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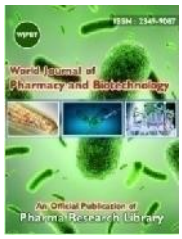

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Salivary diagnostics powered by Nanotechnologies, proteomics and genomics

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A B S T R A C T

The ability to monitor health status, disease onset and progression, and treatment outcome through non-invasive means is a highly desirable goal in health care promotion and delivery. Oral fluid is a perfect medium to be explored for health and disease surveillance. Two prerequisites exist before the goal of salivary diagnostics can be achieved: identification of specific biomarkers associated with a health or disease state and the development of technologies that can discriminate between the biomarkers. A recent initiative of the National Institute of Dental and Craniofacial Research has created a roadmap to achieve these goals through the use of oral fluids as the diagnostic medium to scrutinize the health and/or disease status of patients. This is an ideal opportunity to optimize state-of-the-art saliva-based biosensors for salivary biomarkers that discriminate between diseases. Seven technology groups are developing point-of-care salivary diagnostic technologies. Three groups are working together toward deciphering the salivary proteome.

Keywords: oral fluid, saliva, oral cancer, nanotechnology, Proteomics, genomics.

A R T I C L E I N F O

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1. Introduction

Saliva, an oral fluid that contains an abundance of proteins and genetic molecules and is readily accessible *via* a totally non-invasive approach, has long been recognized as the potential solution to limitation number 2. Through the visionary investment by the NIDCR, the discovery of salivary biomarkers and ongoing development of salivary

diagnostics technologies now provide promising solutions for limitations 1 and 3. There is considerable excitement surrounding the application of saliva-based diagnostics for oral diseases. This realization will enable scientists to bridge oral health research with systemic disease diagnosis. With the additional advantages of an easy, safe, cost-effective, and non-invasive diagnostic approach, saliva shows high potential for monitoring general health and disease, with enormous translational values, and unparalleled opportunities for clinical applications.

Properties of saliva as a diagnostic fluid:

In general, human salivary glands produce about 1–1.5 L of serous and mucinous saliva daily by combining water, salts, and an abundance of molecules from the blood with a cocktail of salivary proteins in the oral cavity to give rise to the multi-constituent whole saliva. Since the advantages of saliva as a diagnostic tool were revealed, the use of saliva for surveillance of disease and general health has become a highly desirable goal in healthcare research and promotion. However, the full power and potential of saliva in medical applications was only recently recognized when saliva was shown to reflect the spectrum of health and disease states and to offer distinctive advantages over serum. Like blood, saliva is a complex fluid containing a variety of enzymes, hormones, antibodies, antimicrobial constituents, and growth factors. Many of these enter saliva from the blood by passing through the spaces between cells by transcellular (passive intracellular diffusion and active transport) or paracellular routes (extracellular ultrafiltration).

Therefore, most compounds found in blood are also present in saliva, thus saliva is functionally equivalent to serum in reflecting the physiological state of the body, including emotional, hormonal, nutritional, and metabolic variations. There has been concern that although saliva contains diverse components with diagnostic properties, their low concentration compared with levels in the blood may prevent salivary diagnostics from being clinically practical; however with the development of new and highly sensitive techniques (*e.g.*, molecular diagnostics, nanotechnology), the low concentration of analytes in saliva is no longer a limitation. Today, a growing number of proof-of-principle assays have been established using saliva to monitor diseases or bodily conditions such as HIV infection, immune responses to viral infections (*e.g.*, hepatitis A, B, and C), systemic levels of drugs, and the detection of illicit drug use.

One of the main advantages of saliva as a diagnostic tool is that sample collection is easy and non-invasive, thus dramatically diminishing discomfort associated with blood collection and privacy issues associated with urine collection. Salivary constituents vary depending on the harvesting method and the degree of salivary flow. The different methods for collecting saliva can be classified according to whether they use stimuli. Stimulated saliva is commonly collected by inducing masticatory action on paraffin wax or chewing gum to increase the salivary flow rate. This method obviously affects the quantity and pH of the saliva, and is generally only used in patients who have difficulty producing enough saliva. Unstimulated saliva is collected without exogenous facilitation, and its flow rate is mostly affected by the degree of hydration.

Figure 1

Collection of whole saliva by the Salivette (absorbent) method:

- Saliva is collected by chewing a cotton wool swab. (b) The swab containing saliva is
- Placed in the tube of Salivette. (c) Centrifugation of the assemblage. (d) Saliva is separated from

Then here are compelling reasons for exploring saliva as a diagnostic tool. It clearly meets the demands for an inexpensive, non-invasive, and easy-to-use screening method. As a diagnostic specimen in the clinic, saliva has many advantages in terms of collection, storage, shipping, and voluminous sampling; all of these processes can be carried out very economically compared with serum or urine. Moreover, for healthcare professionals, a salivary test is safer than using serum, which is more likely to expose operators to blood-borne diseases.

2. Salivary diagnostics

Background: Salivary diagnostic approaches have been developed to monitor oral diseases such as periodontal diseases and to assess caries risk. Recently, due to the combination of emerging biotechnologies and salivary diagnostics, a large number of medically valuable analytes in saliva are gradually unveiled and some of them represent biomarkers for different diseases including cancer, autoimmune diseases, viral diseases, bacterial diseases, cardiovascular diseases, and HIV. These developments have extended the range of saliva-based diagnostics from the simple oral cavity to the whole physiological system. Thus, saliva-based diagnostics is on the cutting edge of diagnostic technology, and may offer a robust alternative for clinicians to use in the near future to make clinical decisions and predict post-treatment outcomes.

Vision and challenge: Salivary diagnostics have received increasing attention as a growing number of high-impact systemic diseases (*e.g.*, cancer, cardiovascular, and metabolic diseases) and physiological conditions were shown to be accurately reflected by the composition of saliva, motivating scientists from academia, government, and industry to invest resources into saliva-based diagnostics. A good diagnostic method should have the characteristics of high sensitivity, specificity, and functionality, and meet the requirements of high

throughput, portability, and low cost for subsequent clinical application. Today, the improved efficiency and accuracy of genomic and proteomic biomarker discovery technologies are turning salivary diagnostics into a clinical and commercial reality. Among the newly developed technologies, the miniaturization technology known as "lab-on-a-chip" provides a new avenue for point-of-care diagnostics since it is able to detect multiple biomarkers in parallel and allows simultaneous assessment of multiple disease conditions.

3. Technologies

Since saliva contains many components also found in serum, and has several advantages over serum as described above, saliva is therefore a unique bodily fluid for the development of molecular diagnostics. However, its potential was frequently underestimated due to technological barriers that did not meet the requirements necessary to screen saliva containing complex constituents with low abundance. Recent studies in salivary protein research have shown that, in addition to the major salivary protein families, saliva contains hundreds of minor proteins or peptides that are present in low concentrations but may play an important role on the discrimination of diseases. Besides proteomes, the salivary transcriptomic technology, the second salivary diagnostic alphabet, further advanced the diagnostic potential of saliva for medical applications.

Discovery of salivary biomarkers by proteomic technology: In principle, a global analysis of the human salivary proteomes can provide a comprehensive spectrum of oral and general health. Furthermore, analysis of salivary proteomes over the course of complications may unveil morbidity signatures in the early stage and monitor disease progression. Proteome-based approaches have been applied over the last three decades to monitor changes in protein expression. Generally, protein expression is primarily analyzed by one- or two-dimensional polyacrylamide gel electrophoresis (PAGE).

To resolve the complex composition of saliva, 2-D PAGE allows separation not only of different molecules with similar molecular weights, but also of different modification patterns or is forms of the same protein. Along with the development and introduction of mass spectrometry (MS), the PAGE-separated proteins can be more accurately characterized and identified, leading to a wider range of applications for proteomic assays. Proteins that are primarily identified by MS can be further characterized by ionization methods such as electro spray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Moreover, coupling ESI and MALDI with mass analyzers, such as quadrupole/linear ion trap, time-of-flight (TOF), quadrupole TOF (QTOF), Fourier transform ion cyclotron resonance (FT-ICR), and the Orbit rap, may improve the sensitivity, resolution, accuracy, and efficiency of protein sequence determination. In some cases, however, simply discriminating up and/or down regulation of the expression of specific proteins may not directly reflect the circumstances of physiological states or disease progression. This is because biological functions of proteins may change due to posttranslational modifications that occur without alteration of protein level. It has been demonstrated that many functional alterations of proteins result from posttranslational modifications such as phosphorylation, glycosylation, acetylation, and methylation. These post-translationally modified proteins may represent signatures in some diseases such as autism spectrum disorder and cervical cancer. As of January 2009, over a thousand salivary proteins have been identified from major salivary glands. For most of these proteins, their expression in saliva is quite distinct from that in serum or tear, and has already demonstrated clinical diagnostic values for diseases manifested in the oral cavity. For example, Sjögren's syndrome (SS), a chronic autoimmune disorder that is clinically recognized by dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca), is associated with changes in specific salivary constituents, such as an increase in inflammatory proteins (*e.g.* α-enolase, carbonic anhydrase I and II, salivary α-amylase fragments) and decrease in acinar proteins (*e.g.*, lysozyme C, polymeric immunoglobulin receptor (pIgR), calgranulin A) compared with the profile in non-SS individuals. Other research efforts showed that saliva is an important tool for the detection of oral squamous cell carcinoma (OSCC). Five salivary proteins (M2BP, MRP14, profilin, CD59, and catalase) were shown to be able to discriminate oral cancer with greater than 90% clinical accuracy. Besides SS and OSCC, salivary proteomic constituents are also capable of detecting high-impact systemic disorders. For example, measurement of antibodies to HIV in saliva has been shown to be as accurate as measurement in serum, and the salivary assay has been commercialized as a product called OraQuick. Moreover, early studies suggest that measurement of salivary CA125 and epidermal growth factor may have diagnostic potential for ovarian cancer and breast cancer, respectively.

Discovery technology of salivary biomarkers by transcriptomic: In addition to salivary proteome, in 2004 we discovered the salivary transcriptomes (RNA molecules) that are unusually stable in saliva. They included mRNA molecules that cells use to convey the instructions carried by DNA for subsequent protein production. This discovery presented a second diagnostic alphabet in saliva and opened a door to another avenue of salivary transcriptomic diagnostics. Although the salivary transcriptome is an emerging concept, we have established a robust platform at UCLA for salivary RNA studies including automated extraction, purification, amplification, and high-throughput microarray screening. Importantly, we have also developed statistical and informatics tools that are tailored for salivary biomarker discovery and validation. Also, Early Disease Research Network (EDRN), an entity within the National Cancer Institute (NCI), has just completed an independent validation study of salivary RNA biomarkers for oral cancer detection.

This investigation confirmed a clinical translational value of salivary RNA for oral cancer detection. In the past 5 years, research into the nature, origin and characterization of salivary mRNA has been actively pursued. At present,

the main strategy for identification of salivary transcriptomic biomarkers is through microarray technology. Although it has been demonstrated that the 3′-based array employing poly-dT priming and two rounds of *in vitro* transcription (IVT) amplification works well for profiling salivary transcripts, some pitfalls still need to be overcome. For instance, much information is lost because approximately 50% of salivary RNA molecules are fragmented, therefore they do not carry the poly-A tail and are not protected against degradation. Furthermore, the random priming approach in the RNA amplification may cause an additional shortening of the fragments resulting in further loss of RNA molecules during the procedure. So far we have defined the salivary exon core transcriptome (SECT), which contains 1,370 probe sets representing 851 unique genes that are present in more than 85% of the tested saliva samples. Pilot studies of oral and pancreatic cancers using AEA are consistent with previous results using conventional assays (*i.e.*, Affymetrix's Human Genome U133A assay), and show that the AEA does indeed expand the numbers of sequences and genes that can be detected.

Quantitative real-time PCR (qPCR) is currently the gold standard for quantification of nucleic acids. It is perfectly appropriate for validation of transcriptomic biomarkers after profiling by microarray, and it is not restricted by the length of the RNA, even for fragmented RNA. However, low amounts of RNA in saliva tremendously hinder their performance in qPCR. To overcome this problem, a new multiplex reverse transcriptase-PCR-based pre-amplification approach was developed that allows accurate quantification of over 50 targets from one reaction. This method dramatically increases the capacity of quantitative analysis that it extends approximately six-fold for the magnitude of target input, and is tailored to the short nature of salivary RNA. It also offers good time- and cost-effectiveness by performing simultaneous reverse transcriptase reactions for different targets, allowing a small volume of pre-amplification product to be used for subsequent qPCR measurement.

Our studies of salivary mRNA biomarkers from patients with primary T1/T2 OSCC showed promising results and demonstrated the diagnostic and translational potential of the salivary transcriptome. Data combining microarray profiling and qPCR validation showed seven mRNA whose expression levels in patients were elevated at least 3.5-fold compared with matched healthy counterparts. These mRNAs are transcripts of DUSP1, H3F3A, OAZ1, S100P, SAT, IL-8, and IL-1β. The study showed that a group of five transcriptomic biomarkers in serum can be consistently validated and distinguished OSCC with 91% sensitivity and 71% specificity (ROC = 0.88).

The salivary transcriptome is a more discriminatory tool for oral cancer detection than the serum transcriptome. So far, over 220 additional oral cancer patients have been tested and the clinical accuracy of the salivary mRNA biomarkers holds up at > 82% (Wang *et al.*, unpublished data), indicating they are among the most discriminatory panels for OSCC screening to date.

Development of point-of-care technologies for salivary diagnostics: In September 1999, the NIDCR initiated a research workshop aimed at applications of micro fluidics and micro/ nanoelectromechanical system (MEMS/NEMS) to saliva-based diagnostics. MEMS/NEMS is an integrated system that consists of a central unit for processing data (*i.e.*, microprocessor) and several other components that connect with the outside interface, such as micro sensors. To proceed with the development of saliva diagnostic technologies, in 2002 the NIDCR funded seven projects that explored different point-of-care systems to detect salivary analytes and provided an overall profile that correlated with a particular disease state: electrochemical sensing, on-chip PCR/RT-PCR, microsphere-based nano-bio chip, microsphere-based optical fiber array, high-throughput DNA microarray (*i.e.*, validation of the first-generation DNA chip), surface plasmon resonance optical system, and microchip electrophoretic immunoassay. In 2006, four groups were awarded another 5 years of support to further develop the respective point-of-care technology for prototype production, analysis, and clinical validation.

UCLA is one of the four funded groups. The UCLA Collaborative Oral Fluid Diagnostic Research Center, partnered with engineers at the UCLA School of Engineering, developed a MEMS-based electrochemical detection platform that is capable of real-time, ultrasensitive, ultra specific multiplex detection of salivary protein and RNA biomarkers. This envisioned product has been labelled the Oral Fluid NanoSensor Test (OFNASET). The OFNASET is a point-of-care, automated, and easy-to-use integrated system that will enable simultaneous and precise detection of multiple salivary proteins and nucleic acids. Oral Fluid Nano Sensor Test (OFNASET), a point-of-care, automated, easy-to-use integrated system that will enable simultaneous, accurate, and rapid detection of multiple salivary protein and nucleic acid biomarkers.

Salivary diagnostics – A new industry in a prospective future: The value of saliva as a diagnostic tool has long been disregarded until the advantages of saliva-based approaches was recognized in the past decade, and led to an evolution from treating saliva as a diagnostic worthlessness to promoting salivary diagnostics. Regarding diagnostic capability, the gap between saliva and other bodily fluids, such as blood, urine, and cerebral spinal fluid, is closing, primarily due to rapid technology development, scientific validation of diagnostic analytes, and advocacy by the NIDCR. Based on the abundance of promising research efforts and the fact that research into salivary diagnostics is currently a priority at NIDCR, saliva-based diagnostics present unparalleled opportunities for research and commercialization opportunities. We foresee that more products to be commercialized; either as new inventions or based on products shown in the With the current rate of progression, salivary diagnostics can become a key player in routine health monitoring in the near future and enable the early detection of disease using a simple and effective assay.

Clinical significance:

Saliva, like blood, contains an abundance of protein and nucleic acid molecules that reflects physiological status; however, unlike other bodily fluids, salivary diagnostics offer an easy, inexpensive, safe, and non-invasive approach for disease detection, and possess a high potential to revolutionize the next generation of diagnostics.

4. Conclusion

The NIDCR initiatives and current research efforts are closing the gap rapidly between the use of saliva and other bio fluids (blood, urine, cerebrospinal fluid, tears, nipple aspirate) for disease diagnostics. Scientific data to establish a benchmark for the diagnostic value of saliva in comparison with that of other bio media will be necessary to assess the disease discriminatory value of saliva. It may well turn out that, similar to the UCLA finding that saliva is more accurate than blood in detecting oral cancer, 18, 19 saliva will outperform other bio media in the diagnosis of other diseases as well.

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