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Salivary diagnostics: enhancing disease detection and making medicine better

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Abstract

To monitor health status, disease onset and progression, and treatment outcome non-invasively is a most desirable goal in the health care delivery and health research. There are three prerequisites necessary to reach this goal:

1. A non-invasive method for collecting biological samples.
2. Specific biomarkers associated with health or disease.
3. A technology platform to rapidly discriminate the biomarkers.

An initiative catalysed by the National Institute of Dental and Craniofacial Research (NIDCR) has created a roadmap to achieve this goal through the use of oral fluids as the diagnostic medium to scrutinize the health and disease status. This is an ideal opportunity to bridge state-of-the-art saliva-based biosensors and disease-discriminatory salivary biomarkers in diagnostic applications. Oral fluid, often called the ‘mirror of the body’, is a perfect medium to be explored for health and disease surveillance. The translational applications and opportunities are enormous. This review presents the translational value of saliva as a credible clinical diagnostic fluid and the scientific rationale for such use.

Keywords

oral fluid; saliva; oral cancer; nanotechnology; proteomics; genomics

Introduction

The central challenge of dental education is to inspire young people to see the magnificence of science and its power to improve health, stop disease and prolong life. Witness how discovery of antibiotics ended the pestilence of bacterial infections which, over millennia, took such a high toll on young and old alike; how invention of anaesthesia heralded the era of tolerable surgery; how perfection of dental restoration ended the age-old torment of caries.

The scientists who spearheaded these developments profoundly changed human existence. They also entrenched in global human consciousness the idea that science marches forward and that this inexorable progress returns to the public miraculous benefits of health and life. Standing on the shoulders of these giants, those of us working in salivary diagnostics envision the potential of this technology to starkly change how health services are delivered and how health research is done. As our work inches forward, we also hope that our success with salivary diagnostics will inspire young people to devote their lives to health sciences.

We need the help of future generations desperately because the pace of scientific progress is not as rapid as it might seem to one learning about it from the nightly news. To those of us working in the lab, that pace is painstakingly, frustratingly slow. The pace grinds to a tragic standstill for those of our patients who are dying as they are waiting for a cure. So each morning we come to work feeling the urgent need to crack the code of salivary biomarkers. The body is asking for help in the language of saliva. We want to learn to speak the body's language.

Vision and challenges

High-impact human diseases, including cancer, cardiovascular, metabolic and neurological diseases, are challenging to diagnose without supplementing clinical evaluation with laboratory testing. Even with the current laboratory tools, definitive diagnosis often remains elusive due to clinical or public health factors. Three roadblocks hold back the realization of clinical diagnostic potential:

1. Simple and inexpensive sampling methods that cause minimal subject discomfort.
2. Definitive disease-associated protein and genetic markers.
3. An accurate, portable and easy-to-use diagnostic platform.

Saliva, a biofluid that is readily available through non-invasive collection, has long been recognized to address the first roadblock (1). With the visionary investment by the National Institute of Dental and Craniofacial Research (NIDCR), saliva biomarker discovery and salivary diagnostic technologies are currently in development, thus addressing the second and third roadblocks.

It is safe to predict that the use of saliva for disease diagnostics and normal health surveillance is about 5 years away. This is an exciting time, as we are seeing the applications of saliva diagnostics for oral diseases. However, we also expect this oral health research to shed light on systemic diseases, since saliva is filtered and processed into the oral cavity from the vasculature that nourishes the salivary glands (Figs 1 and 2). Often called the 'mirror of the body' or 'a window on health status', oral fluid is a perfect medium to be explored for health and disease surveillance. A growing number of proof-of-principle examples have been established for using saliva to monitor systemic diseases and conditions. The translational applications and opportunities are enormous.

The barriers to widespread implementation of saliva diagnostics derive from technological problems such as sensitivity, miniaturization, high throughput, automation, cost and speed. These barriers to detection of markers in saliva have largely been overcome through techniques emerging from many different fields: biology, chemistry, physics and engineering. High-throughput genomics and proteomics approaches are available in the post-genomic era. Miniaturized diagnostic technologies will yield critical patient health information using only minute amounts of body fluids. These 'lab-on-a-chip' platforms will perform multiple operations in parallel and allow the simultaneous assessment of multiple conditions in non-laboratory settings such as hospital, clinic, workplace or home. New discoveries and

technological advances in conjunction with the ability to diagnose disease in a non-invasive biofluid will foment a revolutionary change in medicine.

The diagnostic revolution will spread even to the most remote or impoverished communities where only minimally trained persons are available. The need for non-invasive, simple-to-use point of care (POC) diagnostic tools is particularly acute in the developing world, where many health risks and illnesses remain poorly defined and receive inappropriate treatment. In addition, little information about the burden of disease is available to guide population health decisions. In fact, this technology may have the greatest impact in communities that presently do not receive adequate laboratory or other health services.

Developed countries face different challenges that can also be surmounted with saliva diagnostics. The evolving standards of protection of human experimental subjects are now interfering with paediatric research. Some institutional review boards have taken the position that healthy children, needed to serve as experimental controls, cannot meaningfully consent to venepuncture, a procedure that is quite traumatic for children. The rationale is that healthy children derive no personal benefit from the research, and monetary compensation is thought to benefit parents rather than children. Furthermore, the prevailing attitudes toward child labour also weigh against children submitting to venepuncture for money. In this environment, paediatric researchers can be severely limited or even completely blocked in their efforts to obtain blood from paediatric controls. To the extent that research can be done using salivary rather than serum biomarkers, this problem is circumvented.

The vision and challenge of saliva diagnostics is to discover the diagnostic potential and optimize engineering technologies for this biofluid. To fully understand the diagnostic potential of saliva, we need to establish the scientific foundation and achieve clinical validations. Figure 3 is a Venn diagram illustrating that within the spectrum of total human health and disease (top circle), some states will reflect themselves in saliva proteomically or genomically (lower left circle). The lower right circle represents technology platforms for POC detection of biomarkers in saliva (discussed later). The extent of overlap remains to be determined by the efforts of saliva diagnostics researchers.

Saliva as a diagnostic fluid

Saliva diagnostics is a later bloomer, as only recently has there been a growing appreciation that saliva can reflect virtually the entire spectrum of normal and disease states (1). These include tissue levels of natural substances and a large variety of molecules introduced for therapeutic, dependency or recreational purposes, emotional status, hormonal status, immunological status, neurological effects, and nutritional and metabolic influences.

A major barrier to using saliva as a diagnostic fluid has been the fact that many informative analytes are generally present in lower amounts in saliva than in serum (2). With new and highly sensitive technologies, the lower level of analytes in saliva is no longer a limitation. Almost anything that can be measured in blood can also be measured in saliva. Saliva has been reliably used to detect HIV-1 and -2, and viral hepatitis A, B and C. It can also be used to monitor a variety of drugs including marijuana, cocaine and alcohol (1).

There are compelling reasons to use saliva as a diagnostic fluid to monitor health and diseases. As a clinical medium, saliva has many advantages over serum. Saliva is easy to collect, store and ship and can be obtained at low cost in sufficient quantities for analysis. For patients, the non-invasive collecting techniques dramatically reduce anxiety and discomfort and simplify procurement of repeated samples for longitudinal monitoring over time. For professionals, saliva collection is safer than venepuncture, which could expose health care providers to HIV or hepatitis virus. Saliva is also easier to handle for diagnostic procedures since it does not clot,

lessening the manipulations required. Saliva-based diagnostics are therefore less invasive, less expensive and present less risk to both the patient and the provider than current methodologies.

Developments of technologies for saliva-based diagnostics

Five years ago, in 2002, the NIDCR initiated a concerted research effort in the area of saliva diagnostics. NIDCR funded seven U01 awards to develop microfluidics and microelectro-mechanical systems (MEMS) for saliva diagnostics. The aim is to identify technologically viable systems and support their advance towards commercialization.

MEMS are integrated systems consisting of sensors, actuators, and electronics on a common silicon substrate developed through microfabrication technology. Minute sample and reagent volumes are processed and analysed with integrated detectors. The seven NIDCR-supported U01 awards focused on the development of microfluidic and MEMS technologies for measuring DNA, gene transcripts (mRNA), proteins, electrolytes and small molecules in saliva, as well as overall profile correlates of a particular disease state, such as cardiovascular disease (3,4).

Despite laboratory progress in dental schools and engineering departments, none of the new technologies will become a practical and clinical reality without strong partnerships with industry, early in the development stage. The reasons include the many challenges that such technologies face at every stage including fabrication, integration of individual components, validation, regulatory approval and finally commercialization. This has sparked a new U01-level initiative for the 'Development and Validation of Technologies for Saliva-Based Diagnostics' in order for the currently developed academic saliva diagnostics groups to team up with industrial partners for further development of functional prototypes and testing their robustness for clinical applications. Four of the initial seven groups were recently renewed for 5 years for the second round.

Diagnostic molecular targets in saliva: the proteome and the transcriptome

The salivary proteome

To fully utilize the diagnostic potential of saliva, one needs to comprehensively decipher and catalogue the informative components. In fiscal year 2003, NIDCR funded three U01 awards aiming to identify and catalogue human salivary proteins from the three major salivary glands. It is envisioned that the human salivary proteome (HSP) will be a resource to help elucidate disease pathogenesis and evaluate the influence of medications on the structure, composition and secretion of all salivary secretory constituents.

Multiplexed proteomics platforms are currently explored by the NIDCR-funded Saliva Proteome Consortium in order to collectively decipher the HSP. In general, a 'divide and conquer' bottom-up strategy is used. The proteins from whole or ductal saliva (parotid and SM/SL) are initially fractionated with a variety of separation techniques including reversed-phase liquid chromatography (LC), strong cation exchange (SCX) LC, gel filtration LC, Zoom isoelectric focusing (Zoom IEF), and ultrafiltration. Further, the collected protein fractions are digested with a proteolytic enzyme, e.g. trypsin, and then analysed with 1-D or 2-D LC-MS/MS. Finally, the acquired MS data are processed and submitted for database searching using Mascot database search engine.

We are also comprehensively cataloguing saliva glycoproteins using LC-MS/MS and glycoprotein pull-down method based on hydrazide chemistry. Similar to plasma/serum counterparts, many proteins (e.g. mucins and amylases) in human saliva are glycosylated. In the glycoprotein pull-down approach, glycoproteins are coupled on to a hydrazide resin. The

proteins are then digested and formerly *N*-glycosylated peptides are selectively released with the enzyme PNGase F and analysed by LC-MS/MS. Employing this method, coupled with in-solution isoelectric focusing separation as an additional means for pre-fractionation, we identified 84 formerly *N*-glycosylated peptides from 45 unique *N*-glycoproteins (5).

The multiplexed proteomic platforms have clearly deepened the HSP analysis. As the analysis of parotid and SM/SL saliva nears completion, we have catalogued in whole saliva (WS) more than 1000 proteins (6). We have also developed a saliva proteome knowledge base (SPKB) to centralize the acquired proteomic data and annotate the identified saliva proteins. The SPKB is fully accessible to the public (<http://www.hspp.ucla.edu>) for queries regarding the identified proteins, which are linked to public protein databases.

Elucidation of the normal salivary proteome is only the first step on a road with many forks. Comparison of such a normal protein catalogue with that of a diseased population will reveal diagnostic signatures that can discriminate between normal and diseased individuals. We have started making translational discoveries into the salivary proteome for oral cancer (7) and Sjögren's syndrome patients (8).

Comparative analysis of HSP and human plasma proteome (HPP) suggests that extra-cellular proteins are predominant in HSP, whereas the membrane proteins are predominant in HPP. HSP proteins have significant binding and structural molecular activities whereas the HPP proteins show significant activities of nucleotide/nucleic acid binding. In terms of 'biological processes', a significant percentage of serum proteins are involved in cell cycle or signal transduction whereas a significant percentage of saliva proteins are involved in physiological or response-to-stimulus processes.

The salivary transcriptome

Our laboratory recently made the serendipitous discovery that discriminatory and diagnostic human mRNAs are present in saliva of normal and diseased individuals. The salivary transcriptome presents an additional research and clinical resource, the second saliva-based diagnostic alphabet for disease detection. We found ~3000 mRNAs in the normal salivary transcriptome (9). Of these, 180 are common between different normal subjects, constituting the normal salivary transcriptome core (NSTC).

To demonstrate the diagnostic and translational potential of the salivary transcriptome, saliva from head and neck cancer patients was profiled and analysed. By looking at four genes from the NSTC (*IL8*, *OAZ1*, *SAT* and *IL1B*), we were able to determine whether a saliva sample is from a cancer or normal subject with a sensitivity and specificity of 91% each (ROC = 0.95) (Fig. 4). While head and neck cancer was used as the first proof-of-principle disease for salivary transcriptome diagnostics, data will soon be available for systemic diseases. These data, while early and exploratory, demonstrate the acute need to fully explore the salivary transcriptome for major human disease translational applications.

To benchmark the salivary transcriptome biomarkers for oral cancer detection with that of blood, we examined the serum transcriptome from the same patients who provided saliva for the salivary transcriptome comparison. We found that four serum RNA biomarkers can discriminate oral cancer subjects and have a sensitivity and specificity of 91% and 71%, respectively (ROC = 0.88). However, when we compare the serum transcriptome with the salivary transcriptome, which has a ROC value of 0.95, we can clearly see that for oral cancer detection, saliva transcriptome diagnostics has an edge over serum (10).

There are further advantages to utilizing transcriptome markers for disease diagnostics. The marker discovery process is high-throughput using microarray platforms. While the human

salivary proteome has just been completed, the normal salivary transcriptome has been completed 2.5 years ago (9). As a bio-marker, RNA is as robust and as informative as any other analyte. It is important to note that the Early Disease Research Network (EDRN), an entity within the National Cancer Institute (NCI) has just completed an independent validation study of the oral RNA biomarkers for oral cancer detection. This validation study unequivocally demonstrates the presence of RNA in saliva as well as its clinical translational potential for oral cancer detection.

Thus the salivary transcriptome offers the combined advantages of high-throughput marker discovery in a non-invasive biofluid with very high patient compliance. RNA signatures have been identified for head and neck cancer, and we are working now on major human systemic diseases. Thus saliva is perhaps better than a mirror or a window to the body. Through saliva, like through a magnifying glass, we see the body more clearly than through other biological media.

The growing importance of salivary transcriptome research can also be gauged by the commercial sector launching two products for salivary RNA stabilization and isolation: RNAprotect® Saliva from QIAGEN and Oragen-RNA from DNA Genotek.

The salivary transcriptome vs. the proteome

Bridging our dual interests in salivary ‘omics’ (salivaomics) is our recent study into the relationship of the salivary proteome and transcriptome. We have conducted the concurrent proteomic and transcriptomic profiling of whole saliva samples from three healthy subjects to test if proteins coexist with their counterpart mRNAs in human saliva. Of the function-known proteins identified in WS, more than 60% were also found present as mRNA transcripts. For genes not detected at both protein and mRNA levels, further efforts were made to determine if the counterpart is present. Of 19 selected genes detected only at protein level, the mRNA of 13 (68%) genes was found in saliva by RT-PCR. This study indicates that saliva transcriptome may provide preliminary insights into the boundary of saliva proteome (11).

The UCLA Collaborative Oral Fluid Diagnostic Research Center

In addition to the salivary biomarker initiatives, the UCLA School of Dentistry is also engaged in the parallel development of the saliva diagnostics technology platform. Four years ago, we established the UCLA Collaborative Oral Fluid Diagnostic Research Center to develop nanoscale and microscale technology to detect salivary protein and genomic biomarkers for POC applications of high impact human diseases.

For salivary diagnostic technology development, we have partnered with researchers at the UCLA School of Engineering who are pioneers in micro- and nano-electrical-mechanical systems (MEMS and NEMS) biosensors. These devices exhibit exquisite sensitivity and specificity for analyte detection, down to single molecule level (12,13). Our research consortium has established a firm and committed collaboration toward the development of MEMS/NEMS biosensors for the real-time, ultrasensitive and ultraspecific detection of salivary diagnostic analytes. This robust forum of interactions between engineers and biologists/clinicians will produce in approximately 2 years ‘lab-on-a-chip’ prototypes available for research as well as clinical applications (14).

The envisioned product is the ‘Oral Fluidic NanoSensor Test’ (OFNASET). The handheld, automated, easy-to-use, integrated system will enable simultaneous and rapid detection of multiple salivary protein and nucleic acid targets (Fig. 5) (<http://www.saliva.bme.ucla.edu/>). This saliva biomarker detector can be used in dentist's and other health care provider's offices for POC disease detection.

The cultural, social, behavioural and psychological aspects of saliva as a clinical utility

The rapid advance of saliva diagnostics science and technology leads us to imagine the future, but the reader is now invited to turn the mind's eye to various current and past cultures.

A broad and diverse range of meanings has been attributed to saliva as a bodily fluid. Most people never contemporaneously appreciate both the positive and negative connotations of saliva. The oral fluid may be viewed as grotesque in one culture, yet carry a blessing in others. This dual nature brings up anthropological and psychological connotations showing saliva as the spirited fluid that it is.

Anthropology and animal behaviour

Ivan Pavlov's experiments show how closely salivation is tied to the thought of food, one of life's primary indulgences. Spitting is generally taboo in Western culture, but athletes playing outdoors are exempt from this rule. In many Western cultures people also 'spit' ceremonially, and perhaps superstitiously, to avoid bad luck or to attract good luck (15). While lovers will freely exchange saliva, spitting on a person, including one's lover, is construed as an insult in Western culture. In contrast, in many tribal cultures spit may be used for healing (16-19) or even spiritual and social anointment (20-22).

As just mentioned, in any culture, a kiss is seen as an affectionate gesture, shared in the most intimate relationships. People are generally willing to undergo the act with a loved one, yet are more reserved about the thought of kissing a stranger. Dog owners may allow their own dogs to lick them, even immediately after the dog licks other dogs, yet the affection of other dogs may be rejected. A certain intimate bond is required to get over the idea of sharing saliva with another being.

Throughout the history of mankind, this selectivity may have evolved as protection from the many diseases carried within saliva. However, the persistence of the act hints towards the presence of positive benefits as well. Perhaps kissing spreads not only noxious but also beneficial bacteria. In various animals, a mother will inoculate her offspring within a few hours of birth by covering it with her own saliva. This ensures the passing of vital bacteria and antibodies to assist in the off-spring's digestive and immune functions.

Saliva may play an additional role during infancy in the development of an animal's future social behaviours. Scientists have observed that among Mongolian gerbils, cues within saliva play a key factor in deciding the response behaviours between the young and the old, parents and offspring, and males and females (23).

Psychology of self

Although most people do not have a problem sharing saliva during kissing, there is a much more conservative view in sharing inanimate objects associated with saliva contact. A toothbrush, a spoon and even a straw are all taboo to share, simply due to the fact that contact has been made with someone else's saliva. The psychological disconnection between saliva inside the mouth and saliva exposed to the outer environment may be due to the way our mind sees a difference between saliva that has passed beyond the mouth and saliva that remains within the confines of our bodies. Dr Gordon Allport, a social psychologist from Harvard, asked a human subject to drink from a cup that had previously been filled with subject's own saliva. Paradoxically yet predictably, the individual was hesitant to drink (24). Allport hypothesizes that although the saliva came from the same person, the saliva became non-self – alien to the mind the moment it exited the mouth. This modification in perception may be

the leading factor why people may have difficulties adjusting to the fact that saliva has true and viable diagnostic information about the body from which it came.

Saliva physiology

The cultural attitudes toward saliva just described bear little relation to the true importance of saliva in everyday life, which is rarely appreciated outside the oral biology community. On a molecular level, saliva contains mostly water. It is fitting that saliva is predominantly composed of the essence of life, since this biofluid is the vehicle for so many vital functions. Saliva is also composed of various constituents that confer numerous properties to the rich medium. Many of these properties are crucial in human activities of daily living. Among the top functions of saliva are antibacterial roles through peroxidases, mucins, and cystatins; buffering roles using carbonic anhydrases and histatins; digestive functions using amylases, mucins and lipases; and the more obvious role of lubrication through mucins and statherins. It should also be noted that it was the discovery of epidermal growth factor (EGF) and nerve growth factor (NGF) in saliva in 1986 by Stanley Cohen and Rita Levi-Montalcini that won them the Nobel Prize in Physiology and Medicine. The importance of saliva in medicine and physiology cannot be overstated. Yet, the robust duties of saliva are commonly taken for granted and only appreciated when the precious medium is not found in abundance within the mouth, as in radiation or oral cancer patients.

These individuals suffer speech and severe eating difficulties. Patients with minimal salivary flow, struggle to chew and swallow solid food, such as bread or meat (25). Functional pathologies within the oral cavity also quickly begin to emerge. The abundance of yeast growth topically on the tongue may also lead to halitosis and general bad oral health. Cavities are much more prevalent in patients with lowered salivary flow due to the loss of salivary bathing of the teeth, which normally provides a buffering and antibacterial medium. Fittingly, Frank Oppenheim, chairman of the department of periodontology and oral biology at Boston University, summarizes the importance of saliva's constituents with the statement, 'If saliva were (merely) water, we would have little stumps of teeth or no teeth at all by age 20 – we would have dissolved our teeth away' (26).

Scientific conservatism

In the eyes of oral biologists, the functional value of saliva has long overshadowed the diagnostic possibilities. More recently, however, the use of saliva to diagnose HIV, various forms of cancer, diabetes, arthritis and heart disease has shown that there is much more information in saliva than was previously thought. Scientists, a conservative lot, are slowly transitioning from viewing saliva as a diagnostic outcast in comparison with blood or urine, and are starting to view it as an abundant, valuable scientific resource. However, there may be cultural perceptions that block professional acceptance of salivary diagnostics, and those can be overcome slowly.

The rapidly emerging science, sparked by the NIDCR initiatives, is closing the gap between saliva and other diagnostic biomedica (blood, urine, cerebral spinal fluid, tear, nipple aspirate, faecal matter). Scientific data to benchmark the diagnostic value of saliva against other biomedica will be necessary to assess the disease discriminatory value of saliva. It may well turn out, similar to our recent finding that saliva is more accurate than blood for oral cancer detection (10,14), that saliva diagnostics will outperform other biomedica for other disease diagnostics as well. If the scientific values and diagnostic utilities of saliva are as good as or better than other bio-media, its easy and non-invasive collection will eventually place saliva as the biomedium of choice in clinical diagnostics. The abundance of information in saliva will elevate it to play an even greater role in people's daily lives (27). The day is near when saliva will be considered a diagnostically diverse and charismatic fluid.

Future perspectives

The national agenda to turn saliva diagnostics into a clinical and commercial reality has spawned remarkable progress. But much work remains to be done in identifying many more definitive disease-associated salivary biomarkers to be used with the emerging technology platforms. The scientific community is poised to develop and validate saliva-based tests for POC, chair-side, portable and multiplex diagnostic devices. Dentists are writing a new chapter in the history of health sciences to amaze a new generation of patients worldwide and inspire a new generation of health scientists (Fig. 6).

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Biography

Biosketch

David Wong is Professor and Associate Dean of Research in the Division of Oral Biology and Medicine at the University of California-Los Angeles (UCLA School of Dentistry). He is also the Director of the UCLA Dental Research Institute (DRI). He directs the UCLA Collaborative Oral Fluid Diagnostic Research Center, the UCLA comprehensive T32 Clinical Research Training programme as well as the Laboratory of Head and Neck Oncology Research. He is a fellow of the American Association for the Advancement of Sciences (AAAS).

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Fig. 1.
Anatomical locations of the three major salivary glands: parotid, submandibular and sublingual.

Fig. 2.
Mechanisms of transport of proteins and ions from serum into salivary gland ducts.

Fig. 3. Disease markers manifestation in saliva and their detection by saliva diagnostic biosensors (Oral Fluid NanoSensor Test, OFNASET).

Proof of principle of salivary transcriptome for oral cancer detection

Using 4 Saliva RNA Biomarkers:
IL-1B, OAZ1, SAT and IL-8

Area under ROC curve	Sensitivity	Specificity
0.95	0.91	0.91

Fig. 4.

Receiver operator characteristic (ROC) curve analysis for the predictive power of combined salivary mRNA biomarkers. The final logistic model included four salivary mRNA biomarkers, IL1B, OAZ1, SAT and IL8. Using a cut-off probability of 50%, we obtained sensitivity of 91% and specificity of 91% by ROC. The calculated area under the ROC curve was 0.95.

Fig. 5.
UCLA's Oral Fluid NanoSensor Test (OFNASET).

Saliva diagnostics powered by nano technology, genomics & proteomics

Fig. 6.
A drop of saliva harbours a world of diagnostic information, proteomically and genomically.
A handful of these analytes mark human diseases with great sensitivity and specificity.