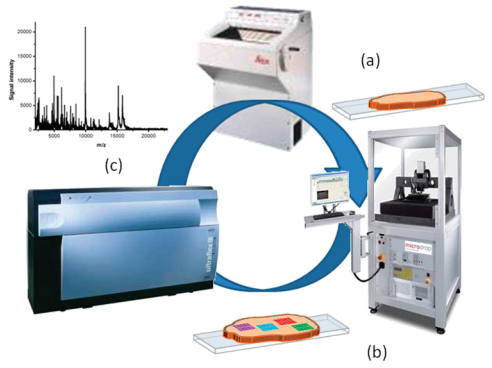

Figure 1. Layout of the approach. (a) A single tissue section is sliced using a cryostat and attached to a conductive ITO glass slide, (b) multiple MALDI matrix materials are deposited onto the single tissue slice using an inkjet printer, (c) and finally the sample is analyzed in a MALDI time-of-flight (TOF) instrument to identify which combination of matrix and printing conditions yield the best protein signals.

attractive as a digital non-contact patterning tool. Inkjet printing describes the use of electrical actuators to eject pico-liter volumes of liquid from microsized apertures onto a substrate in a defined pattern. It has gained wide acceptance in industry as a tool for basic discovery and as a proven means of rapid fabrication. While the most popularly identifiable application of inkjet technology remains that of printing paper documents, it has also been used in fields, such as sensor fabrication, combinatorial chemistry, and biology.[9]

In this contribution, the following approach is reported, in which a single tissue slice is used to evaluate multiple gradient arrays of different MALDI matrices without cross contamination, enabling the identification of optimal conditions for particular MS signals. By taking advantage of the drop-on-demand additive properties of inkjet printing to deliver material selectively and in discrete quantities only where it is programmed, multiple gradients of matrix materials on a single tissue surface were built up. Finally, the advantage of inkjet printing two matrices on a single tissue for MALDI-MSI studies is demonstrated.

RESULTS AND DISCUSSION

The applicability of the approach, as presented in Figure 1, was investigated to characterize the MALDI-MS signals from a single slice of a rabbit liver. In detail, the first step was to cut a 10 μm slice of a rabbit liver using a cryostat, as shown in Figure 1a, and to mount it on a precooled conductive indium tin oxide (ITO) coated glass slide. The second step, as presented in Figure 1b, was to apply four different matrix materials onto the tissue slice using an inkjet printer. And finally the last step (Figure 1c) was to analyze the tissue slice by MALDI-MS to evaluate, which combination of matrix and printing conditions resulted in the best protein signals, such as high signal-to-noise levels.

Four different commonly used UV-MALDI matrix materials were selected, namely 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxy-cinnamic acid (HCCA), ferulic acid (FA), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (see Figure 2).

While DHB and HCCA are both commonly used for characterizing peptides and proteins,[8] FA has also been demonstrated to be effective in characterizing other biomolecules, such as metabolites,[10] and oligosaccharides,[11] and finally sinapinic acid has only been described for protein analysis.[8]

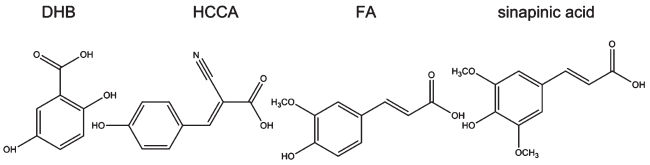

Figure 2. Schematic representation of the structures of matrix material used in this study.

By comparing the mass spectra recorded with DHB, HCCA, FA, and sinapinic acid spatially separated on a single tissue sample, only HCCA gave low signal-to-noise levels (spectrum not shown). A correlation between the total number of droplets deposited on one position and the signal-to-noise level in the recorded mass spectrum was obtained. Figure 3a shows an exemplary embodiment of the matrix sinapinic acid that by doubling the number of droplets deposited on one position an increase in the signal-to-noise level of the acquired mass spectrum was achieved. But not only the total number of droplets spotted, also the number of repeats of spotting a certain number of droplets showed a strong dependency. As depicted in Figure 3b for sinapinic acid, the signal-to-noise ratio is significantly improved when dispensing 20 times a single droplet, instead of two print runs with 10 droplets being dispensed in each run. From these results it can be concluded that the repeated rewetting of the tissue surface improves the extraction of the proteins followed by the cocrystallization with the matrix; as a consequence enhanced mass spectra could be obtained.

Different protein signals were enhanced when comparing the spectrum recorded with DHB with the one obtained with FA, as demonstrated in Figure 3c. The signals which are unique to the DHB matrix include m/z 2348, 7656, 7922, and for FA m/z 3335, 3454, 6210, 7406, 11 305, 11 621, 13 775.

By controlling the number of droplets applied to a given spot, the amount of material added was varied. Small rectangular arrays of spots of each of the matrices were printed onto the tissue surface in rows. Each row represented a different matrix material, and the number of repeats per spot depositing a certain number of droplets on this position was varied from 1 to 12 repeats in steps by 4, staggered arithmetically. Each condition was replicated five times to obtain five spots per condition to accumulate a mass spectrum. The number of droplets per repeat was increased from one over three, five, and ten. Therefore, sixteen conditions were evaluated per matrix. The center-to-center distance between two spots was kept constant at 400 μm. The printing conditions were optimized with regards to spatial resolution; while more matrix material yielded a stronger signal, at a certain point, the spots began to coalesce, and spatial resolution was compromised. The print quality was inspected under a fluorescence light microscopy DAPI filter, as presented in Figure 4. Thus, the printing conditions which enabled separated spots were identified easily. The mass spectra with the best signal-to-noise levels were recorded for DHB and HCCA with twelve times three drops, and for FA and sinapinic acid with 8 times ten droplets, respectively. However, it can be seen in Figure 4 within the red boxes that none of these conditions lead to clear and separated spots on the tissue, except for HCCA.

To perform MALDI-MSI on a rabbit kidney with a lateral resolution of 800 μm the matrices DHB and FA were inkjet printed in an offset grid by using 12 times three drops. The resulting MALDI-MSI images with the corresponding histological

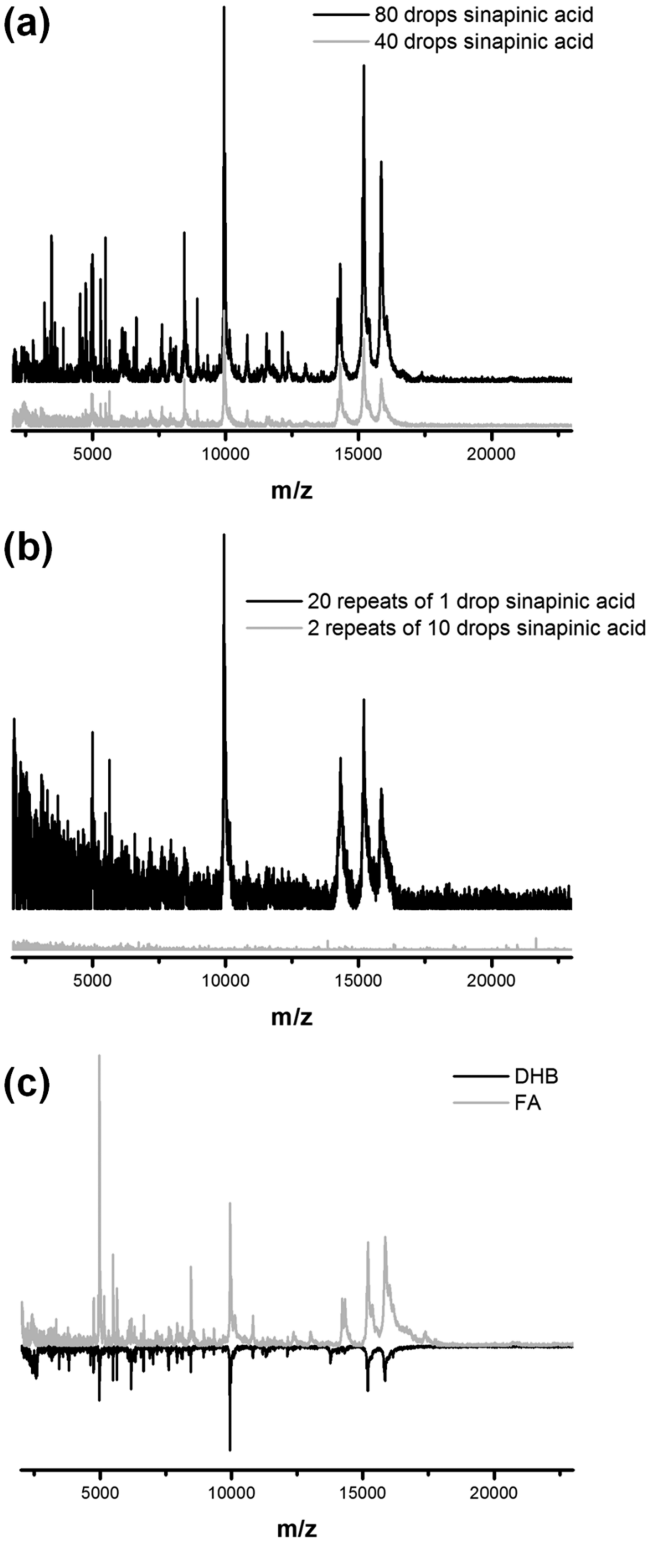

Figure 3. Mass spectra of rabbit liver using different matrices: (a) dependency of the total number of drops spotted (sinapinic acid was employed), (b) dependency of number of spotting repeats (sinapinic acid was employed), and (c) comparison between 2,5-dihydroxybenzoic acid (DHB) and ferulic acid (FA).

H&E stain, the optical image and the averaged mass spectrum obtained for each matrix are presented in Figure 5. In Figure 5b, the matrix spots of FA are shown in dark and the ones of DHB are somehow lighter. The advantage of inkjet printing two matrices on a single tissue slice is shown clearly by comparing the average mass spectra of FA (see Figure 5e) with the one of DHB (see Figure 5g). For example the MALDI-MSI images of the signals at m/z 3702 (red color) and 11 308 (green color) for both matrices are presented in Figure 5c and f, respectively. Figure 5f shows more pixels with low signal intensities than are shown in Figure 5c, where FA was used as matrix. From this it can be concluded that FA is a better matrix for the MALDI-MS measurements of a rabbit kidney than DHB. Another advantage of FA is that the signal at m/z 13 772 is only present when this matrix is chosen, as presented in the average mass spectrum of Figure 5 (e) and the constructed MALDI-MSI image in Figure 5 (d).

CONCLUSION

In this study the potentials and limitations of inkjet printing multiple matrix material on a single tissue slice are presented. Four matrix materials, DHB, HCCA, FA and sinapinic acid were investigated and the number of droplets on one position was varied by maintaining a spatial resolution of 400 μm between the spots. In the case of MALDI-MSI of a rabbit kidney the best quality spectra by maintaining discrete spots were obtained by dispensing twelve times three droplets on one position using DHB or FA as matrix material. The image resolution was 800 μm. Future experiments will be concentrated on improving the image resolution by using a specially developed electronics for the dispenser spray-heads.

EXPERIMENTAL PROCEDURES

Sample Preparation. Cryosections of 10 μm of liver or kidney samples from rabbit were cut on a cryostat and transferred to a precooled conductive ITO-coated glass slide. The sections were briefly washed twice for one minute in 70% ethanol and once for one minute in 100% ethanol and dried in a vacuum desiccator.

MALDI Matrices, Solvents. Several MALDI matrices were prepared: α-cyano-4-hydroxy-cinnamic acid at 10 mg·mL^{-1} in 50% acetonitrile and 50% water with 0.1% trifluoracetic acid; ferulic acid at 40 mg·mL^{-1} in 50% acetonitrile and 50% water with 0.1% trifluoracetic acid, and 2,5-dihydroxybenzoic acid at 30 mg·mL^{-1} in 50% methanol and 50% water with 0.4% trifluoracetic acid, and 3,5-dimethoxy-4-hydroxycinnamic acid at 10 mg·mL^{-1} in 50% acetonitrile and 50% water with 0.1% trifluoracetic acid.

Inkjet Printing of Arrays. Inkjet printing was performed by using a piezoelectric-driven autodrop system (Microdrop Technologies, Norderstedt, Germany). The system was fitted with a 50 μm inner-diameter dispenser-type print head. Droplets for all three inks were generated using a voltage of 158 to 160 V, a pulse width of 38 to 40 ms, and a fixed frequency of 200 Hz. Droplet formation was followed using a fiducial camera, illuminated by a stroboscopic light source, which showed that in all cases droplets with a mean diameter of 45 μm were obtained, corresponding to a volume of approximately 48 pL. Printing was then carried out by dispensing several droplets on a single tissue position, followed by a small waiting time to let the solvent evaporate, after which a next print run is started. The format of the arrays was a series of 1.6 × 1.2 mm squares, where droplets were deposited onto spots that

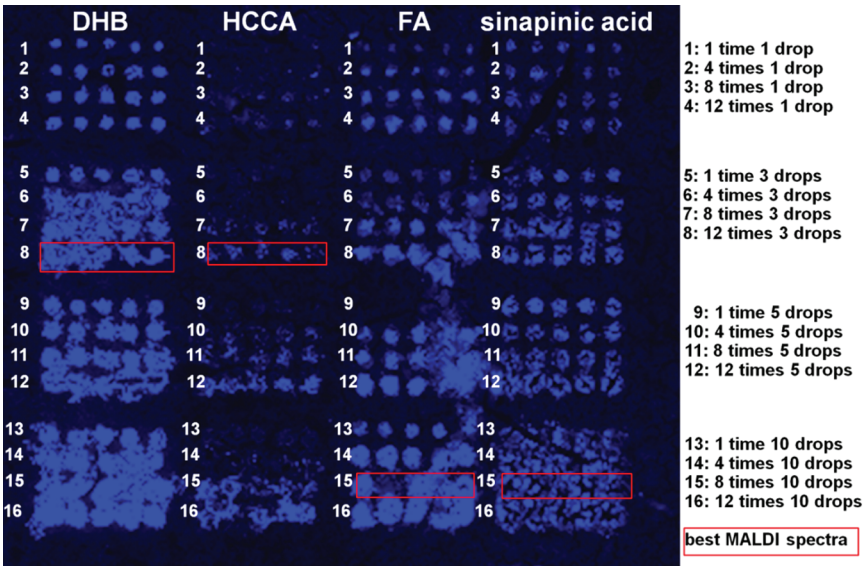

Figure 4. Microscopic image using a fluorescence DAPI filter of the printed matrices on a single rabbit liver tissue.

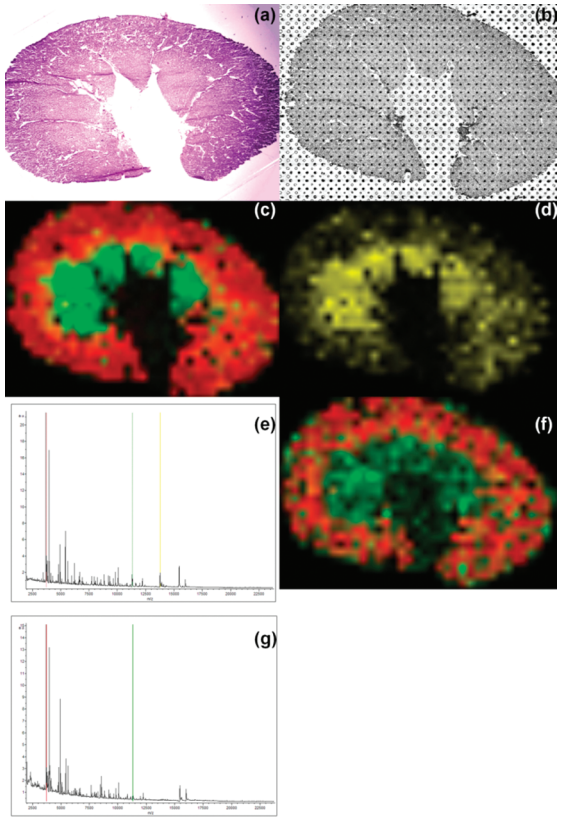

Figure 5. MALDI-MSI of a rabbit kidney. (a) H&E staining, (b) optical image, (c) overlaid MALDI-MSI image of signals at m/z 3702 (red) and 11 308 (green) when FA is used as matrix, (d) MALDI-MSI image of the signal at m/z 13 772 when FA is used as matrix, (e) average mass spectrum when FA is used as matrix, (f) overlaid MALDI-MSI image of signals at m/z 3702 (red) and 11 308 (green) when DHB is used as matrix, and (g) average mass spectrum when DHB is used as matrix, respectively.

were located at a center-to-center distance of 400 μm from each other, such that each array consisted of 20 spots. Each of the arrays consisted of four of these squares, with each line representing a different number of repeats of dispensing a certain number of drops.

MALDI Analysis. Matrix spots on the tissue sections were analyzed on a MALDI-TOF/TOF (Bruker, Ultraflex III TOF/TOF) equipped with a 200 Hz smartbeam laser. The MALDI measurements were done in linear mode in a m/z range of 2000−23 000 with a sampling rate of 0.10 GS/s. Each spot was analyzed with 200 laser shots.

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