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A photonic crystal biosensor assay for ferritin utilizing iron-oxide nanoparticles

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ABSTRACT

Iron deficiency anemia afflicts 1 in 3 individuals, mostly women and children worldwide. A novel application using iron-oxide nanoparticles (IONPs) and a photonic crystal (PC) optical biosensor as an immunodiagnostic platform for detection of serum ferritin, a biomarker for iron deficiency, is presented. Human liver ferritin (450 kDa), clinical serum controls, and three commercially available ferritin ELISA tests were used to evaluate the PC biosensor assay in terms of inter- and intra-assay variability, spike-recovery (%), limit of detection (LOD), and matrix effects on binding. For the PC biosensor, signal response from label-free, sandwich with secondary antibody (pAb), and pAb functionalized with iron-oxide nanoparticles (FpAb) assays were detected using the Biomolecular Interaction Detection (BIND) system. Bland–Altman analysis was used to evaluate agreement between expected values for ferritin in control sera and each of the detection platforms. Inter- and intra-assay variability of the PC biosensor were both $<10\%$. Percent mean recovery ($\pm$%RSD) of ferritin from two control sera samples were 94.3% (13.1%) and 96.9% (7.6%). Use of FpAb in PC biosensor resulted in two orders of magnitude increase in sensitivity compared to label-free assay; capable of measuring serum ferritin as low as 26 ng/mL. In comparison to ELISA tests, the PC biosensor assay had the lowest bias (-1.26; 95% CI [-3.0–5.5]) and narrower limit of agreement (-11.6–9.1 ng/mL) when determining ferritin concentrations from control sera. These proof-of-concept studies support the use of IONPs to enhance detection sensitivity of PC biosensors for determination of biomarkers of nutritional status.

1. Introduction

Iron-deficiency anemia is the most prevalent micronutrient deficiency afflicting 1 in 3 people worldwide; most of them women and children who live in rural areas of developing countries (Black et al., 2008). Iron-deficiency anemia causes deleterious effects on pregnancy outcomes, children's cognitive and physical development, and productivity in working adults (Black et al., 2008). The identification of populations suffering from iron deficiency, however, remains a significant limitation that hinders nutrition and health improvements.

Despite their widespread use and availability, commercial test platforms like enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) are expensive and impractical in field settings considering the high cost of equipment (plate reader, glassware), the need for specialized resources in the field (electricity, radioactive reagents), and the need for highly trained personnel (nurses and technicians) (Nash et al., 2012). On the contrary, robust and field-friendly technologies in biosensing demonstrate potential for point-of-care nutrition diagnostic methodologies.

Photonic crystals are periodic dielectric surface structures, designed to reflect a narrow band of wavelengths when illuminated by a broadband light source (Cunningham et al., 2002). The adsorption of biomolecules on the sensor surface results in an increase in the dielectric permittivity of material in an evanescent electromagnetic field region in the media within ~200 nm of the surface (Arakawa and Kita, 1999), which in turn causes the reflected peak wavelength value (PWV) to shift to a greater value; thus, providing a simple mechanism for biomolecule detection. Unlike ELISAs that use a colorimetric reaction between an enzyme and a substrate to measure analyte concentrations (Voller et al., 1978), and RIAs that determine analyte concentrations based on the change of radioactivity of analyte samples (Marcus and Zinberg, 1975), PC biosensors utilize simple

Abbreviations: Ab, antibody; BIND, Biomolecular Interaction Detection; CI, confidence interval; FpAb, functionalized pAb; IONPs, iron-oxide nanoparticles; LOA, limit of agreement; LOD, limit of detection; mAb, monoclonal Ab; pAb, polyclonal Ab; PC, photonic crystal

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optics and intrinsic physical properties of the analyte as the mechanism of detection. PC detection systems have various diagnostic and screening applications in DNA microarrays (Mathias et al., 2010), cancer cell analysis (Chan et al., 2007), virus detection (Pineda et al., 2009), and pharmaceutical drug screening (Heeres and Hergenrother, 2011). Furthermore, PC biosensors are inexpensively manufactured from plastic materials and incorporated into liquid handling formats such as microplates (Cunningham et al., 2004) and microscope slides (Gallegos et al., 2013) for single-use applications.

One goal of current biomedical and nanotechnology research is to develop biosensor applications for point-of-care diagnostics in field settings (Nash et al., 2012). To achieve field readiness, methods to improve biosensor sensitivity as well as to increase biosensor versatility are needed to detect physiological concentrations of analytes comparable to commercial ELISA and RIA tests, often on the order of 1.0–1000.0 ng/mL. Non-specific binding and inconsistencies in sensitivity due to the proteinaceous nature of complex matrices like whole blood, serum, and plasma limit analysis (Byrne and Diamond, 2006). Current studies aimed at practical applications using optical biosensors acknowledge the difficulty of selective analyte detection in serum (Chung et al., 2005; Kumbhat et al., 2010; Kyprianou et al., 2013) and whole blood (Bonanno and DeLouise, 2007) necessary for point-of-care applications. A promising amplification approach using IONPs has shown to increase the signal-to-noise ratio in surface plasmon resonance optical biosensors in a chocolate bar matrix (Pollet et al., 2011) and serum and stool (Soelberg et al., 2009) matrices. Yet, to our knowledge, no studies with PC optical biosensors have tested IONPs as a method to enhance analyte detection in serum.

In the present work we describe proof-of-concept studies using IONPs to enhance sensitivity for detection of ferritin, a biomarker of iron deficiency anemia, in control serum and quality control samples using a PC biosensor.

2. Materials and methods

2.1. Reagents

3-Glycidoxypropyltrimethoxysilane (GTPMS), NaOH, Tween 20, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, and phosphate buffer saline (PBS) and StartingBlock blocking buffer from Pierce (Thermo Fisher Scientific). Double deionized water (DDW) was used in all experiments.

2.2. ELISA kits, antigen and antibodies (Ab)

Human ferritin ELISA kits were purchased from BioVendor (RCAN-F-4280R), GenWay Biotech, Inc. (GenWay; GWB-F4BE8D), and RayBiotech, Inc. (RayBiotech; ELH-Ferritin-001). Human liver ferritin (US Biological; F4015-21A) and Liquichek™ serum controls (Bio-Rad Laboratories) were dissolved in PBS or BSA to develop standard curves for the BIND and ELISA detection platforms, and act as quality controls for agreement and recovery studies. The monoclonal mouse anti-human liver ferritin Ab (mAb), used as the capture antibody in the BIND assay, were purchased from US Biological (F4015). As the detection Ab in the BIND, polyclonal goat anti-human liver ferritin Ab (pAb) were purchased from US Biological (F4015-17).

2.3. Iron-oxide nanoparticles conjugation protocol

Detection pAb were functionalized (FpAb) to iron-oxide nanoparticles (30 nm) as reported by vendor (Ocean NanoTech, LLC). Aliquots of 0.2 mL of IONPs were combined with 0.1 mL of Activation Buffer. Then, 100 μL of a solution containing 2 mg/ml EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and 1 mg/ml NHS (sulfo-N-hydroxysuccinimide) was added to the IONPs, mixed, and left at room temperature for 5–10 min with continual stirring. The next step mixed 0.4 mL of Coupling Buffer to the activated IONPs and then added at least 1 mg of pAb. Reaction time for conjugation lasted 2 h with continual mixing. Next, 10 μL of Quenching Buffer was left for 10 min at room temperature and then the entire mixture was transferred to a plastic cuvette and 3 mL of Wash/Storage Buffer was added. The cuvette was inserted into a SuperMag Separator™ (Ocean NanoTech, LLC) magnetic separator to allow conjugated magnetic FpAb to separate for 5 h. Liquids were aspirated without taking magnetic contents before FpAb were re-suspended in 3 mL and the last two steps were repeated for higher extraction. Conjugation was verified by gel electrophoresis tests (data not shown).

2.4. PC biosensor and readout system

As described and illustrated in Cunningham et al., 2004, the PC biosensors used in this work are comprised of a plastic replica molded periodic linear grating surface structure that is overcoated with a high refractive index TiO_2 thin film to create a resonant reflection surface that functions as a high efficiency reflector for only a narrow band of wavelengths near $\lambda = 855$ nm when covered with aqueous media. At the resonant wavelength, an optical standing wave is established at the PC surface. Adsorption of biomolecules or iron oxide tags, which have dielectric permittivity that is greater than water, results in displacement of water from the evanescent field region and an increase in the effective refractive index experienced by the optical standing wave. In turn, the augmented refractive index results in an increase in the resonant reflected wavelength from the PC. The PC biosensor structure is fabricated on sheets of plastic film and attached to bottomless standard format microplates. The PWV of the resonant reflection is measured by illuminating the PC at normal incidence with a broadband light source, and measuring the resonantly reflected wavelength with the aid of a spectrometer. Changes in PWV induced by adsorption of biomolecules can be monitored in each well, where the magnitude of the PWV shift can be used to quantify the amount of adsorbed material.

PC microplates (384-well) were purchased from SRU Biosystems, Inc. The Biomolecular Interaction Detection system (BIND; SRU Biosystems, Inc.) was used to measure interactions of ferritin with antibodies. The BIND illuminates the microplate with a broadband light source (λ range 400–700 nm) via an optical fiber positioned below the biosensor microplate. The system contains 8 parallel readout heads, and is capable of measuring the PWV of all 384-well biosensor microplate in ~10 s. The microplate may be re-scanned at preset intervals to generate kinetic plots of biomolecular binding. A ~2 mm diameter region of the biosensor is illuminated. A second parallel optical fiber is bundled with each illuminating fiber to capture reflected light, which is directed into a spectrophotometer. Detailed description of the design and operation of the BIND instrument can be found elsewhere (Cunningham et al., 2002, 2004).

2.5. PC detection procedure

2.5.1. Epoxy-silanization of PC biosensor surface

A 0.1 M NaOH solution was dispensed (20-μL) into the wells of a 384-well biosensor microplate and left to incubate for 1 h at room temperature (23 °C). After incubation, plates were ultra-sonicated (Fisher Scientific Isotemp202 Heater Ultrasonic bath) for 15 min. Wells were then aspirated and dried under nitrogen stream. Next the plate was placed in an oxygen plasma (Planar Plasma System, Texas Instruments Inc.) for 5 min. Then, 2.5% 3-glycidoxypropyltrimethoxysilane and 10 mM acetic acid solution in ethanol of was

added (15 μL) to each well and left to incubate for 1 h at room temperature (23 °C). Finally, wells were aspirated and washed twice with ethanol and dried under nitrogen stream, before they were ready to be assayed.

2.5.2. Capture mAb immobilization

Capture mAb (62.5 μg/mL) was dispensed (15 μL) into all epoxy-silanized wells. The PC microplate was sealed with tape (Pierce, Thermo Fisher Scientific) and left at room temperature (23 °C) overnight. Then, wells were washed with PBS-Tween (0.5%) three times. The PWV corresponding to mAb immobilized on sensor surface was measured relative to baseline. Final mAb concentration in this assay was the result of several tests using different mAb dilutions (data not shown).

2.5.3. Blocking step

Several blocking agents (i.e., BSA, casein, non-protein blocker, ethanolamine, and StartingBlock) were evaluated to limit non-specific antigen binding to the epoxy-silane surface as well as to reduce detachment of blocking molecules. StartingBlock was selected from this group because other blockers such as casein detached from surface to give a negative signal and BSA, ethanolamine, and the non-protein blocker did not effectively prevent nonspecific-antigen binding. Undiluted StartingBlock (20 μL) was pipetted into all wells. PC microplates were incubated for 2 h at room temperature, and then, washed with PBS-Tween three times. The PWV shift was measured relative to baseline.

2.5.4. Antigen preparation

Dose response curves for ferritin were prepared in PBS, BSA, and control serum. For PBS standard curve, ferritin was serially diluted from stock concentrations (4.0 mg/mL) in PBS (137 mM NaCl, 2.7 mM KCl, pH 7.4). Ferritin concentrations ranged from 0.03 to 1.0 μg/mL. To test dynamic range, higher ferritin concentrations were included, 2.0 and 4.0 μg/mL. For BSA response curve, BSA was diluted with DDW to 10 mg/mL and spiked with the same ferritin stock used to make PBS standards. For serum standard curve, Liquicheck™ serum level 3 was diluted in PBS to 10 mg/mL of total protein (based on reported protein content) and spiked with ferritin to reach same concentrations as in the BSA and PBS standard curves. Testing samples (i.e., PBS, BSA and serum) were assayed (15 μL per well) onto PC biosensor. Active binding was measured for 30 min, in 1 min intervals. BioVendor ELISA quality control ferritin was assayed without dilution and had a concentration near 0.35 ± 0.088 μg/mL. This control was also used for accuracy and recovery studies.

2.5.5. Secondary pAb and functionalized pAb (FpAb)

Secondary pAb were used to increase assay sensitivity. Stocks of pAb and FpAb were 5 and 1 mg/mL, respectively. After 30 min kinetic reading of antigen–mAb binding step, either non- or functionalized pAb (250 μg/mL) were dispensed (15 μL) in all wells. Formation of sandwich complexes (mAb–ferritin–F/pAb) was measured for 1 h, in 1 min intervals. Final antibody concentrations in this assay were the result of several tests using different dilutions (data not shown).

2.6. Recovery and accuracy experiments

Based on Liquichek™ manufacturer's reported ferritin concentrations (from 11 different methods of analysis) in each of the three sera levels, we calculated the mean ferritin concentration of all these methods to establish an expected concentration and range for each serum level. Calculated concentrations for each serum level were used as the basis for dilutions and to establish an expected concentration in the analyses of recovery, agreement and accuracy. Liquichek™ levels 1 and 2 were diluted to 10 mg/mL of total protein and spiked to achieve 42–48 ng/mL of ferritin for recovery analysis. Recovery was evaluated using the apparent recovery formula as explained by Burns et al. (2002). Liquichek™ serum level 3 was diluted 10-fold to determine accuracy and agreement of PC biosensor method against reported Liquichek™ ferritin values and ELISA measurements. For evaluation of inter-day and intra-day assay reproducibility in the PC biosensor, all assays were conducted in triplicate, in three separate days, within a 2-week period. Reproducibility within ± 15% RSD was considered appropriate.

2.7. PC biosensor comparison to ELISA

Three commercial ELISAs (BioVendor, GenWay, and RayBiotech) were used as detection platforms to compare against the PC biosensor in terms of detection accuracy, agreement, bias, recovery, dynamic range, limit of detection, inter-assay/intra-assay variability, and matrix effect (i.e. BSA, PBS and serum). Liquichek™ serum controls and BioVendor quality control were tested following the protocols provided by each vendor without modifications.

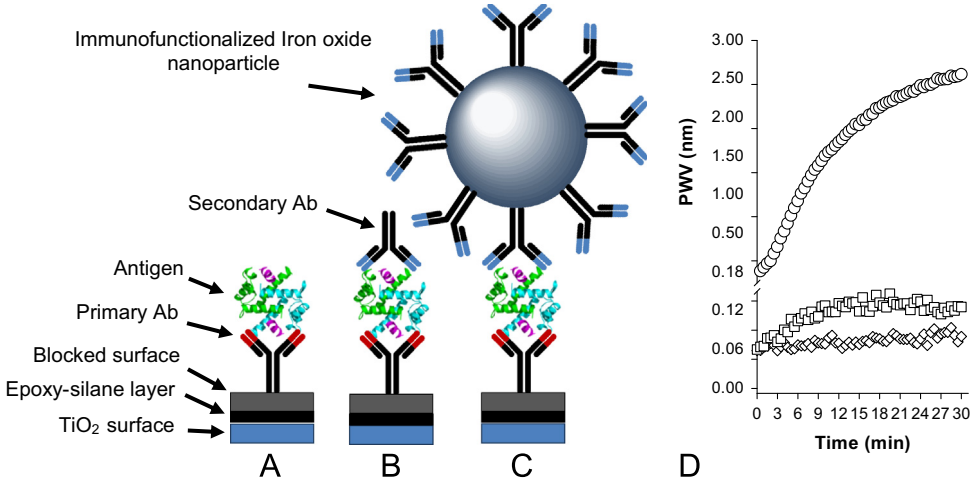

Fig. 1. A schematic of the three immunoassay types on the PC biosensor and their real-time kinetic readings for ferritin (1 μg/mL) in the BIND system. (A) label-free assay; (B) with secondary antibody sandwich assay; (C) immunofunctionalized IONPs with secondary antibodies; (D) kinetic readings: label-free assay (◇), with secondary antibody sandwich assay (□), and immunofunctionalized IONPs with secondary antibodies (○).

2.8. Statistical procedures

Means, standard deviations (SD), replicates (n), confidence intervals (CI), and relative standard deviations (%RSD) presented in figures and tables were calculated using Excel 2008. All figures and linear fitted curves (slope, intercept and determination coefficient) for dose response effects in each diagnostic platform were performed in SigmaPlot (v 11.0, Systat Soft. Inc., San Jose, CA). The limit of detection (LOD) was determined using previously published analytical methods (Armbruster and Pry, 2008). Bland–Altman statistical analysis was employed to determine agreement and bias between each of the methods and reference controls (Bland and Altman, 1986). Mean differences and post hoc analysis were carried out using paired t-test and Tukey honest significant difference test, respectively, in SPSS (v 21.0, SPSS Inc., Chicago, IL). For all tests statistical significance was set at $P < 0.05$.

3. Results

3.1. Development of a PC biosensor platform for analysis of human ferritin in serum

Real time binding of ferritin to antibodies in three PC biosensor modalities was conducted using a set of primary, secondary and functionalized antibodies and was monitored in the BIND system. Fig. 1 shows a schematic of the three binding assays on the PC platform.

Fig. 1 also shows real-time binding data for ferritin (1 μg/mL) in PBS collected in the BIND system. Fig. 2 shows ferritin standard responses for all assay types on the PC biosensor. In the label-free assay (only capture mAb), the PC biosensor resulted in a limit of detection (LOD) of 2.43 μg/mL and was able to detect ferritin up to 4.0 μg/mL. The use of a secondary pAb, "sandwich assay", lowered the LOD (0.38 μg/mL) for ferritin and extended the dynamic range (0.38–4.0 μg/mL). The assay using immunofunctionalized (FpAb) IONPs resulted in the lowest LOD (26 ng/mL), with a dynamic range between 26 and 2000 ng/mL. The LODs and dynamic ranges for all immunoassay platforms are reported in Table 1.

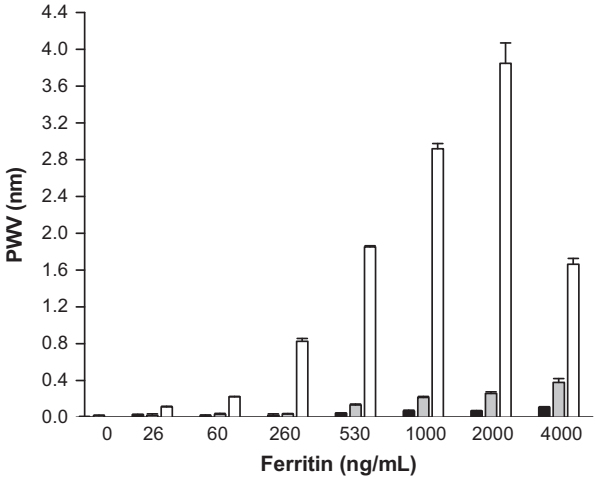

Fig. 2. Comparison of responses from three immunoassay types on the PC biosensor for different ferritin standard concentrations in PBS buffer. Label-free assay (black bars); sandwich assay with secondary antibody (gray bars); immunofunctionalized IONPs with secondary antibodies (white bars). Bars represent mean ± SD (n=3, average of separate assays).

All detection platforms resulted in linear dose–responses (Δsensing units/Δferritin μg/mL) with high coefficients of determination (R^2) (Fig. 3A). Fitted linear equations for ferritin dose response curves (m=slope; b=intercept) were m=0.0028, b=0.11, R^2=0.98 for PC biosensor; m=0.0022, b=0.98, R^2=0.98 for GenWay; m=0.0029, b=0.06, R^2=0.99 for BioVendor; and m=0.021, b=0.49, R^2=0.90 for RayBiotech. Most ELISA assays showed saturation of response below 1 μg/mL. GenWay and RayBiotech ELISAs had the highest sensitivities, but responses quickly saturated detection, especially in the latter. The intra- and inter-day assay variability for all test platforms are reported in Table 1. For the PC biosensor the intra-assay variability was 3.5%. The BioVendor ELISA gave the lowest intra-assay variability (2.4%), whereas the GenWay ELISA gave the highest intra-assay variability (7.9%). With the exemption of GenWay ELISA (14.5%), inter-assay variability was less than 10% for all other platforms.

3.2. Assay agreement and recovery

Liquichek™ control serum level 3 was used to determine agreement between PC biosensor and ELISA detection platforms (Table 2). The PC biosensor and the GenWay ELISA provided results similar to expected range of ferritin (ng/mL) in Liquichek™ serum level 3 (M=32, 95% CI [24.9–39.1l], Tukey, $P > 0.1$). Similarly, analysis of quality control sample from BioVendor on the PC biosensor and BioVendor ELISA resulted in ferritin values (ng/mL) within the expected range (M=350, 95% CI [262–437], Tukey, $P > 0.1$).

Liquichek™ control serum levels 1 and 2 were used to assess recovery from PC biosensor and ELISA detection platforms (Table 3). Expected ferritin concentrations in serum levels 1 and 2 were 47.7 and 45.1 ng/mL, respectively. Ferritin recoveries from analysis of serum levels 1 and 2 on the PC biosensor were 96.9% (7.6% RSD) and 94.3% (13.1% RSD), respectively. Ferritin recoveries from these control sera on the BioVendor's ELISA were similar to those from PC biosensor (Tukey, $P > 0.1$). Detection of ferritin in both serum levels using the GenWay's RayBiotech's ELISAs resulted in the lowest and highest recoveries, respectively.

The Bland–Altman statistical analysis was employed to determine the bias and agreement of the PC and ELISA detection platforms compared to the reported Liquichek™ ferritin value (Fig. 3B). The PC biosensor and BioVendor detection methods had the lowest biases, −1.26 (95% CI[−3.0–5.5]) and 1.34, 95% CI [−15.0–12.3], respectively. Compared against expected values, RayBiotech test had a positive bias of 17.1 ng/mL (95% CI[10.4–23.7]), while the GenWay test had a negative bias of −10.7 ng/m (95% CI[−21.0–0.34]). The limit of agreement (LOA) with the expected ferritin values was −11.6–9.1 ng/mL for the PC biosensor, −28.6–7.3 ng/mL for GenWay, 5.6–28.6 ng/mL for RayBiotech, and −25.2–22.5 ng/mL for BioVendor.

Table 1
Intra-assay and Inter-assay variability of different immunoassay platforms.

Sensing platform	LOD (ng/mL)	Range (ng/mL)	Intra-assay %RSD	n	Inter-assay %RSD	n	Intra-assay %RSD[b]	Inter-assay %RSD[b]
PC biosensor[a]	26	26–2000	3.5	9	6.8	9	–	–
GenWay	5	5–1000	7.9	6	14.5	6	5.6	6.5
RayBiotech	7	7–50	2.8	6	3.5	6	<10	<12
BioVendor	52	52–2000	2.4	6	7.5	6	7.3	4.5

[a] PC biosensor using IONPs functionalized secondary Ab.
[b] Provided by the vendor.

3.3. Matrix effect on PC biosensor response

Detection of ferritin on both the PC biosensor (using FpAb) and BioVendor ELISA was evaluated by spiking concentrated antigen in three matrices: PBS, BSA and serum. Ferritin dose–response curves differed depending on the matrix. On the PC biosensor, ferritin (ng/mL) fitted linear dose response curves in BSA ($m=0.0024$, $b=0.192$, $R^2=0.88$, $P<0.01$) and serum ($m=0.0022$, $b=0.258$, $R^2=0.85$, $P<0.01$) were similar, but different than in PBS ($m=0.0035$, $b=0.062$, $R^2=0.98$, $P<0.01$). Similar trends were observed in experiments using BioVendor ELISA as detection platform; however, coefficients of determination were higher. Fitted linear curves for ferritin (ng/mL) dose response spiked in BSA ($m=0.0033$, $b=0.1817$, $R^2=0.97$, $P<0.01$) and serum ($m=0.0030$, $b=0.1936$, $R^2=0.93$, $P<0.01$) were similar, but different than in PBS ($m=0.0022$, $b=0.0624$, $R^2=0.99$, $P<0.01$).

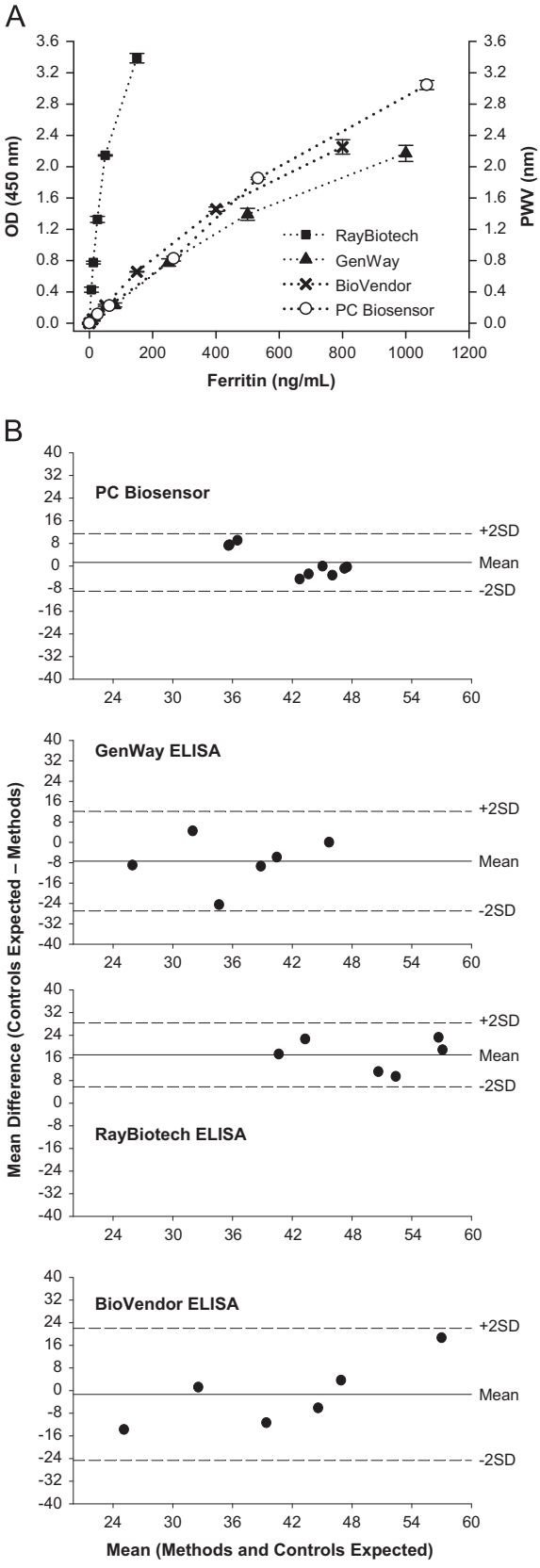

Fig. 3. Standard responses and measurement agreement/bias of four immunoassay platforms. PC biosensor data were obtained from assays with IONPs. (A) Ferritin dose–response curves indicating the linear range and saturation levels of the different sensing platforms. Point-to-point lines are presented to facilitate observation of saturation effects. Data points represent means ± SD ($n=3$, average of separate assays). (B) Bland–Altman plots representing agreement/bias of immunoassay platforms. For each immunoassay type, means between observed values for several ferritin concentrations and their expected control (Liquichek™) sera values (x-axis) are plotted against differences between observed values and expected control sera values (y-axis). Solid lines represent the mean difference. Dotted lines represent upper (+2SD) and lower (−2SD) limits.

4. Discussion

In this work, we describe proof-of-concept studies for the development of a PC biosensor assay for determination of ferritin as a biomarker of iron status. To the authors' knowledge, this is the first known PC biosensor application that uses IONPs to enhance sensitivity for diagnosis of nutritional status. Ferritin, hemoglobin, and soluble transferrin receptor are widely used as diagnostic biomarkers of iron-deficiency anemia. However, alternative low-cost, detection platforms that could identify populations afflicted by micronutrient deficiencies at the point of care in resource-poor environment are still needed. Thus, the objective of this work was to demonstrate the PC biosensor's capability of accurate and precise detection of ferritin down to concentrations close to the cut-off used to differentiate populations with iron deficiency as well as show the ability of IONPs in enhancing the signal-to-noise ratio.

The principle of the PC method for analyte detection is fundamentally different than an ELISA, however both assays use antibodies for antigen recognition and binding. An ELISA (colorimetric assay) uses an enzyme-tagged detection antibody that catalyzes a reaction with an exogenous substrate, producing color as the method for antigen quantification with a spectrophotometer. Different versions of ELISA using fluorescent and luminescent probes are available in microtiter plates (Voller et al., 1978). In contrast, the PC platform uses an intrinsic physical property of the analyte (i.e. dielectric permittivity) that when illuminated with a broadband light source at normal incidence causes a change in the refractive index, which is used to quantify the analyte of interest bound onto the sensor surface (Cunningham et al., 2002). This physical binding is quantified in the form of wavelength shifts (nm). Thus, the PC platform allows for label-free and sandwich applications, without the need of a tagged enzyme. In these studies we found that the immunofunctionalization of IONPs with secondary Ab improved the sensitivity and performance of the PC biosensor when compared with label-free and secondary Ab sandwich assays. As a result, the wavelength shift used to detect biomolecules was enhanced. This mechanism is shown in Figs. 1 and 2, where the kinetic binding and standard responses are presented for all three PC assays. Due to the lower dielectric permittivity in the label-free and antibody sandwich assay, the binding response is nearly constant relative to the enhanced sandwich assay with IONPs.

Table 2
Detection of ferritin in control sera on PC biosensor and commercial ELISAs.

Serum controls		Ferritin (ng/mL)				
		Expected mean and range[1]	PC biosensor[2]	GenWay	RayBiotech	BioVendor
Liquichek™ level 3	Mean	32[a]	39.9[a]	19.9[b]	49.3[c]	18.2[b]
	95% CI	24.9–39.1	36.3–43.6	17.1–22.7	46.8–51.8	11.9–24.5
Quality control	Mean	350[a]	316[a]	421[b]	635[c]	407[a]
	95% CI	262–437	294–338	400–443	623–648	376–439

Means with different superscripts within each row represent statistical differences (Tukey; $P<0.05$).
[1] Concentration based on Liquichek™ serum level 3 and protein buffer quality control provided by BioVendor ELISA kit.
[2] PC biosensor using immunofunctionalized IONPs.

Table 3
Recovery of ferritin from control sera in several immunoassay platforms.

Serum controls	Ferritin (ng/mL)		% Recovery (±%RSD)			
	Unspiked[1]	Spiked	PC biosensor ($n=9$)[2]	GenWay ($n=6$)	RayBiotech ($n=6$)	BioVendor ($n=6$)
Liquichek™ level 1	7.91	47.7	96.9 (7.6)[a]	68.4 (25.9)[b]	130 (1.4)[c]	107 (15.4)[a]
Liquichek™ level 2	25.5	45.1	94.3 (13.1)[a]	75.8 (8.6)[b]	138 (3.5)[c]	90.5 (16.6)[a]

Means with different superscripts within each row represent statistical differences (Tukey; $P<0.05$).
[1] Ferritin concentrations based on reported Liquichek™ serum levels 1 and 2.
[2] Represents number of replicates from three experiments over two weeks.

The use of pAb functionalized with IONPs on the PC biosensor yielded an improvement of two orders of magnitude in sensitivity when compared to the label-free assay. The LOD of ferritin on the PC biosensor (26 ng/mL) fell within the LOD range of the ELISA platforms tested (5–50 ng/mL). Differences among immunoassay platforms were attributed to the characteristics of the assays such as dilution buffers, different protocol steps (e.g., washing and shaking), antibody affinity constants, and the biotinylation of the detection antibodies (Jordan, 2000). The World Health Organization (WHO) has established the cut-off points for ferritin to discriminate iron deficiency and non-iron deficiency at 30 or 15 ng/mL for subjects with or without inflammation, respectively (WHO/CDC, 2007). Although the WHO cut-off point for patients without inflammation falls below the PC biosensor's LOD, our proof-of-concept studies demonstrate potential for further optimization.

Further improvements in signal-to-noise ratio of the PC biosensor could be achieved with the functionalization of slightly larger IONPs (>30 nm) (Tsai et al., 2007; Piletska and Piletsky, 2010) as well as optimization of assay parameters such as adjusting pH, temperature, buffer matrix, and kinetic energy (Jordan, 2000). Although larger IONPs could increase sensitivity, it could also modulate protein interactions. Piletska and Piletsky (2010) demonstrated that binding affinity of streptavidin-coated surface for biotin was two orders or magnitude lower when biotin was bound to silica-based nanoparticles of larger particle size. The density of the nanoparticle and mass transfer of the FpAb may also modulate the amplification of the signal. Tsai et al. (2007) studied the ability of IONP functionalized antibodies for detection of C-reactive protein (CRP), an inflammatory response protein (~115 kDa) in serum. They found that 80 nm IONPs provided better sensitivity than 440 nm IONPs, suggesting steric hindrance interfered with FpAb-antigen binding. In another study using surface plasmon resonance optical biosensors for detection of Ara h1, a peanut allergen (65 kDa), Pollet et al. (2011) found that IONPs of 20 nm in diameter instead of 60 nm were most suitable to increase detection sensitivity. These findings suggest that diameter and density of nanoparticles play a significant role in protein interaction resulting in variable detection sensitivities over a large dynamic range potentially due to lower steric hindrance and facilitated mass transfer. PC biosensors, however, lack systematic optimization studies of IONP size that could lead to improved binding and sensitivity in complex matrices like serum.

Based on the analytical tests used, the PC biosensor performed well in comparison with three commercial ELISAs (Table 2). The PC biosensor demonstrated accuracy and precision in detecting ferritin concentrations in Liquichek™ level 3 and BioVendor's quality control, where the means were similar to those reported by suppliers. Nevertheless, the Liquichek™ level 3 responses on the PC biosensor were on the higher end of the expected range. This result was attributed to the non-specific binding of interfering proteins present in the serum matrix (Pineda et al., 2009). However, the PC biosensor performed within the lower to middle range of the expected value for BioVendor's quality control which had ferritin diluted in an unknown protein buffer solution. Based on the intra-assay and inter-assay variability, the PC biosensor had similar precision (<10%) compared to the ELISAs evaluated. RayBiotech's ELISA had the lowest intra- and inter-assay variability, which was potentially due to the strong interaction of biotin and streptavidin, one of the strongest non-covalent biological interactions used in immunodiagnostics (Guesdon et al., 1979). Immunodiagnostic tests that use biotin–streptavidin interaction normally display stronger binding affinities and lower limits of detection (Gould et al., 1985; Jordan, 2000; Nara et al., 2008).

In spike-recovery experiments with serum, the PC biosensor performed similar to BioVendor's ELISA, and was capable of recovering ferritin within a narrow range of the expected concentrations in Liquichek™ serum levels 1 and 2 (Table 3). In contrast, ferritin recovery in GenWay's ELISA was consistently lower than the expected values. This could be due to the lack of shaking in the GenWay protocol that, as a result, did not provide enough kinetic energy for antibody–antigen interaction in the serum matrix (Yolken, 1982). A high %RSD in GenWay's ELISA was the result of this heterogeneous binding in replicate wells. RayBiotech's ELISA presented consistently higher ferritin recoveries in both Liquichek™ sera, however with the smallest %RSD. This was attributed to the effective interaction between the biotinylated detection antibodies and the HRP-streptavidin (Guesdon et al., 1979; Nara et al., 2008). Also, this

assay required larger sample dilutions for ferritin to fit within the standard curve, whose slope was an order of magnitude higher than the rest of the tests (Fig. 3A).

The dynamic range of detection in the PC biosensor is larger than commercial ELISAs, especially RayBiotech's, even without IONPs functionalization. In the sandwich assay with pAb and without sample dilution the dynamic range for ferritin detection was 0.3–3.2 μg/mL. Pollet et al. (2011) reported similar ranges in studies with surface plasmon resonance. A large dynamic range is useful because it allows more flexibility in modifying the kinetics and timing of the assay. It also reduces the need for dilution steps, which adds variability to final test determination. In our studies the sample matrix affected the dynamic range. The responses in the PC biosensor with IONPs saturated between 0.4 and 0.8 μg/mL ferritin when spiked into BSA or serum. As indicated by the lower R^2 values, these protein matrices may have interfered through steric hindrance with the functionalized IONPs and reduced interaction with capture Ab, which instead affected the linear response (Piletska and Piletsky, 2010). In contrast, ELISA tests showed strong linear responses at those concentrations. It is important to notice that ELISAs use a wash step, whereas the PC biosensor does not. Thus, the reading of ELISA wells is practically conducted on PBS. Despite signal saturation, iron deficient individuals have ferritin levels below 30 ng/mL; thus, signal saturation from PC biosensor has limited implications for iron deficiency diagnosis.

The Bland–Altman bias and agreement analysis was used to evaluate how well the PC biosensor experimental results compared to the expected results of 11 established diagnostic tests used to measure ferritin in the Liquichek™ sera, as well as how well it compared with the ELISAs. In the plot (Fig. 3B) a low bias is reflected in values closer or around the zero reference line. In addition, limits of agreement lines (± 2SD) represent how variable these results are from the mean difference. Thus, the closer these lines are to the mean, the higher the precision. These analyses showed that the PC biosensor assay had low bias and results were within 95% confidence intervals close to those expected values from the 11 established diagnostic tests. GenWay and BioVendor ELISAs yielded ferritin means lower than those expected for the Liquichek™ serum level 3 and thus had a negative bias and lower agreement. Nonetheless, when comparing these values to the AccuBind ELISA ferritin range, one of the 11 Liquichek™ analytical methods reported, the GenWay and BioVendor ELISA results fell within the expected concentration range. Therefore, it can be speculated that ELISAs may yield lower values than other analytical methods like the PC biosensor, among others. This could be due to interfering proteins that prevent the antibody–antigen interaction or the wash steps normally applied in ELISA protocols (Yolken, 1982; Jordan, 2000). In contrast, RayBiotech's ELISA consistently had a positive bias when detecting ferritin values for both control sera, but a small 95% CI of bias and a tight limit of agreement. It is possible that this positive bias was due to analysis of ferritin concentrations on the high-end of the supplier's recommended analyte detection (Nara et al., 2008) as this assay uses an optimized protocol for quantification of low ferritin concentrations in not only serum, but also cell culture supernatants and urine.

Despite promising results supporting the use of PC biosensor and the BIND system as a viable diagnostic test for iron deficiency, certain limitations must be addressed in future experiments. Control sera were used to evaluate assay diagnostic parameters. Further studies will require the use of sera from a large, diverse population pool. Assay optimization is another area of improvement to maximize detection sensitivity and minimize total cost of analysis. Finally, concurrent determination of other biomarkers such as hemoglobin, soluble transferrin receptor and CRP to adequately differentiate types of anemia and inflammatory responses are needed. At the moment few ELISA platforms are capable to measure these biomarkers in tandem (Erhardt et al., 2004). However, the flexible nature of PC biosensors makes them amenable to conduct multiple determinations in a single sensor strip.

The high cost of trained personnel, facilities, sampling storage, laboratory equipment, and detection systems required to conduct ELISA type assays is a significant roadblock for field diagnosis and point-of-care applications in rural or remote areas in developing countries. Recently, the Cunningham group demonstrated the use of a smartphone as a detection instrument for a label-free PC optical biosensor. The smartphone is placed on a cradle that incorporates several inexpensive optical components in alignment with the camera. This allows the phone to work as a highly accurate spectrometer (PWV as low as 0.009 nm) for measuring the transmission spectrum from a PC biosensor (Gallegos et al., 2013). This innovative strategy will streamline our objective to bring point-of-care diagnostics of nutritional status to homes, clinics, or remote areas.

5. Conclusion

Our proof-of-concept studies showed accurate and precise detection of serum ferritin as a biomarker of iron deficiency using a PC biosensor assay along with immunofunctionalized IONPs and the BIND readout system in comparison to commercial ELISAs. Our group is optimizing the current PC assay platform using IONPs of different sizes, biomarker purification, antibodies of higher affinity and avidity, and improved assay protocols. The novel smartphone cradle-reading instrument will be evaluated using the PC biosensor application for diagnosis of serum ferritin along with other biomarkers of nutritional status.

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References

Arakawa, T., Kita, Y., 1999. Anal. Biochem. 271, 119–120.
Armbruster, D., Pry, T., 2008. Clin. Biochem. Rev. 29 (Suppl. 1), S49–S52.
Black, R., Allen, L., Bhutta, Z., Caulfield, L., de Onis, M., Ezzati, M., Mathers, C., Rivera, J., 2008. Lancet 371, 243–260.
Bland, J.M., Altman, D.G., 1986. Lancet 1, 307–310.
Bonanno, L., DeLouise, L., 2007. Biosens. Bioelectron. 23, 444–448.
Burns, D.T., Danzer, K., Townshend, A., 2002. Pure Appl. Chem. 74, 2201–2205.
Byrne, R., Diamond, D., 2006. Nat. Mater. 5, 421–424.
Chung, J.W., Kim, S.D., Bernhardt, R., Pyun, J.C., 2005. Sens. Actuators B: Chem. 111, 416–422.
Chan, L.L., Gosangari, S.L., Watkin, K.L., Cunningham, B.T., 2007. Apoptosis 12, 1061–1068.
Cunningham, B., Li, P., Lin, B., Pepper, J., 2002. Sens. Actuators B: Chem. 81, 316–328.
Cunningham, B.T., Li, P., Schulz, S., Lin, B., Baird, C., Gerstenmaier, J., Genick, C., Wang, F., Fine, E., Laing, L., 2004. J. Biomol. Screen. 9, 481–490.
Erhardt, J.G., Estes, J.E., Pfeiffer, C.M., Biesalski, H.K., Craft, N.E., 2004. J. Nutr. 134, 3127–3132.
Gallegos, D., Long, K., Yu, H., Clark, P., Lin, Y., George, S., Nath, P., Cunningham, B., 2013. Lab on a Chip 13, 2124–2132.
Gould, E.A., Buckley, A., Cammack, N., 1985. J. Virol. Methods 11, 41–48.
Guesdon, J.L., Ternynck, T., Avrameas, S., 1979. J. Histochem. Cytochem. 27, 1131–1139.

Heeres, J.T., Hergenrother, P.J., 2011. Chem. Soc. Rev. 40, 4398–4410.
Jordan, B., 2000. Methods Mol. Med. 40, 373–379.
Kumbhat, S., Sharma, K., Gehlot, R., Solanki, A., Joshi, V., 2010. J. Pharm. Biomed. Anal. 52, 255–259.
Kyprianou, D., Chianella, I., Guerreiro, A., Piletska, E.V., Piletsky, S.A., 2013. Talanta 103, 260–266.
Marcus, M.M., Zinberg, N., 1975. J. Natl. Cancer Inst. 55, 791–795.
Mathias, P.C., Jones, S.L., Wu, H., Yang, F., Ganesh, N., Gonzalez, D.O., Bollero, G., Vodkin, L.O., Cunningham, B.T., 2010. Anal. Chem. 82, 6854–6861.
Nara, S., Tripathi, V., Chaube, S.K., Rangari, K., Singh, H., Kariya, K.P., Shrivastav, T.G., 2008. Talanta 77, 210–216.
Nash, M.A., Golden, A.L., Hoffman, J.M., Lai, J.J., Stayton, P.S., 2012. Smart surfaces for point-of-care diagnostics, Intelligent Surfaces in Biotechnology. John Wiley & Sons, Inc., Hoboken, NJ, pp. 31–70
Piletska, E.V., Piletsky, S.A., 2010. Langmuir 26, 3783–3785.
Pineda, M.F., Chan, L.L., Kuhlenschmidt, T., Choi, C.J., Kuhlenschmidt, M., Cunningham, B.T., 2009. IEEE Sens. J. 9, 470–477.
Pollet, J., Delport, F., Janssen, K.P., Tran, D.T., Wouters, J., Verbiest, T., Lammertyn, J., 2011. Talanta 83, 1436–1441.
Soelberg, S.D., Stevens, R.C., Limaye, A.P., Furlong, C.E., 2009. Anal. Chem. 81, 2357–2363.
Tsai, H.Y., Hsu, C.F., Chiu, I.W., Fuh, C.B., 2007. Anal. Chem. 79, 8416–8419.
Voller, A., Bartlett, A., Bidwell, D.E., 1978. J. Clin. Pathol. 31, 507–520.
World Health / Centers for Disease Control and Prevention (WHO/CDC), 2007. Assessing the Iron Status of Populations. second ed., including literature reviews, WHO Press, Geneva, Switzerland.
Yolken, R.H., 1982. Rev. Infect. Dis. 4, 35–68.