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Refractive Index-Based Detection of Gradient Elution Liquid Chromatography using Chip-Integrated Microring Resonator Arrays

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Abstract

Refractive index-based sensors offer attractive characteristics as non-destructive and universal detectors for liquid chromatographic separations, but a small dynamic range and sensitivity to minor thermal perturbations limit the utility of commercial RI detectors for many potential applications, especially those requiring the use of gradient elutions. As such, RI detectors find use almost exclusively in sample abundant, isocratic separations when interfaced with HPLC. Silicon photonic microring resonators are refractive index-sensitive optical devices that feature good sensitivity and tremendous dynamic range. The large dynamic range of microring resonators allows the sensors to function across a wide spectrum of refractive indices, such as that encountered when moving from an aqueous to organic mobile phase during a gradient elution – a key analytical advantage not supported in commercial RI detectors. Microrings are easily configured into sensor arrays, and chip-integrated control microrings enable real-time corrections of thermal drift. Thermal controls allow for analyses at any temperature and in the absence of rigorous temperature control, obviating extended detector equilibration wait times. Herein, proof of concept isocratic and gradient elution separations were performed using well characterized model analytes (e.g., caffeine, ibuprofen) in both neat buffer and more complex sample matrices. These experiments demonstrate the ability of microring arrays to perform isocratic and gradient elutions under ambient conditions, avoiding two major limitations of commercial RI-based detectors and maintaining comparable bulk RI sensitivity. Further benefit may be realized in the future through selective surface functionalization to impart degrees of post-column (bio)molecular specificity at the detection phase of a separation. The chip-based and microscale nature of microring resonators also makes it an attractive potential detection technology that could be integrated within lab-on-a-chip and microfluidic separation devices.

Keywords

refractive index detector; liquid chromatography; microring resonator; gradient separations; silicon photonics

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

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INTRODUCTION

Refractive index (RI) detectors in high performance liquid chromatography (HPLC) are used to analyze samples that lack strong chromophores in ultraviolet (UV) or visible regions and are non-fluorescent. The most notable advantages of RI detection are that the technique is nondestructive enabling downstream analysis, and RI is a concentration dependent bulk property allowing for universal detection (i.e., all compounds with polarizable electrons are detectable).^{1,2} The differential refractometer^{3,4} was one of the earliest implementations of RI detection in LC, and the design has remained popular over the past half century. This device consists of a sample and a reference flow cell with the temperature of each cell tightly maintained with a thermostat, commonly 30°C so as to circumvent background noise from ambient thermal fluctuations. Comparing changes in bulk RI of sample versus reference cells with identical mobile phase (MP) and temperature allows for sensitivities as low as 10^{-7} refractive index units (RIU) and detection limits of approximately 0.1% sample concentration (e.g., 5 mM for caffeine).² However, the reference cell is also the source of major limitations of RI detection for LC separations, namely the inability to perform gradient separations due to insufficient dynamic range and extended waiting periods to allow for thermal equilibration of the flow cells. Consequently, these limitations have largely restricted the use of the differential refractometer, and RI-based detection in general, to specialty applications, such as lipid,⁵ sugar,⁶ and protein⁷ detection for food analysis.

In the decades following the introduction of RI sensing to the commercial LC detection landscape, there have been multiple attempts to improve upon the original design to increase its overall applicability. Notable modifications include the elimination of the need to operate at elevated temperatures;⁸ incorporation of a second column and pumping system;⁹ continuous alteration of the laser interrogation angle to adjust for RI changes in MP composition to allow for gradient separations;¹⁰ and the use of thermooptic¹¹ and interferometric^{12–14} methods to increase sensitivity. To date, however, refractometer design modifications have yet to result in significant adoption.

Surface plasmon resonance (SPR), a technology most commonly utilized as a surface sensitive biosensor, has previously been interfaced with LC as a detector for oligosaccharide,¹⁵ protein,^{16,17} and carbohydrate^{18–20} analysis. The most notable advantages of SPR over traditional RI detectors for LC are the speed at which the detector can achieve a stable baseline (50 minutes versus >2 hours for commercial detectors¹⁹) and the modification of sensor surface to enhance sensitivity. Notably, all implementations of LC-SPR systems have been applied to isocratic separations.

Adapting RI sensing to gradient separations without complicated optics or additional pumping systems requires expanding the dynamic range of the sensing modality, removing the need for rigorous temperature stabilization, and improving the reproducibility of MP gradients. Microcavity photonic devices are high-Q factor optical sensors that allow for label free analyte detection.²¹ While exact size and geometries may vary, these devices provide circular paths around which light is repeatedly directed to form a resonant microcavity that support well-defined optical modes. The spectral position of the optical modes are extremely responsive to changes in the local dielectric environment surrounding the sensor surface. Most analogous to the studies presented herein, Fan and coworkers²² previously utilized liquid core optical rings resonators (LCORRs) to perform flow speed measurements using electro-osmotic flow and glycerol as a model for use of LCORR as a detector for capillary electrophoresis. The LCORR architecture allows for a RI detection limit of 10^{-6} RIU and sensitivity of 20 nm/RIU. More recently, similar devices have been used as a novel detector for microgas chromatography^{23,24} and for glucose detection in aqueous solutions.²⁵

Silicon photonic microrings are a chip-integrated version of a microcavity resonator that offer advantages in terms of scalability of device fabrication, which allows for sensor arrays to be easily constructed. Each individual microring is located next to an adjacent linear waveguide such that interference between photons circulating the microring and passing down the linear waveguide create a resonant microcavity that supports optical modes only at specific wavelengths, as described by Equation 1,

$$m\lambda=2\pi rn_{eff} \quad [1]$$

where m is an integer, λ is wavelength, r is the radius of the microring, and n_{eff} is the effective refractive index of the propagating optical mode. Based on Equation 1, the resonant wavelength is responsive to changes in the effective refractive index, which is directly affected by changes in the bulk solution, as sampled by the evanescent electromagnetic field.²⁶

Our group has previously applied this technology to the label-free detection of surface-localized binding interactions mediated through target-specific capture agents that recognize particular analytes of interest.^{27–30} However, microring resonator arrays are also responsive to bulk changes in RI. Herein, we demonstrate the application of silicon photonic microring resonator arrays as a bulk RI, universal detector for LC separations. Microring resonators are shown to monitor large fluctuations in refractive index (e.g., moving from an aqueous to organic solvent) and this extremely large dynamic range enables detection across rapid changes in MP. MP gradients are reproducible independent of temperature fluctuations, allowing for detection of analytes in column eluent from gradient elutions. Moreover, thermal controls are integrated into the sensor array, eliminating the need to operate under tightly controlled thermal conditions and obviating extended detector equilibration times. Consequently, microring arrays avoid the major pitfalls of RI-based detection while offering bulk RI sensitivity comparable to conventional detectors.

To demonstrate the utility of the sensor in reference to established commercial detectors, we performed isocratic separations on well-characterized analytes and gradient elution of analytes in pure buffer as well as a more complex matrix of a dissolved liquid gel capsule.

EXPERIMENTAL

Materials

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Ibuprofen liquid gel capsules (NDC 11673-122-80) were purchased from Target Corporation (Minneapolis, MN). Phosphate buffered saline (PBS) was reconstituted from Dulbecco's PBS at 10 mM and the pH adjusted to either 7.4 or 2.3 using 1 M HCl. Oxalic acid dehydrate, DL-malic acid, and succinic acid solutions were prepared at various concentrations in 10 mM PBS pH 2.3. Ibuprofen and simvastatin solutions were prepared in a 50:50 mixture of deionized (DI) water and HPLC-grade acetonitrile. A caffeine solution was prepared in DI water.

Instrumentation

Microring Resonators—Sensor array chips and the Maverick M1 optical scanning instrumentation were purchased from Genalyte, Inc. (San Diego, CA). Sensor chip fabrication and scanning instrumentation operation have been described previously.³¹ Figure S1 shows a scanning electron microscope (SEM) image of a microring, and provides schematics of the sensor geometry as well as an illustration of operation for LC detection. For the experiments in this study, 8 active sensor rings were used for monitoring bulk RI

response, and 4 rings covered by a polymer cladding layer were monitored for use as thermal controls, as they were not exposed to flowing solution during detection experiments.

Before use, sensor chips were briefly rinsed with acetone and then isopropanol to remove a protective photoresist coating, before cleaning with a piranha solution (3:1 H₂SO₄:30% H₂O₂) for 40 seconds. (*Caution! Piranha solutions are extremely dangerous and react explosively with organics.*) The chips were then sonicated in ethanol for 5 minutes and dried under a stream of nitrogen.

A tunable external cavity diode laser centered on 1550 nm was used to serially probe each microring individually, scanning across a 12 nm spectral window. Dips in the transmittance through the adjacent linear waveguide at particular laser wavelengths correspond to a resonance. Resonance wavelengths for each microring were measured as a function of time and relative shifts used to measure small changes in refractive index. The Maverick M1 enables sub-picometer resonance wavelength resolution and a noise floor of approximately 0.1 pm, corresponding to a bulk RI detection limit of 1.5×10^{-6} and sensitivity based on the determined relationship of 63 nm/RIU (See Figure S2).³¹

HPLC—Chromatographic separations were performed using a Dionex Ultimate® 3000 Binary Analytical System (Thermo Fisher Scientific, Waltham, MA) equipped with a HPG-3200SD pump, WPS-3000SL analytical autosampler with 100 μ L sample loop, TCC-3000SD column compartment, MWD-3000 diode array detector, and RefractoMax 521 refractive index detector. The RefractoMax is a deflection type RI detector with tungsten filament light source and 8 μ L flow cells (reference and sample cells). MP composition for isocratic separations were 10 mM PBS pH 2.3, 10 mM PBS pH 7.4, or 90:10 DI water:acetonitrile. Linear MP gradient elutions consisted of 90:10 water:acetonitrile to 0:100 or DI water to 1 M NaCl. Columns utilized included an Acclaim 120 C18 column (3 μ m particle size, pore size of 120 Å, and dimensions of 4.6 mm x 150 mm), and a Hypersil GOLD® aQ polar endcapped C18 column (1.9 μ m particle size, pore size of 175 Å, and dimensions of 4.6 mm x 150 mm). The flow rate when operated in standalone mode was 0.600 mL/min and 0.100 mL/min for the LC-microring resonator interface, unless otherwise noted. The column oven was set to 40°C and 25°C for the Acclaim C18 and Hypersil Gold columns, respectively. The RefractoMax detector was maintained at 30°C.

HPLC-Microring Resonator Interface—Sensor chips were sandwiched between a cartridge holder, 0.007” biaxially-oriented polyethylene terephthalate (Mylar®) gasket, and a polytetrafluoroethylene (Teflon®) cartridge top for fluid delivery. The outlet from the HPLC was connected to a 0.25 mm flangeless 1/4-28 to ZDV 10-32 PEEK low pressure union to convert from HPLC to microring resonator fluidic fittings and a 1/4-28 nut was screwed directly into the Teflon cartridge top. This fluidic interface was limited to flow rates between 0.010 and 0.100 mL/min. A more complete description and image of the fluidic interface is provided in Figure S3.

Data Analysis

All data analysis was performed using Origin Pro 9.0. Active sensor signal responses were adjusted for temperature fluctuations by subtracting responses from 2 or more thermal control rings. Thermally controlled data was subsequently averaged, and the average responses presented represent between 4 and 8 thermally-corrected sensor rings. While data processing and analysis was performed after data acquisition, the data processing could be automated and performed either during or immediately following data acquisition. The current version of the Maverick data acquisition software enables real time control subtraction and averaging of active sensor responses.

RESULTS AND DISCUSSION

Isocratic Separations

The interface between the HPLC and microring resonator platform is depicted in Scheme 1. Initially, isocratic elutions were used to establish the utility of the microring resonator platform for LC detection. A caffeine solution in water is a common standard for verification of system integrity and is often employed as an internal standard.^{2,32} Figure 1a shows a chromatogram corresponding to the microring resonator-based detection of a 10 mg/mL (51.5 mM) caffeine solution at various injection volumes using the Acclaim 120 C18 column and a MP of 90:10 DI water:acetonitrile. Peaks corresponding to the injection volume and caffeine were baseline resolved at the smallest injection volume of 0.1 μ L (corresponding to 1.0 μ g of caffeine injected onto the column), and the peak area is linearly related to the mass of caffeine injected onto the column (See Figure 1b).

As a second demonstration, an equal mass solution of malic acid, oxalic acid, and succinic acid was isocratically separated in 10 mM PBS pH 2.3 on a Hypersil GOLD aQ polar capped C18 column. Peaks for each analyte and the injection were baseline resolved, and Figure 1c shows sample injections ranging from 5 to 20 μ L (between 8.3 and 33.2 μ g of each analyte injected onto column). Peak area correlates strongly with mass injected onto the column for each analyte, as shown in Figure 1d. In comparison to RI data from a commercial detector (Figure S4), the noise levels are slightly higher for the microring resonator platform, most likely due to irregularities in MP flow due to imperfect coupling between the HPLC and scanning instrumentation, but the sensitivities are comparable. Importantly, improvements in microfluidic sample delivery over this initial crude interface should greatly reduce baseline fluctuations.

Gradient Demonstration and Analysis

Early in the development of HPLC, gradient MPs were introduced to increase elution time and sharpen later-eluting peaks.³³ Gradient methods improve the efficiency of overall method development and minimize the need for complicated sample preparation due to interfering species. In addition, large molecules such as proteins and oligonucleotides are difficult to resolve with isocratic methods due to the large changes in retention times that result from minor changes in MP composition.² Gradient elution methods have been widely adopted in the LC community and are routinely used when coupled to optical detection methods such as UV/visible absorption and fluorescence detection. However, RI-based detectors have typically not been amenable to gradient elutions as the overall RI shift surpasses the dynamic range offered by commercial detectors. For example, the reported linear dynamic range of the RefractoMax detector is 600 μ RIU whereas the difference in refractive index (Δ RIU) encountered when transitioning from water to methanol at 25°C is 3200 μ RIUs at a wavelength of 550 nm.

Given the importance of the refractive index dynamic range for gradient elution separations, we sought to probe the capabilities of the microring sensor array using a variety of gradients. Figure 2a demonstrates the reproducibility of a linear gradient encountered when repeatedly cycling between DI water and an aqueous 1 M NaCl solution. Importantly, the stability of the gradient profile in the absence of analyte injection, which is corrected for thermal drift, is sufficiently reproducible so that it can simply be subtracted from the response observed during an actual separation with the same MP gradient profile. A gradient baseline subtraction cannot be performed using commercial instrumentation principally due to the limited dynamic range of the detector. Moreover, the low noise floor of commercial detectors depend upon real time subtraction of reference flow cell containing an identical mobile phase composition as the sample cell. Maintaining identical composition for both

flow cells for gradient elutions is currently not possible since detectors do not allow for simultaneous flow through each of the cells. Figure S5 shows the output of a gradient on the RefractoMax detector. Three other tests of gradient elution compatibility, which are shown in Figure 2b, include transitions from 90:10 water:acetonitrile to acetonitrile, DI water to 1 M NaCl, and DI water to dimethylsulfoxide (DMSO). The DMSO transition shows an enormous resonance shift of over 7 nm, which corresponds to a 100,000 μ RIU change in bulk RI. This measured RI change is more than 3 orders of magnitude greater than that offered by commercial detectors and is more than large enough to accommodate any standard gradient elution.

Separation of Simvastatin and Ibuprofen

While the reproducibility and wide dynamic range of microring resonators in response to large shifts in RI in the form of gradients were demonstrated, it remained to be seen whether small changes in RI associated with the elution of analytes could be resolved from a simultaneous signal increase in response to changes in MP composition. As with the demonstration of isocratic methods, well characterized, model analytes, namely ibuprofen and simvastatin, were used to demonstrate detection of analytes in gradient elutions. The gradient employed was intentionally simplistic to ensure all changes in sensor response were due to injection, MP changes, or sample elution. The gradient from 90:10 water:acetonitrile to acetonitrile over 30 minutes showed clearly resolved peaks for the two model analytes. A large injection volume of 100 μ L was used to demonstrate the upper limit of analyte signal. The samples were dissolved in a 50:50 water:acetonitrile solution before injection. Figure 3 shows the separation both before and after subtraction of a reference gradient.

Analysis of Ibuprofen Liquid Gel

The use of RI based detection is primarily advantageous due to its ability to act as a universal detector responding to essentially all analytes of sufficient concentration. As such, one application of RI detection is in the chromatographic separation of complex sample matrices. In order to demonstrate the application of microring resonator arrays for the analysis of a more complex sample, we used an ibuprofen liquid gel capsule dissolved in a 50:50 water:acetonitrile solution. While less complex than commonly studied matrices, such as serum, cell lysate, or waste water, the ibuprofen capsule served as a proof-of-concept demonstration of the detection platform. In addition to ibuprofen, the liquid gel capsule also contained (per the manufacturer) FD&C green no. 3 (an organic dye), gelatin, mineral oil, pharmaceutical ink (composed of alcohols, shellac, titanium dioxide, and propylene glycol),³⁴ polyethylene glycol, potassium hydroxide, water, sorbitan, and sorbitol.¹⁴ Figure 4a shows the separation of capsule components dissolved in a 50:50 water:acetonitrile solution using a MP gradient from 90:10 water:acetonitrile to acetonitrile over 30 minutes. By comparison with a 10 mg/mL (48.5 mM) ibuprofen solution, the matrix components give a broad response surrounding the injection peak. However, the ibuprofen peaks are comparable between the 10 mg/mL stock and the dissolved capsule. Figure 4b is the same separation with an identical gradient (no injection) subtracted. For reference, data acquired using a commercial UV/Vis detector for an identical separation is provided in Figure S6.

Conclusion

In stark contrast to commercial RI detectors, the vast dynamic range and integrated thermal correction capabilities of microring resonator sensor arrays make them uniquely amenable for high performance LC detection. Specifically, this platform allows for operation without extended thermal equilibration time, operation at ambient temperature, and eliminates the need to simultaneously acquire reference and sample data, as is the case for traditional refractometers. Furthermore, the broad dynamic range of microring resonators firstly allows for RI-based detection of eluents during gradient elution separations, which are essential to

many contemporary LC protocols. Looking forward, improvements in the liquid handling interface as well as automation of data processing will likely reduce sensor noise, increase efficiency and sensitivity, and simplify detector operation for the end user. The potential use of microring resonators in LC might be further developed by modifying sensor surfaces to enhance target-specific sensor sensitivity (e.g., via capture agent immobilization) or to perform quasi-2-D separations (e.g., via silane chemistry) that utilize differentially-functionalized sensors that are matched (or mismatched) to column surface chemistry with interaction kinetics reporting on the chemical properties of eluting species. Finally, since the sensor array has a small footprint (4 mm x 6 mm), the sensors hold potential for incorporation into lab-on-a-chip separation devices as a chip-integrated technology for direct analyte detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) A 2 μ L injection of 10 mg/mL (58.5 mM) caffeine onto an Acclaim C18 column shows a simple isocratic separation using the microring resonator arrays as a bulk RI detector. (B) Plot of peak area versus injection volume shows the reproducibility of peak area and linearity of mass injected onto the column and sensor response ($N = 3$ and error bars represent $\pm$ standard deviation). (C) Various injection volumes of a mixture of 12.4 mM malic acid, 18.4 mM oxalic acid, and 14.1 mM succinic acid (each at 1.66 mg/mL) dissolved in 10 mM PBS pH 2.3. The Hypersil Gold polar-capped C18 column was used. (D) Plot of peak area versus injection volume is similar to panel B, showing a linear relationship with slopes varying slightly dependent upon the analyte.

Figure 2.

(A) A series of 4 identical salt gradients is shown. The MP begins with DI water to 1 M NaCl and back to DI water. A dotted line is added to emphasize gradient reproducibility. (B) Gradients for 3 different MP compositions are shown: (1) water to DMSO, (2) water to 1 M NaCl, and (3) 90:10 water:acetonitrile to acetonitrile. Inset shows a zoomed in plot of the NaCl and acetonitrile gradients.

Figure 3.

(A) A 100 μL injection of 2.5 mg/mL (12.1 mM) ibuprofen and simvastatin in 50:50 water:acetonitrile followed by gradient elution LC separation (90:10 water:acetonitrile to acetonitrile). (B) Data from panel A referenced to an identical MP gradient without a sample injection. Inset shows a zoomed in plot of the ibuprofen and simvastatin peaks.

Figure 4.

(A) A 20 μ L injection of an ibuprofen liquid gel capsule dissolved in 20 mL of 50:50 water:acetonitrile. A 20 μ L injection of 10 mg/mL (48.5 mM) ibuprofen stock is provided for reference. (B) Data from panel A referenced to an identical MP gradient without a sample injection.

Scheme 1.

Outline of instrument setup. For all runs, UV/Vis absorption data was acquired followed by downstream detection using the microring resonator platform. For comparison experiments, the effluent was directed to a commercial RI detector.