Development of a salivary blood contamination nano-bio-chip test targeting transferrin – a putative biomarker of periodontitis

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Honors Thesis Spring 2009

Dean's Scholars Honors Program

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May 2, 2009

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Abstract

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Title: Developing a salivary blood contamination nano-bio-chip test targeting transferrin – a putative biomarker of periodontitis

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With the recent development of salivary biomarker diagnostic tests for clinical settings, the understanding of protein contribution measurements of serum to saliva is crucial for accurate clinical analyses. In this study, the development of a nano-bio-chip (NBC) immunoassay that quantifies transferrin in saliva, a protein found in significantly higher concentrations in the serum than saliva, was established. This transferrin NBC assay demonstrates a high level of precision, accuracy, and sensitivity and correlates well with the current gold standard tests, such as enzyme-linked immunosorbent assay (ELISA). In addition, data from the transferrin NBC test indicate that individuals with periodontal disease exhibit a higher salivary concentration of transferrin than healthy controls. Therefore, transferrin, as measured by the NBC-relevant test, may be useful as a biomarker of periodontal disease and as a protein indicator of the level of serum contribution to salivary measurements.

Contents

Background

Clinical testing commonly includes the drawing of blood to determine protein concentrations in serum by a laboratory technician. These levels of proteins provide a picture to the clinician about the body's internal condition and are used for patient diagnosis. If liver cells are damaged from overuse such as during alcohol abuse, the liver cell walls are weakened and release the protein, alanine transaminase, into the blood. Alanine transaminase is usually found inside healthy liver or heart cells. The elevation of alanine transaminase in the blood compared to normal human levels would indicate possible liver damage and would warrant further testing. Proteins like alanine transaminase are not only useful because of their role as an indicator but often when grouped together with other proteins they can be used to detect the possible presence of certain diseases. In this second role, the protein is called a biomarker. Clinical testing has used blood as the agent primarily for determining biomarkers and proteomic measurements; however, saliva has proven to be an equally useful biological tool.

Much of the saliva utility is due to its likeness to blood.[1,2,3] Blood is composed of mostly plasma, which contains 90 percent water and dissolved proteins, electrolytes, carbohydrates, minerals, and fats. It also contains cell and cell fragments which are categorized into erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). It is also worth mentioning that some tests specify that serum was used and not whole blood. Serum refers to whole blood without the clotting factors. Similarly, saliva is composed of mostly water and then dissolved proteins, electrolytes, carbohydrates, minerals, and fats.[6,7,8] In effect, saliva can be considered a dilute version of the plasma found in blood.

Immunoassay techniques detect and quantify biomolecules, most typically protein, and use molecules from the immune system, namely antibodies, in a very highly specific and sensitive fashion. The enzyme-linked immunosorbent assay (ELISA) is one type of common technique, which uses antibodies produced from B lymphocytes of the humoral component of the immune system, as opposed to the T lymphocytes in the cellular component. These antibodies bind to foreign bodies, tagging them for removal. This two-component system is found in vertebrates and more complex invertebrates and both respond to antigens.

Macrophages consume antigens and present it on the cell surface, stimulating the production of antibodies by B cells. B cells are specific to one type of antibody. The types of immunoglobulins vary depending on what its function is. These types are designated by a letter (example IgG). Antibodies contain a variable region, which provides specificity for a certain antigen. This variability is derived from exon shuffling during the production of the antibody. The different combination of splicing result in a high yield of varieties for different types of antigens. They also contain a constant region that is specific for a certain specie.

Antibodies can be either poly- or monoclonal. Polyclonal antibodies are derived by a population of B-cells that bind to different epitopes (binding sites) on the antigen. This procedure takes several days and depends on the large influx of antibody production from a second injection of the antigen. The first injection creates a memory file in the animal so a second injection receives a larger and quicker response. Monoclonal antibodies stem from a single b cell. All resulting antibodies are clones using cancer cells for replication. While

polyclonal is more cost efficient, it provides less specificity and more variability between different lots of product.

During an ELISA, antigen is bound to a surface. If antigen is introduced, it then binds to the antibody. A detecting antibody with a covalently attached enzyme catalyzes a reaction to indicate concentration. Between a direct, indirect, and sandwich ELISA, the indirect ELISA depends on adsorption of the antigen onto the plastic mictrotiter plate. BSA or blocking agent barricades all remaining active spots. Then a primary antibody incubation tags the bound antigen. For example a rabbit antibody could be used. Then, a secondary antibody attaches to the primary with a HRP enzyme attached. When incubated with ABTS and hydrogen peroxide the resulting product can be measured at 414 nm. The coloration indicates concentration of the antigen. ABTS absorbs at a different wavelength (340nm) so does not interfere.

This ELISA is used to quantify antigen and measure antibody in serum. It is versatile, simple, sensitive, and easily quantified, making it ideal. In order to use it, though, a calibration curve must be set up with known quantities of antigen. This curve is then used to compare with unknowns.

Antibodies contain specificity within a given species. For example, a goat anti-mouse antibody indicates that the antibody will bind all mouse antibodies. This type of antibody that has been tested for specificity of a certain analyte is useful for human samples as this antibody would not normally bind to human antibodies.

Salimetrics is an example of a company that currently creates saliva-based tests used to measure protein concentrations. Its platform consists of an Enzyme-Linked ImmunoSorbent Assay (ELISA) that uses antibodies, highly variable proteins made by the B

lymphocytes in the immune system. These antibodies are originally made to tag foreign objects called antigens inside the body for elimination. These tests allow the technician to perform at most 90 simultaneous tests (assuming that the dose response has six point and is completed without replication), however, tests takes typically several hours when incubation time is factored. It also requires an expensive and bulky plate reader. [8]

The McDevitt laboratories have worked with nanotechnology for the last ten years to develop miniaturized sensors called nano-bio-chips(NBC) sensor systems that are suitable for applications such as salivary diagnostic.[9] These devices are fabricated using the same proven methods implemented to make electronics and have shown to be sensitive enough to correlate well with the current gold standard techniques. However, this particular NBC system is unique compared to other Lab-on-a-chip (LOC) initiatives in that it has already demonstrated the ability of exceptional analytical capacity for a range of analytes. Incorporation of the NBC with other aspects of diagnostic testing is promising for use with fluidic patient samples.

The nano-bio-chip is a hand-held device that integrates various laboratory techniques and machinery into a credit card sized instrument containing a selective membrane or an approximately 1 by 1 cm silicon chip. Very small amounts of patient sample are needed to obtain necessary data. This chip is then inserted into a toaster-sized machine that measures fluorescence using microscope optics and flow cytometry. Due to its size and ease of use, the lab on a chip is appropriate for point of need settings. The hand held glucometers that diabetic patients use may be likened to it.

The NBC system has many redeemable qualities compared to similar assays that make it practical for use in the clinical setting. NBC assays are commonly less than 15

minutes, which is considered rapid compared to ELISA testings that may take over two hours. It also requires a small volume of sample and reagents (in the magnitude of less than 100 ul) without compromising accuracy. This is also comparable to ELISA, which often uses sample sizes in the microliter range. The test itself is a miniaturized assay that is portable and convenient for users. Patients would be able to determine multiple test outcomes at the bedside in minutes. Due to the lower limits of detection, assays can be optimized so they have a higher sensitivity compared to leading ELISA tests. As a result, the NBC system is compatible with non-invasive strategies such as saliva that allows for better patient comfort. The bead-based system also allows for multi-analyte screening, and thus, comprehensive testing. Each chip has the capability of containing up about 20 beads. Each bead could contain antibodies for a different test, and so 20 tests would be performed simultaneously from the same sample. The outcome is a literal finger print of the patient's health at that moment and would allow for more accurate and thorough clinical analysis and determination. Lastly, the NBC platform is a modular sensor and thus, has applications in proteomic, genomic, and cellular fields. The NBC has been used to proven successful in detecting proteins such as C-Reactive Protein (CRP) and cells such as CD4+ cells for HIV detection. These advantages point to the wide possible applications of the NBC assay and its usefulness in the medical field.

Current studies in the McDevitt laboratories indicate that the NBC saliva-based biomarker tests may be suitable for use as a rapid screening method for diagnostic and prognostic cardiac emergencies. This means that an AMI patient may have a cardiac biomarker screening performed in the ambulance with results ready for analysis by the time he or she reaches the emergency room at the hospital. This type of data is valuable for

medical decisions and the faster the physician is able to understand the patient condition and begin the necessary procedures, the less damage to the vital heart tissue the patient will endure. However, in order to use saliva as a diagnostic fluid for systemic disease in the clinical settings, the contribution of blood to the measurements of protein content in saliva must be understood. For example, the high concentration of protein in blood could possibly pollute the concentration of the salivary proteins producing false positives.[10] This type of measurement is of particular concern to those patients that may have poor oral health. An oral disease such as periodontitis, certain infectious diseases such as HIV, and behavior such as smoking all can contribute to the state of the individual's oral health.[11, 12] Thus, a blood contamination companion test was needed for biomarker panels such as the cardiac enzyme described above in order to measure the amount of blood leakage into the oral cavity.

Transferrin is an iron carrying protein normally found in high concentrations (>1000mg/L) in the blood and low concentrations in saliva (<5mg/L). According to Salimetrics elevated levels greater than 10 mg/L correlated with an increase in three other proteins found in low concentrations in saliva, higher concentration in the blood: cortisol, dehydroepiandrosterone (DHEA), and testosterone. Thus, levels of 10mg/L were considered an adequate amount of transferrin to constitute a high amount of blood contamination. The Salimetrics transferrin assay concentration range was from 0.8–66.0 mg/L transferrin. Its high concentration in saliva is predominantly due to the leakage of blood directly into saliva. Thus, an NBC immunoassay for transferrin was developed to measure blood contamination in saliva.

During preliminary testing, a noticeable difference was seen in the levels of transferrin in samples from those with periodontal disease and health individuals. As a result, a study was performed including 48 different patients to see if transferrin could be used an indicator, or biomarker, of periodontitis. Periodontitis may be a particularly relevant disease to analyze because previous studies have shown that patients with periodontitis are at higher risks for cardiac disorders.

Thus, the validation of the NBC trasnferrin immunoassay to detect blood contamination in saliva is described as follows and lucrative data is given to show that transferrin may be found in higher levels in chronic periodontal disease patients than healthy controls.

Materials and Methods

Patient Sample Collection

Approval from the Institutional Review Boards of the University of Kentucky (UK) and the University of Texas at Austin (UT) was granted for the following study. The University of Kentucky College of Dentistry collected unstimulated whole saliva for the periodontitis analysis while the University of Texas at Austin samples were used for NBC transferrin assay validation.

Patients accumulated saliva in the mouth for approximately two minutes, expelling into a test tube until a volume of approximately 5 mL was reached. Samples from the UK were immediately stored at -80°C for no longer than three weeks and then were shipped on dry ice to UT for no more than two days. Upon arrival, samples were centrifuged for ten seconds at 10,000 x g and then aliquoted avoiding any mucous matrices. All samples were then stored at -80°C. Samples from UT were collected and tested on the same day. These samples were filtered and then tested diluted in 1% bovine serum albumin in phosphate buffered saline (PBS).

NBC transferrin assay

Immunoformat

The NBC transferrin is composed of a "sandwich"-type bead-based immunoassay. Thus, a capturing antibody (polyclonal) conjugated to 2% x-linked glycosylated agarose beads is used to bind the target antigen analyte (transferrin), while a detecting antibody (polyclonal) conjugated to a fluorophore (AlexaFluor-488® by Molecular Probes) binds most likely to a separate epitope on the analyte (**Figure 1**).

Reagents

The agarose beads used for the NBC transferrin assay were prepared in the McDevitt laboratory following the same protocol used in previous studies. The transferrin-specific capture and detection antibodies were purchased from Bethyl Laboratories (Montgomery, TX). The transferrin used as the standards was obtained from GenWay Biotech, Inc. (San Diego, CA). The AlexaFluor-488® was conjugated to the detecting antibody using a commercially available AlexaFluor-488® Protein Labeling Kit from Molecular Probes (Eugene, OR). Resulting antibodies were verified using UV light and then stored at -20°C in 10ul aliquots.

Set-up

Design, fabrication, and testing methods for the NBC system has been described in detail in previous reports. The NBC-based transferrin assay is a 2-site immunometric "sandwich-type" test performed on ~280 μm porous agarose beads, positioned in a micro-etched array of wells on a silicon wafer/chip platform (**Figure 1**). Each bead within the array of the chip serves as an independent self-contained "micro-reactor." Included in the array are agarose beads coupled to the antibody that is not specified for transferrin; these beads serve as negative controls. Microfluidic structures included here serve as flow cells that deliver a series of small-volume reagents and washes to the microchip and to each of the beads. The used reagents are pushed to a waste reservoir down the bottom drain element. Images of fluorescent beads are captured with a digital video chip and analyzed to facilitate detection and, ultimately, quantitation of analytes.

The transferrin NBC assay was performed at room temperature under a continuous flow of fluid. PBS is used for an initial wash lasting one minute. Standards or samples were

delivered for three minutes and washed off by a one-minute PBS wash. AlexaFluor-488®-conjugated detecting antibody was introduced for five minutes, followed by a final one minute PBS wash. The total NBC transferrin assay takes eleven minutes.

Analysis

To determine the density of detecting antibody fluorescence from the NBC transferrin assay a software called Image Pro Plus is used to select a specific area of interest (AOI) around the bead following the same steps in previous studies. These intensities are transferred to a data spread sheet where statistical procedures are used to find the average signal intensity according to the intensity of multiple transferrin capturing beads on the same assay. Typically four redundant beads were used per sample or standard. A calibration curve is generated from standard concentrations daily by using a computer software called SigmaPlot. Sample intensity values are then compared to this curve using standard curve fitting protocols to determine the concentration of transferrin.

NBC transferrin assay: characterization

Limit of Detection (LOD)

The LOD for the NBC transferrin assay was determined at the 99.5% confidence level by finding the lowest antigen concentration that provided an average bead signal higher than at least three standard deviations above the mean value recorded for the same assay using no transferrin and only PBS.

Detection Range

The detection range was defined by the NBC transferrin assay's LOD for the lower boundary and the transferrin standard concentration that provided an almost saturated bead signal intensity for the higher boundary (**Figure 2**).

Quantitation Range

The quantitation range of the assay was defined as the linear portion of the dose response curve.

Precision

The intra- and inter-assay precision for the NBC transferrin assay was calculated to describe the precision. The intra-assay precision was found from the amount of agreement of four beads within the same assay run for three runs at two different concentrations. Inter-assay precision was determined by finding the degree of agreement between repeated measurements of an unknown sample. The signal intensities from three runs were compared and precision was calculated as the percent coefficient of variation (%CV).

Linearity

The linearity of the NBC transferrin assay was found by testing serial dilutions of a saliva sample and interpolating the resulting concentrations via dose response curve comparison.

NBC Transferrin Assay: Validation Studies

Methods comparison

The method comparison study was performed by comparing the accuracy of the NBC transferrin assay sample concentrations to those results found using the established commercially-available Salivary Blood Contamination ELISA Kit from Salimetrics, Inc. (State College, PA). Twelve samples were tested using the ELISA and NBC methods. ELISA dose response and samples were tested in duplicate.

Periodontitis Study

Salivary transferrin levels in orally health and periodontal disease patients were tested to determine if a correlation existed with transferrin levels and disease state. The NBC transferrin assay was used to test samples from both types of study groups. One group was comprised of 29 healthy volunteers without any clinically detectable periodontal lesions. All volunteers had at least twenty teeth with less than 10% periodontal sites with bleeding upon probing, less than 2% probing depths greater than or equal to 5 mm, less than 1% of interproximal sites with clinical attachment loss of greater than 2 mm, no evidence of radiographic bone loss as determined by posterior vertical bitewings films, and no pocket depth sites greater than 5mm.

The second group was composed of 19 periodontal patients. These patients had determined 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, they had at least twenty teeth with greater than 30% of periodontal sites with bleeding on probing, greater than 20% probing depths greater than or equal to 4 mm, and greater than 5% of interproximal sites with clinical attachment loss

of greater than 2mm, and evidence of radiographic bone loss as determined by posterior vertical bitewings films.

Results

NBC Transferrin Assay Characterization

The LOD for the transferrin assay on the NBC system was at the low level of 0.2 ug/mL. The detection range of the saliva-based assay was found to be 0.2– 10 ug/mL, while the quantitation range was 0.1-3.0ug/ml. Both intra- and inter-assay precision of the NBC bead-based saliva transferrin assay were determined. Here, NBC transferrin assay exhibits excellent intra-assay precision, in terms of bead to bead variation in signal intensity, at < 0.0647 % CV. The inter-assay precision of the same assay was established at 9.25% CV (**Figure 3**).

Furthermore, the NBC assay for transferrin measurements exhibits adequate linearity with an R^2 value of 0.83111 (**Figure 4**).

NBC Transferrin Assay Validation

Methods comparison studies

The NBC transferrin assay accuracy was determined against the reference ELISA method. The resulting measurements from the two platforms correlated at $R^2 = 0.976$ for 6 samples (**Figure 5**).

Periodontal Disease Study

48 saliva samples of healthy individuals and periodontal disease patients for transferrin were tested on the NBC transferrin assay. As shown in **Figure 6**, the healthy group demonstrated lower transferrin levels with a mean of 2.342 ug/mL compared to the periodontal disease group with a mean of 3.960 ug/mL. Similarly, the same samples were

tested using the Salimetric ELISA kit, and the periodontal group had a mean average of 9.71ug/ml while the healthy group had an average of 3.43 ug/ml. In addition, the ELISA assay efficiently detected differences in transferrin levels between and within each of the groups tested. The transferrin values below 0.5 mg/dl defined the majority (73.9%) of healthy individuals, whereas 60% of PD subjects fell above this line (**Figure 7**).

Discussion and Conclusions

Serum is known to contribute a portion of proteins to saliva. However, levels of serum proteins to the oral mucosa may sometimes be at levels high enough to compromise the actual biomarker concentrations found in the saliva. These instances confound the data used to determine the systemic state of health in a patient and pose an ethical health hazard. Thus, to completely understand indicators from salivary tests, knowledge of the contribution of serum to saliva is imperative to determine the actual biomarker salivary levels. It is possible in some instances to note blood contamination visibly, however, many samples are not visibly obvious and so must be tested by another means.

The NBC transferrin assay developed is efficient and inexpensive and has shown to be suitable for pre-screening of saliva samples to test for blood contamination via characterization and validation. The use of transferrin proved appropriate, as it is a protein found in high concentration in the blood, but low concentration in the saliva. It was also determined that patients with periodontitis exhibit higher salivary transferrin level than the patients who did not have periodontitis. This preliminary data suggests that transferrin may be useful as a biomarker of periodontitis.

In the future, the NBC transferrin assay will be multiplexed in different biomarker panels, starting with that composed of cardiac enzymes, to identify blood contamination in samples and expand the knowledge of variables involved in salivary diagnostic fluids for systemic diseases. [13,14]

Appendix

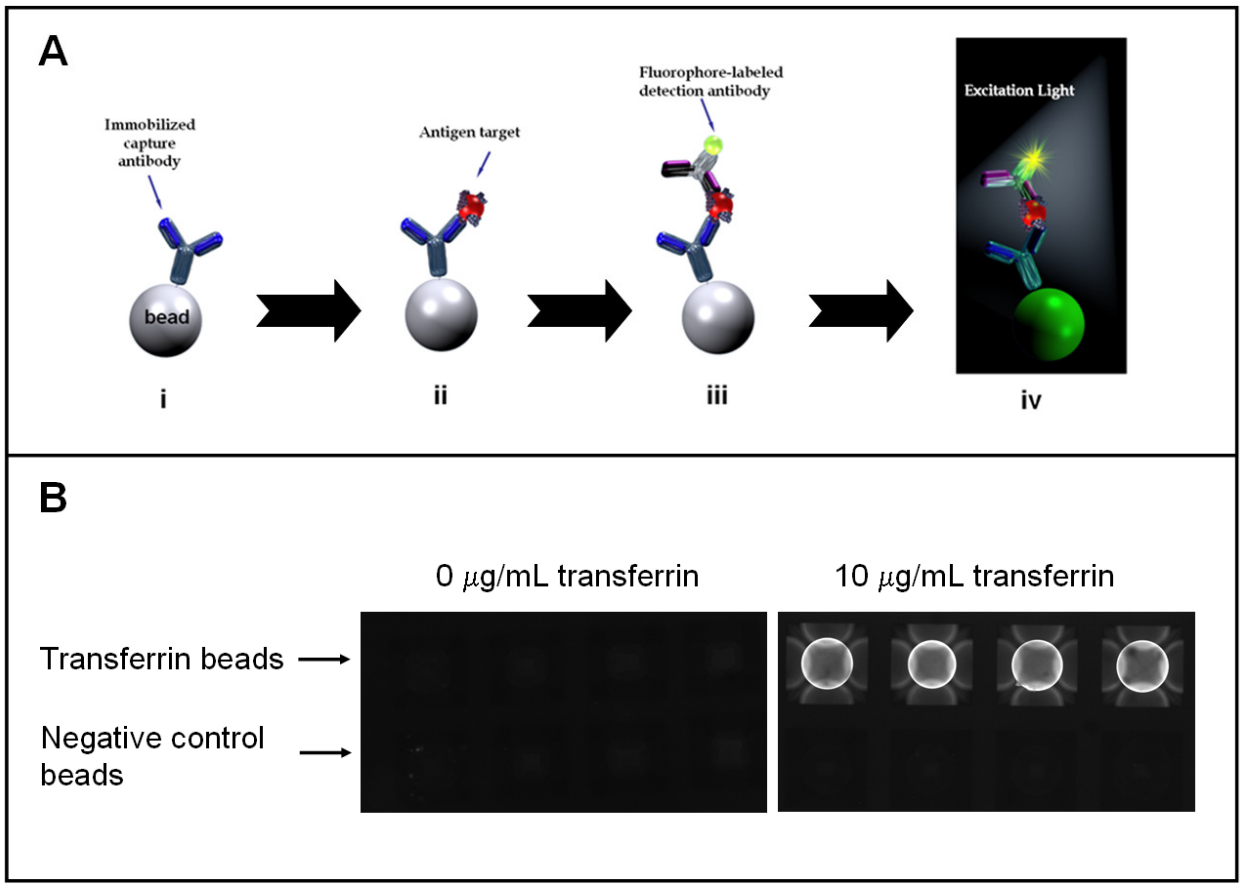

Figure 1. In the NBC structures, "sandwich-type" immunoassays are performed on ~280 μm porous agarose beads arrayed on a microchip. The selectivity of bead sensors is determined by the specificity of the antibody to which they are covalently coupled. The assay steps of a typical NBC immunoassay include the sequential micro-fluidic delivery of antigen (ii) and detection antibody (iii) solutions with an intermittent and final wash steps with phosphate buffered saline (PBS). Images of fluorescent (via epi-illumination) beads are captured with a digital video chip and analyzed to facilitate detection and, ultimately, quantitation of analytes in complex fluids (iv).

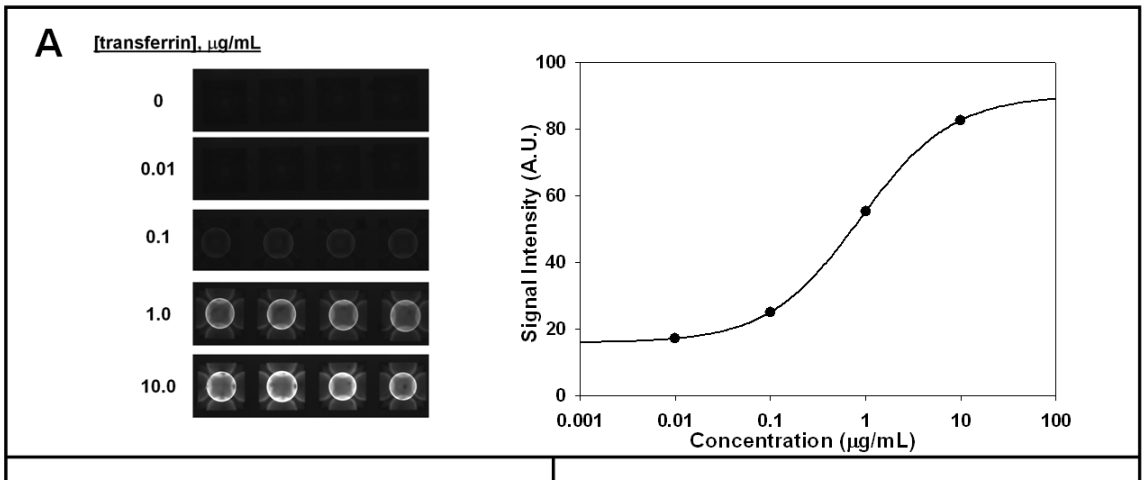

Figure 2. Characterization and validation of NBC-based assays for transferrin. Transferrin capturing bead images are seen on the left hand side while the Transferrin dose response curve on the NBC system is seen on the right.

Conc.	Run	Mean (X)	Std Dev	% CV	$(X-\mu)^2$
1	A	67.4434729	6.8984923	0.102285544	7.96268937
1	B	70.119545	3.18047903	0.045357953	30.22685896
1	C	56.3019295	2.62200844	0.04657049	69.21773478
Data for 1.0 ug precision					
Average Intra-Assay %CV			$\Sigma(\%CV)/n$	0.064737996	
Population Mean		(μ)	$\Sigma(X)/n$	64.62164913	
Population Variance		(σ^2)	$\Sigma(X-\mu)^2/n$	35.8024277	
Population Std Dev.		(σ)	$\sqrt{\sigma^2}$	5.98351299	
Inter-Assay %CV			$(\sigma/\mu)*100$	9.259300978	

Conc.	Run	Mean (X)	Std Dev	% CV	$(X-\mu)^2$
0.1	A	28.677515	0.717916264	0.025034117	1.869322886
0.1	B	27.0316911	0.845437038	0.031275773	0.07761354
0.1	C	26.2216434	1.32413365	0.050497737	1.185136542
Data for 0.1 ug precision					
Average Intra-Assay %CV			$\Sigma(\%CV)/n$	0.035602542	
Population Mean		(μ)	$\Sigma(X)/n$	27.31028317	
Population Variance		(σ^2)	$\Sigma(X-\mu)^2/n$	1.044024322	
Population Std Dev.		(σ)	$\sqrt{\sigma^2}$	1.021775084	
Inter-Assay %CV			$(\sigma/\mu)*100$	3.741356609	

Figure 3. The range of the NBC is greater than that of the ELISA. Inter-assay % CV for a low (0.1 ug/ml) (top chart) and high (1.0 ug/ml) concentration (bottom chart) of transferrin is lower than the ELISA. These values are well within the accepted percent for inter-assay % CV of 20%

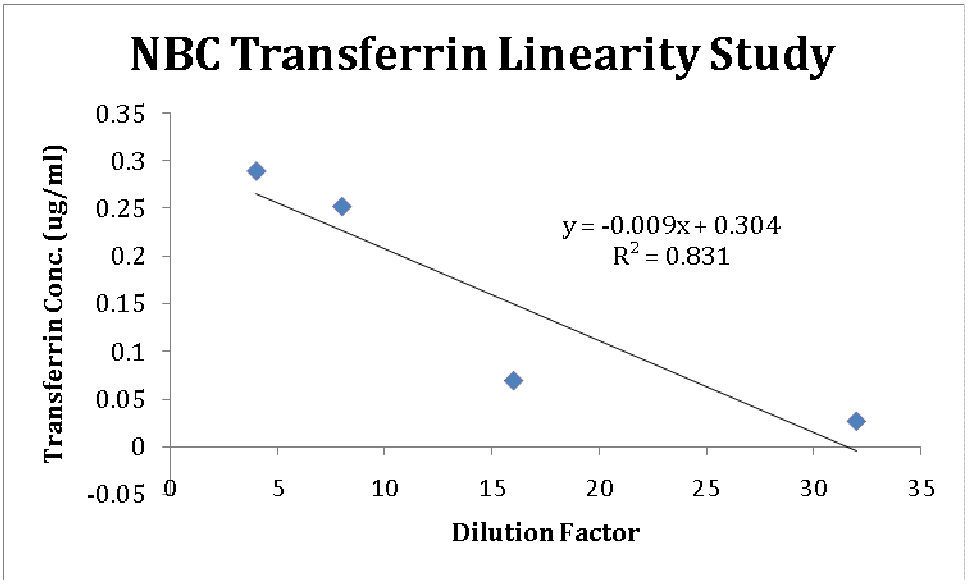

Figure 4. Transferrin linearity study on the NBC system.

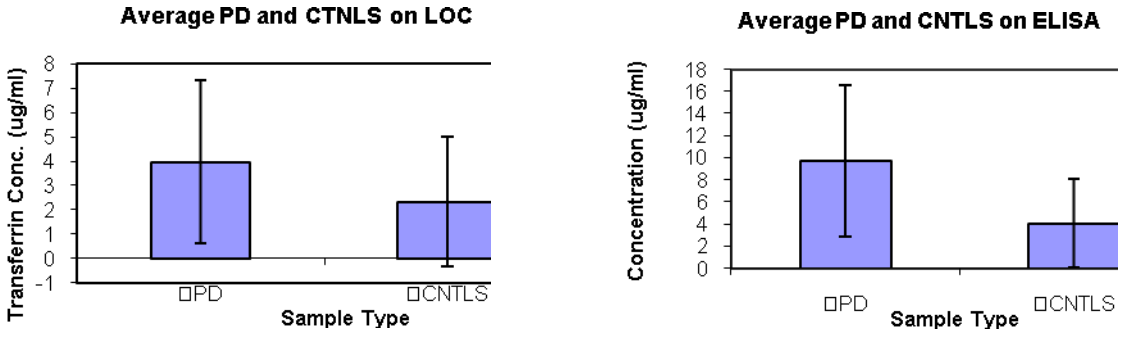

Figure 5. Transferrin levels in 20 periodontal & 23 healthy individuals on the NBC system and ELISA. The higher transferrin salivary level in diseased patients suggests transferrin's possible application as a biomarker of periodontitis

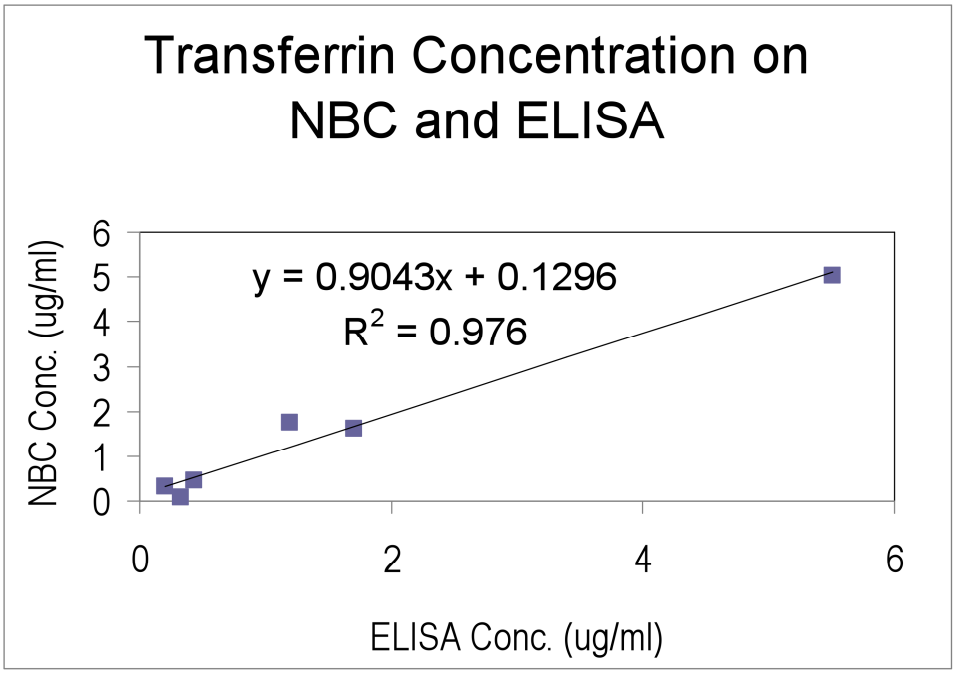

Figure 6. Methods comparison of transferrin samples of known concentration on ELISA and NBC tests. The high correlation value of 0.976 indicate that the NBC is in agreement with those values found in the gold standard ELISA test.

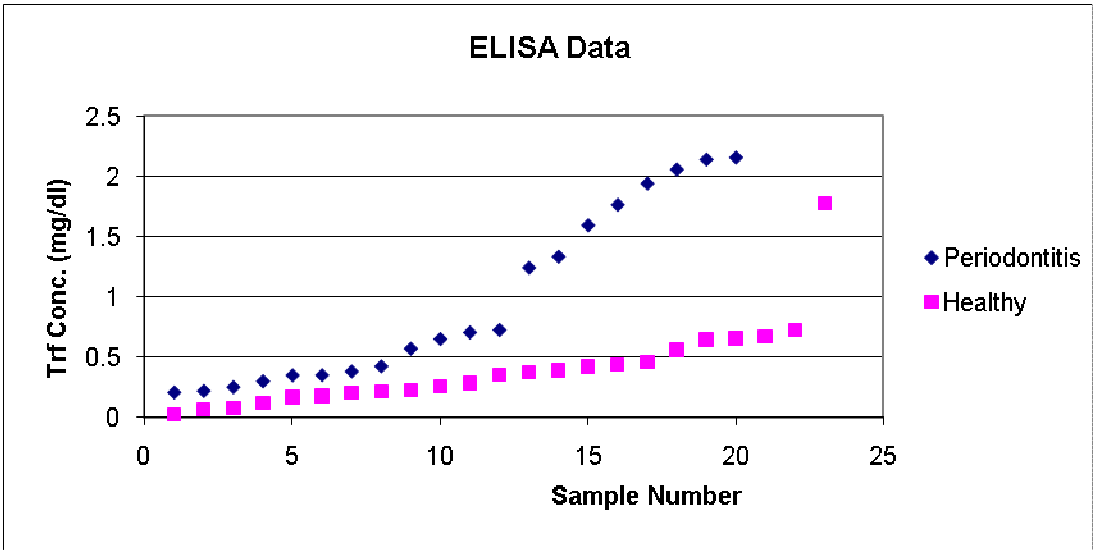

Figure 7. Periodontal studies ELISA data. When 0.5 mg/dl is used as a cut off, the majority of healthy patients are below this boundary, while a majority of diseased patients are above.

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Acknowledgements

Funding for this project was provided by the NIH through the National Institute of Dental and Craniofacial Research (U01DE15017 and U U01 DE017793), General Clinical Research Center at University of Kentucky (M01-RR02602), National Center for Research Resources (P20 RR 020145), and the University of Texas Undergraduate Research Fellowship.

I would like to thank Dr. John McDevitt for not only allowing me to work in his lab but for also taking the time to listen to my ideas and enabling me to merge my Texas Interdisciplinary global health capstone with nano-bio-chip biochemistry research. The overlap of these two projects has made this research that much more valuable to me. I also owe many thanks to Dr. Nick Christodoulides for his on-going, daily guidance and mentorship. Thank you for answering all of my questions and always encouraging my growth as a research scientist. A huge thanks goes to my partner in crime, Eric Yeh, who helped assimilate much of the data. Thank you for your infinite patience and reliable work ethic. I am thoroughly indebted to the entire McDevitt laboratory for all of their assistance and support during my time there. Thank you for always taking the additional time to explain techniques to me and to provide advice.

Many thanks also go to Dr. Jane Richards, Dr. Shelley Payne and Dr. Michael Starbird for guiding me while I wrote this thesis during the past year and for teaching me how to present my research in a way for everyone to appreciate it. Additional thanks goes to Dr. John Stanton for acting as the second reader for this project.

Lastly, thank you to my friends and family for all of the support throughout my college years and especially during these last few months.