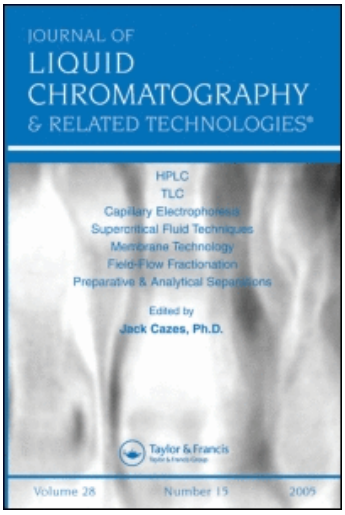


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# Profiling of Secreted Proteins from Human Ovarian Cancer Cell Lines by Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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# Profiling of Secreted Proteins from Human Ovarian Cancer Cell Lines by Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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## ABSTRACT

Surface-enhanced laser desorption ionization (SELDI) time-of-flight mass spectrometry (TOF MS) was used to profile six human ovarian cancer cell lines. Non-confluent cell cultures were exchanged into serum free medium and allowed to grow for either 24 or 48 hours, at which time the medium was collected and analyzed using a Ciphergen SELDI-TOF MS and a QSTAR Pulsar QqTOF mass spectrometer fitted with a SELDI source. The spectra showed the presence of several low molecular weight species, as

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well as differences and similarities in the proteins detected in the media of the six ovarian tumor cell lines. The same species were detected at 24 and 48 hours and reproducible changes in their relative abundances were observed.

*Key Words:* Biomarker detection; Protein profile; Cancer.

## INTRODUCTION

One of the predominant trends in biological sciences today is to devise methods that provide a systems level view of a cell, tissue, or organism. This systems level approach is fueled with information gleaned from the various genome projects that have sought to identify the entire DNA coding sequence of an organism. Following these projects has been the rapid development of methods to characterize the entire gene expression profile at the mRNA level (i.e., transcriptomics), such as serial analysis of gene expression and mRNA arrays. Concomitant with the development of transcriptomic technologies has been the development of methodologies to characterize these gene products at the protein level (i.e., proteomics). Unlike genomics and transcriptomics, which have a defined focus and goal, proteomics encompasses a multitude of different goals with potentially no endpoint. Among these goals, proteomics studies seek to identify proteins on a global level, quantitate their relative (and absolute?) abundances in different cell types, characterize post-translational modifications, determine localization, tertiary, and quaternary structure, and to make all of these measurements as comprehensive and high-throughput as possible.

The field of proteomics also encompasses the study of species not necessarily contained within the cell, per se. Secreted and shed proteins are known to mediate intercellular communication; hence they are responsible for eliciting varying biological responses. For example chemokines and cytokines are families of inducible, secreted proteins involved in coordinating and communicating a variety of immune responses.[1] In addition, there are a large number of secreted growth factors that bind to receptors on the cell surface, resulting in the activation of cell proliferation and/or differentiation.[2,3] Serum, which contains a plethora of cell-secreted and shed proteins, is one of the most actively studied sample type in proteomics today,[4] mainly for the purpose of identifying biomarkers related to specific disease states. While many proteins secreted within serum are readily identifiable using standard proteomics technologies, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS), it is presently not possible to directly discern their specific cell or tissue of origin. Most serum proteome investigations are discovery driven; in which a large number of proteins are identified with the hope that one or more assayable proteins will be found to uniquely be associated with a specific malady or disease state.

While such "cataloging" studies may be fruitful, an alternative hypothesis driven approach in which biomarkers are specifically monitored may provide a more rational alternative. The major difficulty in this approach is the initial identification of unique shed or secreted biomarkers present in situ to assay. Indeed, there has been little reported success from proteomic driven approaches in identifying disease-related secreted or shed proteins.

Sato et al.[5] used surface-enhanced laser desorption ionization (SELDI) time-of-flight mass spectrometry (TOF MS) to profile 16 different cancer cell lines using a hydrophobic chip. They observed that multiple cell lines shared several dominant peaks, though with varied intensities. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry combines retention, not elution, chromatography with TOF MS. The principle of this approach is very simple; proteins of interest are captured by adsorption, partition, electrostatic interaction, or affinity chromatography on a solid-phase protein chip surface. While SELDI provides a unique sample preparation platform, it is similar to matrix-assisted laser desorption/ionization (MALDI) MS, in that a laser is utilized for ionization of samples that have been co-crystallized with a matrix on a target surface. Unlike MALDI target surfaces, the SELDI protein chip chromatographic surfaces are uniquely designed to retain proteins from complex mixtures according to their specific properties. SELDI-TOF MS approach has been used to generate protein patterns for a variety of different applications.[6–10] One of the unique strengths of SELDI-TOF MS is its ability to analyze a variety of crude sample types, ranging from serum to urine, with minimal sample consumption and processing. A recent review of SELDI-TOF MS for diagnostic proteomics was recently published.[11]

The SELDI-TOF MS-based approach was used to profile shed and secreted proteins collected from six different human ovarian cancer cell lines cultured in serum free medium. The objective of this study is to see if distinctive patterns, as well as time-dependent changes in the protein patterns, are produced for each profiled ovarian cancer cell line in conditioned medium. The results illustrate that a SELDI-TOF MS-based approach is a potentially useful method to detect specific proteins present in the medium of cancer cells in culture. The methodology outlined may provide a more general means for the identification of diagnostic biomarkers for serum-based assays.

## EXPERIMENTAL

### Reagents and Instrumentation

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Surface-enhanced laser desorption ionization time-of-flight mass





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spectrometry analysis was carried out using the weak cation exchange ProteinChip Array (WCX-2) from Ciphergen Biosystems, Inc., (Palo Alto, CA). The SELDI-TOF MS Model PBS-II (Ciphergen Biosystems, Inc., Palo Alto, CA) and a QSTAR Pulsar QqTOF MS (Applied Biosystems, Framingham, MA) were used to acquire MS data. All buffers and reagents were prepared in double distilled water using a NANOPure Diamond water system (Barnstead International, Dubuque, Iowa).

## Cell Culture

Six ovarian and one glioblastoma human tumor cell lines from the NCI Developmental Therapeutics Program tumor cell line screening panel[11] were grown in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 5% fetal bovine serum (BioWhittaker, Walkersville, MD) and 2 mM *L*-glutamine (BioWhittaker, Walkersville, MD). The six ovarian cancer cell lines are: OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, IGROV-1, and SKOV-3. Media from the glioblastoma cancer cell line U251 was also profiled. Cells were grown to ~80% confluency in complete medium containing fetal bovine serum and then washed three times in serum-free medium. Cells were subsequently incubated in serum-free medium for 1 hour, washed two more times in serum-free medium, and incubated for either 24 or 48 hours. After incubation the conditioned medium was collected, centrifuged at 1500 rpm for 5 min to remove cellular debris, decanted into clean tubes, and stored at −80°C until assayed. Control flasks with only medium (no cells) were processed in parallel.

## Serum Free Media Application and Analysis

Aliquots (1.5 mL) of conditioned media obtained from each tumor cell line and stored at −50°C until analysis. Weak cation exchange (WCX-2) chips (Ciphergen Biosystems Inc., Palo Alto, CA), having eight similar chromatographic spots, were activated with 10 mM HCl, washed with double distilled water, and equilibrated with 50 mM sodium acetate, pH 4.5. One fifty micro-liter of the conditioned medium was added to the WCX-2 chip surfaces using a bioprocessor (Ciphergen Biosystems Inc., Palo Alto, CA) and incubated for 1.5 hrs at room temperature with gentle agitation. The chips were washed three times with 50 mM sodium acetate, pH 4.5, followed by a final double distilled water wash. The bioprocessor was subsequently removed and the chips air-dried. One micro-liter of a 20% α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile, 0.5% trifluoroacetic acid was added to each spot of the WCX-2 chip and air-dried. The chips were then analyzed using the following





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PBS-II SELDI-TOF MS automated settings: laser intensity 200, detector sensitivity 8, molecular mass range 0–20,000 Da, center mass 10,000, sample position 50, 130 shots per sample. Data was collected and analyzed using Ciphergen ProteinChip software version 3.0. Analyses were also performed on selected samples using an ABI QSTAR Pulsar QqTOF mass spectrometer fitted with a Ciphergen SELDI source. The QSTAR was operated with Analyst QS software and the SELDI interface with the ProteinChip Controller Interface 1000 version 1.20 Beta software. All data were collected with the following general settings: laser intensity 45, laser repetition 20 Hz, $m/z$ range from 800–12,000, 50 multi-channel averaged scans per spectrum. QSTAR data was processed with the Bioanalyst software suite from Applied Biosystems.

## RESULTS AND DISCUSSION

The detection of disease protein biomarkers in biological specimens such as serum, urine, and tissue biopsies is complicated by the presence of unrelated proteins and other interfering metabolites. Serum has garnered the most interest for diagnostic and therapeutic response assay development due largely to its ease of collection and potential utility for rapid and sensitive monitoring of disease state changes within the body because of its intimate role in inter-organ communication. From an analytical standpoint, however, the identification of potential disease biomarkers would be greatly simplified if one could first identify potential disease biomarkers from primary cell culture conducted in serum free medium. This approach is simplified by the virtue that any shed or secreted proteins identified arise solely from the cell in culture. The ability to identify and assay shed and secreted proteins in culture could be an effective first step in defining disease biomarkers in a rational and targeted fashion. In the present study, we demonstrate a simple and effective scheme for profiling shed and secreted proteins from six human ovarian cancer cell lines into serum free medium by SELDI-TOF MS using Ciphergen PBS-II instrument and a selected set by SELDI-QqTOF MS. The PBS-II instrument is a low resolution MS system while the QqTOF instrument is a high resolution one.

### Preliminary Data

In a feasibility experiment, two different cancer cell lines, OVCAR-3, a human ovarian cancer cell line and U251, a human glioblastoma cancer cell line, were selected to evaluate the effectiveness of SELDI-TOF MS for profiling proteins found in the medium recovered from human tumor cell lines. The results shown in Fig. 1 indicates multiple low molecular weight





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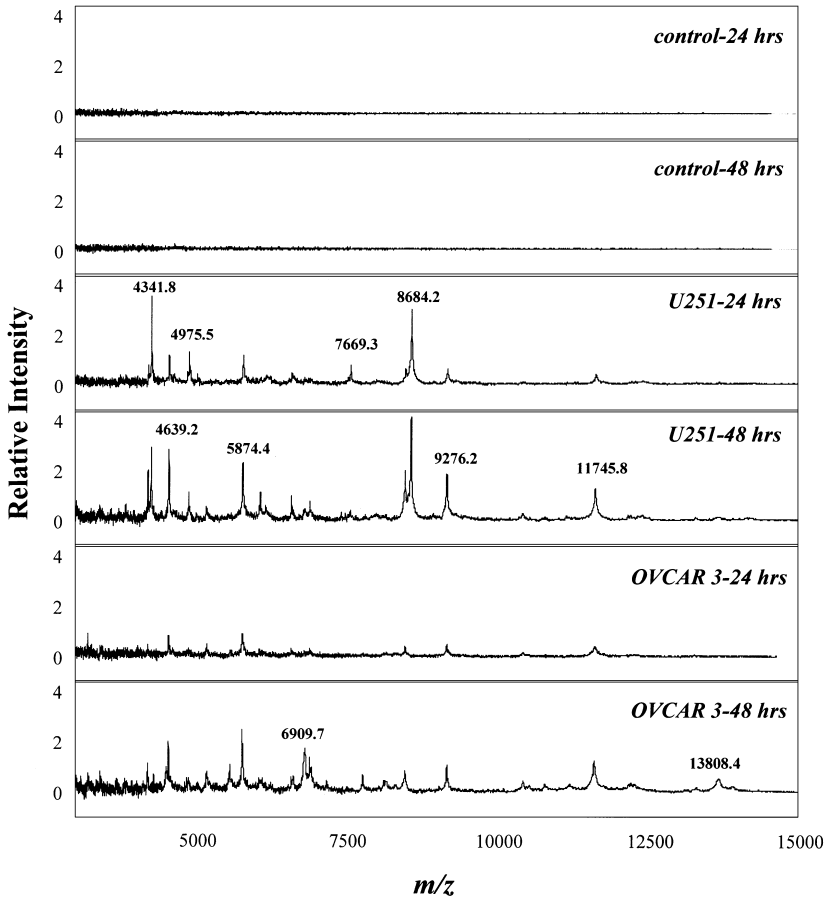


***Figure 1.*** Differential protein profiles of secretions of two human tumor cell lines incubated for 24 and 48 hours in serum free controlled media, using WCX2 ProteinChip Array and SELDI-TOF MS.

species detected within the media of these cells. Control samples of media that had not been introduced to cells did not show the presence of these species, suggesting that the detected peaks represent molecules secreted or shed by the cells. The media was analyzed in duplicate using a weak cation exchange (WCX2) chip. There are similarities and differences between the proteins observed from the two different human cancer cell lines. For example, species present at $m/z$ 4639, 5874, and 11,746 are similar in both cell lines, while the species at $m/z$ 4342 and 8684 are absent from OVCAR 3. The species observed





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at $m/z$ 4342/8684, and $m/z$ 5874/11,764 are the same species (doubly/singly charged ions). Based on these preliminary results, we decided to profile the media of six human ovarian cancer cell lines and to see what significant similarities and differences, if any, can be detected.

## Ovarian Cancer Cell Line Profiles

The serum free media of the six human ovarian cancer cell lines were each prepared as outlined in Experimental and analyzed using SELDI-TOF MS. The profiles for the 24 and 48 hr incubation times along with the control sample are shown in Figs. 2 and 3, respectively. The results show that the relative intensities of the peaks are higher at the 48 hr incubation period than the 24 hr incubation period. Careful examination of the data reveals many similarities and differences in the proteins expressed by the six cell lines. For example, OVCAR 4, OVCAR 5, and OVCAR 8 gave the lowest number of species. In actuality these three cell lines gave one significant protein; $m/z$ 5862/11,710 (OVCAR 4), $m/z$ 4565/9127 (OVCAR 5), and 4569/9132 (OVCAR 8), and few minor species. The $m/z$ 4565/9127 and $m/z$ 4569/9132 are within the resolution of the PBSII and can be considered the same protein. There are distinct differences in the proteins observed within the media of the six different cell lines. For example, the $m/z$ 5872/11,704 is present in OVCAR 3, IGROV 1, and SKOV 3 but not in the other three cell lines. It is clear that the profile of OVCAR-3 is different from those of the other five cell lines in that more peaks are observed. Analysis of the data of OVCAR 3, Figs. 2 and 3, shows multiple singly and doubly charged ions, 4632/9257 and 5265/10,522. Note that these $m/z$ values are within the resolution of the PBSII instrument. In SKOV 3, Fig. 3, the low intensity peaks at $m/z$ 8916, 9132, 9258 are mirrored by their 2+ versions at $m/z$ 4460, 4568, and 4632. The peaks at 3906–3908 in OVCAR 3, OVCAR 4, and SKOV 3, Fig. 2, could be the +3 ion of mass 11,706.

## Instrument-to-Instrument Reproducibility

To evaluate the instrumental reproducibility of the technique we selected four of the ovarian cancer cell line secretions and analyzed them using an API QSTAR Pulsar QqTOF mass spectrometer fitted with a Ciphergen SELDI source. Figures 4 and 5 show the observed profiles for the media collected from cells incubated for 24 and 48 hr in serum free medium, respectively. Comparison of Figs. 2 and 4, and Figs. 3 and 5 show that the reproducibility of the profiles obtained by the two instruments for the four cell line secretions are comparable in mass and relative intensity. While the spectra acquired from





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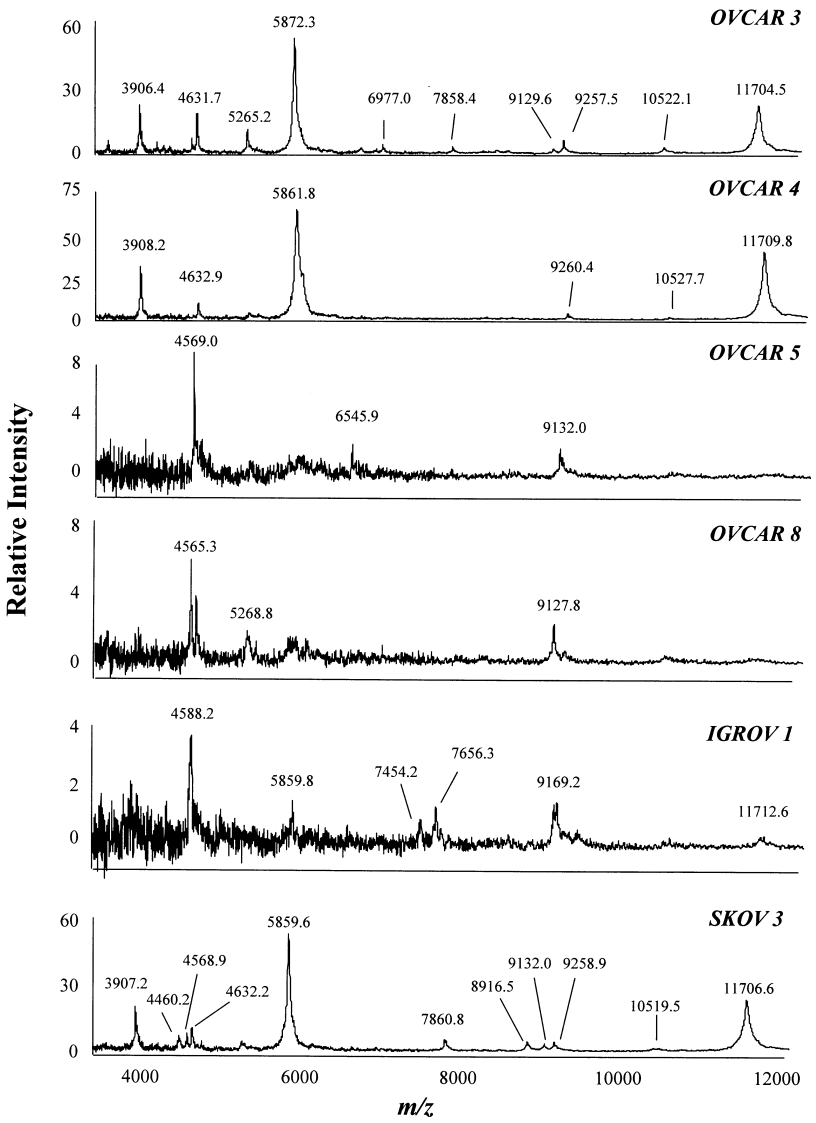


***Figure 2.*** Differential protein profiles of secretions of six human ovarian tumor cell lines incubated for 24 hours in serum free controlled media, using WCX2 ProteinChip Array and SELDI-TOF MS.





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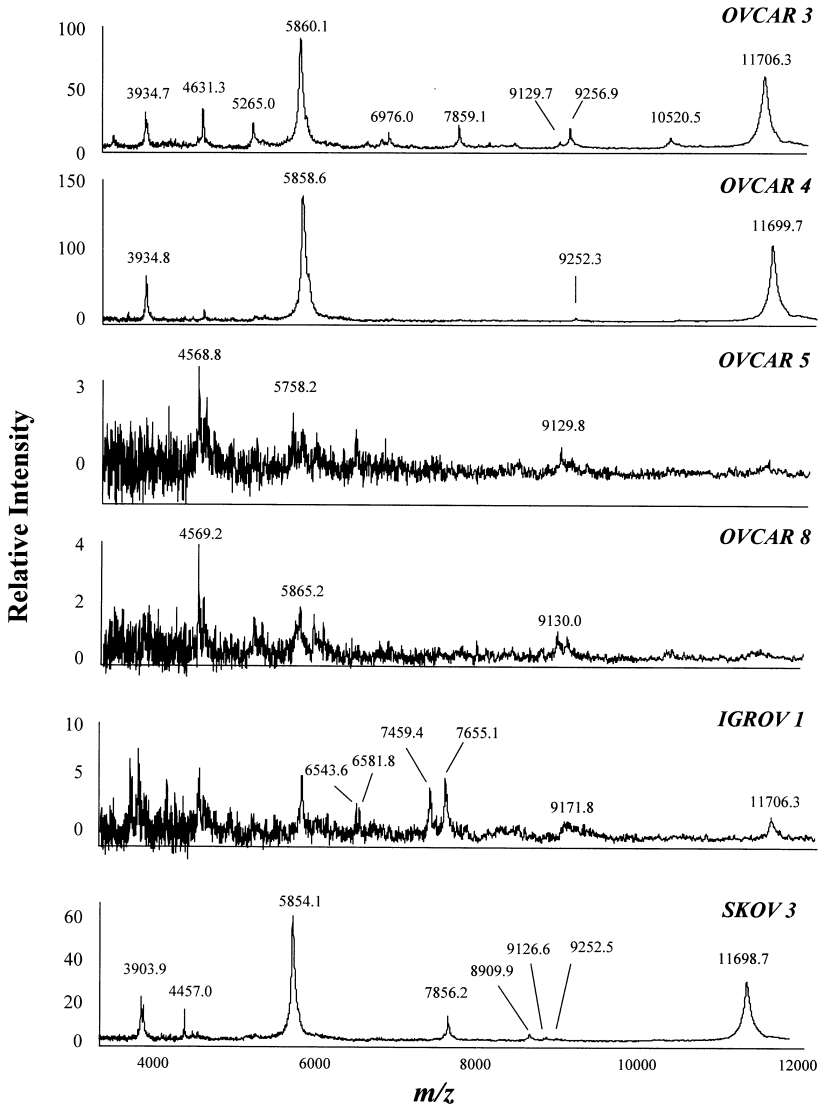


*Figure 3.* Differential protein profiles of secretions of six human ovarian tumor cell lines incubated for 48 hours in serum free controlled media, using WCX2 ProteinChip Array and SELDI-TOF MS.





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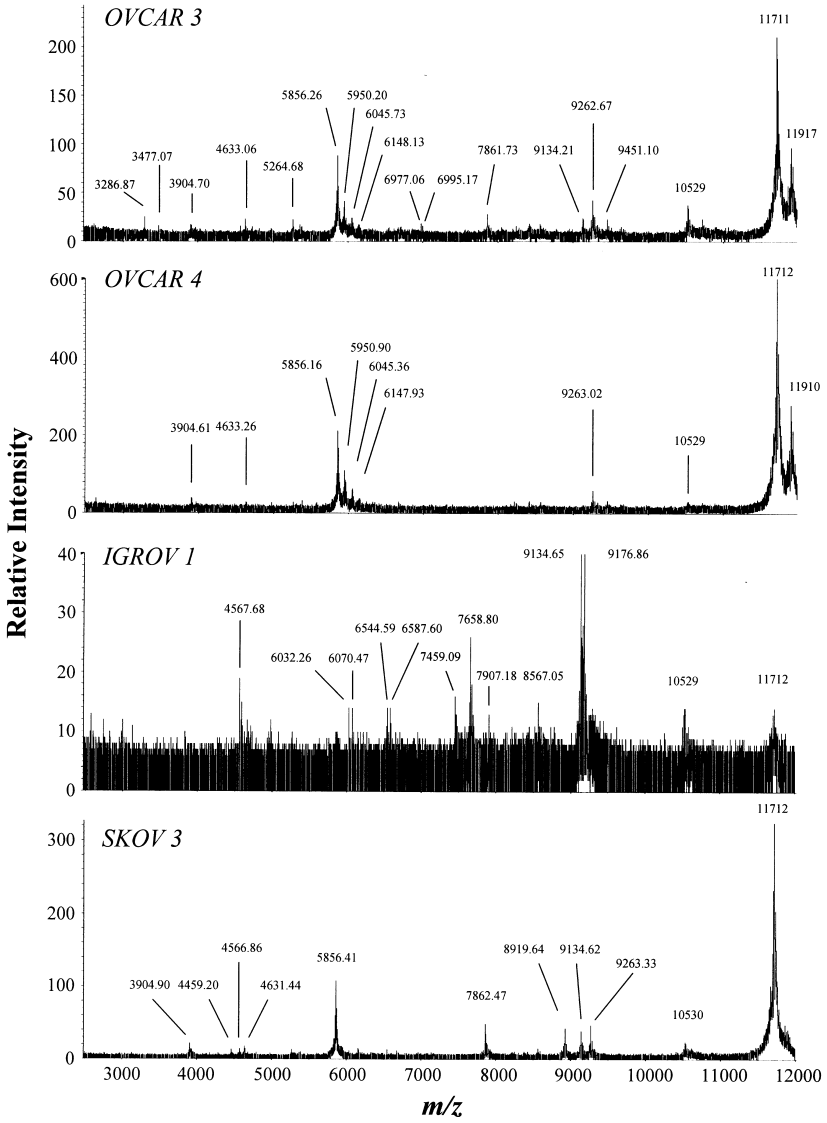


***Figure 4.*** Differential protein profiles of secretions of four human ovarian tumor cell lines incubated for 24 hours in serum free controlled media, using WCX2 ProteinChip Array and SELDI-QqTOF MS.





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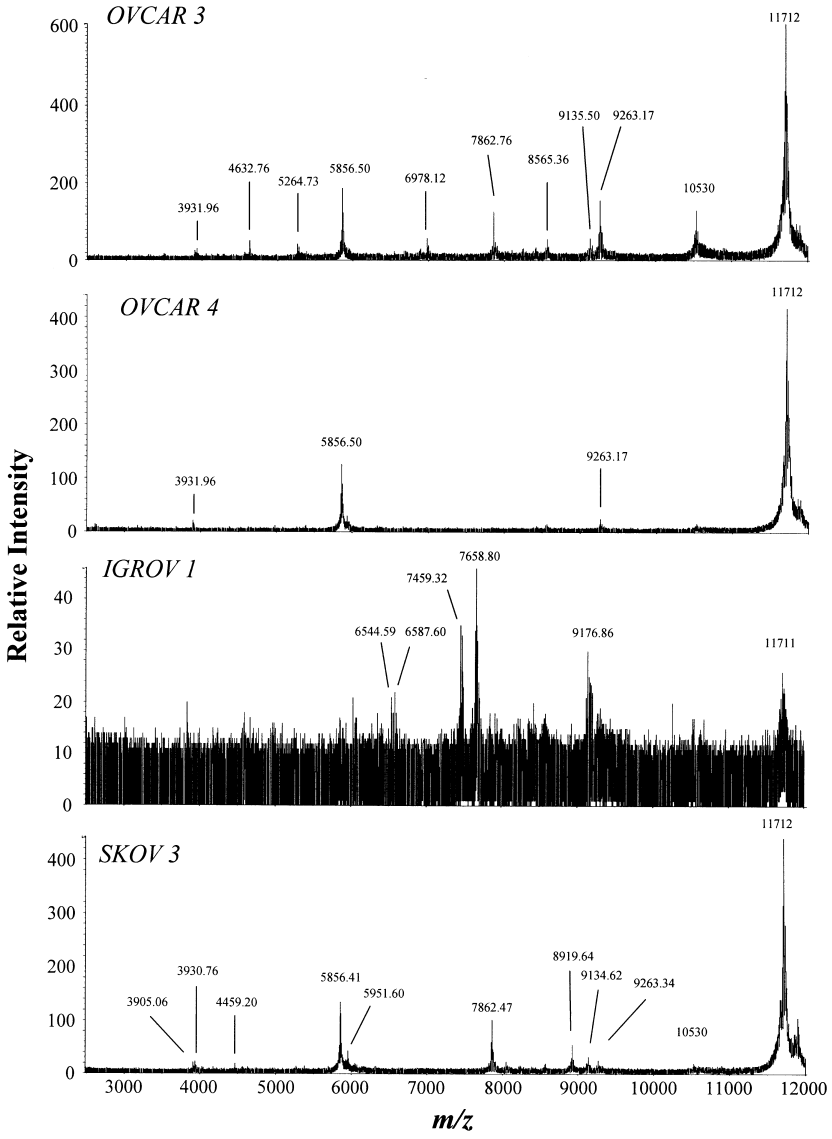


***Figure 5.*** Differential protein profiles of secretions of four human ovarian tumor cell lines incubated for 48 hours in serum free controlled media, using WCX2 ProteinChip Array and SELDI-QqTOF MS.





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both instruments are qualitatively similar, the higher resolution afforded by the Qq-TOF MS is readily apparent (Figs. 4 and 5). This increased resolution allows species with similar $m/z$ values that are unresolved by the PBS-II TOF MS to be distinctly observed in the Qq-TOF mass spectrum. Indeed, simulations demonstrate the ability of the Qq-TOF MS (routine resolution ~8000) to completely resolve species differing in $m/z$ of only 0.375 (e.g., at $m/z$ 3000), whereas complete resolution of species with the PBS-II TOF MS (routine resolution ~150) is only possible for species that differ by $m/z$ of 20 (data not shown).

A unique capability afforded by the use of a QqTOF mass spectrometer in SELDI-based screening is the potential to identify species retained on the chromatographic surface. Identification of analytes is possible by conducting tandem MS where specific precursor ions can be accumulated and subjected to collision-induced dissociation with an inert gas in the quadrupole section of the mass spectrometer; measurement of the product ions in the TOF section then provides a CID spectrum of the selected precursor. The resulting CID spectrum often contains sufficient sequence specific information resulting from the predictable low-energy fragmentation of peptides to permit protein database searching using conventional bioinformatic software. Conventional QqTOF mass spectrometers, however, are capable of conducting CID of ions up to $m/z$ ~3000 routinely. Since many of the ions of interest observed in the present experiments are above this upper $m/z$ limit, efforts are under way in our laboratory to establish on-chip digestion protocols to generate lower mass ions that are more amenable to conventional CID analysis.

## CONCLUSIONS

Surface-enhanced laser desorption ionization time-of-flight mass spectrometry has proven to be a useful and efficient technique for the profiling of proteins found in serum-free media of cultured cells. The profiling of six human ovarian tumor cell lines indicates the presence of similarities and differences in the proteins expressed by each cell line. Also, the levels of intensities were different among the six cell lines, which indicates that some proteins are up-regulated in some cell lines while down-regulated in others. The comparison of an ovarian cancer and glioblastoma cell line showed common, as well as unique proteins, were observed within the media of the different cell types. Overall, the technique is efficient and provides a useful method to detect proteins within the media of cells grown in culture.





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## ABBREVIATIONS

| | |
|---|---|
| SELDI | surface-enhanced laser desorption ionization |
| MS | mass spectrometry |
| TOF | time-of-flight |
| QqTOF | hybrid quadrupole time-of-flight |

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