Identification of effective substrates for the direct analysis of lipids from cell lines using desorption electrospray ionization mass spectrometry

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RATIONALE: Various disease conditions, particularly tumours, can be understood easily by studying changes in the lipid profile of cells. While lipid profiles of tissues have been recorded by desorption electrospray ionization mass spectrometric (DESI-MS) imaging, there is paucity in standardized protocols for sample preparation involving cell cultures to generate reliable results. In this study, we report a method for the direct analysis of lipids from cultured cells by incorporating them onto Whatman 42 filter paper.

METHODS: The WERI-RB1 cell line was spotted on commonly used substrates for DESI-MS analysis, such as glass slides, Teflon coated glass slides, thin layer chromatography (TLC) plates, and Whatman 42 filter paper. A comparison of mass spectrometric images with two different lipids was made to understand the behaviour of different surfaces when the same sample was spotted on them. Relative intensities of different lipid peaks in the WERI-RB1 cell line were compared and relative lipid abundances were also compared across two different human retinoblastoma cell lines; WERI-RB1 and Y79.

RESULTS: The study demonstrates that good lipid signals can be obtained by DESI-MS when the cells are spotted on Whatman 42 filter paper. Tandem mass spectrometry was performed to identify the lipids as glycerophosphocholines (PC). Better lipid images from assembly of cells were obtained with distinct boundary when they were spotted on Whatman 42 filter paper than other surfaces.

CONCLUSIONS: We demonstrate the use of a simple substrate for reliable DESI-MS analysis of cultured cells. This method has the potential to understand various interactions of cells with other external agents. The current method would help in the application of DESI-MS for biology in general and medical sciences in particular.

Desorption electrospray ionization (DESI) is an ambient mass spectrometric technique used for the analysis of surfaces. In this method, a stream of charged solvent droplets is directed towards a sample surface. It forms a thin film over the surface which dissolves the analytes. Subsequent impact of droplets creates secondary droplets which contain dissolved analytes. These secondary droplets enter into the mass spectrometer through the atmospheric interface and ions are mass analyzed. Being a surface desorption ionization process, it enables imaging of analytes from a given surface by directing the spray to small segments of the surface, systematically. Spatial resolution of such imaging depends primarily on the spot size of the primary droplets impacting the surface and the spot size can be controlled by various instrumental and experimental parameters of the source during the experiment.

Being an ambient ionization process, the experiments are carried out in open atmosphere and minimal/no sample preparation is involved prior to the experiment. As a consequence, the process is fast, simple and enables the analysis of biological samples in their almost native state without much distortion. For this reason, DESI-MS, after its introduction in late 2004, has found a wide range of applications in biology, medicine and related disciplines. They include study of molecules from plant tissues, rapid identification of drug molecules from plants, distribution of metabolites in leaves and petals, direct detection of drugs from human skin and animal tissues, etc. However, the most important and promising use of DESI-MS in biology is the analysis of lipids directly from biological specimens.

Different lipids can be identified by DESI-MS in positive and negative ionization modes depending on their structures. For example, fatty acids (FA), glycerophosphoinositols (PI), glycerophosphoserines (PS), glycerophosphoethanolamines (PE), plasm-enyl glycerophosphoethanolamines (plasm-enyl-PE), sulfatides (ST), etc., give signals in negative ionization mode whereas glycerophosphocholines (PC), glycerophosphoethanolamines (PE), ceramides (Cer), etc., give...
signals in positive ionization mode. The ease of ionization of lipids in DESI-MS by choosing the appropriate ionization mode and solvent has been reflected in a large number of applications reported in the literature. DESI-MS has been used to identify and/or monitor changes in lipid profiles in tissue samples of mouse brain,[2,16] human brain with different degrees of tumour,[17] colorectal adenocarcinoma,[18] human prostate,[19] human lens,[20] tumorous and non-tumorous canine urinary bladder,[21] and human gastric cancer[22] as well as bacteria.[23,24] This list can be extended further. A somewhat old review presents a comprehensive discussion on lipid analysis by DESI-MS.[25] Discussion about lipid analysis by other mass spectrometric methods including ambient ionization techniques can be found elsewhere.[26]

Cells contain different kinds of lipids and they play different biological roles therein. During a diseased condition, the lipid profile changes from the normal. Alteration in lipid profile has been reported in different diseases like human brain cancer,[27] colon cancer,[28,29] stomach cancer,[30] and other diseases.[31] Thus, studying the lipid profile can be used to understand the diseased condition and it can be used for diagnostic purposes. DESI-MS provides scope for rapid lipid analysis and has been used successfully to differentiate tumorous and non-tumorous tissues in many different human organs such as brain,[32] meninges,[33] stomach,[22] colon,[18] testis,[34] bladder,[35] kidney,[36] prostate,[19] etc., by lipid profiling. Although in all the above-mentioned examples direct tissues are used, study of cell lines for lipid analysis is also equally important due to its easy availability compared to tissues from subjects. Furthermore, during new drug discovery and evaluation of existing drugs for new diseases, cell lines are the primary objects where trial is conducted. From this perspective, it is important to study cell lines for rapid analysis of lipids.

In our present work, we examined different surfaces like glass slides, Teflon-coated glass slides, thin layer chromatography (TLC) plates, Whatman filter paper, etc., which are commonly used in DESI-MS, for analysis of retinoblastoma cell lines, WERI-RB1 and Y79. It is reported that in retinoblastoma, a childhood eye cancer, fatty acid synthase, a lipogenic enzyme, is over-expressed[37,38] and lipid profile changes from normal to tumor cells.[39] From this perspective, retinoblastoma cell lines are important and chosen for our work. Comparison of different surfaces is shown in terms of spectra and image quality. We show that when Whatman 42 filter paper was used as a substrate, good quality spectral data and lipid images could be obtained. Moreover, our method is completely independent of cell culture procedure; cells can be cultured, treated or made to undergo any other modifications elsewhere but still the experimental procedure will remain the same for all kinds of cells. Such reliable sample-handling procedures are highly beneficial in rapid and reliable screening of cell lines.

**EXPERIMENTAL**

**Reagents**

HPLC grade methanol was purchased from Sigma-Aldrich, Germany, and used as such without any purification. RPMI 1640, antibiotic antimycotic solution, fetal bovine serum (FBS) and trypsin were purchased from Gibco Life Technologies, USA.

**Materials**

Whatman 42 filter paper was purchased from GE Healthcare UK Limited, UK. The TLC plates (TLC Silica gel 60 F254) were purchased from Merck KGaA, Germany. Teflon-coated glass slides were purchased from Prosolia, USA, and normal glass slides from Polar Industrial Corporation, India.

**Cell culture**

Retinoblastoma WERI-RB1 and Y79 cells were obtained from RIKEN BioResource Center, Ibaraki, Japan, and they were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640), with 10% FBS and supplemented with antibiotic antimycotic solution (containing 100 μg/mL penicillin, streptomycin and 250 ng/mL amphotericin B). Cells were seeded in a T25 flask and maintained in a 5% CO2 environment at 37 °C till they reached 100% confluence. After that, the cells were removed from the flask by adding 1 mL of trypsin. To stop the action of trypsin, 1 mL of complete media (RPMI 1640) with 10% FBS was added, completely resuspended and collected in a centrifuge vial.

**Spotting of cells on different surfaces**

Cells collected in the centrifuge tube were washed twice with 1x phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 5 min. Then 50 μL of 1x PBS was added to the pellet to resuspend it completely. From this 10 μL was pipetted out and spotted on different surfaces (filter paper, glass slide, Teflon-coated glass slide and TLC plate) and left under the laboratory condition for drying. During the spotting, precautions were taken so that pipette tip did not touch the surface and hands did not shake. In the case of the filter paper, when the solution containing the cells contacted the surface, first it wetted the surface forming a circle. Then slowly it diffused outwards in a circular manner. Cells did not move in this process and stayed within the initially formed circle. The paper was kept for drying for about 5 min. After that, the filter paper was cut into a square containing the cells and pasted on a glass slide. This glass slide was placed on the stage of the DESI mass spectrometer and a spectrum was collected. Figure 1 schematically illustrates this procedure. In the case of the TLC plate, similar events were observed. In the case of the glass slide and the Teflon-coated
glass slide, diffusion was absent and for the Teflon-coated glass slide, initial wetting was also absent due to the hydrophobic nature of Teflon.

**DESI-MS measurement**

Mass spectrometric measurements were performed in a LTQ XL ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA). The DESI source used for ionization was from Prosolia. Data were acquired in the positive ion mode with a spray voltage of 5 kV. Methanol was used as spray solvent and it was sprayed at a flow rate of 5 μL/min. Dry nitrogen was used as the nebulization gas and the pressure was kept at 150 psi. The inlet capillary of the mass spectrometer was kept very close to the surface, the spray tip was kept at 2 mm above the surface, the distance between the inlet capillary and the spray tip was 3 mm and the spray angle was maintained at 60° to the surface. Tandem mass spectrometry was performed with collision-induced dissociation (CID). The following parameters were used for CID experiments; isolation width: 1 m/z, normalized collision energy: 20 (manufacturer’s unit), activation Q: 0.250 (manufacturer’s unit) and activation time: 30 ms. For imaging experiments by DESI-MS, a pixel size of 250 μm × 250 μm was chosen throughout.

**RESULTS AND DISCUSSION**

**Lipid analysis from the WERI-RB1 cell line by DESI-MS**

The human retinoblastoma cell line, WERI-RB1, was tested for direct identification of lipids by DESI-MS after spotting them on Whatman 42 filter paper. Figure 2 shows the positive ion mass spectrum of the WERI-RB1 cells from the filter paper in the region of m/z 700–900. The mass spectrum was acquired in this particular region because it is rich in information about lipids. Several lipid peaks corresponding to the glycerophosphocholine (PC) class were observed in the spectrum. The inset of Fig. 2 shows the general structure of glycerophosphocholine lipids where R1 and R2 represent two similar or dissimilar hydrocarbon chains coming from saturated or unsaturated fatty acids. Identification of the lipids was made by tandem DESI-MS analysis and using the database as reference. The highest abundance peak appeared at m/z 782.6 and it corresponds to [PC(34:1)+Na]+. Other prominent peaks observed in the spectrum were at m/z 754.6 [PC(32:1)+Na]+, 768.6 [PC(33:1)+Na]+, 780.6 [PC(34:2)+Na]+, 808.6 [PC(36:2)+Na]+, 810.6 [PC(36:1)+Na]+, 832.7 [PC(38:4)+Na]+, and a few more. Beside these, several other low intensity peaks were also observed in the spectrum.

**Performance evaluation of different surfaces for DESI-MS analysis of cells in terms of signal intensity, noise and image quality**

To evaluate the efficacy of different substrates on the DESI-MS signal intensity and image quality, four different, commonly used substrates were chosen. They were, Whatman 42 filter paper, glass slide, Teflon-coated glass slide and TLC plate. Among them, the surfaces of the glass slide and the Teflon coated glass slide are smooth and devoid of any porosity of macroscopic diameter whereas the filter paper and the TLC plate possess porous structures. When samples (WERI-RB1 cells) were spotted on these four substrates and kept under the laboratory condition to dry excess water, it took different times to dry each substrate. For example, due to the porous nature of the filter paper and the TLC plate, water diffused to peripheral regions of the initial drop spotted on the surface. This fact is illustrated in more details in the Experimental section with the help of Fig. 1. This accounted for the fast evaporation of water from the filter paper and the TLC plate and it took a maximum of 5 min to dry it completely. In the case of the glass slide, there was no provision of diffusion and it took almost 15–20 min to dry the spot completely. For the Teflon-coated slide also, diffusion was restricted. Moreover, owing to the hydrophobic nature of Teflon, the drop spotted on this surface tended to shrink unlike a normal glass surface where it got spread. For this reason, it took more time (30–40 min) to dry the spot from the Teflon surface than from the glass surface, though both of them were smooth surfaces without any porosity to help fast evaporation. The spot size on the surface mostly depends on the volume of the drop coming out of the pipette tip and the way it is spotted. For example, if properly handled, and solution-containing cells were spotted without touching the pipette tip on the surface, it normally created a circular

![Figure 2. DESI-MS spectrum of WERI-RB1 cells from Whatman 42 filter paper. Inset shows the generalized structure of glycerophosphocholine lipids.](image-url)
spot containing cells with a diameter of 5–6 mm when 10 µL solutions were used. In case of porous surfaces (TLC plate and filter paper), the spot got spread and it formed a circle of ~10 mm with the initial inner circle of ~6 mm diameter. In this process, only the water medium was spread and not the cells; they remained in the inner circle. For the glass surface the cells did not get spread and a spot size of ~6 mm diameter was observed. However, when a Teflon surface was used, the spot size obtained was 2–2.5 mm diameter because of the obtuse contact angle of the drop on the hydrophobic Teflon surface. It is noteworthy to mention that if during the spotting the hand shook or the pipette tip with the protruding drop touched the surface, the spot shape could change from a regular, almost circular shape to an elongated shape.

After drying the spots on different substrates, they were subjected for DESI-MS imaging experiments. Figure 3 shows a comparison of the mass spectrometric images taken from different surfaces and two lipids are chosen here for detailed discussion. These two lipid ions are m/z 782.6 (highest in intensity) and 810.6 (relatively lower in intensity) (Fig. 2). The top row of Fig. 3 (Figs. 3(A), 3(B), 3(C), and 3(D)) shows the distribution of the m/z 782.6 ion over the spot from different surfaces. Spots appeared almost circular in shape as expected and were visible to the naked eye before the experiments. For the Teflon surface, during the long time of solvent evaporation, cells became more settled in one direction and this could happen if the surface was tilted minutely by a few degrees which may not be prominent to detect normally. However, the spot looked like a circle to the naked eye. Intensities obtained from the filter paper, Teflon surface and TLC plate were comparable and are evident from the images (Figs. 3(A), 3(C), and 3(D)). For the glass surface (Fig. 3(B)), however, the signal intensity was low. Almost uniform distribution is noticed from all the four surfaces (Fig. 3(A), 3(B), 3(C), and 3(D)). If we consider the signal-to-noise ratio, noise is much less for the filter paper (Fig. 3(A)) and glass slide (Fig. 3(B)). Distinct circular shapes are obtained with sharp contrast in the outside regions where the cells are absent. For the Teflon surface (Fig. 3(C)) and the TLC plate (Fig. 3(D)), more noise is evident from the images and boundary regions of the spots do not contain sharp contrast. To understand the reason behind these observations regarding noise, we need to first group all the surfaces into two different categories depending on how solvent evaporates from these surfaces. In the group of porous surfaces there are filter paper and TLC plate. The porosity of filter paper is more than the TLC plate. When solution is spotted on the filter paper, it is absorbed very quickly and cells do not get any time to spread. However, the TLC plate is not as porous as the filter paper. After spotting solution on it, a thin film is formed before complete absorption of the solvent and this thin solvent film slowly diffuses radially. During this radial diffusion, cells also move outwards and that is the reason for more noise, the absence of sharp contrast in the boundary region and the gradient in the intensity distribution (red > yellow > green > cyan > blue, Fig. 3(D)). In the other group of non-porous surfaces we have the glass slide and the Teflon-coated slide. In both these cases, solvent does not diffuse after spotting and it evaporates slowly. For the glass slide, there is an initial spread of solvent (wetting of surface) just after spotting and for the Teflon surface that too is absent due to the hydrophobic nature of Teflon. Cells do not get any chance to spread in these cases. As a result, a good quality image is obtained from the glass slide (Fig. 3(B)). The noise associated with the Teflon can be attributed to the DESI spray. Probably during the spray, secondary droplets as splashes can go outside of the spot and contribute to the noise. This particular phenomenon cannot contribute to the noise of porous surfaces as splashing is less due to rapid absorption of solvent from the DESI spray. Considering the images (Figs. 3(A), 3(B), 3(C), and 3(D)), signal intensities obtained and time required for drying the spots, it is clear that the filter paper is the most suitable substrate than the others for cell line analysis by DESI-MS. It should also be noted that it
makes no difference which grade of filter paper is used. Filter papers other than Whatman 42 can also be used for the same purpose as long as they have no organic impurities to interfere during the DESI-MS experiments and can absorb water rapidly. Whatman 40 filter paper was also used for DESI-MS imaging experiments. Supplementary Fig. S3 (see Supporting Information) shows images of different lipids from WERI-RB1 cells spotted on Whatman 40 filter paper. Other fibrous surfaces like nanofibre mat or cloth have the potential to become suitable substrates as they can help fast evaporation of the solvent and restrict cell movement.

Noise in the images can be reduced to some extent during the processing of the images in BioMAP. To reduce noise, we need to put some cut-off value in the minimum intensity field. This will reduce noise but the overall peak intensity will also be reduced and for low intensity peaks this process will not help much to reduce noise as it will significantly reduce the peak intensities. This process was applied to reduce noise of the images and the middle row of Fig. 3 (Figs. 3(E), 3(F), 3(G), and 3(H)) shows the distribution of the same ion, m/z 782.6, on different substrates. For the Teflon surface (Fig. 3(G)) and the TLC plate (Fig. 3(H)), noise are reduced to some extent and sharp boundaries appear in some regions. This noise reduction has very little effect on the filter paper (Fig. 3(E)) as it does not produce significant noise. However, the effect of this method on peak intensity is prominently observed in the case of the glass surface (Fig. 3(F)) as signal intensity is already less here compared to the other surfaces. Overall intensity is also reduced due to the noise reduction process in BioMAP. Another interesting fact regarding the glass surface becomes prominent during this exercise. The image obtained from the glass slide (Fig. 3(F)) shows somewhat more intensity of the lipid of m/z 782.6 on the circumference of the spot. This can be attributed to the ‘coffee ring effect’. When solvent evaporates from a drop situated at some surfaces, outward flow of solvent occurs which drives dispersed particles towards the edge. This is the reason why the cells move towards the circumference of the spot and more signal intensity is observed. Reduction of noise during the experiment is more desirable than reduction by data processing. The bottom row of Fig. 3 (Figs. 3(I), 3(J), 3(K), and 3(L)) shows the distribution of another ion at m/z 810.6 which is almost 50% less in intensity than the base peak at m/z 782.6. These images are made without any noise reduction in BioMAP. In this situation of comparatively low intensity peak, noise becomes much more prominent for the Teflon surface (Fig. 3(K)) and the TLC plate (Fig. 3(L)) and the data cannot be modified much by processing. For the filter paper (Fig. 3(I)) and the glass slide (Fig. 3(J)), increase in noise is small. Again the coffee ring effect is prominent for the glass slide (Fig. 3(J)).

DESI-MS imaging experiments were done in triplicate for each substrate and the data were consistent for each substrate. Almost similar intensities from each substrate were obtained. Sensitivity of the substrates has been evaluated by spotting lower numbers of cells. Approximately 10<sup>5</sup> cells in 10 μL solution were required to obtain proper signals from a filter paper, Teflon surface and TLC plate. Reducing the number of cells resulted in poor signals which were not resolved properly from the background. For the glass surface, 10<sup>5</sup> cells in 10 μL solution itself was not sufficient to give proper signals as expected from the earlier discussion. Considering all the facts discussed, it is clear that filter paper is a better choice as a substrate for DESI-MS analysis of cells.

### Intensity comparison of different lipids from the WERI-RB1 cell line

After choosing the filter paper as an ideal substrate for imaging lipids derived from cells, distribution of different lipids was mapped from Whatman 42 filter paper when the WERI-RB1 cell line was spotted on them. Six major lipids shown in Fig. 2 are considered and the images are shown in Fig. 4. It is clear from the images that m/z 782.6 (Fig. 4(C)) is the most abundant ion in the WERI-RB1 cells. Next abundant ion is m/z 810.6 (Fig. 4(E)) followed by m/z 808.6 (Fig. 4(D)), 754.6 (Fig. 4(A)) as well as 780.6 (Fig. 4(B), both having almost similar abundance) and 832.7 (Fig. 4(F)). This observation is in agreement with the spectrum shown in Fig. 2. A similar pattern for lipid distribution was observed when WERI-RB1 cells were spotted on Whatman 40 filter paper (Supplementary Fig. S3, see Supporting Information).

**Figure 4.** DESI-MS images of different lipids from WERI-RB1 cells spotted on Whatman 42 filter paper. The scale bar applies to all the images. Intensity is colour coded; from black (low) to red (high).
Intensity comparison of different lipids from WERI-RB1 and Y79 cell lines

Not only different lipids of a particular cell line, but also different lipids across the cell lines can be compared by this technique. For this purpose, another human retinoblastoma cell line, Y79, was chosen. The mass spectrum of the Y79 cell line from the filter paper is shown in Fig. 5. The lipids observed were similar to that of WERI-RB1 cells and lipids identification was confirmed by tandem DESI-MS. Major lipids that appeared were \( m/z \) 754.6 [PC(32:1)+Na]+, 768.6 [PC(33:1)+Na]+, 780.6 [PC(34:2)+Na]+, 782.6 [PC(34:1)+Na]+, 808.6 [PC(36:2)+Na]+, 810.6 [PC(36:1)+Na]+ and 832.7 [PC(38:4)+Na]+ with \( m/z \) 782.6 as the base peak.

Though the relative intensities of different peaks can be readily observed from the mass spectrum, comparison across the cell lines cannot be made directly from them and images are required for that. Fig. 6 shows a comparison of four lipids from the WERI-RB1 and Y79 cell lines when both were spotted on Whatman 42 filter paper. Ion intensities are more in WERI-RB1 for \( m/z \) 782.6 (Figs. 6(B) and 6(F)), 808.6 (Figs. 6(C) and 6(G)) and 810.6 (Figs. 6(D) and 6(H)) whereas both the cell lines possess similar ion intensity of \( m/z \) 754.6 (Figs. 6(A) and 6(E)). This kind of observation can be made directly from the images.

CONCLUSIONS

In the present study we have successfully demonstrated that Whatman filter papers can be used as a suitable substrate for direct lipid imaging from cells reliably when they are spotted on them. Other common substrates like Teflon-coated glass slides and TLC plates give a considerable amount of noise in the images and from the glass slides the signal intensity is lower. The relative intensities of different lipids from a given cell line can be discovered easily. Moreover, lipid intensities across different cell lines can also be compared by this imaging technique. It should be noted that other surface-sensitive sampling techniques like liquid extraction surface analysis (LESA) can also be used to study lipids from cells. The method has already demonstrated its capability for studying lipids from human atherosclerotic plaques, drugs and their metabolites from rat tissue, etc. This method has also been used to study lipids from single cells. Furthermore, electrospray ionization mass spectrometry (ESI-MS) of direct lipid extracts can also be used to study lipids from cells. These methods can produce similar data and can potentially be used as complementary techniques to DESI-MS. As both the above-mentioned methods are based on the extraction of lipids, suitable solvent/solvent mixtures have to be chosen carefully and more time will be required due to the extraction process compared to DESI-MS where the extraction step is not necessary. A very recent report also shows that lipids from a cell line can be imaged from a glass surface by DESI-MS. In that study, cells were grown on the modified glass plate and imaged. Our study does not require any kind of modifications of the surface. Filter paper can be used directly. Moreover, the current method allows cells grown elsewhere by any process to be imaged by transferring them onto filter paper for imaging. The method is also advantageous in the case of cells that do not adhere properly on glass surfaces. We believe that in the foreseeable future
this method will find its own place in the field of biomedical research with respect to drug development and screening where initial trials of new drugs are carried out on cultured cells.

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REFERENCES


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