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Aluminium foil as a single-use substrate for MALDI-MS fingerprinting of different melanoma cell lines†

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Herein, we present the intact cell matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for the fingerprinting of human melanoma cancer cell lines grown on aluminium foil. To perform the MALDI-MS assay, melanoma cells were cultured on a flat and thin foil, which was directly transferred to the target plate of MALDI-MS for analysis. The influence of a wide range of cell fixation protocols (*i.e.* formalin-based and alcohol-based methods) and MALDI matrices on the obtained characteristic spectra was investigated. For the optimization of the MALDI-MS protocol, the MS fingerprints of the melanoma WM-239 cell line with and without an overexpressed enhanced green fluorescent protein were employed. The fingerprints obtained from WM-239 cells grown on aluminium foil were compared with the intact cell MALDI-MS of the cell pellet and presented higher sensitivity in a high *m*/*z* range. The optimized protocol was subsequently applied to characterise melanoma cell lines derived from different cancer stages and allowed identification of unique MS signals that could be used for differentiation between the studied cell lines (*i.e.* molecular weight equal to 10.0 kDa and 26.1 kDa).

Introduction

Mammalian cells cultured *in vitro* have been widely employed in medicine and biology as a simple model of complex living organisms to develop new strategies for diagnosis and treatment of different diseases.[1–4] With this aim various approaches to characterize cells have been developed based on chemical sensing, optical microscopy and mass-spectrometry (MS).[5–9] In comparison with other strategies, MS is a label free technique where the analytical signal depends on the molecular weight and charge of the analysed species after ionization. Typically, MS experiments for the characterization of *in vitro* cultured cells include cell lysis followed by the MS analysis of the obtained extract with or without enzymatic protein cleavage.[10] However, the full cell proteome analysis is very challenging and therefore, MS is often combined with separation techniques, *i.e.* electrophoresis or liquid chromatography.[11] To ionize cellular constituents without fragmentation, soft ionization techniques, *e.g.* electrospray ionization (ESI)[12] and matrix-assisted laser desorption/ionization (MALDI)[13,14] are widely used.[15–18] Although these methods allow the detection and identification of a wide range of proteins with high sensitivity, they are complex and time-consuming.

Another approach for analysis of *in vitro* cultured mammalian cells is the intact cell (IC) analysis, typically performed by MALDI-MS. In this case, cells can be either grown directly on a MALDI target plate[19] or collected by centrifugation after culturing in a classical Petri dish.[11,20–23] The latter allows cell pellets to be either transferred directly to the target plate, where they are dried and covered with a matrix solution,[22,24] or mixed with a matrix solution prior to the transfer.[20,21,23,25,26] As a result, instead of individual protein peaks, a number of signals representing the MS fingerprint characteristic for a specific cell type or physiological state can be obtained.[11] This approach has been successfully applied for the identification of two different pancreatic cell lines,[22] the differentiation between stimulated and non-stimulated macrophages,[20] differentiation of toxic effects generated by different compounds,[24] the prediction of mammalian cell phenotypes,[21] the rapid detection of apoptosis in mammalian cells,[26] the monitoring of histone deacetylase drug target engagement[25] and the characterization of neural cell types.[23] Moreover, it was reported that the analysis of on-target-grown cells resulted in mass spectra of higher peak intensity in comparison with the protocols with whole cells placed on top of a matrix layer and with cellular extracts analysed using the conventional

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sample-matrix mixture technique.[19] However, it is important to note that when culturing cells directly on MALDI target plates the surface can be contaminated with high concentrations of salts present in the culturing medium, which negatively influence the ionization efficiency. Furthermore, direct sample washing with deionized water becomes difficult due to the strong osmotic pressure. In previous studies, this problem was solved by chemical fixation of cells,[27] which is a well-established method in cell biology, histology and MALDI-MS imaging of tissues[28–30] and cells.[31–33] Cells, treated in such a way can be further washed without losing any intracellular protein content.

Herein, we present an intact cell MALDI-MS protocol for characterizing differences in the high-abundant protein content of human melanoma cells derived from different cancer stages. With this aim, cells were grown *in vitro* and chemically fixed on a flat and thin aluminium foil, which was directly transferred to a MALDI target plate for MS analysis. As a proof of concept and for optimization of the sample preparation, MS fingerprints of the melanoma WM-239 cell lines with and without a recombinantly overexpressed enhanced green fluorescent protein (EGFP) were recorded. Different chemical fixatives including cross-linkers (*i.e.* paraformaldehyde and paraformaldehyde–methanol) and dehydrators (*i.e.* methanol, methanol–acetone and methanol–ethanol) were tested and compared to the non-fixed cell samples. The optimized protocol was subsequently applied to investigate mass spectra differences between three melanoma cell lines, *i.e.* Sbcl2, WM-115 and WM-239 corresponding to the radial growth phase (RGP), vertical growth phase (VGP) and metastatic melanoma stages, respectively.

Materials and methods

Chemicals

Trifluoroacetic acid (TFA) (99.0%) was obtained from Acros Organics (New Jersey, USA). Cytochrome C (CytC), trypsin, 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (HCCA) and sinapic acid (SA) were purchased from Sigma-Aldrich (St Gallen, Switzerland). Methanol, acetone, ethanol and acetonitrile were obtained from Merck (Dietikon, Switzerland) and formaldehyde solution (4% in PBS) was from Alfa-Aesar (Karlsruhe, Germany). Deionized water was produced by the Alpha Q Millipore system (Zug, Switzerland).

The matrices containing 10 mg per mL of SA, 10 mg per mL of DHB or 10 mg per mL of HCCA were prepared in the solution containing 70% of acetonitrile, 29.9% of water and 0.1% of TFA in terms of v/v.

WM-239, WM-115 and Sbcl2 human melanoma cell lines were purchased from the American Type Culture Collection (ATCC).

Cell culture preparation

Human melanoma cell lines WM-239, WM-115, and Sbcl2 as well as WM-239 with overexpressed EGFP were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Basel, Switzerland), supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere with 5% CO_2. Twenty hours before the experiment, 200 μL of cell suspension (10^5 cells per mL) were placed on a sterile aluminium foil (~1 cm wide and ~2 cm long) and incubated over 4 hours for attachment on the surface (37 °C, 5% CO_2). Finally, 2 mL of medium was gently added into the system and the cells were incubated overnight before starting the experiment (37 °C, 5% CO_2). The optical images of cell culturing steps are presented in ESI-I.† In order to obtain WM-239 cells with overexpressed EGFP, transient transfection was performed by using Lipofectamine 2000 (Invitrogen, Basel, Switzerland). Twenty hours before transfection WM-239 cells were split in a 75 cm^2 T-flask (TPP, Trasadingen, Switzerland) reaching 80% confluence. The transfection mixture was obtained by mixing 24 μg of EGFP-N1 plasmid DNA (Clontech, Basel, Switzerland) in 1.5 mL of OptiMEM medium (Gibco Life Technologies, Basel, Switzerland) and 60 μL of Lipofectamine 2000 reagent in 1.5 mL of OptiMEM medium. After 20 min of incubation at room temperature (RT) the transfection mixture was added to the cells. The transfection efficiency calculated at 20 h after transfection was approximately 80%.

For MALDI-MS fingerprinting of melanoma cells without Al foil, the cells were washed with PBS, collected by centrifugation and kept at −80 °C. Before MS experiments, PBS was added to the cell pellets in order to have a final concentration equal to 10^4 cells per μL. These samples were further deposited on MALDI plates using matrix premixing or layer-by-layer deposition approaches (for more details, see ESI-II†).

Fixation protocols

To fix cells with dehydrating agents, the aluminium foil with adherent cells was submerged for 5–7 min in the cooled down (−20 °C) solution of pure methanol (methanol protocol), methanol/acetone (50%/50% v/v, methanol–acetone protocol) or methanol/ethanol (50%/50% v/v, methanol–ethanol protocol). Cross-linking fixation was performed by placing the aluminium foil inside an ice-cold 4% formaldehyde solution for 15 min (formaldehyde protocol). To permeabilize cells fixed with formaldehyde, the foil was additionally placed for 10 min in methanol cooled down to −20 °C (formaldehyde–methanol protocol). Thereafter, all fixed samples were submerged in deionized water for 5 min and finally dried at RT. In order to obtain the non-fixed samples adherent cells grown on aluminium foil were placed into the deionized water for 5 s and then dried at RT.

MALDI experiments

Molecular mass fingerprints of melanoma cells were obtained by a Microflex LRF MALDI-time-of-flight (TOF) instrument (Bruker Daltonics, Bremen, Germany) operated in a positive linear mode. Before each experiment, the samples on aluminium foil were positioned on a MALDI target plate by using a double-sided tape and flattened by pressing it with a microscopic glass slide. Thereafter, 1 μL of CytC aqueous solution

(2 mg mL^{-1}) was positioned onto each aluminium foil in a cell-free region and dried at RT for the external calibration of the TOF analyser by using its $[M + H]^+$ and $[M + 2H]^{2+}$ peaks. Finally, 1 μL of the matrix solution was deposited over the cells and the calibration spots and crystalized at RT. Calibration of the instrument was performed separately for each cells-on-aluminium sample. An average cell spectrum was collected from 500 random laser shots at 20 Hz laser frequency. The instrumental parameters were fixed as the following: laser attenuator – 90% within the range of 30% to 70% laser intensity; delayed ion extraction time – 400 ns; detector gain – 19.4×; electronic gain – enhanced (100 mV). The ion source voltages were at the optimized values: ion source 1 – 20.0 kV; ion source 2 – 18.5 kV; lens – 8.5 kV. The MS spectra were analysed by mMass – Open Source Mass Spectrometry Tool (http://www.mmass.org). Peaks with S/N ≥ 3 were considered as significant. A tolerance of 500 ppm was set for identical peaks.

Results and discussion

The intact cell MALDI-MS approach is a simple method that allows distinguishing differences in the mass spectra of distinct cell types without the need of performing the full proteome identification. Sample preparation for the intact cell MALDI-MS typically involves only washing of cells which are directly cultured on the target plate, as was presented by Bergquist *et al.*[19] The main limitations of this strategy are related to the MALDI plate contamination, and the large consumption of reagents due to the MALDI target plate size. To overcome these problems and as an alternative to cell pellet collection methods,[11,20–23] we demonstrate for the first time the concept of growing cells on a disposable thin aluminium foil for MALDI-MS experiments. Previously the aluminium foil layer has been shown as an ideal disposable substrate for MALDI-MS presenting good sensitivity for the detection of proteins and peptides.[34] However, this approach has thus far not yet been applied for whole cell analysis. In contrast to the on-plate cell culturing, growing mammalian cells on a disposable thin aluminium foil requires a smaller amount of both cells and growth medium, and allows working with different cell types simultaneously by placing a few samples on the same target plate (ESI-I†). Furthermore, adherent cells grown on aluminium foil can be easily transferred between various solutions (*e.g.* washing and fixation solutions). However, it is of note to mention that estimation of cell density in such samples becomes difficult due to the non-transparency and strong light reflection of the foil. Therefore, equal and reproducible cell culturing conditions are highly required.

Moreover, positioning the aluminium foil on the MALDI target plate changes the distance that ions have to pass in the TOF mass analyser, and thus the energy obtained by the ions from the extraction/acceleration electric fields. Therefore, the MALDI-TOF-MS instrument has to be calibrated for each experiment, *e.g.* by analysing a spot of CytC positioned on the same aluminium foil. An important step for MALDI-MS experiments is the optimization of the sample ionization, which can be significantly influenced by (i) the applied MALDI matrix, (ii) the presence of salts in the sample and (iii) the application of organic solvents. With the aim of optimizing the ionization process of cellular proteins on aluminium foils, the MS-spectra of WM-239 cells overexpressing EGFP (M = 26.9 kDa) were collected for several matrix combinations (*i.e.* SA, DHB and HCCA) and cell fixation protocols (*i.e.* formaldehyde, formaldehyde–methanol, methanol, methanol–acetone and methanol–ethanol). Additionally, non-fixed cells directly washed with deionized water were also analysed by MALDI-MS (Fig. 1).

Fixation is a process commonly used in biology that eliminates the biological activity inside cells and tissues, but preserves the cellular ultrastructure as well as proteins, carbohydrates and other bio-active moieties in their original spatial organization within the cells.[35] Fixation procedures will trap protein components in a matrix of insoluble proteins and therefore, allows us to immobilize cells on a surface and to wash them in order to remove salts present in the growth medium without any risk of sample destruction. Conversely, washing of intact cells without fixation should be performed fast and gently in order to minimise the removal and damaging of cells that can lead to a loss of intracellular protein content. Nevertheless, previous studies indicate that fixation can drastically affect the final ionization efficiency and therefore, the most widely used biological fixation approaches were tested here in order to determine the optimal one for the intact cell MALDI-MS.

As can be seen from the spectra collected for different matrix and fixation combinations (Fig. 2), when HCCA or DHB matrices were applied no ionization of molecules with molecular weight higher than 15 kDa was observed irrespective of the fixation method (Fig. 2 columns 2 and 3). Additionally, poor ionization efficiency was also obtained when a DHB matrix was used (Fig. 2 column 2), *i.e.* the highest number of peaks was achieved when analysing methanol and methanol–acetone fixed cell samples and was equal to 20. In contrast, the SA matrix showed both the highest ionization efficiency and number of detected species within an m/z range from 4000 to 40 000 (Fig. 2 column 1), *i.e.* up to 50 well resolved peaks can be distinguished depending on the fixation protocol.

Indeed, the fixation protocol significantly influences the results. For instance, formaldehyde fixation with and without permeabilization was not suitable for the detection of molecules with a molecular weight higher than 15 kDa (Fig. 2d and g), while all types of alcohol fixation protocols (Fig. 2j, m and p) and the non-fixed samples (Fig. 2a) allowed the detection of species within the 25 000–40 000 m/z range. The latter can be due to: (i) the different nature of the formaldehyde and alcohol fixation, *i.e.* chemical cross-linking and physical precipitation, respectively and (ii) additional cell membrane permeabilization by alcohols. Moreover, when working with formaldehyde fixed cells, the molecular weight of the cross-linked proteins might be too high and its concentration too low to be detected by MALDI-MS.

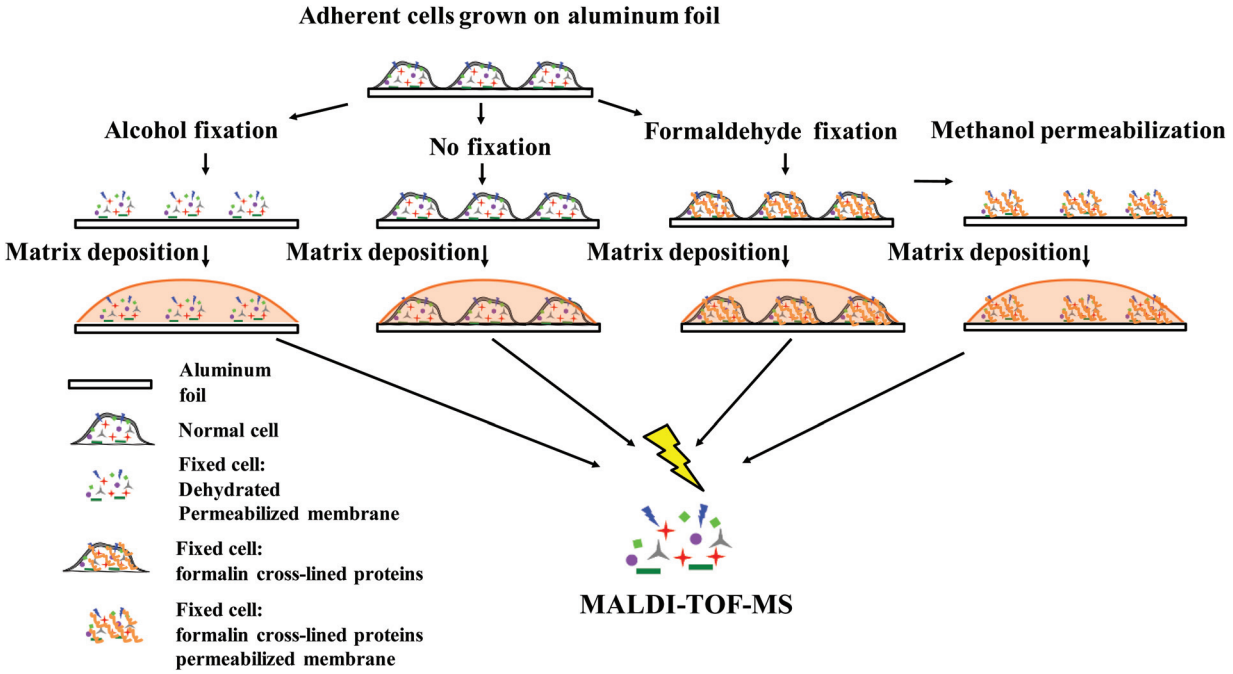

Fig. 1 Schematic representation of different sample preparation protocols applied for MALDI-MS analysis of mammalian cells grown on disposable aluminium foils.

It is also of note to mention that the overexpressed EGFP was only detected in non-fixed and methanol–acetone permeabilized cells (Fig. 2 red arrows). Despite a higher EGFP peak intensity being observed in the non-fixed sample, the reproducibility of the signal under these experimental conditions was low (*vide infra*).

Indeed, the MALDI-MS fingerprints obtained from a set of non-fixed WM-239 cells-on-aluminium samples showed significant differences in the ionization efficiency and MS spectra (Fig. 3a). Thus, only 22 well-resolved peaks with S/N $\geq$ 3 can be distinguished on the upper spectrum in Fig. 3a, while 37 peaks are presented on the lower one. The latter can be explained by the strong influence of the salts present in the analysed sample and the irreproducibility of the cell washing step due to the time-restrictions when working with live cells.

Conversely, alcohol fixation results in a good reproducibility of 32 resolved peaks with a signal to noise ratio $\geq$3 in all collected spectra (Fig. 3b). Therefore, the intact cell MALDI-MS of samples pretreated with a methanol–acetone solution can be compared in a more reliable way.

The intact cell MALDI-MS fingerprints of the WM-239 cell line with and without overexpressed EGFP are presented in Fig. 3c. An additional peak corresponding to a molecular weight equal to 26.9 kDa with a 3% relative abundance can be clearly detected, confirming the potential ability of our approach to detect differences in protein expression profiles in whole cells.

Furthermore, the comparison of the intact cell MALDI-MS on aluminium foil with the cell pellet collection approach presented significant differences in the obtained MALDI-MS fingerprints. The cell pellet fingerprints were obtained by following two approaches: (i) premixing of the pellet with the SA matrix[20,21] and (ii) deposition of the pellet and matrix layer-by layer.[22] As a result, the fingerprints obtained by the premixing approach presented better resolution (ESI-II†). Additionally, no species with an *m*/*z* higher than 15 000 can be detected for the cell pellet independently of the matrix deposition procedure, illustrating the better ionization of large molecules in the case of cells grown on aluminium foil (Fig. 3d).

The optimized protocol for the intact cell MALDI-MS on aluminium foil was further applied to characterize three melanoma cell lines, *i.e.* Sbcl2, WM-115 and WM-239 derived from RGP, VGP and metastatic melanomas, respectively. In order to characterize the reproducibility of the obtained fingerprints, each of the cell type was grown in triplicate and thereafter, three MALDI-MS spectra were collected from each of the obtained samples. Further, all the collected spectra (*i.e.* 27 spectra in total) were analysed in terms of *m*/*z* and the intensity of all characteristic peaks. As a result, MALDI-fingerprints obtained from cells grown on aluminium foil presented high reproducibility, when collected from the same sample (thus, the standard deviation of the intensity of most of the peaks was less than 5). However, significant variation of peak intensity can be observed when comparing fingerprints from different samples (but for the same cell type), leading to high values of standard deviation (*i.e.* up to 30). The last can be due to some differences in cell density on the investigated surfaces. A detailed list of all the characteristic peaks with their intensities and standard deviation as well as all the obtained spectra are given in ESI-III.†

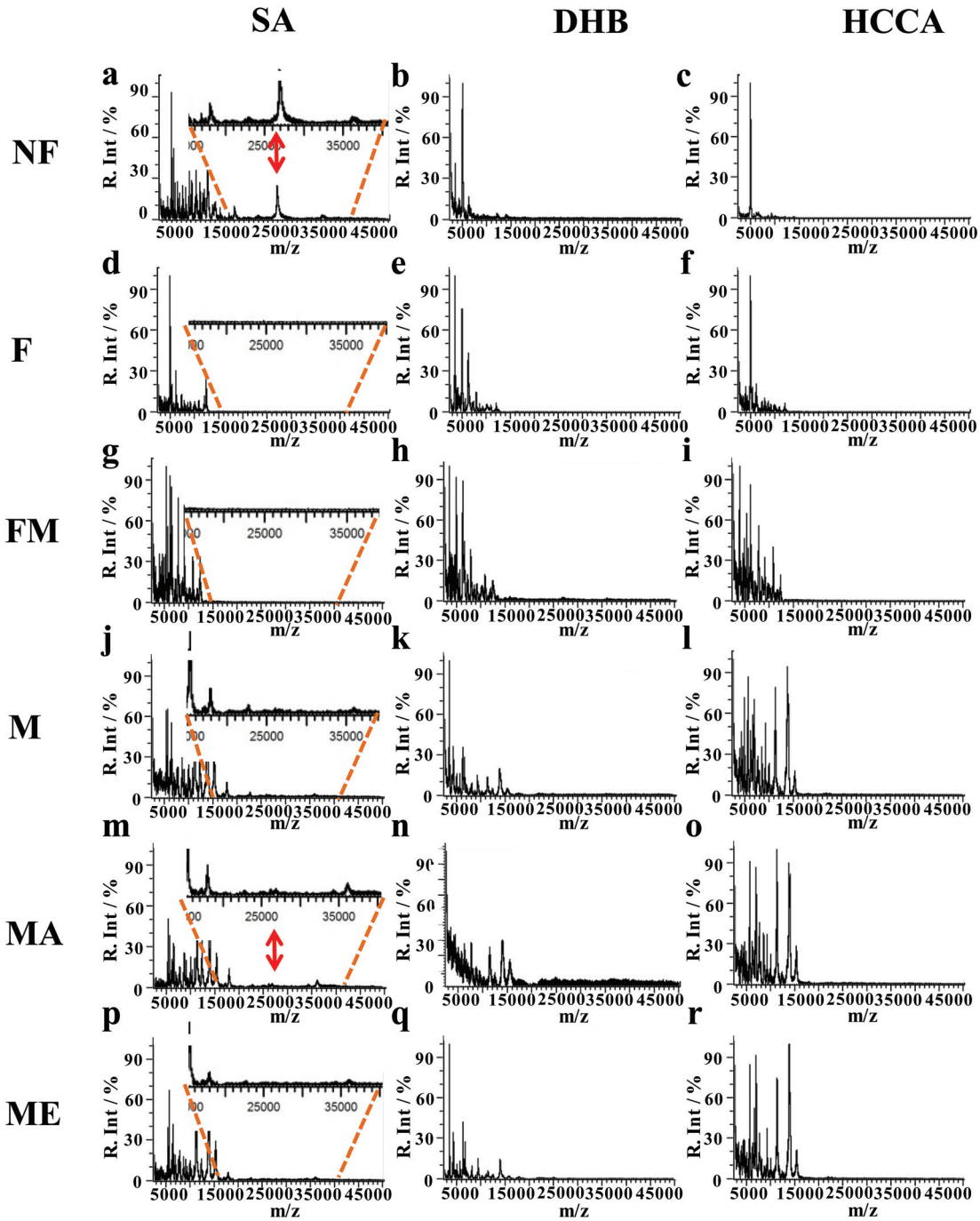

Fig. 2 Optimization of the MALDI matrices and the cell fixation protocols for obtaining characteristic MS fingerprints of WM-239 melanoma cells with an overexpressed EGFP. MALDI matrices were SA (a, d, g, j, m and p), DHB (b, e, h, k, n and q) and HCCA (c, f, i, l, o and r). Fixation protocols were: non-fixed cells, NF (a–c); formaldehyde, F (d–f); formaldehyde–methanol, FM (g–i); methanol, M (j–l); methanol–acetone, MA (m–o); and methanol–ethanol, ME (p–r).

Comparison of the fingerprints collected from different cell lines (*i.e.* Sbcl2, WM-115 and WM-239) presented a very similar peak distribution except for 2 peaks of *m*/*z* equal to 10 000 and 26 100. Thus, plotting the intensity of *m*/*z* equal to 26 100 over 10 000 presents the possibility of differentiating all the investigated cell lines based on these peaks (ESI-III and Fig. S7†). Indeed, the *m*/*z* equal to 10 000 was only detected in the early stage melanoma cells (*i.e.* RGP and VGP), but not in the metastatic stage cells (Fig. 4a and b). Furthermore, the signal of 26 100 *m*/*z* was absent only in the RGP stage cells.

It is also interesting to notice that the MALDI-MS fingerprints obtained from pellets of different cell types also present some differences in the fingerprints of the investigated cell

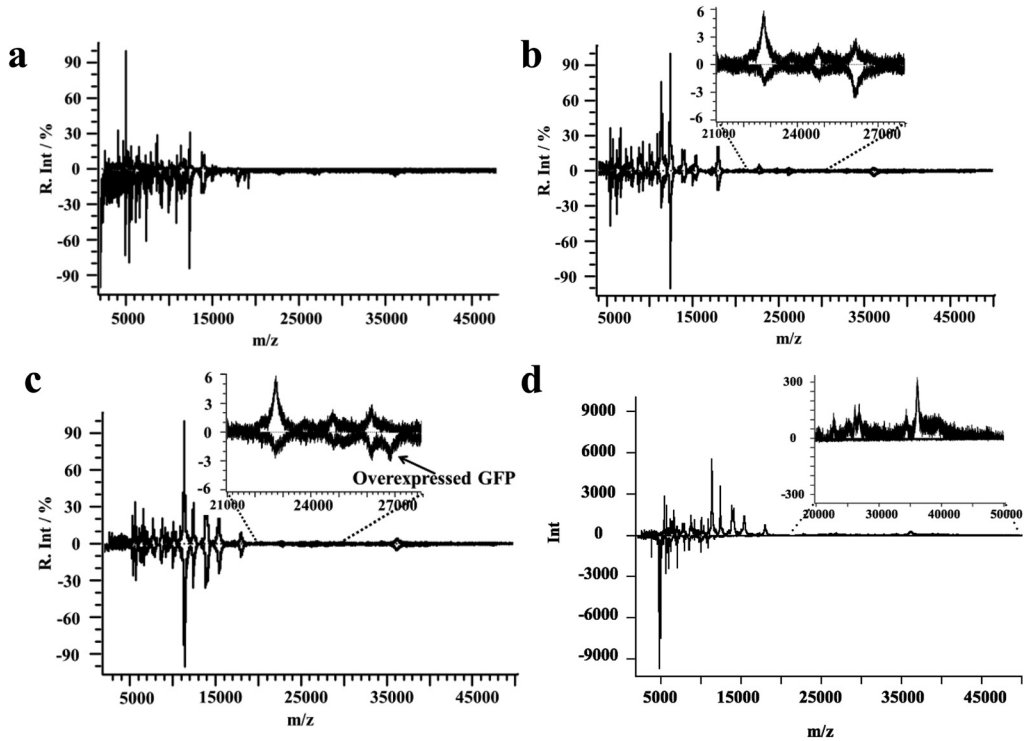

Fig. 3 Reproducibility of the MS spectra obtained for non-fixed (a) and methanol–acetone fixed (b) WM-239 cells. The upper and lower spectra represent MALDI-MS results for 2 samples prepared in the same way (*i.e.* culturing conditions, fixation and washing). Characteristic MALDI-MS fingerprints of WM-239 cells without (upper spectrum) and with (lower spectrum) the overexpressed EGFP in the optimized conditions (*i.e.* methanol–acetone fixation protocol) are presented in (c). Comparison of the fingerprints obtained from WM-239 cells grown on aluminium foil (upper spectrum) and intact cell MALDI-MS of the cell pellet (lower spectrum; 10^4 cells per μL premixed with the matrix) are presented in (d). SA was employed as the MALDI matrix in all the experiments.

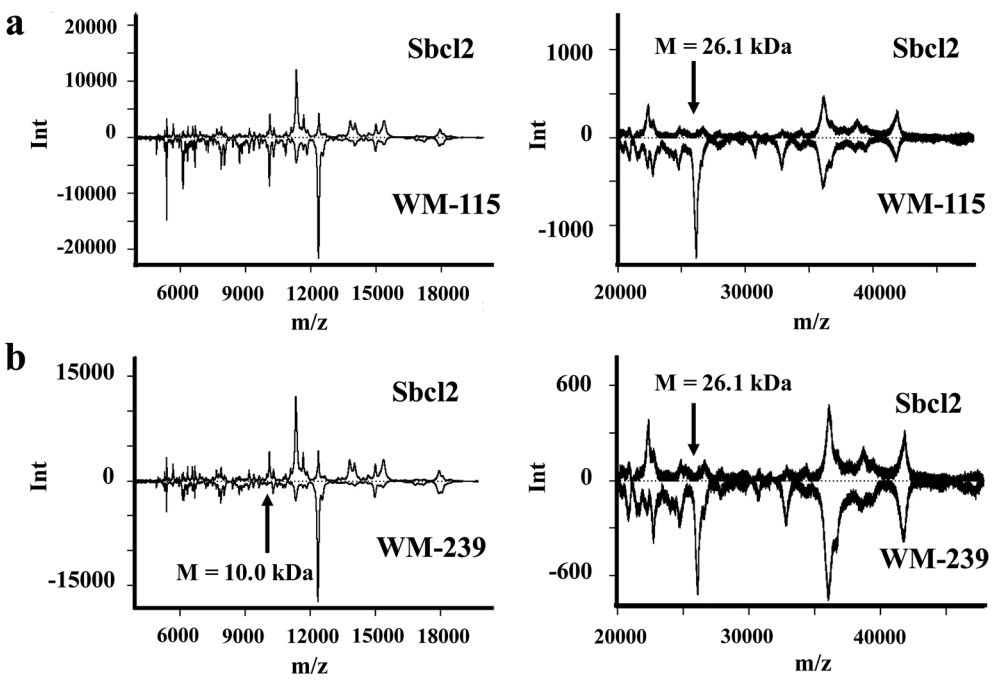

Fig. 4 The characteristic MALDI-MS fingerprint spectra obtained for Sbcl2, WM-115 and WM-239 melanoma cell lines representing cancer progression (RGP, VGP and metastatic, respectively). For better visualisation of the obtained MS spectra, the obtained full spectra are separated in two *m*/*z* ranges (*i.e.* *m*/*z* intervals from 5000 to 19 000 and from 19 000 to 50 000), and data are presented as the following pairs: Sbcl2 *vs*. WM-115 (a) and Sbcl2 *vs*. WM-239 (b). Experimental conditions: methanol/acetone fixation protocol, SA matrix.

lines (ESI-IV†). However, no correlation of these results with fingerprints on aluminium foil can be detected.

The fingerprints of melanoma cells grown on the aluminium foil were shown to be applicable in following the differences between different cell lines. Despite this method not allowing the identification of the observed peaks, the comparison of our data with the previously published proteomic characterization of melanoma cell lines could be interesting.[36,37] In the literature, northern blot analysis revealed a significant expression of the S100 family protein mRNAs in the early melanoma stages (*i.e.* Sbcl2 and WM-115), while a lower level in the metastatic WM-239 cell line.[36] The molecular weight of S100 proteins is 10 kDa, which correlates well with the characteristic MS peak with m/z equal to 10 000 observed only with Sbcl2 and WM-115. On the other hand, the m/z = 26 100 peak may represent the transmembrane protein V-ATPase B2 identified by Baruthio *et al.* using liquid chromatography coupled to MS/MS.[37] This protein was shown to be characteristic for late primary and metastatic cancer stages (*i.e.* WM-115 and WM-239), but not for early tumour cells (*i.e.* Sbcl2) pointing to its usefulness as a potential cancer progression biomarker.[37] The ability to follow its expression by the detection of its characteristic peak at m/z = 26 100 using the intact cell MALDI-MS approach was demonstrated for the first time in the present study. Thus, the approach can be potentially interesting for the investigation of cellular processes.

Conclusions

A fast and simple intact cell MALDI-MS approach was successfully implemented as a tool for mammalian cell fingerprinting. For this purpose, different adherently growing melanoma cell lines were cultured on disposable aluminium foils, which allowed their facile transfer to the MALDI target plate for consecutive analysis. The influence of a wide range of cell fixation protocols (*i.e.* formalin-based and alcohol-based methods) as well as the impact of different MALDI matrices on the obtained characteristic spectra were investigated. Optimization of the intact cell MALDI-MS protocol was performed based on the MS fingerprints of the melanoma WM-239 cell line with and without overexpression of EGFP (molecular weight equal to 26.9 kDa). We found that a methanol–acetone fixation protocol in combination with an SA matrix allowed the reliable and reproducible detection of the recombinant EGFP. Furthermore, in comparison with the cell pellet collection method, this approach presented a higher ionization efficiency of large molecules. The optimized protocol was used to differentiate melanoma cell lines derived from different cancer stages, *i.e.* RGP, VGP and metastatic cells.

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