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Rapid and Label-Free Detection of Breast Cancer Biomarker CA15-3 in Clinical Human Serum Samples with Optofluidic Ring Resonator Sensors

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Sensitive and specific detection of breast cancer biomarker CA15-3 in human serum is an important step toward successful evaluation of clinical treatment and prediction of breast cancer recurrence. In this work, we developed an optofluidic ring resonator (OFRR) sensor and the corresponding sensing protocols for label-free CA15-3 detection without any additional signal amplification steps. Nonspecific serum protein adsorption was minimized with effective surface blocking methods. The sensor performance for CA15-3 detection was first characterized in phosphate-buffered saline (PBS) buffer and in fetal calf serum. Then the potential use of the OFRR as a simple clinical laboratory testing device for breast cancer diagnostics was tested by measuring the CA15-3 level in clinical human serum samples, and the results were compared with those of standard clinical lab tests. It was found that the OFRR was capable of detecting approximately 1 unit/mL CA15-3 in both PBS buffer and diluted serum within approximately 30 min. Our work marks the first demonstration of the optical ring resonator biosensor in real clinical applications that features low cost, simple detection procedures, rapid response time, low sample consumption, and high specificity.

Breast cancer affects one of eight women in the United States, and the incidence of breast cancer continues to increase. The American Cancer Society (ACS) estimated approximately 182 460 women in the United States would be found to have invasive breast cancer in 2008. About 40 480 women will die from the disease this year.[1] The early diagnosis of the disease combined with the effective treatment offers the best chance of survival. Up to date, X-ray mammography is still the most effective and widely available method for breast cancer detection. However, mammography has a lower sensitivity in young women (<40 years) due to their higher glandular breast density. Compared with X-ray mammography, magnetic resonance imaging (MRI) is more sensitive for breast cancer, but it is quite expensive and time-consuming, which limits its applications in mass screening, and especially it may not be affordable in many resource-limited regions. Consequently, there is an urgent demand for developing rapid, cost-effective, sensitive, and specific detection methods as tools complementary to those traditional methods to improve the effectiveness of breast cancer detection and the follow-up of breast cancer patients.

Advances in molecular biology have provided considerably increased understanding of breast cancer at molecular levels. Several breast cancer biomarkers have already been identified, including estrogen receptor (ER),[2] progesterone receptor (PR),[2] carbohydrate antigen 15-3 (CA15-3),[3] carcinoembryonic antigen (CEA),[4] Her-2/neu,[5] etc. Often these biomarkers are overexpressed in breast cancer patients, and they can be detected in tumor cells, blood, and other body fluids. While these biomarkers may not have enough specificity for early screening or diagnosis, their presence and concentration in body fluids may provide important information to assist clinicians in assessing prognosis, monitoring the effectiveness of therapy, and predicting cancer recurrence.[6] Among those breast cancer biomarkers, serum biomarkers are most attractive, since a simple blood test is minimally invasive to patients and can yield valuable information about the breast cancer status. CEA is the first widely used serum biomarker for breast cancer, but it has been found to be a non-organ-specific biomarker. CA15-3 with much better clinical specificity has emerged as a more important biomarker for breast cancer.[3] CA15-3 is a mucin-like glycoprotein defined by two

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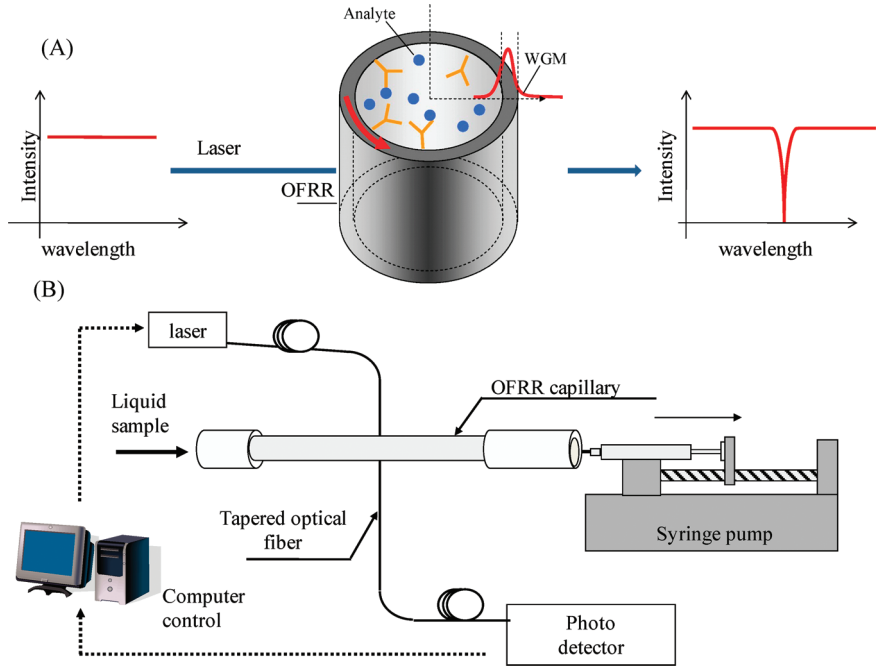

Figure 1. (A) Conceptual illustration of the OFRR sensing principle. (B) OFRR detection experimental setup.

monoclonal antibodies: DF3 and 115D8.[7,8] It is a heterogeneous molecule with highly variable molecular weight from 300 000 to 450 000.[5] In healthy people, CA15-3 concentrations are usually below 30 units/mL.[9,10] Patients with primary breast cancer or metastatic breast cancer show elevated CA15-3 levels.[3] Thus, CA15-3 is one of the important biomarkers commonly used in monitoring therapy outcomes and disease progression in metastatic breast cancer patients.[11] CA15-3 has been detected with commercialized enzyme-linked immunosorbent assay (ELISA), radioimmunometric assay (RIA),[12] and microparticle enzyme immunoassay (MEIA).[13] These sandwich-based immunoassays indirectly detect CA15-3 with enzyme or radioisotopically labeled secondary antibodies. Despite excellent sensitivity, these methods inevitably suffer from shortcomings such as lengthy processes and high reagent cost due to the use of labeled secondary antibodies. Additionally, they require sophisticated detection systems and well-trained personnel to perform the assay. As a result, these assays are usually carried out at a central laboratory rather than at the local clinical laboratory. A rapid and inexpensive device with simple operation procedures and reasonable sensitivity for CA15-3 detection is highly desirable to deliver a rapid result, significantly reduce the patients' turnaround time, and relieve the patients' anxiety caused by a prolonged waiting time for results.

The optofluidic ring resonator (OFRR) is a novel type of affinity optical biosensor developed recently[14] and has been applied in detection of a wide range of biomolecules, including proteins,[15,16] DNA,[17] and viruses[18] with excellent sensitivity and a short detection time. As shown in Figure 1A, the OFRR employs a glass capillary approximately 100 μm in diameter. The capillary functions as a microfluidics device for sample delivery, while the capillary circular cross section forms an optical ring resonator. The light is circulated along the curved surface in the form of whispering gallery modes (WGMs), which is launched by an optical fiber taper perpendicularly in contact with the OFRR. The capillary wall is sufficiently thin (<4 μm) so that the evanescent field of the WGM extends into the capillary core to interact with the analytes repeatedly.

The OFRR performs label-free sensing by detecting refractive index (RI) changes on the inner surface induced by the binding of the biomolecules. The relationship between the WGM spectral position or resonant wavelength, λ, and the RI is shown as follows:[19]

$$2\pi n_{\mathrm{eff}} r = m\lambda \qquad (1)$$

where n_{eff} is the effective RI experienced by the WGMs, r is the capillary radius, and m is an integer representing the WGM angular momentum. The WGM spectral position shifts when analytes are captured by those biorecognition elements im-

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mobilized on the OFRR inner surface, generating the real-time quantitative and kinetic information about the binding of biomolecules to the OFRR surface. The OFRR has achieved a sensitivity of 10^{-7} refractive index unit (RIU), which corresponds to a mass detection limit of subpicograms per square millimeter,[15] competitive with or even better than other commercialized label-free sensors such as surface plasmon resonance (SPR) sensors. In addition, as compared to other label-free sensors, the OFRR sensor is more suitable as a cheap and disposable device due to its low fabrication cost, small physical size, easy operation principle, multiplexing capability, and small sample consumption volume.[20]

In this study, we demonstrated a sensitive, rapid, and label-free sensor platform based on the OFRR and developed the corresponding sensing protocols for direct detection of CA15-3 in human serum samples without any signal amplification steps. First, we tested different surface blocking reagents on the OFRR and optimized surface blocking protocols. Subsequently, we demonstrated the feasibility of the OFRR to detect the CA15-3 in PBS buffer in a range of 1−200 units/mL. Meanwhile, the OFRR sensor specificity and cross-reactivity were tested with a negative control antigen (CA12-5) and negative control antibody (anti-ER antibody), respectively. Then we detected CA15-3 spiked into the fetal calf serum (FCS) and established a concentration calibration curve. Finally, we applied the OFRR to measure the CA15-3 concentration in stage IV breast cancer patients' sera without any sample pretreatment steps. The results compare favorably with standard clinical lab tests.

EXPERIMENTAL SECTION

Materials. All of the materials and reagents were used as received. Ethanol (98%), hydrofluoric acid (HF; 48%), 12 N hydrochloric acid (HCl), (3-aminopropyl)trimethoxysilane (3-APS), 50% glutaraldehyde, phosphate-buffered saline (PBS) tablets, bovine serum albumin (BSA), and casein were purchased from Sigma-Aldrich (St. Louis, MO). *Caution! Hydrofluoric acid is a particularly dangerous inorganic acid that should be handled with consideration for safety.* All chemical agents were used without purification and were prepared in 18 MΩ water generated by the Easypure-UV system from Barnstead (Dubuque, IA). Fetal calf serum (FCS) was purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-CA15-3 antibody (clone number 2F16 and product number C0050-22A) and mouse monoclonal anti-ER antibody (clone number 4E73 and product number E3565-09) were purchased from USBiological (Marblehead, MA). Purified CA15-3 antigen (product number 150-12A) and CA125 (product number 150-11) were purchased from Lee Biosolutions (St. Louis, MO). Tween-20 surfactant was purchased from Thermo Scientific (Rockford, IL). Amine−PEG−amine (MW = 1000) was purchased from Laysan Bio, Inc. (Arab, AL). Silica capillary tubing (857 μm o.d. and 699 μm i.d.) was purchased from Polymicro (Phoenix, AZ).

OFRR Fabrication and Experimental Setup. The OFRR fabrication procedures and experimental setup were described in detail in our previous work.[21] Briefly, the OFRR was fabricated by stretching a glass capillary under intense heat provided by CO_2 lasers. Then the OFRR was connected to Tygon tubing (shown in Figure 1B) so that the liquids can pass through it using a syringe pump. To further reduce the capillary wall thickness, a diluted HF solution (~10%) in DI water was pumped through the OFRR to reduce the wall thickness to a few micrometers. The overall experiment setup for the biomolecule detection is simply shown in Figure 1B. The OFRR was perpendicularly in touch with an optical fiber taper that has a diameter of approximately 3 μm. The fiber taper delivered the light from a 980 nm tunable diode laser from New Focus (San Jose, CA; line width <3 fm). The output wavelength of the laser was periodically scanned at a rate of 5 Hz. A photodetector was placed at the output end of the optical fiber to measure the transmission optical intensity. When the laser wavelength met the resonant condition described in eq 1, the light was evanescently coupled into the OFRR, causing a reduction in transmission power measured at the fiber output, which was used to indicate the WGM spectral position. The measurement system was controlled by a computer through a data acquisition card from National Instruments (Austin, TX), and the WGM spectral position was recorded for postanalysis.

Calibration of the OFRR Bulk Refractive Index Sensitivity. The OFRR is a refractometric sensor. Therefore, it is essential to characterize its sensitivity to the bulk refractive index change in the capillary. The bulk refractive index sensitivity (BRIS), as shown in Figure S1 in the Support Information, was obtained by flowing through the OFRR various concentrations of ethanol−water mixtures with known RI. For biomolecule sensing experiments, the observed WGM spectral shift can be normalized to the BRIS ($\delta\lambda$/BRIS) to minimize the slight BRIS variations among the different OFRR sensors.

Biomolecule Surface Density Calculation. In our previous work we have established a linear relationship among the BRIS, the amount of WGM spectral shift, and the biomolecule surface density:[15]

$$\frac{\delta\lambda}{\lambda} = \sigma_p \alpha_{ex} \frac{2\pi\sqrt{n_2^2 - n_3^2}}{\varepsilon_0 \lambda^2} \frac{n_2}{n_3^2} S \qquad (2)$$

where $\delta\lambda$ is the WGM spectral shift due to the biomolecules captured on the surface, σ_p is the molecular surface density, α_{ex} is the excess polarizability of the analyte and is proportional to the molecular weight,[22] n_2 (1.45) and n_3 (1.33) are the RIs of the OFRR wall and the aqueous medium in the capillary core, ε_0 is the vacuum permittivity, S is the BRIS, and λ is the wavelength of the WGM (~980 nm). Equation 2 allows us to quantify the biomolecule surface density on the OFRR inner wall using the experimentally measurable sensing signal, i.e., the WGM spectral shift normalized to the BRIS ($\delta\lambda$/BRIS).

Monoclonal Antibody Immobilization. Anti-CA15-3 antibodies were immobilized on the OFRR surface through standard amine coupling chemistry as reported previously.[16] Briefly, a

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freshly HF etched OFRR surface was cleaned with a HCl/methanol (v:v = 50:50) mixture for 10 min and rinsed with DI water thoroughly. 3-APS (1%) in 10% ethanol was flowed through the OFRR for 30 min to aminate the surface, followed by DI water rinsing. The surface was activated with 5% glutaradehyde solution in PBS buffer (10 mM, pH 7.4) for 30 min. Then the OFRR was rinsed with PBS buffer and soaked in PBS buffer to establish a stable detection baseline. Anti-CA15-3 antibody at a concentration of 50 μg/mL was flowed through the OFRR at a flow rate of 5 μL/min. The optimal time for antibody immobilization is discussed later. The nonspecifically bound antibodies were washed away with PBS buffer. Finally, the surface was deactivated with surface blocking solution, as shown in the next section.

Surface Blocking Methods. To detect CA15-3 in the human serum, it is crucial to minimize the nonspecific serum protein adsorption. In our work, several different surface blocking reagents were tested on an antibody-functionalized OFRR surface, and the optimized method was chosen. Those reagents included 1 mg/mL BSA in PBS buffer, 1% casein in PBS buffer, 1 mg/mL amine−PEG−amine in PBS buffer, and 10% FCS in PBS buffer containing 0.5% Tween-20 (10% FCS−PBS/0.5% Tween-20). First anti-CA15-3 antibodies were immobilized on the OFRR surface using the procedures described before, and then one of those reagents was flowed through the OFRR to block the surface for 30 min.

Label-Free Detection of CA15-3 Spiked in PBS Buffer. The antibody-functionalized OFRR surface was blocked with 1 mg/mL amine−PEG−amine for 30 min and rinsed with PBS buffer. Then various concentrations of purified CA15-3 spiked in PBS buffer were introduced into the OFRR at a flow rate of 5 μL/min. The flow was switched for PBS buffer rinsing when the sensing signal reached the plateau. Negative control experiments were performed to test the system specificity and cross-reactivity. In the first negative control experiment, anti-ER antibody was immobilized on the OFRR surface and the OFRR surface was blocked with the same procedures described before. Then 100 units/mL CA15-3 in PBS buffer was flowed through the OFRR. In the second negative control experiment, anti-CA15-3 antibody was immobilized and the OFRR surface was blocked. Then 100 units/mL CA12-5 in PBS buffer was flowed through the OFRR for 20 min.

Generation of Calibration Curves for the OFRR CA15-3 Sensor. To quantitatively measure CA15-3 concentrations in clinical human serum, it is essential to generate a calibration curve first. To achieve this, artificial serum samples were prepared by spiking various amounts of CA15-3 into FCS. The lack of pre-existing CA15-3 or any other heterophilic antibodies in FCS avoided potential interference. Compared to PBS buffer, serum is a complex biological medium. Consequently, dual surface blocking steps were used to enhance the sensor nonspecific adsorption resistance ability. Briefly, 1 mg/mL amine−PEG−amine was first applied to the OFRR surface for 30 min, followed by PBS buffer rinsing. Then 10% FCS−PBS/0.5% Tween-20 solution was flowed through for 10 min. Finally, the OFRR was soaked in running buffer (3% FCS−PBS buffer/0.5% Tween-20) to establish a stable detection baseline. The artificial serum samples were first diluted with running buffer and then measured with the OFRR.

Label-Free Detection of CA15-3 in Clinical Human Serum with the OFRR. Stage IV breast cancer patients' blood samples were collected from the Ellis Fischel Cancer Center at the University of Missouri. The blood samples were centrifuged. Sera were stored at −80 °C and thawed before use. The serum was diluted with running buffer and measured with the same procedures described in the previous section.

RESULTS AND DISCUSSION

Monoclonal antibody immobilization. Figure S2A (Supporting Information) shows a real-time anti-CA15-3 antibody immobilization sensorgram. Upon the injection of the antibody at a concentration of 50 μg/mL (~323 nM) in PBS buffer, the WGM spectral position shifted rapidly to a longer wavelength within the first couple of seconds. This huge rapid shift was due mainly to the bulk RI change inside the OFRR, because the antibody solution contained 40% glycerol. The WGM spectral position continued to shift slowly due to the actual antibody binding to the surface. After a certain amount of time, the flow was switched back to PBS buffer for surface rinsing, and consequently, the WGM spectral position shifted back. The difference between the final WGM spectral position and the initial baseline was the actual amount of spectral shift caused by antibody immobilized on the OFRR interior surface. From the amount of WGM spectral shift and the OFRR sensor BRIS, we can estimate the antibody surface density using eq 2, in which $(\alpha_{ex})_{antibody} = 4\pi\varepsilon_0(9.04 \times 10^{-21})$ cm^3 [22] (MW of antibody = 155 000).

Figure S2B (Supporting Information) shows that the antibody surface density is directly proportional to the immobilization time for the first 40 min and gradually saturated. The antibody surface density was about $(4-5) \times 10^{11}/cm^2$ after 30−40 min of immobilization time. This result is similar to the typical antibody density on ELISA ($\sim 5.8 \times 10^{11}/cm^2$),[23] which amounts to only 20−25% of surface coverage, given the surface density of approximately $2 \times 10^{12}/cm^2$ expected for a fully packed protein monolayer on the sensing surface.[15] Although a higher antibody surface density is achievable, in practical applications a relatively low antibody surface density is preferred to minimize steric hindrance, antibody aggregation, and the mass transport limitation.[24] Therefore, for all the experiments performed we employed the amine−glutaradehyde covalent attachment method with 40 min of antibody immobilization.

Minimizing Nonspecific Serum Protein Adsorption. Because the OFRR detects the RI change near the sensing surface, nonspecific serum protein adsorption is a major obstacle to using the OFRR to detect CA15-3 in complex biological media such as serum or plasma. Traditional blocking reagents (e.g., BSA, casein, or PEG derivatives such as amine−PEG−amine) can block the surface well enough for detecting the target molecules in pure buffer solution, but they may not be able to block the surface sufficiently to resist the nonspecific serum protein adsorption. Recently, several research groups have developed new surface blocking strategies that allow them to detect biomarkers in diluted serum or even undiluted serum. Those approaches include using

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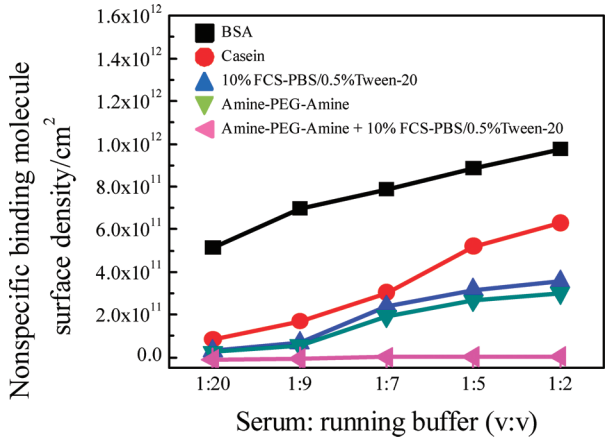

Figure 2. Surface densities of nonspecifically adsorbed serum protein molecules vs FCS concentration using different surface blocking methods. The FCS concentration was 33%, 16.7%, 12.5%, 10%, and 4.8% for 1:2, 1:5, 1:7, 1:9, and 1:20 dilution, respectively.

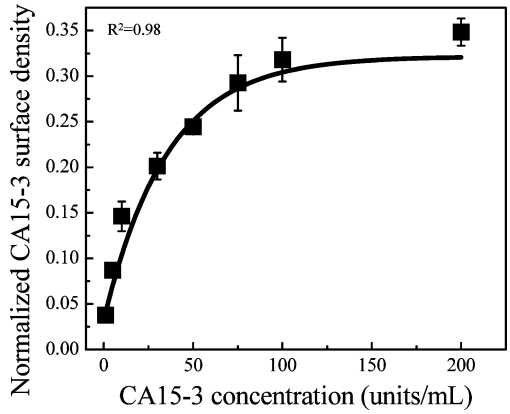

Figure 3. Dose−response curve for CA15-3 concentration ranging from 1 to 200 units/mL. Each data point was measured three times. The solid curve is fitted with eq 3, where $\sigma_{max} = 0.36$ and $K_d = 19$ units/mL.

nonfouling materials such as poly(ethylene glycol) (PEG) based copolymers[25] and zwitterionic polymers,[26] which have shown remarkable reduction in nonspecific protein adsorption. Another approach is to expose the sensor surface to the serum in the absence of analyte prior to detection, thus preventing any further nonspecific adsorption.[27,28]

Here, to find the most effective surface blocking reagent for the OFRR, we tested several different surface blocking reagents and evaluated their surface blocking effects. First, we immobilized the antibodies on the surface. Then the surface was blocked with BSA, casein, amine−PEG−amine, or 10% FCS−PBS/0.5% Tween-20 prior to exposure to diluted FCS. Figure 2 shows the density of nonspecifically adsorbed biomolecules on the OFRR surface for various concentrations of FCS. Because albumin is the most abundant protein in serum, the nonspecific adsorption molecule surface density shown in Figure 2 is estimated with eq 2 by simply assuming most adsorbed proteins are albumin ($(\alpha_{ex})_{BSA} = 4\pi\varepsilon_0(3.85 \times 10^{-21})$ cm^3, MW = 66 000). Among those methods, the BSA blocked surface shows the strongest nonspecific adsorption. The casein blocked surface also shows large nonspecific adsorption at low dilutions of FCS (<6-fold). In contrast, amine−PEG−amine and 10% FCS−PBS/0.5% Tween-20 solution blocked surfaces have much lower nonspecific adsorption. However, the surface density of the nonspecific protein adsorption with either of these two methods was about 3×10^{11}/cm^2 (or 33 ng/cm^2) for 3-fold diluted FCS. Typically the total nonspecific protein adsorption on the ultralow fouling surface should be less than 5 ng/cm^2 for complex media.[26,29] Therefore, the amine−PEG−amine blocked OFRR surface was not able to meet this requirement yet.

On the basis of the results discussed above, in our experiments, we developed a new surface blocking strategy involving dual blocking steps. We first blocked the surface with amine−PEG−amine, followed by 10% FCS−PBS buffer/0.5% Tween-20. As shown in Figure 2, the nonspecific protein adsorption was significantly suppressed with this method. The nonspecific protein density for 3-fold and 6-fold diluted FCS was only about 4.5×10^9/cm^2 (or 0.5 ng/cm^2) and 2.7×10^9/cm^2 (or 0.3 ng/cm^2), respectively, which met the ultralow fouling criteria.[29] Since the OFRR sensor can resolve a 0.1 pm WGM spectral shift,[30] which corresponds to the albumin surface density of 1.7×10^9/cm^2, the nonspecific protein density from the 6-fold dilution was already very close to the system detection limit and the nonspecific protein densities from other higher dilutions (8-fold to 21-fold) were not detectable, thus allowing us to detect CA15-3 in diluted serum directly without any additional reference channels or amplification steps.

Label-Free Detection of CA15-3 Spiked in PBS Buffer. After solving the nonspecific adsorption problem, we started to investigate the CA15-3 detection. First, we detected CA15-3 in PBS buffer. After immobilization of the capture antibodies and blocking of the remaining active functional groups with amine−PEG−amine, CA15-3 in PBS buffer was flowed through the OFRR. Figure S3A (Supporting Information) plots the representative sensorgrams obtained from three different OFRRs for the direct detection of CA15-3 at concentrations of 1, 5, and 30 units/mL with a flow rate of 5 μL/min. For the 1 unit/mL CA15-3 sample, it took around 20 min to reach 90% of the maximum signal, indicating that the whole detection can be completed in 30 min even taking into account the subsequent rinsing step.

Then we explored the detection limit and the dynamic range of the OFRR sensor. Similar to the previous experiments, the anti-CA15-3 antibody coated OFRR surface was first blocked and then PBS buffer was flowed to establish the detection baseline. CA15-3 samples with incremental concentrations were flowed through the OFRR in sequence. Each sample was flowed through the OFRR at a flow rate of 5 μL/min. After around 20 min, the flow was switched for PBS buffer rinsing for about 5 min before the injection of the subsequent sample into the OFRR sensor (see the real-time sensorgram shown in Figure S3B in the Supporting Information). After each injection, the gradual increase of the

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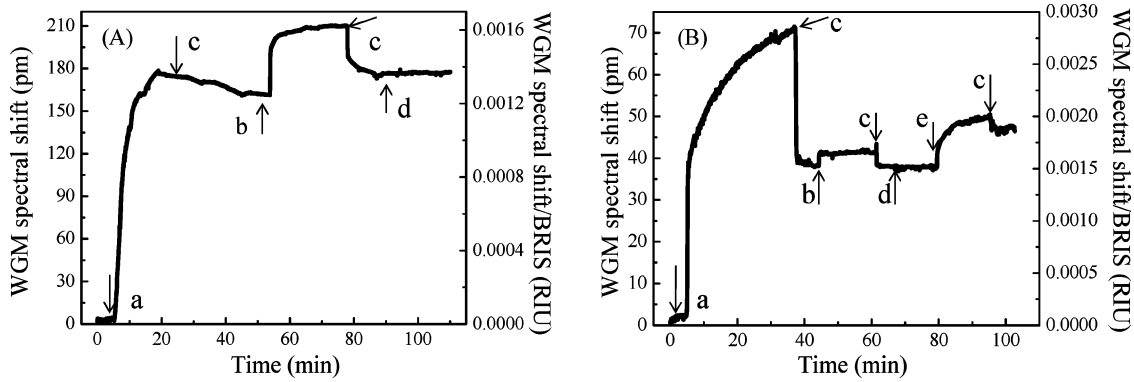

Figure 4. Demonstration of the OFRR specificity for CA15-3 detection. (A) Demonstration specificity of the CA15-3 antigen: sensor response to (a) immobilization of anti-ER antibody (20 μg/mL), (b) surface blocking with amine−PEG−amine, (c) PBS buffer rinsing, and (d) CA15-3 antigen (100 units/mL). (B) Demonstration of no cross-reactivity of anti-CA15-3 antibody: sensor response to (a) immobilization of anti-CA15-3 antibody (50 μg/mL), (b) surface blocking with amine−PEG−amine, (c) PBS buffer rinsing, (d) CA12-5 antigen (100 units/mL), and (e) CA15-3 antigen (100 units/mL).

sample concentration tended to re-equilibrate bound CA15-3 and free CA15-3 in solution and hence increased the CA15-3 density on the OFRR surface. Note that the normalized CA15-3 densities obtained through this accumulative method in Figure S3B agree well with those obtained separately using individual OFRRs in Figure S3A and that both parts A and B of Figure S3 show that the OFRR is capable of detecting CA15-3 in buffer below 1 unit/mL. The titration curve based on Figure S3B is plotted in Figure 3. It can be fitted with the following equation:

$$\sigma = \frac{\sigma_{max}[\text{CA15-3}]}{K_d + [\text{CA15-3}]} \quad (3)$$

where σ is the normalized CA15-3 surface density, σ_{max} is the maximum of the normalized CA15-3 surface density, K_d is the dissociation constant for antibody−antigen binding, and [CA15-3] is the CA15-3 concentration. The sensing signal approached saturation at approximately 100 units/mL, corresponding to the normalized CA15-3 density of approximately 36%. This suggests that only 36% of the antibody binding sites were active, which was much lower than the antibody activities achieved in other studies.[24] This low activity may be due to the covalent attachment method used in experiments for antibody immobilization. In our studies, antibodies were immobilized onto the surface by glutaraldehyde through their lysine residue, resulting in multipoint attachment to the surface and thus reducing their available binding sites.[24,31,32] The reduction in antibody activity may also result from the fact that CA15-3 is a large antigen (MW ≈ 300000−450000), which makes it difficult for them to simultaneously occupy the antibody binding sites due to the lateral packing effects.[24] A simple solution to improving the antibody activity is to uniformly orient the antibody on the surface through a protein A or protein G intermediate layer.[31] However, through systematic investigations (data not shown), we found that this method is not applicable when target molecules are in serum due to the large amount of nonspecific bindings. Another antibody orientation method requires labeling antibodies with biotin and subsequently linking them to the surface through

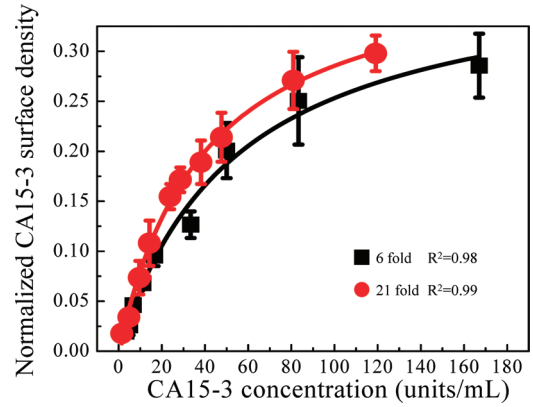

Figure 5. OFRR sensor response for the detection of CA15-3 spiked into FCS. Samples were diluted 6-fold or 21-fold with 3% FCS−PBS/0.5% Tween-20 buffer. Each point was measured five times. The solid curves were fitted with eq 3, where σ_{max} = 0.389 (0.399) and K_d = 54 units/mL (40 units/mL) for 6-fold (21-fold) dilution.

biotin−streptavidin binding,[33] which will likely increase the experimental time and cost. Therefore, from a practical perspective, the covalent immobilization method used here was still the most cost-effective way for antibody immobilization, even at the expense of the antibody activity.

OFRR Sensor Specificity Study. In Figure 4, we examined the specificity and cross-reactivity of the OFRR sensor for CA15-3 detection. Figure 4A shows the response of the anti-ER antibody coated OFRR to a high concentration of CA15-3 (100 units/mL) in PBS buffer. No noticeable shift was observed in the WGM spectral position, indicating that only anti-CA15-3 antibody could bind the CA15-3 specifically. Meanwhile, it also shows that amine−PEG−amine can block the surface to eliminate the nonspecific binding when the sample is in PBS buffer. Figure 4B shows the cross-reactivity study results, in which anti-CA15-3 antibodies were immobilized and the surface was also blocked with amine−PEG−amine. Interfering antigen CA12-5, a glycoprotein used as an ovarian cancer biomarker,[5] was flowed through the OFRR at a concentration of 100 units/mL for 20 min. No WGM spectral shift was observed. Immediately after the OFRR surface was rinsed with PBS buffer, 100 units/mL CA15-3 was introduced

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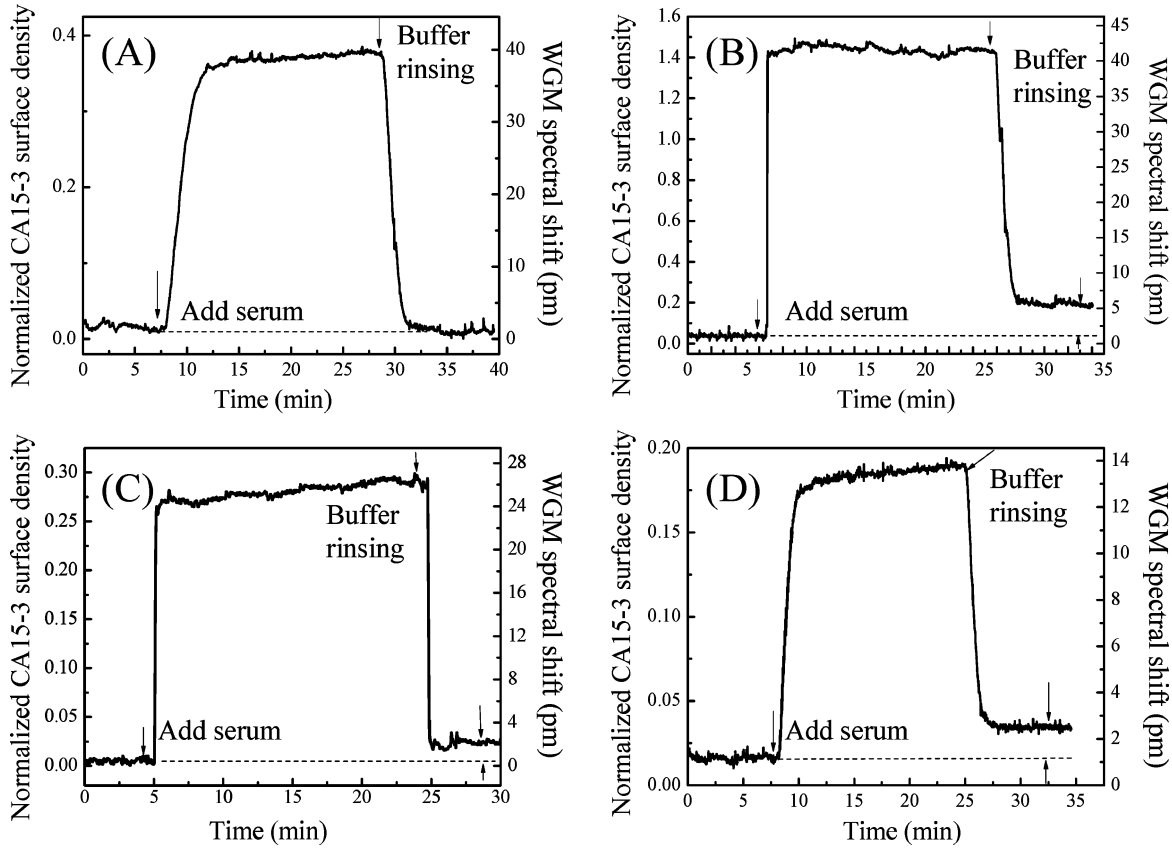

Figure 6. OFRR sensor response to diluted human sera. (A) No observable signal from 6-fold dilution of healthy male serum. (B) Sensor response from 6-fold dilution of stage IV breast cancer patient no. 4 serum. Original concentration 211 units/mL (standard clinical test result). (C) Sensor response from 21-fold dilution of stage IV breast cancer patient no. 1. Original concentration 19 units/mL (standard clinical test result). (D) Sensor response from 21-fold dilution of stage IV breast cancer patient no. 2. Original concentration 25 units/mL (standard clinical test result).

into the OFRR and a clear WGM spectral shift was measured, suggesting that anti-CA15-3 antibody has high specificity toward CA15-3.

Generation of Calibration Curves for the OFRR CA15-3 Sensor. To generate a calibration curve, we spiked purified CA15-3 into FCS to prepare sample concentrations ranging from 20 to 2500 units/mL, which covered the most useful clinical concentration range. Those samples were further diluted with running buffer by 6-fold or 21-fold. The detection process was very similar to what was described earlier for Figure S3B (Supporting Information); i.e., the calibration sample with incremental concentrations was flowed through the OFRR sequentially, followed by running buffer rinsing after each sample injection. The flow rate was kept at 5 μL/min.

Figure 5 shows the titration curve for CA15-3 in serum after 6-fold or 21-fold dilution. The curves are fitted with eq 3. The dissociation constant was 54 units/mL for 6-fold dilution and 40 units/mL for 21-fold dilution. These calibration curves were used to estimate the CA15-3 concentrations in an unknown clinical sample. Experimentally, we have demonstrated the detection of 1 unit/mL CA15-3 in diluted serum shown in Figure 5. To determine the CA15-3 concentration in an unknown sample, a clinical serum sample can be first diluted until the measured normalized antigen surface converges within the linear range of those calibration curves, which is a common procedure used in many commercialized detection kits such as ELISA. The actual CA15-3 concentration will be obtained by multiplying the dilution factor by the measured concentration.

Label-Free Detection of CA15-3 in Clinical Breast Cancer Patients' Sera. To study the feasibility of using OFRR for actual clinical diagnosis, we carried out preliminary experiments in which clinical patients' sera were diluted by either 6-fold or 21-fold and then tested with the OFRR. The test results were further compared with hospital pathology test results. Figure 6 shows some of the real-time sensorgrams for the serum test. The serum samples were flowed through the OFRR sensor, followed by running buffer rinsing. The healthy male serum did not generate an observable signal (Figure 6A) after buffer rinsing. The signals from four stage IV breast cancer patients' sera were significantly greater than that of the healthy male serum (Figure 6B−D). These results suggested OFRR could easily differentiate breast cancer patients from healthy people. Additionally, the detection can be completed in 20 min, much quicker than the detection using a commercialized CA15-3 ELISA kit, which usually takes approximately 3 h from serum sample incubation to getting the final test results.

On the basis of the calibration curves provided in Figure 5, we are able to estimate the CA15-3 concentration for those patients. Figure 7 compares the results obtained with clinical standard tests performed at ARUP Lab (Salt Lake City, UT) and with the OFRR tests. In Figure 7A, the patients' sera were diluted 6-fold, and it is shown that the OFRR results were 2−3 times higher than those from the standard tests. While we are unclear

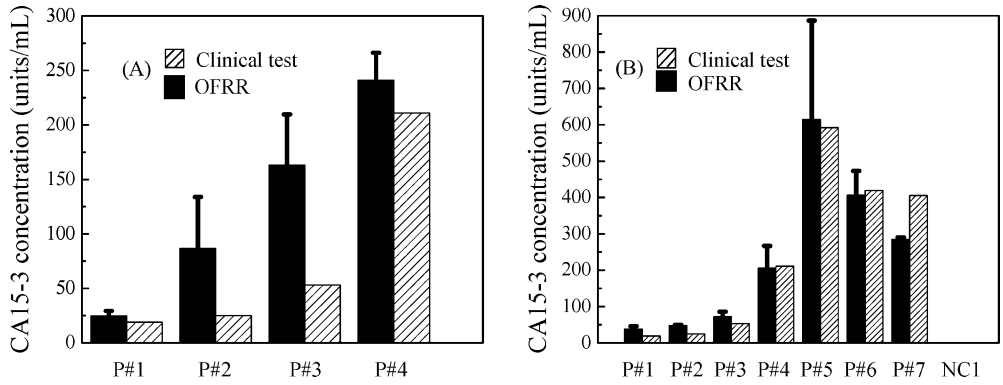

Figure 7. Comparison of CA15-3 levels of breast cancer patients measured by the OFRR and standard clinical test. Each point was measured three times. (A) P1−P4 serum samples were diluted 6-fold with running buffer and tested by the OFRR. (B) P1−P7 samples and the normal control serum sample (NC1) were diluted 21-fold with running buffer and tested by the OFRR. Note that the NC1 result is nearly zero in the figure.

about the real cause at this exploration stage, one possibility is the interference from the heterophilic antibodies in the human serum.[34,35] In contrast, the OFRR test results by diluting patients' sera 21-fold (Figure 7B) match the standard test results much better, and Figure S4 (Supporting Information) shows a good correlation between the OFRR detection and standard clinical tests. These results suggest that higher dilutions might reduce the interference and result in better antigen recovery, thus providing more accurate measurement. We have experimentally demonstrated the detection of approximate 1 unit/mL CA15-3 in diluted serum. If we choose 21-fold dilution as the optimal dilution factor for future clinical studies, we should be able to detect CA15-3 around 20 units/mL in undiluted human serum samples, as has been demonstrated by Figure 6C. Therefore, the OFRR can be used as a clinical diagnostic tool to quantitatively measure the CA15-3 concentration in breast cancer patients' sera below and above the 30 units/mL cutoff value.

CONCLUSION

In this work, we developed label-free OFRR sensors that were applied for the first time to clinical samples. In particular, we developed the sensing protocols to detect breast cancer biomarker CA15-3 in PBS buffer and in human serum. Those results showed that the OFRR was capable of rapidly detecting CA15-3 in less than 30 min. Effective surface blocking methods were developed, which minimized the serum protein nonspecific adsorption. The OFRR achieved good sensitivity and specificity. Experimentally we have detected CA15-3 of 1−200 units/mL in PBS buffer and 20−2500 units/mL in FCS with proper dilutions. Clinical stage IV breast cancer patients' serum samples were also tested by the OFRR. It is shown that the OFRR was capable of detecting CA15-3 in human serum in the clinically relevant range, thus providing a rapid and cost-effective tool for clinicians to monitor their cancer patients during therapy and for long-term follow-up. Future studies will first focus on optimizing the OFRR sensing performance (e.g., antibody density, serum sample dilution factor, etc.) and carrying out the systematic clinical trials to further validate the OFRR's applications as a simple clinical laboratory test device. OFRR arrays will also be designed and fabricated to simultaneously detect multiple breast cancer biomarkers, which will significantly improve the breast cancer diagnosis and prognosis.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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