Video Article

Sampling Human Indigenous Saliva Peptidome Using a Lollipop-Like Ultrafiltration Probe: Simplify and Enhance Peptide Detection for Clinical Mass Spectrometry

Wenhong Zhu¹, Richard L. Gallo^{2,3}, Chun-Ming Huang^{2,3,4}¹Sanford-Burnham Medical Research Institute²Division of Dermatology, University of California, San Diego³VA San Diego Healthcare Center⁴Moores Cancer Center, University of California, San DiegoCorrespondence to: Chun-Ming Huang at chunming@ucsd.eduURL: <http://www.jove.com/video/4108/>

DOI: 10.3791/4108

Keywords: Medicine, Issue 66, Molecular Biology, Genetics, Sampling, Saliva, Peptidome, Ultrafiltration, Mass spectrometry,

Date Published: 8/7/2012

Citation: Zhu, W., Gallo, R.L., Huang, C.M. Sampling Human Indigenous Saliva Peptidome Using a Lollipop-Like Ultrafiltration Probe: Simplify and Enhance Peptide Detection for Clinical Mass Spectrometry. J. Vis. Exp. (66), e4108, DOI : 10.3791/4108 (2012).

Abstract

Although human saliva proteome and peptidome have been revealed¹⁻² they were majorly identified from tryptic digests of saliva proteins. Identification of indigenous peptidome of human saliva without prior digestion with exogenous enzymes becomes imperative, since native peptides in human saliva provide potential values for diagnosing disease, predicting disease progression, and monitoring therapeutic efficacy. Appropriate sampling is a critical step for enhancement of identification of human indigenous saliva peptidome. Traditional methods of sampling human saliva involving centrifugation to remove debris³⁻⁴ may be too time-consuming to be applicable for clinical use. Furthermore, debris removal by centrifugation may be unable to clean most of the infected pathogens and remove the high abundance proteins that often hinder the identification of low abundance peptidome.

Conventional proteomic approaches that primarily utilize two-dimensional gel electrophoresis (2-DE) gels in conjugation with in-gel digestion are capable of identifying many saliva proteins⁵⁻⁶. However, this approach is generally not sufficiently sensitive to detect low abundance peptides/proteins. Liquid chromatography-Mass spectrometry (LC-MS) based proteomics is an alternative that can identify proteins without prior 2-DE separation. Although this approach provides higher sensitivity, it generally needs prior sample pre-fractionation⁷ and pre-digestion with trypsin, which makes it difficult for clinical use.

To circumvent the hindrance in mass spectrometry due to sample preparation, we have developed a technique called capillary ultrafiltration (CUF) probes⁸⁻¹¹. Data from our laboratory demonstrated that the CUF probes are capable of capturing proteins *in vivo* from various microenvironments in animals in a dynamic and minimally invasive manner⁸⁻¹¹. No centrifugation is needed since a negative pressure is created by simply syringe withdrawing during sample collection. The CUF probes combined with LC-MS have successfully identified tryptic-digested proteins⁸⁻¹¹. In this study, we upgraded the ultrafiltration sampling technique by creating a lollipop-like ultrafiltration (LLUF) probe that can easily fit in the human oral cavity. The direct analysis by LC-MS without trypsin digestion showed that human saliva indigenously contains many peptide fragments derived from various proteins. Sampling saliva with LLUF probes avoided centrifugation but effectively removed many larger and high abundance proteins. Our mass spectrometric results illustrated that many low abundance peptides became detectable after filtering out larger proteins with LLUF probes. Detection of low abundance saliva peptides was independent of multiple-step sample separation with chromatography. For clinical application, the LLUF probes incorporated with LC-MS could potentially be used in the future to monitor disease progression from saliva.

Video Link

The video component of this article can be found at <http://www.jove.com/video/4108/>

Protocol

1. Creation of LLUF Probes

1. The polyethersulfone membranes (2 cm²) were sealed with triangle polypropylene paddles (University of California, San Diego) by gluing membranes with epoxy on the borders of paddles. A negatively charged polyethersulfone membrane with a molecular weight cut-off (MWCO) at 30 kDa was used.
2. A teflon fluorinated ethylene propylene tube (inner diameter/outer diameter, 0.35/0.50 cm) was attached to a cylinder exit of a triangle polypropylene paddle so the LLUF probe can be connected to a 20 ml syringe.
3. After soaking the polyethersulfone membrane into human saliva in a culture dish (50 mm diameter), negative pressure was created by withdrawing a syringe. The syringe with negative pressure drove the ultrafiltration process for sampling proteins from saliva.
4. The probes were sterilized with 70% alcohol overnight prior to use. To demonstrate proper sealing, we positioned LLUF probes into a solution containing blue dextran (50 mg/ml) with an average molecular mass of 2,000 kDa for 2 h. The absence of blue dextran in the collected samples indicated that no leaks developed during probe fabrication and sampling.

2. Saliva Collection

1. Whole saliva was collected from three healthy volunteers (two males and one female between the ages 20 and 40) taking no medications, with no overt signs of gingivitis or cavities⁶.
2. After rinsing the mouth with water, the whole saliva samples were collected by spitting, without chemical stimulation, into an ice-cooled vessel.
3. All samples were pooled and kept on ice during the collection procedure.
4. Immediately after the collection, saliva (200 μ l) was applied for the sampling with LLUF probes. The sampling was performed at a 4 °C room.
5. Saliva proteins (1.0 μ g/ μ l) with or without LLUF probe collection were directly subjected to nano LC-mass spectrometry analysis without tryptic digestion. Protein concentrations were determined using a Bio-Rad Protein Assay¹².

3. NanoLC-LTQ MS Analysis

1. The un-digested saliva samples (5 μ l) were directly loaded to the trap column of the Eksigent NanoLC system by the autosampler, using 100% buffer A (2% acetonitrile/0.1% formic acid). The NanoLC was on-line coupled with a Finnigan LTQ mass spectrometer.
2. After sample loading and washing, the valve was switched and a 500 nl/min linear gradient was delivered to the trap and the separation column (10 cm in length, 100 μ m i.d., in-house packed with Synergi 4 μ m C18). The gradient was from 0 to 50% buffer B (80% acetonitrile/0.1% formic acid) in 45 min.
3. The nanoLC-LTQ MS instruments were operated in the data dependent mode by Xcalibur. MS/MS spectra of the four strongest MS ions above an intensity of 1×10^5 were collected with dynamic exclusion enabled and the collision energy set at 35%.
4. Each sample was run twice by the NanoLC-LTQ MS system. Samples from three separate preparations were used. Those peptides detectable in three separate samples were listed in **Supplemental Tables 1 and 2**. Representative of NanoLC-LTQ MS spectra was illustrated in **Figure 3**.

4. Data Analysis and Protein Database Searching

1. Each RAW file was converted to a mzXML file using Readw.exe.
2. The mzXML file was input into a SEQUEST Sorcerer 2 system and searched against a human database generated from the corresponding National Center for Biotechnology Information (NCBI) protein database using non-enzyme specificity. The mass tolerance of precursor ion was set at 1.5 Da. A molecular mass of 16 Da was added to methionine for differential search to account for oxidation.
3. After SEQUEST searching, the results were automatically filtered, validated and displayed by PeptideProphet and ProteinProphet [Institute for Systems Biology (ISB)]. PeptideProphet estimates a comprehensive probability (P) score that a peptide assignment is "correct" vs. "incorrect" on the bases of it's SEQUEST scores (Xcorr, Δ Cn, Sp, RSp) and additional information of each peptide sequence identified. ProteinProphet computed a probability score from 0 to 1 for each protein on the basis of peptides assigned to MS/MS spectra.
4. To minimize false positive identifications we used stringent filter criteria. First, the minimum P score cutoff is set at 0.8 for any accepted peptide to assure very low error (much less than 3%) and reasonably good sensitivity. Second, all peptides with >0.8 P score must have high cross-correlation (Xcorr) scores at the same time: 1.9, 2.2, and 3.0 for +1, +2, and +3 charge.

5. Removal of Oral Bacteria with LLUF Probes

1. To determine the capability of LLUF probes to remove the oral bacteria, saliva before and after LLUF probe collection (Section 3) was spread on agar plates for bacterial detection.
2. Aerobic bacteria were grown on an antibiotic-free Lauria-Bertani (LB) agar plate at 37 °C for one day.
3. Anaerobic bacteria were grown on an antibiotic-free Brucella broth agar plate (BD, Sparks, MD) under anaerobic conditions using Gas-Pak (BD Biosciences, San Jose, CA) at 37 °C for one day.

6. Representative Results

1. Fabrication of LLUF probes and sampling saliva in an imitated oral environment

If sampling saliva can be performed as sucking a lollipop, the procedure will avoid the degradation of spitted saliva in collection devices¹³⁻¹⁴. Importantly, it will also become possible to monitor patients dynamically and locally from oral cavities. In addition, if sample preparation using saliva could be simplified, clinicians would easily facilitate the procedure to expedite their decision on the next clinical operation. Mass spectrometry is one of the most sensitive techniques to detect and even sequence proteins in a very short period of time. However, complicated procedures for sample preparation have hampered using this technique in clinic. Furthermore, it is known that higher abundance proteins or proteins with high molecular weights in clinical samples (e.g. amylase in saliva) mask low abundance proteins in mass spectrometric analysis¹⁵⁻¹⁶. To overcome the hurdles mentioned above, we developed a lollipop-like ultrafiltration device named LLUF probes (**Figure 1**). A negatively charged polyethersulfone membrane with a MWCO at 30 kDa (**Figure 1A, a**) was glued to a polypropylene paddle (**Figure 1A, b**). It was positioned in front of the LLUF probe with the intention of filtering out larger proteins in saliva. To mimic the human oral environment (**Figure 1A, g**), a sponge (**Figure 1A, e**) was soaked into saliva in a culture dish (**Figure 1A, f**). After fully withdrawing the syringe (**Figure 1A, d**), filtered saliva started moving along a connected tube (**Figure 1A, c**) and was collected.

2. Identification of indigenous saliva peptidome by NanoLC-LTQ MS

Comparing LC chromatograms, we found distinct chromatograms of saliva before and after LLUF sampling (**Figure 2**), indicating that there are different protein compositions in saliva after LLUF sampling. To determine the protein compositions, we employed NanoLC-LTQ mass spectrometry that is known to be able to promptly sequence peptides from a multiple protein mixture. More importantly, to simplify sample preparation for clinical purposes, whole saliva without chemical or enzymatic digestion was applied for NanoLC-LTQ MS analysis. Unexpectedly, 131 peptides were identified in undigested saliva (**Supplemental Table 1**). These peptides are fragments derived from various proline rich proteins, actin, alpha amylase, alpha 1 globin, beta globin, histain 1, keratin 1, mucin 7, polymeric immunoglobulin receptor, satherin, and S100A9. Twenty-six unique peptides were identified in saliva after filtering with LLUF probes (**Supplemental Table 2**). These peptides are fragments mainly derived from various proline-rich proteins. Peptides derived from proteins such as the polymeric immunoglobulin receptor (83.24 kDa) and alpha amylase, were undetectable, demonstrating the capability of LLUF probes in removal of larger and abundant proteins. A

MS/MS spectrum of the PFIAIHAEESKL peptide corresponding to an internal peptide of alpha-amylase is illustrated in **Figure 3A**. Most intriguingly, after removing larger proteins, 18 of 26 sequenced peptides became detectable in the LLUF probe-sampled saliva (**Table 1**). These 18 peptides were derived from proline-rich proteins or hypothetical proteins that ended with a proline (P)- glutamine (Q) (-PQ), -SR, -SP or -PP C-terminus. **Figure 3B** showed a MS/MS spectrum of the PQGPPQQGGHPRPP peptide that was detected in the LLUF probe-sampled saliva. The peptide could be derived from proline-rich protein HaeIII subfamily 1 and 2 (**Table 1**).

Figure 1. Compositions of LLUF probes and sampling of whole saliva from mimic human oral cavity. *Panel A:* (a) a semi-permeable polyethersulfone with a MWCO of 30 kDa; (b) a polypropylene paddle; (c) a teflon fluorinated ethylene propylene tube; (d) a 20 ml syringe. *Panel B:* a sponge (e) (a mimicked tongue) was soaked into a culture dish (f) containing human saliva to create an artificial human oral cavity (g). The resulting negative pressure created by fully withdrawing a syringe drives the collected fluid to move along a connected tube (arrow) and towards a created space (arrowhead) within syringe. Bar: 2.0 cm.

Figure 2. Differential LC/MS/MS chromatograms of saliva before and after LLUF sampling. Proteins (1.0 µg/µl) in human whole saliva were filtered without or with a LLUF probe. Proteins without tryptic digestion were directly subjected to NanoLC- LTQ MS that was conjugated with an Eksigent Nano LC system as described in Material and Methods. The base-peak chromatograms (with 44-min retention times) of saliva before (A) and after (B) LLUF sampling were illustrated.

Figure 3. Detection of saliva peptidome by NanoLC- LTQ MS sequencing. Saliva proteins before (A) and after (B) sampling with LLUF probes were analyzed by NanoLC- LTQ MS as described in Experimental Procedures. Saliva peptidome derived from natural human saliva without tryptic digestion were demonstrated in **Supplemental Tables 1 and 2**. A peptide (PFIHAEAESKL) derived from alpha-amylase was exclusively detected in a saliva sample without collection with LLUF probes (A), illustrating that the capability of LLUF probes in removing large proteins. Many peptides with -PQ, -SR, -SP or -PP C-termini were solely in samples after ultrafiltration LLUF probes. One (PQGPPQGGHPRPP) of peptides derived from various proline rich proteins were shown (B). MS/MS spectra with characteristic "y" and "b" series ions confirmed the identities of both peptides.

Figure 4. Removal of oral bacteria using during LLUF probes. A LLUF probe was constructed as described in Materials and methods. The probe was positioned into the human saliva within an imitated oral environment (Figure 1). The syringe at the end of LLUF probe was withdrawn to create a negative pressure that drove the ultrafiltration process for saliva sampling. During sampling, whole saliva crossed selectively through the polyethersulfone membrane and accumulated inside a syringe. Whole saliva before the sampling with a LLUF probe served as a control. Saliva (10 μ l) before and after LLUF probe sampling was spread side-by-side on an antibiotic-free LB agar plate at 37 $^{\circ}$ C for one day. **Panel A:** Saliva with (+LLUF) and without (+LLUF) LLUF probe sampling was spread side-by-side on an antibiotic-free LB agar plate at 37 $^{\circ}$ C for one day. **Panel B:** Saliva with and without LLUF probe sampling was spread on an antibiotic-free Brucella broth agar plate under anaerobic conditions using Gas-Pak (BD Biosciences, San Jose, CA) at 37 $^{\circ}$ C for one day. Bacteria did not grow on agar plates spread with LLUF probe-sampled saliva, demonstrating the capability of LLUF probes in eliminating aerobic as well as anaerobic oral bacteria. Bar: 1.0 cm.

	Peptide sequence/ Measured peptide mass	Accession number	Name
1	AGNPQGSPQGGNKPQ	gi 41349484	Proline-rich protein BstNI subfamily 1 isoform 2 precursor

	GPPPPPGKPQ		
	2485.3		
		gi 41349482	Proline-rich protein BstNI subfamily 1 isoform 1 precursor
		gi 60301553	Proline-rich protein BstNI subfamily 2
2	GGHQGGPPPPPGKPQ	gi 4826944	Proline-rich protein HaeIII subfamily 2
	1576.9		
		gi 9945310	Proline-rich protein HaeIII subfamily 1
3	GPPAGGNPQQPQAPPA	gi 37537692	Proline-rich protein BstNI subfamily 4 precursor
	GKPQGPPPPQGGRRP		
	3126.2		
4	GPPPPGGNPQQPLPPPAGKPQ	gi 113423660	PREDICTED: hypothetical protein
	2028.3		
5	GPPPPGKPQGPPQGDKSRSP	gi 113423262	PREDICTED: hypothetical protein isoform 5
	2077.8		
		gi 41349482	Proline-rich protein BstNI subfamily 1 isoform 1 precursor
		gi 60301553	Proline-rich protein BstNI subfamily 2
		gi 113423663	PREDICTED: hypothetical protein
		gi 41349484	Proline-rich protein BstNI subfamily 1 isoform 2 precursor
		gi 41349486	Proline-rich protein BstNI subfamily 1 isoform 3 precursor
6	GPPPPPGKPQGPPPPQ	gi 9945310	Proline-rich protein HaeIII subfamily 1
	GGRPQGPPQGQSPQ		
	2918.5		
7	GPPQEGNKQRPPPPGRPQ	gi 113423660	PREDICTED: hypothetical protein
	2131.3		
8	GPPQGGNKQGGPPPPGKPQ	gi 113423663	PREDICTED: hypothetical protein
	2030		
		gi 41349482	Proline-rich protein BstNI subfamily 1 isoform 1 precursor
		gi 113423262	PREDICTED: hypothetical protein isoform 5
		gi 60301553	Proline-rich protein BstNI subfamily 2
9	GPPQGGRPQGPPQGQSPQ	gi 4826944	Proline-rich protein HaeIII subfamily 2
	1866.6		
10	GPPQGGGHPPPPQGRPQ	gi 9945310	Proline-rich protein HaeIII subfamily 1
	1713.8		
		gi 4826944	Proline-rich protein HaeIII subfamily 2
11	GPPQGGGHQQGPPPPPGKPQ	gi 9945310	Proline-rich protein HaeIII subfamily 1
	2083.1		
		gi 4826944	Proline-rich protein HaeIII subfamily 2
12	GRPQGPPQGGGHQQGP	gi 4826944	Proline-rich protein HaeIII subfamily 2
	PPPPPGKPQ		
	2512.6		
		gi 9945310	Proline-rich protein HaeIII subfamily 1
13	NKPQGPPPPGKPQGPP	gi 113423262	PREDICTED: hypothetical protein isoform 5
	PQGGSKSRSSR		
	2720.2		
		gi 113423663	PREDICTED: hypothetical protein
14	PQGPPQGGHPRPP	gi 9945310	Proline-rich protein HaeIII subfamily 1

	1450.1		
15	QGRPQGPPQGGHPRPP	gi 4826944	Proline-rich protein HaeIII subfamily 2
		gi 4826944	Proline-rich protein HaeIII subfamily 2
	1791.1		
		gi 9945310	Proline-rich protein HaeIII subfamily 1
16	SPPGKPQGPPQ	gi 113423262	PREDICTED: hypothetical protein isoform 5
	1186.9		
		gi 41349482	Proline-rich protein BstNI subfamily 1 isoform 1 precursor
		gi 60301553	Proline-rich protein BstNI subfamily 2
		gi 113423663	PREDICTED: hypothetical protein
		gi 41349484	Proline-rich protein BstNI subfamily 1 isoform 2 precursor
		gi 41349486	Proline-rich protein BstNI subfamily 1 isoform 3 precursor
17	SPPGKPQGPPQGGNQ	gi 113423663	PREDICTED: hypothetical protein
	PQGPPPPGKPPQ		
	2720.3		
		gi 41349482	Proline-rich protein BstNI subfamily 1 isoform 1 precursor
		gi 113423262	PREDICTED: hypothetical protein isoform 5
		gi 60301553	Proline-rich protein BstNI subfamily 2
18	SPPGKPQGPPQEGNKPQ	gi 37537692	Proline-rich protein BstNI subfamily 4 precursor
	1870.9		

Table 1. Peptides were exclusively detectable in LLUF-collected samples.

Supplemental Table 1. [Click here to view supplemental Table 1.](#)

Supplemental Table 2. [Click here to view supplemental Table 2.](#)

Discussion

We have found that many peptide fragments exist in human undigested saliva. These peptide fragments are derivatives from various forms of proline-rich proteins, actin, alpha amylase, alpha 1 globin, beta globin, histain 1, keratin 1, mucin 7, polymeric immunoglobulin receptor, satherin, S100A9. There could be many factors contributing to the production of peptides with undetermined cleavage sites. For example, some peptide fragments may be naturally present in human whole saliva. Many peptides with -PQ C-termini were identified (**Table 1 and Supplemental Tables 1 and 2**). Proline-rich proteins can be classified as acidic, basic or glycosylated proteins and are encoded by six genes clustered in a single region¹⁷. More than thirty different proline-rich proteins result from allelic variation, differential RNA splicing, proteolytic processing, and post-translational modifications¹⁷. After secretion, the acidic proline-rich proteins are rapidly attached to tooth surfaces and degraded into potential innate immunity peptides by dental plaque proteolysis¹⁸⁻¹⁹. Both gram-negative and gram-positive bacteria express a variety of glycosidases and proteases¹⁸. It has been reported that acidic proline-rich proteins can be degraded into potential innate-immunity-like peptides by oral *Streptococcus* and *Actinomyces* species¹⁸. The Glutamine (Gln) - Glycine (Gly) cleavage is biologically critical with respect to the bacterial degradation of salivary acidic proline rich proteins and production of a bacteria-binding -PQ C-terminus^{18, 20-22}. The binding of -PQ termini of proline-rich proteins to bacteria was confirmed by an *in vitro* experiment using a synthetic RGRPQ pentapeptide¹⁸. In agreement with our data, S. J. Fisher's group was able to identify several peptides with -PQ C-termini in the undigested human parotid saliva²³, suggesting several peptides with -PQ C-termini may indigenously exist in human whole saliva. Human saliva contains multiple peptides with -PQ C-termini which may function as innate-immunity-like peptides^{18, 19}. When oral bacteria are present, proline-rich proteins may instantly break down to various fragments with -PQ C-termini in order to efficiently kill the bacteria.

Another reason for peptide fragments existing in undigested saliva are that some of the endogenous proteases present in whole saliva may remain active during sample preparation²⁴⁻²⁵. The cleavage occurring in these proteins by endogenous proteases in the oral cavity likely continued before mass spectrometry analysis. For example, mucin could be cleaved by salivary protease down to a smaller form that is more competent in bacterial clearance²⁶. It is also possible that proteases from oral bacteria may cleave the oral proteins before or after saliva collection. It has been documented that several saliva proteins can be chopped by proteases from oral *Streptococcus* and *Actinomyces* species²⁷⁻²⁹.

The semi-permeable membrane is a key component of LLUF probes. The membrane is necessary to selectively remove larger substances including proteins, oral bacteria and debris. LLUF acts as a selective barrier that permits the passage of certain components and rejects other components within an artificial oral cavity. The semi-permeable membrane allows small molecule to pass through the membrane while excluding macromolecules. Recent data from our laboratory showed that LLUF probes can effectively remove both aerobic and anaerobic oral bacteria (**Figure 4**). Saliva samples before and after collection with LLUF probes were spread onto antibiotics-free agar plates and incubated under aerobic and anaerobic conditions. Bacteria did not grow when agar plates were spread with LLUF probe-sampled saliva, demonstrating the capability of LLUF probes in removing aerobic and anaerobic oral bacteria. We searched all spectra with a human database that did not include protein databases of human oral microbes. Identification of proteome/peptidome of oral microbes will become possible if a comprehensive protein database with all oral microbes can be established. An organic (polyethersulfone) membrane was used to fabricate the LLUF probes. Although the membrane was known to have a 30 kDa MWCO, the sampling performance also depended on other factors such as interaction of saliva proteins to negative charges on the membrane surface³⁰. The changes in pH values and temperature as well as protein complexity in oral cavity also influence the sampling performance. Our data demonstrated that 18 peptides were exclusively present in LLUF probe-sampled saliva (**Table**

1). The peptides ended with -PQ, -SR, -SP or -PP C-termini are mainly derived from proline-rich proteins. It has been reported that the net negatively charged proline-rich proteins displayed a strong adsorption to a negatively charged surface³¹.

In summary, in an attempt to detect specific groups of proteins in the future, semi-permeable membranes in front of LLUF probes may be altered using various pore sizes and materials that have different surface charges. For example, nanofiltration membranes with pore sizes range from 0.05 microns to 1 nanometer can separate viruses from saliva samples³². LLUF probes also can be applied for monitoring the concentration of various substances such as glucose and lactate in saliva samples. Although ultrafiltration membranes have made use of protein separation via centrifugal ultrafiltration³³, they have rarely been used to collect proteins via application of negative pressure across a semi-permeable membrane. Linking the LLUF probes with advanced mass spectrometer such as fourier transform ion cyclotron resonance (FT-ICR) may allow the identification of the intact saliva proteins³⁴⁻³⁵. Notably, on-line analysis of the dynamic patterns of saliva proteome and peptidome may be vital for the clinical application of LLUF probes. After collection with LLUF probes, many peptide fragments derived from proline-rich proteins were identified from undigested whole saliva. Proline-rich proteins can interact with oral bacteria to influence the development of dental caries³⁶ and may be significant in protecting mucosal surfaces from viral infection³⁷. Furthermore, it has been documented that the expression of proline-rich proteins was altered in the rheumatoid arthritis patients³⁸. These studies strongly support that proline-rich proteins collected by LLUF probes may serve as biomarkers for monitoring various human diseases.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by National Institutes of Health Grants (R01-AI067395-01, R21-R022754-01, and R21-158002-01). We thank C. Niemeyer for critical reading of the manuscript.

References

- Denny, P., *et al.* The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome Res.* **7**, 1994-2006 (2008).
- Hu, S., Loo, J.A., & Wong, D.T. Human saliva proteome analysis. *Ann. N.Y. Acad. Sci.* **1098**, 323-329 (2007).
- Ng, D.P., Koh, D., Choo, S.G., Ng, V., & Fu, Q. Effect of storage conditions on the extraction of PCR-quality genomic DNA from saliva. *Clin. Chim. Acta.* **343**, 191-194 (2004).
- Wade, S.E. An oral-diffusion-sink device for extended sampling of multiple steroid hormones from saliva. *Clin. Chem.* **38**, 1878-1882 (1992).
- Hu, S., *et al.* Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics.* **5**, 1714-1728 (2005).
- Huang, C.M. Comparative proteomic analysis of human whole saliva. *Arch. Oral Biol.* **49**, 951-962 (2004).
- Guerrier, L., Lomas, L., & Boschetti, E. A simplified monobuffer multidimensional chromatography for high-throughput proteome fractionation. *J Chromatogr. A.* **1073**, 25-33 (2005).
- Huang, C.M., Wang, C.C., Kawai, M., Barnes, S., & Elmets, C.A. Surfactant sodium lauryl sulfate enhances skin vaccination: molecular characterization via a novel technique using ultrafiltration capillaries and mass spectrometric proteomics. *Mol. Cell Proteomics.* **5**, 523-532 (2006).
- Huang, C.M., Wang, C.C., Kawai, M., Barnes, S., & Elmets, C.A. *In vivo* protein sampling using capillary ultrafiltration semi-permeable hollow fiber and protein identification via mass spectrometry-based proteomics. *J. Chromatogr. A.* **1109**, 144-151 (2006).
- Huang, C.M., Wang, C.C., Barnes, S., & Elmets, C.A. *In vivo* detection of secreted proteins from wounded skin using capillary ultrafiltration probes and mass spectrometric proteomics. *Proteomics.* **6**, 5805-5814 (2006).
- Huang, C.M., *et al.* Mass spectrometric proteomics profiles of *in vivo* tumor secretomes: capillary ultrafiltration sampling of regressive tumor masses. *Proteomics.* **6**, 6107-6116 (2006).
- Ahmed, N., *et al.* An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum. *Proteomics.* **3**, 1980-1987 (2003).
- Michishige, F., *et al.* Effect of saliva collection method on the concentration of protein components in saliva. *J. Med. Invest.* **53**, 140-146 (2006).
- Kruger, C., Breunig, U., Biskupek-Sigwart, J., & Dorr, H.G. Problems with salivary 17-hydroxyprogesterone determinations using the Salivette device. *Eur. J. Clin. Chem. Clin. Biochem.* **34**, 926-929 (1996).
- Luque-Garcia, J.L. & Neubert, T.A. Sample preparation for serum/plasma profiling and biomarker identification by mass spectrometry. *J. Chromatogr. A.* **1153**, 259-276 (2007).
- Ramstrom, M., *et al.* Depletion of high-abundant proteins in body fluids prior to liquid chromatography fourier transform ion cyclotron resonance mass spectrometry. *J. Proteome Res.* **4**, 410-416 (2005).
- Messana, I., *et al.* Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. *J. Proteome Res.* **3**, 792-800 (2004).
- Li, T., *et al.* Possible release of an ArgGlyArgProGln pentapeptide with innate immunity properties from acidic proline-rich proteins by proteolytic activity in commensal streptococcus and actinomyces species. *Infect. Immun.* **68**, 5425-5429 (2000).
- Davtyan, T.K., Manukyan, H.A., Mkrtchyan, N.R., Avetisyan, S.A., & Galoyan, A.A. Hypothalamic proline-rich polypeptide is a regulator of oxidative burst in human neutrophils and monocytes. *Neuroimmunomodulation.* **12**, 270-284 (2005).
- Jonsson, A.P., *et al.* Gln-Gly cleavage: correlation between collision-induced dissociation and biological degradation. *J. Am. Soc. Mass Spectrom.* **12**, 337-342 (2001).
- Gibbons, R.J., Hay, D.I., & Schlesinger, D.H. Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of *Streptococcus gordonii* to apatitic surfaces. *Infect Immun.* **59**, 2948-2954 (1991).
- Li, T., Johansson, I., Hay, D.I., & Stromberg, N. Strains of *Actinomyces naeslundii* and *Actinomyces viscosus* exhibit structurally variant fimbrial subunit proteins and bind to different peptide motifs in salivary proteins. *Infect Immun.* **67**, 2053-2059 (1999).
- Hardt, M., *et al.* Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry.* **44**, 2885-2899 (2005).
- Wilmarth, P.A., *et al.* Two-dimensional liquid chromatography study of the human whole saliva proteome. *J. Proteome Res.* **3**, 1017-1023 (2004).

25. Saitoh, E., Isemura, S., & Sanada, K. Complete amino acid sequence of a basic proline-rich peptide, P-D, from human parotid saliva. *J. Biochem.* **93**, 495-502 (1983).
26. Slomiany, B.L., Piotrowski, J., Czajkowski, A., Shovlin, F.E., & Slomiany, A. Differential expression of salivary mucin bacterial aggregating activity with caries status. *Int. J. Biochem.* **25**, 935-940 (1993).
27. Juarez, Z.E. & Stinson, M.W. An extracellular protease of *Streptococcus gordonii* hydrolyzes type IV collagen and collagen analogues. *Infect Immun.* **67**, 271-278 (1999).
28. Lo, C.S. & Hughes, C.V. Identification and characterization of a protease from *Streptococcus oralis* C104. *Oral Microbiol. Immunol.* **11**, 181-187 (1996).
29. Harrington, D.J. & Russell, R.R. Identification and characterisation of two extracellular proteases of *Streptococcus mutans*. *FEMS Microbiol. Lett.* **121**, 237-241 (1994).
30. Huang, C.M. *In vivo* secretome sampling technology for proteomics. *Proteomics Clin. Appl.* **1**, 953-962 (2007).
31. Skepo, M., Linse, P., & Arnebrant, T. Coarse-grained modeling of proline rich protein 1 (PRP-1) in bulk solution and adsorbed to a negatively charged surface. *J. Phys. Chem. B.* **110** (31), 12141-12148 (2006).
32. Losic, D., Rosengarten, G., Mitchell, J.G., & Voelcker, N.H. Pore architecture of diatom frustules: potential nanostructured membranes for molecular and particle separations. *J. Nanosci. Nanotechnol.* **6**, 982-989 (2006).
33. Linhares, M.C. & Kissinger, P.T. Pharmacokinetic monitoring in subcutaneous tissue using *in vivo* capillary ultrafiltration probes. *Pharm Res.* **10**, 598-602 (1993).
34. Li, W., Hendrickson, C.L., Emmett, M.R., & Marshall, A.G. Identification of intact proteins in mixtures by alternated capillary liquid chromatography electrospray ionization and LC ESI infrared multiphoton dissociation Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **71**, 4397-4402 (1999).
35. Whitelegge, J.P., *et al.* Protein-Sequence Polymorphisms and Post-translational Modifications in Proteins from Human Saliva using Top-Down Fourier-transform Ion Cyclotron Resonance Mass Spectrometry. *Int. J. Mass Spectrom.* **268**, 190-197 (2007).
36. Castro, P., Tovar, J.A., & Jaramillo, L. Adhesion of *Streptococcus mutans* to salivary proteins in caries-free and caries-susceptible individuals. *Acta Odontol. Latinoam.* **19**, 59-66 (2006).
37. Challacombe, S.J. & Sweet, S.P. Oral mucosal immunity and HIV infection: current status. *Oral Dis.* **8** Suppl. 2, 55-62 (2002).
38. Jensen, J.L. Salivary acidic proline-rich proteins in rheumatoid arthritis. *Ann. N.Y. Acad. Sci.* **842**, 209-211 (1998).