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PAPER

Rapid detection of apoptosis in mammalian cells by using intact cell MALDI mass spectrometry†

Hongjuan Dong,[a] Wei Shen,[a] Myra Ting Wai Cheung,[a] Yimin Liang,[a] Hon Yeung Cheung,[a] Günter Allmaier,[b] Oscar Kin-Chung Au[c] and Yun Wah Lam*[a]

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Detection of cell death has extensive applications and is of great commercial value. However, most current high-throughput cell viability assays cannot distinguish the two major forms of cell death: apoptosis and necrosis. Many apoptosis-specific detection methods exist but they are time consuming and labour intensive. In this work, we proposed a novel approach based on Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) for the specific detection of apoptosis in cultured mammalian cells. Buffer washed cells were directly mixed with a matrix solution and subsequently deposited onto the stainless steel target for MALDI analysis. The resulting mass spectrometric profiles were highly reproducible and can be used to reflect cell viability. Remarkably, the mass spectrometric profiles generated from apoptotic cells were distinct from those from either normal or necrotic cells. The apoptosis-specific features of the mass spectra were proportional to the percentage of apoptotic cells in the culture, but are independent of the drugs used to stimulate apoptosis. This is the first report on the utilization of intact cell MALDI mass spectrometry in detecting mammalian cell apoptosis, and can be used as a basis for the development of a reliable, fast, label-free and high-throughput method for detecting apoptotic cell death.

Introduction

Detection of cell death is one of the most fundamental experimental techniques in both basic and applied biological research. There are two major types of cell death.[1] One type is termed apoptosis, a physiological cell death characterized by specific morphological and biochemical features. It is different from the other type of cell death, necrosis, caused by physical disruption of cellular integrity. There are many inexpensive, quick and convenient methods for cell death detection, but most of them cannot distinguish these two types of death. Most high-throughput assays for cell death are based on the detection of declined cell metabolism, as in MTT assays,[2] or compromised membrane integrity, as in dye exclusion assays.[3] Apoptosis-specific detection methods do exist, *e.g.*, electron microscopy,[4] TUNEL assay[5,6] and flow cytometry,[4–7] but most of them involve relatively complex and tedious procedures, with the need for multiple staining and washing steps which make these techniques difficult to be automated.

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI TOF MS) has found many applications in biology, mainly in the proteomics field.[8,9] MALDI is a soft desorption/ionization technique, generating ions from intact molecules without fragmentation of the analyte. It also offers relatively high tolerance against contaminants such as salts or detergents. Researchers recognized the capability of this technique and were quick to develop related methodologies to analyze intact cells. The term "intact cell" here refers to the analysis of whole microbial cells without any prior sample extraction or purification, but does not suggest that the cells are viable or architecturally intact at the time of MS analysis. In fact, the exposure to water, organic solvent or the strong organic acid in the MALDI matrix solution causes cell wall ablation to a certain extent.[12] Intact cell MALDI TOF MS (ICM-MS) is widely applied to identify microorganisms based on their specific biomarkers[10] or spectral patterns.[11] This technique requires small sample volume, minimal sample preparation and can be automated readily. It can yield very specific peptide/protein profiles with high speed. With all these advantages, ICM-MS is emerging as one of the most popular techniques for the rapid analysis of microorganisms.[13–18]

Despite the wide application of ICM-MS in microbiology, mostly in the identification of bacteria[19–22] and fungi,[23–29] there

[a]*Department of Chemistry and Biology, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong. E-mail: yunwlam@cityu.edu.hk; Fax: +852-2788-7406; Tel: +852-3442-6347*
[b]*Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164-IAC, A-1060 Vienna, Austria*
[c]*School of Creative Media, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong*
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are relatively few attempts to apply this technology to mammalian cells.[30–32] The desorption/ionization properties of mammalian cell components on an ICM-MS platform are not well characterised, but recent studies have shown that different mammalian cell types appear to exhibit ICM-MS with distinct patterns.[30] This suggests that ICM-MS can be used to display gross biochemical differences in mammalian cells. In this study, we discovered a new application of ICM-MS that can readily distinguish viable and dead cells with only hundreds of cells per test—about 100 times less than what traditional flow cytometry requires—from a mass spectrum generated in only a few seconds. Since the method developed in this study measures the changes in accessibility of cellular proteins to desorption/ionization, it is a "label-free" technique, forgoing any need for probes and washing steps. Remarkably, we showed that this ICM-MS method could give specific readouts for apoptotic but not necrotic cells. To our knowledge, this is the only single-step, label-free, apoptotic-specific detection method.

Materials and methods

Chemicals

Acetonitrile (ACN) HPLC grade was purchased from RCI Labscan. Trifluoroacetic acid (TFA), sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma (St Louis, MO).

Cell culture

The following cell lines were used in this study: cervical cancer cells HeLa, kidney epithelial carcinoma MDCK, retinal pigmented epithelial cells RPE, breast cancer cells MCF-7 and mouse fibroblast cells 3T3. All cell lines were obtained from ATCC and grown as adherent mono-layers in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and antibiotics (all from Invitrogen, Carlsbad, CA), in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C.

In this paper, three drugs were used to induce apoptosis: etoposide (Sigma E1383), camptothecin (Sigma C9911) and andrographolide (Sigma 365645). After cells were grown to 90% confluence, normal medium was replaced with medium containing drugs at different concentrations (see below) and incubated for 24 hours before cell harvesting. For all experiments, controls (mock-treated) and drug treated cells were run in parallel.

Measurement of cell death

Cells were harvested and pelleted using a centrifuge at 2000*g*, 4 °C for 5 min. The supernatant was removed and cells were washed three times with 10 mL ice-cold phosphate-buffered saline (PBS). Finally the cell pellet was re-suspended in PBS buffer. The percentage of dead cells was calculated by using a TC 10™ automated cell counter (Bio-Rad Laboratories Inc.) which auto-detected the presence of the trypan blue dye in the sample to access cell viability. For apoptosis-specific detection, washed cells were resuspended in ice-cold 70% ethanol in water and incubated for at least 1 h at 4 °C. Afterwards, cells were washed with PBS as above, and then resuspended in PBS containing propidium iodide (Sigma, St Louis, MO) and RNase A (Sigma, St Louis, MO), both at 50 μg mL^{-1}. After a 30 min incubation at RT, the cells were analysed by a flow cytometer (FACScan, BD), following the manufacturer's instruction.

MALDI TOF MS

A 10 mg mL^{-1} solution of α-cyano-4-hydroxycinnamic acid (CHCA) diluted in ACN/aqueous 0.1% TFA (7/3, v/v) was used as matrix solution. The mixed volume technique was used for matrix/sample preparation. Briefly, the mammalian cells and matrix solution were placed into an Eppendorf tube with volume ratio 1/1 and mixed well by vortex. Then 1 μL of the mixture was deposited onto the MALDI target, and the solvent was evaporated at RT.

All MALDI mass spectra were obtained on an Applied Biosystems 4800 MALDI TOF/TOF analyzer. This instrument is equipped with a Nd:YAG laser with 355 nm wavelength. The instrument was operated at an acceleration potential of 20 kV. All mass spectra were recorded in positive ion linear mode with delayed extraction (optimized for *m/z* 10 000) by accumulating up to 5000 single unselected laser shots in the *m/z* range of 2000 to 20 000.

Data visualization

To better visualize and compare the mass spectral features of viable, necrotic and apoptotic cells, we linearly mapped the

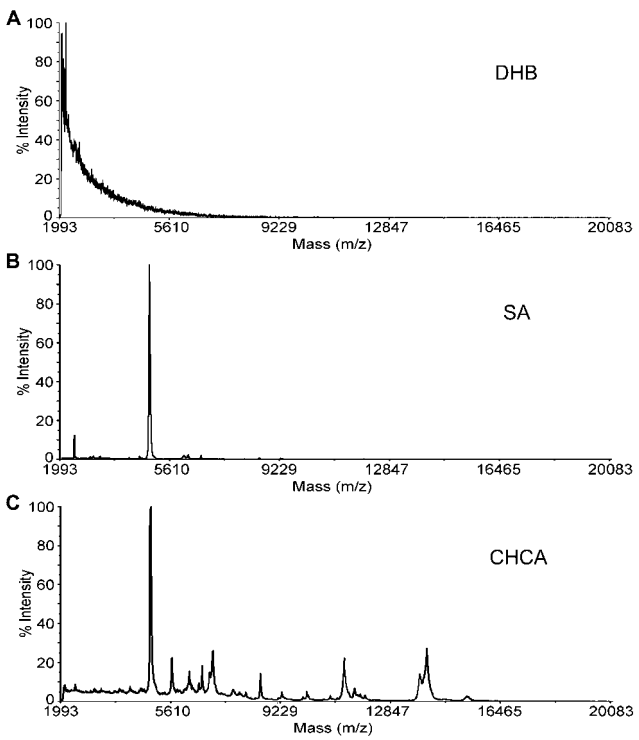

Fig. 1 MALDI mass spectra of HeLa cells with different matrix solutions. (A) 10 mg DHB per mL ACN/0.1% TFA (7/3, v/v), (B) 10 mg SA per mL ACN/0.1% TFA (7/3, v/v), and (C) 10 mg CHCA per mL ACN/0.1% TFA (7/3, v/v).

generated spectra to the colour domain with the standard cold-to-hot colour ramp. Lowest and highest spectral values are represented with blue and red colors, respectively. In order to introduce larger color variation and maximize the distinguishing power we use the colours cyan and yellow for intermediate values (*i.e.* colour ramp of blue-cyan-green-yellow-red). Local peaks are emphasized with bars with colours different from the relatively smooth background.

Result and discussion

Apoptotic cells exhibit specific MALDI mass spectral features

It is well known that sample preparation is critical to a successful MALDI MS analysis. In the process of generating MALDI mass spectra from a mammalian cell line, there are many experimental factors that can influence the final appearance of mass spectra. In order to detect apoptotic cells specifically and at the same time obtain reproducible MALDI mass spectra, we carefully identified and controlled these factors by using HeLa cells as a model.

In the first series of experiments, several matrices were tested for the analysis of mammalian cell lines by MALDI MS. After HeLa cells were washed and re-suspended in PBS, they were deposited onto the MALDI target using the mixed volume technique with various matrices. The mixed volume technique was chosen because matrix/sample crystals evenly covering the whole sample spot can be formed, so that automatic rastering of the sample spot can result in homogeneous desorption/ionization of the analyte ions.[33] As shown in Fig. 1, mass spectra were generated from intact HeLa cells in both CHCA and SA matrix

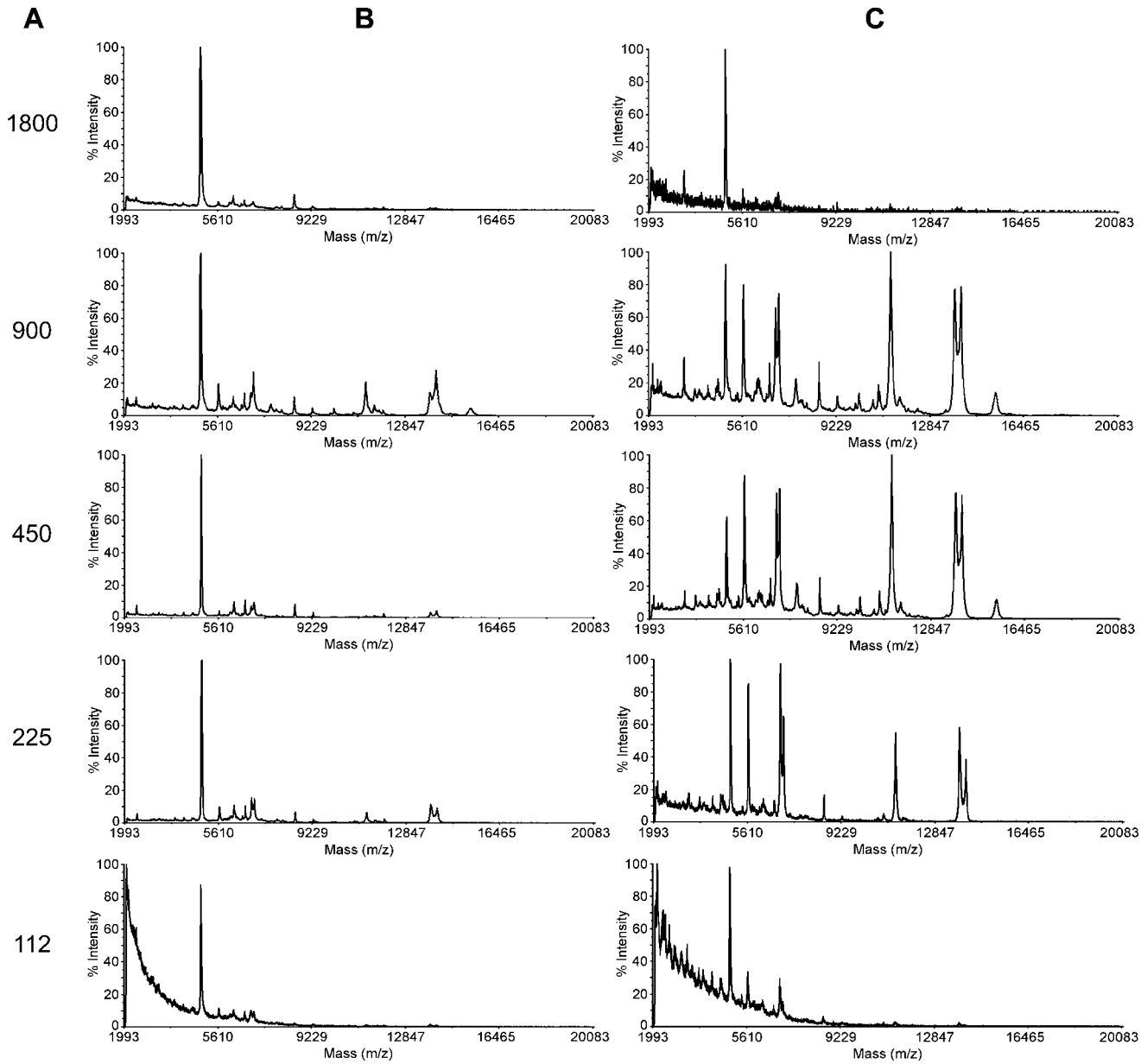

Fig. 2 Comparison of MALDI mass fingerprints of HeLa cells at different cell numbers per spot on the MALDI target. (A) Cell numbers on each spot, (B) MALDI TOF mass spectra of viable cells, and (C) MALDI TOF mass spectra of apoptotic cells.

solutions but no peaks were observed when using DHB (Fig. 1). Since the mass spectra obtained using the CHCA matrix solution contained more informative peaks, with higher reproducibility than the ones using SA, we chose CHCA as the matrix for the rest of this study. Although the salts in PBS may interfere with either the crystallization or ionization process, our result indicates that MALDI mass spectra can readily be acquired with CHCA. In fact, the quality of the mass spectra did not

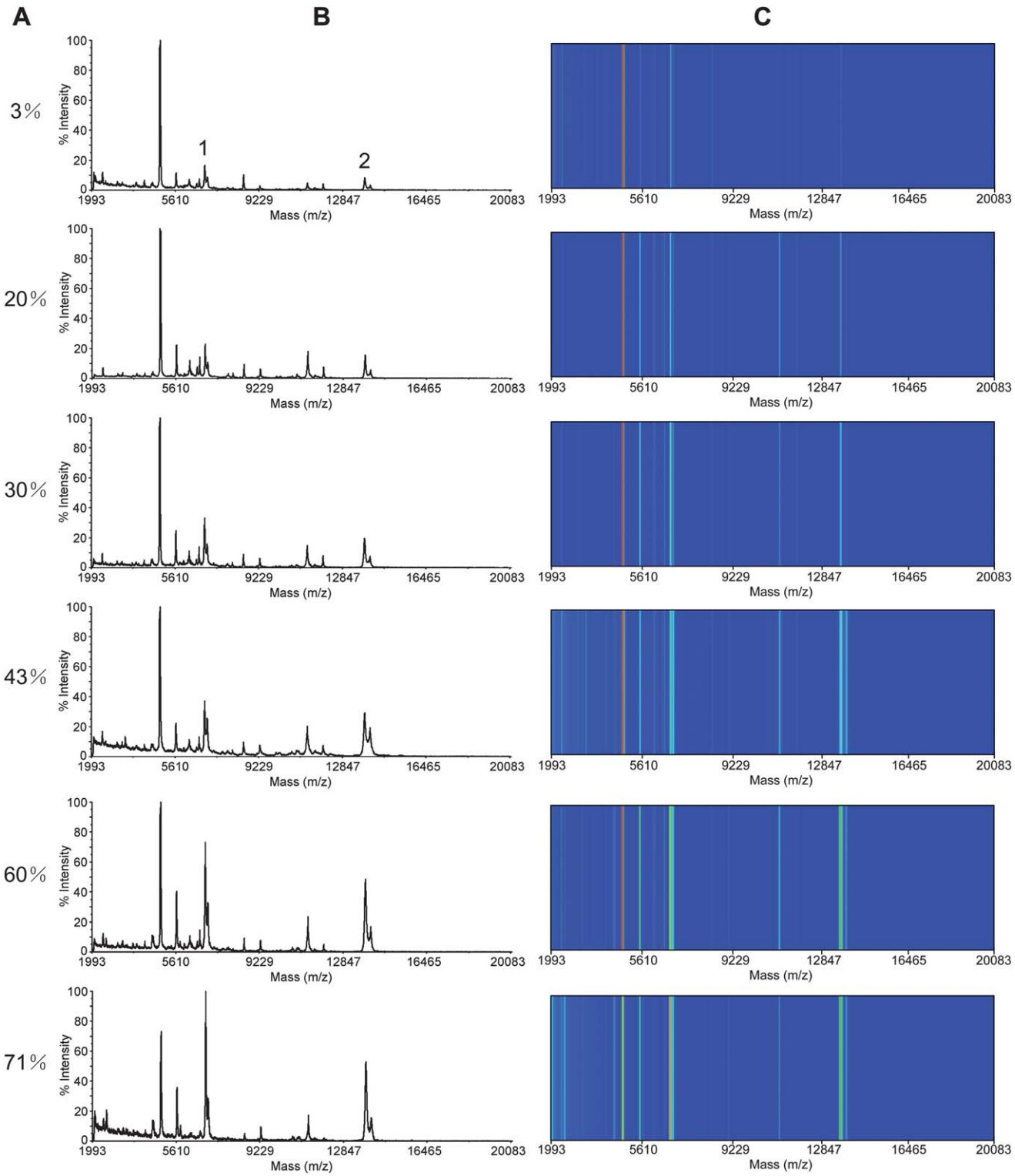

Fig. 3 Intact cell MALDI TOF MS analysis of the mixture of viable cells and dead cells. (A) The percentage of dead cells in the mixture, (B) intact cell MALDI TOF mass spectra, and (C) MALDI mass spectra presented in barcode view. Numbers 1 and 2 denote representative peaks correlating with the percentage of dead cells.

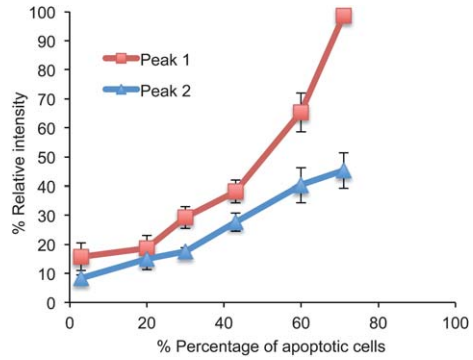

Fig. 4 Correlation of the relative intensities of peaks 1 and 2 and the percentage of dead cells contained in the sample. Averages and SD from 6 replicated experiments.

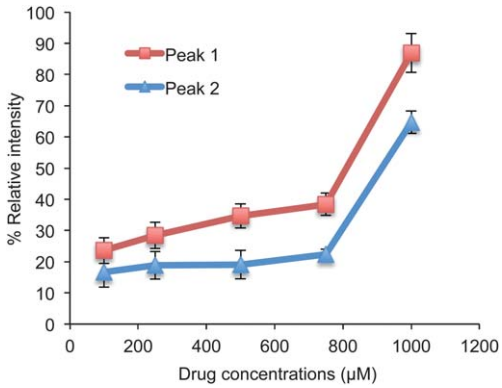

Fig. 6 Correlation of the relative intensities of peaks 1 and 2 and the concentrations of andrographolide used in the *in vitro* toxicity test. Averages and SD from 6 replicated experiments.

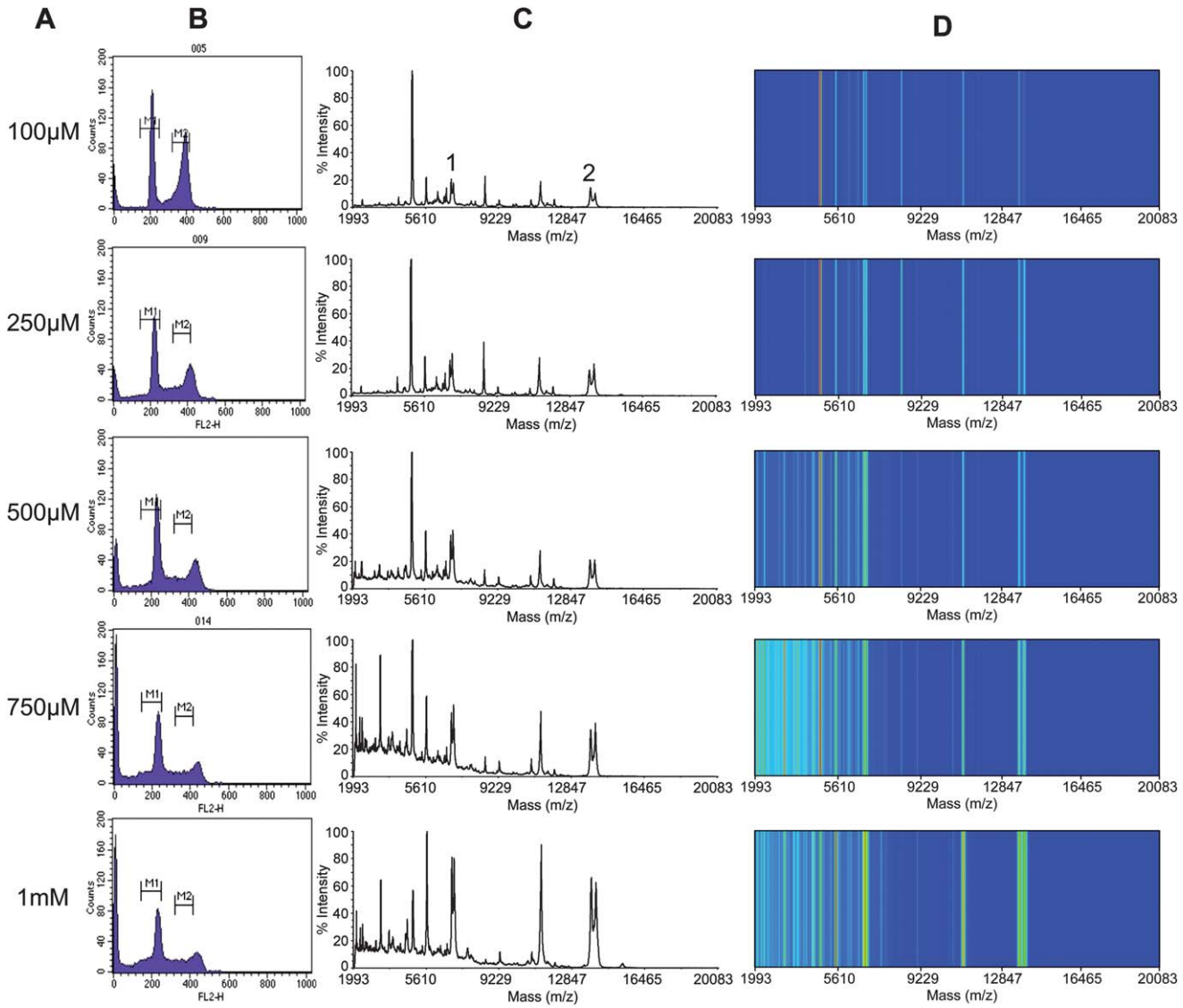

Fig. 5 Comparison of flow cytometry and intact cell MALDI TOF MS in dead cell detection. (A) Concentrations of pro-apoptotic compound andrographolide used to treat HeLa cells. (B) Propidium iodide-labeled DNA profiles of the treated cells as measured by flow cytometry. (C) Intact cell MALDI TOF MS of the same samples. (D) MALDI mass spectra presented in barcode view.

significantly improve if the cells/matrix spots on plate were rinsed to remove salts (data not shown). All the subsequent experiments were therefore performed with the mixed volume technique without additional washing steps.

Remarkably, when apoptotic HeLa cells (generated by pre-treating the cells with the herbal diterpene lactone andrographolide[34] at the concentration of 1 mM) were used, the MALDI mass spectra were significantly different from those of viable cells (Fig. 2). Comparing with the untreated cells, apoptotic cells generated a much more complex mass spectrometric profile. The differences can best be viewed in a barcode format, which converts the MS peaks into colour-coded lines

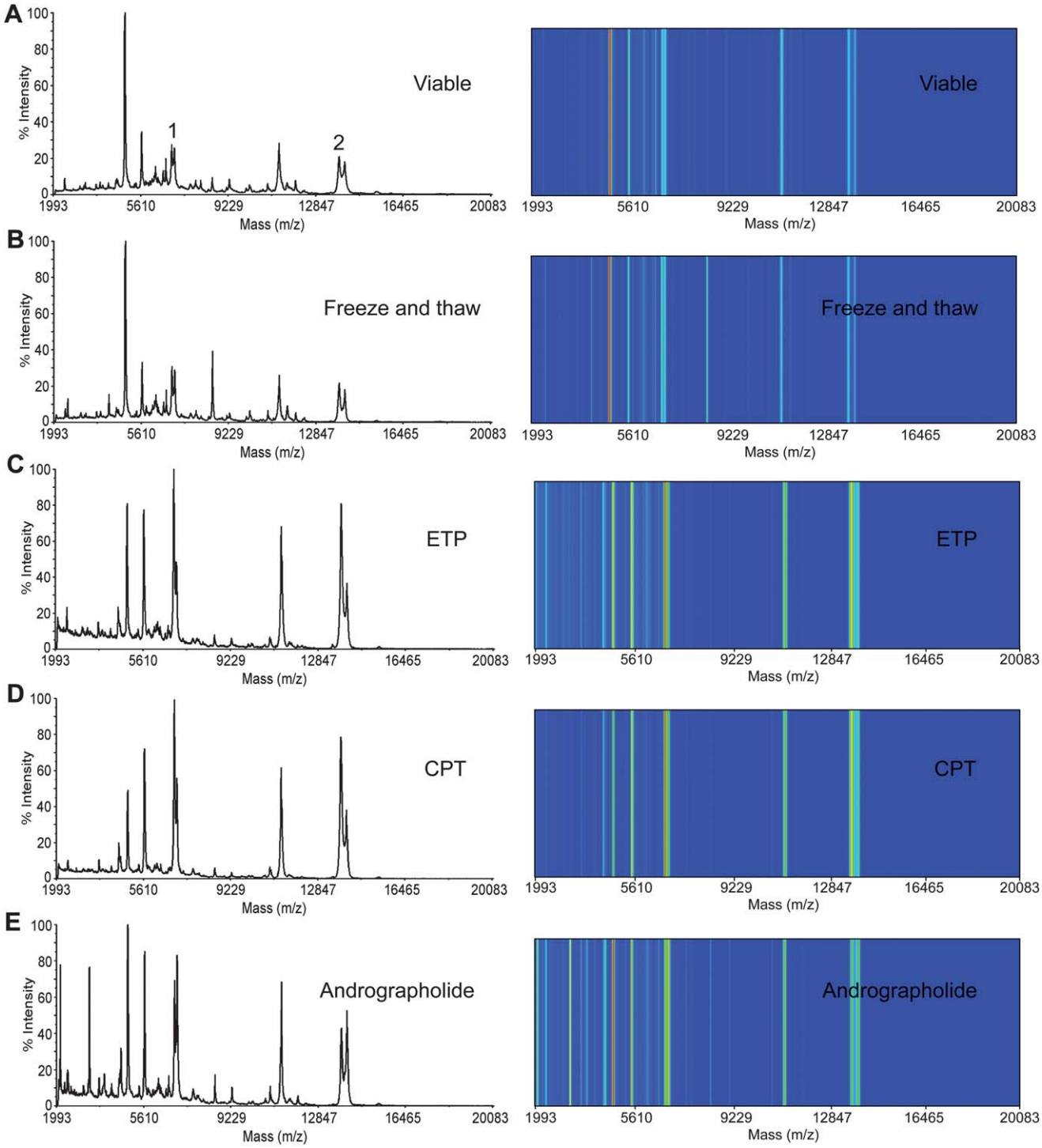

Fig. 7 Intact cell MALDI TOF mass spectra of HeLa cells treated in different ways, and their corresponding barcode view. (A) Viable cells, (B) necrotic cells generated by freeze–thaw cycles, (C) apoptotic cells generated by etoposide (ETP) toxicity, (D) apoptotic cells generated by camptothecin (CPT) toxicity, and (E) apoptotic cells generated by andrographolide toxicity.

(Fig. 3). We titrated the number of cells per spot in order to determine the optimal cell loading for ICM-MS that can maximize the distinction between live and dead cells. Fig. 2 shows that, for HeLa cells, MALDI spectra exhibited many peaks with 200–1000 cells per spot, but tended to generate less informative spectra for apoptotic cells if more or fewer cells were used. All the subsequent experiments with HeLa cells were performed with a cell concentration at around 900 cells per spot.

The remarkable difference in MS generated from drug-treated and control cells is unexpected and intriguing. Regardless of the viability of the cells at the moment of harvest, all the cells would be killed and disrupted on the addition of the matrix solution. The additional MS peaks detectable in the drug-treated samples are therefore not likely due to membrane leakage associated with cell death. Rather, it may represent drug-induced biochemical changes that are particularly sensitive to MALDI detection. We are actively investigating the identity of these drug-induced MS peaks. Even without any molecular information, these peaks may still be utilised as signature markers for cell death. To establish the reproducibility of ICM-MS on mammalian cells, MALDI mass spectra from eight replicated preparations of both viable and apoptotic HeLa cells were compared for reproducibility (ESI, Fig. S1A and B†). The result showed that they could generate quite reproducible MALDI mass spectra when they were treated in the same well-defined way and were recorded with the same instrumental operating conditions.

The optimised protocol of sample preparation for the detection of apoptotic HeLa cells by ICM-MS is summarized as follows: cells were collected and washed three times with PBS. Then the cell pellet was resuspended in PBS with a final concentration at around 1800 cells per μL. The matrix (10 mg CHCA dissolved in 1 mL ACN/0.1% TFA (7/3, v/v)) and cell solutions were premixed in an Eppendorf tube at ratio 1/1 (v/v) and subsequently 1 μL of the mixture was deposited onto the MALDI target.

ICM-MS as a cytotoxicity test

We next asked whether our optimised ICM-MS protocol can be used to assess the percentage of cell death in cell culture. In a proof-of-concept experiment, we mixed untreated HeLa cells with andrographolide-treated, apoptotic HeLa cells at various ratios, and subjected the mixtures to ICM-MS (Fig. 3). Although the general appearance of the MS looked similar, some MS peaks, as exemplified by peaks 1 and 2, appeared to reflect more sensitively the percentage of apoptotic cells in the mixture. As shown in Fig. 4, the relative intensities of both peaks 1 and 2 are consistently proportional to the percentage of apoptosis, suggesting that these MS features can be used as markers for cell death. To test the feasibility of using ICM-MS in an actual *in vitro* toxicity testing scenario, we treated HeLa cells with andrographolide at different concentrations (100 μM, 250 μM, 500 μM, 750 μM and 1 mM). This compound is known to induce apoptosis by perturbating the intracellular levels of reactive oxygen species.[34] After 24 hours of treatment, all the cells in culture, including both the attached and floating cells, were collected. The percentage of apoptosis was determined by the size of the "sub-G1 peak" by using flow cytometry, a classical method for measuring the extent of DNA fragmentation associated with apoptosis. Their corresponding MALDI TOF mass spectra were also obtained (Fig. 5). The results showed that with an increase of numbers of apoptotic cells, some of the MS peaks, notably the apoptosis-correlated peaks 1 and 2, were progressively changed. This suggests that the MS profiles can be used to reflect the level of cell death quantitatively. Fig. 6 shows correlation of the intensities of these two MS peaks with the concentrations of andrographolide used. Interestingly, ICM-MS could apparently distinguish cells treated with the drug at 250 and 500 μM, respectively, whereas flow cytometry could not.

ICM-MS patterns are apoptosis-specific

We observed that the treatment of cells with an increasing concentration of andrographolide caused the emergence of MS peaks, especially in the low mass region (Fig. 5), which is not seen in the cell mixing experiment (Fig. 3). These features may be due to specific cellular responses to andrographolide. We asked whether the detected changes in MALDI mass spectra represent a general feature of apoptotic cells, or merely an exclusive characteristic of andrographolide-treated HeLa cells. We did the MALDI-MS analysis of HeLa cells treated with two other common anti-cancer compounds, etoposide (at the concentration of 1 mM) and camptothecin (at the concentration of 10 μM). These compounds induce apoptosis through inhibition of topoisomerases in mammalian cells,[35] and are unlikely to share the same cytotoxic pathway as andrographolide. Fig. 7 shows that while there are unique spectral features in each of the MALDI mass spectra, many peaks are common to cells treated with different drugs. In particular, peaks 1 and 2 were found increased in the apoptotic cells induced by all three drugs (Fig. 8). Since these common features are not found in untreated HeLa cells, we concluded that the ICM method developed in this paper can be used to distinguish live and dead cells, regardless of the identity of the drug used to induce apoptosis.

Apart from apoptosis, an alternative form of cell death is necrosis. These two types of cell death are different in regulation, morphology and molecular features. Since ICM-MS can reveal biochemical differences in intact cells, we questioned whether this technique could be used to distinguish apoptosis and necrosis. To obtain necrotic cells, viable HeLa cells were suspended in PBS and were frozen at −80 °C for 1 hour and then completely thawed to RT. The freeze and thaw cycles were performed two

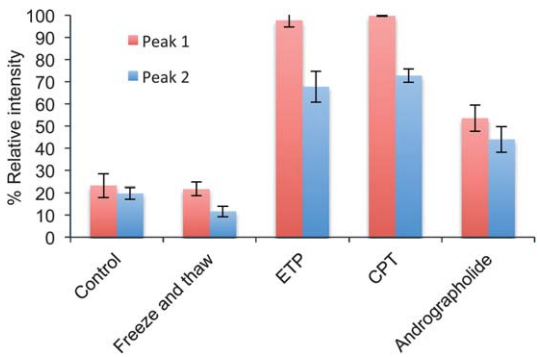

Fig. 8 Relative intensities of representative peaks 1 and 2 in various samples described in Fig. 7. Averages and SD from 6 replicated experiments.

more times until all the cells were killed by the ice crystals formed during freezing. The use of freezing conditions completely avoided the occurrence of apoptosis, which is an active, physiological process requiring energy production.[36] The resulting MALDI mass spectral patterns are shown in Fig. 7. Remarkably, cells killed by repeated freeze–thaw cycles exhibited very small differences in the MS profile compared with that of untreated cells. This suggests the MALDI sample preparation procedure has probably disrupted the untreated cells to the same extent as those that have been previously killed by necrosis. However,

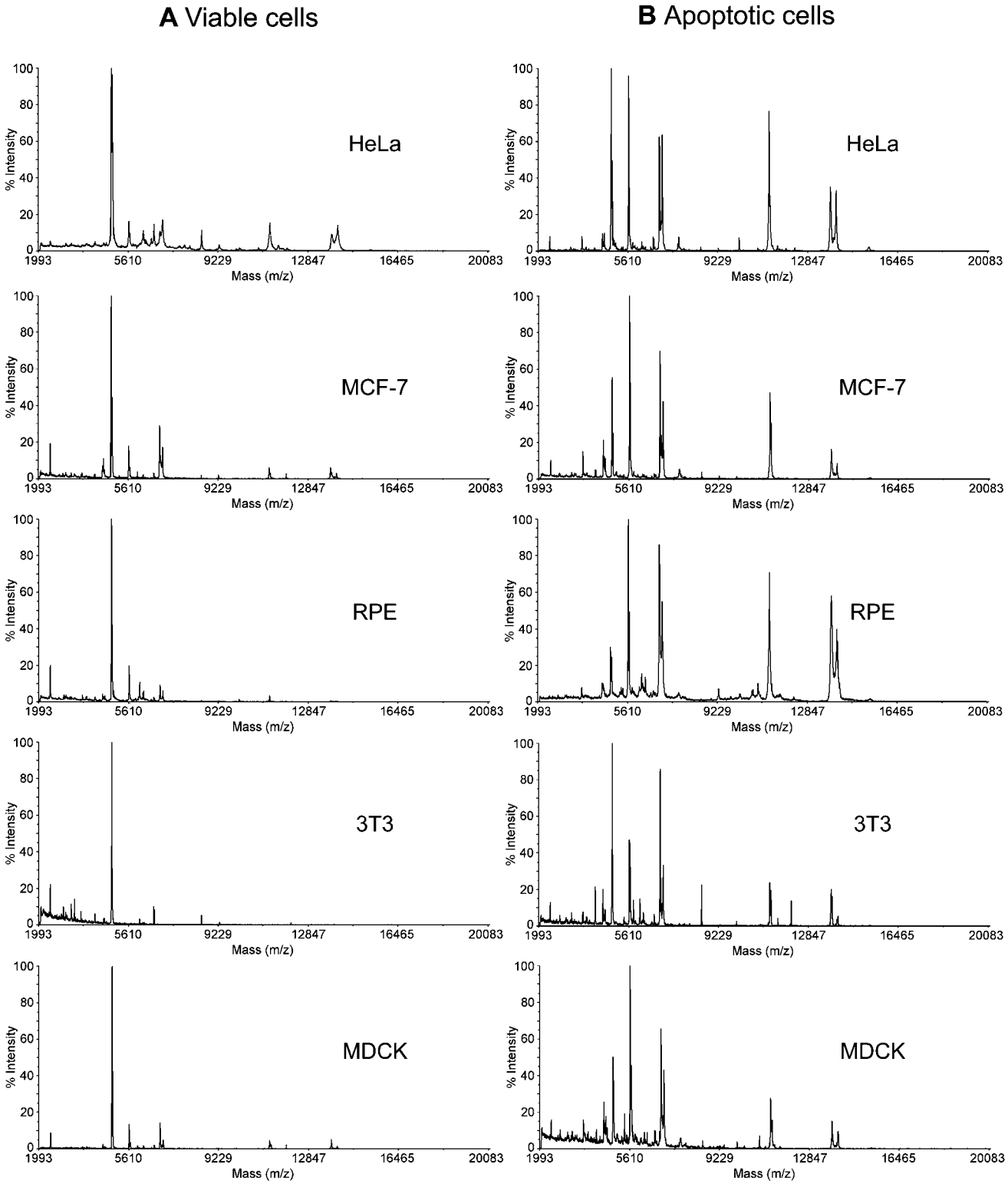

Fig. 9 Apoptosis detection of different mammalian cell lines by using intact cell MALDI MS analysis. (A) Viable cells and (B) apoptotic cells generated by andrographolide toxicity.

drug-induced apoptosis produced a highly distinct mass spectrum from both viable and necrotic cells. This indicates that ICM-MS can allow a fast distinction of apoptosis and necrosis.

ICM-MS-based apoptosis detection can be used in a wide diversity of mammalian cell lines

In order to test if this apoptosis detection method is also applicable to other mammalian cell and cancer types, we analysed the following cell lines: Madin-Darby Canine Kidney epithelial cells MDCK, retinal pigmented epithelial cells RPE, breast carcinoma MCF-7, and mouse embryonic fibroblast cells 3T3. The result shown in Fig. 9 demonstrates that ICM-MS could distinguish live and apoptotic cells for all the cell lines tested. Some peaks were commonly detected in all apoptotic cells, regardless of the cell lines used. We interpret this as an indication that these ICM-MS peaks can be used as the "biomarkers" specific to apoptosis, as they were detected in all apoptotic cells, independent of cancer types, histological origins and species of origin of these cell lines, and were undetectable in live or necrotic cells.

Conclusions

We have demonstrated in this study a novel application of ICM-MS, for the specific detection of apoptosis in mammalian cells. The MALDI mass spectra of living, necrotic and apoptotic cells were different significantly, allowing for a fast differentiation of apoptotic cells from living or necrotic cells. Furthermore, similar apoptosis-specific mass spectra can be generated from various cell lines and different apoptosis-causing conditions, indicating that this method can generally be applied for apoptosis detection. In addition, we showed that the intensities of some "signature" peaks in the mass spectra can be correlated with the percentage of apoptotic cells in the sample. ICM-MS can therefore be developed into a quantitative assay of apoptosis. At this stage, the detection of these peaks relies on the use of sophisticated MALDI-TOF/TOF equipment. In future, we believe the data reported in this study can be used as the basis for the development of relatively inexpensive devices that measure the intensities of representative apoptosis-specific ions. This method requires minimal sample processing, completely avoids the use of any additional dyes or probes, and uses less than 1000 cells per test. To our knowledge, this method is the fastest and simplest apoptosis-specific detection method to date, and is readily compatible with miniaturization and automation that are essential for high-throughput platforms.

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