Novel possibilities in the study of the salivary proteomic profile using SELDI-TOF/MS technology (Review)

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Abstract. There is currently an increasing interest in exploring human saliva to identify salivary diagnostic and prognostic biomarkers, since the collection of saliva is rapid, non-invasive and stress-free. Diagnostic tests on saliva are common and cost-effective, particularly for patients who need to monitor their hormone levels or the effectiveness of undergoing therapies. Furthermore, salivary diagnostics is ideal for surveillance studies and in situations where fast results and inexpensive technologies are required. The most important constituents of saliva are proteins, the expression levels of which may be modified due to variations of the cellular conditions. Therefore, the different profile of proteins detected in saliva, including their absence, presence or altered levels, is a potential biomarker of certain physiological and/or pathological conditions. A promising novel approach to study saliva is the global analysis of salivary proteins using proteomic techniques. In the present study, surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry (SELDI-TOF/MS), one of the most recent proteomic tools for the identification of novel biomarkers, is reviewed. In addition, the possible use of this technique in salivary proteomic studies is discussed, since SELDI technology combines the precision of matrix-assisted laser desorption/ionization-TOF/MS proteomic analysis and the high-throughput nature of protein array analysis.

Contents

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1. Introduction

Human saliva is a complex biological fluid that contains electrolytes, enzymes such as amylase and carbonic anhydrase, proteins (including glycoproteins such as mucins and proline-rich glycoproteins) and peptides such as statherin, cystatins, histatins and proline-rich proteins (1).

Human saliva participates in important biological functions in the mouth, and is essential for mastication and digestion (2). Furthermore, it protects the oral health by means of lysozymes, cystatins, immunoglobulins and histatins present on the saliva, which prevent the growth of microorganisms in the oral cavity (3).

The protein content of whole saliva derives from the three major paired salivary glands, which comprise the contralateral major (parotid, submandibular and sublingual) and minor salivary glands (4). The protein composition of whole saliva depends on the circadian rhythm, diet, age, gender and physiological status of the individual (5).

The study of the salivary proteome may aid to identify all the proteins present in the saliva, and may detect alterations in protein levels that occur in specific physiological and possibly pathological conditions (6).

The classical proteomic approach for the study of proteins is based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to compare and identify differences in the protein expression patterns between diseased and normal samples (7,8). Following 2D-PAGE fractionation and staining, the proteins of interest are removed, digested (proteolytically or chemically) and identified by matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) (7,8). However, this approach presents several limitations, since it is time consuming, expensive and requires difficult and laborious tasks (7,8).

Proteomic analysis has recently benefited from the introduction of surface-enhanced laser desorption/ionization (SELDI)-TOF. SELDI-TOF/MS requires the use of chips, and constitutes a simple and high-throughput technique to rapidly identify a large number of differentially expressed peptides and proteins in saliva samples, particularly proteins of low molecular weight (<10 kDa) that are difficult to detect effectively with other methods (9). In addition, since SELDI-TOF/MS requires minimal quantities of a biological

sample to generate an accurate protein profile in a relatively short period of time, it is suitable for mapping protein profiles in samples derived from healthy and diseased individuals in order to identify differential protein expression patterns between the two groups (10). The results of a SELDI-TOF/MS analysis are usually presented as a list of proteins that are upregulated or downregulated in healthy versus diseased subjects (11).

SELDI technology is also useful for the protein profiling of a variety of complex biological tissues and fluids, including serum, blood, plasma, intestinal fluid, urine, cell lysates and cellular secretion products (12). The working principle and preparation of the protein chip in SELDI are the same for every biological fluid analyzed, whereas the pretreatment differs according to each biological matrix (12).

SELDI-TOF/MS has revealed numerous novel biomarkers for different types of cancer, including ovarian (13), breast (14), pancreatic (15) and prostate cancer (16). In addition, this technology has been used for obtaining proteomic patterns for the diagnosis of bladder cancer from urine samples (17), and for the diagnosis of cervical cancer from captured cell lysates obtained by laser capture microdissection of tissue samples (18). SELDI-TOF/MS has also been used for identifying and monitoring biomarkers of Alzheimer's disease in cerebrospinal fluid (19), and for analyzing saliva samples of patients with Sjögren's syndrome (SS) (20). Therefore, SELDI-TOF/MS technology may aid the identification of potential prognostic or predictive markers for oral squamous cell carcinoma (OSCC).

2. SELDI technique

The ProteinChip® (Bio-Rad Laboratories, Inc., Hercules, CA, USA) biology system used by SELDI-TOF/MS enables the retention of proteins on a solid-phase chromatographic surface prior to their subsequent ionization and detection by TOF/MS (21). The chromatographic surfaces in the various ProteinChip® arrays commercially available are uniquely designed to retain proteins from a complex sample mixture according to general or specific properties such as hydrophobicity and charge (21). Each spot in the array contains a chemically treated surface, including anionic, cationic, hydrophobic, hydrophilic and metal surfaces (21). In the case of ionic exchange ProteinChip® arrays, the operating mechanism is the reversible binding of charged molecules to the surface of the chip (21). For analysis of the expression of multiple samples simultaneously, 12x8-spot chips assembled in 96-well bioprocessors have been developed (21). The fraction of the proteome bound to the chips may be subsequently analyzed by MS on the same chip, resulting in a pattern of proteins characterized by their mass-to-charge ratio (m/z) (22).

Currently, four types of chips exist: i) CM10 (Bio-Rad Laboratories, Inc.), which is a weak cation exchanger whose surface has been negatively charged, thus enabling the binding of proteins that are positively charged at a certain pH; ii) Q10 (Bio-Rad Laboratories, Inc.), a strong anion exchanger with quaternary ammonium groups positively charged on its surface, which binds proteins that are negatively charged at alkaline conditions; iii) IMAC30 (Bio-Rad Laboratories, Inc.), which binds proteins on its surface by metal affinity; and iv) H50 (Bio-Rad Laboratories, Inc.), which captures proteins through hydrophobic or reverse-phase interaction (21).

The SELDI-TOF/MS instruments consist of three major components, namely the ProteinChip® array, the reader and the software. The ProteinChip® reader is an LDI-TOF/MS unit equipped with a laser source. Upon activation of the laser, the sample becomes irradiated, and is then subjected to DI, which liberates gaseous ions from the ProteinChip® array (11). Next, these gaseous ions enter the TOF/MS region of the instrument, which measures the m/z of each protein based on its velocity through an ion chamber (11). Subsequent signal processing is accomplished by a high-speed analog-to-digital converter linked to a personal computer, whereby the detected proteins are displayed as a series of peaks (11). The output generated from the TOF/MS analysis of the samples is a trace representing the relative abundance of the detected proteins vs. their molecular weight (11). Thus, the end result of a SELDI-TOF/MS analysis is a list of the molecular weights of the detected proteins (11). Next, the proteomic profiles of all the samples are analyzed by ProteinChip® Data Manager™ software version 3.5, in order to identify mass peaks (also known as clusters) that are differentially expressed between two different groups (for example, healthy vs. pathological samples).

3. SELDI technique and saliva

Sample collection and storage. The saliva samples for a SELDI-TOF/MS analysis must be produced between 9:00 a.m. and 10:00 a.m. by prior mouth rinsing with water (23). Donors must abstain from eating, drinking, smoking or using oral hygiene products for ≥2 h prior to collection (24). The saliva must be spitted directly into a clean 15 ml conical tube, and a protease inhibitor cocktail must then be added to the samples prior to further processing (24). Subsequently, the saliva samples should be centrifuged for 50 min at 13,000 x g, and the supernatants divided into aliquots and frozen for storage at -80˚C.

Reagents. Each chip must be pretreated with a specific binding buffer, depending on its surface. Thus, 100 mM sodium acetate pH 4 must be used for CM10, while 100 mM Tris-HCl pH 8.8 and pH 7.4 must be used for Q10 and IMAC30, respectively. The latter chip requires preliminary loading with 0.1 M Cu^{2+}, followed by neutralization with 0.1 M sodium acetate (pH 4), prior to the addition of 100 mM Tris-HCl (pH 7.4) as binding buffer. Milli-Q® water (Merck Millipore, Darmstadt, Germany) may be used as solvent.

Similarly, the sample must be diluted in the binding buffer that is specific for the particular ProteinChip® tested, or mixed (2:3 v/v) with a denaturing buffer solution such as DB3 (9 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 100 mM dithiothreitol; Sigma-Aldrich, St. Louis, MO, USA) (3).

For all types of chips, 1 μl of matrix must be used, which consists of 50% sinapinic acid (SPA) (Ciphergen Biosystems, Inc., Fremont, CA, USA) solubilized in 50% acetonitrile (ACN)/0.5% trifluoroacetic acid (TFA) (Sigma-Aldrich).

SELDI-TOF/MS analysis. Each chip must be pretreated at room temperature with vigorous agitation with its specific binding buffer, according to the manufacturer's protocol (Bio-Rad Laboratories, Inc.). Next, the sample must be incubated on the chip in the presence of the corresponding

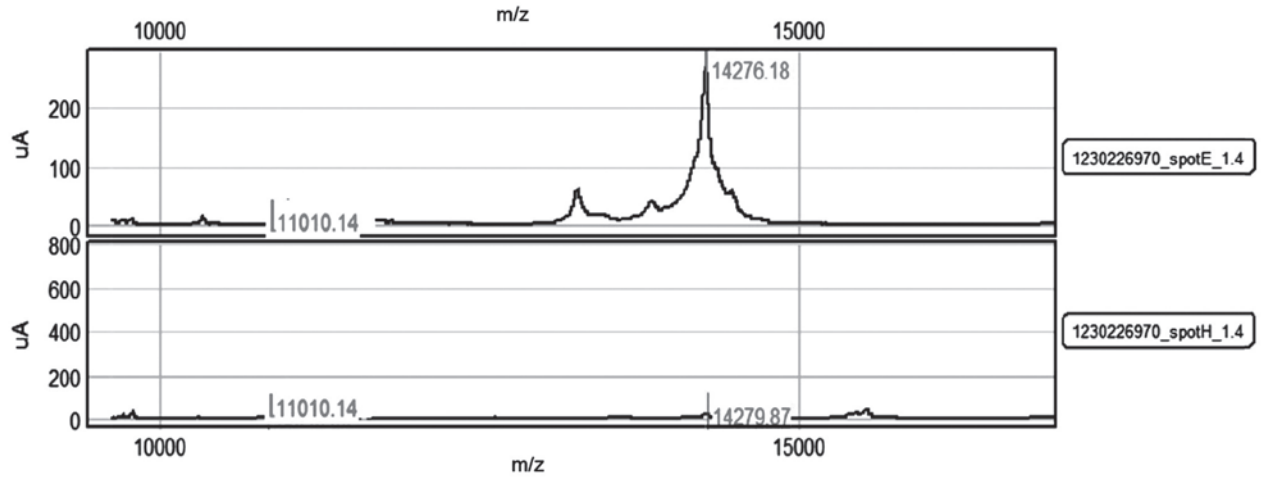

Figure 1. Surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry analysis of the salivary proteome profile of a healthy subject (upregulated peak, bottom panel), compared with that of a patient with oral squamous cell carcinoma (downregulated peak, top panel).

binding buffer for 30 min with continuous agitation (3). Upon removing the sample from the wells, the chip must then be washed three times with 150 μl washing buffer (0.1 M sodium acetate [pH 4] for CM10; 0.1 M Tris HCl [pH 8.8] for Q10; and 0.1 M Tris-HCl [pH 7.4] for IMAC30; Sigma-Aldrich) while agitating, in order to remove the majority of the salts present, which otherwise interfere with the subsequent MS analysis (6).

A final wash should be performed with 200 μl deionized Milli-Q® water, prior to air-dry the chip for 20 min (3). Next, a saturated solution of SPA must be prepared in 50% ACN/0.5% TFA, and diluted by 50% in this solvent, in order to generate the matrix (3). Subsequently, 1 μl of this matrix must be applied to each spot, and allowed to dry (25). The above procedure should be repeated twice (25).

The matrix, also known as energy-absorbing matrix (EAM), is a solution that crystallizes and promotes the ionization of the proteins present in the sample once these have been dried on the chip. Next, the chip must be placed in the SELDI ProteinChip® reader for MS analysis (25).

Unless otherwise specified, it should be possible to read all the chips by an automated protocol using the same instrument conditions, namely, laser energy, 6,000 nJ; matrix attenuation, 2,500 Da; focus mass, 10,000 Da; sample rate, 800 MHz; coverage of the surface area of the spot, 25%; and acquired mass range, 2,500-25,000 m/z) (24). All the experiments must be performed in duplicate.

The proteomic profiles of all the samples must be subsequently analyzed with ProteinChip® Data Manager™ software version 3.5, in order to identify differentially expressed mass peaks (clusters) among groups of different samples (Fig. 1). These differences must be verified by Mann-Whitney U test, whereby $P<0.05$ is considered to indicate a statistically significant difference.

The analysis software employs classification and regression tree, a multivariate analysis where the samples are divided into two groups, termed the training and the testing set, respectively. The intensity in μA units of all the mass peaks identified in the training set is then used by Biomarker Pattern Software (BPS)® (version 5.0; Ciphergen Biosystems, Inc.) to build a classification tree.

The intensity of the peaks selected from the BPS® analysis is regarded as a 'root node'. Furthermore, it is possible to develop an optimal threshold value to improve the stratification of the two groups, based on the intensity of the mass peak measured in each sample.

4. Discussion

The interest on saliva as diagnostic material has markedly increased in recent years, since the information associated with various substances present in this biological fluid may aid the surveillance of general and oral health, and contribute to the understanding of the pathogenesis of certain diseases (26-30).

Proteins are the most important constituents of saliva. Therefore, the full analysis and identification of the human salivary proteome constitutes the first step towards the identification of novel biomolecules associated with the salivary state of health or disease (26-30).

As diagnostic material, saliva presents certain disadvantages, including the influence exerted by the method of collection and the degree of stimulation of the salivary flow on the composition of the saliva samples (31,32). In addition, saliva contains analytes at concentrations 1,000 times lower than in plasma (33). Thus, sensitive detection systems are required when using saliva as diagnostic material (9).

SELDI-TOF/MS was originated by combining MALDI-TOF/MS with surface chromatography, and has acquired an important role in proteomic analysis in recent years (9). SELDI-TOF/MS is a highly efficient technique that is particularly suitable for the study of small peptides and proteins, since its fmol sensitivity complements the results of 2D-PAGE analysis (9).

Previous studies have employed the SELDI technique to search for potential biomarkers in the salivary proteome (3,6,34-48) (Table I).

In 2004, Baskova *et al* (34) performed the first study on saliva using the SELDI technique, and detected 45 individual compounds of molecular masses ranging from 1,964 to 66.5 kDa in salivary gland secretions of the medicinal leech *Hirudo medicinalis*.

Table I. Possible applications of surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry for clinical research on saliva.

Reference	Disease	Proteins as putative biomarkers in saliva
34	Salivary gland secretion of *Hirudo medicinalis*	Identification of 45 compounds of 1,964-66.5 kDa
35	Breast cancer	Peaks at 18, 113, 170, 228 and 287 m/z
20	SS	Peaks at 11.8, 12.0, 14.3, 80.6 and 83.7 kDa were increased, while peaks at 17.3, 25.4 and 35.4 kDa were reduced in SS
6	HC	Improvement of technology
38	HC	Improvement of technology
37	Allo-HCT and GVHD	Peaks at 11,760, 11,691, 11,946, 5,864, 15,149, 18,711, 17,556, 11,041, 94,737, 11,524, 38,788, 13,386, 80,197 and 27,885 m/z were increased post-HCT
36	HC	Salivary proteome associated with satiety and body mass index
3	HC	Improvement of technology
39	HC	Improvement of technology
40	OSCC and OLK	Identification of peaks at 5,797, 2,902, 3,883 and 4,951 m/z in OSCC, and peaks at 5,818, 4,617 and 3,884 m/z in OLK
41	OSCC	Peak at 1,400 m/z identified as truncated cystatin SA-I
42	OSCC and OLK	Peaks at 3,738 and 11,366 m/z were differentially expressed in OSCC vs. OLK
43	Fibromyalgia	Detection of RhoGDI2 and calgranulin A and C
47	Orthodontics	Modifications of the saliva proteome
44	Periodontitis in obese patients	Peaks at 66,000, 15,200 and 15,900 m/z, and at 3,492, 3,448, 3,492 and at 5,378 m/z, were increased in obese patients with and without periodontitis, respectively
45	DS II	Peaks at 12,679 Da (IMAC30), 13,264 Da (CM10) and 3,822 Da (Q10) were increased, while peaks at 35,125 Da (CM10) and 12,954 Da (Q10) were reduced in DS II
48	HC	Influence of sodium chloride and sucrose on the presence of proteins of 2-20 kDa in whole saliva following stimulation with different tastants
46	PSS	Differential expression of peaks at 7,149, 7,192, 13,517, 13,714, 16,547 and 24,059 m/z in patients with PSS vs. HC

m/z, mass-to-charge ratio; SS, Sjögren's syndrome; HC, healthy controls; allo, allogeneic; HCT, hematopoietic stem cell transplantation; GVDH, graft-versus-host disease; OSCC, oral squamous cell carcinoma; OLK, oral leukoplakia; RhoGDI2, Rho GDP-dissociation inhibitor 2; GDP, guanosine diphosphate; DS II, Denture stomatitis type II; PSS, primary Sjögren's syndrome.

In 2006, Ryu *et al* (20) used parotid saliva to identify the most significant salivary biomarkers in SS using SELDI-TOF/MS. The results revealed eight peaks of molecular weights in the range of 10-200 kDa whose levels were >2-fold altered in the SS group, compared with the non-SS group ($P<0.005$). Thus, the levels of the peaks at 11.8, 12.0, 14.3, 80.6 and 83.7 kDa increased, while those at 17.3, 25.4 and 35.4 kDa reduced in SS samples (22).

Using SELDI, Streckfus *et al* (35) identified the presence of proteins at 18, 113, 170, 228 and 287 m/z in the saliva samples of women with breast cancer, and hypothesized that these proteins may be efficient salivary biomarkers of breast cancer.

In 2007, Harthoorn *et al* (36) studied the human saliva proteome in regards to satiety and body mass index using SELDI-TOF/MS, since this technique provides a valuable and non-invasive way of profiling, which enables the characterisation of novel and differentially expressed peptides and proteins that may be used as biomarkers of satiety and overweight.

Also in that year, Imanguli *et al* (37) evaluated by SELDI-TOF/MS the alterations that occur in salivary proteins following allogeneic hematopoietic stem cell transplantation (allo-HCT) in 41 patients undergoing allo-HCT. The authors detected significant increases and reductions in the levels of multiple salivary proteins.

The studies by Schipper *et al* (6,38) in 2007 demonstrated the possibility of using the SELDI technique to interrogate the salivary proteome. In addition, the authors reported the procedures required for the correct treatment of saliva samples, while Papale *et al* (3,24) reported a protocol that improved the quality and reproducibility of SELDI-TOF/MS analysis.

In 2008, Esser *et al* (39) used SELDI to study the salivary proteome in healthy subjects, revealing the presence of 218 proteins, 84 of which were also present in plasma. Based on a comparison with previous proteomics studies on whole saliva, the authors also identified 83 novel salivary proteins.

In 2009, Sun and Ping (40) studied the salivary proteome by SELDI, and identified novel potential salivary biomarkers that may aid the early diagnosis of oral cancer and forecast the transformation from oral leukoplakia (OLK) to oral cancer and metastasis. The study identified a differentiated pattern

between patients with OSCC and healthy subjects consisting of four biomarker peaks of 5,797, 2,902, 3,883 and 4,951 m/z, with a sensitivity of 88.24% and a specificity of 93.33%. The study also identified a differentiated pattern between patients with OSCC and patients with OLK, which consisted of three biomarker peaks of 5,818, 4,617 and 3,884 m/z, with 100.00% sensitivity and specificity. In addition, the results of the study revealed a differentiated pattern between patients with OSCC and those with local metastatic oral cancer, which consisted of two biomarker peaks of 55,809 and 5,383 m/z, with a sensitivity of 94.12% and a specificity of 85.71%.

In 2010, Shintani *et al* (41) also studied the salivary proteome of patients with OSCC using SELDI analysis, and detected 26 proteins with significantly different expression levels in patients with OSCC, compared with healthy controls. In particular, the authors identified the presence of a truncated cystatin SA-I, characterized by the deletion of three amino acids from its N-terminus, in the saliva of patients with OSCC.

These results are supported by the findings of He *et al* (42), who in 2011 identified by SELDI-TOF/MS technology differential proteomic patterns in serum, saliva and tissue samples of patients with OSCC, compared with patients with OLK.

Recently, several SELDI-TOF/MS studies on the saliva proteome have identified potential biomarkers for fibromyalgia (43), periodontitis in obese patients (44), denture stomatitis (45), primary SS (46) and post-transplant complications, including infections and graft-versus-host disease (37).

Previous studies on the saliva proteome using SELDI technology have evaluated the modifications affecting the oral mucosa and bones in patients undergoing orthodontic treatment (47). In addition, a previous study employed SELDI-TOF/MS to investigate the influence of sodium chloride and sucrose solutions on the presence of proteins of 2-20 kDa in whole saliva (48). The results revealed that oral stimulation with different tastants affects the composition of salivary proteins in a protein- and stimuli-dependent way, regardless of the glands of origin (48).

In conclusion, SELDI technology combines the precision of MALDI-TOF/MS proteomic analysis and the high-throughput nature of protein array analysis (9). Furthermore, the analysis of saliva may enable the screening of a large number of patients, since the collection of saliva samples is easy, non-invasive, inexpensive and reduces the risk of cross-infections, contrarily to that of blood and serum samples (37). Therefore, the analysis of the salivary proteome by SELDI-TOF/MS may aid to identify prognostic or diagnostic biomarkers, complement the results of 2D-PAGE analysis and confirm the findings of MALDI-TOF/MS analysis.

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