Size-selective analyte detection in an integrated optical Young interferometer biosensor

Harmen Mulder
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Harmen K.P. Mulder

Members of the dissertation committee:

Prof. dr. ir. J.W.M. Hilgenkamp	University of Twente (<i>chairman and secretary</i>)
Prof. dr. V. Subramaniam	University of Twente (<i>promotor</i>)
Dr. ir. J.S. Kanger	University of Twente (<i>assistant promotor</i>)
Prof. dr. K.J. Boller	University of Twente
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Dr. ir. C. Blum	University of Twente
Prof. dr. H. Salemink	Radboud University Nijmegen
Prof. dr. L.M. Lechuga Gómez	Institut Català de Nanociència i Nanotecnologia

Cover: Schematic representation of a cross section of a waveguide with propagating modes of three different wavelengths which can be used to discriminate between different sized substances.

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Harmen Klaas Peter Mulder

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SIZE-SELECTIVE ANALYTE DETECTION IN AN INTEGRATED OPTICAL YOUNG INTERFEROMETER BIOSENSOR

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Dit proefschrift is goedgekeurd door:

Prof. dr. V Subramaniam
promotor

Dr. ir. J.S. Kanger
assistant promotor

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Chapter 1

Introduction

Abstract

This thesis presents a Young interferometer (YI) biosensor that can perform size-selective measurements using multiple wavelength excitation. This size-selective detection can be used to improve the specificity of evanescent field-based optical biosensors and is based on the various sensitivities of the evanescent fields of multiple wavelengths. The approach of using multiple wavelengths is, in addition to YI biosensors, also applicable to other types of evanescent field-based optical sensors. In this chapter, we first introduce the general concept of biosensors, followed by a discussion of important criteria for biosensors. The most widely-used evanescent field-based optical sensors are reviewed. The limiting specificity of these sensors is addressed, together with techniques to improve the specificity. Finally, we propose the new approach of size-selective detection, based on the use of multiple wavelengths to improve the specificity of evanescent field-based optical biosensors.

1.1 What is a biosensor?

A sensor can be defined as a device that detects a change in a physical stimulus and converts this into a measurable signal. If a sensor is used for detection and quantification of the detected material in a biological sample we speak of a biosensor. The detected material, which is called the analyte, could, for example, be a disease biomarker, an enzyme, a virus or a protein. Biosensors generally consist of a sensitive recognition element that recognizes a specific analyte and as a result produces a physicochemical signal. Enzymes, antibodies, nucleic acids, cell receptors, tissue, and microorganisms are several examples of biological elements used in biosensors. Furthermore, the biosensor contains a transducer element that converts the detected physicochemical signal into a measurable signal. The intensity of this signal is directly or inversely proportional to the analyte concentration in the measurement sample. Fig. 1.1 shows a schematic overview of a biosensor.

Fig. 1.1: Schematic overview of a biosensor

1.2 Biosensor criteria

Depending on its purpose, a biosensor must meet several criteria. If, for example, the purpose of the biosensor is to measure very low concentrations of a specific analyte, the biosensor must be very sensitive and specific. Other criteria are important if high concentrations of the analyte need to be measured rapidly and inexpensively. The six main criteria for biosensors are:

- Sensitivity
- Specificity
- Scalability to smaller dimensions
- Measurement time
- Measurement costs
- Multiplexing

and are discussed here.

1.2.1 Sensitivity

The more sensitive the biosensor, the lower the measurable concentration of analyte. The sensitivity of the biosensor is strongly determined by the transducer element of the biosensor. Widely used transducers in biosensors are optical, electrochemical, micromechanical, thermal or piezoelectric. However, optical transducers play a predominant role, because of their high sensitivity and high bandwidth [1]. Additionally, optical transducers have the advantages of being non-invasive and non-destructive, free of electrical or explosion risks and immune to electromagnetic interference. Therefore, we focus on optical transducers.

Apart from the type of transducer element, the sensitivity of a biosensor is also determined by the recognition element of the sensor. Depending on which type of element is used, biosensors can be classified as labelled or label-free. In a labelled detection scheme, a label (for example fluorophores, enzymes or radionuclides) is usually attached to the analyte to allow sensing. The main advantage of labelled biosensors is their high potential to detect low concentrations. With fluorescence-based detection, the sensitivity can even go down to single molecules [2]. However, labels have their own inherent problems; for example, fluorophores can quench and photobleach, and radioisotopes have a short lifetime, high costs and can produce hazardous contaminants. Furthermore, quantitative measurements are challenging when using labelled sensing, because it is difficult to control the exact number of labels on each molecule [3]. Most importantly, the label has to be conjugated to the recognition element, which requires further sample handling steps, so no direct on-site measurement is possible. Conversely, label-free optical detection is highly suited for kinetic and quantitative measurements of molecular interactions. For label-free optical detection, a bioreceptor layer is usually used. The sensitivity of these label-free optical sensors is also determined by the specificity of this bioreceptor layer, the stability of the linker between the molecule and the surface, and the number of available binding sites. In conclusion, the selection of the recognition element is crucial for determining the sensitivity of the biosensor.

The sensitivity of a biosensor can be expressed as a limit of detection (LOD). The LOD of an optical biosensor can be expressed in refractive index units (RIU) or in surface mass density (pg/mm^2) for any biosensor that is sensitive to any accumulation of mass on its surface.

1.2.2 Specificity

The specificity of a biosensor is a measure for how specific the measured signal is when detecting the analyte. The specificity of a biosensor is mainly determined by the biological element of the biosensor. For labelled biosensors it is important to correctly label the analyte, so a suitable label should be found for each analyte. The label must not have any effect on the function of the molecule and must not block the active

binding sites of the molecules. On the other hand, for label-free detection, the bioreceptor layer must be highly specific toward the analyte and must have a high affinity toward the analyte. Furthermore, the interaction event between the element and analyte must be detectable by the transducer element. Proteins, nucleic acids and antibodies are most commonly used as bioreceptor layers. Such bioreceptor layers can be immobilized at the surface on the basis of physical adsorption, covalent binding, non-covalent interactions to a previously deposited layer (for example, biotin-streptavidin or protein A for antibodies), physical entrapment, and self-assembled monolayers. It is important that the specificity and affinity of the element must not be altered significantly by its immobilization on the surface of the optical transducer. Reference channels, blocking techniques and washing steps are used to improve the specificity of label-free biosensors, as the specificity of the bioreceptor layer is not always sufficient to accurately determine the analyte concentration. Reference channels are used to cancel out bulk effects, where blocking techniques prevent non-specific binding and washing steps are used to remove non-specific bound particles.

1.2.3 Scalability to smaller dimensions

Lab-on-a-chip (LOC) devices are miniaturized devices in which all functionalities are integrated on the same platform and that offer significant advantages over conventional analytical methods [4]. Such LOC devices have to be both small and portable, and therefore the biosensor should preferably be scalable to small dimensions. For that reason, integrated optical (IO) devices offer an ideal solution for LOC platforms [1]. Apart from the advantages for all optical sensors, IO devices combine mechanical stability with scope for miniaturization. Besides the advantages of being small and portable, smaller devices also lead to reductions in volumes of sample and reagents required.

1.2.4 Measurement time

The measurement time is also important for any biosensor that has to detect analytes rapidly and in real-time. Many techniques, such as enzyme-linked immunosorbent assay (ELISA) [5, 6] and polymerase chain reaction (PCR) [7], are very sensitive and specific, but require multiple processing steps and therefore considerable time to perform. Label-free biosensors usually require much less time to perform compared to labelled biosensors, because no time is needed for labelling the analyte, amplification steps or purification of samples. Moreover, label-free techniques can be used to measure in real-time and on-site, because analytes can be detected in their natural forms. If the label-free techniques are combined with IO devices that incorporate microfluidics, the response time of the biosensor can be reduced even further.

1.2.5 Measurement costs

Biosensors should preferably be as inexpensive as possible without compromising the essential properties of the biosensor. Required labels and sometimes laborious labelling processes represent a substantial part of the costs of labelled biosensors. On the other hand, label-free techniques entail the costs of a bioreceptor layer. The advantage of IO optical devices is that they can be both small and mass-produced, and therefore have the potential to be inexpensive.

1.2.6 Multiplexing

Multiplexing is the ability to simultaneously measure various analytes from the same sample. Optical transducers have a potential for parallel sensing which means that they can be used for multiplexing [1, 8]. Nevertheless, multiplexing for labelled biosensors can be complicated, as it requires multiple labels and suitable detection. On the other hand, label-free detection needs suitable bioreceptor layers. However, label-free detection can be combined with IO devices, which are highly suited for multiplexing, because of the possible fabrication of arrays of sensors with the same characteristics within the same chip and their great flexibility in the choice of materials and the structures selection [4].

1.3 Label-free integrated optical biosensors

Based on the previously discussed selection criteria, the focus in this thesis will be on label-free IO detection. Most IO sensors rely on the evanescent field detection principle. In such sensors, the electromagnetic waves, called guided modes, are confined in a waveguide due to total internal reflection (TIR). However, part of the electromagnetic waves, the evanescent field, penetrates the interfaces of the different materials of the waveguide. Interaction between the analyte and the bioreceptor coated on the waveguide surface results in a change in refractive index (RI). This in turn changes the propagation of the guided mode through the waveguide by changing the evanescent field. The evanescent field-based sensors can be classified as non-linear when guided modes are generated that have a different wavelength to that of incident light (e.g. Raman detection). Linear evanescent field-based sensors measure the change in the RI in the evanescent field as a modification of the intensity, phase or polarization of the output signal. The linear evanescent field sensors can be divided into two categories, both measuring the RI change in the evanescent field. On one hand, there are the absorption sensors, in which the change in the imaginary part of the RI results in a modification of the light intensity at the end of the device. On the other hand, the changes can also be created in the real part of the RI, resulting in a change in the propagating velocity of the guided mode. We focus on the linear

evanescent field-based sensor, based on the detection of changes in the real part of the RI. The most important examples of this type of sensor are reviewed in this subsection. As a comparison to these IO sensors, the commonly-used optical evanescent-field based biosensor, Surface Plasmon Resonance (SPR), is added. Table 1.1 on page 17 shows a comparison of the LOD of various biosensors.

1.3.1 Surface Plasmon Resonance sensors

The SPR sensor is a widely used type of biosensor. Surface plasmons can be excited when an incident beam of transverse magnetic (TM)-polarized light strikes a thin electrically conducting layer at interface of a thin metal film and a dielectric material. Under conditions of TIR, the incident light will be coupled to a surface plasmon (SP) if the incident light wave vector component parallel to the surface matches the surface plasmon wave vector ($k_i=k_p$). The incident light wave vector component parallel to the surface, k_i , is given by:

$$k_i = \frac{2\pi}{\lambda} n_p \sin \theta_i, \quad (1.1)$$

where λ the wavelength of the incident light, n_p the refractive index of the prism and θ_i the angle of the incident light. The SP wave vector, k_p is expressed as:

$$k_p = \frac{2\pi}{\lambda} \left(\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2} \right)^{1/2}, \quad (1.2)$$

where, ε_1 and ε_2 are the dielectric permittivity constants of the metal film and the dielectric exit medium, respectively. Coupling is performed by a coupling device. Grating couplers, waveguide couplers and prisms couplers are examples of coupling devices. One can speak of the Kretschmann configuration if there is a thin metal film at the surface of the prism, as shown in Fig. 1.2a. A bioreaction, causing an RI change in the dielectric and thereby a change in ε_2 , results in a change to the SP wave vector. This can be quantified by measuring the changes in the characteristics of the reflected light, commonly achieved by measuring the coupling angle, coupling wavelength or intensity. Depending on which variable is measured, the SPR sensor is classified in terms of angular, wavelength, intensity or phase modulation [9]. Those SPR sensors based on wavelength, intensity or phase modulation (see Fig. 1.2b) provide the highest RI resolutions [10]. The best SPR sensor based on prism coupling with a limited number of channels (<10) provides a RI resolution of 10^{-7} RIU [10]. SPRs based on grating coupling typically achieve resolutions of 10^{-5} - 10^{-6} RIU [11, 12] and waveguide based SPRs, typically achieve resolutions of 10^{-5} - 10^{-6} RIU [13, 14]. A resolution of 3×10^{-8} RIU has also been reported [15]. However, these long-range surface plasmons, which are called long-range because of their low attenuation, so

propagation over centimetres, penetrate much further (1400 nm vs. 200 nm for conventional SPR) into the sensing region; therefore the sensor can only be fully used when large analytes are targeted or biorecognition elements are immobilized on the surface and combined with an extended coupling matrix [10]. Average mass detection limits of 1-5 pg/mm² were reported [10]. The SPR biosensor is successfully commercialised by GE Healthcare as Biacore™ [16]. SPR is widely used due to its robustness and simplicity. However, its large size makes it complex to achieve miniaturisation suitable for LOC devices.

Fig. 1.2: a) SPR in Kretschmann configuration based on b) angular, wavelength or intensity modulation.

1.3.2 Grating couplers

A grating coupler consists of a single-mode waveguide containing periodic disturbances. At a certain angle, at which the incoupling condition is fulfilled, the grating permits the excitation of a guided mode. The incoupling condition is given by:

$$N_{eff} = n_{air} \sin \theta_i + q \frac{\lambda}{\Lambda}, \quad (1.3)$$

where N_{eff} is the effective refractive index of the waveguide, n_{air} the RI of air, θ_i the angle of the incident light, q the diffraction order, λ the wavelength of the incident light and Λ the grating period. If the RI changes, for example due to a bioreaction, this results in a change in N_{eff} . As a consequence, the optimal coupling angle changes. Photodetectors at the end of the waveguide measure the intensity of the coupled light as a function of the angle θ_i , which angle is changed using a rotation stage with precise mechanical movement. Alternatively, the outcoupling angle can be used for sensing, which does not require a rotation stage [17]. Both the incoupling and outcoupling configuration show an LOD of 10⁻⁶ RIU. Furthermore, a setup configuration based on reflection-mode operation has been developed [18], which

avoids moving parts in the setup. A lens is used to simultaneously irradiate a range of angles onto the grating. Due to the effective coupling into guided modes under the coupling angle, a minimum of the reflected light is observed and followed by a CCD camera. Applying this method, an LOD of 3×10^{-6} RIU is reported. Besides, a so-called wavelength interrogated optical sensor (WIOS) based on grating couplers has been developed [19, 20]. One grating coupler is used for coupling the light into a single-mode waveguide. The other grating is used for light outcoupling. At a fixed angle of incidence, which can be set using an adjustable mirror, the RI changes are monitored by scanning the resonance peak, by using a tuneable laser diode. At the resonance wavelength, light is coupled into the waveguide. Next, the light is coupled out via another grating and collected by a multimode fiber, which directs the light to a photodiode. An LOD of $<10^{-6}$ RIU was reported and by measuring small molecules, a mass coverage of 100 fg/mm^2 was obtained, corresponding to a detection limit of 0.3 pg/mm^2 [19]. Grating couplers are suitable for multiplexing and due to its simplicity and ability to measure label-free, the technology has been commercialised by MicroVacuum Ltd [21]. Fig. 1.3 shows a schematic overview of two types of grating coupler sensors.

Fig. 1.3: Two types of grating couplers, both using a grating to couple the light into the waveguide, where one uses a detector at the end of the waveguide measuring the intensity as a function of the coupling angle and the other uses a second grating to couple the light out of the waveguide, measuring the change in resonant wavelength.

1.3.3 Ring resonator sensors

In ring resonator sensors, light is coupled into a circular waveguide via the evanescent field of an input waveguide. As a result of TIR along the curved boundary, the waves propagate through the waveguide in the form of whispering gallery modes (WGM). The sensitivity is increased by constructive interference over the multiple round-trips. The WGM circulates along the resonator surface many times where it interacts with the analyte via the evanescent field. In contrast with straight waveguides, the interaction is no longer determined by the length of the waveguide, but by the number

of light circulations within the ring, which is characterized by the resonator quality factor (Q-factor). The effective length L_{eff} , is given by:

$$L_{eff} = \frac{Q\lambda}{2\pi n}, \quad (1.4)$$

where λ is the resonant wavelength and n is the RI of the ring resonator. Q-factors of 10^6 can be reached in resonators resulting in an L_{eff} of several centimetres.. The detection is based on RI changes, which are related to the resonant wavelength by:

$$\lambda = 2\pi N_{eff} \frac{r}{M}, \quad (1.5)$$

where N_{eff} is the effective refractive index experienced by the WGM, r the radius of the ring, and M is an integer describing the WGM angular momentum. Therefore, a bioreaction – causing an RI change near the surface of the ring resonator – changes the effective RI, leading to a spectral shift in the WGM. This can be monitored by scanning the wavelength or by measuring the intensity profile at a fixed wavelength. Fig. 1.4 shows a schematic overview of a ring resonator sensor. Ring resonators have the potential to be combined into highly dense arrays, which is a valuable feature for multiplexing. Sensitivities of 7.6×10^{-7} RIU [22] and 1.5 pg/mm^2 [23] have been achieved and the Maverick Detection System ring resonator sensor has been commercialized by Genalyte [24].

Fig. 1.4: Schematic overview of a ring resonator sensor, in which RI changes are measured as a change in transmission wavelength, as the light coupled from the input waveguide to the circular waveguide changes, because of the change in the resonant wavelength of the circular waveguide.

1.3.4 Photonic crystal based sensors

Photonic crystal (PC) based sensors are well-defined nanostructures, which consist of a periodicity in the order of the wavelength of the light. Photonic bandgaps (PBG) are generated; the light whose wavelength lies within the PBG cannot propagate through the PC. This results in a wide stopband in the transmission or reflection spectrum. However, by locally disturbing the structure of the PC, a photonic “defect” within the bandgap can be introduced, leading to the formation of a defect mode. Consequently, light resonant with this defect mode can propagate through the PC, resulting in a peak within the bandgap. The spectral position of this peak strongly depends on the local environment around the defect. For that reason, this can be used for sensing molecules binding to the defect, which is illustrated schematically in Fig. 1.5. Parallel detection and light interaction with volumes down to femtolitres are possible, because light can be localized and concentrated in very small volumes. LODs of 7×10^{-5} RIU [25], 2.4×10^{-3} RIU, 4.9×10^2 pg/mm² [26] and 2.1 pg/mm² [27] have been achieved. These LODs were achieved with typically achieved sensitivities of around 100 – 175 nm/RIU, where Liu et al. show a significantly higher sensitivity of 460 nm/RIU and a design with multiple peaks in the transmission spectra which enables simultaneous detection of analytes [28]. Kita et al. presented an LOD of 9.0×10^{-5} RIU with an expectation of a resolution of $<10^{-6}$ RIU [29]. As the PC based sensors are quite recent, compatible microfluidics, surface chemistry and detection procedures to minimize non-specific binding still need to be optimized. Nevertheless, it was possible to specifically detect protein with PC's [30].

Fig. 1.5: Schematic overview of a photonic crystal based sensor, where binding of an analyte to the antibody results in a shift in transmission wavelength.

1.3.5 Interferometric waveguide sensors

Interferometric waveguide biosensors are the most attractive of various IO sensors due to their high sensitivity and broad dynamic range [4]. Next to their outstanding sensitivity, advantages of the interferometric waveguides are the cost-effective and simple production and the ability of multiplexing and miniaturization [31]. Two or more confined light waves form an interference pattern which is measured over time.

Interactions with the external medium (sample) via the evanescent field (see. Fig. 1.6) result in changes in velocity between the waves, which can be analysed from the interference pattern. High sensitivities can be achieved, due to the option of using long interaction lengths. Mach-Zehnder, bimodal, dual polarization, Hartman and Young interferometers are examples of interferometric devices used as biosensors and are described below.

Fig. 1.6: Schematic overview of a typical side view of a waveguide used for interferometric waveguide sensors, where the binding of the analyte to the antibody results in a change in velocity of the guided mode (red line), which can be analysed from an interference signal. The evanescent field, which is guided mode that penetrates into the sample, is used for sensing.

Mach-Zehnder interferometer

In a Mach-Zehnder interferometer (MZI), coherent light is coupled into a single-mode waveguide. A single-mode waveguide is required as higher order modes respond differently than single-mode on RI changes in the evanescent field. If a single-mode and higher order modes simultaneously propagate through the waveguide, their different sensitivities result in a misleading signal. A schematic overview of a Mach-Zehnder setup is shown in Fig. 1.7. Via a Y-junction the light is split up into two channels: one reference arm and one measuring arm. A bioreaction on the sensing area within the evanescent field of the measuring arm will cause a change in N_{eff} . This induces a phase change and consequently a change in the interference when the two arms are recombined. At the output of the sensor, a photodiode measures the intensity I_b which is expressed as:

$$I_t = I_s + I_r + 2(I_s I_r)^{1/2} \cos(\Delta\phi(t)), \quad (1.6)$$

where I_s is the intensity of the signal arm, I_r is the intensity of the reference arm and $\Delta\phi$ the phase change described by:

$$\Delta\varphi(t) = \frac{2\pi}{\lambda} l (N_{eff,s} - N_{eff,r}), \quad (1.7)$$

where λ the wavelength of the incident light, l the detection length and $N_{eff,s}$ and $N_{eff,r}$ the effective refractive index of the signal and reference arms, respectively. Due the cosine dependency of the intensity, the sensitivity depends on the position of the interferometric curve. The signal change is lower near the minima and maxima of the cosine compared to the quadrature points (points where $\Delta\varphi = (m + 1/2)\pi$, with $m = 1, 2, 3, \dots$) of the cosine. However, if the quadrature points move both continuously and linearly during a sensing event, the quadrature points can be tracked instead of the output intensity. Following this improvement, an LOD of 5×10^{-8} RIU was reported by Heideman and Lambeck [32]. Other LODs reported are 1.9×10^{-7} RIU by Dante et al. [33] and 1×10^{-7} RIU by Zinoviev et al. [34]. MZI's were also combined with grating couplers by Kozma et al. [35] resulting in an LOD of surface mass density of 1 pg/mm^2 and by Duval et al. [36] resulting in an LOD of 1.6×10^{-7} RIU. Lambeck reported an LOD of surface mass density of 0.01 pg/mm^2 [37]. The MZI is commercialised into a biosensor by Optisense [38] and Creoptix GmbH [39] which also combines the MZI with grating couplers.

Fig. 1.7: Schematic overview of a Mach-Zehnder interferometer, in which an analyte binding to an antibody causes a change in velocity in the measuring arm compared to the reference arm, resulting in a change in the interference signal.

Hartman interferometer

The Hartman interferometer [40] uses a grating to couple linearly polarized light into a single mode waveguide. Next, the light propagates through both a measuring arm and a reference arm. Again, the evanescent field is used for sensing a bioreaction. After passing through the sensing region in the measuring arm the light is combined with the light from the reference arm by an array of optical elements, creating an interference pattern. Finally, the interference signal is coupled out by another grating into a photodiode. Fig. 1.8 shows a schematic overview of the Hartman interferometer. A Hartman interferometer based sensor achieved an LOD of 10^{-6} RIU [41]. The main advantages of the Hartman technique are its simplicity and the ability to implement multiplexing without increasing the complexity of the setup. However, additional optical elements, which are required for multiplexing, and off-chip detection can be drawbacks for LOC devices based on Hartman interferometers.

Fig. 1.8: Schematic overview of a Hartman interferometer, in which an analyte binding to an antibody changes the velocity in the measuring arm compared to the reference arm, causing a change in the interference signal.

Bimodal waveguide

A bimodal waveguide (BiMW) is also based on interferometry. However, instead of using two arms (a signal arm and a reference arm), a single waveguide is used. The single waveguide consists of two different zones: a starting single-mode section and a second bimodal section that accepts two modes; for example, a zero-order mode and a first-order mode which form an interference pattern. RI changes can be sensed by the evanescent field in the sensing region which is located within the cladding. A

bioreaction causing such an RI change can be detected as a change in the interference pattern. The configuration of this type of interferometer (see Fig. 1.9) is less complex than for example the MZI interferometer. However, the reported sensitivities with the bimodal interferometer of $2\text{--}4 \times 10^{-7}$ RIU [42–44], are lower compared to those achieved using the MZI interferometer.

Fig. 1.9: Schematic overview of a bimodal interferometer, in which an analyte binding to an antibody results in various changes of the velocities of the different propagating modes, causing a change in the interference signal.

Dual polarization interferometer

The dual polarization interferometer (DPI) consists of a structure of five layers with various RI, one on top of the other. Fig. 1.10 is a schematic overview of the DPI. By means of TIR, the light is confined to the second and fourth layers which both have a higher RI than the first, third and fifth layers. These two waveguides are used as a reference and a sensing arm. The evanescent field of the sensing arm penetrates into a sensing region and is used for the detection of RI changes, induced by a bioreaction for example. The light propagating through the reference arm remains constant, as the evanescent field does not reach the surface of the device that is used as the sensing region. Both waveguides are excited simultaneously and at the output of the device, the light will form an interference pattern which is detected by a photodiode array. The polarization of the laser is modulated over time in order to excite TM and transverse electric (TE) modes. The change in propagation velocity due to a bioreaction is not the same for both polarizations. Therefore, the RI change and the change in thickness of a protein layer can be determined without ambiguity and real-

time [45]. The DPI was commercialised as AnaLight® interferometer by the Farfield Group who can reach an LOD of 10^{-7} RIU [4] and a surface mass density LOD of <0.1 pg/mm^2 [46].

Fig. 1.10: Schematic overview of a dual polarization interferometer, in which an analyte binding to an antibody causes a change in velocity in the measuring arm compared to the reference arm, resulting in a change in various shifts of the interference patterns of the two polarizations, that are then used to determine the RI change and thickness change of a protein layer.

Young interferometer

The YI is another widely used biosensor. In a YI, light is coupled into a single mode waveguide structure. Next, a y-splitter is used to split up the light into two waveguide arms, which are used as reference arm and sensing arms. Subsequently, the light is coupled out of the chip, after which it will recombine and form an interference pattern which is then detected by a charge-coupled device (CCD) camera. A schematic overview is shown in Fig. 1.11. The evanescent field senses RI changes near the surface of the sensing arm. This means that a bioreaction that causes an RI change results in an effective refractive index change ΔN_{eff} of the sensing arm with respect to the reference arm. Consequently, the interference pattern changes, from which the following phase difference between the different interfering beams is given by:

$$\Delta\varphi(y) = \frac{2\pi}{\lambda} \left(\frac{d \cdot y}{L} - (N_{eff,s} - N_{eff,r})l \right), \quad (1.8)$$

where λ is the wavelength of the incident light, d the distance between the two channels, L the distance between output of the sensor and the camera, y the position of the camera, l the detection length and $N_{eff,s}$ and $N_{eff,r}$ the effective refractive index of the signal arm and reference arm, respectively. If the RI changes in the signal arm, this results in a change in $N_{eff,s}$, resulting in phase change which is measured from applying a Fast Fourier Transform (FFT) on the interference pattern and reading out the phase signal at the correct spatial frequency. Advantages of the YI method include the detection of the whole interference pattern, contributing to the simplicity of the device. Furthermore, the identical lengths of the arms reduce the effects of wavelength drift and temperature. Moreover, the YI biosensors are among the most sensitive biosensors. An LOD of 6×10^{-8} RIU, corresponding to a surface mass density of 0.020 pg/mm^2 , was reported by Ymeti et. al [47]. Furthermore, Schmitt et. al reported an LOD of 9×10^{-9} RIU, corresponding to a surface mass density of 0.013 pg/mm^2 [48]. Additionally, multiplexing can be achieved, by using more than two waveguides [49]. The distance required from the output of the chip to the detector for detecting interference and in order to get a maximum resolution can be a drawback for LOC platforms. Moreover, this distance L as well as l , d and λ should be kept stable to only be sensitive to changes in N_{eff} .

Fig. 1.11: Schematic overview of a Young interferometer, in which an analyte binding to an antibody causes a change in velocity in the measuring arm compared to the reference arm, resulting in a shift of the interference pattern. The core of the waveguide consists in this case of the layer above the substrate and the ridges (which provides confinement in lateral direction) on top of this layer which are made from the same material and therefore coloured the same.

Table 1.1: Comparison of detection limits of optical biosensors

Device	Surface detection limit	RI detection limit (RIU)	References
SPR	1-5 pg/mm ² (averaged) 2 fg/mm ² (combined with imaging)	10 ⁻⁵ - 10 ⁻⁷	[10, 50]
Grating couplers	0.3 pg/mm ²	<10 ⁻⁶	[19]
Ring resonator sensors	1.5 pg/mm ²	7.6 x 10 ⁻⁷	[22, 23]
PC based sensor	2.1 – 490 pg/mm ²	2.4 x 10 ⁻³ - 7 x 10 ⁻⁵	[25-27, 29]
Interferometric waveguide sensors			
Mach-Zehnder interferometer	0.060 - 1 pg/mm ²	1.9 x 10 ⁻⁷ - 5 x 10 ⁻⁸	[32-37]
Hartman interferometer	n.d.	10 ⁻⁶	[41]
Bimodal waveguide	n.d.	2-3 x 10 ⁻⁷	[42-44]
Dual polarization interferometer	< 0.1 pg/mm ²	10 ⁻⁷	[4, 46]
Young interferometer	0.013 - 0.020 pg/mm ²	6 x 10 ⁻⁸ - 9 x 10 ⁻⁹	[47, 48]

1.4 Methods for improving specificity

As discussed previously, the specificity is an important criterion of a biosensor. Despite the application of a bioreceptor layer and the techniques for improving specificity of a biosensor (reference channels, blocking techniques and washing steps), the specificity is often insufficient. When measuring a human sample, such as blood or serum, bulk effects and non-specific binding limit the specificity. Therefore, Worth et al. developed a method to reduce the contribution to RI changes attributable to non-specific binding [51]. By tuning the evanescent field of two different polarization modes, a thin layer (20-30nm) was desensitized and the response to non-specific binding was reduced by a factor of a hundred or more. Their polarimetric waveguide interferometer uses the differential phase shift between two orthogonal modes, which were tuned by changing the core thickness such that both modes have equal sensitivity in the first 20-30 nm of the evanescent field. Therefore, the contribution due to non-specific binding was reduced. However, any RI change in this layer cannot be detected, resulting also in a reduction of the contribution due to specific binding. Furthermore, dual-wavelength operation of an integrated-optical difference interferometer was used to discriminate between the binding of molecules and bulk RI changes, or between binding of molecules and temperature changes [52]. However, the various background contributions to the signal are usually present simultaneously and therefore the existing methods that allow distinguishing only one of the various background contributions from the signal are in practice not always sufficient.

In this thesis, we present a new method to discriminate between analytes based on their size (size-selective detection) which can be used to improve the sensor specificity. We use the size-selective detection in combination with the extremely

sensitive YI biosensor, but it is also applicable to other types of evanescent field-based optical sensors. Previously, we expanded the existing dual-wavelength approach [52] to a three-wavelength approach that allows discrimination of several different background contributions (bulk and temperature induced RI changes) simultaneously [53]. Here, we use multiple wavelengths for size-selective detection of analytes. The multiple wavelengths enable the probing of RI changes at various distances from the sensor surface, allowing the simultaneous discrimination of larger particles (e.g. viruses) from both smaller particles (e.g. proteins) and bulk contributions, which improves the specificity of the sensor. The method can be used in combination with a bioreceptor layer and the existing methods for improving the specificity of the biosensors.

1.5 Outline of the thesis

In Chapter 2 a theoretical analysis of size-selective detection is presented. Numerical calculations are used to optimize sensor design and the detection method. Next, Chapter 3 presents the design, realisation and characterization of the setup which is used for size-selective detection. Chapter 5 present different analysis approaches which can be used for size-selective detection. Subsequently, chapter 4 presents the application of these analysis approaches on measurements, where among other things binding of beads (size ≈ 85 nm, representing specific binding) is discriminated from binding of protein A (size ≈ 2 nm, representing non-specific binding) and bulk refractive index changes induced by D-Glucose (which occurs in the whole evanescent field of a few hundreds of nanometres which is used for detection). In order to optimize the size-selective detection method, the use of multiple wavelengths in combination with multiple polarizations was investigated in Chapter 6. Moreover, it shows proof-of-principle experiments of the realization of size-selective detection based on multiple wavelengths and polarizations. The measured data of chapter 5 were analysed carefully and showed a timing difference between bulk changes and binding of proteins which was investigated in Chapter 7. Finally, Chapter 8 presents a forward look with possible new applications of size-selective detection based on constructive technology assessment. It was investigated if the method is suitable for biosensing or if there are other more interesting or promising applications or markets.

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Chapter 2

Size-selective detection in integrated optical interferometric biosensors

Abstract

In this chapter we present a size-selective detection method for integrated optical interferometric biosensors that could strongly enhance their performance. We demonstrate that by launching multiple wavelengths into a Young interferometer waveguide sensor it is feasible to derive refractive index changes (Δn) from different layers above the waveguide surface, enabling one to distinguish between bound particles (e.g. proteins, viruses, bacteria) based on their differences in size and simultaneously eliminating interference from a Δn in the bulk (region of a few hundred nanometres above sensor surface). Numerical calculations are used to optimize sensor design and the detection method. Adding size-selectivity to the sensor reduces the sensitivity of the sensor. However, the theoretical sensitivity remains still comparable to other existing biosensors when discriminating between Δn 's in three different layers above the waveguide based on simultaneous detection of effective refractive index changes (ΔN_{eff}) at three different wavelengths. Assuming a particle of 80 nm in size as the specific analyte to detect, the theoretically determined minimum detectable mass coverage is 4×10^2 fg/mm² (assuming a phase noise of 10^{-4} fringes). This is approximately one order of magnitude higher than the minimum detectable mass coverage with the YI using a single wavelength. However, with size-selective detection it is now possible to discriminate the 80 nm sized analyte binding from non-specific bound particles of 10 nm size and simultaneously occurring bulk changes.

2.1 Introduction

Integrated optical (IO) biosensors have been demonstrated as a powerful detection and analysis tool for biosensing. Main advantages of IO biosensors are its high sensitivity, real-time and label-free measurements. Interferometric sensors [1-6], grating couplers [7, 8], resonant optical microcavity sensors [9-11], and photonic crystal waveguide sensors [12, 13], are several IO sensors which have been developed. Integrated optical interferometric biosensors sense refractive index (RI) changes, induced by analyte binding, occurring in the evanescent field. These sensors, including the Mach Zehnder interferometer and the Young interferometer (YI), show extremely high (10^{-7} - 10^{-8} refractive index units (RIU)) RI sensitivity. The YI is a strong candidate for point-of-care viral diagnostics, because of this high sensitivity and its multiplexing capability [14]. Measurements show short time delays, because no extensive sample treatment is needed. However, the utilization of the high sensitivity is often hampered by background signals arising from non-specific RI changes within the evanescent field. Any RI change within the evanescent field will contribute to the measured signal. Consequently, in addition to specific binding of the analyte, also non-specific binding and RI changes (e.g. due to temperature changes) in the fluid covering the waveguide (bulk) will be detected. To distinguish between specific and non-specific binding, selective chemical binding techniques are used in combination with washing steps and/or differential measurements. Nevertheless, non-specificity and bulk background changes still hamper successful application of these type of biosensors. Measurements done in body fluids such as blood serum show a high variability in background and large non-specific binding. For that reason, a method to reduce the contribution to RI changes attributable to non-specific binding was developed [15]. By tuning the evanescent field of two different polarization modes a thin layer (20-30nm) was desensitized and the response to non-specific binding was reduced by a factor of hundred or more. Furthermore, a dual-wavelength operation of an integrated-optical difference interferometer was used to discriminate between binding of molecules and bulk RI changes or between binding of molecules and temperature changes [3]. In general however, the various background contributions to the signal are present simultaneously and therefore the existing methods that allow distinguishing only one of them from the signal are in practice not always sufficient. Previously we expanded the existing dual-wavelength approach [3] to a three wavelength approach that allows to discriminate several different background (bulk and temperature induced RI changes) contributions simultaneously [16]. Here we explore a similar approach for size-selective detection of analytes. The use of multiple wavelengths (3 or more) enables to probe RI changes at different distances from the sensor surface allowing to discriminate larger particles (e.g. viruses) from both smaller particles (e.g. proteins) and bulk contributions. We provide a theoretical basis for this method, we optimize

the method for application to a YI sensor and we calculate the achievable detection limit. We anticipate that using the size-selective multiple-wavelength approach as presented here should significantly improve the background suppression. It should be noted that the method presented here will most likely not replace existing methods like bio-receptor layers and antifouling strategies to reduce non-specific binding [17], but is rather to be used in combination with these methods to yield enhanced specificity.

A detailed theoretical analysis is given on the performance of this new method for two cases. The first case is aimed to distinguish between specific binding of analytes (~ 80 nm, e.g. virus particles) and bulk changes by using two wavelengths. The second case uses three wavelengths to distinguish specific binding of larger particles (~ 80 nm) from bulk contributions and non-specific binding (smaller particles 10 nm, e.g. proteins). Optimized waveguide structures are calculated for each case. Although we specifically develop this method for a YI sensor, the method is also applicable to other types of IO interferometric sensors.

2.2 Theoretical aspects

This section starts with the theory required to calculate the precision with which the RI change can be determined from the phase changes measured for the different wavelengths. This approach is used to optimize waveguide properties. It should be noted that the precision (defined as the standard deviation $\sigma_{\Delta n}$ of subsequent measurements of the RI change) is the relevant parameter for indicating the performance of the sensor. Induced RI change due to specific binding of analytes should exceed $2 \times \sigma_{\Delta n}$ (95 % confidence interval).

2.2.1 General theory

YI sensors are based on the evanescent field sensitivity of guided modes propagating through the waveguide structure of the sensor [18]. Fig. 2.1 illustrates the working of the YI sensor. Monochromatic light is coupled into an optical channel waveguide and split into two channels, including a measurement and a reference channel. Binding events near the surface of the measurement channel result in an RI change Δn at this surface. Consequently, the phase of the beam in the measuring channel changes, resulting in an alteration of the interference pattern that exist in the region of overlapping beams from the two channels. Assuming small RI changes such that the electric field distribution of the guided mode (mode profile) is not affected, the phase change $\Delta\phi$ between two beams, propagating through any two channels, can be described by [19]:

$$\Delta\varphi = \frac{2\pi}{\lambda} \cdot l \cdot \Delta N_{eff} = \frac{2\pi}{\lambda} \cdot l \cdot \left(\frac{\partial N_{eff}}{\partial n} \right)_{\lambda} \cdot \Delta n \quad (2.1)$$

where l is the length of the sensing window, λ is the vacuum wavelength of the guided light, ΔN_{eff} the effective RI change of the guided mode, Δn the RI change in the region probed by the evanescent field and $\left(\frac{\partial N_{eff}}{\partial n} \right)_{\lambda}$ the sensitivity coefficient of N_{eff} with respect to n , for a wavelength λ . Although not explicitly written, chromatic dispersion is taken into account (see Appendix 2.A). Next, we define multiple layers above the core of the waveguide of which the RI change has to be determined (Fig. 2.2). Thicknesses of the defined layers can be chosen arbitrarily depending on the experiment, e.g. three layers to discriminate between non-specific binding (e.g. proteins), specific binding (e.g. viruses) and bulk RI changes (see Fig. 2.3).

Fig. 2.1: The principle of a Young interferometer, where light is coupled in and guided through an integrated channel waveguide structure and projected onto a CCD camera by a cylindrical lens, giving an interference pattern. The core of the waveguide consists in this case of the layer above the substrate and the ridges (which provides confinement in lateral direction) on top of this layer which are made from the same material and therefore coloured the same.

Fig. 2.2: Structure definition of waveguide with on top N introduced imaginary layers and a guided mode profile (dashed line), where d is the thickness and n the refractive index.

Fig. 2.3: Guided mode profiles of three different wavelengths propagating through a waveguide structure with three layers introduced on top of the sensing window to distinguish between the non-specific protein binding of smaller molecules, the specific binding of larger particles, and the bulk solution changes.

The electric field distribution of the guided mode depends on the wavelength of the light (shorter wavelengths are more confined to the core than longer wavelengths, see Fig. 2.3). Consequently, the RI changes in the different layers can be determined by measuring the phase changes at a number of different wavelengths, provided the number of layers does not exceed the number of used wavelengths.

Consider N_{layer} layers (see Fig. 2.2), and N_λ number of different wavelengths. The measured phase changes can be written as (in analogy with Eq. (2.1)):

$$\overline{\Delta\varphi} = \overline{\overline{M}_s} \cdot \overline{\Delta n} \text{ with } \overline{\Delta\varphi} = \begin{bmatrix} \Delta\varphi_1 \\ \vdots \\ \Delta\varphi_j \\ \vdots \\ \Delta\varphi_{N_\lambda} \end{bmatrix}, \quad \overline{\Delta n} = \begin{bmatrix} \Delta n_1 \\ \vdots \\ \Delta n_i \\ \vdots \\ \Delta n_{N_{layer}} \end{bmatrix} \quad (2.2)$$

with $\Delta\varphi_j$ the phase change measured at λ_j , Δn_i the RI change in layer i , and $\overline{\overline{M}_s}$ (sensitivity matrix) defined as:

$$\overline{\overline{M}_s} = 2\pi \cdot l \cdot \begin{bmatrix} \frac{1}{\lambda_1} S_{1,1} & \frac{1}{\lambda_1} S_{2,1} & \frac{1}{\lambda_1} S_{3,1} & \cdots & \frac{1}{\lambda_1} S_{N_{layer},1} \\ \frac{1}{\lambda_2} S_{1,2} & \ddots & \vdots & \cdots & \frac{1}{\lambda_2} S_{N_{layer},2} \\ \frac{1}{\lambda_3} S_{1,3} & \cdots & \frac{1}{\lambda_j} S_{i,j} & \cdots & \frac{1}{\lambda_3} S_{N_{layer},3} \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ \frac{1}{\lambda_{N_\lambda}} S_{1,N_\lambda} & \frac{1}{\lambda_{N_\lambda}} S_{2,N_\lambda} & \frac{1}{\lambda_{N_\lambda}} S_{3,N_\lambda} & \cdots & \frac{1}{\lambda_{N_\lambda}} S_{N_{layer},N_\lambda} \end{bmatrix} \quad (2.3)$$

with $S_{i,j} = \left(\partial N_{eff} / \partial n_i \right)_{\lambda_j}$ the sensitivity coefficient of the i^{th} layer and j^{th} wavelength (see Appendix 2.B for an explicit expression of $S_{i,j}$). Eq. (2.2) is rewritten to find the RI change in each layer:

$$\overline{\Delta n} = \overline{\overline{M}_s}^{-1} \overline{\Delta\varphi}, \quad (2.4)$$

where $\overline{\overline{M}_s}^{-1}$ is the inverse of $\overline{\overline{M}_s}$ for a square matrix and the right inverse of $\overline{\overline{M}_s}$ for a non-square matrix (for $N_\lambda > N_{layer}$). Eq. (4) has a unique solution if $\det(\overline{\overline{M}_s}) \neq 0$. In that case, the RI change in layer i can be determined with a precision $\sigma_{\Delta n_i}$ (defined as the standard deviation in Δn_i) depending on the precision $\sigma_{\Delta\varphi_j}$ of the measurement

of $\Delta\varphi_i$, which is determined by experimental factors such as laser noise, camera noise, and temperature fluctuations. If the matrix $\overline{\overline{M}}_s$ gets more singular the precision $\sigma_{\Delta n_i}$ will worsen. Therefore, it is essential to optimize the experimental configuration such that $\overline{\overline{M}}_s$ does not get singular. $\overline{\overline{M}}_s$ is determined by the sensitivity coefficients which in turn depend on the guided mode profiles. If the mode profiles are similar, the sensitivity coefficients will be almost equal and as a consequence, the matrix $\overline{\overline{M}}_s$ gets more singular. Therefore, the wavelengths should differ as much as possible in the workable wavelength range.

We define the relative precision Φ_i (describing the relative precision in determining the RI change in the i^{th} layer) as the ratio between $\sigma_{\Delta n_i}$ and $\sigma_{\Delta\varphi_i}$. Assuming $\sigma_{\Delta\varphi_k} = \sigma_{\Delta\varphi} \quad \forall \quad 1 \leq k \leq N_\lambda$, the relative precision is a vector $\overline{\Phi}$ with the i^{th} element Φ_i given by (see Appendix 2.C):

$$\Phi_i = \frac{\sigma_{\Delta n_i}}{\sigma_{\Delta\varphi}} = \left[\sum_{j=1}^{N_\lambda} (M_{s,ij}^{-1})^2 \right]^{1/2}. \quad (2.5)$$

Φ_i is evaluated for different wavelengths, waveguide refractive indices and layer thicknesses. Given an experimentally determined precision in the phase measurements ($\sigma_{\Delta\varphi} \approx 10^{-4}$ fringes @ 1 Hz for the reported YI sensor [18]), the precision $\sigma_{\Delta n_i}$ with which a RI change of a given layer i can be measured, can be calculated by multiplying the relative precision Φ_i of the corresponding layer with the experimentally determined value of $\sigma_{\Delta\varphi}$.

2.2.2 Surface mass coverage

Next, we convert the RI changes obtained from a measurement into the more relevant mass coverage of the specific bound particles C_s . To discriminate between non-specific binding of smaller molecules (e.g. proteins), specific binding of larger molecules (e.g. viruses) and bulk solution changes we introduce three layers on top of our waveguide (see Fig. 2.3). We assume that layer 2 changes due to specific binding and bulk RI changes, whereas changes in layer 3 are only caused by bulk RI changes. It therefore follows that the RI changes due to specific binding are given by the difference in RI changes between layer 2 and 3. Multiplying this RI change with a constant β converts an RI change into a mass coverage (see Appendix 2.D):

$$C_s = \frac{1}{\beta}(\Delta n_2 - \Delta n_3), \quad (2.6)$$

and the minimal detectable mass coverage given by:

$$\Delta C_s = \frac{\sqrt{2}}{\beta} \sigma_{\Delta n_{\text{virus}}} \quad (2.7)$$

where β is given by:

$$\beta = (n_{\text{analyte}} - n_{\text{solution}}) \cdot d^2 / m. \quad (2.8)$$

Assuming that the specific bound particles is a virus, the RI of the analyte is $n_{\text{analyte}} = 1.41$ [20] and the RI of a buffer solution $n_{\text{solution}} = 1.33$, a molecular weight of a single Adenovirus particle, $m_{\text{analyte}} = 1.75 \times 10^8$ Da [21] ($= 2.91 \times 10^{-1}$ fg), and the diameter of the specific bound virus particle $d_{\text{analyte}} = 80$ nm, β is equal to 1.76×10^{-9} mm²/fg.

2.3 Results and discussion

2.3.1. Optimization of the waveguide structure

First, the two layers case is treated, which is used to discriminate between analyte binding (layer 1) and RI changes of the bulk (layer 2). Layer 1, the “specific binding layer” has a defined thickness of 80 nm. The second layer is called “bulk” and is all the space above layer 1. The dependence of the relative precision on the core thickness d_{core} is given (Fig. 2.4a,b) for three different core refractive indices. The refractive indices are chosen such that they correspond to those of real materials that are often used for waveguide fabrication; $n \approx 1.77$: aluminium oxide (Al_2O_3), $n \approx 2.02$: silicon nitride (Si_3N_4) and $n \approx 2.65$ titanium oxide (TiO_2). The used wavelengths are $\lambda_1 = 400$ nm and $\lambda_2 = 700$ nm. More details of the waveguide can be found in Table 2.1. For all simulations only zeroth order transverse electric (TE0) modes are taken into account. From Fig. 2.4 it is clear that the best performance (lowest value of $\bar{\Phi}$) is obtained for high values of n_{core} combined with low core thicknesses. This holds for both the precision achieved for the specific binding layer and the bulk layer (note that at optimal thickness, the dependence on the core RI is marginal). This conclusion is important in choosing the waveguide materials, i.e. choosing Si_3N_4 ($n(\lambda = 500 \text{ nm}) = 2.034$) as a core material is justified as it combines a high RI with excellent cleanroom processing possibilities. The interpretation of the calculated $\bar{\Phi}$ and its consequences for the mass coverage are discussed later.

Fig. 2.4: Relative precision as a function of the core thickness and core refractive index (given at a wavelength of 550 nm) for a) the specific layer using two layers, b) the bulk using two layers, c) the specific binding layer using three layers, d) the bulk using three layers and e) the non-specific binding layer using three layers.

Table 2.1: Waveguide structure details for all the simulations, where the RI of the layers is determined by the Sellmeier equations (see Appendix A).

Simulation description	Substrate material	Core material	$n_1 - n_{N-1}$	d_{core} (nm)	$d_1 - d_{N-1}$ (nm)	d_1 (nm)	d_2 (nm)
$\overline{\Phi}$ vs. d_{core} (two layers)	SiO ₂ (n=1.461 @ λ =550 nm), [21]	Al ₂ O ₃ , (n=1.770 @ λ =550 nm), [22] Si ₃ N ₄ , (n=2.024 @ λ =550 nm), [23] TiO ₂ , (n=2.648 @ λ =550 nm), [22]	Water @ 20°C, (n=1.335 @ λ =550 nm), [24]	30- 200	-	80	-
$\overline{\Phi}$ vs. d_{core} (three layers)	SiO ₂	Al ₂ O ₃ , Si ₃ N ₄ , TiO ₂	Water @ 20°C	30- 200	-	10	80
$\overline{\Phi}$ vs. N_{layer}	SiO ₂	Si ₃ N ₄	Water @ 20°C	70	random values	-	-
$\overline{\Phi}$ vs. N_λ	SiO ₂	Si ₃ N ₄	Water @ 20°C	70	-	80	-

In order to distinguish between specific analyte binding, non-specific binding and bulk RI changes, three layers are required, a non-specific binding layer, a specific binding layer, and a bulk layer. For details see Table 2.1. Fig. 2.4c-e shows Φ for the specific binding layer, the bulk and the non-specific binding layer respectively as a function of d_{core} for the different core materials. The three wavelengths are: $\lambda_1 = 400$ nm, $\lambda_2 = 550$ nm and $\lambda_3 = 700$ nm. For optimal detection of specific binding, the relative precision of the specific binding layer and the bulk should be minimal. Fig. 2.4c shows a minimum value of $\Phi_{specific} = 6.12 \times 10^{-4} \text{ rad}^{-1}$ for a core material of TiO₂ and $d_{core} = 35$ nm, and a two-fold higher value of $\Phi_{specific} = 1.63 \times 10^{-3} \text{ rad}^{-1}$ for Si₃N₄ as core material and $d_{core} = 70$ nm. Fig. 2.4d shows a similar relative precision of the bulk for both conditions. For each core material, the core thickness should be chosen carefully.

2.3.2. Expanding the number of layers

With the use of an increasing number of wavelengths it is, in theory, possible to determine a complete RI change profile, i.e. the RI change as a function of distance to the waveguide surface. However the use of an increasing number of wavelengths results in mode profiles which are increasingly similar and therefore result in a worsening precision $\sigma_{\Delta n}$. For example, by comparing the relative precisions for the two and three layers cases, we see an increase in $\overline{\Phi}$ of approximately one order of magnitude. In order to explore the limits, we calculated the $\overline{\Phi}$ as a function of the

number of layers N_{layer} . The RI change cannot be determined at distances from the waveguide surface where the evanescent field becomes very small. Therefore, we define our layers in the region starting at the surface and ending at 200 nm from the surface. The space above the 200 nm limit is considered as bulk. The thicknesses of the layers are randomly chosen with a minimum of 10 nm. $\bar{\Phi}$ is calculated for all layers and this calculation is repeated 1000 times (each time with different randomly chosen layer thicknesses). Next, the mean of all these calculated relative precisions $\bar{\Phi}$ is calculated. The number of wavelengths is chosen equal to N_{layer} . For the core we chose Si_3N_4 with a thickness of 70 nm. For optimal settings, the wavelengths are spread maximally. This means that $\lambda_{\text{min}} = 400$ nm and $\lambda_{\text{max}} = 700$ nm. The wavelengths in between are equally divided within this range. Fig. 2.5 shows $\bar{\Phi}$ as a function of N_{layer} , where bulk is included in N_{layer} , but not in $\bar{\Phi}$. The error bars are based on a 95% confidence interval of the 1000 calculated relative precisions and give an indication about the spread in the calculated relative precisions. A one-layer situation is included as a solid line in Fig. 2.5 (wavelength is set at 550 nm). Fig. 2.5 shows that $\bar{\Phi}$ increases exponentially with, meaning that $\bar{\Phi}$ can be determined less precisely. For a situation with three layers and assuming a phase precision of approximately 10^{-4} fringes for the YI sensor [18], the detection limit (which is defined here as the minimum reliably detectable induced RI change which equals two times the precision) of Δn is on average 5×10^{-6} RIU, which is still comparable to other optical biosensors [25].

Fig. 2.5: Mean relative precision as a function of the number of layers, which is equal to the number of wavelengths, for a core thickness of 70 nm and Si_3N_4 as core material. The mean relative precision multiplied by the phase precision gives the minimal reliably detectable induced Δn .

2.3.3. Expanding the number of wavelengths

The number of wavelengths should be equal to or larger than the number of layers that needs to be resolved. In the previous sections we explored several configurations of various number of layers and equal number of wavelengths. Here $\bar{\Phi}$ is evaluated for the case $N_{\text{layer}} = 2$ and $N_{\text{layer}} \geq N_{\lambda}$. The two layers are defined as a “specific binding layer” of 80 nm and a “bulk” layer covering all the space above 80 nm. The wavelengths are in each case equally divided between 400 nm and 700 nm (for waveguide details see Table 2.1). Fig. 2.6 shows $\bar{\Phi}$ as a function of the number of wavelengths for $N_{\text{layer}} = 2$. The Φ for the “specific binding layer” only decreases a factor of ≈ 2 by adding eight extra wavelengths, where Φ of bulk layer decreases even less. It can be concluded that $\bar{\Phi}$ decreases with an increasing number of wavelengths. In conclusion, adding more wavelengths than layers results in a marginal gain in $\bar{\Phi}$.

Fig. 2.6: Relative precision as a function of the number of wavelengths for two layers, a core thickness of 70 nm and SiO_2 as the substrate material, Si_3N_4 as the core material, and water on top of this core.

2.3.4. Surface mass coverage

To illustrate the possibilities of the size-selective detection scheme, we calculate the surface mass coverage for the specific case of virus detection. Using Eq. (2.7) in combination with the results from the three layer case, we calculate the minimal detectable surface mass coverage ΔC_s . Multiplying the relative precisions from Fig. 2.4c for the specific binding layer with the previously reported phase precision of approximately 10^{-4} fringes for the YI sensor [18] gives $\sigma_{\Delta n_{\text{analyte}}}$. For a core thickness of 70 nm and a core material of Si_3N_4 and assuming that the specific analyte is a virus, we

find $\Delta C_s = 8 \times 10^2 \text{ fg/mm}^2$. It should be noted that different analytes have different sizes. In Fig. 2.4 we analysed a specific situation of an 80 nm sized analyte. However, calculations show that the values of the relative precision for analytes of different sizes do not differ significantly, e.g. for analytes of 160 nm the relative precision differs a factor of ≈ 2 .

2.3.5. Detection of analytes in complex matrices

Here we would like to discuss several issues related to the use of this method for the detection of analytes in complex matrices such as blood, serum, urine or sputum. In our analysis we assumed a thin and uniform layer of particles that are non-specifically bound to the sensor surface. However, the particles responsible for non-specific binding can have various sizes, with some of them being larger than 10 nm, exceeding the thickness of the non-specific binding layer assumed in the calculations. In general, the majority of particles in e.g. blood or serum are however much smaller than the analyte, i.e. a virus particle. It is this majority, the excess of smaller particles compared to the number of analyte molecules that leads to measurable background due to non-specific binding. Small numbers of very large particles next to the analytes can be discriminated by the specificity of the bio-receptor coating of the sensor surface.

Another concern is that several types of particles can be present in a real sample all having the same size. The method presented here obviously cannot distinguish between particles with the same size and therefore it is important to always combine this method with a traditional affinity bio-receptor coating of the sensor surface in order to strongly enhance the binding of the specific analyte of interest. Only in extreme cases where the concentration of the analyte of interest is much lower than the concentration of similar sized particles could this result in the non-specific binding of other these particles exceeding the specific binding of the analyte of interest. In this case our method obviously cannot distinguish between the analytes and the non-specific bound similar sized particles.

Finally, in a real sample the molecules are moving rather than being static. Brownian motion of molecules in the sampling volume gives rise to fluctuations in the refractive index. The expected fluctuations in the refractive index in a 10 nm thin layer above the sensor surface is calculated to be $\sim 10^{-9}$ RIU (corresponding to a phase noise of $\sim 10^{-6}$ fringes) for an average protein content of 10 g/ml (typical for blood). The calculated fluctuations are well below the detection limit of the sensor (two orders of magnitude smaller than the value used in the calculations) and as such we can conclude that fluctuations in the signal due to Brownian motion of proteins in the sample fluid can be neglected.

2.3.6. Implementation

For the implementation of this new method two aspects need special attention. First, multiple lasers emitting at different wavelengths should be coupled into a single channel waveguide of the YI sensor. This can be accomplished by combining the output of multiple monochromatic lasers using either free space optics (e.g. using appropriate dichroic mirrors) or by using fiber-optical combiners. Secondly, for each wavelength the phase change should be measured independently from the recorded interference pattern. This can be done in two ways. First, the interference pattern of each wavelength is measured separately. This can be done using either a multicolour video camera, or by introducing a dispersive element (e.g. a grating) in the detection path to separate the different interference patterns (one for each wavelength) spatially on a monochrome video camera. Alternatively, the same detection setup as used for single wavelength sensors can be used [1]. In this case a single interference pattern is recorded. To obtain the phase changes for each wavelength separately one can make use of the fact that the spatial frequency of the interference pattern depends on the wavelength of the used light. Therefore, the amplitude spectrum of the Fourier-transformed interference pattern now consists of well-separated spatial frequency peaks (one for each wavelength). Consequently, the phase change for a given wavelength can be monitored independently from the other wavelengths by selecting the corresponding spatial frequency in the phase spectrum of the Fourier-transformed interference pattern. We have successfully used a similar approach to simultaneously measure multiple phase changes from a multi-channel integrated optical YI sensor [6].

2.3.7 General discussion

Comparing the calculated detection limit (which is defined here as the minimum reliably detectable induced RI change which equals two times the precision) with existing methods we find that our method is comparable to the detection limit of optical biosensors based on SPR ($\approx 10^{-6}$ RIU) [26]. The detection limit also compares to reported results obtained with grating couplers ($\approx 10^{-6}$ RIU, 0.3 pg/mm²) [8], photonic crystals ($\approx 10^{-2}$ RIU) [13] and resonant optical microcavities sensors ($\approx 10^{-5}$ - 10^{-6} RIU) [10, 11]. Although in this method the detection limit gets worse compared to single wavelength YI sensors, the performance is comparable to existing methods with the advantage that the sensor is capable of size selective detection which we believe yields a strong improvement in the specificity of the sensor.

We further envision the potential of implementing improvements or alternative configurations to improve sensor performance. One possible improvement is to make use of other than TE₀ modes. As discussed, the precision with which different layers can be resolved requires the use of mode profiles that have different field strengths in the different layers. Here we used different wavelengths to achieve this. Alternatively one can use dual polarizations [27], however it will not be possible to distinguish

between specific binding, non-specific binding, bulk changes simultaneously using only two polarizations. On the other hand, higher order modes can be used. The difference between mode profiles of e.g. TE0 and TE1 at the same wavelength is larger than the difference between mode profiles of e.g. a TE0 mode at different wavelengths. Measuring the phase changes for different order modes at the same wavelength is possible [28] but is less trivial than detecting the same mode at different wavelengths. Alternatively it is possible to use a TE0 mode at one wavelength and a TE1 mode at another wavelength. The combination of modes and/or wavelengths that give the best results requires a systematic experimental investigation and is beyond the scope of the current paper.

Currently methods to increase specificity of the sensor are aimed to reduce non-specific binding by chemical treatment of the sensor surface (e.g. blocking of vacant positions by bovine serum albumin). The method described here can be used in addition to this blocking approach to further improve the specificity. Approaches to eliminate bulk RI changes also exist. Usually after interaction of the analyte with the sensor surface, the sample fluid is replaced by a clean buffer and the bulk contribution is obtained by comparing the RI before and after fluid replacement. However this approach has some disadvantages. First, no significant changes in binding of the analyte to the sensor surface should occur during the time of fluid replacement, which usually means long measuring times. Second, this method is prone to errors as a change from sample to buffer solution sets a new equilibrium of the free analyte vs. surface-bound analyte. Also quite common in interferometric sensors is to use the reference channel to eliminate common RI changes. In this case a reference channel is modified identically to the sensing channel without the analyte specific antibody. Applying the sample to both channels simultaneously strongly reduces the bulk and non-specific contributions, however in practice this approach is not always sufficient especially in complex matrices like blood. Also here the size-selective approach can be used either alone or in addition to a reference channel to further increase the specificity.

2.4. Conclusions

In this chapter we have described a new approach, based on the use of multiple wavelengths in combination with an integrated optical Young interferometer sensor, which allows detecting analytes based on size. The use of multiple wavelengths allows discriminating between RI changes from different locations. To simultaneously distinguish between specific binding, non-specific binding and bulk RI changes, a three layer system with three wavelengths is used. The required precision of the RI determines the spatial resolution of this new method. Assuming a phase precision of $\approx 10^{-4}$ fringes, and an analyte of size 80 nm diameter, this new method has a minimum

detectable mass coverage of $4 \times 10^2 \text{ fg/mm}^2$. With a better phase precision it is even conceivable to reach a higher precision of $\overline{\Delta n}$ or to introduce an extra imaginary layer, resulting in a possibility to distinguish from another type of particle with a different size. Furthermore, applying this new method to the current sensor gains in specificity while the detection limit is still comparable to the detection limit of other existing methods. Therefore, we believe that this method can strongly improve the performance of IO interferometric.

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Appendix 2.A Chromatic dispersion

Chromatic dispersion is included in the determination of the sensitivity coefficients. The sensitivity coefficients are determined by the mode profile of the light source, which depends on the wavelength of the light and the waveguide structure (d_{core} , n_s , n_{core} , n_c). The refractive indices of the chosen materials as a function of the wavelength are given by the Sellmeier equations, which are listed below in Table 2.A.1. Chromatic dispersion in the formation of a layer of particles (e.g. viruses, proteins) on the surface is assumed to be negligible.

Table 2.A.1: Sellmeier equations for the different materials used in the waveguide structure

Material	Sellmeier equation	Reference
Al_2O_3	$n(\lambda) = \left(1 + \frac{A\lambda^2}{\lambda^2 - B^2} + \frac{C\lambda^2}{\lambda^2 - D^2} + \frac{E\lambda^2}{\lambda^2 - F^2} \right)^{1/2}, \lambda \text{ in } [\mu\text{m}]$ <p>$A=1.4313493, B=0.0726631, C=0.65054713, D=0.1193242, E=5.3414021,$ $F=18.028251$</p>	[22]
Si_3N_4	$n(\lambda) = \left(1 + \frac{A\lambda^2}{\lambda^2 - B^2} \right)^{1/2}, \lambda \text{ in } [\mu\text{m}]$ <p>$A=2.8960, B=0.14010$</p>	[23]
TiO_2	$n(\lambda) = \left(5.913 + \frac{A}{\lambda^2 - B} \right)^{1/2}, \lambda \text{ in } [\mu\text{m}]$ <p>$A=0.2441, B=0.0803$</p>	[22]

Material	Sellmeier equation	Reference
SiO ₂	$n(\lambda) = \left(1 + \frac{A\lambda^2}{\lambda^2 - B^2}\right)^{1/2}, \lambda \text{ in } [\mu\text{m}]$	[23]
	$A=1.1008, B=0.094025$	
Water (20°C)	$n(\lambda) = \left(1 + \frac{A\lambda^2}{\lambda^2 - B} + \frac{C\lambda^2}{\lambda^2 - D} + \frac{E\lambda^2}{\lambda^2 - F} + \frac{G\lambda^2}{\lambda^2 - H}\right)^{1/2}, \lambda \text{ in } [\mu\text{m}]$	[24]
	$A=5.684027565 \cdot 10^{-1}, \quad B=5.101829712 \cdot 10^{-3}, \quad C=1.726177391 \cdot 10^{-1},$ $D=1.821153936 \cdot 10^{-2}, \quad E=2.086189578 \cdot 10^{-2}, \quad F=2.620722293 \cdot 10^{-2},$ $G=1.130748688 \cdot 10^{-1}, A=1.069792721 \cdot 10^1$	

Appendix 2.B Derivation sensitivity coefficient

In this section we derive the sensitivity coefficient S_{ij} . Based on a three layer waveguide with a substrate (n_s), a core (n_{core}), and a cladding (n_c), the sensitivity coefficient is given by the N_{eff} dependence on a RI change in the region probed by the evanescent field (cladding) and can be determined for TE polarization by [29]:

$$\left(\frac{\partial N_{eff}}{\partial n_c}\right)_{\lambda_j} = \left(\frac{n_c}{N_{eff}}\right) \left(\frac{n_{core}^2 - N_{eff}^2}{n_{core}^2 - n_c^2}\right) \left(\frac{Y_{0c}(\lambda_j)}{d_{core} + Y_{0c}(\lambda_j) + Y_{0s}(\lambda_j)}\right), \quad (2.B.1)$$

where d_{core} is the core thickness. Y_{0c} is the penetration depth of the electric field into the covering region on top of the waveguide, and Y_{0s} is the penetration depth of the electric field into the substrate, which are given by:

$$Y_{0c,s}(\lambda_j) = \left(\frac{\lambda}{2\pi}\right) \left(N_{eff}^2 - n_{c,s}^2\right)^{1/2}, \quad (2.B.2)$$

where λ the vacuum wavelength of the guided light. The sensitivity of the i^{th} layer in the evanescent region can now be calculated as a fraction of the complete evanescent mode power present in this layer [30]:

$$S_{i,j} = \frac{\int_{z_{i-1}}^{z_i} e^{\left(\frac{2z}{Y_{0c}(\lambda_j)}\right)} dz}{\int_0^\infty e^{\left(\frac{2z}{Y_{0c}(\lambda_j)}\right)} dz} \left(\frac{\partial N_{eff}}{\partial n_c}\right)_{\lambda_j}, \quad (2.B.3)$$

where $z_0 = 0$, $z_i = \sum_{n=1}^i d_n$, where d_n is the thickness of layer n . This finally results in this expression for the sensitivity coefficient for the i^{th} layer and the j^{th} wavelength:

$$S_{i,j} = \left[e^{\left(\frac{2z_{i-1}}{Y_{0c}(\lambda_j)} \right)} - e^{\left(\frac{2z_i}{Y_{0c}(\lambda_j)} \right)} \right] \left(\frac{n_c}{N_{eff}} \right) \left(\frac{n_{core}^2 - N_{eff}^2}{n_{core}^2 - n_c^2} \right) \left(\frac{Y_{0c}(\lambda_j)}{d_{core} + Y_{0c}(\lambda_j) + Y_{0s}(\lambda_j)} \right). \quad (2.B.4)$$

Appendix 2.C Derivation relative precision

Here we derive an expression for the relative precision Φ , where Φ is defined as the standard deviation in the RI change $\sigma_{\Delta n_i}$ normalized by the standard deviation of the measured phase change. From equation (2.4) of the main text we have:

$$\overline{\Delta n} = \overline{\overline{M}_s}^{-1} \overline{\Delta \varphi}. \quad (2.C.1)$$

The variance of $\overline{\Delta n}$ is given by:

$$\overline{\sigma^2(\Delta n)} = \left(\overline{\overline{M}_s}^{-1} \circ \overline{\overline{M}_s}^{-1} \right) \overline{\sigma^2(\Delta \varphi)}, \quad (2.C.2)$$

where $\circ$ is the Hadamard product, which is an element-wise multiplication:

$$\left(\overline{\overline{M}_s}^{-1} \circ \overline{\overline{M}_s}^{-1} \right)_{ij} = \left(\overline{M_{s,ij}^{-1}} \right)^2. \quad (2.C.3)$$

So, the i^{th} element of vector $\overline{\sigma(\Delta n)}$ can be described by:

$$\left[\overline{\sigma(\Delta n)} \right]_i = \sigma_{\Delta n_i} = \left[\left(\overline{\overline{M}_s}^{-1} \circ \overline{\overline{M}_s}^{-1} \right) \overline{\sigma^2(\Delta \varphi)} \right]_i^{1/2} = \left[\sum_{j=1}^{N_j} \left(\overline{M_{s,ij}^{-1}} \right)^2 \sigma_{\Delta \varphi_j}^2 \right]^{1/2}. \quad (2.C.4)$$

By assuming an equal phase noise for every laser ($\sigma_{\Delta \varphi_j} = \sigma_{\Delta \varphi}$), it is possible to define the i^{th} element of the relative precision as:

$$\Phi_i = \frac{\sigma_{\Delta n_i}}{\sigma_{\Delta \varphi}} = \frac{1}{\sigma_{\Delta \varphi}} \left[\sum_{j=1}^N \left(\overline{M_{s,ij}^{-1}} \right)^2 \sigma_{\Delta \varphi}^2 \right]^{1/2} = \left[\sum_{j=1}^N \left(\overline{M_{s,ij}^{-1}} \right)^2 \right]^{1/2}. \quad (2.C.5).$$

Appendix 2.D Derivation surface mass coverage

We derive here a set of RI changes into a specific surface mass coverage C_s . On top of the core of the waveguide three imaginary layers are defined. The RI change in layer one Δn_1 , is caused by non-specific binding of particles (e.g. proteins), specific analyte binding (e.g. viruses) and bulk changes. Specific binding and bulk changes result in an RI change in layer two and the RI change in layer three is only caused by concentration changes of the bulk. In addition, we convert the RI change of the different layers into a non-specific mass coverage, C_n , a specific mass coverage, C_s , and a concentration change of the bulk C_b . Therefore, the relation between C_n , C_s , C_b and $\overline{\Delta n}$ is given by:

$$\begin{bmatrix} \Delta n_1 \\ \Delta n_2 \\ \Delta n_3 \end{bmatrix} = \overline{\overline{M_c}} \cdot \begin{bmatrix} C_n \\ C_s \\ C_b \end{bmatrix}, \text{ with } \overline{\overline{M_c}} = \begin{bmatrix} \alpha & \beta & \gamma \\ 0 & \beta & \gamma \\ 0 & 0 & \gamma \end{bmatrix}, \quad (2.D.1)$$

where α , β , and γ , convert an RI change into respectively a non-specific mass coverage, a specific mass coverage and a bulk concentration change. We assume small concentration changes, so we can neglect changes in α , β , and γ , because of the growth of analytes to the surface, which results in less space on the surface which can be covered by new analytes. The non-specific mass coverage, specific mass coverage and bulk concentration change can be calculated by:

$$\begin{bmatrix} C_n \\ C_s \\ C_b \end{bmatrix} = \overline{\overline{M_c}}^{-1} \cdot \begin{bmatrix} \Delta n_1 \\ \Delta n_2 \\ \Delta n_3 \end{bmatrix}, \text{ with } \overline{\overline{M_c}}^{-1} = \begin{bmatrix} 1/\alpha & -1/\alpha & 0 \\ 0 & 1/\beta & -1/\beta \\ 0 & 0 & 1/\gamma \end{bmatrix}. \quad (2.D.2)$$

From this the mass coverage C_s is given by:

$$C_s = \frac{1}{\beta} (\Delta n_2 - \Delta n_3), \quad (2.D.3)$$

where Δn_2 is composed of two contributions: $\Delta n_2 = \Delta n_{\text{specific}} + \Delta n_{\text{bulk}}$, whereas $\Delta n_3 = \Delta n_{\text{bulk}}$, which results in an equation for mass coverage:

$$C_s = \frac{1}{\beta} \Delta n_{\text{analyte}}. \quad (2.D.4)$$

Next, we determine β . We define an expression for C_s as:

$$C_s = \frac{N_{analyte} \cdot m_{analyte}}{w \cdot l}, \quad (2.D.5)$$

where $N_{analyte}$ is the number of analytes bound the surface, $m_{analyte}$ the mass of a single analyte, w the sensitivity window width ($= 4 \mu\text{m}$ for current chip), and l the sensitivity window length ($= 4 \text{ mm}$ for current chip). The maximum number of analytes $N_{\max}$ that can be bound to the surface is determined as the ratio of the surface area of the sensor and the surface area of a single analyte and can be approximated as $N_{\max} = w \cdot l / d^2$, where d is the diameter of the analyte. If we define a layer of thickness equal to the diameter of the analyte, than the corresponding maximum RI change $\Delta n_{\max}$ in that viral layer equals $\Delta n_{\max} = n_{analyte} - n_{solution}$. If in an experiment a Δn is introduced by specific analyte binding (denoted as $\Delta n_{analyte}$), the number of analytes $N_{analyte}$ bound to the surface of the chip is calculated as:

$$N_{analyte} = \frac{\Delta n_{analyte}}{\Delta n_{\max}} N_{\max} = \frac{\Delta n_{analyte} \cdot w \cdot l}{(n_{analyte} - n_{solution}) \cdot d^2}. \quad (2.D.6)$$

Combining Eq. (D.4), Eq. (D.5), and Eq. (D.6), this results in an expression for β :

$$\beta = (n_{analyte} - n_{solution}) \cdot d_{analyte}^2 / m_{virus}. \quad (2.D.7)$$

Based on Eq. (D.4) (so $C_s = f(\Delta n_2, \Delta n_3)$) the minimal detectable specific mass coverage is given by:

$$\Delta C_v = \left[\left(\frac{\partial f}{\partial (\Delta n_2)} \right) \cdot \Delta(\Delta n_2) \right]^2 + \left[\left(\frac{\partial f}{\partial (\Delta n_3)} \right) \cdot \Delta(\Delta n_3) \right]^2 \Bigg]^{1/2} = \frac{1}{\beta} \left[|\Delta(\Delta n_2)|^2 + |\Delta(\Delta n_3)|^2 \right]^{1/2}. \quad (2.D.8)$$

If we assume that the error in Δn_2 , $\Delta(\Delta n_2)$, is given by the precision in Δn_2 (relative precision multiplied by the precision in the phase change), $\sigma_{\Delta n_2}$, and that $\sigma_{\Delta n_2} = \sigma_{\Delta n_{analyte}} \approx \sigma_{\Delta n_{bulk}}$, than the minimal detectable specific mass coverage of an analyte can be approximated by:

$$\Delta C_v = \frac{\sqrt{2}}{\beta} \sigma_{\Delta n_{analyte}}. \quad (2.D.9)$$

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Chapter 3

Design, realization and characterization of a size-selective Young interferometer sensor

Abstract

This chapter presents the design, realization, and characterization of a Young interferometer sensor setup capable to measure simultaneously effective refractive index changes at multiple wavelengths to achieve size-selectivity. First, the requirements and an overview of the setup are presented. Next, we describe the realization and characterization of the six main modules of the setup: light sources, incoupling, sensing platform, imaging, detection and data processing. Finally, we characterise the phase noise and drift of the setup (unexpected phase changes on a longer time scale). It was found that the measured phase noise on short time scales is determined by photon shot noise. A CCD camera with a high dynamic range was implemented in the setup such that the measured phase noise is smaller than 10^{-4} fringes @ 1 Hz. Phase drift is smaller than 5×10^{-3} fringes per 1000 s for the setup with end-fire coupling. The incoupling was made more time efficient by using a fiber and butt-end coupling. However, the drift increased to values smaller than 1.5×10^{-2} fringes per 1000 s, partly caused by positional drift of the fiber. Drift in distance between fiber and chip resulted in phase oscillations which could be solved using a matching index gel. It was also found that air flow can strongly influence the phase signal, so the setup was covered. Moreover, changes in the ambient temperature have an effect on the phase stability as it can lead to thermal expansion of components of the setup and the chip itself. This can lead to changes in the position of the chip with respect to the camera which results in phase changes. Next to drift also artefacts are observed in the phase signal. The origin of these artefacts is investigated in this chapter. Possible reasons of the artefacts are aberrations of grating and lenses in the imaging part of the setup and boundary effects of the shifting interference pattern. The artefacts show up as oscillations in the phase change with an amplitude in the order of 10^{-2} fringes. Induced signals should be significantly higher than drift and artefacts to minimize the influence of drift and artefacts on the determined Δn 's.

3.1 Introduction

To measure size-selectively with the YI using multiple wavelengths there are some requirements for the setup. First, to discriminate between three different RI changes at least three different monochromatic coherent light sources with each a different wavelength are required. Second, these light sources should be coupled into an existing integrated optical YI. Third, after coupling out of the YI, the light sources will form three interference patterns which should be detected independently from each other. Fourth, the three interference patterns should be read out and fast Fourier transform (FFT) should be applied to each of them to determine phase changes ($\Delta\varphi$ ($\sim \Delta N_{eff}$), see formula 2.1) for each wavelength and each channel combination. Fifth, Chapter 2 showed that with adding size-selectivity to the YI, the sensitivity of the YI is negatively affected. Any noise in ΔN_{eff} will be enhanced by determining multiple Δn 's from ΔN_{eff} 's determined from measured $\Delta\varphi$'s at multiple wavelengths. However, Chapter 2 also showed that if $\Delta\varphi$ is measured with a precision of 10^{-4} fringes the detection limit of the YI in combination with size-selective detection is still comparable to the detection limits of other existing methods. Therefore, the phase noise should preferably be as low as possible and in the order of 10^{-4} fringes to be comparable to other existing methods in terms of sensitivity.

A setup was designed, realized and characterized such that it meets all the requirements named above. For convenience of comparison, the setup is divided up to six main parts which are presented in a function block diagram as shown in Fig. 3.1. Each of the sections 3.2 – 3.7 belongs to a single block in the function diagram. Next, section 3.8 presents a final overview of the self-built setup. Finally, section 3.9 presents experiments which were done to characterize phase noise (on short time scale of seconds) and phase drift (on longer time scale of hundreds to thousands of seconds) of the self-built setup.

Fig. 3.1: Schematic overview of the setup presented as a function block diagram illustrating in which section which part of the setup is described and that the final overview is presented in section 3.8.

3.2 Sensing platform

3.2.1 Four-channel integrated optical Young interferometer

First, we describe the sensing platform of the setup, which was developed in earlier work [1], for the size-selective measurements using multiple wavelengths. The sensing platform is a four-channel integrated optical Young interferometer. Light is first coupled into a single channel with a core width of 10 μm at the beginning of the chip followed by a 100 μm long taper to a final width of 4 μm . Next, the single channel splits up into four different channels. The waveguide consists of three layers, a PECVD SiO_2 layer, on top of a 70 nm thick LPCVD Si_3N_4 core, on top of a Thermal SiO_2 substrate. A schematic top view and lateral view of the end part of the YI interferometer are shown in Fig. 3.2a and b respectively. The YI is a ridge waveguide which means that lateral confinement of the channels is realized by the 0.7 nm high and 4 μm wide ridges shown in Fig. 3.2b. Therefore, the modes propagate at the ridges resulting in four channels distanced at 60 μm , 80 μm and 100 μm . The distances of the channels are chosen such that there are 6 different combinations of distances between the channels, resulting in 6 different spatial frequencies which can be read out individually from the interference pattern after applying an FFT on it. At the sensing windows of the four different channels, the top layer of PECVD SiO_2 is etched away such that the sample can reach the core of the waveguide. A cross-section of the waveguide at the sensing windows is presented in Fig. 3.3.

The YI chips have a single mode (in height) wavelength range of approximately 170 - 800 nm. However, the chips are optimized for TE modes of 632.8 nm light. Therefore, more losses can be expected for especially shorter wavelengths than the optimal 632.8 nm. Incoupling will be more critical for shorter wavelengths as the mode size is smaller for shorter wavelengths. Moreover, propagation losses due to scattering (SiO_2 and Si_3N_4 layers are amorphous and can have small defects) will be higher for shorter wavelengths ($\sim \lambda^{-4}$) and at the y-splitters there might be some extra losses for smaller wavelength which have smaller propagation modes. At $\lambda = 632.8$ nm only TE00 or TM00 modes can propagate through the waveguides. We calculated using an effective index method [2] that this also holds for the current YI waveguide at wavelengths longer than 540 nm. However, for shorter wavelengths than 540 nm the TE01 and TM01 do also fit in the waveguide. This means that in height the propagating modes are single mode, but in width, the zeroth and first order mode fit into the waveguide. Using the same effective index method we calculated that the sensitivities of zeroth and first order are almost equal. The effective index method has limitations, but for a weak lateral confinement, which is the case with this waveguide, the procedure is usually well defined [2]. Consequently, the multi modes should not give any problem and the sensitivities of the TE00 and TE01 mode are assumed equal. However, for the incoupling the multiple modes do matter, because

the position of the zero order mode (from the fiber or focussed with lens) which is coupled into the waveguide does determine if mainly TE₀₀ or TE₀₁ modes are coupled into the waveguide. This will result in non-equal distribution of the light in the channels occurring at the y-splitters. Details about the incoupling will be discussed in section 3.4.

Fig. 3.2: a) Top view illustrating the sensing windows (black rectangles) and position of fluid cuvette (black dotted rectangles) and b) lateral view of the end part waveguide structure, where the 0.7 nm and 4 μm ridges define the lateral confinement of the light and can therefore be seen as channels. Images are not to scale.

Fig. 3.3: Cross-section of the YI waveguide structure guiding a TE₀ mode of 660 nm. In height the image is to scale, which means that the evanescent field penetration depth is a couple of hundred nanometres.

3.2.2 Sample transportation

To bring the sample to the evanescent field of one of the four sensing windows of the YI waveguide, a 4 channel fluid cuvette with each a single inlet and outlet is positioned on top of the waveguide as illustrated with the black dotted rectangles in Fig. 3.2. The fluid cuvette consists of four flow chambers, each of a volume of 1.2 μl (2 mm wide, 6 mm long and 100 μm high). The sample is brought to the sensing windows of the YI using a 4 tube Reglo Digital MS-CA4/12-100 peristaltic pump with 0.57 mm inner diameter Tygon tubing. Inside the cuvette the sample is transported by laminar flow. As a consequence, the velocity at the surface is zero which means that the analytes will be transported to the sensor surface by diffusion perpendicular to the flow. The flow speed during the measurements is set at 100 $\mu\text{l}/\text{min}$. Before the samples reach the sensing windows it is degassed by a 4 channel Biotech Degasi® Classic to prevent unwanted air bubbles which can result in a high Δn on a very short time scale which can significantly disturb or even ruin the experiment.

3.3 Light sources

3.3.1 Requirements

As stated before, the light sources that are required for the YI should be monochromatic and coherent. The light source should be a single transverse mode to effectively couple the light into the YI waveguide. For a stable incoupling, the light source should also have a stable beam pointing. Furthermore, a single longitudinal mode with a high level of stability is required to prevent mode-hopping which results in unstable intensity and wavelength and therefore an unstable phase signal. To be able to measure an interference pattern and stable phase signals the light source should have a sufficiently long coherence length. Besides, calculations from Chapter 2 show that best results are obtained using an as large as possible spread between the wavelengths. On the other hand, the spread is limited by the YI waveguide structure, which should only be guiding zero order modes of the light source of specified wavelength.

3.3.2 Realization

The Cobolt diode-pumped solid state (DPSS) lasers have an excellent performance, in terms of power stability, wavelength stability, beam pointing and coherence length [3, 4] and therefore the (457.0 $\pm$ 0.3) nm Cobolt Twist™ and the (561.2 $\pm$ 0.3) nm Cobolt Jive™ from the (660 $\pm$ 0.3) nm Cobolt 04-01 Series and the Cobolt Flamenco™ from the 05-01 Cobolt series were implemented in the experimental setup. The wavelengths were chosen such that they are well spread and that single order modes of these wavelengths do still fit into the waveguide. Light sources of 50 mW were chosen to be

able to compensate for possibly high coupling losses into the waveguide, especially for shorter wavelengths than the 632.8 nm for which the waveguide was optimized. Thorlabs NDC-100C-4M mounted variable ND filters are used to regulate the power of the transmitted light of the light sources as the DPSS laser perform optimally at maximum output power. A couple of minutes are required for the laser to stabilise its temperature and to achieve power, wavelength and beam pointing stability which makes modulating of the laser impracticable. By implementing these lasers into the setup all requirements of the light sources were fulfilled to measure size-selectively using multiple wavelengths.

3.1.3 Characterization

The power of the lasers was verified using a Thorlabs PM121D power meter and the polarization of the laser was verified using a Thorlabs LPVISE100-A linear polarizer with N-BK7 Protective Windows (400-700 nm). The measured data is presented in Table 3.1. These powers were sufficient to overcome coupling losses and still be able to detect the interference patterns coupled out of the chip. Moreover, with polarization ratios (TM:TE) larger than 100:0.009 it is possible to couple well defined polarized modes into the waveguide, which is required as modes of different polarizations respond differently on RI changes in the evanescent field (see Chapter 6).

Phase oscillations were observed at the 660 nm interference pattern, indicating that the oscillations were caused by the 660 nm laser. After weeks we found together with the Cobolt company that the temperature controllers of the 660 nm laser were causing the oscillations. Overcompensating the temperature by the laser causes temperature oscillations possibly resulting in oscillations in wavelength, beam pointing or power of the laser. By lowering multipliers of the PID controller of the laser these oscillations were not seen again so this problem solved.

Table 3.1: Measured specifications of the light sources

	Cobolt Twist™	Cobolt Jive™	Cobolt Flamenco™
Power (mW)	49.5	49.5	57.7
P_{TE} (mW)	0.360	0.115	0.151
P_{TM} (mW)	38.0	38.8	46.5
Polarization ratio (TM:TE)	100:0.009	100:0.003	100:0.003

3.4 Incoupling

3.4.1 Requirements

The light coming from the three lasers should be simultaneously coupled into a ridge waveguide with a (70 ± 1) nm thick core and a ridge of 0.7 nm high and 10 μm wide

(which is tapered to a width of 4 μm). The linear polarization of the light source should be maintained and its orientation with respect to the waveguide determines whether TM (perpendicular to core layer, vertical) or TE (parallel to core layer, horizontal) modes are coupled into the waveguide structure.

3.4.2 Realization

End-fire coupling, butt-end coupling, prism coupling, grating coupling and directional coupling are different ways to couple the light sources into the waveguide [5]. First, the incoupling was realized using optics in free space to overlap the three lasers and using an achromatic lens to focus the light into the core structure of the waveguide (end-fire coupling) to be flexible in adding or removing components in the setup. Later on in the project, the light was coupled into the waveguide using single mode fiber and butt-end coupling to go towards a faster and easier incoupling of the three wavelengths and a more application driven solution. Prism coupling, grating coupling and directional coupling were not used because of simultaneously incoupling of multiple wavelengths into the waveguide and the given waveguide structure of the YI. The end-fire coupling and the butt-end coupling are described in section 3.4.2.1 and 3.4.2.2 respectively.

3.4.2.1 End-fire coupling

To use end-fire coupling the wavelengths should first be overlapped. A schematic overview of how this was done is shown in Fig. 3.4. The light sources as described in section 3.3 are first directed to Thorlabs NDC-100C-4M mounted variable ND filters to regulate the transmitted power per laser. The transmittance can be regulated from 1% to 100%, because of a varying optical density of 0 to 2.0. For safety reasons, the reflected light is captured by BT500 beam dumps. The transmitted light first passes Thorlabs Mounted Zero-Order Half-Wave Plates (Thorlabs, WPH05M-488, WPH05M-546 and WPH05M-670) to change the linear polarization of the light sources to be able to couple either TE or TM modes into the waveguide. Next, a set of visible broadband dielectric mirrors (Thorlabs, BB1-E02) and dichroic mirrors (Semrock, Di01-R561-25x36 (DM1) and Di01-R442-25x36 (DM2)) are used to overlap the three light sources. DM1 has an average transmission of 94% for wavelength higher of 582.4 nm till 1200 nm and an average reflection of 94% of wavelengths in between 554 nm and 568 nm. Therefore, it will reflect most of the light of the 561 light source and transmit most of the light of the 660 nm light source. The DM2 has an average transmission of 93% for wavelengths in between 469.3 nm and 900 nm and an average reflection of 94% of wavelengths in between 439 nm and 457.9 nm. Therefore, it will transmit most of the light coming from the 561 nm and 660 nm laser and reflect most of the light coming from the 457 nm laser which makes it possible to overlap the laser beams. Next, the collinear beams are pointed via a mirror to

Fig. 3.4: Schematic overview of the end-fire coupling to couple the light into the YI waveguide, where ND stands for neutral density filter, M stands for dielectric mirror, HWP stands for half-wave plate, DM stands for dichroic mirror, GM for a dielectric mirror placed in Gimbal mirror mount and L stands for achromatic lens.

the centre of another mirror positioned in a Gimbal mirror mount whose point of rotation for both axes is located at the centre of the mounted optic's surface. Therefore, changing the angle of the beam by tilting this mirror, the position of the beam at the mirror is not changed, which is required to position the beam at the centre of lens L3. The mirror in the Gimbal mirror mount is positioned at the first focal plane of a $4f$ lens system consisting of two achromatic doublets (AC127-019-A-ML, $f_1 = 19$ mm and AC254-100-A-ML, $f_2 = 100$ mm). At the final focal plane of the $4f$ lens system a third achromatic doublet (AC254-040-A-ML, $f_3 = 40$ mm) is placed to focus the light into the waveguide structure. Ray tracing shows that the x_f and y_f position can be adjusted by changing the angle of the beam with respect to the horizontal plane $\alpha_{0x,y}$ by (see Appendix 3.A):

$$x_f, y_f = \frac{-f_3 \cdot f_1}{f_2} \alpha_{0x,y} \quad (3.1)$$

where f_n is the focus of the n^{th} lens. The position x_f can be set by adjusting the angle α_{0x} and the position of y_f can be set by adjusting α_{0y} . The lenses L1 and L2 also function as a beam expander which determines the focal beam width w_f given by:

$$w_f = \frac{4\pi f_1 f_3}{\pi w_0 f_2} \quad (3.2)$$

where λ is the wavelength of the light, w_0 the beam width at the gimbal mirror. The focal beam width w_f is calculated to be $7.61 \mu\text{m}$, assuming $\lambda = 550$ nm, the focal lengths of the lenses named before and $w_0 = 700 \mu\text{m}$ ($1/e^2$) which is the beam width of

the laser. This does not fit perfectly to the single mode of the waveguide which is easily a factor of ten times smaller in height, which can lead to coupling losses as was stated before. In width, the mode profile is wider because of the 10 μm wide ridge, which leads to a higher coupling efficiency it means also that in the beginning first order modes can be coupled into the waveguide. Coupling of these first order modes in width can be minimized by focussing the light in the centre of ridge. Using the 50 mW lasers, the coupling losses were overcome and a sufficient amount of light was coupled into and finally out of the waveguide to detect three interference patterns.

3.4.2.3 Fiber butt coupling

The incoupling method was changed to butt-end coupling to go towards a more application driven solution and a more time-efficient incoupling. The setup of Fig. 3.4 was maintained up to and including mirror M5 after which an apochromatic fiber collimator (60FC-4-RGBV11-47, Schäfter + Kirchhoff GmbH, Hamburg, Germany) placed on a xyz-stage (MAX361D/M Fiber Launch, Thorlabs) is used to couple the light into a Thorlabs PM460-HP single mode polarization maintaining (SMPM) optical fiber. Instead, it is also possible to use fiber pigtailed lasers in combination with an Integrated Laser Beam Combiner [6] to couple all the light sources in one single fiber which can be positioned with respect to the waveguide to couple light into the waveguide via butt-end coupling. As we already bought the non-pigtailed lasers this was not an option, but for a final product which could be brought onto the market this option should be taken into account.

For fiber butt-end coupling the half-wave plates are taken out of the setup because they are not required as the polarization of the light can be changed by rotating the fiber. The end of the fiber is positioned on top of an ULTRAlign™ Precision XYZ Positioning Stage with DS-4F High Precision Adjusters (8.0 mm Coarse Travel, 0.3 mm Fine Travel) to position the fiber with respect to the waveguide in order to efficiently couple the light into the waveguide. The single mode fiber has a core diameter of 3.0 μm and a mode field diameter of $3.3 \pm 0.5 \mu\text{m}$ ($1/e^2$ fit near field, [7]) at 515 nm and is single mode above 450 nm. To reach the waveguide which is embedded in a chip holder and to protect the fiber core, the end of the fiber is glued into a glass ferrule with a size of 1 mm and a hole of 125 μm by XiO Photonics BV. After gluing the fiber in the ferrule, the ferrule and fiber are polished together. Consequently, the ferrule does not affect the mode profile. The coupling of the single mode of the fiber is especially critical in height as the size of the single mode of the waveguide in height is approximately 0.5 μm ($1/e^2$) at 515 nm. In width it is also important to align the fiber at the centre of the waveguide taper such that zero order modes are coupled in most efficiently to get the most equal distribution of the light over the four channels. Using a USB camera for the alignment of the fiber we were able to couple the 457 nm, 561 nm and the 660 nm light simultaneously into the fiber. However, changing the incoupling of the system from end-fire coupling to fiber butt-end coupling, oscillations in the

phase signal appeared with an amplitude of approximately 3×10^{-3} fringes (see Fig. 3.5a). Therefore, the phase precision of 10^{-4} fringes could not be achieved. Oscillations were also measured in the mean signal (mean total number of counts of the whole interference pattern) of the measured interference patterns. The period of the observed oscillations in phase and mean number of counts were wavelength dependent, indicating that they are caused by an interference effect. Using a piezo xyz-stage to actively change the position of the fiber by a triangular wave with respect to the waveguide the origin of the oscillations was traced to a change in distance of the fiber with respect to the chip. Similar oscillations in phase and mean counts are measured when changing the distance (z-direction) and not for changing the height (y-direction) and width (x-direction) as shown Fig 3.5b-d. The period of the oscillations shown in Fig. 3.5d are not constant over time, which means that the drift is not constant over time. This was not expected because of the use of the triangular wave. However, this can be explained by the fact that the piezo stage was positioned on top of another stage which was used to do the coarse alignment and which also slightly drifts with a non-constant velocity over time. The oscillations in the mean number of counts can be explained by the fact that the fiber tip and waveguide behave like two partly reflective mirrors in a Fabry-Perot cavity. It was expected that the phase signal would not show oscillations, because the incoming beam is coupled into one channel which splits finally up into four channels in which beams should propagate with the same phase, independent of the incoming phase or intensity. Slab light which is not coupled into the channels but is coupled out at the end face of the chip might be a reason why oscillations are also seen in phase signal.

Oscillations can be prevented by using a xyz-stage with a sufficiently small drift. Over 2000 s the fiber was displaced 2 μm and approximately 13 oscillations were detected for the 660 nm laser. This means that one oscillation corresponds to approximately 154 nm. Consequently, for a maximum phase noise of 10^{-4} fringes and the measured amplitude of approximately 3×10^{-3} fringes, only a thirtieth of an oscillation is allowed. This means that the stage should not drift more than approximately 5 nm. On the other hand, the stage should be able to displace the fiber for a couple of millimetres. It is hardly possible to find a stage which meets these requirements. Therefore, the problem was solved placing an index matching gel, which matches the refractive index of the glass fiber tip, in between fiber and waveguide and bring the fiber in contact with the waveguide. Therefore, the light will not be back reflected at the interface of fiber and matching gel. An index matching fluid also was sufficient to get rid of oscillations but did leak into the measurements chambers of the waveguide structure and was therefore not suitable. Fig. 3.6 shows that using a matching index gel the oscillations in the phase signal disappeared.

Fig. 3.5: a) Phase change and mean number of counts of interference patterns measured at $\lambda = 561$ nm and 660 nm showing typical oscillations and the phase change and mean number of counts when changing position of the fiber in b) y-direction (height), c) x-direction and d) z-direction (distance to the chip).

Fig. 3.6: a) Phase change and mean number of counts for a) fiber not in contact with waveguide, b) fiber in contact with waveguide, c) fiber in contact with waveguide with index matching gel in between.

So using the matching index gel and the butt-end coupling a sufficient amount of light was coupled into and out of the YI waveguide to measure a phase change of the three interference patterns without the oscillations such that this does not restrict the required phase precision of 10^{-4} fringes.

3.4.3 Characterization

The lasers were coupled into a PM460-HP SMPM optical fiber. This fiber is a “Panda” style PM fiber which means it has a fast and a slow axis which should be aligned with the polarization of the light such that fiber is polarization maintaining. If it is not aligned properly, there is also a small TE component which propagates with a slightly different velocity and then the length of the fiber determines what fraction TE/TM comes out of the fiber. Also bending of the fiber and temperature changes can change the output, because the velocity of the TE and TM modes are changed differently. Coupling efficiencies achieved using the 60FC-4-RGBV11-47 apochromatic fiber collimator were 37% for the 457 nm laser, 51% for the 561 nm laser and 50% for the 660 nm laser coupling all the three lasers into the fiber. Higher coupling efficiencies could be reached by coupling a single laser into the fiber. Using the MAX361D/M Fiber Launch xyz-stage the incoupling was very stable after settling of the adjustment screws. No further adjustment was required after once optimising the incoupling. Laser light with a ratio TE/TM of 100:1 was coupled into the fiber. The measured TE/TM ratio out of the fiber was varied from 1:100 to 5:100 for all the lasers. The TM polarized light was filtered out after the waveguide using a polarizer. The TE/TM ratio was strongly influenced by the bending of the fiber. Therefore, the fiber should be stably positioned during the measurements. By stable positioning the fiber and using and polarizer behind the waveguide, we were able to measure phase changes of the three wavelengths of either TE or TM propagating modes.

3.5 Imaging

3.5.1 Requirements

To measure size-selectively with multiple wavelengths it is necessary to detect the interference patterns of the multiple wavelengths such that the phase change of the various wavelengths can be measured independently. With the current waveguide structure it is required to image interference patterns on different pixel rows of the camera. Imaging the interference patterns on the same pixel rows would give an overlap in spatial frequencies ($k_{ij} = d_{ij}/\lambda L$) for some wavelengths in combination with various distances between the channels. For example, the phase change of the 457 nm laser in combination with 100 μm distance between the channels can hardly be distinguished from a phase change of the 660 nm laser in combination with the 140

μm distance between the channels, because they both have approximately the same spatial frequency at which the phase signal should be red out. In order to realize the separation of the wavelengths, a diffraction grating can be used in combination with a couple of achromatic lenses. The three interference patterns should be imaged onto a CCD camera with a chip height of 6.656 mm (see section 3.6). For optimal spread the interference patterns should approximately be imaged on 1/6, 3/6 and 5/6 on the camera. This means that the distance between the outer interference pattern is 2/3 of the camera pixels, so $2/3 \times 6.656 \text{ mm} = 4.437 \text{ mm}$. The position y on the camera is determined by the focal lens and the diffraction grating that are used to split up the interference patterns and described by:

$$y = f \tan \theta_m, \quad (3.3)$$

where f is the focal length of the lens and the incident angle of the lens θ_m can be determined from the grating formula for incident angles of 0° [8]:

$$a \sin \theta_m = m \lambda \quad (3.4)$$

where a is the distance between the centres of two adjacent slits, m the order of the various principal maxima and λ the wavelength of the light. Assuming a diffraction grating of 300 lines/mm, $\lambda_1 = 457 \text{ nm}$ and $\lambda_2 = 660 \text{ nm}$, $m = 1$, the f corresponding to $y = 4.437 \text{ mm}$ is equal to 69.8 mm.

Next to the different wavelengths, different polarizations can be coupled into the waveguide which also have a different response on RI changes in the evanescent field. Therefore, the TM and TE modes should be measured independently. It was noticed that the TE modes of the 457 nm light do sometimes convert to TM modes on the chip. To prevent these modes interfering with the measured TE modes, a polarizer was placed between camera and waveguide. Alternatively, TE and TM modes can be measured both by splitting them up using a Wollaston prism (see Chapter 6).

3.5.2 Realization

The light coupled out of the chip is first collimated using a cylindrical achromatic lens with a focal length $f = 75 \text{ mm}$ (Thorlabs, ACY254-075-A). Next, the light is directed to a visible transmission grating of 300 lines/mm (Thorlabs, GT-25-03, 25mmx25mm). Subsequently, a second cylindrical achromatic lens with focal length $f = 50 \text{ mm}$ (Thorlabs, ACY254-050-A) is used to image the interference patterns on the CCD camera. For a lens of $f = 50 \text{ mm}$, the difference in x between the first orders of $\lambda_1 = 457 \text{ nm}$ and $\lambda_2 = 660 \text{ nm}$ is 3.2 mm. A schematic side view is shown in Fig. 3.7. A Linear Polarizer with N-BK7 Protective Windows (400-700 nm) (Thorlabs, LPVISE100-A) is used to filter out TE or TM polarized light. Light is only collimated in the vertical direction using cylindrical lenses. In lateral direction no lenses are

required as the interference pattern is measured at a distance $L \gg a$. Fig. 3.8 shows the three interference patterns images on top of each other on different pixels rows of the camera, illustrating that we are now able to independently measure phase changes of the three interference patterns. Interference patterns are imaged on multiple rows to increase the dynamic range of the interference patterns to reduce the phase noise (see section 3.6).

Fig. 3.7: A schematic side view of how tow lenses and a grating are used to image the interference patterns of the three different wavelengths separately on a CCD camera, where the white part represent the overlapped light three wavelengths. Image is not to scale.

Fig. 3.8: Typical camera image illustrating three measured interference patterns from three different wavelengths, 660 nm, 561 nm and 457 nm, from top till bottom.

3.6 Detection

3.6.1 Requirements

Simulations were done to determine which camera settings are important to accurately detect a phase change from an interference pattern. First, an interference pattern ($\lambda = 550 \text{ nm}$) based on 4 point sources is generated consisting of a certain number of pixels and with a certain dynamic range. Independent of any camera, there

will always be photon shot noise and therefore shot noise (Poisson noise) is added to the interference pattern. No phase change is induced in the interference pattern, which means the determined phase should be zero. Consequently, the determined phase is equal to the error in phase. The error in the phase is determined as a function of the dynamic range of the camera and the number of pixels of the camera. For each dynamic range and number of pixels an interference pattern was generated 1000 times and a phase error was determined. The standard deviation of these values is taken as a final measure for error in phase. The results shows that the error in phase shows a linear relationship with the dynamic range and number of pixels on a log-log scale (see Fig. 3.9). The slope is given by approximately $-\frac{1}{2}$, which means that the noise can be decreased by measuring more counts (higher dynamic range and number of pixels) and that the decrease of noise is limited by the photon shot noise ($\sim N^{0.5}$). Increasing the number of pixels has the same effect as increasing the dynamic range. To realize an error in the determined phase considerably lower than 10^{-4} fringes, such that the camera is not the limiting factor, a camera with a dynamic range of 16 bits (65536) combined with a number of pixels of 4096 is required. Alternatively, the dynamic range can artificially be increased by imaging the interference pattern on multiple pixel rows and then adding up these pixel rows. As was stated before it is also required to have a camera with multiple pixel rows onto which the various interference patterns can be imaged.

Fig. 3.9: Standard deviation of the determined phase of 1000 generated interference patterns (including shot noise) as a function of the number of pixels and the dynamic range of a camera.

3.6.2 Realization

Because of its high dynamic range in combination with acceptable number of pixels in width and height, an Alta U30-OE CCD camera is used. Specifications of the camera are shown in Table 3.2. At least 10 rows of 1024 pixels with a dynamic range of 12489 were selected per interference pattern such that the phase noise is much lower than the required 10^{-4} fringes.

Table 3.2: CCD camera specifications [9]

CCD chip	E2V CCD30-11
Array Size (pixels)	1024x256
Pixel Size	26 μm x 26 μm
Linear Full Well (typical)	271K electrons
RMS Noise	21.6 electrons
Dynamic Range (SNR)	12489 (13 bits=8192, 14 bits=16384)
Digital Resolution	16 bits
Gain	4.05 ± 0.01 electrons per count (measured)
Bias level	1236 (measured)
120 second Dark current	1244 counts (measured)
Dark current	0.1494 counts per pixel per second (measured)
Test temperature	-30 $^{\circ}\text{C}$, $\Delta=51$ $^{\circ}\text{C}$
Exposure Time	30 milliseconds to 183 minutes

3.6.3 Characterization

3.6.3.1 Shutter

The Alta U30-OE charge-coupled device (CCD) camera has a mechanical shutter to block incoming light when reading out the pixel rows. Phase stability tests showed that the using the camera shutter leads to a phase noise in the order of 10^{-3} fringes which is higher than the required 10^{-4} fringes. This phase noise might be caused by movement of the camera due to this mechanical shutter. Maximally 80 fringes fall onto a camera width of $26 \mu\text{m} \times 1024 = 2.6624 \text{ cm}$, which means that 1 fringe corresponds to 0.033 cm. Consequently, a phase change of 10^{-3} fringes corresponds to a movement of 330 nm. Fig. 3.10 shows the phase change can be reduced significantly to a phase noise of $<10^{-4}$ fringes when not using the shutter of the camera. However, the mechanical shutter is required to block the light during readout to prevent vertical smear. Fig. 3.11 shows a camera image when no shutter is used, illustrating the vertical smear which makes it impossible to measure the interference patterns independently. The lasers cannot be used optimally when modulating it by turning it on and off, so the light is blocked during the readout of the camera using a mechanical shutter (Uniblitz, LS2ZM2, Vincent Associates, Rochester, New York), driven by a VCM-D1 shutter driver (Uniblitz) to prevent vertical smear on the CCD camera. The shutter is placed on a position where all the wavelengths overlap and the shutter driver is driven by a Arduino Uno board which also drives the readout of the CCD

camera. Using the external shutter the phase change on a short time scale is lower than the required phase precision of 10^{-4} fringes.

Fig. 3.10: Phase change over time for a stability measurement with a shutter on (red) and without using a shutter (black).

Fig. 3.11: An example of a camera image of three interference patterns using a) the camera mechanical shutter and b) no shutter, resulting in vertical smear.

3.5.3.2 CCD Camera temperature

To determine the influence of the camera cooler, the phase stability was measured with the cooler and fan off and on. Turning off the cooler of the camera results in a very unstable temperature, resulting in a non-stable phase signal as seen in Fig. 3.12 a/b. Fig 3.12 c/d show the temperature and corresponding phase stability when the fan was turned off. When turning the fan off, the temperature starts increasing resulting in a phase drift, probably caused by expansion of materials, resulting in movement of the CCD chip. As expected, the phase change is the highest for the highest spatial frequency, which has the highest number of fringes on the chip. Therefore, one fringe is the smallest at the highest spatial frequency and consequently a movement of the CCD chip results in the highest phase change for this spatial

Fig. 3.12: a) Temperature of CCD camera and b) corresponding phase change over time for the 660 nm laser when turning the camera fan and cooler on and off, and c) temperature of CCD camera and d) corresponding phase change over time for the 660 nm laser when turning off only the camera fan.

frequency. To keep the phase change stable, the temperature of the camera should be kept stable and this was done during the measurements by setting cooler of the camera at $-30\text{ }^{\circ}\text{C}$ and the fan on in the highest mode (data of different fan modes is not shown, but best results were obtained by highest fan mode).

3.5.3.3 Measured camera noise

The noise of the camera was characterized using a photon transfer curve (PTC) as was also illustrated by [10, 11]. The CCD camera can be seen as a system block with light as input and digital data as output. Shot noise due to the nature of photons is the only noise at the input. So any difference between the noise at the input and output must be caused by the CCD camera. The CCD camera was exposed with an illumination field using a Farnell 1814434 green light-emitting diode (LED) (525 nm). For a range of intensities (~ 1260 -64000 counts), two consecutive frames were captured and subtracted to remove frame-to-frame offsets and photo-response non-uniformity. The root mean square (RMS) noise of the camera is calculated by taking the square root of the variance and is given by [10]:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N_p} ([X1_i - M1] - [X2_i - M2])^2}{2 \times N_p}}, \quad (3.5)$$

where N_p is the number of pixels, $X1_i$ the individual pixel values of the first frame, $X2_i$ the individual pixel values of the second frame, $M1$ the mean of all the pixels of the first frame and $M2$ the mean of all the pixels of the second frame. Because the noise was initially doubled by subtraction of the two frames, there is a factor of two in the divisor. A PTC curve is now generated at $T_{CCD} \approx -30\text{ }^{\circ}\text{C}$ by plotting the RMS noise as a function of the mean signal in a log-log plot which is shown in Fig. 3.13. Fig. 3.13 shows that for a number of counts larger than 1000 the shot noise is the limiting factor as the slope of the figure is equal to 0.5. Shot noise is equal to the square root of the number of counts which appears as a 0.5 slope in a log-log plot. To confirm that this camera can be used to measure a phase precision of 10^{-4} fringes an interference pattern based on 4 point sources (representing a 4 channel waveguide) was imaged onto the CCD by an achromatic lens using a 525 nm Farnell 1814434 LED source, setting the maximum number of counts measured by the CCD camera to ≈ 60000 by tuning the intensity of the LED. With an exposure time of 0.03 s and the $T_{CCD} \approx -30\text{ }^{\circ}\text{C}$, the interference patterns were measured over a multiple number of rows N_{rows} (4, 16, 50, 100 and 200 rows) which were added up. The phase change over time was determined of these selected interference patterns and for $N_{rows} = 100$ plotted in Fig. 3.14a. To determine the short time scale noise and excluding long time scale (thermal) drift, the phase change was smoothed with a $N = 65$ linear Savitsky-Golay filter. This

signal was subtracted from the originally measured signal (see Fig. 3.14b) and from the difference (Fig. 3.14c) the noise was defined by determining its standard deviation. The phase noise was averaged for all spatial frequencies and determined as a function of N_{rows} and compared with simulated data which is shot noise limited. For spatial frequency d_{12} which belongs to the distance between channel 1 and 2, the measured phase noise is close to the simulated shot noise limited case. For larger spatial frequencies, the noise increases as shown in Fig. 3.14a. This might indicate that imaged interference pattern vibrates with respect to the camera, because the lower spatial frequencies are less affected because of their lower number of fringes on the camera and therefore a larger size per fringe. The measured phase noise corresponding to d_{14} was $\approx 5 \times 10^{-5}$ which corresponds to a vibration of ≈ 5 nm (for d_{14} approximately 65 fringes fall onto the 6.656 mm which means that 1 fringes is equal to 100 μ m). The absolute phase drift on a large time scale also scales linearly with the spatial frequencies which indicates that the interference pattern image slightly drifts ($<10^{-3}$ fringes ≈ 416 nm over ≈ 4000 s) with respect to the camera. As a conclusion we can say that the position of the camera with respect to the camera should be very stable (≈ 10 nm for a phase noise of $\approx 10^{-4}$), but if phase drift or noise is caused by movement it will scale linearly with the measured phase noises at the spatial frequencies belonging to different distances between the YI channels. Moreover, it can be confirmed that the camera noise on a short time scale is shot noise limited and measuring the interference patterns over more than 10 pixels, the phase noise is much lower than the required 10^{-4} fringes.

Fig 3.13: Photon transfer curve showing the RMS noise as a function of the mean signal measured for the CCD camera measured at $T_{CCD} = -30$ °C. The red line has a slope of 0.5 indicating that the measured points fit the shot noise. The blue line with slope of zero represents the read noise level.

Fig. 3.14: a) Measured phase change over time when interference pattern was imaged onto CCD camera for all six spatial frequencies and after summation of 100 rows of the camera, b) the phase change over time for $N_{rows} = 200$ and spatial frequency (sf) d_{12} , smoothed with a $N = 65$ Savitsky-Golay filter, c) the subtracted signal of the measured and smoothed signal for d_{12} and $N_{rows} = 50, 100$ and 200 , and d) the phase noise (standard deviation) as a function of N_{rows} of the measured data (average of all sf's and d_{12} only) and simulated data (average of all sf's).

3.7 Data processing

3.7.1 Requirements

The phase change should be detected as accurately as possible from the collected interference patterns with the CCD camera. First, an FFT should be applied to the interference patterns. Next, the phase change should be red out at the spatial frequencies belonging to the wavelengths, camera distance and channel distances. The phase error (PE = difference between expected induced phase change and measured phase change) and crosstalk (CT = deviation where no phase change expected) should

be minimized. PT and CT can be reduced by an artificial increase of spatial frequency resolution and application of a sufficient windowing [1]. The spatial frequencies can be reduced by a factor of ~ 2 using an artificial increase of the spatial frequency [1]. The spatial frequency resolution cannot be increased indefinitely, because the error introduced by a decrease of the peak amplitudes, which is also a result of the artificial increase, will dominate the effect of spatial frequency increase. Using a Hanning window, the PE and CT can also be reduced by a factor of ~ 5 [1].

3.7.2 Realization

To readout the interference pattern, to determine the phase change over time and to apply the above named techniques a Labview program was written. First, the CCD chip is read out as a 256×1024 (rows $\times$ columns) matrix with individual pixel values. Next, a selected number of rows of this matrix is added up separately for each interference pattern of each wavelength. Also the next steps are done separately for each wavelength. Subsequently, a window is applied on the summed interference patterns after which 1024 zeros are added to decrease the PE and CT. Then, an FFT is applied after which the phase signal is read out at a selected spatial frequency amplitude corresponding to a certain channel combination and wavelength. Phase jumps of 2π are corrected using a self-written unwrap function. Moreover, the spatial frequency amplitude and camera temperature T_{CCD} are recorded.

3.7.3 Characterization

Computer generated images of interference patterns (based on our four channel Young interferometer) were used to verify the correct working of the Labview program which is used to collect interference patterns and determine the corresponding phase changes during an experiment, to investigate artefacts when determining a phase change from a finite interference pattern and to test windowing functions to reduce artefacts. Calculations of the generated interference patterns are based on four point sources distanced at 60 μm , 80 μm and 100 μm (see Fig. 3.2), an imaged distance of 17.5 cm, and wavelengths of 457 nm and 660 nm. The generated images have a width of 1024 pixels, a height of 100 pixels and a dynamic range of 60000 counts and include shot noise (Poisson noise). To simulate a real experiment, a time varying ΔN_{eff} ($\sim \Delta\varphi$) is introduced (see Fig. 3.15a). For each corresponding time point an image is calculated and stored for later analysis. Next, the Labview program loads the images, adds up the selected number of rows on which the interference patterns are imaged and subsequently adds 1024 zeros to increase spatial frequency resolution to improve phase readout. Optionally, a windowing function is applied before a Fast Fourier Transform (FFT) is executed. From the resulting spectrum, the phase and amplitude of the six different spatial frequencies are determined of which we now analyse one peak belonging to one channel pair. Fig. 3.15a shows an example

of the induced and calculated ΔN_{eff} for the two wavelengths. The same figure also shows the difference in ΔN_{eff} between the induced and determined values. Fig. 3.15b shows the determined amplitude and Fig. 3.15c shows the $\overline{\Delta n_s}$ determined with the ratio-based approach (see Ch. 4, page 79 for explanation of ratio-based approach and $\overline{\Delta n_s}$) based on the induced (ind) and determined (det) ΔN_{eff} . Fig. 3.15d, e and f show the $\overline{\Delta N_{eff}}$, the amplitude and the determined $\overline{\Delta n_s}$ of a real measurement. Similar fluctuations (which we call artefacts) are seen in the amplitude and $\overline{\Delta n_s}$ when no windowing is applied on the simulated interference patterns, indicating that boundary effects of the shifting interference pattern are causing the measured artefacts. These artefacts are most prominent during (fast) changes in the induced ΔN_{eff} .

Fig. 3.15: Comparison of fluctuations in the ΔN_{eff} as determined by using simulated data (a,b,c) and measured data (d,e,f) in a typical measurement composed of a bulk signal followed by a combination of a bulk signal and a signal arising from surface binding. a) ΔN_{eff} induced (ind) and determined by analysing simulated data without applying a window (det) and the difference between detected and induced ΔN_{eff} , b) the amplitude determined by the FFT corresponding to the $\Delta\phi$ from which ΔN_{eff} is determined, c) Δn as induced and as determined using the ratio-based approach, d) the ΔN_{eff} of a measurement where first D-glucose and later D-glucose and 85 nm beads are added to the sensor surface, e) the corresponding amplitude of the FFT-spectrum at the measured spatial frequency and f) the determined Δn due to binding of the beads and bulk changes due to the D-glucose.

To investigate this further, a linear $\Delta\varphi$ ($\sim \Delta N_{eff}$) was induced and the peak-to-peak (pk-pk) values of the artefacts in ΔN_{eff} were determined to test the influence of the applied window on the size of the artefacts (both in amplitude and $\Delta\varphi$ ($\sim \Delta N_{eff}$)). Fig. 3.16a shows the linear ΔN_{eff} determined from the linear $\Delta\varphi$, and the difference between the determined and induced ΔN_{eff} ($= \Delta N_{eff}$ error, abbreviated as NE in the remainder of the text) for the case in which no window is applied and the case in which a in Labview build-in Blackman-Nuttall window is applied. Fig. 3.16b shows the mean pk-pk values of the NE of different channel combinations, determined from $\Delta\varphi$ measured at the spatial frequencies (sf) belonging to the channel combination in which a Δn was generated in one of the channels as well as from the channel combinations in which no Δn occurred (cross-talk, CT). To demonstrate the effect of windowing, results are plotted for different type of windows. For example, applying a Blackman-Nuttall or a Dolph-Chebyshev window, both CT and NE can be completely suppressed down to the shot-noise level as the artefacts were not visible above the shot-noise level.

Next, the use of different windows on real experimental data is evaluated. The mean pk-pk values of CT are similar to the mean pk-pk values of NE. Therefore, the mean pk-pk values of CT are used as a measure for the pk-pk values of NE for the real experiments, as it is not possible to determine the fluctuations in the measured signal of ΔN_{eff} , because the slope of the measured signal is much higher than the slope of the artefacts. Also no induced ΔN_{eff} can be determined and consequently it is not feasible to determine the difference between a measured and induced ΔN_{eff} to determine the NE. Raw data (interference patterns) were saved and different windows were applied on the measured data. Fig. 3.16c shows the fluctuations in ΔN_{eff} due to CT for a typical measurement where first D-glucose only (between $t \approx 500$ and 1000 s) and subsequently D-glucose plus 85 nm beads (between $t \approx 1500$ and 2000 s) were added simultaneously to the sensor. Fig. 3.16d shows the pk-pk value of the first artefact in ΔN_{eff} (at $t \approx 500 - 700$ s) due to CT as a function of the applied window. Applying different windows did not lead to significant differences in performance. Probably the measured interference patterns already contains a hardware-induced windowing. The non-ideal properties of the lenses, the grating and beam shape cause an ellipse-shaped image of the interference pattern as shown in Fig. 3.8. Before applying an FFT on the measured patterns, pixel intensities of each column in the recorded image are summed and therefore the ellipse-shaped image results in an interference pattern in which the intensities are suppressed at the edges which means that it already consists of a certain window. Furthermore, it was seen that different optics resulted in change in artefacts. Therefore, it should be tested if larger lenses and grating can reduce the

artefacts in the signals of ΔN_{eff} . The analysis of an experiment with a two channel YI (two channels were blocked with a mask) showed similar artefacts as for a four channel YI, which means the artefacts are in the measured signal itself and not only induced by CT as there is only one spatial frequency peak for a two-channel YI. The amplitude of the measured oscillations in CT are in the order of 10^{-2} fringes. Induced signals should be significantly larger than the artefacts. The artefacts influence the final amplitude of the signal with a maximum deviation equal to the amplitude of the oscillation.

Fig. 3.16: The effect of applying different windows on the data on the error in the determined phase change ($\sim \Delta N_{eff}$). a) ΔN_{eff} induced and the difference between determined and induced ΔN_{eff} (NE) applying no window (magenta) and a Blackman-Nuttall window (green), b) the peak-to-peak (pk-pk) values of the fluctuations in ΔN_{eff} (for both NE and CT) for the different applied window, c) the measured ΔN_{eff} of a measurement channel and the CT in a side channel and d) the pk-pk values of the fluctuations in ΔN_{eff} due to CT as a function of the applied window and selected pixels of the interference pattern.

3.8 Overview setup

An overview of the setup is given in Fig. 3.17. The setup is covered with Thorlabs enclosure accessories (see Fig. 3.17a) to protect it from air currents, dust and light interfering with the measurements. It was measured that air current can strongly influence the phase signal as shown in Fig. 3.18 which also shows that the lasers covering box should stay closed during measurements. Fig. 3.17b/c show an overview

Fig. 3.17: a) Setup covered with Thorlabs enclosure accessories to protect in from air current, dust and light interfering with the measurements, b/c) the setup when using end-fire coupling and d/e) the setup when using fiber butt-end coupling and f) the 660 nm light coupled out the 4 channel waveguide structure.

of the setup used for end-fire coupling. Fig. 3.17d/e show an overview of the setup used for fiber butt-end coupling. Fig. 3.17f shows 4 point sources of the 660 nm light coupled out of the waveguide structure which correspond to the output of the 4 channels of the YI.

Fig. 3.18: Phase change over time while turning fan on and off and opening and closing of the laser covering box.

3.9 Characterization of phase noise and drift

The phase noise (short time scale of seconds) and the phase drift (noise on long time scale of hundreds of seconds) were measured for both incoupling methods as shown in Fig. 3.19. The determined phase noise on a short time scale was determined by smoothing the phase with a $N = 15$ Gavitsky-Golay smoothing filter and subtracting this from the original phase signal and by taking the standard deviation of this subtracted signal. This lead to a determined average phase noise of the measurements of 2×10^{-5} and 6×10^{-5} fringes for butt-end coupling and end-fire coupling respectively which is typical for all the measurements. It can be concluded that this sufficient as it is lower than the required 10^{-4} fringes.

The usual phase drift is smaller than 5×10^{-3} fringes per 1000s for the end-fire coupling and 1.5×10^{-2} fringes per 1000s for the butt-end coupling. The higher drift with the butt-end coupling might be caused by the ULTRAlign™ Precision XYZ Positioning Stage and the long fiber tip which might be not perfectly stable. It was seen that after stabilising the stage for a couple of hours the drift was reduced to values comparable with the end-fire coupling. It should be noticed that there is a difference between the phase drift of the different spatial frequencies.

An idea was to use the correlation between the measured phase changes of the different wavelengths in different channels as illustrated in Fig. 3.20. The phase noise of the three wavelengths is presented for all the six spatial frequencies belonging to

the distances between the four channels. The Pearson product-moment correlation coefficients were determined for all the phase signals and varied from 0.441 (d_{34} for 457 nm and 660 nm) to 0.998 (d_{24} for 561 nm and 660 nm), where a correlation coefficient of 1 is fully correlated). This means that there is a correlation between these phase signals. However, this correlation is not constant. Sometimes, the drift in the 660 nm laser is the highest, but also sometimes the 561 nm or the 457 nm laser. No clear trend between the phase signal of the wavelengths could be observed. Therefore, the wavelengths could not easily be used to cancel out phase drifts. Lateral movement of the camera with respect to the chip can lead to phase drift, but this can clearly be detected as it scales inversely with the spatial frequencies. It is also known that temperature plays an important role as it can lead expanding materials and stress in materials which can lead to RI changes and changes in distances. Channels in the YI waveguide were placed close together to have a relatively constant temperature over the chip. To have a better heat sink for the chip the plastic chip holder was replaced by a brass chip holder, but this did not lead to any reduction of the phase drift. Moreover, phase measurements without a waveguide but with two slits, but this did also not gave inside in the origin of the drift. This means that the measurements presented in the next chapter contain drift. Simulations in Chapter 4 show that linear drift results in a linear drift in the signal of Δn . The drift in the measurements should be determined by determining the baseline. If measurements show a linear phase drift the drift can be compensated for by fitting it linearly and subtracting it. Preferably, induced signals should be significantly higher than the phase drift.

Fig. 3.19: a) Phase change of 660 nm laser measured for fiber incoupling and free space incoupling for two measurements per incoupling methods measured each at six spatial frequencies belonging to the six channel combination of the YI and b) a zoom-in off two signals of a).

Fig. 3.20: Typical phase noise over time of 457, 561 and 660 nm laser coupled into waveguide using fiber butt-end coupling for sf's belonging to distances between channels a) d_{12} , b) d_{23} , c) d_{13} , d) d_{34} , e) d_{24} and f) d_{14} .

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Appendix 3.A Focal position using a 4f lens system

Assuming small angles and small distances relatively to the optical axis of the system (paraxial approximation), the angle and position at a certain point can be determined with Ray transfer matrix analysis. For the Ray transfer matrix analysis, a thin lens can be described by the following matrix:

$$\begin{bmatrix} 1 & 0 \\ -\frac{1}{f} & 1 \end{bmatrix}, \quad (3.A.1)$$

where f is the focal length of the lens. A distance d can be described by the following matrix, assuming propagation in air ($n = 1$):

$$\begin{bmatrix} 1 & d \\ 0 & 1 \end{bmatrix}. \quad (3.A.2)$$

The 4f lens system which is used to focus the light into the waveguide structure is shown in Fig. 3.A.1 and consists of lenses and distances between the lenses and can therefore be described using equations 3.A.1 and 3.A.2 in reverse order of the propagation of the light through these components. The position and angle at f are determined using the following equation:

$$\begin{bmatrix} y_f \\ \alpha_f \end{bmatrix} = \begin{bmatrix} 1 & f_3 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} 1 & 0 \\ -\frac{1}{f_3} & 1 \end{bmatrix} \begin{bmatrix} 1 & f_2 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} 1 & 0 \\ -\frac{1}{f_2} & 1 \end{bmatrix} \begin{bmatrix} 1 & f_1 + f_2 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} 1 & 0 \\ -\frac{1}{f_1} & 1 \end{bmatrix} \begin{bmatrix} 1 & f_1 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} y_0 \\ \alpha_0 \end{bmatrix}, \quad (3.A.3)$$

where f_1 , f_2 and f_3 are the focal distances of lens L_1 , L_2 and L_3 respectively and y_0 and α_0 are the position and angle at the Gimbal mirror. Equation 3.A.3 can be simplified to:

$$\begin{bmatrix} y_f \\ \alpha_f \end{bmatrix} = \begin{bmatrix} -\alpha_0 \frac{f_1 f_3}{f_2} \\ \frac{f_2 y_0}{f_1 f_3} - \frac{f_1 \alpha_0}{f_2} \end{bmatrix}. \quad (3.A.4)$$

This means that the position y_f is determined by the angle α_0 in y-direction at the Gimbal mirror and the focal distances of the used lenses. In a similar way x_f is a function of the angle α_0 in x-direction.

Fig. 3.A.1: Schematic overview of 4f lens system.

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Chapter 4

Different analysis approaches for size-selective analyte detection

Abstract

This chapter presents a description and a detailed study of different signal analysis approaches that are required to obtain the signal from the different substances from the measured phase changes at the different wavelengths. It was found that a theoretical approach as presented in Chapter 2 is exact but in practice difficult to implement because of the many parameters that have to be tuned. Therefore, we also developed a much more practical ratio-based approach based on the ratios of ΔN_{eff} 's measured at different wavelengths. These ratios were determined independently for 85 nm carboxylated polystyrene beads (representing specific binding of e.g. viruses which have approximately this size), protein A (representing non-specific binding) and D-glucose (representing bulk changes). These ratios are used to discriminate between the Δn 's induced by these substances which are applied in the proof-of-principle experiments for size-selective detection. On the other hand, using this ratio-based approach it is not possible to determine an absolute value of Δn . However, this is not strictly required, because calibration measurements, which are usually also carried out by a single wavelength YI, can be used to calibrate the measured values of Δn with an analyte concentration. The theoretical and ratio-based approach were combined to determine the absolute value of Δn and the corresponding surface mass coverage. Moreover, the approaches were combined to determine the thicknesses of 85 nm beads to be ≈ 72.6 nm and protein A to be ≈ 2.1 nm which are in agreement with literature. Furthermore, the influence of the parameters that are required for the analysis approaches (e.g. waveguide core thickness, waveguide refractive indices, ratios of ΔN_{eff} 's) on the determined Δn 's caused in different layers or by different substances was tested. Finally, simulations of a real experimental setting confirm the theoretical analysis given in Chapter 2 that noise, drift and artefacts show up enhanced in the determined Δn 's.

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4.1 Introduction

Usually, a YI is used to determine an effective refractive index change ΔN_{eff} at a single wavelength. Consequently, an average refractive index change Δn is determined, based on any Δn in the whole evanescent field. Determining the ΔN_{eff} at multiple different wavelengths enables discrimination between multiple different Δn 's or determine analyte thicknesses. In this chapter we present three approaches which can be used for this: a theoretical, a ratio-based and a combined theoretical and ratio-based approach (combined approach).

4.2 Theoretical analysis approach

Determining the ΔN_{eff} at two different wavelengths, enables the discrimination between Δn 's originating from two arbitrarily chosen layers within the evanescent field as shown in Chapter 2, using the following equation:

$$\overline{\Delta n_l} = \overline{S}^{-1} \cdot \overline{\Delta N_{eff}}; \quad (4.1)$$

where vector $\overline{\Delta n_l}$ is composed of the elements $\Delta n_{l,j}$ which is the RI change in the j^{th} layer, the coefficients of matrix $\overline{S}$ are given by sensitivity coefficients $S_{i,j} = (\partial N_{eff,i} / \partial n_j)$, which are the derivatives of N_{eff} of the i^{th} wavelength with respect to n occurring in the j^{th} layer and the vector $\overline{\Delta N_{eff}}$ is composed of the elements $\Delta N_{eff,i}$ which are the ΔN_{eff} measured at the i^{th} wavelength. The sensitivity coefficients are determined by the structure of the waveguide (core thickness and RIs of the various layers of the waveguide), the wavelengths, the polarization and two arbitrarily chosen layers (within the evanescent field). The $\overline{\Delta n_l}$ can be determined by measuring $\overline{\Delta N_{eff}}$ and multiplying this by the inverse of matrix $\overline{S}$. A homogeneous Δn_l per layer is determined and therefore this method is less suitable for situations where Δn 's induced by different substances occur in the same layer. However, assuming a low concentration of analytes, for example bulk effects can be cancelled out by subtraction of Δn determined from multiple layers to finally arrive at an analyte concentration as shown in Chapter 2. Nevertheless, this method requires tuning of many parameters to determine the correct values for the sensitivity coefficients that are required to determine the correct value of Δn and $C_{analyte}$. Hence, this method is exact but in practice difficult to implement because of the many parameters that have to be tuned.

4.3 Ratio-based analysis approach

4.3.1 Theory

We also developed a much more practical ratio-based analysis approach. This approach uses the ratios of ΔN_{eff} at multiple wavelengths, caused by Δn 's originating from different substances Δn_s . Each substance with a different size which causes a Δn_s in the evanescent field results in different ΔN_{eff} 's for each wavelength because of the different electric field distributions of the respective wavelengths. When a substance s_m is added to the sensor it induces a Δn_{s_m} (m enumerates the substances, $m = 1, 2, 3 \dots$), this results in an effective refractive index change $\Delta N_{eff, \lambda_k}$ at the wavelength λ_k ($k = 1, 2, 3 \dots$). Assuming that the response of the sensor has a linear relationship with the amount of substance s_m that is added to the sensor, this can be written as:

$$\Delta N_{eff, \lambda_k} = \frac{\partial N_{eff, \lambda_k}}{\partial n_{s_m}} \Delta n_{s_m}, \quad (4.2)$$

where $\partial N_{eff, \lambda_k} / \partial n_{s_m}$ is the sensitivity coefficient of the sensor for a RI change induced by s_m . Assuming that substances do not influence each other's response of the sensor, multiple substances inducing different ratios can be discriminated from each other using :

$$\overline{\Delta N_{eff}} = \overline{S_{sub}} \cdot \overline{\Delta n_s}, \quad (4.3)$$

where the vector $\overline{\Delta N_{eff}}$ is composed of the elements $\Delta N_{eff, \lambda_k}$, the vector $\overline{\Delta n_s}$ is composed of the elements Δn_{s_m} , and the coefficients of matrix $\overline{S_{sub}}$ are given by $s_{k,m} = \partial N_{eff, \lambda_k} / \partial n_{s_m}$.

Next, we define the ratio of two ΔN_{eff} 's measured at two wavelengths λ_k and λ_l , induced by a substance s_m as:

$$R_{\lambda_k / \lambda_l}^{s_m} = \frac{\Delta N_{eff, \lambda_k}^{s_m}}{\Delta N_{eff, \lambda_l}^{s_m}} = \frac{\partial N_{eff, \lambda_k}^{s_m} / \partial n_{s_m} \cdot \Delta n_{s_m}}{\partial N_{eff, \lambda_l}^{s_m} / \partial n_{s_m} \cdot \Delta n_{s_m}} = \frac{s_{k,m}}{s_{l,m}}, \quad (4.4)$$

where we neglect the dispersion in Δn_{s_m} . We can express the elements of matrix $\overline{\overline{S_{sub}}}$ in terms of the ratios $R_{\lambda_k/\lambda_l}^{s_m}$. Due to the fact that $R_{\lambda_k/\lambda_l}^{s_m} = 1/R_{\lambda_l/\lambda_k}^{s_m}$ and $R_{\lambda_k/\lambda_l}^{s_m} = R_{\lambda_k/\lambda_j}^{s_m} / R_{\lambda_l/\lambda_j}^{s_m}$ we can rewrite equation (4.3) as:

$$\overline{\Delta n_{eff}} = \overline{\overline{R}} \cdot \overline{\overline{\Sigma}} \cdot \overline{\Delta n_s}, \quad (4.5)$$

where $\overline{\overline{\Sigma}}$ is a matrix with theoretical sensitivity coefficients of which the elements are given by $\sigma_{k,m} = \partial N_{eff, \lambda_k} / \partial n_{s_m} \forall k=m$ and $\sigma_{k,m} = 0 \forall k \neq m$, and the coefficients of matrix $\overline{\overline{R}}$ are given by $r_{k,m} = R_{\lambda_k/\lambda_m}^{s_m}$. To determine $\overline{\Delta n_s}$ equation (4.5) can be rewritten as:

$$\overline{\Delta n_s} = \overline{\overline{\Sigma}}^{-1} \cdot \overline{\overline{R}}^{-1} \cdot \overline{\Delta n_{eff}}.^2 \quad (4.6)$$

The experimental procedure is now as follows. First a characterization step is required to determine matrix $\overline{\overline{R}}^{-1}$ by measuring the response of the sensor for each individual substance. The matrix $\overline{\overline{\Sigma}}^{-1}$ can be regarded as a scaling matrix that allows to get absolute values of $\overline{\Delta n_s}$. When the coefficients of this matrix are unknown, $\sigma_{k,m} \forall k=m$ can be set as 1 such that $\overline{\overline{\Sigma}}^{-1}$ becomes the identity matrix, which means that $\overline{\Delta n_s}$ is fully determined by the measured ratios $R_{\lambda_k/\lambda_m}^{s_m}$. Without the scaling it is possible to compare the unscaled Δn_{s_m} with Δn_{s_m} of a different measurement. However, the amplitude of Δn_{s_m} should not be compared with Δn_{s_n} as scaling is required. Now that we know the matrices of equation (4.6), we can use this equation for analysis of a real experiment in which we add multiple substances simultaneously to the sensor to find the contribution of each substance independently.

The absolute value of $\overline{\Delta n_s}$ can be determined by combining the ratio-based approach with the theoretical model used for the theoretical approach (see section 4.4). In this way $\overline{\overline{\Sigma}}$ can be determined. However, this requires again tuning of a lot of

² This equation is used for Fig 5.2. For the analysis of the other data based on the ratio-based approach we used a slightly different shape of matrix $\overline{\overline{R}}^{-1}$ (see Appendix 4.A) which yields slightly different results in terms of scaling of Δn_s , which makes comparison of the absolute value of Δn_s from experiment to experiment not possible. The shape of Δn_s over time is the same.

parameters. Therefore, it might be easier to determine scaling parameters for $\overline{\Delta n_s}$ using calibration experiments, which are usually also carried out by a single wavelength. Alternatively, this method can also be used as a quick screener, providing only a yes or no answer on the presence of the analyte and which therefore does not require knowing the absolute values of $\overline{\Delta n_s}$.

An advantage of the ratio-based method is that direct discrimination between different substances is possible, also when Δn_s 's occur in same layer in the evanescent field, as long as the ratios in wavelengths are different from each other and as long as the substances do not influence each other's ratio. The analysis of two substances which induce a Δn_s is relatively easy as only two ratios are determinable parameters which determine the shape of the signal of $\overline{\Delta n_s}$ over time. For three substances inducing a Δn_s , already six ratios need to be determined, illustrating the rapidly increasing complexity when increasing the number of distinguishable substances inducing a Δn_s . However, these ratios can be determined independently for each substance to discriminate these substances from each other. This was done for three different wavelengths ($\lambda_1 = 457$ nm, $\lambda_2 = 561$ nm and $\lambda_3 = 660$ nm), and three different substances: 85 nm carboxylated polystyrene beads, protein A and D-glucose as shown in section 4.3.2.

4.3.2 Effective refractive index ratios measured at various wavelengths

The ratios $R_{\lambda_k/\lambda_m}^{S_m}$ are experimentally determined for three different wavelengths ($\lambda_1 = 457$ nm, $\lambda_2 = 561$ nm and $\lambda_3 = 660$ nm), and for three different substances: 85 nm carboxylated polystyrene beads, protein A and D-glucose, which are substances measured in Chapter 5, representing specific binding, non-specific binding and bulk changes respectively. Fig. 4.1 shows an example of a measurement with D-glucose and 85 nm beads measured at $\lambda_1 = 457$ nm and $\lambda_3 = 660$ nm, illustrating the difference in $R_{660/457}$ for both substances. A single value for the ratio for a given substance is determined by calculating the mean value of the ratios over the time interval in which the ratios have a relatively stable value for that substance. In practice it was found that the time intervals that show a stable ratio correspond to intervals in which N_{eff, λ_1} was between 80 - 100 % of its maximum value for D-glucose, 20 - 100 % for 85 nm beads and 10 - 100 % for protein A. Therefore these intervals were applied for determination of all ratios. The determined ratios are presented in Table 4.1. The

error margins of the ratios are determined by twice the standard deviation (95% confidence interval) of the measured ratios from different experiments.

More research is required to determine what the origin of the spread of the ratios is. In order to do this, it should be investigated if the spread can be explained by the artefacts or other noise sources of the measurements (see Chapter 3), the cleaning of the surfaces of the chip (larger spread is seen in binding of substances compared to bulk changes due to D-glucose) or the substances self (spread in protein A is smaller compared to beads). For each substance which should be distinguished from other substances, the ratios should be measured.

Fig. 4.1: The ΔN_{eff} measured at 457 nm (blue line) and at 660 nm (red line) when adding 6.16 mg/ml D-glucose and 1.0 $\mu\text{g/ml}$ 85 nm carboxylated polystyrene beads to the sensor plotted on the left axis and the corresponding ratio $R_{660/457}$ (orange line) plotted on the right axis.

Table 4.1: Experimentally determined ΔN_{eff} ratios at $\lambda_1 = 457$ nm, $\lambda_2 = 561$ nm and $\lambda_3 = 660$ nm determined for D-glucose, 85 nm carboxylated polystyrene beads and protein A.

	$R_{660/457}$	$R_{561/457}$	$R_{660/561}$
D-glucose	1.218 ± 0.016	1.160 ± 0.012	1.050 ± 0.008
85 nm beads	0.918 ± 0.045	0.998 ± 0.037	0.920 ± 0.040
Protein A	0.680 ± 0.022	0.830 ± 0.022	0.820 ± 0.023

4.4 Combined analysis approach

To be able to determine the absolute values of $\overline{\Delta n_s}$ or a corresponding surface mass coverages C the theoretical approach and ratio-based approach were combined. For this combined approach, the measured ratios of ΔN_{eff} at multiple wavelengths can be

fitted to the theoretical ratios of ΔN_{eff} at multiple wavelengths by changing the waveguide parameters. Assuming that D-glucose causes a homogeneous Δn_s in the evanescent field of the sensor, the measured ratios can be fitted to the expected ratios based on the theoretical model by changing waveguide parameters (e.g. n_{core} , n_{sub} , d_{core}) such that the expected and measured ratios correspond. Next, using the waveguide parameters determined from the D-glucose ratios, the ratios of the substances which do not induce a homogeneous Δn_s can be fitted by varying the layer thickness d_1 , such that we can say that the Δn_s occurred in that layer. Consequently, the determined values for waveguide parameters and d_1 can be inserted in the theoretical model and combined with the measured ΔN_{eff} 's to determine the absolute values of Δn_s .

The ratios can be fitted by changing the n_{core} and n_{sub} , resulting in an optimal fit for $\Delta n_{core} = -0.068$ and $\Delta n_{sub} = +0.04$ with respect to the specifications of RI of the materials. Although the RI of the materials can be slightly different due to contaminations, these differences in RI of these materials (SiO_2 and Si_3N_4) are not realistic. The theoretical ratios were also fitted with the measured ratios by changing n_{sol} . However, n_{sol} could not be changed such that the measured and theoretical values correspond. Alternatively, it is possible to fit the theoretical values with the measured ratios by changing the wavelength of the light sources, but the wavelengths had to be changed in the order of tens of nanometres to give a fit within the standard deviations of the measured ratios. This is very unrealistic, because the lasers have a specified wavelength within 0.3 nm [1, 2]. The ratios can also be fitted by changing the d_{core} between 62.4 nm and 63.4 nm remaining within the standard deviation of the measured ratios for D-glucose. The optimal fit was found for $d_{core} = 62.9$ nm, based on $\chi = \left| R_{660/457}^{theory} - \text{mean}(R_{660/457}^{meas}) \right| + \left| R_{561/457}^{theory} - \text{mean}(R_{561/457}^{meas}) \right| = \min$. These values are from a fabrication perspective not very realistic, because the specified thickness of the d_{core} of the used waveguide is 70 ± 1 nm. Instead of fitting only one parameter, it is also possible to fit the ratios by tuning multiple parameters, which is plausible because it is very likely that the waveguide behaviour is an effect of the sum of multiple errors in specified waveguide parameters. However, even then it is not guaranteed that the exact values are found and that this results in a better fit. Therefore, we base our results on the best fit with $d_{core} = 62.9$ nm (the fit of n_{core} and n_{sub} resulted both in a higher χ compared to the fit with d_{core}) and assume that our waveguide behaves like a waveguide with an effective $d_{core} = 62.9$ nm.

Fig. 4.2: Measured ratios $R_{\lambda_k/\lambda_m}^{S_m}$ (dots, diamonds and triangles) for protein A, 85 nm carboxylated polystyrene beads and D-glucose for all combinations of the wavelengths 457 nm, 561 nm and 660 nm. Also indicated is the average of the measured ratios and the corresponding error bars representing the 95% confidence interval. The pink squares represent the theoretically determined ratios for a homogeneous Δn based on $d_{core} = 70$ nm, and all layers starting at the surface with a thickness of $d_{glucose} = \text{infinity}$, $d_{protein} = 2$ nm and $d_{bead} = 85$ nm. The black lines are the theoretical ratios fitted to the measured ratio, by changing first the d_{core} to 62.9 to fit the ratios of the D-glucose and changing d_1 to 2.1 nm and 72.6 nm to fit the ratios of the protein A and the 85 nm beads respectively.

Next, the ratios, $R_{660/457}^{bead}$ and $R_{561/457}^{bead}$ can be fitted to the theoretical model by changing the thickness d of the layer which starts at 0 (surface) and ends at d and in which the Δn occurred, assuming a homogeneous Δn in this layer. This resulted in an optimal fit ($\chi = \min$) for $d_{bead} = 72.6$ nm. Fitting such that the theoretical ratios stayed within the error margins of the measured ratios resulted in a minimum and maximum d_{bead} of 57.5 nm and 88.4 nm respectively. In similar way fitting $R_{660/457}^{protein}$ and $R_{561/457}^{protein}$, resulted in optimal $d_{protein}$ of 2.1 nm and a maximum $d_{protein}$ of 7.3 nm. Those values are close to the exact values of 1 nm [3] to 2.5 nm [4] for the protein A and 85.0 ± 6.5 nm for the beads. Differences for the optimal fitted bead thickness might be explained by the fact that they do not give a homogeneous Δn , because they are spherical instead of the assumed cubical particles. Moreover, differences between the theoretical and experimental waveguide configuration and artefacts in the measurements can also lead to differences between the determined thickness and real thickness of the beads. More research is required to determine the correctness of the determined thicknesses. The measured ratios from Table 4.1, the theoretical ratios (based on specified waveguide parameters) and the fitted theoretical ratios based on $d_{core} = 62.9$ nm, $d_{bead} = 72.6$ nm and $d_{protein} = 2.1$ nm are shown in Fig. 4.2. The individual points plotted in the same figure represent the mean $R_{\lambda_k/\lambda_m}^{S_m}$ for each single measurement, all performed

with the same waveguide. The fitted thicknesses d_{bead} , d_{protein} and d_{core} can be inserted in the theoretical approach to determine scaling factors which can be used to determine the absolute value of Δn for these substances and a corresponding surface mass coverage of the beads and the proteins. Finally, we can say the average measured ratios correspond to the theoretical ratios when choosing $d_{\text{core}} = 62.9$ nm, $d_{\text{bead}} = 72.6$ nm and $d_{\text{protein}} = 2.1$ nm. Moreover, the measured ratios show a spread and as was stated before, more research is required to determine the origin of this spread. The spread is low enough such that ratios of the different substances do not overlap. Consequently, it is possible to discriminate between the different substances using these ratios. The more different the ratios, the less singular the analysis matrix, which means that the drift, the aforementioned artefacts and noise will be less enhanced.

4.5 Influence input parameters in analysis approaches on determined RI change

Fig. 4.3: Effective refractive index change over time at $\lambda_1 = 457$ nm and $\lambda_2 = 660$ nm for addition of 6.16 mg/ml D-glucose only and addition of 6.16 mg/ml D-glucose and 1.0 $\mu\text{g/ml}$ 85 nm beads simultaneously. After adding D-glucose and applying a washing step the ΔN_{eff} comes back to its baseline as expected as the D-glucose does hardly bind to the surface and will fully be replaced by the PBS buffer again.. After adding beads and D-glucose simultaneously and applying again a washing the step the ΔN_{eff} does not go back to zero because beads stay attached to the surface of the chip.

An extensive study on the application of the analysis approach on different measurements can be found in Chapter 5. Here, we only pick out one measurement which we will not discuss in detail but only use to illustrate the influence of the input parameters of the theoretical and ratio-based approaches on the end result of Δn . Fig.

4.3 shows the measurement where first D-glucose only and later on 85 nm carboxylated polystyrene beads plus D-glucose simultaneously were added to phosphate buffered saline (PBS) in the sensor. For this analysis it is only important to know the expected end result and the influence of the input parameters on this result, which will be discussed for the theoretical approach and ratio-based approach in sections 4.5.1 and 4.5.2 respectively. We focus here on the shape of Δn and not the absolute values of Δn .

4.5.1 Theoretical approach

For the theoretical approach we determine Δn_{i_1} and Δn_{i_2} which should increase equally when only D-glucose is added (between $t \approx 500$ s and $t \approx 1000$ s) independent of the chosen layer thickness d_i as D-glucose is expected to give a homogenous Δn in the whole evanescent field. When a washing step at $t \approx 1000$ s both Δn_{i_1} and Δn_{i_2} should go back to zero as all the D-glucose is replaced by PBS again. When beads are added the d_i is ideally chosen equally to the height of the beads such that the Δn due to the beads only occurs in layer 1 and therefore can be discriminated from the Δn due to the D-glucose. Consequently, when d_i is chosen right, Δn_{i_2} should go back to zero after the washing step is applied at $t \approx 2000$ s and the Δn_{i_1} should be equal to the Δn due to the beads.

The theoretical analysis approach was applied to the experiment of Fig. 4.3 to illustrate the influence of the parameters d_{core} and d_i on the end result of Δn . Here we use the following strategy. Varying the d_{core} results in a varying level of the both the value of Δn of layer 1 and layer 2 at the D-glucose step. Independent of the layer thickness d_i , the Δn at the addition of only D-glucose should be the same in both layers, which is realized by choosing $d_{core} = 63$ nm. Again, these values are from a fabrication perspective not very realistic, because the specified thickness of the d_{core} of the used waveguide is 70 ± 1 nm. However, other waveguide parameters were also tuned to fit the measurement data, but did not give better results. Alternatively, it is also possible to tune multiple waveguide parameters, but also then guaranteed that the exact values of the waveguide parameters are found and that this results in a better fit. Therefore, we characterize our chips by introducing a homogeneous Δn due to addition of D-glucose and fit this by changing the d_{core} of the waveguide.

Fig. 4.4: Determined Δn with the theoretical analysis approach varying a, b, c) the core thickness d_{core} and d, e, f) the thickness of the first layer d_1 , illustrating the influence of input parameters on the end result of Δn . Layer 1 (L1) starts at zero and ends at d_1 , whereas layer 2 (L2) starts from d_1 and ends at infinity. The black line is the Δn of layer 1 minus the Δn of layer 2 and represents the Δn due to the binding of the beads assuming a low surface coverage of the beads.

Next, the end level of both signals can be tuned by varying d_1 . The end level of the bulk should be zero, because there was no Δn for the bulk compared to the starting situation where the bulk was also PBS. In the case of linear drift, the end level will not be zero but in line with the linear drift. This was realized with a $d_1 = 65$ nm, assuming a $d_{core} = 63$ nm. Next, we investigate the results if we choose slightly different values for these two parameters. Fig. 4.4 shows some examples of determined signals of Δn using different values of d_{core} and d_1 . It shows that the $\overline{\Delta n_i}$ is influenced by the different input parameters. Changing the correctly fitted $d_{core} = 63$ nm with 1 nm and using the same d_1 results in a percentage-wise difference in $\Delta n_1 - \Delta n_2$ (black dotted line) of maximally 2%. With different fitted d_1 's this can increase. Therefore, a D-glucose characterization step was applied in each measurement to verify the response of each chip and to be able to determine its d_{core} .

4.5.2 Ratio-based approach

For the ratio-based approach the Δn_{bead} should not change when only D-glucose is added (between $t \approx 500$ s and $t \approx 1000$ s), Δn_{bead} should increase when the beads and D-glucose are added simultaneously at $t \approx 1500$ s and Δn_{bead} stabilise when a washing step is applied at $t \approx 2000$ s. The $\Delta n_{\text{glucose}}$ should increase when D-glucose was added at $t \approx 500$ s and $t \approx 1500$ s and come back to approximately its zero level when a washing step was applied at $t \approx 1000$ s and $t \approx 2000$ s.

Fig. 4.5: Determined Δn with the ratio-based analysis approach varying a) the ratio $R_{\lambda_k/\lambda_l}^{\text{glucose}}$ resulting in a change of the beads signal and b) the ratio $R_{\lambda_k/\lambda_l}^{\text{bead}}$ resulting in a change of the D-glucose signal.

In this case the ratio $R_{660/457}^{\text{bead}}$ was varied from 0.885 till 0.905 and the ratio $R_{660/457}^{\text{glucose}}$ was varied from 1.210 till 1.230. The results are shown in Fig. 4.5. The ratio $R_{660/457}^{\text{bead}}$ determines the signal of the D-glucose and the ratio $R_{660/457}^{\text{glucose}}$ determines the signal of the beads. In this case the best fit for the signal of the beads was found for $R_{660/457}^{\text{glucose}} = 1.220$, having a zero level for the beads when only D-glucose was added. To fit the end level of the D-glucose with the expected value, assuming a constant linear drift, the $R_{660/457}^{\text{bead}} = 0.895$. Again, different values result in different amplitudes of in this case the Δn_s . A difference in $R_{660/457}^{\text{glucose}}$ of 0.02 results in a Δn_{bead} of approximately 4%, whereas A difference of 0.02 in $R_{660/457}^{\text{bead}}$ resulted in an approximately 14% difference in $\Delta n_{\text{glucose}}$. Therefore, it is important to know how accurately the ratios can be determined. More research is required to determine what is causing the spread in the ratios as was stated before.

4.6 Influence of noise, drift and artefacts on determined RI change

Simulations were done to determine the influence of drift, noise and artefacts (due to boundary effects of shifting interference patterns, see Chapter 3) in a measured signal of ΔN_{eff} on the result of Δn . For the determination of Δn , the matrix of the ratio-based analysis approach is used together with $R_{660/457}^{binding} = 0.919$, $R_{660/457}^{bulk} = 1.217$. Fig. 4.6a and b show a signal of ΔN_{eff} and the corresponding Δn when no drift, noise and artefacts are added to the signal. Next, Poisson distributed noise with different standard deviations (see labels in Fig. 4.6) is added to the signal. Fig. 4.6c/d show confirms the results from Chapter 2 that noise in ΔN_{eff} is enhanced in the result Δn . Artificial fluctuations (similar as artefacts due to boundary effects of shifting interference pattern, see Chapter 3) were also added to the signal of ΔN_{eff} and also show up enhanced in the result Δn as they are not seen in ΔN_{eff} but are seen in Δn , especially in the signal for the binding as shown in Fig. 4.6e/f.

The influence of linear drift in ΔN_{eff} on Δn is shown in Fig. 4.7. Linear drift in the signal ΔN_{eff} is translated to a linear drift in the result of Δn . The sign of the linear drift of Δn is the determined by the sign and ratio of the drift of ΔN_{eff} at the different wavelengths. This can be explained from a theoretical perspective:

$$\begin{bmatrix} \Delta n_1 \\ \Delta n_2 \end{bmatrix} = M \begin{bmatrix} \Delta N_{eff,1} + c_1 \cdot t \\ \Delta N_{eff,2} + c_2 \cdot t \end{bmatrix} = M \begin{bmatrix} \Delta N_{eff,1} \\ \Delta N_{eff,2} \end{bmatrix} + M \begin{bmatrix} c_1 \\ c_2 \end{bmatrix} t, \quad (4.7)$$

where c_1 and c_2 are constants which determine the amplitude of the linear drift of ΔN_{eff} . One can see that this finally results in linear drift in the signal of $\overline{\Delta n}$ which is determined by the coefficients of the matrix M and constants c_1 and c_2 .

Fig. 4.6: a) Simulated signal of ΔN_{eff} over time comparable with a typical measurement but without any noise and b) the corresponding $\Delta n_{unscaled}$ over time determined with ratio-based approach, c) the simulated signal of ΔN_{eff} over time with noise of different standard deviations (see black outlined boxes for zoom in) and d) the corresponding $\Delta n_{unscaled}$ over time illustrating the enhancement of noise in the signal of Δn , e) the ΔN_{eff} over time with added artefacts (black line) and f) the corresponding $\Delta n_{unscaled}$ over time.

Fig. 4.7: a, c, e, g) ΔN_{eff} over time with various added linear drifts and b, d, f, h) the corresponding determined $\Delta n_{\text{unscaled}}$ for binding and bulk signal, determined with ratio-based approach, illustrating that linear drift in ΔN_{eff} results is enhanced linear drift in $\Delta n_{\text{unscaled}}$.

4.7 Discussion and conclusions

In this chapter we showed two analysis approaches which also could be combined into a third analysis approach. The theoretical approach is exact, but requires tuning of many parameters to determine the correct values for the sensitivity coefficients that are required to determine the correct value of Δn and surface mass coverage of an analyte. Hence, this method is exact but in practice difficult to implement because of the many parameters that have to be tuned. Therefore, we developed a much more practical ratio-based approach based on the ratios of the measured ΔN_{eff} 's measured at different wavelengths. These ratios can be determined independently for each substance to discriminate these substances from each other. This was done for three different wavelengths ($\lambda_1=457$ nm, $\lambda_2=561$ nm and $\lambda_3=660$ nm), and three different substances: 85 nm carboxylated polystyrene beads, protein A and D-glucose such that it can be used for the application of this approach in the next chapter. On the other hand, using this ratio-based approach it is not possible to determine an absolute value of Δn as scaling factors are unknown. However, to determine e.g. an analyte concentration it is not required to know these, because calibration measurements, which are usually also carried out by a single wavelength YI, can be used to calibrate the unscaled values of Δn with an analyte concentration. Additionally, this method can also be used as a quick screener, providing only a yes or no answer on the presence of the analyte. To determine the absolute value of Δn or a corresponding surface mass coverage, a combined theoretical and ratio-based approach was developed, which was also used to determine thickness of 85.0 ± 6.5 nm beads to be in between 57.4 nm and 88.4 nm and with an optimal fit of 72.6 nm. Protein A was determined to be smaller than 7.3 nm, with an optimal fit of 2.1 nm which is in relatively good agreement with the measured thicknesses of 1 nm [3] and 2.5 nm [4].

To apply the theoretical approach or combined approach a more elaborate study is required to characterize the waveguide structure as it was shown that the input parameters have a great influence on the outcome of Δn or thicknesses. Well defined beads (in size and RI) with different sizes can be used to study if there is a structural difference between the theoretical and experimentally determined sizes. Additionally, alternatives for D-glucose which are well defined in RI and give a homogeneous Δn , and do not bind the sensor surface, can be used to determine the waveguide parameters. These characterization experiments should also be used to test the robustness of the analysis methods.

Finally, the influence of noise, drift and artefacts in ΔN_{eff} on the result of Δn was tested. The simulations confirmed the calculations in Chapter 2 which showed that there is a trade-off between size-selectivity and sensitivity. Simulations showed that noise (e.g. shot noise) and artefacts (oscillations which occur due to boundary effects

of shifting interference pattern) in ΔN_{eff} are found back enhanced in the end result of Δn , which means that artefacts and noise should be as low as possible. Furthermore, it was shown that simulated linear drift in ΔN_{eff} results in enhanced linear drift in Δn . So assuming linear drift in ΔN_{eff} , it is possible to compensated for that in Δn . In this case we used the ratio-based approach to test the influence of noise, drift and artefacts in ΔN_{eff} on the end result of Δn . However, the influence will be similar for the theoretical approach as both approaches use a similar methods (both use a matrix multiplication) to determine Δn from ΔN_{eff} .

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Appendix 4.A Slightly different matrix for ratio-based approach

Here, we show the slightly different forms of the matrix $\overset{=-1}{R}$ which are used for the analysis of some experiments using the ratio-based analysis approach. The expression of equation (4.6) of the main text was found in the final stage of this project such that no time was left to apply the matrix of this equation to all the measurements. The $\overset{=-1}{R}$ which was used at an earlier stage in this project can also be inserted in equation (4.6) of the main text and will be given in this appendix. The slightly different matrices $\overset{=-1}{R}$ result in slightly different results in terms of scaling of Δn_s , which makes comparison of the absolute value of Δn_s from experiment to experiment are used not possible when different matrices are used. However, the shape of the signals of Δn_s are the same. The main text states for which figures $\overset{=-1}{R}$ of the main text is used. The analysis of other experiments analysed with the ratio-based approach are based on the matrices which are given in this appendix.

For the situation of two substances which should be discriminated based on ΔN_{eff} measured at two wavelengths, the slightly different matrix is given by:

$$\stackrel{=-1}{R} = \begin{bmatrix} 1 & \frac{-1}{R_{\lambda_2/\lambda_1}^{s_2}} \\ 1 & \frac{-1}{R_{\lambda_2/\lambda_1}^{s_1}} \end{bmatrix}. \quad (4.A.1)$$

For the situation of three substances which should be discriminated based on ΔN_{eff} measured at three wavelengths, the slightly different matrix is given by:

$$\stackrel{=-1}{R} = \begin{bmatrix} 1 & \frac{-1}{R_{\lambda_2/\lambda_1}^{s_2}} \left(\frac{(R_{\lambda_2/\lambda_1}^{s_3} - R_{\lambda_2/\lambda_1}^{s_2}) R_{\lambda_3/\lambda_1}^{s_2}}{(R_{\lambda_3/\lambda_1}^{s_3} \cdot R_{\lambda_2/\lambda_1}^{s_2} - R_{\lambda_2/\lambda_1}^{s_3} \cdot R_{\lambda_3/\lambda_1}^{s_2})} + 1 \right) & \frac{1(R_{\lambda_2/\lambda_1}^{s_3} - R_{\lambda_2/\lambda_1}^{s_2})}{(R_{\lambda_3/\lambda_1}^{s_3} \cdot R_{\lambda_2/\lambda_1}^{s_2} - R_{\lambda_2/\lambda_1}^{s_3} \cdot R_{\lambda_3/\lambda_1}^{s_2})} \\ 1 & \frac{-1}{R_{\lambda_2/\lambda_1}^{s_1}} \left(\frac{(R_{\lambda_2/\lambda_1}^{s_3} - R_{\lambda_2/\lambda_1}^{s_1}) R_{\lambda_3/\lambda_1}^{s_1}}{(R_{\lambda_3/\lambda_1}^{s_3} \cdot R_{\lambda_2/\lambda_1}^{s_1} - R_{\lambda_2/\lambda_1}^{s_3} \cdot R_{\lambda_3/\lambda_1}^{s_1})} + 1 \right) & \frac{1(R_{\lambda_2/\lambda_1}^{s_3} - R_{\lambda_2/\lambda_1}^{s_1})}{(R_{\lambda_3/\lambda_1}^{s_3} \cdot R_{\lambda_2/\lambda_1}^{s_1} - R_{\lambda_2/\lambda_1}^{s_3} \cdot R_{\lambda_3/\lambda_1}^{s_1})} \\ 1 & \frac{-1}{R_{\lambda_2/\lambda_1}^{s_1}} \left(\frac{(R_{\lambda_2/\lambda_1}^{s_2} - R_{\lambda_2/\lambda_1}^{s_1}) R_{\lambda_3/\lambda_1}^{p_1}}{(R_{\lambda_3/\lambda_1}^{s_2} \cdot R_{\lambda_2/\lambda_1}^{s_1} - R_{\lambda_2/\lambda_1}^{s_3} \cdot R_{\lambda_3/\lambda_1}^{s_2})} + 1 \right) & \frac{1(R_{\lambda_2/\lambda_1}^{s_2} - R_{\lambda_2/\lambda_1}^{s_1})}{(R_{\lambda_3/\lambda_1}^{s_2} \cdot R_{\lambda_2/\lambda_1}^{s_1} - R_{\lambda_2/\lambda_1}^{s_3} \cdot R_{\lambda_3/\lambda_1}^{s_2})} \end{bmatrix}. \quad (4.A.2)$$

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Chapter 5

Experimental application of analysis approaches for size-selective analyte detection

Abstract

In this chapter we present the experimental application of the size-selective analyte approaches presented in Chapter 4. We use the different analysis approaches to discriminate between binding of 85 nm beads from binding of protein A, and binding of 85 nm beads or protein A from D-glucose bulk changes by measuring ΔN_{eff} at two wavelengths. Three wavelengths were used to discriminate between three substances simultaneously, however this led to noisy results which leaves the applicability useless at this moment. For a successful application of these analysis approaches used to determine three independent Δn 's, the artefacts (due to boundary effects of the shifting interference pattern or lens or grating aberrations in the imaging path of the setup) and drift in ΔN_{eff} should be reduced significantly. Alternatively, it was shown that other techniques to improve specificity, such as the use of a reference channel to compensate for bulk changes, can be used next to size-selective detection. The working of the ratio-based approach to discriminate between two substances was verified by a blind experiment with samples containing different concentrations of protein A and 85 nm beads. Furthermore, a triplicate of measurements series with different bead concentrations and a constant protein A concentration showed that we could discriminate refractive index changes caused by binding of protein A and 85 nm beads, even when the Δn of the protein was approximately 20 times higher. This can for example be used to discriminate specific analyte binding of larger particles from non-specific binding of smaller particles to improve the performance of the YI sensor and IO interferometric sensors in general.

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5.1 Introduction

In this chapter we first test the reliability of the sensor in section 5.2 (tested with setup with end-fire coupling, see Chapter 3). Next, we apply the different analysis approaches to measurements with 85 nm carboxylated polystyrene beads which bind to the sensor surface (representing specific binding of e.g. viruses which have approximately this size), protein A which also binds to the sensor surface but is smaller (representing non-specific binding) and D-glucose which hardly binds to the sensor surface but induces a bulk change. These measurements were done with the fiber butt-end coupling as presented in Chapter 3. These substances induce Δn 's at different places within the evanescent field and can therefore be discriminated from each other. All the measurements are done using a 1x phosphate buffered saline (PBS) buffer to which the different samples were added. The measurements starts with a baseline of approximately 400 s to determine the drift of ΔN_{eff} signal. This can be used to compensate for drift, as linear drift in ΔN_{eff} results in linear drift in Δn (see Chapter 4). All the graphs of ΔN_{eff} and Δn over time which are shown in this thesis are raw data (ΔN_{eff}) or directly determined from the raw data (Δn) with the analysis approaches, so are not corrected for drift. Only the determined values of ΔN_{eff} and Δn at one time point in the graph are corrected for linear drift by fitting a linear curve to the baseline of 400 s which is shown in the graphs and subtracting the value at the time point of interest based on the linear drift from the value of the raw data at that same time point. To minimize the effect on linear drift and artefacts (due to boundary effects of moving interference pattern), the applied concentrations are relatively high compared to these drift and artefacts. Next, the measurements continue with a characterization step, containing an addition of 6.16 mg/ml D-glucose for 500 s to the PBS, which can be used to see if the sensor responds as expected or to fit a theoretical model by changing waveguide parameters such that the determined curves of Δn fulfil the expectations (see Chapter 4). In section 5.3 we discriminate between two different substances using the theoretical and ratio-based approach, after which we discriminate between three substances using the same approaches in section 5.4. In section 5.5 we show that other techniques to improve specificity of the sensor can be used next to the size-selective detection using multiple wavelengths. Next, in section 5.6 we verify the ratio-based approach by adding unknown concentrations of 85 nm beads and protein A to the sensor in a blind experiment and discriminating them from each other. Subsequently, in section 5.7 the combined approach is applied to discriminate between 85 nm beads and protein A and determine its absolute values of Δn and the corresponding surface mass coverage. Finally, we end this chapter with a discussion and conclusions.

5.2 Reliability of sensor

To determine the reliability of the sensor with the simultaneous detection of zero order TE modes of multiple wavelengths, nine concentration series with D-glucose were measured. For the concentration series D-glucose solutions of 6.16 mg/ml, 12.32 mg/ml and 18.48 mg/ml concentration were successively added to *Milli-Q* water. This was done in three different channels and the experiment was repeated three times using a different chip each time. A typical example of one measurement in one channel is shown in Fig. 5.1a.

Fig. 5.1: a) A measurement of ΔN_{eff} over time for a concentration series of D-glucose measured at spatial frequency d14 in measurement M3, b) the corresponding ΔN_{eff} for all the three measurements compared with the theoretical ΔN_{eff} . For visibility reasons, the data points of the three different measurements are plotted slightly offset, but they all correspond to the glucose concentrations of 6.16, 12.32 or 18.48 mg/ml.

Next, the ΔN_{eff} due to D-glucose is determined by the difference between ΔN_{eff} when D-glucose was added to the chip and the value of ΔN_{eff} which it would have based on linear drift when no D-glucose would have been added. Subsequently, the ΔN_{eff} is plotted in Fig. 5.1b as a function of the glucose weight percentage. Furthermore, the theoretical ΔN_{eff} [1] is plotted as a function of the weight percentage in Fig. 5.1b. For visibility reasons, the data points belonging to the three different measurements (M1, M2, M3) done on different chips are slightly offset, but they all correspond to the glucose concentrations of 6.16, 12.32 or 18.48 mg/ml. Equal symbols belong to measurements done on the same chip, but measured at different

channels. The measured ΔN_{eff} corresponds relatively well to the theoretical values for ΔN_{eff} . However, there is a spread in the measured values for ΔN_{eff} which can be caused by slightly different core thicknesses or sensing window lengths of the different chips. Chips were fabricated from the same wafer. The spread between channels is smaller than the spread between chips. Furthermore, small differences in ΔN_{eff} may be attributed to artefacts due to boundary effects of the shifting interference pattern (see Chapter 3).

It can be concluded that these measurements show that the sensor in combination with the multiple wavelength works as expected, but that comparative measurements should preferably be done with a single waveguide and preferably in the same channel, because of the different response of ΔN_{eff} in each different channel and waveguide. Alternatively, measurements can be done on different chips or channels by applying a characterization step where D-glucose only is added to the sensor before measuring the real sample (see Chapter 4).

5.3 Discrimination between two different-sized substances

In this section we present measurements in which we discriminate between two of the three named different-sized substances. First, 6.16 mg/ml D-glucose and 2.0 $\mu\text{g/ml}$ protein A were added simultaneously to the sensor (see Fig. 5.2a) and ΔN_{eff} is measured at $\lambda_1 = 457 \text{ nm}$ and $\lambda_2 = 660 \text{ nm}$. As a characterization step 6.16 mg/ml D-glucose only was added to the sensor, which results in an increase of the ΔN_{eff} as D-glucose has a higher n than PBS. As expected, the ΔN_{eff} is the highest at λ_2 as it is more sensitive to bulk changes (D-glucose does hardly bind to the surface) compared to λ_1 . After applying a washing step, the ΔN_{eff} comes back to zero as the D-glucose bulk solution is completely replaced by PBS. After simultaneously adding D-glucose and protein A the ΔN_{eff} increases again differently at λ_1 compared to λ_2 . For both ΔN_{eff} 's the increase consist of a steep slope due to the bulk effect and a less steep slope due to the binding of the protein A to the surface. After again applying a washing step the signal drops due to the fact that the D-glucose and protein A in the bulk are replaced by PBS. The ΔN_{eff} does not go back to zero as protein A is left at the surface. In this case the ΔN_{eff} at λ_1 is higher than at λ_2 which is expected since

Fig. 5.2: The ΔN_{eff} measured over time by adding to the sensor surface: a) 85 nm carboxylated polystyrene beads and D-glucose, d) protein A and D-glucose and g) protein A and 85 nm carboxylated polystyrene beads and corresponding determined Δn using the theoretical analysis approach (b, e, h) and the ratio-based analysis approach (c, f, i).

shorter wavelengths are more confined to the core of the waveguide and therefore more sensitive to changes close to the surface compared to longer wavelengths. Fig. 5.2d shows a similar experiment where after addition of only 6.16 mg/ml D-glucose, 6.16 mg/ml D-glucose was added simultaneously with 1.0 μ g/ml 85 nm beads. Fig 5.2g shows a similar measurement where after addition of only 6.16 mg/ml D-glucose, 1.0 μ g/ml protein A was added simultaneously with 2.0 μ g/ml 85 nm beads. In the next subsections, the Δn 's of these different measurements determined with the

theoretical and ratio-based approach. The signal determined from the ratio-based approach is called $\Delta n_{unscaled}$ as this signal is related to a Δn induced by the aforementioned substances. However, information the absolute value is lost using the ratio-based approach as ratio of a substance is constant independent of its concentration (assuming low concentrations and therefore no aggregation of protein and beads on top of each other). Therefore, only the shape of unscaled RI change ($\Delta n_{unscaled}$) and not the absolute values of $\Delta n_{unscaled}$'s should be compared with the Δn 's of the theoretical model.

Protein A and D-glucose

Fig. 5.2b shows the Δn corresponding to Fig. 5.2a determined with the theoretical approach. Assuming a homogeneous Δn in the whole evanescent field for the D-glucose characterization step, the determined Δn_{l_1} should be the same as Δn_{l_2} , independent of the arbitrarily chosen thickness of the layers. The d_{core} , which determines the theoretical sensitivity coefficients and therefore Δn , can be adjusted such that both Δn 's of both layers for the glucose step reach an equal level independent of the selected layers (see Chapter 4). The best fit of Δn was achieved by a d_{core} of 61.8 nm. The Δn for the first D-glucose step based on this d_{core} of 61.8 nm is equal to $8.78 \cdot 10^{-4}$ RIU which agrees well with the $8.88 \cdot 10^{-4}$ RIU determined from the ΔN_{eff} measured at 660 nm assuming the same d_{core} .

Next to the d_{core} , also the layer thickness of the first layer (d_1), of which one can determine a Δn , can be adjusted. Changing d_1 results in a varying end level of Δn_{l_1} and Δn_{l_2} as layer 1 is defined from 0 - d_1 and layer 2 from d_1 - infinity. In the case of simultaneous addition of D-glucose and protein A, the expected end level of Δn_{l_2} after the washing step back to PBS is zero. The layer thickness that provides the expected bulk levels for the experiment with the protein A and D-glucose is 3.5 nm, which is reasonably close to the determined height of an absorbed layer of protein A of approximately 1 nm [2] - 2.5 nm [3]. Assuming low concentrations and bulk changes occurring in both layers, the Δn_{l_2} was subtracted from Δn_{l_1} resulting in a signal which should belong to only the protein A (see black dotted line in Fig. 5.2b). The protein A signal at the end is approximately equal to a seventeenth of a full coverage of protein A.

The determined Δn corresponding to Fig. 5.2a and based on the ratio-based approach is shown in Fig. 5.2c. The ratios $R_{660/457}^{protein}$ and $R_{660/457}^{glucose}$ that were used for this ratio-based approach are taken from Table 4.1. Amplitudes of Δn_{s_m} can be compared with Δn_{s_m} of other measurements, however Δn_{s_m} should not be compared with

amplitudes of Δn_{s_n} as scaling is required (see Chapter 4). The $\Delta n_{\text{glucose}}$ fulfils the expectations as the signal goes up when D-glucose was added and comes down to the baseline when applying a washing step. The $\Delta n_{\text{protein}}$ goes up when protein A was added and slowly decreases after applying a washing step, which is probably caused by partly desorption of the protein A from the sensor surface. However, $\Delta n_{\text{protein}}$ goes also slightly up when D-glucose was added. With an $R_{660/457}^{\text{glucose}}$ of 1.220 the $\Delta n_{\text{protein}}$ goes back to the expected level based on linear drift of $\Delta n_{\text{protein}}$. This $R_{660/457}^{\text{glucose}}$ of 1.220 remains within the error margins given in Table 4.1 which are a result of the differences in the measured ratios of the same single substances measured at different experiments.

Beads and D-glucose

In a similar way as for the protein A and D-glucose, Fig. 5.2d was analysed with the theoretical model as shown in Fig. 5.2e. The optimal result was realized based on a d_{core} of 63.4 nm and d_1 of 70 nm, which is lower than the expected 85 ± 6.5 nm. This might partly be explained by the fact that the beads are spherical and not cubical, as the response of the Δn_{eff} 's induced by a cubic shaped particle binding on the sensor surface is similar to the response of the Δn_{eff} 's induced by a larger spherical shaped bead binding on the same sensor surface. By subtracting the Δn_{i_2} from Δn_{i_1} and assuming low concentrations and bulk changes occurring in both layers it was again possible to determine a signal which should belong to only the beads.

Using the ratio-based approach, $\Delta n_{\text{glucose}}$ and Δn_{bead} were determined, again based on the measured ratios of Table 4.1. As expected, the Δn_{bead} does not increase when adding D-glucose, increases when adding the beads and remains constant after applying a washing step. The $\Delta n_{\text{glucose}}$ reaches a level which is approximately the same as in Fig. 5.2c as expected because the added D-glucose concentrations were the same. However, after the washing step the $\Delta n_{\text{glucose}}$ is a bit lower than expected. An optimal result is given at $R_{660/457}^{\text{bead}} = 0.900$ which again remains within measured error bars of $R_{660/457}^{\text{bead}}$.

Protein A and beads

For the measurement of simultaneous addition of beads and protein A the theoretical approach is not very suitable, especially because Δn_{i_2} is far from homogeneous as the evanescent field penetrates deeper than the thickness of the bead. Moreover, the expected end levels of the protein A and the beads are not known which was known for the bulk effect of the D-glucose. Consequently, the fitting of d_1 is not feasible.

Using the ratio-based approach it is possible to discriminate between the protein A and the beads. The result based on ratios of Table 4.1 is shown in Fig. 5.2i. As was mentioned, amplitudes between $\Delta n_{\text{protein}}$ and Δn_{beads} should not be compared, as scaling factors of substances are individual. Calibration measurements or the combined approach are required for that. However, $\Delta n_{\text{protein}}$ of Fig. 5.2i can be compared with $\Delta n_{\text{protein}}$ of Fig. 5.2c. In Fig. 5.2i half of protein A was added to the sensor, but the measured $\Delta n_{\text{protein}}$ is only 1.6 times lower. An even lower signal of $\Delta n_{\text{protein}}$ is expected as now also beads can cover part of the surface where beads can bind. Furthermore, the Δn_{beads} of Fig. 5.2f is approximately 2.6 times higher compared to Δn_{beads} of Fig. 5.2i, where the added beads concentration was two times lower. The differences might be explained that different chips and different stock solutions were used for the different measurements. Furthermore, all the measurements are based on the non-specific adhesion of the protein A and the beads to the sensor surface. Therefore, small differences between sensor surfaces can make a large difference. The result from D-glucose in which no binding is involved, the signals were in nice agreement with each other.

Besides, Fig. 5.2i shows that using two ratios, excluding $R_{660/457}^{\text{glucose}}$, it is not possible to suppress the first D-glucose step from the protein and beads signal. Three wavelengths are required to discriminate between protein A, 85 nm beads and D-glucose in a single measurement, as presented in the next section.

5.4 Discrimination between three different-sized substances

To test if both analysis approaches are also applicable when using three wavelengths to discriminate three Δn 's induced by three different substances, D-glucose (6.16 mg/ml), 85 nm carboxylated polystyrene beads and protein A were added to the sensor after each other. The ΔN_{eff} was measured at $\lambda_1 = 457$ nm, $\lambda_2 = 561$ nm and $\lambda_3 = 660$ nm as shown in Fig. 5.3a. Fig. 5.3b shows the Δn corresponding to Fig. 5.3a determined with the theoretical approach after fitting the d_{core} , d_1 and d_2 based on analysing the results based on two wavelengths. An example of this is shown in Fig. 5.3c where d_{core} and d_2 were determined as 62.7 nm and 64 nm respectively, based on λ_1 and λ_3 . It can be seen that at the D-glucose step the Δn_{i_1} and Δn_{i_2} are equal and when adding the beads at t_3 there is an increase of Δn_{i_1} and Δn_{i_2} stays approximately constant. Similarly, the fitting was done with λ_1 and λ_2 , and for the

combination of D-glucose and protein A and the combination of protein A and beads, based on the wavelength combination λ_1 with λ_2 and λ_1 with λ_3 . This resulted in a $d_{core} = 62.55 \pm 0.15$ nm, $d_1 = 5 \pm 2$ nm and $d_2 = 60 \pm 4$ nm, illustrating that different wavelength combinations give different fit values, meaning that the parameters in the theoretical model are probably not completely right. The result in Fig. 3b is based on the average values for d_{core} , d_1 and d_2 and three wavelengths to discriminate between the Δn of three layers. The overall signals determined with the theoretical approach based on three wavelengths/layers are much more noisy compared to the two wavelengths/layers case, which makes the optimization more difficult. The increase in noise, artefacts and drift can be explained by the fact that the matrix to determine Δn_{i_1} from $\overline{\Delta N_{eff}}$ becomes more singular and therefore all noise sources are enhanced which was already shown in Chapter 2. However, ignoring the noise, drift and artefacts, all signals increase roughly with the same amplitude when adding D-glucose at t_1 (Δn_{i_1} has a much higher amplitude, so is plotted on a different scale). When adding the beads, an increase in Δn_{i_1} and Δn_{i_2} is expected, but there is also an increase of Δn_{i_3} . Also when adding protein A at t_5 there is an unexpected decrease of Δn_{i_2} and unexpected increase of Δn_{i_3} as it was only expected that Δn_{i_1} would increase. These observations probably mean that the signals Δn_{i_1} , Δn_{i_2} and Δn_{i_3} are not sufficiently decoupled. Optimizing the theoretical matrix further is more complex compared to the two wavelengths/layers situation, because changing one parameter will result in change of all the signals Δn_{i_1} , Δn_{i_2} and Δn_{i_3} , illustrating the complexity and limits of the theoretical approach. Therefore, we ratio-based approach is better used for this analysis.

Fig. 5.3d shows the determined $\overline{\Delta n_s}$ with the ratio-based approach. The ratios used for the analysis are again based on the determined ratios for the individual substances. Between t_1 and t_2 only $\Delta n_{glucose}$ changes apart from some fluctuations seen for Δn_{bead} and $\Delta n_{protein}$, which can be explained by boundary effects of the shifting interference pattern (see Chapter 3). At t_3 the beads were added, but besides the increase of Δn_{bead} there is also an increase of $\Delta n_{protein}$ and $\Delta n_{glucose}$ which means that the signals of $\overline{\Delta n_s}$ are not perfectly decoupled. This is also the case at t_5 when protein A is added to the sensor which mainly result in an increase of $\Delta n_{protein}$, but also in a small increase of $\Delta n_{glucose}$. Therefore, the ratios were also optimized by analysing the measurement based on two wavelengths/substances. An example is shown in Fig. 5.3f

Fig. 5.3: a) The ΔN_{eff} measured over time starting with a PBS buffer, adding 6.16 mg/ml D-glucose at t_1 resulting in higher ΔN_{eff} for the longer wavelengths as expected. After applying a washing step at t_2 , 1.0 μ g/ml 85 nm beads was added at t_3 . The response of the shorter wavelengths is now larger. After washing again with PBS at t_4 , 2.0 μ g/ml protein A was added at t_5 resulting in a relatively stronger response of the shorter wavelengths. After applying a washing step at t_6 the signal decreases due to desorption of the protein A, b) the corresponding Δn_i determined with the theoretical approach fitting d_{core} , d_1 and d_2 based on analysing the measurement with two wavelengths (see example shown in c)), the Δn_s determined with ratio-based approach based on d) the ratios measured with the individual substances and e) after tuning the ratios based on analysing the measurement with two wavelengths and two substances of which an example is given in f).

which shows that $\Delta n_{\text{protein}}$ was sufficiently decoupled from $\Delta n_{\text{glucose}}$ based on λ_1 and λ_3 . Because only two wavelengths were used to discriminate between $\Delta n_{\text{protein}}$ and $\Delta n_{\text{glucose}}$ it was not possible to discriminate them from Δn_{bead} which is shown between t_3 and t_4 , where both $\Delta n_{\text{protein}}$ and $\Delta n_{\text{glucose}}$ increase. Fig 3e shows the result of $\overline{\Delta n_s}$ after optimization of the ratios which led to $R_{561/457}^{\text{protein}}=0.837$, $R_{660/457}^{\text{protein}}=0.698$, $R_{561/457}^{\text{bead}}=0.967$, $R_{660/457}^{\text{bead}}=0.885$, $R_{561/457}^{\text{glucose}}=1.158$ and $R_{660/457}^{\text{glucose}}=1.215$ from which $R_{660/561}^{S_m}$ can be calculated by dividing $R_{660/457}^{S_m}$ by $R_{561/457}^{S_m}$. All these values fall within the error margins of the experimentally determined ratios of the individual substances presented in Table 4.1. Using the slightly changed ratios, the three substances are nicely decoupled, illustrating that it is possible to discriminate between three different substances. However, the enhanced drift and artefacts in $\overline{\Delta n_s}$ makes it difficult to analyse these results. Moreover, when substances were added simultaneously, fitting based on two substances/two wavelengths is not possible. Therefore, the ratios used for the analysis should be measurable from measurements with the single substances and not be tuned afterwards. To realise this the spread in ratios should be reduced. More research is required to determine what the origin of the spread of the ratios is. In order to do this, it should be investigated if the spread can be explained by the artefacts or other noise sources of the measurements, the cleaning of the surfaces of the chip (larger spread is seen in binding of substances compared to bulk changes due to D-glucose) or the substances self (spread in protein A is smaller compared to beads).

The protein A, 85 nm beads and D-glucose were also added simultaneously to the sensor which is shown in Fig 5.4. In the first measurement protein A was added to the measurement channel and in the second measurement protein A was added to the reference channel such that there is no interference between binding of protein A and binding of beads. Fig. 5.4a, c shows the determined Δn_{eff} measured at 457, 561 and 660 nm. The D-glucose step was used to determine the $R_{660/457}^{\text{glucose}}$, because another chip was used for this measurement. Next, the measured ratios of the substances from Table 4.1 are used and corrected percentagewise based on the difference in ratios of D-glucose measured on this chip and in Table 4.1 (multiplied by $R_{660/457}^{\text{glucose, current chip}} / R_{660/457}^{\text{glucose, Table 4.1}}$).

Fig 5.4b, d show the Δn determined with ratio-based approach, based on the percentagewise corrected ratios. Scaling factors are chosen such that $\Delta n_{\text{protein}}$, Δn_{bead} and $\Delta n_{\text{glucose}}$ have similar amplitudes and can be seen clearly in the graph, but should not be compared. Again it is clear that the $\overline{\Delta n_s}$ determined for three different

Fig. 5.4: a) The ΔN_{eff} measured over time where 6.16 mg/ml D-glucose was added simultaneously with 1 μ g/ml 85 nm beads and 1 μ g/ml protein A to PBS, b) the ΔN_{eff} over time for a measurement where 6.16 mg/ml D-glucose was added simultaneously with 1 μ g/ml 85 nm beads to 1x PBS in the measurement channel and 1 μ g/ml protein A to 1x PBS in the reference channel and b, d) the corresponding Δn for protein A, beads and D-glucose determined with the joint theoretical and ratio-based approach. The dashed lines are fitted to the baselines of the signal assuming linear drift and neglecting the artefacts due boundary effects of the shifting interference pattern.

substances gives noisy signals. The aforementioned artefacts that show up in the data are in the order of the signal of the beads and protein A. Neglecting the artefacts, the dashed lines in the graphs of Δn are fitted to the assumed linear phase drift. Overall the signals of $\overline{\Delta n_s}$ correspond roughly to the expected signals, however the drift artefacts are in the same order of magnitude as the signal. Therefore, it is not possible to analyse Δn_{bead} and $\Delta n_{protein}$ properly. Thus, with the current setup and current drift

and artefacts it is not possible to discriminate between three Δn 's based on three wavelengths. Artefacts and drift in ΔN_{eff} should be reduced significantly for successful application of the size-selective detection when discriminating between three Δn 's based on three wavelengths. Sufficiently wide optical components behind the waveguide to image interference patterns on camera (e.g. lenses and grating) are required. Alternatively, other techniques to improve specificity can be used next to size-selective detection, such that it is only required to discriminate between two substances based on two wavelengths. For example, to discriminate the binding of beads from binding of protein A and bulk changes due to D-glucose, D-glucose can also be added to the reference channel to cancel out the contribution due to D-glucose as is shown in the section 5.5. In that case only two wavelengths are required to discriminate between binding of beads and proteins, resulting in less enhancement of noise.

5.5 Simultaneous use of other techniques next to size-selective detection

In this section we illustrate the possibility to use alternative techniques next to the size-selective detection in order to discriminate between e.g. two instead of three substances. For example non-specific binding can be reduced by using specific antibodies and blocking agents. Moreover, a bulk contribution can be cancelled out by applying the same bulk solution in measurement and reference channels. An example of simultaneous addition of a bulk contribution to a measurement channel (channel 2) and reference channel (channel 4) is shown in Fig. 5.5. Fig. 5.5a shows the ΔN_{eff} of 457, 561 and 660 nm over time belonging to spatial frequency corresponding to d_{12} (channel combination 1 - 2) when adding 2.0 $\mu\text{g/ml}$ 85 nm beads, 0.5 $\mu\text{g/ml}$ protein A and 6.16 mg/ml D-glucose simultaneously to channel 2. Fig. 5.5b shows the corresponding Δn contribution of the beads, protein and D-glucose using the ratio-based approach. The characterization step (not shown in Fig. 5.5) resulted in a fit for $d_{core} = 68$ nm. Consequently, the ratios used for the analysis were again the ratios measured in Chapter 4 (see Table 4.1) and percentagewise corrected based on the measured difference in ratio for the D-glucose step. Fig. 5.5b shows the resulting $\overline{\Delta n_s}$, which is a very noise signal, mainly caused by the aforementioned artefacts. Fig. 5.5c shows the ΔN_{eff} of 457, 561 and 660 nm over time corresponding to spatial frequency d_{24} (channel combination 2 - 4) when adding 2.0 $\mu\text{g/ml}$ 85 nm beads, 0.5 $\mu\text{g/ml}$ protein A and 6.16 mg/ml D-glucose simultaneously to channel 2 and simultaneously 6.16 mg/ml D-glucose to channel 4. This measurement was also analysed with the

Fig. 5.5: a) The measured ΔN_{eff} over time at 457, 561 and 660 nm of the spatial frequency corresponding to d₁₂ adding 2.0 $\mu\text{g/ml}$ 85 nm beads, 0.5 $\mu\text{g/ml}$ protein A and 6.16 mg/ml D-glucose simultaneously to channel 2, b) the corresponding Δn 's of protein A, beads and D-glucose determined with the joint theoretical and ratio based approach, c) the ΔN_{eff} over time at 457, 561 and 660 nm of the spatial frequency corresponding to d₂₄ adding 2.0 $\mu\text{g/ml}$ 85 nm beads, 0.5 $\mu\text{g/ml}$ protein A and 6.16 mg/ml D-glucose simultaneously to channel 2 and 6.16 mg/ml D-glucose simultaneously to channel 4, d) the corresponding Δn 's of protein A, beads and D-glucose determined with the ratio based approach based on 3 wavelengths and e) the corresponding Δn 's of protein A, beads determined with the ratio based approach based on 2 wavelengths.

ratio approach using the same ratios as for Fig. 5.5b and using three wavelengths to discriminate between three Δn 's (Fig. 5.5d) and using two wavelengths to discriminate between two Δn 's (Fig. 5.5e). Fig. 5.5d shows that the determined bulk contribution is approximately zero as expected because the D-glucose in reference and measurement channel cancel out each other. Fig. 5.5e shows that when only analysing the signal with two wavelengths to determine the bead and protein A contribution, the noise in $\overline{\Delta n_s}$ is reduced significantly. Moreover, it illustrates that other techniques used for improving specificity of the sensor can be used next to the size-selective detection approach.

5.6 Blind experiment with 85 nm beads and protein A

To approve the usability and verify the correctness of the ratio-based approach a blind experiment was carried out. Six combined samples of protein A (in the range of 0.1 - 1.0 $\mu\text{g/ml}$) and 85 nm beads (in the range of 0.4 - 4.0 $\mu\text{g/ml}$) were made and labelled by an independent person. Before measuring, the samples were carefully mixed to ensure homogeneous solutions. Next, the samples were measured in two measurement sets performed on the same chip using three measurement channels and one reference channel. In between the measurement sets the waveguides were cleaned using a cleaning protocol presented in Appendix 5.A. A D-glucose characterization step was applied to verify the correct response of the different wavelengths. This resulted in a different ratio the measured ΔN_{eff} 's of 1.263 compared to the 1.218 ± 0.016 . This difference can be explained by the fact that these measurements were performed with a different chip which can have for example a slightly different d_{core} . To correct for this deviation, we changed the average measured ratios of Table 4.1 percentagewise (multiplied by $1.263 / 1.218$) and used this for the ratio-based approach to analyse this experiment. Fig. 5.6a shows an example of a measurement with the D-glucose characterization step and adding sample A and Fib. 5.6b shows the corresponding determined Δn_{bead} and $\Delta n_{\text{protein}}$ over time, which are read out before at $t = 2400$ s when the beads signal was constant. The expected values of Δn based on linear drift (baseline determined between $t = 1320$ s and $t = 1380$ s) is subtracted from this $\Delta n_{t=2400 \text{ s}}$ and plotted as a function of the applied concentration in Fig. 5.6c and Fig. 5.6d for the 85 nm beads and the protein A respectively.

The applied bead concentration and the determined Δn_{bead} show a linear trend which means the Δn_{bead} was determined correctly using the ratio-based approach, assuming a linear relation between the number of beads in the sample and response of the sensor (see Appendix 5.B for verification). Moreover, it shows that it is possible

Fig. 5.6: Blind experiment with six samples (A-F) containing 85 nm beads and protein A showing a) the measured ΔN_{eff} over time of one measurement of the set of blind experiments b) the corresponding determined Δn_{bead} and $\Delta n_{protein}$ using the ratio-based approach and c) the determined Δn as a function of the by forehand unknown applied concentrations of the 85 nm beads and d) the determined Δn as a function of the by forehand unknown applied concentrations of the protein A. For visibility reasons, the data points A and F of the applied protein concentration are slightly offset but they both correspond to 0.9 $\mu\text{g/ml}$.

to do a calibration experiment to find the relations between the beads concentration and the response of the sensor. Furthermore, also a linear trend is seen between the measured $\Delta n_{protein}$ and the applied protein concentration. Only at higher applied protein A concentrations, the determined Δn_{bead} flattens off, which can be explained by the fact that for these measurements the surface was saturated with protein A which can be seen in Fig. 4b. That the signal of Δn_{bead} still increases might be explained by the beads replacing the protein A of the surface. The be able to compare

Fig. 5.7: Blind experiment with six samples (A-F) containing 85 nm beads and protein A, showing the determined surface mass concentrations as a function of the by forehand unknown applied concentrations of a) the 85 nm beads and b) the protein A.

Δn_{bead} and $\Delta n_{\text{protein}}$, the signals were both read out at the same time point. The amplitude of the signals of Δn_{bead} , $\Delta n_{\text{protein}}$ and the corresponding surface mass coverage can be compared by applying the combined approach. The absolute values Δn_{bead} and $\Delta n_{\text{protein}}$ were determined using the optimal fits of d_{core} , d_{bead} and d_{protein} found in Chapter 4. The corresponding C_{bead} was determined assuming cubical beads with an average determined height of 72.6 nm, a density of 1.05 g/cm³ [4] and $n_{\text{polystyrene}}(\lambda = 561 \text{ nm}) = 1.595$ [5], $n_{\text{PBS}}(\lambda = 589 \text{ nm}) = 1.334$ [6] and using equation 2.6 from Chapter 2 assuming that the RI change is given by Δn_{bead} instead of $\Delta n_2 - \Delta n_3$ as was determined for the layer case. The corresponding C_{protein} to $\Delta n_{\text{protein}}$ was determined assuming protein A with an effective size of 2.1 nm (= average determined height), a mass of 42 kDa [7] and $n_{\text{protein}} = 1.41$ [8]. The C_{protein} and C_{bead} are plotted in Fig. 5.7 which shows that it was possible to discriminate the binding of beads from the binding of proteins, even when the C_{protein} was approximately 20 times higher than C_{bead} (sample A).

5.7 Validation and reproducibility of size-selective detection

To validate the working of the size-selective detection method and to determine its reproducibility a triplicate of a measurement series where 85 nm beads and protein A were added simultaneously to the sensor was performed. Samples were made with a varying bead concentration (0, 0.75, 1.5 and 3 μg/ml) and a fixed protein A concentration of 0.5 μg/ml and added to the sensor. The combined approach was

applied to with determine how much beads we can discriminate from how much protein A in terms of absolute RI changes and corresponding surface coverages. Inserting the optimal fits of d_{core} , d_{bead} and $d_{protein}$ found in Chapter 4 in the theoretical model and using $R_{660/457}^{protein} = 0.680$ and $R_{660/457}^{beads} = 0.918$ resulted in a determined Δn_{bead} and $\Delta n_{protein}$ shown Fig. 5.8. Fig. 5.8a shows a typical example of the combined approach applied on a measurement where protein A and 85 nm beads were added simultaneously. The Δn_{bead} and $\Delta n_{protein}$ were determined at $t = 1050$ s when the signal of Δn_{bead} was stable. The signal of the protein A was not always stable at this time point. Sometimes the protein A was still increasing (see Fig. 5.8a) and sometimes, when no beads were added next to the protein A, already saturated, so the Δn_{bead} of the different measurements cannot exactly be compared. However, to be able to compare $C_{protein}$ and C_{bead} , the signal of Δn_{bead} and $\Delta n_{protein}$ were read out at the same time. Furthermore, both were corrected for a linear drift based on the first 400 s. The determined Δn_{bead} and $\Delta n_{protein}$ for all measurements of the triplicate are shown in Fig. 5.8b. Fig. 5.8b shows that each measurement series has a linear trend ($R^2 \geq 0.996$) which was expected as it was shown that a two times higher bead concentration lead to approximately twice as many beads bound to the surface (see Appendix B). This verifies that we can use size-selective detection to discriminate binding of 85 nm beads sufficiently from binding of protein A. The signal measured for the protein is decreasing with an increasing bead concentration. This might be explained by the fact that for higher bead concentrations more beads are covering the sensor surface, resulting is less space for the proteins to bind.

Despite the three differences show a linear trend, there is a difference (which is smaller than a factor two) between the triplicates. The measured difference between the triplicates might be explained by the fact that fewer beads and protein A bind to the surface of the chip if it was not cleaned sufficiently between runs. The same chip had to be reused, and thus cleaned (see Appendix 5.A for cleaning protocol) between experiments, to be able to determine a d_{core} and d_s which can be used for the analysis of all the experiments. As was mentioned before, different chips resulted in different ratios, possibly due to slightly different core thicknesses or RI's or as a result of artefacts in the measurements. To be able to use different chips for reproducibility studies artefacts should be reduced and if still differences are measured with different chips, improvement of the chips is required. Carboxylated fluorescents beads were used to verify that the beads were sufficiently removed after cleaning (see Appendix 5A). However, it is not trivial to check for any residual protein A as the surface of the sensing window is embedded in the waveguide and the used protein A is smaller than the diffraction limit and not fluorescent, and thus not easy to visualise. However, as the protein A is desorbing more compared to the beads when applying a washing step, it is expected that the protein A is removed sufficiently by this protocol. Nevertheless,

it should be noted that we use non-specific adhesion of particles which means that small surface changes can lead to different amounts of binding of protein A and the beads. Alternatively, it is possible that beads or protein A stick to the tube where they are stored before the measurement starts or to the tubes which bring the substances to the sensor surface. The latter argument can also result in a baseline which slightly increases due to the fact that analytes which were attached to the tube walls during previous experiments become loose and subsequently are transported to the sensing window. To test the influences of these factors, measurements should be repeated where substances are kept in tubes an equal amount of time before measurement and tubes should be replaced for each measurement. In these measurements this was not the case and only substances in tubes were mixed by pipetting the solution in and out of the tubes just before adding to the sensor. More measurements are required to determine the origin of the spread of the triplicates.

Fig. 5.8: Example of Δn determined with combined approach for a measurement where 85 nm beads and protein A where added simultaneously at the sensor and b) the determined Δn for three measurement series where protein A and 85 nm beads where added simultaneously with a varying bead concentration and a fixed protein concentration of 0.5 $\mu\text{g/ml}$.

To determine maximal distinguished difference in surface mass coverage C in this measurement (which is not equal to the maximal distinguishable C in general), we determine here the C (in a similar way as before) corresponding to highest ratio of $\Delta n_{\text{protein}} / \Delta n_{\text{beads}}$ and using formula 2.D.5 (see Appendix 2.D) , resulting in $C_{\text{beads}} = 3.3 \cdot 10^2 \text{ pg/mm}^2$ and $C_{\text{protein}} = 5.6 \text{ ng/mm}^2$. This means 85 nm beads are discriminated from protein A with an approximately 17 times higher C . We did not push it to the limit here, so in principle even a higher difference between surface mass coverage could be reached as was already shown in the blind experiment. The maximal resolvable difference in C which is different for each model system, so for each model system measurements are required to determine the maximal resolvable difference in C .

Furthermore, it was not possible to verify the number of beads and protein bound to the chip surface as they were non-fluorescent and so small they are not easily imaged.

5.8 Discussion and conclusions

By measuring the ΔN_{eff} at two different wavelengths it was possible to discriminate between two different substances inducing a Δn in the evanescent field of the sensor. The binding of 85 nm beads or protein A was discriminated from D-glucose and the binding of 85 nm beads was discriminated from the binding of protein A. The determined Δn induced by D-glucose corresponds to the Δn due to D-glucose as measured with a single wavelength whereas the Δn 's due to bound protein A and 85 nm beads to the surface could not be verified. The protein A and beads could not be imaged as they are smaller than the detection limit and non-fluorescent. However, it was not our goal to determine the exact amount of bound protein A or beads to the surface. Using three ΔN_{eff} 's to discriminate between three different substances inducing a Δn , the result became more noisy because the drift and artefacts in ΔN_{eff} (due to boundary effects of the shifting interference pattern) show up more enhanced when discriminating between three Δn 's based on ΔN_{eff} 's measured at three wavelengths compared to two Δn 's based on ΔN_{eff} 's measured at two wavelengths, as the analysis matrix which is used for this becomes more singular. For a successful application of these analysis approaches used to determine three independent Δn 's, the artefacts and drift in ΔN_{eff} should be reduced significantly. Alternatively, other techniques to improve specificity can be used next to size-selective detection. It was shown that when applying equal bulk changes due to D-glucose in both the measurement and reference channels it was possible to use only two wavelengths to discriminate between binding of beads and proteins, resulting in less enhancement of drift and artefacts compared to the three wavelength/three substances case.

Application of a theoretical analysis approach made it possible to discriminate between Δn 's occurring in different layers in the evanescent field, assuming a homogeneous Δn in each layer. However, to determine the correct value for Δn , this method requires multiple parameters (e.g. the waveguide core thickness, waveguide RIs, layer thicknesses, wavelengths) to be tuned that is difficult and labour-intensive. Therefore, also a much more pragmatic ratio-based analysis was applied to discriminate between 85 nm beads, D-glucose and protein. For the ratio-based approach it was possible to use measured ratios of individual measurements of the substances (see Chapter 4) to discriminate between the substances when using the same chip. Using a different chip it was possible to use the measured ratio of Chapter 4 when correcting them percentagewise based on the ratios in ΔN_{eff} measured when

D-glucose was added only. This was verified by a blind experiment with different samples containing different concentrations of protein A and 85 nm beads and a triplicate of measurements series with different bead concentrations and a constant protein A concentration. Information on the absolute amplitude of Δn_s is lost using the ratio-based approach, however for determining an analyte concentration calibration measurements can be performed which are usually also required using an YI with a single wavelength. Alternatively, the ratio-based approach can be combined with the theoretical approach. This was done for the blind experiment which showed that we could discriminate Δn_{bead} from $\Delta n_{\text{protein}}$, even when the protein RI change was approximately 20 times higher, resulting in an expected linear trend of the Δn_{bead} with the applied concentration. The blind experiment illustrates that the size-selective detection using the ratio-based approach or combined approach can be used to discriminate specific analyte binding of larger particles from non-specific binding of smaller particles.

Moreover, we checked the reproducibility of the size-selective detection using the combined approach with the triplicate of the measurement series with 85 nm beads and protein A. The triplicates showed differences in Δn_{bead} and $\Delta n_{\text{protein}}$ up to approximately a factor of two. This spread is not very high if we take into account that our signal is determined by non-specific adhesion of beads and protein A, which means that small changes on the surface can affect the amount of bound substances. Alternatively, it is possible that beads or protein A stick to the tube where they are kept in before the measurement starts or the tubes which bring the substances to the sensor surface. More measurements are required to determine the origin of the spread of the triplicates and to verify that it is not caused by size-selective detection.

In summary, we believe that with adding size-selectivity using the different presented approaches, we can strongly improve the performance of our YI sensor and IO interferometric sensors in general. Especially the ratio-based approach is an easy approach to distinguish between different substances. With the current setup it is possible to discriminate between maximally two substances causing a Δn , but when the artefacts in the measurements are reduced significantly it is also possible to discriminate between three substances and keep a sensitivity comparable to other biosensors.

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Appendix 5.A Cleaning protocol

The following steps describe the cleaning procedure of the waveguide chips after and before a measurement with beads, protein A and or D-glucose in PBS:

- 1) rinse chip with water
- 2) rinse chip with acetone and wash chip with acetone and cotton (4x)
- 3) rinse chip with isopropanol (99.8%, filtered with 0.22 μm filter) and wash chip with same isopropanol and cotton (4x)
- 4) rinse chip with isopropanol (99.8%, filtered with 0.22 μm filter)
- 5) blow dry with instrumental air or nitrogen gas (N_2)

Fluorescent carboxylated beads were added to the sensor surface and after the measurement and after the cleaning protocol imaged with a fluorescence microscope to verify that the cleaning protocol was sufficient for removing the beads (see Fig. 5.A.1). As the protein A was not fluorescent and is smaller than the diffraction limit, the protein A could not be imaged and the cleaning protocol was not verified for the protein A. However, as we see that beads bind stronger to the surface than protein A (for protein A see more desorption after applying a washing step compared to the beads), we expect that the protein A is sufficiently removed from the surface using this cleaning protocol.

Fig. 5.A.1: Images of sensing windows after measurements with fluorescent beads a) before and b) after cleaning. Both images are taken with a 10x Plan Apo objective and an Andor DU-885 camera at an exposure time of 2 seconds and a multiplier of 80.

Appendix 5.B Relation between applied bead concentration and measured surface mass coverage

To verify if there is a linear relation between the sample bead concentration and Δn and therefore also C , 60 nm carboxylated fluorescent yellow [9] polystyrene beads (SPHERO™ CFP-00552-2) were added to the sensor. After measuring the binding of the beads to the sensor surface (only at 660 nm as it was shown that the 457 nm light was absorbed by the beads) they were imaged with a fluorescence microscope. The

fluid cuvette was removed from the top of the chip which was turned upside down to be able to measure them in EPI mode. The yellow fluorescent beads were excited with a 457 nm laser line and imaged using a filter cube with 450-460 nm excitation and 470-500 nm emission.

The measured $\Delta N_{eff}(660\text{ nm})$ was calculated into a surface mass coverage. A layer of 60 nm changing from 1x PBS to a homogeneous polystyrene layer $n_{polystyrene}(\lambda = 660\text{ nm}) = 1.586$ results in a $\Delta N_{eff}(660\text{ nm})$. Assuming a tight packing of beads ($V_{bead} / (2 \cdot r_{bead})$), 8.49×10^6 beads can bind to the surface and a single bead results in a $\Delta N_{eff}(660\text{ nm}) = 4.42 \times 10^{-9}$. Assuming a loose packing (surface area bead = $4r_{bead}^2$), 4.45×10^6 can bind to the surface, resulting $\Delta N_{eff}(660\text{ nm}) = 2.31 \times 10^{-9}$ for a single bead. The C can now be determined by dividing the measured $\Delta N_{eff}(660\text{ nm})$ by the $\Delta N_{eff}(660\text{ nm})$ for a single bead and dividing this by the surface area of the sensitive part of the sensing window (4 mm x 4 μm).

Fig. 5.B.1a, c, e show the measured $\Delta N_{eff}(660\text{ nm})$ for three different concentrations of beads. The $\Delta N_{eff}(660\text{ nm})$ was determined after signal was relatively constant ($t = 1100\text{ s}$) compensating for linear phase drift based on the of the first 300 s. Fig. 5.B.1b, d, f show an example of corresponding 200 x 200 pixels images taken with the fluorescent microscope with the 40X objective plus 1.5 magnification. Four different regions of the sensing window were imaged of which an average C_{bead} is determined using a local maxima algorithm (see end of Appendix), which is compared C_{bead} measured with the YI (see Fig. 5.B.2). The determined surface concentrations correspond relatively well. However, it should be taken into account that the four different areas are only a small part of the whole sensing region which was shown not to be homogeneously covered with beads. Moreover, a spread in intensities was measured for detected beads with the fluorescent microscope. This could not be explained by a spread in fluorescence intensities or sizes of the beads regarding the company. Therefore, it is possible that the detected spots are not single beads but aggregates of beads. The histograms of the intensities did however not show clear different populations of aggregates, which might indicate that there were no aggregates of 2, 3 or 4 beads, but it might be possible that there were aggregates of a higher number of beads with a large spread. This means that the exact number of beads per surface area cannot be determined. However, assuming that the aggregation is a linear process with respect to the concentration of the beads, it can be concluded that the surface concentration has a linear relationship with the sample bead concentration. To be sure about the exact surface concentration, dynamic light scattering, fluorescent correlations spectroscopy or flow cytometry can be used to check the size distribution of the beads.

Fig. 5.B.1: Fluorescent carboxylated beads (60 nm) were added in different concentrations to the sensor showing a, c, e) the measured ΔN_{eff} at 660 nm and b, d, f) one of the four corresponding images of a part of the sensing window imaged with a fluorescent microscope with each red circle a detected bead using a local maxima algorithm.

Fig. 5.B.2: The surface concentration determined with a fluorescent microscope and calculated based on a measured phase change at 660 nm with the YI, assuming a tight and a loose packing, all as a function of the initial bead sample concentration.

Local maxima algorithm

```
function out=pkfind(im,th,sz)
% finds local maxima in an image to pixel level accuracy.
% this provides a rough guess of particle
% centers to be used by cntrd.m. Inspired by the lmx subroutine of Grier
% and Crocker's feature.pro
% INPUTS:
% im: image to process, particle should be bright spots on dark background with little noise
%      often an bandpass filtered brightfield image (fbps.m, fflt.m or bpss.m) or a nice
%      fluorescent image
% th: the minimum brightness of a pixel that might be local maxima.
%      (NOTE: Make it big and the code runs faster
%      but you might miss some particles. Make it small and you'll get
%      everything and it'll be slow.)
% sz: if your data's noisy, (e.g. a single particle has multiple local
%      maxima), then set this optional keyword to a value slightly larger than the diameter of your blob. if
%      multiple peaks are found within a radius of sz/2 then the code will keep
%      only the brightest. Also gets rid of all peaks within sz of boundary
% OUTPUT: a N x 2 array containing, [row,column] coordinates of local maxima
%         out(:,1) are the x-coordinates of the maxima
%         out(:,2) are the y-coordinates of the maxima
% CREATED: Eric R. Dufresne, Yale University, Feb 4 2005
% MODIFIED: ERD, 5/2005, got rid of ind2rc.m to reduce overhead on tip by
%           Dan Blair; added sz keyword
% ERD, 6/2005: modified to work with one and zero peaks, removed automatic
%           normalization of image
% ERD, 6/2005: due to popular demand, altered output to give x and y
%           instead of row and column
% ERD, 8/24/2005: pkfind now exits politely if there's nothing above
%           threshold instead of crashing rudely
% ERD, 6/14/2006: now exits politely if no maxima found
% ERD, 10/5/2006: fixed bug that threw away particles with maxima
%           consisting of more than two adjacent points

%find all the pixels above threshold
im=im/max(max(im));
ind=find(im > th);
[nr,nc]=size(im);
tst=zeros(nr,nc);
n=length(ind);
if n==0
    out=[];
    display('nothing above threshold');
    return;
end
mx=[];
%convert index from find to row and column
rc=[mod(ind,nr),floor(ind/nr)+1];
for i=1:n
    r=rc(i,1);c=rc(i,2);
    %check each pixel above threshold to see if it's brighter than it's neighbors
    % THERE'S GOT TO BE A FASTER WAY OF DOING THIS. I'M CHECKING SOME MULTIPLE TIMES,
    % BUT THIS DOESN'T SEEM THAT SLOW COMPARED TO THE OTHER ROUTINES, ANYWAY.
    if r>1 & r<nr & c>1 & c<nc
        if im(r,c)>im(r-1,c-1) & im(r,c)>im(r,c-1) & im(r,c)>im(r+1,c-1) & ...
           im(r,c)>im(r-1,c) & im(r,c)>im(r+1,c) & ...
           im(r,c)>im(r-1,c+1) & im(r,c)>im(r,c+1) & im(r,c)>im(r+1,c+1)
            mx=[mx,[r,c]'];
            %tst(ind(i))=im(ind(i));
        end
    end
end
```

```

end
end
%out=tst;
mx=mx';

[npks,crap]=size(mx);

%if size is specified, then get ride of pks within size of boundary
if nargin==3 & npks>0
    %throw out all pks within sz of boundary;
    ind=find(mx(:,1)>sz & mx(:,1)<(nr-sz) & mx(:,2)>sz & mx(:,2)<(nc-sz));
    mx=mx(ind,:);
end

%prevent from finding peaks within size of each other
[npks,crap]=size(mx);
if npks > 1
    %CREATE AN IMAGE WITH ONLY PEAKS
    nmx=npks;
    tmp=0.*im;
    for i=1:nmx
        tmp(mx(i,1),mx(i,2))=im(mx(i,1),mx(i,2));
    end
    %LOOK IN NEIGHBORHOOD AROUND EACH PEAK, PICK THE BRIGHTEST
    for i=1:nmx
        roi=tmp( (mx(i,1)-floor(sz/2)):(mx(i,1)+floor(sz/2)+1), (mx(i,2)-floor(sz/2)):(mx(i,2)+floor(sz/2)+1)) ;
        [mv,indi]=max(roi);
        [mv,indj]=max(mv);
        tmp( (mx(i,1)-floor(sz/2)):(mx(i,1)+floor(sz/2)+1), (mx(i,2)-floor(sz/2)):(mx(i,2)+floor(sz/2)+1))=0;
        tmp(mx(i,1)-floor(sz/2)+indi(indj)-1,mx(i,2)-floor(sz/2)+indj-1)=mv;
    end
    ind=find(tmp>0);
    mx=[mod(ind,nr),floor(ind/nr)+1];
end

if size(mx)==[0,0]
    out=[];
else
    out(:,2)=mx(:,1);
    out(:,1)=mx(:,2);
end

```

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Chapter 6

Size-selective analyte detection using multiple wavelengths and polarizations

Abstract

Until now, size-selective detection with the YI sensor was done using multiple wavelengths based on their different mode profiles and their different sensitivities in different layers in the evanescent field. Chapter 6 shows that in a similar way, multiple polarizations can also be used to discriminate between refractive index changes (Δn 's) of multiple layers or multiple different-sized substances. Calculations from chapter 2 showed that the more different the sensitivity coefficients of each mode, the less singular the matrix to solve Δn 's in multiple layers or induced by multiple substances, meaning that all noise sources in ΔN_{eff} show up less enhanced. We show that it is possible to measure multiple polarizations and multiple wavelengths simultaneously, resulting in a matrix which is less singular for some cases and therefore representing an improvement of the approach in these cases. However, it should be noticed that measuring multiple wavelengths and polarizations also lead to an increase of the artefacts (due to boundary effects of the shifting interference pattern or due to grating or lens aberrations in the imaging path of the setup) in the measurements. For a successful application of the use of multiple polarizations and wavelengths for size-selective detection, the artefacts in the measurements should be reduced significantly.

6.1 Introduction

We have demonstrated in Chapter 5 that size-selective discrimination of analytes can be done using multiple wavelengths. However, such discrimination can also be done using different polarizations or a combination of wavelengths and polarizations, because different polarizations also have different mode profiles and therefore different sensitivities in different layers in the evanescent field. It is also possible to determine both the thickness and density (RI) of a protein adlayer using multiple polarizations, as was illustrated by Cross et. al [1] and Swann et. al [2] using dual polarization interferometry. Calculations from chapter 2 showed that wavelengths should be chosen widely spread such that the sensitivity coefficients of each wavelength differ the most. Consequently, the matrix to solve Δn in multiple layers or induced by multiple substances is less singular, meaning that all noise sources in ΔN_{eff} will be less enhanced. Therefore, using multiple polarizations, either at one or at multiple wavelengths, results in a less singular matrix, thus leading to an improvement in the experimental realization.

To determine a N number of Δn 's in multiple layers or induced by multiple substances from a N number of ΔN_{eff} 's (measured at multiple different wavelengths and/or polarizations), using any analysis approach, a $N \times N$ matrix is required. To see how well the solution of Δn can be determined from the matrix equation, the condition number κ of the matrix can be determined. For a linear system $\bar{\bar{A}}\bar{x} = \bar{b}$, the condition number gauges the transfer of error from matrix $\bar{\bar{A}}$ and vector $\bar{b}$ into vector $\bar{x}$ [3]. For a small condition number the problem is well-conditioned, whereas a large condition number corresponds to an ill-conditioned problem. The rule of thumb is that the expected loss in solving a problem of $\bar{\bar{A}}\bar{x} = \bar{b}$ is at least k digits of precision for a condition number $\kappa(\bar{\bar{A}}) = 10^k$ [3]. When the determined condition number of the matrix is lower using the multiple polarizations and wavelengths, this will improve the setup as the errors in $\bar{\Delta n}$ and $\bar{S}$ will be less transferred into $\bar{N}_{eff}$. However, the implementation of the use of multiple polarizations and wavelengths requires extra components in the setup to measure the transverse electric (TE) and transverse magnetic (TM) polarized light independently. Therefore, the improvement of precision of Δn should be weighed against the drawbacks (e.g. extra costs, complexity of the instrumental setup, alignment overhead). To illustrate the feasibility of measuring simultaneously multiple wavelengths and polarization, the experimental realisation and proof-of-principle experiments are shown in section 6.2 and 6.3 respectively. The chapter ends with a general discussion and conclusions in section 6.4.

6.2 Experimental realisation

The setup with butt-end coupling using a SM-PM fiber as described in Chapter 3 is used for the experiments shown in this chapter. The fiber was rotated 45 degrees to couple both TE and TM modes into the waveguide. After coupling out of the YI, a Wollaston prism was used to split up the polarizations to be able to independently detect the interference patterns. In order to determine the required splitting angle of the Wollaston prism which is positioned after the first cylindrical lens and before the grating, the formula of a transmission grating [4] is required:

$$a(\sin \theta_m - \sin \theta_i) = m\lambda, \quad (6.1)$$

where a is the distance from the centre of one slit to the centre of the adjacent slit, θ_m the angle of the m^{th} maximum and θ_i the angle of the incident beam which is determined by the Wollaston prism. This can be combined with the formula which describes the height of the focus h as a function of the angle of the incident beam at the lens θ_m , which is given by the expression:

$$h = f \tan \theta_m, \quad (6.2)$$

where f is the focal distance of the lens. The maximal distance between the outermost interference patterns should be smaller than $256 \times 26 \mu\text{m} = 6.656 \text{ mm}$, which is the size of the CCD chip. Combining formula 6.1 and 6.2 and assuming $f = 50 \text{ mm}$, and the wavelengths 457, 561 and 660 nm, a sufficient incident angle θ_i which should be realised by the Wollaston prism was determined to be 1° . Therefore, a PWQ 60.25 Wollaston prism (Bernhard Halle Nachfl. GmbH, Berlin) made of quartz crystal with a divergence angle of 1° @ 550 nm with a clear aperture of 24.5 mm was placed into the setup in between the first cylindrical lens and the transmission grating.

Fig. 6.1: CCD image of four interference patterns corresponding to TE and TM polarized light of $\lambda = 457 \text{ nm}$ and 561 nm .

Fig. 6.1 shows four interference patterns of TE457, TM457, TE561 and TM561 imaged on the CCD (note that the colours are artificial and correspond to the lookup table used). It was also possible to measure phase changes of six different interference patterns to discriminate between three substances, but this does not improve the end result very much compared to measuring the phase changes of three different interference patterns (see Chapter 2). Therefore, it was chosen to image only two wavelengths with two polarizations such that these four interference patterns fall over large number of pixels rows but still do not overlap such that they can be measured independently and with the lowest possible phase noise.

6.3 Proof-of-principle experiments

Protein A and D-glucose

To illustrate if two substances can be discriminated from each other using a combination of different wavelengths and polarizations, an experiment was done where 6.16 mg/ml D-glucose and 1 μ g/ml protein A were added simultaneously to the sensor as shown in Fig. 6.2a. The ΔN_{eff} was measured over time for TE polarized light at $\lambda = 457$ nm and 660 nm simultaneously with TM polarized light at $\lambda = 457$ nm. First, a 1x PBS buffer is completely replaced by D-glucose which is after 500s completely replaced by PBS again, illustrated by the ΔN_{eff} level which comes back to the original level before adding the D-glucose. Next, D-glucose is added simultaneously with protein A and again 500s later a washing step is applied. The ΔN_{eff} does not go back to its original level, because protein A is left at the surface. After the washing step, there is a slight decrease of the ΔN_{eff} signal which is probably be caused by desorption of the protein A.

Three different combinations of two of the three measured ΔN_{eff} 's were used to determine Δn for the D-glucose and the protein A and shown in Fig. 6.4b-d. The analysis was done with the ratio-based approach. The measured ratios were fitted to the theoretical model to determine a thickness and consequently determine the amplitude of the signal (combined approach). For the TM modes, the measured ratios for the protein A were very different from the theoretical ratios. Therefore, the theoretical model used to determine sensitivity coefficients in Chapter 2 [5] was compared with an effective index approximation based mode solver program [6]. The correctness of the used theoretical model to determine ΔN_{eff} was verified as the ΔN_{eff} 's of TE and TM modes gave the same values as an effective index approximation based mode solver program. The sensitivity coefficients were also determined by the same mode solver program, by calculating a ΔN_{eff} , subsequently inducing a Δn (in a

layer or in bulk), then calculating again the ΔN_{eff} , and dividing the difference in the two N_{eff} 's by the induced Δn . The values for the sensitivity coefficient for TE where approximately (<0.1% for bulk and <1% for layers) the same compared to the sensitivity coefficients based on the theory in Chapter 2. However, the sensitivity coefficients of TM did not correspond well. For the bulk the deviation was small (<1%), but for differently chosen layers the deviation varied from 19-27%. Therefore, we did not use the same model as in Chapter 2, but we used the effective index method to determine theoretical ratios and compare them with the determined ratios.

From the measurement we determine the ratios of the different wavelengths/polarizations by fitting the measured data to the expected values (see Chapter 4). The $\Delta n_{protein}$ should be approximately zero when adding D-glucose and that $\Delta n_{glucose}$ should be approximately zero after the second washing step (approximately zero means in this case that the value should be in line with the value expected based on linear drift). Based on this assumptions, the ratios were changed and this resulted for TE457 with TE660 in optimal ratios of $R_{TE660/TE457}^{glucose} = 1.20$ and $R_{TE660/TE457}^{protein} = 0.69$, which correspond to a $d_{core} = 62.0$ nm and a $d_{protein} = 2.0$ nm. For TE457 and TM457 the fitting with the ratio-based approach resulted in optimal ratios of $R_{TM457/TE457}^{glucose} = 1.44$ and $R_{TM457/TE457}^{protein} = 1.06$, corresponding to a $d_{core} = 66.5$ nm and a $d_{protein}$ which could not be fitted (ratio for 2.1 nm = 1.075). Fitting based on TM457 and TE660 resulted ratios of $R_{TE660/TM457}^{glucose} = 0.825$ and $R_{TE660/TM457}^{protein} = 0.65$, agreeing with a $d_{core} = 70.5$ nm and a $d_{protein} = 24.0$ nm (ratio for 2.1 nm = 0.618). The values for $d_{protein}$ are not realistic. The differences might be explained by the artefacts in the measurement (due to boundary conditions of the shifting interference pattern, see Chapter 3) which are much larger for TE457 compared to measurements without the Wollaston prism in the setup (see Fig. 6.2e/f). The artefacts are different in amplitude for each polarization/wavelength and therefore probably also different in the measured values of ΔN_{eff} for each polarization/wavelength, which results in different ratios between them. This can also be seen in the strong artefacts in Δn (see Fig. 6.2d) which were not observed in the analysed data of the measurements without the Wollaston prism in the setup. This illustrates that the artefacts are present to a greater extent with the Wollaston prism in the setup. Due to the increasing artefacts in the measurements, it was not possible to discriminate the protein A completely from the D-glucose when adding them simultaneously when analysing the results on TE457 and TE660 or TM457 and TE457. The D-glucose level is too high (should be the same as the first D-glucose step) and the protein A shows an unexpected drop. When the artefacts are

Fig. 6.2: a) ΔN_{eff} over time when adding 6.16 mg/ml D-glucose alone and together with 1 $\mu\text{g/ml}$, b, c, d) the analysed signal of Δn for TE 457/TM457, TE457/TE660 and TM457/TE660 respectively, all determined with the ratio-based approach, e) the measured relative amplitude and f) the typical relative amplitude for a measurement with the TE457 and TE660 without the Wollaston prism in the setup.

lower, in the case of TM457 and TE660, it was possible to sufficiently discriminate between protein A and D-glucose, illustrating that it is possible to do the discrimination based on TE and TM modes of different wavelengths.

The condition number of the matrix used with the ratio-based approach were also determined. The κ 's were 13.5, 7.65 and 17.8 for TE457/TM457, TE457/TE660 and TE660/TM457 respectively. This showed that in this case the combination of polarizations and wavelengths was not an improvement compared to measuring with TE457 and TE660 nm only, which shows that wavelengths and polarizations should be chosen carefully.

85 nm beads and D-glucose

To discriminate binding of 85 nm beads from a D-glucose bulk change, ΔN_{eff} was measured at TE457, TE561 and TM561 when adding 6.16 mg/ml D-glucose only and together with 1 μ g/ml 85 nm beads as shown in Fig. 6.3a. Fig. 6.3 b shows that the amplitudes of the artefacts in the measurements are again relatively high (up to 40% for TM561). The artefacts show up predominantly in the analysed signal of Δn based on TM561 (see Fig. 6.3b-d), which is expected as the κ 's of the matrices used are 18.95, 61.35 and 13.74 for TE457/TM561, TE561/TM561 and TE457/TE561 respectively and the artefacts are the highest for TM561. Comparing Fig. 6.3b and c the artefacts in the end result in Δn based on TE561/TM561 are clearly higher despite comparable normalized amplitudes of TE457 and TE561 (see Fig. 6.3b), illustrating the effect of the higher condition number of the matrix of TE561/TM561.

The ratios in ΔN_{eff} were again fitted as stated before and are $R_{TM561/TE457}^{glucose} = 1.025$, $R_{TM561/TE457}^{bead} = 0.828$, $R_{TE561/TM561}^{glucose} = 1.091$, $R_{TE561/TM561}^{bead} = 1.022$, $R_{TE561/TE457}^{glucose} = 1.125$ and $R_{TE561/TE457}^{bead} = 0.950$. The ratios were fitted to the effective index method resulting in $d_{core} = 66.6$ nm, 68.0 nm and 58.2 nm, and a $d_{bead} = 97$ nm, an unfittable value and 62 nm corresponding to TM561/TE457, TE561/TM561 and TE561/TE457 respectively. However, because of the large artefacts in the measurements these values are very unreliable and not usable. Artefacts should be reduced significantly for accurate discrimination between substances and determination of substance thicknesses.

Fig. 6.3: a) ΔN_{eff} over time when adding 6.16 mg/ml D-glucose alone and together with 1 $\mu\text{g/ml}$ 85 nm beads, b) the corresponding signal of the relative amplitude and c, d, e) the analysed signal of Δn for TE561/TM561, TE561/TM457 and TM457/TM561 respectively, all determined with the ratio-based approach.

Protein A, 85 nm beads and D-glucose

To illustrate the possible discrimination of three substances based on multiple polarizations and wavelengths, protein A, 85 nm beads and D-glucose were also added to sensor within one measurement as shown Fig. 6.4. First, at t_1 only D-glucose is added to the sensor and at t_2 a washing step is applied. Next, at t_3 85 nm beads were added to the sensor after again applying a washing step at t_4 . Between t_5 and t_6 protein A was added to the sensor. Next, Δn was determined with the ratio-based approach. The ratios were again fitted to the expected values of Δn , resulting in $R_{TM457/TE457}^{protein} = 1.045$, $R_{TM561/TE457}^{protein} = 0.53$, $R_{TM457/TE457}^{bead} = 1.39$, $R_{TM561/TE457}^{bead} = 0.819$, $R_{TM457/TE457}^{glucose} = 1.465$, $R_{TM561/TE457}^{glucose} = 1.017$. These ratios correspond to $d_{core} = 67.0$ nm, 66.5 nm and 65.8 nm, an unfittable $d_{protein}$ (2.1 nm corresponds to $R_{TM457/TE457}^{protein} = 1.091$, $R_{TM561/TE457}^{protein} = 0.563$ and $R_{TM561/TE457}^{protein} = 0.516$) and $d_{bead} = 131$ nm, 110 nm and 69 nm for TE457/TM457, TE457/TM561 and TM457/TM561 respectively. The differences between measured

Fig. 6.4: a) ΔN_{eff} over time when adding 6.16 mg/ml D-glucose (t_1 - t_2), 1 μ g/ml 85 nm beads (t_3 - t_4) and 2 μ g/ml protein A (t_5 - t_6) to the sensor, all measured at TE457, TM457 and TM561, b) the corresponding signal of the relative amplitude, c) the analysed signal of Δn using the ratio-based approach, d) ΔN_{eff} over time when adding 6.16 mg/ml D-glucose (t_1 - t_2), 1 μ g/ml 85 nm beads (t_3 - t_4) and 2 μ g/ml protein A (t_5 - t_6) to the sensor, all measured at TE457, TE561 and TE660, e) the corresponding signal of the relative amplitude and f) the analysed signal of Δn using the ratio-based approach.

and theoretical values are probably again explained by the artefacts in the measurements. Despite these measured values do not match the expected thickness of ≈ 2.1 nm for the protein and 85 nm for the beads, it was still possible to discriminate between the three independent sources using the ratio-based approach, illustrating the strength of the ratio-based approach. Theoretical parameters are not required for the ratio-based approach and just normal calibration measurements can be used to determine the ratios of the individual substances.

The matrix based on the determined ratio has a $\kappa = 68.94$, which is more than three times lower than the matrix based on measurements with TE457, TE561 and TE660, which had a $\kappa = 209.41$. The artefacts in the relative amplitude in the case when measuring with multiple wavelengths and polarizations is higher (standard deviation is 8.74×10^{-2}) compared to measuring with only multiple wavelengths (standard deviation is 3.75×10^{-2}) as shown Fig. 6.4b/e. However, the artefacts that show up in Δn are comparable (see Fig. 6.4c/f), as the artefacts are less enhanced for the multiple wavelengths and polarizations because of the lower condition number of the matrix which is used to determine $\overline{\Delta n_s}$ from $\overline{\Delta N_{eff}}$. At the addition of D-glucose, the artefacts are relatively low for $\Delta n_{protein}$ and Δn_{beads} for the polarization/wavelength case. On the other hand, the artefacts in $\Delta n_{glucose}$ are a bit higher for the polarization/wavelength case. So, when lowering the artefacts in ΔN_{eff} when measuring with multiple wavelengths and polarizations it is possible to improve the setup as the artefacts are less enhanced in this specific case due to the better conditioned matrix (κ is 3x lower).

6.4 Discussion and conclusions

Using different wavelengths and polarizations it was possible to discriminate between D-glucose, 85 nm beads and protein A. However, the polarizations and wavelengths should be chosen carefully when they are used for improvement of the setup. Choosing the wrong polarization/wavelength combination results in a higher κ resulting in more enhancement of all noise sources. Measuring with TE4567, TM457 and TM561 we were able to reduce the κ three fold compared to TE457, TE561 and TE660, corresponding to an approximately 4.8 times lower enhancement of noise. More measurements or theoretical calculations are required to determine if there are even more favourable combinations of wavelengths and polarizations to reduce the κ , which strongly depends on the measurable substances, even more.

Despite the decrease of κ , the artefacts due to boundary effects of the shifting interference patterns were increased using a Wollaston prism to split up the interference patterns, illustrating the optical components influence artefacts.

Therefore, the determined signals of $\overline{\Delta n_s}$ showed noisy signals despite the decrease of κ . Consequently, the artefacts should be strongly reduced for a successful application of the size-selective detection using multiple wavelengths and polarizations, using for example larger optical components and detecting more and larger fringes of the interference pattern. Alternatively, it is possible to alternately measure TE and TM modes by switching between these modes using a ferroelectric liquid crystal 1/2 wave plate [1].

The measured ratios between ΔN_{eff} of different wavelengths and polarizations do not match with the theoretical expected ratios, especially for the TM modes. Furthermore, it seems that the theory used in Chapter 2 [5] for determination of sensitivity coefficients for TE modes for different regions in the evanescent field is not applicable for TM modes as there is a large (up to almost 30%) mismatch with the effective index method from Hammer [6]. Therefore, the approximation to determine the sensitivity coefficients per layer by Schipper [5] should not be applied using TM modes. Fitting the measured ratios to the theoretical effective index method did lead to fitted ratios for the protein A and beads which were not reliable because of the very high artefacts in the measured $\overline{\Delta N_{eff}}$. Next to the influence of artefacts on the end result of $\overline{\Delta n_s}$, it might be possible that some light is converted on the chip. Light converted from TE to TM modes or the other way around could not be filtered out as the polarizer had to be removed from the setup to be able to measure two polarizations. To determine if light was converted from TE to TM modes, separate measurements with only TM light with polarizer in imaging path are required to compare them with the results of the combined TE/TM measurements. Measurements with TE light show comparable results with the combined TE/TM measurements. It should be noted that when only measuring with TE or TM light, modes that are converted twice (e.g. TE to TM and back to TE) cannot be filtered out. For successful application the artefacts should be strongly reduced. Subsequently, repeatability studies are required to determine if measured ratios are constant when artefacts in $\overline{\Delta N_{eff}}$ are reduced.

Notwithstanding the mismatch between theory and measured ratios, it was still possible to discriminate between the three independent sources using the ratio-based approach. Theoretical parameters are not required for the ratio-based approach illustrating the strength of the ratio-based approach. However, for a successful application of the use of multiple polarizations for size-selective detection, the artefacts in the measurements should be reduced significantly.

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Chapter 7

Timing difference of refractive index changes induced by binding of proteins and bulk changes

Abstract

From the determined Δn 's using size-selective detection we saw that the Δn due to binding of protein A to the surface occurred earlier than the Δn due to protein A in the bulk (a few hundreds of nanometres above the sensors surface). However, it was expected that protein A should arrive in the bulk before it can attach to the surface. In Chapter 7 this unexpected time delay is discussed. Finite element method simulations on the diffusion and binding of molecules show that a time delay between surface binding of protein A and protein A bulk changes can arise when protein A has a high affinity for the surface. The bulk region is depleted due to the fact that protein A is strongly attracted towards the surface. The time delay increases with an increasing maximum surface capacity $\gamma_{\max}$ and increasing adsorption rate k_a . For a k_a of 10^5 $\text{m}^3/\text{mol}/\text{s}$ and a $\gamma_{\max}$ between 4.4×10^{-3} and 1.7×10^4 fg/mm^2 (corresponding to a protein A radius of 1 and 2 nm respectively which agrees well to the measured thickness of 2.1 nm (see Chapter 4)), a time delay of approximately 20 s to 100 s is found. This is comparable to the observed time delay. To verify the results of the simulations, protein A and D-glucose were added simultaneously to the sensor. Due to the fact that D-glucose has a much higher diffusion than protein A it was expected that Δn due to D-glucose bulk changes would appear earlier now. However, again the signal of Δn due to binding of protein A occurred earlier than the Δn due to D-glucose. Experiments hint that this time delay is related to the transport towards the sensing window as increasing the transporting tube length resulted in an increasing time delay. Experiments showed that the time delay also occurred for bovine serum albumin, suggesting that the time delay is independent of the protein used. However, the exact origin of the time delay between D-glucose bulk changes and surface binding of protein A is not found.

7.1 Observed time delay of bulk signal compared to binding

7.1.1 Protein A binding earlier than protein A bulk

Protein A and D-glucose were added separately from each other to the sensor. Fig. 7.1a,b show the ΔN_{eff} over time measured at 457, 561 and 660 nm of two similar experiments and Fig. 7.1c-f the corresponding Δn determined for D-glucose and protein A using the combined approach (see Chapter 4). The protein A was added in concentrations in the order of 100 $\mu\text{g/ml}$, resulting in two measurable effects: a signal in the first ≈ 2 nm due to the binding of protein A to the surface and a bulk signal in the region of ≈ 200 nm due to the protein A in solution. Both signals have a different slope which is already seen in the measured signal of ΔN_{eff} in Fig. 7.1a, b, but this shows up more clearly in the determined signal of Δn (see Fig. 7.1e, f), in which it was observed that the signal due to binding of protein A started earlier than the bulk signal due to protein A. The bulk signal due to the high concentration of protein A results in the same ratios of ΔN_{eff} as the D-glucose bulk signal, which means that both bulk signals could be analysed using a single ratio for bulk effects.

7.1.2 Protein A binding earlier than D-glucose bulk

Also experiments were done where protein A and D-glucose were added simultaneously to the waveguide. Fig. 7.2a, b show the ΔN_{eff} over time measured at 457, 561 and 660 nm of two experiments where D-glucose and protein A were added simultaneously to the waveguide and Fig. 7.2c-f shows the corresponding Δn determined for the bulk signal and the protein A using the combined approach. For these experiments a lower protein A concentration was used, such that the bulk contribution due to the protein A can be neglected (checked when protein A was added alone to the sensor in these low concentrations) and that the bulk contribution is only caused by the D-glucose. Also for these experiments the signal to the binding of protein A to the surface starts earlier compared to the signal due to the bulk effect of D-glucose. Fig. 7.2 e, f show that this delay is longer for protein A concentration of 26.7 $\mu\text{g/ml}$ compared to 1 $\mu\text{g/ml}$. However, because of the artefacts due to boundary effects of the shifting interference pattern (see Chapter 3), it is difficult to determine exactly when the signal starts increasing and therefore it is difficult to determine the exact time delay. Furthermore, the time delay could be an artefact from the method. To answer these questions, a timing experiment is done where protein A and D-glucose were added to the waveguide at the same time, but in different channels. Fig. 7.3 shows the results of this measurement. First, a timing step of water to

Fig. 7.1: a, b) Two experiments where showing ΔN_{eff} over time at 457, 561 and 660 nm adding protein A and D-glucose separately to the waveguide, c, d) the corresponding Δn due to the bulk and the binding of the proteins determined with the combined approach and e, f) a zoom-in of c, d).

Fig. 7.2: a, b) Two experiments where showing ΔN_{eff} over time at 457, 561 and 660 nm adding protein A and D-glucose simultaneously to the waveguide, c, d) the corresponding Δn due to the bulk and the binding of the proteins determined with the combined approach and e, f) a zoom-in of c,d).

phosphate buffered saline (PBS) is done, to cancel out timing differences due to different tube lengths belonging to the different channels. The timing differences were compensated for by shifting the signal by the delay found for the water - PBS step. After compensation of time differences due to tube length, also a time delay of D-glucose compared to the binding of protein A is observed. Therefore, it can be concluded that the measured time delay of bulk effects compared to binding is not an artefact of the analysis method but is real.

But why do we see a time delay of bulk signal due to protein A or D-glucose compared to binding of proteins? Intuitively one would say that the molecules should arrive first in the bulk (≈ 200 nm above the surface) before they can bind to the surface. Moreover, assuming laminar flow, the transport of molecules near the surface is determined by diffusion of molecules as the velocity is nearly zero. Glucose molecules diffuse faster ($D_g = 6.7 \times 10^{-10} \text{ m}^2/\text{s}$ [1]) than protein A molecules ($D_p = 4.3 \times 10^{-11} \text{ m}^2/\text{s}$ [2]) and therefore, the glucose molecules should arrive earlier at the bulk region than the proteins reach the surface. Simulations with COMSOL were done to find an explanation for especially the time delay of protein bulk signal with respect to the binding of protein A, but also the D-glucose is considered in the model. Results of this COMSOL simulation are presented in section 7.2.

Fig. 7.3: a) Timing experiment showing ΔN_{eff} over time at 660 nm measured at different channels to which protein A and D-glucose were added separately and b) a zoom-in of a).

7.2 Simulations D-glucose and protein A in sensing window

The theory in this section is based on research on convection, diffusion and adsorption in surface-based biosensors of Hansen et. al [3]. We slightly changed their

model, because their research only considered unimolecular systems. We study a model with both proteins and glucose in solution. COMSOL Multiphysics 4.3 was used for simulations and a model called 'transport and adsorption' was used as a basis for our model.

7.2.1 System geometry

A flow chamber is placed on top of the sensing windows of the chip. The dimensions of the flow chamber, including the sensing window, are shown in Fig. 7.4. The scales of the flow chamber are $l = 6$ mm, $w = 2$ mm, $h = 100$ μ m. The length of the sensing window l_s is 4 mm and the width of the sensing window w_s is 4 μ m. The flow through the flow chamber is in x -direction and because of the low Reynolds number the flow is laminar. For that reason and because of the large aspect ratio $w/h \approx 15$ (side walls in z -direction are expected to not influence the sensing window), invariance in z -direction is assumed and consequently the system can be described by a 2D model [3] as shown in Fig. 7.4. The velocity of the laminar flow v_{lam} can be described by the following equation:

$$v_{lam} = 4v_{max} (y/h)(1 - y/h) \quad (7.1)$$

where v_{max} is the maximum velocity.

Fig. 7.4: Flow chamber and sensing window dimensions in a 3D and 2D configuration, where h is the height of the flow chamber, w the width of the flow chamber, l the length of the flow chamber, l_s the sensing window length, w_s the sensing window width and v_{lam} the velocity of the laminar flow.

7.2.2 Theoretical equations

Three important variables that are studied are defined as: c_g , which is the average concentration of D-glucose in a region of 200 nm high above the sensing window; c_p , which is the average concentration of protein A in a region of 200 nm high above the

sensing window; and γ , which is the average protein A surface coverage on the sensing window. First, the spatiotemporal development of c_g can be described by:

$$\frac{\partial c_g}{\partial t} + v_{lam} \frac{\partial c_g}{\partial x} = D_g \nabla^2 c_g, \quad (7.2)$$

where $\nabla^2 = \partial^2/\partial x^2 + \partial^2/\partial y^2$ is the Laplacian and D_g is the diffusion coefficient of glucose. For glucose the assumption is made that there is no adsorption of molecules at the walls of the flow chamber, which means that there is a non-flux condition at those non-adsorbing domains, which is given by:

$$\frac{\partial c_g}{\partial y} = 0. \quad (7.3)$$

Second, the spatiotemporal development of c_p is described by a similar equation:

$$\frac{\partial c_p}{\partial t} + v_{lam} \frac{\partial c_p}{\partial x} = D_p \nabla^2 c_p, \quad (7.4)$$

where D_p is the diffusion equation of the proteins. The boundary equations are given by:

$$\frac{\partial c_p}{\partial y} = 0, \quad (7.5)$$

which is a no-flux condition which holds for the non-adsorbing regions (flow chamber walls except for the sensing window) and

$$-D_p \frac{\partial c_p}{\partial y} = -R(\gamma, c_p) \quad (7.6)$$

which is a balance between diffusive flux perpendicular to the surface and the adsorption rate (which is given by equation (7.9)) and holds for the sensing window, to which proteins can adsorb.

Third, the spatiotemporal development of γ is described by adsorption-diffusion equation:

$$\frac{\partial \gamma}{\partial t} - \frac{\partial}{\partial x} \left[D_s \frac{\partial \gamma}{\partial x} \right] = R(\gamma, c_p), \quad (7.7)$$

where D_s is the surface diffusion coefficient of the proteins and $R(\gamma, c_p)$ describes the net rate of change of γ , due to adsorption and desorption of molecules. Molecules can only leave the sensing window by desorption, which results in the following boundary condition at both ends of the sensing window:

$$\frac{\partial \gamma}{\partial x} = 0. \quad (7.8)$$

Finally, the adsorption rate can be described by the Langmuir model. It is a first order scheme between bulk molecules at the interface $c|_{y=0}$ and free surface sites $(\gamma_{\max} - \gamma)$, and can be described by:

$$R(\gamma, c_p) = k_a c|_{y=0} (\gamma_{\max} - \gamma) - k_d \gamma, \quad (7.9)$$

where $\gamma_{\max}$ is the maximum surface capacity, k_a the adsorption rate constant and k_d the desorption rate constant. The k_a and k_d values are assumed to be independent of the surface density. At high density it could be expected that there will be interaction between the bound molecules. Nevertheless, this model can explain in general the majority of adsorption and desorption processes in molecular biology [4].

7.2.3 Parameter values

In this section we summarize all the parameters and their values and we make an estimation of the unknown values, which will all be used in the simulations. The input parameters and variables are shown in Table 7.1. The surface diffusion coefficient D_s was set at zero, assuming irreversible adsorption of the proteins. For the same reason, the desorption of proteins k_d is set zero. An estimate of the maximum surface capacity was made by dividing the number of moles per protein ($1/N_A$) by the diameter of the protein squared ($d_p^2 = 4r_p^2$). The radius of protein A was varied during the simulation. One of the values is 2 nm, which is approximately equal to the measured height of protein A adsorbed on a surface [5, 6] and one value is 5 nm which is equal to the Stokes radius of protein A [2]. For steady, laminar flow between two fixed parallel plates, the maximum velocity is given by $3/2 v_{mean}$ [7], where v_{mean} is equal to the flow rate Q divided by the cross-sectional area, which is given by the height h times the width w of the flow chamber.

7.2.4 Simulation results and discussion

The protein A surface concentration, the protein A bulk concentration and the glucose bulk concentration are analysed over time. The parameters that were put into the simulation can be found in the section 7.2.3 in Table 7.1. The variables k_a and $\gamma_{\max}$ were varied, where $\gamma_{\max}$ was varied by changing r_p in the maximum surface capacity formula (see Table 7.1). The c_g , c_p and γ are shown in Fig. 7.5 and Fig. 7.6 for the different adsorption rates and different maximum surface capacities respectively.

Table 7.1: Simulation parameters and variables

Parameter variables	Description	Expression	Value
h	flow chamber height		100 μm
l	flow chamber length		6 mm
w	flow chamber width		2 mm
l_c	sensing window length		4 mm
w_c	sensing window width		100 μm
D_g	Diffusion coefficient glucose		$6.7 \times 10^{-10} \text{ m}^2/\text{s}$ [1]
D_p	Diffusion coefficient proteins		$4.825 \times 10^{-11} \text{ m}^2/\text{s}$ (based on Stokes radius of 5 nm and Stokes-Einstein-Sutherland equation $D = \frac{k_b T}{6\pi\eta r_{\text{stokes}}}$, with k_b the Boltzmann constant $1.3806488 \times 10^{-23} \text{ J/K}$, $T = 20^\circ\text{C}$, and the viscosity of water $\eta = 8.90 \times 10^{-4} \text{ Pa}\cdot\text{s}$.)
D_s	Surface diffusion coefficient		0
γ_{max}	Maximum surface capacity	$\frac{1}{4N_A r_p^2}$	varied in simulation: (69, 17, 4.4, 1.9, 1.1, 0.69, 0.17) $\times 10^3 \text{ fg/mm}^2$ based on values of protein A radius r_p of 0.5, 1, 2, 3, 4, 5, 10 nm (Protein A Stokes' radius = 5 nm [2])
v_{max}	Maximum velocity	$\frac{3}{2}v_{\text{mean}} [7] = \frac{3}{2} \frac{Q}{hw}$	1.25 cm/s
k_a	Adsorption rate constant of protein A (adsorption rate constant of glucose is set at zero)		varied in simulation: 0.1, 1, 10, 50, 100, 200, 400, 600, 800, 1000, 2000 $\text{m}^3/\text{mol/s}$
k_d	Desorption rate constant		0
Q	Flow rate		100 $\mu\text{l/min}$
M_p	Molecular weight protein A		42 kDa [2] = $42 \times 10^3 \text{ g/mol}$
N_A	Avogadro constant		$6.02214 \times 10^{23} \text{ 1/mol}$
$c_{0,g}$	Start concentration of glucose		0.616 % (weight percentage) = 6.16 mg/ml = 34.193 mol/ m^3
$c_{0,p}$	Start concentration of proteins		40 $\mu\text{g/ml}$ = $9.52 \times 10^{-4} \text{ mol/m}^3$

Fig. 7.5: a) Development of C_p over time for different adsorption rates of protein A, b) the development of γ over time for different adsorption rates of protein A, c) the development of C_g over time as a function of the adsorption rates of protein A and d) the C_p and γ for $k_a = 10^5$ $\text{m}^3\text{mol}^{-1}\text{s}^{-1}$ illustrating the time delay of the bulk concentration compared to the surface coverage.

Fig. 7.5 shows that the protein A bulk concentration starts increasing very quickly. The proteins arrive quickly in the centre of the flow chamber because of the high velocity in the centre of the flow chamber. However due to the laminar flow, the velocity at the surface is zero. This means that in the region that we consider as bulk (≈ 200 nm above the surface) the velocity is nearly zero and that diffusion transports the proteins in this bulk region. The diffusion coefficient of glucose in water is higher than proteins in water [1, 2]. Therefore, it is likely that the diffusion of glucose in PBS is also higher than the diffusion of proteins in PBS. As a result, the bulk concentration

Fig. 7.6: a) The C_p over time as a function of $\gamma_{\max}$ determined by changing the radius of the protein A, b) a zoom in of the first 30 seconds of a), c) the corresponding γ and d) the C_g over time as a function of the protein A $\gamma_{\max}$.

of the glucose should increase earlier than the bulk concentration of the protein A, which is also found as a result from the simulations as shown in Fig. 7.5c. The adsorption rate of glucose is set at zero as we expect it not to bind to the sensing window. From the simulations seen in Fig. 7.5c, we can conclude that the glucose bulk concentration is almost not affected by the k_a of protein A. On the other hand, the protein A bulk concentration as well as the protein A surface concentration do strongly depend on the adsorption rate k_a (see Fig. 7.5a). As expected, for higher k_a values, the slope of the surface concentration is higher (see Fig. 7.5b), because the proteins have a higher affinity to bind to the surface. Moreover, for high k_a values, the

protein A bulk concentration gets a time delay compared to the surface concentration (see Fig. 7.5d). Due to the strong adsorption rate, the proteins bind to the surface. When the first proteins reach the surface, the protein A surface concentration starts to increase, which continues until the maximum surface capacity is reached. As a consequence, no proteins can bind to the surface anymore. The bulk region (≈ 200 nm above the surface) gets depleted for the time that the proteins can still bind to the sensor surface. After saturation of the surface, the protein A bulk concentration starts increasing as it equilibrates with the centre region of the flow chamber in which constantly protein A with concentration of $4 \mu\text{g/ml}$ arrives.

For $k_a = 10^5 \text{ m}^3\text{mol}^{-1}\text{s}^{-1}$, the simulations were repeated, but for different maximum surface capacities γ_{max} (see Table 7.1). Fig. 7.6a/b show that for a higher γ_{max} , it takes more time before the protein A bulk concentration increases. Fig. 7.6c show that there is no timing difference for the protein A surface concentration as expected, because the adsorption rate was constant for this set of results. Consequently, a higher γ_{max} results in larger time delays of protein A bulk concentration compared to protein A surface concentration. Furthermore, it can be noticed that the end level of the surface concentration is equal to the maximum surface capacity γ_{max} . Again, the glucose bulk concentration is almost not affected by the protein A binding at the surface as shown in Fig. 7.6d.

To conclude, we can say that the simulation results can explain the time delay of the protein A bulk compared to the protein A surface concentration, as was found in the analysis of the measurements where only protein A was added to the buffer solution in high concentration (see Fig. 7.1). This delay strongly depends on the adsorption rate k_a and the maximum surface capacity γ_{max} . For a high k_a , all proteins which reach the surface bind to the surface, which causes a depletion of the bulk region (≈ 200 nm region above surface) until the surface is fully covered (maximum surface capacity γ_{max} is reached). On the other hand, the time delay of the D-glucose bulk signal compared to the protein A binding signal is a different story. As expected the D-glucose signal always starts earlier than the protein A signal, because of the higher diffusion coefficient of D-glucose, and is not affected by the protein A (independent of k_a and γ_{max} of protein A).

7.3 Time delay BSA and D-glucose

To verify that the time delay of bulk signal of D-glucose compared to binding of protein A is not protein A specific, D-glucose was also added simultaneously to 66.4 kDa A7906-100g bovine serum albumin (BSA) in a 1x PBS buffer. This protein was

Fig. 7.7: a, b) Two experiments where showing ΔN_{eff} over time at 457, 561 and 660 nm adding protein A and D-glucose simultaneously to the waveguide, c, d) the corresponding Δn due to the bulk and the binding of the proteins determined with the combined approach and e, f) a zoom-in of c, d).

dissolved in a salt buffer which was replaced by the 1x PBS buffer using a Zeba™ Desalt Spin Column. Because some loss of protein can be expected after using the spin column, the concentration was determined afterwards by a NanoDrop® UV-Vis ND-1000 Spectrophotometer. Results of two experiments where D-glucose was also added simultaneously to two different concentrations of BSA are shown in Fig. 7.7. Also for this measurement it is difficult to determine the exact time delay because of the features in the measurements due to cutting of the interference pattern. However, it can be concluded that there is a time delay of the bulk signal compared to the binding signal due to BSA which is concentration dependent. This means that the time delay is not only observed for protein A.

7.4 Time delay as function of tube length

As the simulations confirm that time delay of the bulk signal due to D-glucose compared to the binding of proteins is not caused in the flow chamber, the time delay should be caused before the molecules reach the flow chamber. To check if the time delay is influenced by the tubes which guide the buffer containing the molecules to the flow chamber, the tube length was varied. D-glucose (6.16 m/ml) and protein A (1 µg/ml) were added simultaneously to the different flow chambers which were connected to tubes with different lengths. To enlarge the tube length, extra tubes of 40 cm were interposed between the two normally used tubes of 40 cm each. The results of the measurements are shown in Fig. 7.8. The measurement of Fig. 7.8j does not show the ΔN_{eff} of the 660 nm laser, because this signal was very noisy and unusable, but it could be analysed based on the measured ΔN_{eff} 's at 457 and 561 nm.

The time delay was determined as the time difference of the start of increase of the Δn bulk signal and the start of increase of the Δn signal due to the binding. The time delay is plotted as a function of the extra added tube length in Fig. 7.9. The error bars are quite large, because it is difficult to determine the exact time point when the signal starts to increase. Despite the large error bars it is clearly observable that the time delay increases with increasing tube length. Therefore, it is likely that the time delay is caused in the tubing, before the molecules reach the sensing window.

Fig. 7.8: The ΔN_{eff} over time where first D-glucose was added separately and later simultaneously with protein A to the sensor for an extra tube length of a) 0 cm, d) 40 cm, g) 80 cm and j) 160 cm and b, e, h, k) the corresponding determined Δn for the bulk and the protein A binding using the combined approach and c, f, i, l) a zoom-in of b, e, h, k).

Fig. 7.9: Time delay of D-glucose bulk signal compared to protein A binding signal as a function of the extra length of the tube guiding the buffer and molecules to the sensing window of the waveguide.

7.5 Conclusions and forward look

From the simulations we can conclude that the evolution of the bulk and surface concentration of protein A strongly depends on the adsorption rate k_a and the maximum surface capacity $\gamma_{\max}$. For a k_a of 10^5 m³/mol/s and a $\gamma_{\max}$ between 4.4×10^{-3} and 1.7×10^4 fg/mm² (corresponding to a protein A radius of 1 and 2 nm (see Table 7.1) respectively which agrees well to the measured thickness of 2.1 nm (see Chapter 4)), a time delay of approximately 20 s to 100 s is found. This is comparable with the time delay between the bulk and binding signal of protein A determined with the size-selective detection analysis approach. As the time delay can be explained by the simulations which show a time delay between of the bulk signal due to the proteins compared to the binding of the proteins, the time delay determined with the size-selective detection appears real and not an artefact of the analysis approach.

To verify the results of the simulations, protein A and D-glucose were added simultaneously to the sensor. Simulations show that due to the high diffusion of D-glucose this signal should be earlier than the binding of protein A, independent of k_a and $\gamma_{\max}$. However, again the determined signal of Δn due to binding of protein A occurred earlier than the Δn due to D-glucose. Therefore, this time delay must be caused before the protein A and D-glucose reach the flow chamber. This was confirmed by an experiment which showed that the time delay is influenced by the tube length. Longer tube lengths result in a longer time delay. The delay of D-glucose compared to binding of protein A also occurs when adding the D-glucose and protein A separately to different tubes guiding the molecules to different flow chamber with

sensing windows. Therefore, the time delay cannot be explained by phase separation of the two different molecules in the tubes, which can occur for example for two incompatible polymers in capillary tubes [8]. This was also verified in an experiment where the same protein A concentration (80 µg/ml) and D-glucose concentration (6.16 mg/ml) were added in amount of $\approx$ 3.5 ml to a 5 ml, 12 mm wide transparent tube. After mixing the molecules and waiting for a day no RI differences were observed between different areas. Also after adding a Coomassie Brilliant Blue G-250 dye (is used for Bradford protein assays), which changes from a brown colour to a more blue colour when binding to a protein, no clear colour differences were observed between possible areas with D-glucose and possible areas with protein A. Consequently, there is no strong phase separation of protein A and D-glucose, meaning that this cannot explain the time delay of the D-glucose compared to the protein A measured with the YI sensor in combination with size-selective detection.

Alternatively, there might be a mechanism which can transport the protein A faster through the tubes, possibly along the walls of the tubes. This mechanism is not protein A specific, as the delay was also observed using BSA instead of protein A. More research is required to study the effect of the tubes and their walls on the time delay. To visualize the difference in the tubes it might be possible to label (dye) the proteins and D-glucose. It is also possible to determine the influence of the tubing by using different tubing materials such as (PEEK) Polyetheretherketone instead of Tygon tubing. However, in view of time, these experiments were not done and this research could not be continued.

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Chapter 8

Applications for size-selective detection: a forward look

Abstract

This chapter explores what are the most relevant applications for the Young interferometer (or a similar) sensor in combination with size-selective detection based on multiple wavelengths. In this chapter we also address what should be next steps in future research based on the conclusions drawn from the other chapters. For that purpose, a small technology assessment study was performed and a mini-workshop was organised to identify possible innovation pathways and relevant factors to consider from a technological, product and commercialization point of view. From the assessment it was determined that the added value of the sensor is to measure size-selectively (discriminate between substances based on their size) and that the gain in size-selectivity should be worth the loss in sensitivity and stability and the increasing costs. Therefore, pharmacy and the medical sector are probably less suitable markets for this sensor, as in these application areas, specific biochemistry and sensitivity are more important than size-selectivity. A research platform for universities or companies was found to be the most promising market, but it must be noticed that markets should be analysed in more depth for a more complete picture. Possible applications in this market are measurement tools to measure the kinetics of layer growth, a signal enhancer, a quick screener or an alternative for a Scanning Electron Microscope, an alternative for Dynamic Light Scattering (to measure thickness of layers or particles) and a research tool to study fundamental research on binding or other surface effects or flow dynamics. This resulted in research questions *“What are the minimum and maximum size which can be detected and determined?”* and *“What differences in sizes could be discriminated from each other?”*. Further research is required to answer these questions. When a certain application or market is identified, then the sensor should be optimized to determine the limits of the device for the specific application in mind.

8.1 Introduction

Integrated optical (IO) sensors have been demonstrated as a powerful detection tool for biosensing, because of their ability to detect label-free, in real-time and very sensitive. The Young interferometer (YI) is a very promising biosensor because of its capability of multiplexing and its extremely high refractive index (RI) sensitivity (10^{-7} - 10^{-8} RIU) [1, 2]. However, non-specific signals like bulk changes and non-specific binding still hamper a full utilization of the high sensitivity and therefore also a successful application of these type of biosensors. We have extensively studied how the use of multiple wavelengths can contribute to an increased specificity (defined here as the ability to correctly detect analytes, meaning not measuring false positives). It was found that the different evanescent fields of the different wavelengths can be used to differentiate between particles based on their size and therefore add size-selectivity to the sensor. Experimental data showed that 85 nm beads could be discriminated from protein A, which is found to be ≈ 2 nm in height by other studies [3, 4], and D-glucose inducing a homogeneous RI change in the whole evanescent field (few hundred nanometres). Therefore, for specific cases, it is possible with size-selective detection to discriminate between specific binding of larger molecules, non-specific binding of smaller molecules, and/or bulk effects, to increase the specificity of the sensor. However, it should be explored what will happen if the size of the substances is not 40 times larger but only 2 or 3 times larger. Nevertheless, we were also able to observe timing differences between the protein A and the D-glucose which was not possible before when measuring with a single wavelength.

Moreover, we presented a theoretical approach to discriminate between RI changes from different layers in the evanescent field based on measurements with different wavelengths. This method requires input of theoretical waveguide parameters which do not always fit with the measurements. Some measurements with similar beads did not fit the theoretical model and were different when they were measured in different solutions. Therefore, a much more pragmatic wavelength ratio-based approach was developed which only requires calibration experiments of the substances alone. This method does not require any input of theoretical sizes and RI's of the waveguide and the measured substances, but is just based on the response (ratios of ΔN_{eff} 's of the different wavelengths) of the sensor to the substances which are measured. Information on the absolute amplitude of Δn is lost using this approach, however calibration measurements which are usually also required using an YI with a single wavelength can be performed to determine an analyte concentrations. D-glucose, 85 nm beads and protein A were discriminated from each other using this ratio-based approach. The combined theoretical and ratio-based approach was developed to determine the absolute value of Δn , the corresponding surface mass coverages and/or the size of the measured substances.

Calculations show that by improving the specificity in the form of size-selectivity, the sensitivity is reduced. Moreover, experiments and simulations show that the more different sources (each inducing a Δn) you want to discriminate from each other, the more all noise sources are enhanced and emerge in the data. At the moment the setup is limited to use two wavelengths to reliably discriminate between two substances. When discriminating between three substances is required, the setup needs to be improved. Especially artefacts due to insufficiently reduced boundary effects of the interference pattern should be removed from the measured phase changes. Measuring with multiple wavelengths and polarizations, we tried to improve the setup. Because of the more different response of certain wavelength/polarization combinations the loss in sensitivity can be reduced. This was realised for discrimination between three substances, however, the aforementioned artefacts in the measurement were also increased by these measurements meaning we were not able to improve the setup in this way. Further research is required to realise optimal detection with multiple wavelengths and polarisations.

As the sensitivity is reduced when discriminating between multiple substances, the technique of size-selective detection might be less suitable for the application of biosensing, as generally high sensitivities are required to detect low concentrations of biologically relevant analytes. To explore what are the most relevant applications for the YI (or a similar sensor) in combination with size-selective detection based on multiple wavelengths and what should be next steps in future research, a small technology assessment (TA) study was performed. A mini workshop was organised together with Dr. Haico te Kulve and Dr. Verena Stimberg, both postdoctoral fellows in the department of Science, Technology and Policy Studies (STePS) at the University of Twente. A general introduction to TA, the method (workshop) and the results and findings of this workshop are presented in section 8.2. Section 8.3 presents the reflections on this workshop and the thesis in general and presents a forward look.

8.2 Technology assessment

The main goal of the TA mini-workshop was to obtain input for further research and to look for possible applications and/or innovation pathways. This means that we wanted to know what are interesting applications for the sensor as we look at its capabilities and what questions needed to be answered for that and what future research needs to be performed. In this section we give a small introduction into TA and we explain why we chose for a mini-workshop and what preparations were taken for this workshop. After that we report the most important findings of the workshop and we present conclusions from the TA exercise together with the implications for further research.

8.2.1 Introduction

To further the development and understanding of the YI in combination with size-selective detection, we can and will build upon the insights gained during this project. However, what is interesting from a scientific point of view may not necessarily be important from a valorisation or knowledge utilization perspective. Working with the Dutch NanoNextNL program we were inspired by its ambition to not only perform cutting-edge fundamental and technology research but also to innovate and bring micro- and nanotechnology- enabled technologies closer to the market. *That is, what could be possible applications of the YI with its size-selectivity possibilities and what would this imply in terms of further research questions?* These questions open-up a range of considerations for the introduction of new technologies on the market (i.e. their introduction and acceptance by firms, regulatory authorities and the general public). Thus, another set of evaluation criteria and perspectives come into play compared with a researcher's purely scientific perspective. Examining and anticipating the perspectives of other actors involved in the introduction and development of new technologies such as the YI with size-selective detection then helps to enrich a view on what are societally relevant issues associated with the YI as well as related research questions. Taking into account these issues and questions may support acceleration of commercialization of the YI with size-selective detection capabilities. The perspectives of a broader set of actors may help to open up new research avenues which have not yet been considered before.

To broaden the perspective on possible routes to further research and develop the YI with size-selective detection capabilities, we make use of techniques and approaches from the field of TA. TA involves "the early identification and assessment of eventual impacts of technological change and applications, performed as a service to policy making and decision making more generally" [5]. There exists a broad variety of approaches in this field. Here, we make use of a particular perspective, namely constructive technology assessment (CTA) [6] and how it is applied and developed for emerging technologies such as nanotechnologies [7]. What distinguishes CTA from other TA forms is that it has a strong focus on technological developments and their dynamics and aims to feedback insights of assessments to ongoing technology developments. As we are interested in enriching our perspective on interesting research questions with considerations of societal aspects, CTA seems well suited for our purposes. In the next section we will elaborate the approach we followed to apply a CTA perspective to this project.

8.2.2 Method

To explore possible applications of the YI with size-selective detection, we organized a small interactive workshop. To support discussions during the workshop we applied a so called, 'multi path mapping' tool [8]. This tool shares similarities with what is

known as roadmapping. Roadmapping is a well-known technique within companies as part of their long term planning strategies [9]. A famous example is the International Technology Roadmap for Semiconductors. Specific for the multi path mapping tool is that it (1) identifies different routes, 'paths', towards the development and introduction of products and (2) that it starts from ongoing research activities and takes into account the inherent uncertainty and open-ended character of scientific work. The mapping starts from identifying research lines or functionalities of technologies, then their integration into a platform or product, followed by a specific application area (a product/market combination), and finally the consideration of broader societal aspects regarding the specific application. Mapping potential applications from research lines or envisioned functionalities of specific technologies then results in a variety of possible routes towards applications. The function of this tool is threefold. Firstly, it supports a researcher, or in this case a group of workshop participants, to articulate these different steps and consider puzzles and challenges in each step. Secondly, it supports them to think about possible approaches to deal with these puzzles and challenges and offers an overview. Thirdly, it gives indications of what could be possible main pathways along which these envisioned applications could develop. As these technologies become more developed, and all kinds of technological and social relationships come into place, it becomes increasingly difficult to deviate from these routes.¹ Mapping such pathways then enables the researcher or workshop participants to identify and become aware where it becomes more difficult to deviate from such application routes (e.g. when do you get at the point where it becomes really difficult to change gears, because of sunk investments?).

Constructing a multi-path map can be done by oneself, but it becomes more robust when more people and organizations are involved with different perspectives to the technology. Ideally all stakeholders related to, in our case the YI, would be involved. However, practically and because of the uncertainties involved with new technologies and therefore the challenges to assess future applications, this may be difficult. This was the case for this project, where there was a strong focus on fundamental research with many open-ended questions. Furthermore, an expansive exploration of applications was beyond the scope of this research project. We thus chose to focus in particular on the lower layers of the multi path mapping tool. That is, assessing technological building blocks, possible functionalities and application areas. Therefore, we opted for inviting a mix of researchers with varying disciplinary backgrounds and research orientations, and companies interested in the YI with size-selective detection. This approach looked promising and productive within the scope of this project in order to take into account a broader set considerations for further research.

¹ This phenomenon is known as 'path dependency' and 'path creation'.

We designed a workshop based on a multi-path map in which we presented a first round assessment of different routes towards possible different applications based on research findings. During the workshop the participants of the workshop were encouraged to change the multi-path map and add inputs to the multi-path map by placing post-it notes on the multi-path map (see Appendix 8.B). Furthermore, we made a workshop program including questions per session which could be asked to the participants to encourage the discussion. The multi-path map, workshop program and the workshop participants are presented in Appendix 8.A. The goals of the workshop were:

- I. Joint development of a multi-path map for commercialisation of the Young interferometer in combination with multiple wavelengths.
- II. Identification of factors to consider in the multi-path map, including a) technological/product development issues, b) commercialization aspects, including user/industry requirements; c) possible broader societal aspects such as (health, environmental and safety) risks, regulations (standards, rules/regulations in particular application domains), public acceptance.
- III. Implications of the assessment in terms of (most) plausible and promising innovation pathways, important next steps for further research.

Considering the focus of the workshop and the limited amount of time, priority was given to points i, ii a-b. The workshop participants were invited by sending them a general letter including the goals of the workshop, the workshop program and the multi-path map. Furthermore, a presentation was prepared including background information on the YI in combination with the size-selectivity based on multiple wavelengths and the most important experimental and computational results, to inform workshop participants about this research project.

8.2.3 Findings workshop

In this section we discuss the outcomes of the workshop. The findings from the workshop are presented in Table 8.1 of which we discuss the most important findings, based on their impact on possible applications, here.

A crucial point during the workshop discussion was the meaning of specificity and size-selectivity, because there were different interpretations of the meaning of specificity which could lead to different interesting applications. In biochemistry the specificity relates to the selective attachment or influence of one substance on another, for example an antibody and its specific antigen, but in general it can also mean the ability to correctly detect specific analytes, meaning not measuring false positives. The size-selective measurements can improve the specificity of the sensor using the second more general definition. However, using the first definition from the biochemistry perspective, this is not possible as size-selectivity does not improve the

selective attachment of substances². Therefore, it was decided to not talk about the specificity of the sensor during the workshop, but using the term size-selectivity when talking about discrimination between substances based on their size using the multiple wavelengths in combination with the YI. Furthermore, it was mentioned that the YI using a single wavelength is generally a factor of ten times more sensitive than SPR, but the SPR is a more established technique. The advantage of the YI with multiple wavelengths is the ability to measure size-selectively. However, there is a trade-off: the gain in terms of size-selectivity results in enhancement of noise and drift and therefore in a less stable signal and a reduced sensitivity (comparable to SPR when using two wavelengths to discriminate between two substances). Therefore, it is important to find applications which require size-selectivity and fast detection (which is still possible measuring with multiple wavelengths) and where sensitivity is less important. Thereby, it is important to notice that discriminating between a continuous distribution of size is not possible, so it is important to search for applications where two (or three, when setup is improved) particles with measurable different sizes or locations have to be discriminated. Alternatively, the size-selective YI can be used to determine the size of certain substances.

With all these issues and possibilities in terms of measurement time, sensitivity, specificity and size-selectivity, this led to a lot of suggestions of participants for potential applications for the YI sensor with size-selective detection. A possible application is the deviation based sensor for water quality where they use RI as an indicator of the water quality [10]. Size-selective measurements might be interesting, as surface and bulk effects can be separated. One can also think of applications where size of particles is very important, for example in air pollution, because smaller particles are more hazardous than larger particles. It can also be used for detection and/or characterisation of nanoparticles used in rubber tires to reduce their abrasion. Otherwise, it might be possible to detect or characterise nanoparticles in seawater, asbestos or new materials in material science. Furthermore, ascertaining the authenticity of products in a pharmaceutical plant or a food company was suggested as an application. The size-selective detection could be used to determine if products are indeed the products which were expected by verifying the size of the products e.g. in combination with specific antibodies. Another application might be monitoring the production of certain chemicals at high speed which require a specific size.

The sensor might also be used as alternative for or an addition to other techniques. It can be used to measure kinetics of layer growth or determine layer or particle thickness such that it can be used as an alternative for dynamic light scattering (DLS). Alternatively, the sensor might be used as a quick screener with a yes/no answer based on size before a more elaborate study is done with for example a

² In general it is important to discuss in detail important characteristics of the sensor like sensitivity and specificity during a mapping exercise as it can lead to different outcomes.

scanning electron microscope (SEM) or even as an alternative of SEM as the YI with size-selective detection is an easier and faster method. However, the SEM can still give additional information as it allows to visualise particles, where the YI does not.

Biochemistry plays an important role in biosensors, so there were some suggestions to combine biochemistry with size-selectivity. The sensor might be applicable for virus (50-200 nm) detection where non-specific binding of proteins (1-10 nm) plays a role. Alternatively, specific antibodies could be connected to a spacer to separate them physically from non-specific binding of particles at the surface, such that a difference in size between specific analytes and non-specific particles is not required. By rearranging the linker quickly, so bringing the analyte from a certain distance to very close to the surface, the signal gets enhanced quickly as the signal of the bulk will decrease and the signal at the surface increases. In this case it is required to separate the bulk signal from the signal at the surface which is possible measuring with two different wavelengths. A washing step can be used to get rid of non-specific bound particles to the surface and particles in the bulk, but this is probably not required as the specific signal, coming from bringing the analyte bound to the antibody with a linker close to the surface, can be realised very fast compared to non-specific binding of particles.

It might also be possible to use the sensor to do fundamental binding studies as the sensor is very sensitive for changes at the surface and by using the multiple wavelengths even more information can be obtained. In a similar way it might be possible to study flow dynamics such as laminar flow profiles. According to a participant the sensor might be relevant for work on lab-on-a-chip and research in the field of microfluidics or nanofluidics. The sensitivity is not so important here, but the location of particles in the channel is very important. Finally, it was suggested to use the sensor as a thermometer, because it is possible to suppress surface effects and use bulk signals as a thermometer as the bulk RI is very sensitive for temperature changes.

Next to the applications, also possible markets were suggested for the YI with size-selective detection. Drugs detection was mentioned with the example of heroin and cocaine which are difficult to distinguish. As the difference in average mass is only 66 Da [11], it is probably much smaller than a couple of nanometres which was found for the thickness of protein A that has a molecular weight of 42 kDa [12]. So the YI with size-selective detection is probably not able to discriminate between them based on their size, but there might be other substances which are distinguishable based on their size.

As there are a couple of applications in fundamental research, it was also suggested that the sensor can be used as a research platform in universities or companies in which RIVM or TNO might be interested. In pharmacy, biochemistry is often more important than size-selectivity, so this market seems less suitable when only taking into account the size-selectivity. Furthermore, medical applications seem

to be less suitable as high sensitivity is very important. However, these findings are not definitive as there might always be certain applications where size-selectivity might be more important than biochemistry or sensitivity.

The discussion in the workshop was based on the multi-path map which is presented in Fig. 8.1, where the red coloured parts were added during the workshop. There was not a lot of discussion on the level of societal embedding as the focus was more on the technology and functionality of the sensor and the consequent applications, which is probably a consequence of the background of the participants. It was determined that the unique selling point of the sensor and its advantage over other techniques is the possibility to measure size-selectively. Furthermore, the trade-off between sensitivity and size-selectivity is an important conclusion for commercialisation but also for further research. Moreover, the discussion during the workshop resulted in a lot of new research questions which will be discussed in section 8.2.4, where also the most promising pathway in the multi-path map is presented.

Table 8.1: Issues and solutions per level of the multi-path map of which the points in bold are discussed in the text. Table continues on next page.

	Issues	Solutions
Technology	1) The term specificity might be misleading, it can mean different things. 2) Stability (for long measurements) and sensitivity of the device are the largest challenges in a step to commercialization, however there is a trade-off for size-selectivity and sensitivity and stability. 3) Interferometer performs generally 10x better in terms of sensitivity as the SPR in case of single wavelength measurement. However, SPR is more established technique	1) Use the name of size-selectivity instead of specificity. 2) The added value of YI with multiple wavelengths can be the addition of size-selectivity (vertical resolution) 3) Find application(s) which require(s) size-selectivity and fast detection and where high sensitivity is less important
Functionality	1) A broad particle size distribution makes it more difficult to distinguish between particles. Measuring continuous distribution is not possible. 2) When speaking about sensitivities of fg/mm ² , be very clear what assumptions (e.g. spherical particles, RI, coverage, etc.) are made.	1) Search for applications to discriminate between two or three 'particles' with measurable differences in size

	Issues	Solutions
Product	1) In the case of biosensors, biochemistry plays a more important role than size-selectivity, but these technique can be combined.	<p>Potential applications:</p> <ul style="list-style-type: none"> a) Deviation based sensors for water quality b) Measure particles in gas, air pollution (nanoparticles, size is important, smaller=more hazardous) c) Environmental problem: e.g. nanoparticles in sea water or asbestos d) Nanoparticles in rubber e) Material science f) Pharmacy/food: known products g) Production chemicals at high speed h) Kinetics of layer growth i) Quick screener j) Alternative for/addition to SEM k) Thickness of layers (alternative for DLS) l) Virus detection with a lot of non-specific binding m) Signal enhancer n) Fundamental research on binding or other surface effects o) Flow dynamics p) Thermometer (~bulk signal)
Market	<ul style="list-style-type: none"> 1) Medical applications need mostly high sensitivity 2) Participant: start from the technology and see what it can do, and what advantages it has over existing technologies. Another participant argues that one can also start from promising applications and reason back to functionalities and technologies. 3) In the case of pharmaceuticals, e.g. detecting active pharmaceutical ingredients, biochemistry is more important than size-selectivity 	<ul style="list-style-type: none"> 1) Issue 1) and 3) are not definitive, because there might also be applications for which quick and size-selective measurements are more important than biochemistry or sensitivity, in which it can be combined with biochemistry. 2) In the world of drugs detection, the YI with multiple wavelengths is not probably not able to discriminate between heroin and cocaine (it seems to be difficult to discriminate between them) based on their size as their mass difference is around 66 Da. 3) A research platform which could be used at universities or companies appeared to be the most promising path way. Organizations such as TNO and RIVM might be interested according to participant.
Societal embedding	1) Is it a problem if the IP already is protected and would it be possible to commercialise this platform (in general)?	1) This might still be possible, but at the moment we focus on the technology and functionality and consequent applications of the sensor. An important question is where does this technology offer an advantage over existing technologies. So what are unique selling points? This is the added value of size-selectivity.

Fig. 8.1: Multi-path map after workshop where changes and additions made to the multi-path map during the workshop are marked in red.

8.2.4 Conclusions from technology assessment and implications for further research

From the discussion during the workshop and the issues and solutions resulting from this discussion we selected, according to us, the most promising pathway in the multi-path map as shown in Fig. 8.2. However, it must be noticed that innovation pathways, including impacts on society and implications for research, should be analysed in more depth for a more complete picture. Furthermore, the discussion and its resulting issues and solutions also led to research questions which are presented in Table 8.2. The research questions related to the most promising route are discussed here.

First of all, it is important to know what are the minimum and maximum sizes which can be detected with the devices if a size of a particle or layer should be determined. When discriminating between different sized particles it is important to know what differences in sizes could be measured. So, this means that it is required to know if the cut-off values differ between 1 and 10 nm or between 1 and 100 nm. Further research is required to determine these values which should be compared with the resolution of alternative techniques like SEM or DLS. In terms of sensitivity, we know that we lose approximately an order of magnitude compared to the single wavelength YI when adding an extra wavelength to discriminate between two substances or determine the size of a particle. However, this should be less relevant for the selected applications from Table 7.1 where fast and size-selective detection is more important than sensitivity. With fast measurements we mean that with the current, far from optimized setup, measurements can be done within 5-10 minutes, but this can be optimized further to measurement times smaller than a minute. But most important, the gain in size-selectivity should be worth the loss in sensitivity and stability, and extra costs.

From a market point of view, it is important to know what the requirements are of the particular markets, if the requirements match with the device specifications and if this is not the case what could be done to reach the requirements. When a certain application or market is found, then the sensor should be optimized to determine the limits of the device. As most of the participants were focussed more on the technology, its functionalities and to which product this could lead and not one clear application was discussed, no research questions were constructed in terms of societal embedding.

Fig. 8.2: Illustration of the most promising route of the multi-path map based on the finding from the workshop.

Table 8.2: Research questions per level of the multi-path map.

Research questions	
Technology	1) Can the use of multiple wavelength to add size-selectivity also be applied to other techniques? 2) How fast can you measure your signal? 3) What are applications which require size-selectivity and fast detection and where sensitivity is less important? 4) Is it possible to tune the spatial resolution of the device?
Functionality	1) What are the sizes (minimum and maximum) that can be detected with the device? 2) What differences in size can you determine? Is the cut-off value the difference between 1 nm and 10 nm or 1 nm and 100 nm? 3) What is the positional accuracy? 4) How sensitive can you measure when using size-selective detection? 5) What is the sensitivity/mass coverage of own experiments?
Product	1) What are applications which require size-selectivity above specificity due to biochemistry or sensitivity? 2) For which application does size-selective detection play an important role and could it be an advantage that the YI platform can both catch particles and be able to discriminate them? 3) Is the gain in size-selectivity worth the loss in costs, sensitivity, stability? 4) How can DLS be compared with YI with multiple wavelengths in terms of resolution?
Market	1) What are requirements of the particular markets? 2) Do the requirements match with the device specifications? 3) What is needed to reach the requirements of a specific device?
Societal embedding	

8.3 Conclusions, reflections and forward look

A TA workshop resulted in promising applications for the YI (but also applicable to other evanescent field based sensors) combined with size-selective detection. First of all, it became clear that term specificity can mean different things. Therefore, it was decided to avoid using the term specificity, but to speak about size-selectivity as we discriminate between substance based on their size. If we compare our sensor to other techniques the added value of the sensor is the size-selectivity. However, as the size-selectivity negatively influences the sensitivity and stability of the sensor, we have to look for applications which require fast and size-selective detection and for which sensitivity is less important. The gain in size-selectivity should be worth the loss in costs, sensitivity and stability. Therefore, pharmacy and the medical sector are probably less suitable markets for this sensor, as usually biochemistry and sensitivity are more important than size-selectivity. Different market and applications were discussed during the workshop, resulting in a research platform for universities or companies to be the most promising market, but it must be noticed that markets should be analysed in more depth for a more complete picture. Possible applications in this market are measurement tools to measure the kinetics of layer growth, a signal enhancer, a quick screener for SEM, an alternative for DLS (to measure thickness of layers or particles), a research tool to study fundamental research on binding or other surface effects or flow dynamics. This resulted in research questions *“What are the minimum and maximum size which can be detected and determined?”* and *“What differences in sizes could be discriminated from each other?”*. Further research is required to answer these question and to determine these values which should be compared with the resolution of alternative techniques. When we look at it from a market perspective, it is important to define what the requirements are of the particular markets, if the requirements match with the device specifications and if not, what should be done to reach the requirements. When a certain application or market is found, then the sensor should be optimized to determine the limits of the device. This search process is an interactive process. That is, technological options generate ideas on applications, and vice versa. The discussion focused primarily on technology and performance, where application and market specific considerations and especially societal embedding considerations were backgrounded. Thinking on the multi-path map started from the technology side rather than the application side, which is understandable considering that few participants were present who could discuss an application field. This was intentional in the design of the meeting as the application areas for this device were still very open. From this discussion we analysed the performance characteristics of the device and in which applications and markets this would be seen as useful. A second round could be organised where actors from these markets are present and can thus stimulate more thinking from the application side.

The interactions during the workshop helped to put some prioritization in the pathways identified in advance and those that were added later during the workshop. The identification of applications had the character of opening-up (more applications) and closing-down articulation processes (reducing amount of applications). This occurred mainly from anticipating future performance of the device and assessing where it could make a difference. While few concrete applications were identified, the characteristics of such applications became increasingly clear (size-selectivity and rapid detection more important than sensitivity).

Personal reflection on technology assessment

The TA workshop was a useful tool for me to get a broader look on my research project. The discussions with different people from different fields resulted in new ideas and a clearer view on the future perspectives of the YI using multiple wavelengths to measure size-selectively. By taking the helicopter view it was easier to determine what are most important results and what is the added value of this sensor. Furthermore, I got a clearer view on possible applications and which research questions are important to answer for these applications. To make a TA workshop more useful in general, I think it is better to do such an exercise closer to the beginning (beginning second year) of the project and not in the final year as I did, such that the output of the TA exercise can help to guide the researcher in the right direction and makes him/her more aware of the possible directions he or she could or should take. Finally, it should be noted that the TA exercise takes a lot of time to do it properly. If PhD's are asked to do this, this time should be given to them and also sufficient help from TA people is required. When the TA exercise is done properly, this time can be regained, because it is easier to see where the opportunities lie and therefore better decisions can be made.

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Appendix 8.A Workshop preparations

Fig. 8.A.1: First draft of the multi-path map illustrating possible applications of size-selective detection.

Table 8.A.1: Group composition³

Participant	Function
Ron Gill	Expert biosensors
Christian Blum	Measurement expert, temporary supervisor Harmen
Roy Kolkman	Business development UTwente, Director High tech factory
Daan Sprünken	Physicist, Ostendum R&D b.v., PA Imaging
Vinod Subramaniam	Expert biophysics, director AMOLF, promotor Harmen
Verena Stimberg	Chair, TA researcher
Haico te Kulve	Organizer, TA researcher
Harmen Mulder	Organizer, PhD research project Young interferometer

Table 8.A.2: Workshop program

Time	What	Goals	Comments
10:00	Welcome,	- Everyone gets to know	- Welcome by Harmen
-	introduction	background of participants	- Announce goal of the workshop
10:10	round		- Mapping exercise based on cooperation with Verena and Haico of STEPS (Science, Technology & Policy Studies)
			- Emphasize informality: as much discussion as possible, brainstorm character, asking underlying ideas and considerations
			- Verena will chair the workshop and will watch over the time
			- Introduction round
10:10	Presentation	- Explain research to	- Participants are allowed to ask questions
-	Harmen	participants such that they	for clarification
10:30	about:	know enough about it to	
	- research	participate in the discussion	
	(introduction to research)	- Inform participants on	
	- goals of	program and goals of	
	workshop	workshop	
		- Introduce multi-path map	
		(see Fig. 8.A.1)	
10:30	Identifying	- Identify possible	- 5 minutes: participants write down on
-	applications	applications if multiple	post-it note: functionality, product, market.
10:55	(make use of	wavelengths are used to	One per post-it note. Or they make post-it
	multi-path	improve specificity of YI	not to change the current multi-path map.
	map, post-it	sensor	- Round: participants explain post-it note.
	notes can be	- Identify possible	Other participants are asked to respond on
	added by	applications of YI sensor with	this, ask to assumptions, underlying
	participant /	multiple wavelengths next to	thoughts about the assessment.
	organizer)	using it for improvement of	Possible questions to participants:
		specificity of het sensor	- Are functionalities/products/markets
			relevant? Why or why not?
			- Are functionalities/products/markets
			desirable? Why or why not?
			- Is sensing of diffusion possible and is this
			interesting?

³ Participants agreed with mentioning their names

Time	What	Goals	Comments
10:55 - 11:35	Assessing innovation pathways (make use of multi-path map, post-it notes can be added by participant / organizer)	<ul style="list-style-type: none"> - Identify accompanying pathways of possible applications (from technology to market (possibly societal embedding)) - Assess pathways: identify technological/commercial and broader challenges/opportunities in aligning technologies – functionalities – products – markets – societal embedding. - Iterations: identification of new applications, new pathways and challenges 	<ul style="list-style-type: none"> - 5 minutes: participants write down on post-it note: points of interest and solutions of functionality/product/market - Round: participants explain post-it note. Other participants are asked to respond on this, ask to assumptions, underlying thoughts about the assessment.
11:35 - 11:50	Implications of assessment (make use of multi-path map: arrows, exclamation marks)	<ul style="list-style-type: none"> - Identification of most plausible and promising pathways - Identification of next steps in research - Identification of possible collaborations? 	<ul style="list-style-type: none"> Possible questions to participants: - What are the most promising routes? - What has to be done to realise this? - What is the vision of the experts? - What is the vision of the business developer?
11:50 - 12:00	Conclusions	<ul style="list-style-type: none"> - write down most important conclusions - summarize answer to research questions - thank participants 	<ul style="list-style-type: none"> - What are the most important findings? - What are most striking insights?

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List of abbreviations

Abbreviation	Meaning
BiMW	Bimodal waveguide
CCD	Charge-coupled device
CT	Crosstalk
CTA	Constructive technology assessment
DLS	Dynamic light scattering
DPI	Dual polarization interferometer
DPSS	Diode-pumped solid state
ELISA	Enzyme-linked immunosorbent assay
FFT	Fast Fourier transform
IO	Integrated optical
IP	Intellectual property
LED	Light-emitting diode
LOD	Limit of detection
MZI	Mach-Zehnder interferometer
NE	Effective refractive index error
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phase error
pk-pk	Peak-to-peak
PTC	Photon transfer curve
Q-factor	Quality factor
RI	Refractive index
RIU	Refractive index units
RMS	Root mean square
SEM	Scanning electron microscope
SNR	Signal-to-noise ratio
SP	Surface plasmon
SPR	Surface plasmon resonance
TA	Technology assessment
TE	Transverse electric
TIR	Total internal reflection
TM	Transverse magnetic
WGM	Whispering gallery modes
WIOS	Wavelength interrogated optical sensor
YI	Young interferometer

Summary

This thesis presents a Young interferometer (YI) biosensor that can perform size-selective measurements using multiple wavelength excitation. This size-selective detection can be used to improve the specificity of evanescent field-based optical biosensors and is based on the various sensitivities of the evanescent fields of multiple wavelengths. The approach of using multiple wavelengths is, in addition to YI biosensors, also applicable to other types of evanescent field-based optical sensors. In Chapter 1 we first introduce the general concept of biosensors, followed by a discussion of important criteria for biosensors. The most widely-used evanescent field-based optical sensors are reviewed. The limiting specificity of these sensors is addressed, together with techniques to improve the specificity. Finally we propose the new approach of size-selective detection, based on the use of multiple wavelengths to improve the specificity of evanescent field-based optical biosensors.

In Chapter 2 we present a size-selective detection method for integrated optical interferometric biosensors that could strongly enhance their performance. We demonstrate that by launching multiple wavelengths into a YI waveguide sensor it is feasible to derive refractive index changes (Δn) from different layers above the waveguide surface, enabling one to distinguish between bound particles (e.g. proteins, viruses, bacteria) based on their differences in size and simultaneously eliminating interference from a Δn in the bulk (region of a few hundred nanometres above sensor surface). Numerical calculations are used to optimize sensor design and the detection method. Adding size-selectivity to the sensor reduces the sensitivity of the sensor. However, the theoretical sensitivity remains still comparable to other existing biosensors when discriminating between Δn 's in three different layers above the waveguide based on simultaneous detection of effective refractive index changes (ΔN_{eff}) at three different wavelengths. Assuming a particle of 80 nm in size as the specific analyte to detect, the theoretically determined minimum detectable mass coverage is 4×10^2 fg/mm² (assuming a phase noise of 10^{-4} fringes). This is approximately one order of magnitude higher than the minimum detectable mass coverage with the YI using a single wavelength. However, with size-selective detection it is now possible to discriminate the 80 nm sized analyte binding from non-specific bound particles of 10 nm size and simultaneously occurring bulk changes.

Chapter 3 presents the design, realization, and characterization of a YI sensor setup capable to measure simultaneously ΔN_{eff} 's at multiple wavelengths to achieve size-selectivity. First, the requirements and an overview of the setup are presented. Next, we describe the realization and characterization of the six main modules of the setup: light sources, incoupling, sensing platform, imaging, detection and data processing. Finally, we characterise the phase noise and phase drift (unexpected

phase changes on a longer time scale) of the setup. It was found that the measured phase noise on short time scales is determined by photon shot noise. A CCD camera with a high dynamic range was implemented in the setup such that the measured phase noise is smaller than 10^{-4} fringes @ 1 Hz. Phase drift is smaller than 5×10^{-3} fringes per 1000 s for the setup with end-fire coupling. The incoupling was made more time efficient by using a fiber and butt-end coupling. However, the phase drift increased to values smaller than 1.5×10^{-2} fringes per 1000 s, partly caused by positional drift of the fiber. Drift in distance between fiber and chip resulted in phase oscillations which could be solved using a matching index gel. It was also found that air flow can strongly influence the phase signal, so the setup was covered. Moreover, changes in the ambient temperature have an effect on the phase stability as it can lead to thermal expansion of components of the setup and the chip itself. This can lead to changes in the position of the chip with respect to the camera which results in phase changes. Next to drift also artefacts are observed in the phase signal. The origin of these artefacts is investigated in this chapter. Possible reasons of the artefacts are aberrations of grating and lenses in the imaging part of the setup and boundary effects of the shifting interference pattern. The artefacts show up as oscillations in the phase change with an amplitude in the order of 10^{-2} fringes. Induced signals should be significantly higher than drift and artefacts to minimize the influence of drift and artefacts on the determined Δn 's.

In Chapter 4 we present a description and a detailed study of different signal analysis approaches that are required to obtain the signal from the different substances from the measured phase changes at the different wavelengths. It was found that a theoretical approach as presented in Chapter 2 is exact but in practice difficult to implement because of the many parameters that have to be tuned. Therefore, we also developed a much more practical ratio-based approach based on the ratios of ΔN_{eff} 's measured at different wavelengths. These ratios were determined independently for 85 nm carboxylated polystyrene beads (representing specific binding of e.g. viruses which have approximately this size), protein A (representing non-specific binding) and D-glucose (representing bulk changes). These ratios are used to discriminate between the Δn 's induced by these substances which are applied in the proof-of-principle experiments for size-selective detection. On the other hand, using this ratio-based approach it is not possible to determine an absolute value of Δn . However, this is not strictly required, because calibration measurements, which are usually also carried out by a single wavelength YI, can be used to calibrate the measured values of Δn with an analyte concentration. The theoretical and ratio-based approach were combined to determine the absolute value of Δn and the corresponding surface mass coverage. Moreover, the approaches were combined to determine the thicknesses of 85 nm beads to be ≈ 72.6 nm and protein A to be ≈ 2.1 nm which are in agreement with literature. Furthermore, the influence of the

parameters that are required for the analysis approaches (e.g. waveguide core thickness, waveguide refractive indices, ratios of ΔN_{eff} 's) on the determined Δn 's caused in different layers or by different substances was tested. Finally, simulations of a real experimental setting confirm the theoretical analysis given in Chapter 2 that noise, drift and artefacts show up enhanced in the determined Δn 's.

In chapter 5 we present the experimental application of the size-selective analyte approaches presented in Chapter 4. We use the different analysis approaches to discriminate between binding of 85 nm beads from binding of protein A, and binding of 85 nm beads or protein A from D-glucose bulk changes by measuring ΔN_{eff} at two wavelengths. Three wavelengths were used to discriminate between three substances simultaneously, however this led to noisy results which leaves the applicability useless at this moment. For a successful application of these analysis approaches used to determine three independent Δn 's, the aforementioned artefacts and drift in ΔN_{eff} should be reduced significantly. Alternatively, it was shown that other techniques to improve specificity, such as the use of a reference channel to compensate for bulk changes, can be used next to size-selective detection. The working of the ratio-based approach to discriminate between two substances was verified by a blind experiment with samples containing different concentrations of protein A and 85 nm beads. Furthermore, a triplicate of measurements series with different bead concentrations and a constant protein A concentration showed that we could discriminate Δn 's caused by binding of protein A and 85 nm beads, even when the Δn of the protein was approximately 20 times higher. This can for example be used to discriminate specific analyte binding of larger particles from non-specific binding of smaller particles to improve the performance of the YI sensor and IO interferometric sensors in general.

Until now, size-selective detection with the YI sensor was done using multiple wavelengths based on their different mode profiles and their different sensitivities in different layers in the evanescent field. Chapter 6 shows that in a similar way, multiple polarizations can also be used to discriminate between Δn 's of multiple layers or multiple different-sized substances. Calculations from chapter 2 showed that the more different the sensitivity coefficients of each mode, the less singular the matrix to solve Δn 's in multiple layers or induced by multiple substances, meaning that all noise sources in ΔN_{eff} show up less enhanced. We show that it is possible to measure multiple polarizations and multiple wavelengths simultaneously, resulting in a matrix which is less singular for some cases and therefore representing an improvement of the approach in these cases. However, it should be noticed that measuring simultaneously with multiple wavelengths and polarizations also led to an increase of the aforementioned artefacts in the measurements. For a successful application of the use of multiple polarizations and wavelengths for size-selective detection, the artefacts in the measurements should be reduced significantly.

From the determined Δn 's we saw that the Δn due to binding of protein A to the surface occurred earlier than the Δn due to protein A in the bulk. However, it was expected that protein A should arrive in the bulk before it can attach to the surface. In Chapter 7 this unexpected time delay is discussed. Finite element method simulations on the diffusion and binding of molecules show that a time delay between surface binding of protein A and protein A bulk changes can arise when protein A has a high affinity for the surface. The bulk region is depleted due to the fact that protein A is strongly attracted towards the surface. The time delay increases with an increasing maximum surface capacity $\gamma_{\max}$ and increasing adsorption rate k_a . For a k_a of 10^5 $\text{m}^3/\text{mol/s}$ and a $\gamma_{\max}$ between 4.4×10^{-3} and 1.7×10^4 fg/mm^2 (corresponding to a protein A radius of 1 and 2 nm respectively which agrees well to the measured thickness of 2.1 nm (see Chapter 4)), a time delay of approximately 20 s to 100 s is found. This is comparable to the observed time delay. To verify the results of the simulations, protein A and D-glucose were added simultaneously to the sensor. Due to the fact that D-glucose has a much higher diffusion than protein A it was expected that Δn due to D-glucose bulk changes would appear earlier now. However, again the signal of Δn due to binding of protein A occurred earlier than the Δn due to D-glucose. Experiments hint that this time delay is related to the transport towards the sensing window as increasing the transporting tube length resulted in an increasing time delay. Experiments showed that the time delay also occurred for bovine serum albumin, suggesting that the time delay is independent of the protein used. However, the exact origin of the time delay between D-glucose bulk changes and surface binding of protein A is not found.

Chapter 8 explores what are the most relevant applications for the Young interferometer (or a similar) sensor in combination with size-selective detection based on multiple wavelengths and/or polarisations. In this chapter we also address what should be next steps in future research based on the conclusions drawn from the other chapters. For that purpose, a small technology assessment study was performed and a mini-workshop was organised to identify possible innovation pathways and relevant factors to consider from a technological, product and commercialization point of view. From the assessment it was determined that the added value of the sensor is to measure size-selectively (discriminate between substances based on their size) and that the gain in size-selectivity should be worth the loss in sensitivity and stability and the increasing costs. Therefore, pharmacy and the medical sector are probably less suitable markets for this sensor, as in these application areas, specific biochemistry and sensitivity are more important than size-selectivity. A research platform for universities or companies was found to be the most promising market, but it must be noticed that markets should be analysed in more depth for a more complete picture. Possible applications in this market are measurement tools to measure the kinetics of layer growth, a signal enhancer, a quick screener or an

alternative for a Scanning Electron Microscope, an alternative for Dynamic Light Scattering (to measure thickness of layers or particles) and a research tool to study fundamental research on binding or other surface effects or flow dynamics. This resulted in research questions “*What are the minimum and maximum size which can be detected and determined?*” and “*What differences in sizes could be discriminated from each other?*”. Further research is required to answer these questions. When a certain application or market is identified, then the sensor should be optimized to determine the limits of the device for the specific application in mind.

Samenvatting

In dit proefschrift wordt een Young interferometer (YI) gepresenteerd die selectief kan meten op basis van grootte, gebruikmakende van detectie met meerdere golflengtes. De selectiviteit op basis van grootte kan worden gebruikt om de specificiteit van optische biosensoren (die gebruik maken van detectie met het evanescente veld) te verbeteren en is gebaseerd op de verschillende gevoeligheden van de evanescente velden van de verschillende golflengtes. De benadering van het gebruik van meerdere golflengtes is naast de YI ook toepasbaar op andere types van evanescente-veld-gebaseerde optische sensoren. In hoofdstuk 1 introduceren we eerst het algemene concept van biosensoren, gevolgd door een discussie van de belangrijkste criteria van biosensoren. De meest gebruikte evanescente-veld-gebaseerde sensoren worden besproken. Daarnaast wordt de gelimiteerde specificiteit van deze sensoren aan de orde gesteld, samen met technieken die gebruikt kunnen worden om de specificiteit te verbeteren. Ten slotte wordt de nieuwe benadering van selectiviteit op basis van grootte (grootte-selectieve detectie) gebaseerd op detectie met meerdere golflengtes voorgedragen om de specificiteit van evanescente-veld-gebaseerde optische sensoren te verbeteren.

In hoofdstuk 2 presenteren we een grootte-selectieve detectiemethode voor geïntegreerde optische interferometrische biosensoren die hun presentaties sterk kunnen verbeteren. We demonstreren dat het mogelijk is om brekingsindexverschillen (Δn) te bepalen in verschillende lagen boven het oppervlakte van de golfgeleider door middel van detectie met meerdere golflengtes. Hiermee maken we het mogelijk om gebonden deeltjes (bijvoorbeeld eiwitten, virussen, bacteriën) te onderscheiden van elkaar op basis van grootte en dit tegelijkertijd te onderscheiden van een homogene Δn in het complete evanescente veld (bulk). Numerieke berekeningen zijn gebruikt om het sensorontwerp en de detectiemethode te optimaliseren. Het toevoegen van grootte-selectiviteit leidt tot reductie van de gevoeligheid van de sensor. De theoretische gevoeligheid blijft echter nog steeds vergelijkbaar met de gevoeligheid van andere vergelijkbare biosensoren wanneer we onderscheid maken tussen Δn 's van drie verschillende lagen, gebaseerd op simultane detectie van drie verschillende golflengtes. Als we aannemen dat de deeltjes die we detecteren 80 nm groot zijn, dan is de theoretisch bepaalde minimaal detecteerbare oppervlaktetmassadekking 4×10^2 fg/mm² (een faseruis aannemende van 10^{-4} fringes). Dit is ongeveer één ordegrrootte hoger dan de minimaal detecteerbare oppervlaktetmassadekking van de YI op basis van detectie met een enkele golflengte. Echter met grootte-selectieve detectie is het nu mogelijk om de binding van de 80 nm grote analyten te onderscheiden van non-specifieke binding van deeltjes van 10 nm groot en tegelijkertijd plaatsvindende bulkveranderingen.

Hoofdstuk 3 presenteert het ontwerp, de realisatie en de karakterisering van de YI sensor opstelling die het mogelijk maakt om simultaan effectieve brekingsindexverschillen (ΔN_{eff}) te detecteren bij verschillende golflengtes om grootte-selectief te kunnen meten. Eerst worden de eisen en een overzicht van de opstelling gepresenteerd. Daarna beschrijven we de realisatie en karakterisering van de zes belangrijkste onderdelen van de opstelling: de lichtbronnen, de inkoppeling, het sensorplatform, het afbeelden, de detectie en de gegevensverwerking. Ten slotte karakteriseren we de faseruis en fasedrift (onverwachte faseveranderingen op een grotere tijdschaal) van de opstelling. De gemeten faseruis op korte tijdschaal wordt bepaald door foton-hagelruis. Een CCD-camera met een hoog dynamisch bereik is geïmplementeerd in de opstellingen zodat de gemeten faseruis kleiner is dan 10^{-4} fringes. De fasedrift is gebruikelijk lager dan 5×10^{-3} fringes over 1000 s voor de opstelling met inkoppeling van het licht in de golfgeleider via een lens. De inkoppeling is tijdefficiënter gemaakt door het licht in te koppelen via een fiber. Echter de bijkomende fasedrift nam toe tot waardes lager dan 1.5×10^{-2} fringes over 1000 s, gedeeltelijk bepaald door het langzaam wegdrijven van de fiber. Drift in afstand tussen de fiber en de chip resulteerde in faseoscillaties. Dit probleem kon opgelost worden door gebruik te maken van een matching-index-gel. Verder is gemeten dat luchtstromingen het fasesignaal sterk kunnen beïnvloeden, waardoor we de opstelling volledig hebben bedekt. Tevens hebben omgevingstemperatuurveranderingen invloed op de fasedrift, omdat het kan leiden tot thermische uitzetting van componenten in de opstelling en de chip zelf. Dit kan leiden tot verandering in de positie van de chip ten opzichte van de camera, wat resulteert in faseveranderingen. Naast de drift zijn ook artefacten gemeten in het fasesignaal. De oorsprong van deze artefacten zijn in dit hoofdstuk onderzocht. Mogelijke oorzaken zijn de afwijkingen van de tralie of lenzen tussen de chip en de camera of randeffecten van een verschuivend interferentiepatroon. De artefacten zijn te zien als oscillaties in het fasesignaal met een amplitude in de ordegrrootte van 10^{-2} fringes. Geïnduceerde signalen moeten significant hoger zijn dan de drift en de artefacten om de invloed van de drift en artefacten op de bepaalde Δn 's te minimaliseren.

In hoofdstuk 4 presenteren we de beschrijving en een gedetailleerde studie van verschillende analysemethodes die nodig zijn voor het verkrijgen van de Δn 's van meerdere substanties/laagjes op basis van de gemeten faseveranderingen met meerdere verschillende golflengtes. Het is bevonden dat de methode van hoofdstuk 2 exact is, maar in de praktijk moeilijk te implementeren vanwege het feit dat er vele parameters afgesteld moeten worden. Daarom hebben we een veel praktischere methode ontwikkeld gebaseerd op de ratio's van de ΔN_{eff} gemeten bij verschillende golflengtes. Deze ratio's zijn afhankelijk gemeten voor 85 nm gecarboxyleerde polystyrene bolletjes (representeren specifieke binding van bijvoorbeeld virussen die ongeveer deze grootte hebben), het eiwit proteïne A (representeert non-specifieke

binding) en D-glucose (representeert bulkveranderingen) zodat deze ratio's gebruikt kunnen worden om onderscheid te maken tussen deze substanties die gebruikt zijn voor de experimenten om aan te tonen dat grootte-selectieve detectie werkt. Aan de andere kant is het niet mogelijk om, op basis van de ratio-gebaseerde methode, absolute waarden van Δn te meten. Dit is echter ook niet noodzakelijk, omdat kalibratiemetingen, die normaal ook gebruikt worden bij detectie met een enkele golflengte, gebruikt kunnen worden om de gemeten waarden van Δn te kalibreren met een analytconcentratie. De theoretische methode en de ratio-gebaseerde methode zijn ook gecombineerd om de absolute waarden van Δn en de corresponderende oppervlaktetotaledekking te bepalen. Verder zijn deze methodes gecombineerd om de grootte van de 85 nm bolletjes te bepalen als ≈ 72.6 nm en proteïne A als ≈ 2.1 nm, wat overeenkomt met de literatuur. Verder is de invloed van inputparameters, die nodig zijn voor de analysebenaderingen (bijvoorbeeld golfsnelheidsindex en golfsnelheidsbrekingsindices, ratio's van ΔN_{eff}), op de bepaalde Δn 's van de verschillende laagjes of substanties getest. Ten slotte bevestigen simulaties van een echte experimentele setting dat ruis, drift en artefacten versterkt tevoorschijn komen in de bepaalde Δn 's.

In hoofdstuk 5 presenteren we de experimentele toepassing van de bovengenoemde analysemethoden voor grootte-selectieve detectie. We gebruiken de verschillende analysemethoden om te onderscheiden tussen de binding van 85 nm bolletjes en de binding van proteïne A en om D-glucose bulkveranderingen te onderscheiden van de binding van 85 nm bolletjes of de binding van proteïne A, door ΔN_{eff} te meten bij twee golflengtes. Drie golflengtes worden gebruikt om te onderscheiden tussen alle drie substanties tegelijkertijd. Dit leidde echter tot resultaten waarin de ruis versterkt zichtbaar werd, waardoor grootte-selectieve detectie, om te onderscheiden tussen Δn 's van drie substanties/laagjes op basis van drie golflengtes, met de huidige opstelling op dit moment onbruikbaar is. Voor succesvolle applicatie van de analysemethoden om drie Δn 's te onderscheiden, zullen eerdergenoemde artefacten in ΔN_{eff} en drift significant gereduceerd moeten worden. Als alternatief is het laten zien dat het ook mogelijk is om andere technieken om specificiteit te verbeteren, zoals het gebruik van een referentiekanaal om bulkeffecten te compenseren, te gebruiken naast de grootte-selectieve detectie. De werking van de ratio-gebaseerde methode is geverifieerd met een blind experiment met verschillende monsters bestaande uit verschillende concentraties van proteïne A en 85 nm bolletjes. Daarnaast liet een drievoud van meetseries met verschillende 85 nm bolletjes concentraties en een constante proteïne A concentratie zien dat we konden onderscheiden tussen Δn 's veroorzaakt door de binding van bolletjes en de binding van eiwitten, zelfs als de Δn van het eiwit 20 keer zo hoog was als de Δn van de bolletjes. Dit kan bijvoorbeeld worden gebruikt om te onderscheiden tussen specifieke analytbinding van grotere deeltjes en non-specifieke binding van kleinere deeltjes om

de presentaties van de YI sensor en geïntegreerde optische sensoren in het algemeen verbeteren.

Tot nu toe werd de grootte-selectieve detectie met de YI sensor uitgevoerd met meerdere golflengtes gebaseerd op hun verschillende gevoeligheden in verschillende lagen in het evanescente veld. Hoofdstuk 6 laat zien dat op een vergelijkbare manier ook verschillende polarisaties gebruikt kunnen worden om te onderscheiden tussen verschillende Δn 's. Berekeningen van hoofdstuk 2 lieten zien dat hoe meer de gevoeligheden van de verschillende golflengtes van elkaar verschillen, hoe minder singulier de matrix (die nodig is om Δn 's te bepalen) is. Dit betekent dat de ruis in de ΔN_{eff} 's minder versterkt tevoorschijn komt in de bepaalde Δn 's. We laten zien dat het mogelijk is om meerdere polarisaties en golflengtes simultaan te meten, resulterende in een minder singuliere matrix voor sommige gevallen en daarom een verbetering van de benadering in deze gevallen. Het moet echter worden opgemerkt dat het simultaan meten van meerdere golflengtes en polarisaties leidde tot een toename van artefacten in de metingen. Voor een succesvolle toepassing van grootte-selectieve detectie op basis van verschillende polarisaties en golflengtes zullen de artefacten in de metingen significant gereduceerd moeten worden.

Uit de bepaalde Δn 's zagen we dat de Δn door binding van proteïne A aan het oppervlakte eerder plaats vond dan de Δn door de proteïne A in de bulk. Het was echter verwacht dat de proteïne A eerst in de bulk zou moeten arriveren voordat het kan binden aan het oppervlakte. In hoofdstuk 7 wordt deze onverwachte tijdsvertraging bediscussieerd. Eindige-elementenmethode-simulaties naar de diffusie en binding van moleculen laten zien dat de tijdsvertraging kan ontstaan wanneer proteïne A een hoge affiniteit heeft met het sensoroppervlakte. Doordat de proteïne A sterk wordt aangetrokken door het oppervlakte zal er depletie van de bulkregio plaatsvinden. De tijdsvertraging neemt toe met een toenemende maximale oppervlaktecapaciteit γ_{max} en een toenemende adsorptieconstante k_a . Voor een k_a van $10^5 \text{ m}^3/\text{mol/s}$ en een γ_{max} tussen 4.4×10^{-3} en $1.7 \times 10^4 \text{ fg/mm}^2$ (komen overeen met respectievelijk een proteïne A radius van 1 en 2 nm, wat ongeveer overeenkomt met de bepaald dikte van 2.1 nm in hoofdstuk 4) is er een tijdsvertraging van 20 s tot 100 s gevonden. Deze tijdsvertragingen zijn vergelijkbaar met de gemeten tijdsvertragingen. Om de resultaten van de simulaties te verifiëren, zijn D-glucose en proteïne A tegelijkertijd aan de sensor toegevoegd. Doordat D-glucose een veel hogere diffusie heeft dan proteïne A, werd verwacht dat de Δn door D-glucose eerder zou plaatsvinden. Echter opnieuw vond de Δn door de binding van proteïne A eerder plaats dan de Δn van D-glucose. Experimenten hintten ernaar dat de vertraging wordt veroorzaakt tijdens het transport naar het sensorgebied toe, omdat de vertraging toeneemt naarmate de slanglengte van het transport werd vergroot. Ook laten experimenten zien dat de tijdsvertraging optreedt bij het eiwit bovine serum albumine, suggererende dat de tijdsvertraging onafhankelijk is van het gebruikte

eiwit. Een precieze oorzaak van de tijdsvertraging tussen bulkveranderingen door D-glucose en de binding van proteïne A is echter niet gevonden.

Hoofdstuk 8 zoekt wat de meest relevante toepassingen zijn voor de YI (of een vergelijkbare) sensor in combinatie met grootte-selectieve detectie gebaseerd op detectie met meerdere golflengtes en/of polarisaties. Verder onderzoeken we in dit hoofdstuk wat vervolgstappen zouden moeten zijn in vervolgonderzoek, gebaseerd op de conclusies getrokken uit de vorige hoofdstukken. Hiervoor is een klein technologisch aspectenonderzoek uitgevoerd en is een mini-workshop georganiseerd, om mogelijke innovatiepaden en relevante factoren op het gebied van technologie, product en commercialisatie te identificeren. Uit dit onderzoek is vastgesteld dat de toegevoegde waarde van de sensor is dat het grootte-selectief kan meten (dus kan onderscheiden tussen substanties op basis van hun grootte) en dat de winst die hierin te behalen valt moet opwegen tegen het verlies in gevoeligheid en stabiliteit en de toename in kosten. Daarom zullen de farmacie en de medische sector waarschijnlijk minder geschikte markten zijn, omdat in deze toepassingsgebieden specifieke biochemie en gevoeligheid belangrijker zijn dan grootte-selectiviteit. Een onderzoeksplatform voor universiteiten of bedrijven bleek de meest veelbelovende markt, maar het moet in acht worden genomen dat markten dieper geanalyseerd moeten worden voor een completer beeld. Mogelijke toepassingen in deze markt zijn meetgereedschappen om kinetiek van laaggroottes te bepalen, een signaalversterker, een snelle scanner of een alternatief voor een rasterelectronmicroscop (SEM). Tevens kan het gebruikt worden als een alternatief voor Dynamic Light Scattering (om grootte van lagen of deeltjes te meten) of als een onderzoeksmiddel om fundamenteel onderzoek te doen naar binding, andere oppervlakte-effecten of stromingsdynamica. Dit resulteerde in de volgende onderzoeksvragen: *“Wat zijn de minimale en maximale grootte die gedetecteerd en bepaald kunnen worden?”* en *“Wat zijn de verschillen in grootte die van elkaar onderscheiden kunnen worden?”*. Meer onderzoek is vereist om deze vragen te beantwoorden. Wanneer een bepaalde toepassing of markt is geïdentificeerd, zal de sensor moeten worden geoptimaliseerd om vast te stellen wat de limieten van het instrument zijn voor deze specifieke toepassing.

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