Lab-on-a-Chip Methods for Point-of-Care Measurements of Salivary Biomarkers of Periodontitis

NICOLAOS CHRISTODOULIDES,[a] PIERRE N. FLORIANO,[a] CRAIG S. MILLER,[b,c] JEFFREY L. EBERSOLE,[b,c] SANGHAMITRA MOHANTY,[a] PRIYA DHARSHAN,[a] MICHAEL GRIFFIN,[a] ALEXIS LENNART,[a] KARRI L. MICHAEL BALLARD,[a] CHARLES P. KING, JR.,[b] M. CHRIS LANGUB,[d,e] RICHARD J. KRYSCIO,[f] MARK V. THOMAS,[b] AND JOHN T. McDEVITT[a,g,h]

[a]*Department of Chemistry & Biochemistry, The University of Texas at Austin, Austin, Texas, USA*

[b]*Department of Oral Health Practice and Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, Kentucky, USA*

[c]*Department of Microbiology, Immunology & Molecular Genetics, and Department of Internal Medicine, University of Kentucky, Lexington, Kentucky, USA*

[d]*Office of Extramural Programs, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Atlanta, Georgia, USA*[i]

[e]*Department of Internal Medicine, College of Medicine, University of Kentucky, Lexington, Kentucky, USA*[j]

[f]*Department of Biostatistics, College of Public Health, University of Kentucky, Lexington, Kentucky, USA*[j]

[g]*Center for Nano and Molecular Science and Technology, The University of Texas at Austin, Austin, Texas, USA*

[h]*Texas Materials Institute, The University of Texas at Austin, Austin, Texas, USA*

ABSTRACT: Salivary secretions contain a variety of molecules that reflect important pathophysiological activities. Quantitative changes of specific salivary biomarkers could have significance in the diagnosis and management of both oral and systemic diseases. Modern point-of-care technologies with enhanced detection capabilities are needed to implement

Address for correspondence: Dr. John T. McDevitt, University of Texas at Austin, Department of Chemistry and Biochemistry, 1 University Station - A5300, Austin, TX 78712. Voice: 512-471-0046; fax: 512-232-7052.
mcdevitt@mail.utexas.edu
[i]Current affiliation.
[j]Affiliation at the time the work was performed.

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a significant advancement in salivary diagnostics. One such promising technology is the recently described lab-on-a-chip (LOC) assay system, in which assays are performed on chemically sensitized beads populated into etched silicon wafers with embedded fluid handling and optical detection capabilities. Using this LOC system, complex assays can be performed with small sample volumes, short analysis times, and markedly reduced reagent costs. This report describes the use of LOC methodologies to assess the levels of interleukin-1β (IL-1β), C-reactive protein (CRP), and matrix metalloproteinase-8 (MMP-8) in whole saliva, and the potential use of these biomarkers for diagnosing and categorizing the severity and extent of periodontitis. This study demonstrates that the results achieved by the LOC approach are in agreement with those acquired with standard enzyme-linked immunosorbent assay (ELISA), with significant IL-1β and MMP-8 elevations in whole saliva of periodontitis patients. Furthermore, because of the superior detection capacities associated with the LOC approach, unlike those with ELISA, significant differences in CRP levels between periodontitis patients and normal subjects are observed. Finally, principal component analysis (PCA) is performed to yield an efficient method to discriminate between periodontally healthy and unhealthy patients, thus increasing the diagnostic value of these biomarkers for periodontitis when examined with the integrated LOC sensor system.

KEYWORDS: lab-on-a-chip; salivary diagnostics; inflammation; biomarkers; periodontitis

INTRODUCTION

Periodontitis is a chronic inflammatory disorder of the tissues supporting the teeth and represents one of the most widely distributed and prevalent microbial diseases of humans. Current figures estimate that 10–15% of adults worldwide have advanced periodontal disease.[1] Although implementation of preventive measures during the past 30 years has significantly improved the oral health of many individuals, periodontal disease still contributes to widespread oral health dysfunction and increased susceptibility to other systemic health risks.[2–4]

The World Health Organization (WHO) recently published a global overview of oral health, and described its approach to promoting further improvement in oral health during the 21st century.[1] The report emphasized that despite great improvements in the oral health status of populations across the world, major problems still persist for historically under-served groups in both developed and developing communities. The WHO recognizes oral health as an integral part of general health, as recent data support that chronic infection of the periodontium with chronic stimulation of inflammatory responses contributes to systemic sequelae, such as preterm delivery of low-birth-weight babies, cardiovascular disease (CVD), and diabetes.[5–7] Indeed, numerous case–control and cohort studies have indicated that patients with periodontitis have an increased

risk of CVD, that is, acute myocardial infarction (AMI), stroke, and peripheral arterial disease, when compared with subjects with healthy periodontium.[8–11]

Although the diagnosis and treatment of periodontitis has historically focused on mechanical approaches, research in molecular mechanisms and the outcomes of the Human Genome Project have provided increasing information demonstrating the specific biological pathways and biomolecules that could be used as biomarkers for risk assessment, diagnosis, and prognosis. Obviously, saliva, because of its locality, represents a key and very relevant diagnostic fluid for periodontal disease. Many important biological substances including electrolytes[12,13] drugs,[14–20] proteins (e.g., cytokines, hormones, enzymes),[21–24] antibodies,[25–27] microbes,[28–30] and RNAs[31–34] have been identified in saliva. However, significant correlates that could be used as adjunctive diagnostic/prognostic information by clinicians remain elusive. In contrast to medicine's rapid use of point-of-care (POC) diagnostic devices with focus on biomarkers, or risk factors, in serum, urine, and cerebrospinal fluid, progress in oral health studies targeting the use of saliva as a diagnostic fluid for local and/or systemic diseases has been slow. Impediments to the use of oral fluids have been the relatively low concentration of various important biomolecules in saliva, compared with serum or plasma, and the lack of sufficiently sensitive and simple assays and equipment that could be used in the dentist's office.

The development and implementation of modern technologies that use specific biomarkers at the "chair-side" are likely to increase clinical diagnostic and prognostic insights. Their application in broad health-care settings and in populations that lack access to necessary medical and dental infrastructure should be a substantial benefit to public health. In particular, biomarkers used for the diagnosis of periodontitis should lead to early identification with increased potential for referral, early intervention, and prevention that could contribute to improved overall oral and systemic health.

Tremendous advances have been made recently in the area of lab-on-a-chip (LOC) devices exploiting the advantages offered by miniaturization, such as reduced reagent and sample volume requirements, rapid analysis times, and cost-effective assays that can be operated with fewer technological constraints, making them amenable to POC testing. Most importantly, these characteristics, when fully developed into a functional system, have the potential to lead to a significant reduction in the time that is needed for an accurate diagnosis and treatment.

Over the past five decades, the microelectronics industry has sustained tremendous growth and has become what is arguably the most dominant industrial sector for our society. The availability of powerful microfabrication tools based on photolithographic methods that can be used to process these devices in highly parallel manner has led to this explosive growth. Our group has combined and adapted the tools of nano-materials and microelectronics for the practical implementation of miniaturized sensors that are suitable for application to a variety of health-care areas. Importantly, the performance metrics

of these miniaturized sensor systems have been shown to correlate closely with established macroscopic gold-standard methods, making them suitable for use as subcomponents of highly functional detection systems for analysis of complex fluid samples. These efforts remain unique in terms of functional LOC methods having a demonstrated capacity to meet or exceed the analytical characteristics of mature macroscopic instrumentation for a variety of analyte measures, including pH, DNA oligonucleotides, metal cation, biological cofactors, and inflammatory mediators.[35–47]

These LOC methods offer the ability to perform multiplex assays in small sample volumes, with enhanced sensitivity, thus making them amenable to applications involving a variety of bodily fluids, including saliva. Salivary biomarkers that were previously undetectable by standard methods can now be targeted to assess periodontal disease in a noninvasive fashion. Here, we report the application of the LOC system for the concomitant measurement of salivary biomarkers C-reactive protein (CRP), matrix metalloproteinase-8 (MMP-8), and interleukin-1β (IL-1β) as related to the clinical expression of periodontitis. Our results suggest the LOC approach is suitable for the detection of three important biomarkers in saliva, which makes this technology relevant to the diagnosis, staging, and management of periodontal disease.

METHODS AND MATERIALS

Patient Populations and Collection of Salivary Fluids

Orally healthy and periodontitis patients were recruited from the population at the University of Kentucky College of Dentistry. The protocol and consent forms were approved by the Institutional Review Boards of the University of Texas at Austin and the University of Kentucky. Subjects included were $\geq$18 years of age, had a minimum of 20 teeth, and no medical history of chronic illnesses (e.g., diabetes, rheumatoid arthritis).

Two groups of adult patients matched by race and age participated. The first group consisted of 29 normal healthy volunteers without any clinically detectable periodontal lesions. Specifically, this group of patients had at least 20 teeth with fewer than 10% of gingival sites with bleeding on probing (BOP), no probing pocket depths (PD) $\geq$5 mm, $<$1% of interproximal sites with clinical attachment loss $>$2 mm, and no evidence of radiographic bone loss as determined by posterior vertical bitewings films, and no PD sites greater than 5 mm. The second group was derived from 28 patients with moderate-to-severe periodontal disease. This group of patients had noticeable loss of connective tissue attachment and bone around the teeth in conjunction with the formation of periodontal pockets due to the apical migration of the junctional epithelium. Specifically, this group comprised individuals with at least 20 teeth and $>$30% of gingival sites with BOP, $>$20% probing depths $\geq$4 mm, and

>5% of interproximal sites with clinical attachment loss (CAL) of >2 mm, and evidence of radiographic bone loss as determined by posterior vertical bitewings films.

Unstimulated whole saliva was collected from each of the subjects as described previously.[48] In brief, after the mouth was rinsed with water, saliva was allowed to accumulate in the floor of the mouth for approximately 2 min and was repeatedly expectorated into a test tube to collect 5 mL. Following collection, the samples were aliquoted and immediately stored at –80°C either until evaluated by enzyme-linked immunosorbent assay (ELISA) at the University of Kentucky or transported on dry ice to the University of Texas at Austin for LOC testing.

ELISA Analyses

ELISA analyses of IL-1β, MMP-8, and CRP were performed using commercial kits (Human IL-1β Quantikine kit and Human Quantikine MMP-8 ELISA kit, R&D Systems, Minneapolis, MN, USA and ALPCO, Windham, NH, USA, respectively) and following the manufacturers' instructions. The whole saliva was evaluated in duplicate using a microQuant plate reader using KC4 Kineticalc software for curve fitting and calculation of the levels.

LOC Procedures and Analyses

Bead Preparation, Sieving, Activation, and Antibody Conjugation

Agarose beads used in the LOC system in past studies were purchased from a commercial source.[46] More recently, we have acquired the expertise and an in-house capacity to produce large batches of agarose beads. All the experiments described in this study utilized exclusively beads developed in our laboratories. As the precision of the LOC assays is highly dependent on the size homogeneity of its component sensor beads, the beads are exposed to a sieving process that produces a consistent population of microspheres 280 ± 10 μm. Reactive aldehyde groups within the agarose matrix are generated by mixing gently overnight 1 mL of the sieved beads in a 10-mL solution of 1-M sodium hydroxide containing 20 mg of sodium borohydride and 3 mL of glycidol. The beads are then washed copiously with water and exposed to 0.16-M sodium periodate solution followed with successive water washes. Analyte-specific and control (analyte-irrelevant) antibodies are coupled to the beads by reductive amination, as described previously.[35]

Reagents for LOC Assays

CRP, tumor necrosis factor (TNF)-α, IL-6, myeloperoxidase (MPO), MMP-8, and IL-1β protein standards were obtained from Cortex Biochemicals

(San Leandro, CA, USA), Biomol International (Plymouth, PA, USA), eBioscience (San Diego, CA, USA), Biodesign International (Saco, ME, USA), Chemicon International (Temecula, CA, USA) and BD Biosciences Pharmingen (Chicago, IL, USA), respectively. Capturing and detecting antibodies for CRP assay were from Accurate Chemical (Westbury, NY, USA). Capture and detection antibodies for the TNF-α assay were from R&D Systems (Minneapolis, MN) and Sigma Aldrich (St. Louis, MO, USA), respectively. Capture and detection antibodies for the MPO assay were from Biodesign International and Abcam (Cambridge, UK), respectively. Capture and detection antibodies for both the MMP-8 and IL-1β assays were from R&D Systems.

Description of Assay Run on LOC System

Prior to each assay, beads are placed in addressable regions within the array. The bead-loaded chip is encased into the flow cell, which is located at a fixed position with regards to the optical station and the charge-coupled device (CCD). Wash buffer (i.e., phosphate-buffered saline [PBS]), detecting antibody, blocking agent (3% bovine serum albumin [BSA] in PBS), and sample are then primed into the system to minimize the introduction of air bubbles into the analysis area.

During each assay, bead components are first blocked with 3% BSA/PBS, to eliminate nonspecific binding. Protein standards, or the unknown sample, are then delivered into the array for analysis. Following a brief rinse with PBS, the presence of captured analyte on the beads is achieved with an analyte-specific detecting antibody conjugated to AlexaFluor-488® (Molecular Probes). Excess antibody reagent is removed from the flow cell by washing with PBS, and final image of the bead array is acquired with a CCD camera and stored digitally for analysis.

Image and Data Analysis

Digital information from each array/run is obtained using Image Pro Plus software and analyzed with SigmaPlot® (Systat Software Inc [SSI], San Jose, CA, USA). The concentration of the unknown sample is extrapolated from the generated standard curve, as described below.

First, an area of interest is drawn on the periphery of each bead of the array, for each run. The intensity in the green channel for each bead, for standards and unknowns, is measured and recorded as density of green (average intensity per pixel). Data from all beads are then exported to a SigmaPlot® datasheet. The signal intensity obtained from redundant beads of the array is averaged for each assay run. A *Q*-test is then applied to identify and discard outliers. The remaining data are analyzed using a four-parameter logistic equation process within the SigmaPlot® environment to generate a standard, dose-response curve and to predict concentrations of the unknowns.

Statistical Analyses

Evaluation of the analyte levels in saliva emphasized the use of nonparametric Wilcoxon-Mann-Whitney *U* rank sum analyses based upon the small number of samples and the nonnormal distribution of the data. As such, correlations were determined using a nonparametric Spearman rank correlation analysis. Logistic and multiple regressions were performed on the analyte levels in the saliva. All statistical analyses were performed using SigmaStat 3.0 (Chicago, IL, USA).

Principal component analysis (PCA) was performed using Statistica 5.5 (Tulsa, OK, USA). Two principal components were extracted from a total of six measured variables across 48 patients. Patients with incomplete measurements across all six variables were excluded from this study. The raw data were normalized to zero mean and unit variance. Only factors with eigenvalues greater than 1.0 were utilized.

RESULTS AND DISCUSSION

Previously, we have described studies of the design, fabrication, and testing of nano-biochip structures whereby immunoassays are performed on chemically sensitized beads that are arranged in an array of wells etched on silicon wafers with integrated fluid handling and optical detection capabilities (FIG. 1).[35,46] Each bead within the array serves as its own independent self-contained microreactor sensor, with specificity determined by the antibody element that it hosts. The bead-loaded nano-biochip is sandwiched between two optically transparent poly-methyl-methacrylate (PMMA) inserts, packaged within a casing described here as the "flow cell." The flow cell allows for

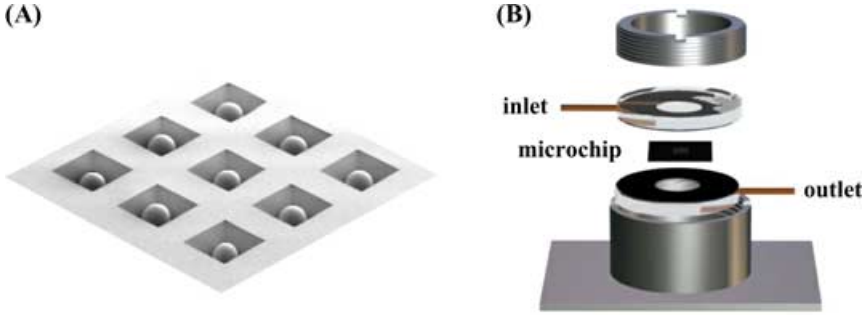

FIGURE 1. A scanning electron micrograph of the assay platform of the LOC system composed of a silicon microchip with a microetched array of addressable wells that host beads sensitized to analytes of interest (**A**). The bead-loaded microchip is sandwiched between two PMMA inserts and packaged within a casing to create the analysis flow cell of the LOC system (**B**).

delivery of sample and detecting reagents to the nano-biochip and the associated beads. Fluids are delivered to the beads via the inlet of the top PMMA insert, soaking evenly the beads located therein, while unspent reagents are directed to a waste reservoir through the bottom drain element of the bottom PMMA element.

In this study, sandwich-type immunoassays are used for the measurement of CRP, IL-1β, and MMP-8 biomarkers on the LOC system, as shown in the immunoschematic in FIGURE 2A. Here, the capture antibody-coated beads are sequentially exposed to the analyte of interest (protein standard or sample) and to a detection antibody conjugated to Alexafluor-488 to produce an analyte/dose-dependent fluorescent signal within and around the bead. The top insert of the flow cell allows for the microscopic evaluation of signals generated within the array, which are subsequently captured by a CCD video chip along with the use of transfer optics (FIG. 2B). Here, after each assay run, the final image of the bead array is captured with the CCD (FIG. 2C), digitally processed and analyzed, and the signal intensity converted for each bead into a quantitative measurement based on the generated standard curve.

Typical dose-response (standard) curves for LOC-based assays for MMP-8, IL-1β, and CRP are shown in FIGURE 3. Highly sensitive immunoassays targeting these analytes were developed to accommodate efficient measurement of diluted saliva samples (1:20, 1:100, and 1:1,000 for MMP-8, IL-1β, and CRP, respectively). With these dilutions, possible interference problems associated with the viscous nature of saliva are thus avoided. Likewise, these assays were designed to provide a useful quantitation range, consistent with the reported pathophysiological levels of these analytes in saliva.[49] Here, the quantitation ranges of the MMP-8 and IL-1β assays (after dilution) were at 20–20,000 ng/mL and 10–10,000 pg/mL, respectively.

As described in a previous report, the detection limit of the LOC-based assay for CRP is significantly lower than that of ELISA, which is the current gold standard for high-sensitivity (hs) measurements of CRP.[46] From a comparison of the two methods, it is clear that the LOC approach yields a five orders of magnitude lower limit of detection than that exhibited by the hsCRP ELISA method. Furthermore, the LOC assay was linear for three orders of magnitude, whereas ELISA was only linear for two orders of magnitude. Here, the LOC assay procedure demonstrates a detection limit at 5 fg/mL and a useful range between 10 fg/mL to 10 pg/mL. Therefore, even with 1:1,000 dilution of saliva samples, the LOC assay affords a useful quantitation range of 10–10,000 pg/mL. In contrast, the hsCRP ELISA method yields a detection limit of 2 ng/mL and a useful detection range between of 2–100 ng/mL CRP.

It is well known that the utility of an assay is directly associated with its capacity to detect low concentrations of the analyte it targets. From this perspective, the diagnostic utility of the LOC assay system was recently demonstrated with the application of ultrasensitive CRP measurements in human saliva from both healthy and periodontal disease patients.[46]

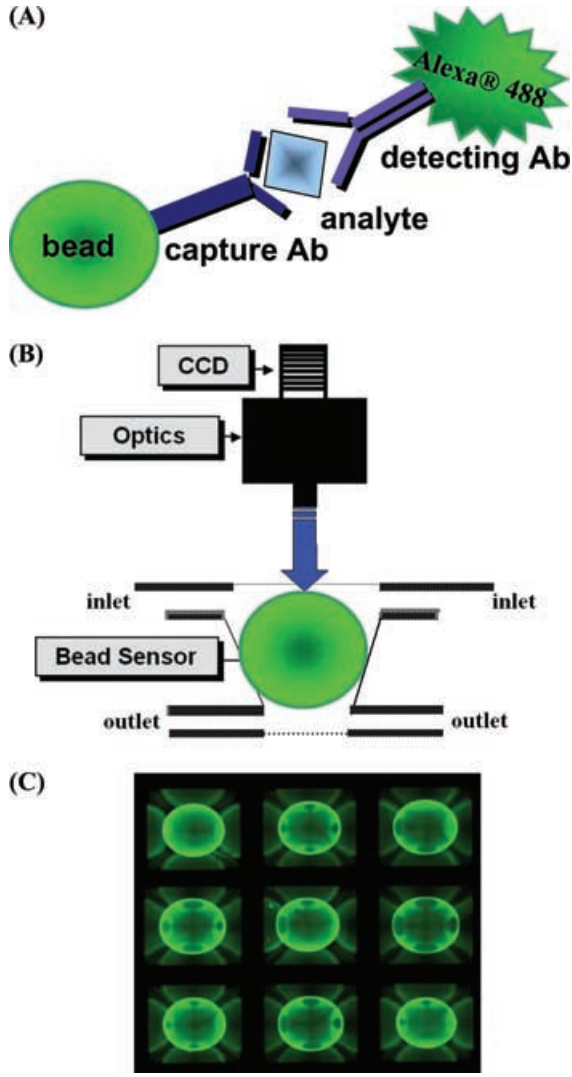

FIGURE 2. The relevant immunocomplexes of the bead-based, sandwich-type of immunoassays on the LOC system are shown in (**A**). Here, an analyte-specific capturing antibody sequesters the antigen on the beads. Detection of the captured antigen is achieved in fluorescence mode with an Alexafluor-488®-conjugated detecting antibody. Completion of assays on the LOC system relies on the integrated function of its microfluidic and optical components (**B**). Here, during each assay, sample and detecting reagents are delivered to the beads via the top inlet of the flow cell, while the bottom drain provides an outlet for the direction of unspent reagents to a waste reservoir. The flow cell allows for the microscopic analysis and capture of signals generated on the array in conjunction with an optical station equipped with a CCD camera. Shown in (**C**) is a typical image of an array of beads captured in the last step of an LOC assay run.

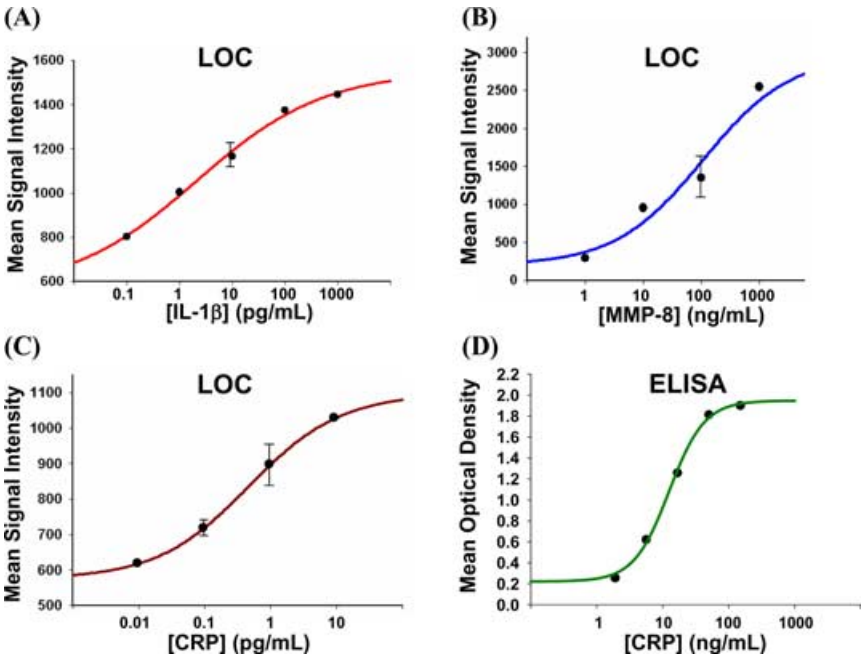

FIGURE 3. Dose-dependent curves obtained for IL-1β (**A**), MMP-8 (**B**), and CRP (**C**), as achieved by the LOC method. Shown in (**D**) is a CRP dose-response curve achieved with a commercial high-sensitivity ELISA method.

Having demonstrated the capacity of the LOC system to measure CRP, and use it to discriminate between healthy and periodontitis patients, we next evaluated the capacity of the LOC method to assess multiple proteins as biomarkers of periodontal disease. Here, both LOC and ELISA methodologies were applied in parallel for the measurement of salivary levels of biomarkers CRP, IL1-β, and MMP-8 in healthy and periodontitis patients. Ten patients who had at least 20 erupted teeth provided whole expectorated saliva and were clinically examined for oral disease. Subjects ranged in age from 29 to 58 years. The study group with oral disease was homogeneous for type II (moderate) periodontitis and was demographically similar to the nine healthy controls. Concentrations of IL1-β and MMP-8 in whole expectorated saliva were successfully measured by both systems. Salivary levels of IL-1β and MMP-8 were higher in subjects who had periodontitis compared with healthy controls, using both LOC and ELISA methods (FIG. 4). Here, salivary levels of IL-1β in the periodontal disease group were 2.6 times higher (as measured by LOC) and 1.5 times higher (as measured by ELISA) than the controls. Salivary MMP-8 levels were 2.0 times higher (as measured by LOC) and 2.6 times higher (as measured by ELISA) in the periodontal disease group compared with the healthy

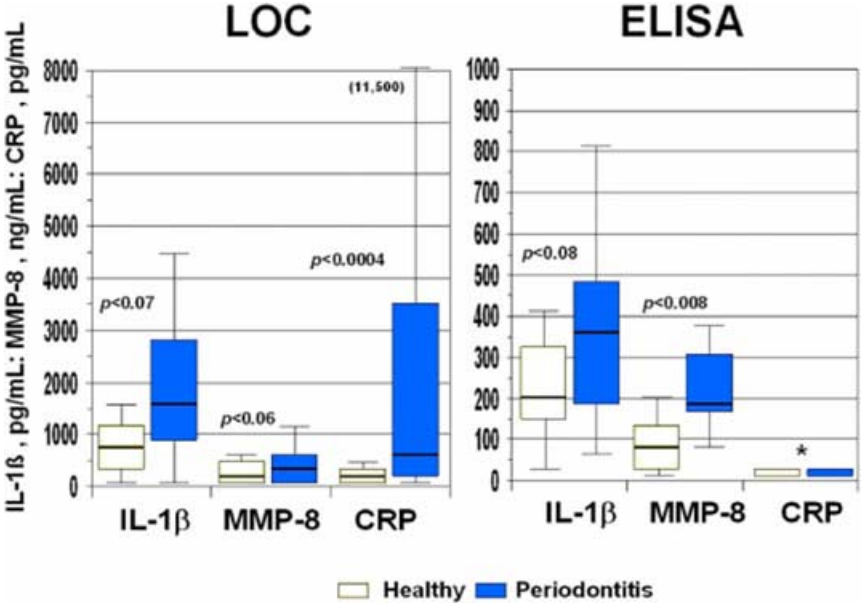

FIGURE 4. Comparison of (**A**) IL-1β (pg/mL), (**B**) MMP-8 (ng/mL), and (**C**) CRP (pg/mL) levels between healthy and periodontitis groups, as achieved by LOC and ELISA methods. The *bars* denote the 95% confidence interval, the *horizontal line* denotes the median of the data, and the *vertical brackets* provide the range of values for each mediator. *Salivary CRP levels were below the level of detection of ELISA.

controls. Inasmuch as salivary CRP levels were below the level of detection of high-sensitivity ELISA, only the LOC approach measured CRP successfully in both groups. Consistent with our previous report,[46] whole saliva from patients with periodontitis contained 18.2 times higher CRP levels than those of healthy patients.

The diagnostic value of strategic biomarkers of periodontitis is expected to increase significantly when these biomarkers are evaluated in a multiplexed manner. Multiplexing is consistent with miniaturization efforts and a multimarker screening strategy, which target a reduction in sample and reagent volumes, and assay time. From a diagnostic point of view, multiplexing offers the opportunity to eliminate the main disadvantage of parallel testing in lowering costs, while also improving turnaround time, usually an issue for sequential testing. Further, this approach promotes monitoring of multiple parameters in a simultaneous manner with the generation of large amounts of complex data that no longer necessarily behave in a linear way. The unique opportunity to probe for several markers as a multivariate observation improves diagnostic efficiency by minimizing the tradeoff between high specificity and sensitivity characteristic of combinations of parallel or sequential testing strategies.[50]

The nonlinear relationships in the data can be deciphered and thus generate patterns, or fingerprint, of disease, which can be expanded to include both biomarker and physical data.

Previous work from Diehl *et al*.[51] has shown that it is possible to apply PCA and discriminant function analysis (DFA) to classify quantitative measurements of periodontitis based on the evaluation of physical parameters. PCA is an unsupervised mathematical method used to simplify a multivariate dataset by choosing a new coordinate system that maximizes the variance represented by the initial dataset. Rather than being limited to the graphical representation of two or three different variables, PCA can be used to effectively represent the information from multiple variables in a two-dimensional plot.

In order to take full advantage of the multianalyte testing capacity of the LOC method, PCA was used here as a multivariate analysis method to discriminate between healthy and periodontitis patients using data aggregated from both physical parameters and biomarker measurements. PCA was applied to a large dataset that included clinical measures of percent BOP, percent CAL >2 mm, percent PD >4 mm, and the recently described biomarkers MMP-8, IL-1β,[49] and CRP.[35,46] For this study, MMP-8 and IL-1β were measured with ELISA, while CRP was measured by means of the more sensitive LOC system. FIGURE 5 shows a principal component plot using all six variables with complete separation between healthy ($N = 24$) and periodontitis patients ($N = 22$). The tight

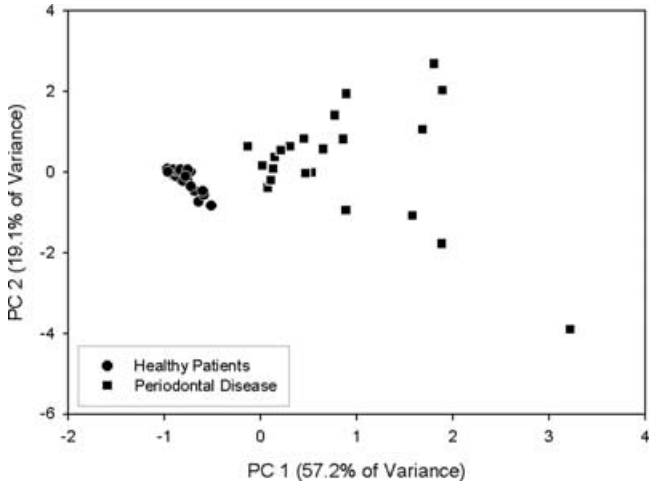

FIGURE 5. A principal component plot of 46 patients showing distinct clustering of healthy patients from the periodontal disease population. Two factors were extracted to account for 76.3% of the total variance. For the principal component analysis, a total of six variables was used including percent of teeth with probe depth greater than 4 mm (% > 4 mm), percent of teeth with blood on probe (% BOP), percent of teeth with CAL >2 mm (% CAL > 2 mm), IL-1β, MMP-8, and CRP.

grouping of healthy patients is indicative of minimal distribution of measured values, while the scatter in the unhealthy patients may be indicative of additional, unclassified subgroups. Work is currently under way to see whether additional clusters can be identified in the diseased population to characterize systemic versus localized disease and to identify the different stages of periodontitis.

In addition to being able to discriminate between different patient populations, it is possible to assign a loading score to each marker that is indicative of its contribution to the magnitude of variance in the dataset. For two well-separated groups, the loading value can be used as one potential indicator of the diagnostic efficacy of that biomarker. Aided by this information, a subset of biomarkers can be chosen for effective diagnosis. Here, CRP, IL-1β, MMP-8, as measured by LOC and ELISA, and oral physical parameters were analyzed with PCA to rapidly identify the biomarkers with near-perfect discrimination between healthy and unhealthy patients. These initial successes bode well for the future use of such methods for the classification of patient samples.

Having demonstrated the capacity of the LOC approach to measure and differentiate three biomarkers of periodontal disease in saliva, the expansion to a larger panel of analytes was initially attempted with a multiplexed assay for six biomarkers, CRP, IL-1β, IL-6, MMP-8, MPO, and TNF-α, in phosphate buffer. Prior to attempting simultaneous detection of all six analytes, each analyte is first captured by its specific bead and then detected individually with a cocktail of antibodies specific for all six antigens (FIG. 6A). Here noted is the absence of signal on control (CTL) beads and of any "cross talk" between irrelevant bead sensors and detecting reagents. In a separate assay run, requiring the same amount of time and reagents as the individual test, all analytes are detected concurrently from the same solution without an apparent loss in signal or a significant increase in nonspecific background level (FIG. 6B). These results demonstrate the capacity of the bead array system to detect simultaneously six biomarkers, all of which have been shown to be associated with periodontal disease.[10,46,49,52–54] Our team is currently working on augmenting further the LOC salivary analyte panel with inclusion of additional biomarkers for both local and systemic disease.

CONCLUSIONS

In conclusion, the selection of salivary biomarkers for the diagnostic assessment of chronic inflammatory diseases, including periodontal disease, from the array of potential markers, has benefited from use of evidence-based information describing biochemical, physiological, and immunological phases of inflammation and tissue destruction. To be useful, salivary biomarkers must be accurate, biologically relevant, discriminatory, and at measurable concentrations. The development and implementation of modern technologies that

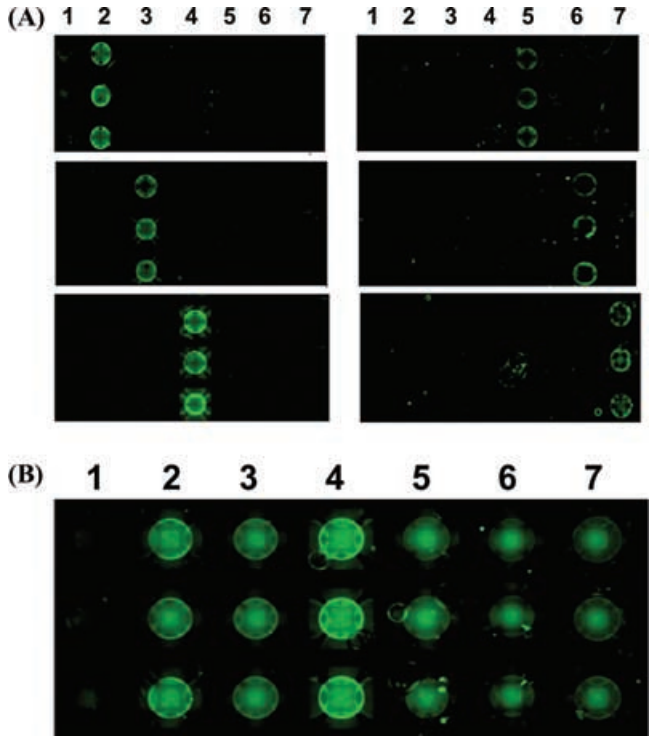

FIGURE 6. Expansion of the multiplexed panel of LOC system to six analytes. Detection of CRP, TNF-α, IL-6, MPO, MMP-8, and IL-1β protein standards is shown first in a single-analyte assay format (**A**). Here, an array of beads sensitized to the six analytes, as indicated below, is exposed to six assay runs. A different analyte is introduced to a fresh array of beads during each assay run. The bead-captured analyte is then probed with a cocktail of detection antibodies. Note the absence of signal on control (CTL) beads loaded with an irrelevant antibody to the analyte, as well as the absence of "cross talk" between irrelevant bead sensors and detecting reagents. The simultaneous detection of all six analytes accomplished in one assay run is shown in (**B**). Here, the bead array is sequentially exposed to a solution containing a mixture of the six analytes and to a cocktail solution of detecting antibody probes specific for all six analytes. The positioning of each bead type (with a threefold redundancy per column) in the array is as follows: column 1, CTL beads; column 2, CRP; column 3, TNFα; column 4, IL-6; column 5, MPO; column 6, MMP-8; and column 7, IL-1β.

use specific biomarkers at the chair-side appear to be on the horizon and are likely to increase clinical diagnostic and prognostic insight. In this study, the LOC methodology is shown to serve as a useful analytical tool that promises to extend the potential diagnostic value of salivary biomarkers of disease. Future opportunities that are afforded through the marriage of the bead-based LOC system with salivary diagnostics are promising. Indeed, attractive goals for

future research include the technological abilities to evaluate both local and systemic diseases from their onset and progression and the ability to influence treatment outcomes through noninvasive means.

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