Biosensing by Direct Observation of Leaky Waveguide Modes

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Abstract. The resonance angles of leaky waveguides (LWs) can be observed directly as exponentially decaying interference fringes in waveguides with refractive index that is 0.001–0.01 higher than cover, and when illuminated with wedge-shaped light beam. This work for the first time shows that such LWs can be realised using hydrogels of acrylamide copolymerised with N-(3-aminopropyl)methacrylamide hydrochloride (APMA). LWs made of 4.5% (w/v) acrylamide/APMA crosslinked with poly(ethylene glycol) diacrylate (PEGDA, Mₙ: 700Da) were ~59% and ~56% porous to 100kDa and 300kDa species, which are typical molecular weights of biomolecules, and hence were selected for biosensing of immunoglobulin G (IgG) with protein A–biotin (PAB) as biomolecular recognition element. The limit of detection of the acrylamide/APMA LW for IgG was 3.69±0.37 nM, which is at least 1/100th of the dissociation constant of the PAB–IgG binding partners used in this work. Future work will investigate the application of acrylamide/APMA LW for biosensing of clinically relevant analytes.

1. Introduction
Leaky waveguides (LWs) comprise of a few micron thick film of refractive index (RI) that is lower than the substrate, but higher than the cover, which is typically a sample solution. Light is therefore partially confined in LWs via Fresnel reflection and total internal reflection (TIR) at waveguide/substrate and waveguide/cover interfaces, respectively (1). The angles of incidence at which light is partially confined in LWs are called resonance angles (θₚ). The position of θₚ is determined by the effective mode index of the waveguide, which in turn depends on the RI of the cover and waveguide (2). Thus, shifts in resonance angle (Δθₚ) are observed because of changes in the RI on/in the waveguide. The RI changes on/in the waveguide may be caused by analytes to be measured, forming the basis of sensing using LWs. It is therefore essential to determine θₚ and measure Δθₚ. Previously, θₚ of LWs has been observed indirectly by using the following approaches: (i) depositing a metal layer between substrate and waveguide (3), (ii) introducing known amount of optical losses in the waveguide by covalent attachment of dye molecules in the waveguide (4), and (iii) making an array of strips of LWs to obtain a leaky waveguide grating (LWG) (5). Interposing a metal layer requires vacuum processing and can cause problems with adhesion of the waveguide layer. Incorporating absorbing species such as dyes in the waveguide can reduce the number of available binding sites for recognition elements and can increase non-specific binding. The fabrication of LWGs is more challenging than LWs with a continuous waveguide layer. Thus, being able to observe LW resonances directly simplifies the fabrication of the sensor and avoids problems arising from the additional elements introduced by the visualisation method.

In 2020, we showed that the θₚ can be observed directly (6) by illuminating LWs with a range of angles of incidence simultaneously using a wedge-shaped light beam. Qualitatively, when LWs are illuminated using a wedge-shaped beam, light at the θₚ of LWs is coupled into the waveguide and propagates along
while coupling out as exponentially decaying substantially plane wavefronts that are \( \pi \) phase shifted with respect to incident light. Light at angles other than the \( \theta_R \) of LWs is not coupled into the waveguide and is reflected. This reflected light has cylindrical wavefronts and undergoes a \( 2\pi \) phase shift with respect to incident light around the resonance angle(s). As shown in Figure 1, the curved wavefronts of the reflected uncoupled light and the plane wavefronts coupled out of the waveguide interfere, producing exponentially decaying interference fringes at the \( \theta_R \) of LWs. In essence, the LW acts as a phase object in incident angle space.

![Figure 1. Diagram showing the interference between the reflected curved wavefronts and the plane wavefronts coupled out of the LW](image)

We also showed that the \( \theta_R \) of LWs can be observed directly only when the RI difference between the waveguide and cover is 0.001–0.01. This is because as the RI of the waveguide increases, the distance over which light propagates in the waveguide decreases and hence \( w \) (marked in Figure 1) reduces. This in turn decreases the overlapping region between the reflected uncoupled light and the plane wavefronts coupled out of the waveguide, resulting in fewer exponentially decaying interference fringes. Eventually, as the RI between the waveguide and cover increases to >0.01, no interference fringes are observed. LWs that result in exponentially decaying interference fringes at the \( \theta_R \) are called diffraction-based LWs (1).

The realisation of diffraction-based LWs requires that the waveguide layer is made of materials with low RI difference with respect to the cover, and hydrogels can fulfil this requirement. It is also beneficial to make LWs out of hydrogels because they provide a physiologically similar environment conducive for biosensing (2). Equally, it is advantageous to make LWs of porous hydrogels so that biomolecular recognition elements can be immobilised in the volume of waveguides to increase the number of available binding sites for analytes, and analyte-biomolecular recognition elements can interact with a large fraction of light confined in waveguides. As a result, the sensitivity of porous LWs is \( \sim \)9 times better than non-porous LWs (7). Finally, it is preferable to use synthetic hydrogels because they offer a higher degree of control over the density of functional groups (and hence binding sites for analytes) and better batch-to-batch reproducibility than natural materials (8). This work demonstrates the feasibility of using hydrogel waveguides of acrylamide copolymerised with N-(3-aminopropyl)methacrylamide hydrochloride (APMA) to serve as waveguide layers for diffraction-based LWs. We discuss the parameters that affect the porosity of acrylamide/APMA hydrogel waveguides. Finally, this work shows the suitability of acrylamide/APMA diffraction-based LWs for biosensing using protein A–biotin (PAB) and immunoglobulin G (IgG) as an exemplar biomolecular recognition element and analyte system.

2. Experimental
2.1. Chemicals and materials
1 mm thick glass microscope slides were purchased from VWR. Decon 90 was purchased from Fisher Scientific. The following were bought from Sigma-Aldrich: acrylamide, bis-acrylamide, N-(3-
aminopropyl)methacrylamide hydrochloride (APMA), ammonium persulphate (APS), N,N,N',N'-tetramethylethlenediamine (TEMED), poly(ethylene glycol) diacrylate (PEGDA, $M_\text{w}$: 700Da), 3 µm latex beads, hexamethyldisilazane (HMDS), trimethoxy(3,3,3-trifluoropropyl)isilane (TMPTS), chloro(dimethyl)vinylsilane (CDVS), toluene, glycerol, poly(ethylene glycol) (PEG) of molecular weight 35kDa, 100kDa and 300kDa, sodium phosphate monobasic monohydrate, sodium phosphate dibasic dodecahydrate, protein A–biotin (PAB) (P2165) and sheep immunoglobulin G (IgG) (I5131). Biotin-PEG$_{12}$-NHS carbonate ester (biotin-PEG-NHS) was purchased from Thermo Scientific. Streptavidin (2-0203-100) was purchased from IBA Lifesciences (Germany).

2.2. Fabrication of LWs

The glass slides were cut into 25.4 by 25.4 mm squares and cleaned via UV ozone treatment (Ossila, UV ozone cleaner) for 10 mins. The silane groups of glass petri dishes were blocked by treating them with 1% (v:v) HMDS solution in toluene for 10 min followed by a wash in toluene. Cleaned glass squares were placed in HMDS blocked petri dishes. A few of the slides were immersed in 1% (v:v) CDVS solution in toluene, while others were immersed in 5% (v:v) TMPTS solution in toluene, for 10 mins. The slides were washed in toluene and allowed to dry. 4 drops of a suspension of 1.25 µg/ml latex beads prepared in water were placed on the corners of TMTFS slides and allowed to dry.

Nitrogen was bubbled through de-ionised water for 30 min and was used to prepare stock solutions of monomers (i.e. acrylamide, APMA), crosslinkers (i.e. bis-acrylamide, PEGDA), APS and TEMED. Volumes of stock solutions of monomers and crosslinkers including the dilution factor to be used were determined using Equation 1 and Equation 2.

$$\frac{71.08 \text{m}_{\text{APMA}}}{178.66 \left(W - m_{\text{APMA}}\right)} \times 100 = M \tag{1}$$

$$\frac{71.08 \text{m}_{\text{crosslinker}}}{M_{\text{w,crosslinker}} \left(W - m_{\text{APMA}}\right)} \times 100 = 1.59 \tag{2}$$

Where $m_{\text{APMA}}$ and $m_{\text{crosslinker}}$ are masses of APMA and the crosslinker (i.e. bis-acrylamide or PEGDA) respectively in grams, $M_w$ is the molecular weight of the crosslinker (i.e. bis-acrylamide or PEGDA), $W$ is percentage total (w:v) monomer concentration and $M$ is percentage molar ratio of APMA with respect to acrylamide. The value of $W$ was either 4% (w:v), 4.5% (w:v) or 5% (w:v). The value of $M$ was 4%. Unless stated otherwise, the concentrations of APS and TEMED were 12.5% (v:v) and 1.25% (v:v) respectively. The resulting solution was immediately cast between CDVS and TMPTS treated glass squares with latex beads as the spacer. The diameter of the latex beads determined the thickness of the hydrogel film formed between the two glass substrates. The solution polymerised in a few minutes and then the glass squares were detached, resulting in a hydrogel films deposited on CDVS treated glass squares, which served as LWs. The LWs were stored in 100 mM phosphate buffer, pH 8.0 until used.

2.3. Instrumentation

A schematic of the instrumentation used to test LWs is provided in Figure 2. The instrumentation comprised of a BK7 equilateral prism (Qiotic Photonics) with a base of 30 mm by 30 mm on to which a LW was placed with a thin layer of refractive index (RI) matching oil between the prism and the glass substrate. Unpolarised light from a point source red LED (TL-6, iC-Haus, 640 nm) was collimated by passing it through an achromatic doublet (40 mm focal length, Comar Optics). The collimated light was then passed through a cylindrical lens (40 mm focal length, Comar Optics) to obtain a wedge-shaped beam, which was used to illuminate the LW with a range of angles of incidence simultaneously. The light reflected from the LW was passed through a cylindrical lens and then captured using a 20 Mpixel CMOS camera (MER-2000-19U3M-L, Daheng Imaging, China). A two-channel flow cell was mounted on top of the LW and held in place using a clamping plate connected to a temperature-controlled recirculating water bath (LT ecocool 100, Grant) maintained at 20 °C. The flow cell had two channels each of which were 2 mm wide, 0.25 mm deep and 15 mm long with a centre-to-centre separation of 4
4 mm (see inset in Figure 2). The solutions used for testing the LWs were pumped through the flow cell using a peristaltic pump (Minipuls® 3, Gilson) at a flow rate of 0.2 ml/min. Unless stated otherwise, all solutions were prepared in 100 mM phosphate buffer, pH 8.0. The RI of the solutions were measured using a RFM900-T refractometer (Bellingham and Stanley) with an accuracy of ±1 × 10⁻⁵ RIU.

![Figure 2](image)

**Figure 2.** Schematic of the LW instrumentation where the insets show a two-channel flow cell and ray diagram showing partial confinement of light in LWs (TIR is total internal reflection)

3. Results and discussion

Figure 3 (a) gives a typical camera image of a LW with a waveguide made of 4.5% (w:v) total monomer concentration where the x-axis is the angle of incidence and y-axis is the distance across the width of the LW. To obtain the reflectivity curve (i.e. intensity of reflected light versus angle of incidence) of the LW, the gray scale values across the image were normalised with respect to the gray scale values between 63.5° and 63.8° because the reflectivity in this angular range is expected to be unity. The resulting reflectivity curve (black trace) is provided in Figure 3 (b) and clearly shows the presence of exponentially decaying interference fringes at the θ'R of LWs. The reflectivity of the interference peak is greater than one because of constructive interference, while the reflectivity of the interference dip is less than one because of destructive interference. At angles > θ'R, the reflectivity is unity because of TIR.

![Figure 3](image)

**Figure 3.** (a) camera image of the output of a LW sensor consisting of a layer of 4.5% APMA-acrylamide copolymer crosslinked with bis-acrylamide; (b) intensity profile taken from (a) (black trace) and theoretical best fit (red trace)

3.1. Mathematical modelling. The quantitative model starts by using the theory of simple LWs, which has been derived elsewhere (9), to determine if the LW can support one or more modes. Once a structure that can support mode(s) has been found, transfer matrix modelling can be used to determine the complex reflectance amplitude function over a suitable range of angles that encompasses the mode(s) of interest. The transfer matrix method is well known (10-12) and hence its derivation will not be given.
here. Once the complex amplitude transmittance function has been determined, Fresnel’s approximation can be used to propagate the optical field represented by the reflectance function to a suitable detector. To generate the intensity distribution at the detector, the shifted FFT of the amplitude transmittance coefficient is multiplied with Equation 3:

\[ H = e^{ikz} e^{-in\lambda u^2} \]  

(3)

Where \( k \) is the wave vector (\( 2\pi/\lambda \), m\(^{-1} \)), \( z \) is the distance to the detector (m), \( \lambda \) is the wavelength (m) and \( u^2 \) is the quadratic phase term. The resulting product is then inverse FFT transformed and the absolute value of the result gives the intensity at the detector. This mathematical model forms the basis of a simplex optimisation used to fit the experimental reflectivity curve (black trace) to a theoretical reflectivity curve (red trace) in Figure 3 (b). The simplex optimisation algorithm was integrated into an in-house developed transfer matrix modelling package that also incorporated the Fresnel’s approximation method to generate the intensity profiles. Because no windowing is used on the FFT, the theoretical reflectivity profile does contain artefacts that are not present in the experimental reflectivity profile. The best fit reflectivity profile gave a waveguide thickness of 3.68 \( \mu \)m and the RI difference between the waveguide and cover was 3.523x10\(^{-3} \) – 2.6x10\(^{-5} \)i. The imaginary refractive index of the acrylamide/APMA films is a measure of the scattering losses in the waveguide and is ~3.8 times lower than chitosan waveguides (13).

3.2. Porosity of hydrogel films used to make LWs. The porosity of the waveguide of LWs plays a significant role in determining their sensitivity. The molecular weight of biomolecular recognition elements such as antibodies is 150kDa, so the waveguide must have significant porosity to species of at least this molecular mass. We therefore investigated the factors influencing the porosity of acrylamide/APMA waveguides. The \( \Delta \theta \) of LWs is proportional to percentage porosity of the waveguide to species of certain molecular weight. Thus, the porosity of the waveguides was tested by introducing solutions of species of different molecular weights on LWs and measuring the corresponding \( \Delta \theta \), \( \Delta \theta \) of LWs for PEGs of different molecular weights was measured with respect to a low molecular weight species, glycerol, which is likely to be able to diffuse freely in the waveguides.

Firstly, we varied the concentrations of APS and TEMED. The \( \Delta \theta \) of LW with a waveguide made of 5% (w:v) total monomer concentration was monitored in real-time as glycerol and 35kDa PEG solutions were flowed on the waveguide. The \( \Delta \theta \) was then normalized with respect to the \( \Delta \theta \) observed for glycerol solutions, and the resulting data is summarized in Table 1. For low concentrations of APS and TEMED, the \( \Delta \theta \) to 35 kDa PEG is low, and based on modelling is likely to arise just from sensing the solution in the evanescent field above the waveguide. Faster polymerization with higher concentrations of APS and TEMED increased the porosity significantly.

| Analyte         | (\(\Delta \theta /\Delta \theta_{\text{gly}}\))\times100\% |
|-----------------|-----------------------------------------------------|
| 35kDa PEG       | 18.1±0.3%                                             |

We then investigated the effect of percentage total monomer concentration (W, w:v) on the porosity of analytes of different molecular weights. As shown in Figure 4, the highest porosity to PEGs of 35, 100 and 100 kDa was at 4.5% (w:v) total monomer concentration. This observation is consistent with the work of Kizilay et. al. (14), where a maximum gel spatial inhomogeneity is observed at similar total monomer concentration and crosslinker molar ratio. This would lead to a higher fraction of larger pores (15), increasing the porosity to PEGs. The effect on porosity of the crosslinker length was also
investigated using bis-acrylamide and PEGDA of $M_n$ 700 Da for total monomer concentrations of 4.0%, 4.5% and 5.0% (w:v). Using a one-tailed (because we expect the longer PEGDA crosslinker to increase porosity) pooled t-test at the 95% confidence level, the porosities to all three PEGs for 4.0% and 4.5% (w:v) hydrogels crosslinked with bis-acrylamide and PEGDA were significantly different. For 5% (w:v) hydrogels, the porosity for 35 and 300 kDa PEGs were significantly different, with only the porosity for 100 kDa PEG not being significantly different. From this, we can say that the length of crosslinker does have a significant effect on the porosity of the hydrogel.

Figure 4. Shifts in resonance angles normalised to that observed for 0.5% glycerol (a) recorded in real-time for different total monomer concentrations, (b) versus total monomer concentration (bis-acrylamide was used as a crosslinker)

Table 2. Relative sensitivity to 35, 100 and 300 kDa PEG solutions for 4.0, 4.5 and 5.0% (w:v) total monomer concentration hydrogels using bis-acrylamide and PEGDA crosslinkers

| Crosslinker | Total monomer concentration: 4% (w:v) | Total monomer concentration: 4.5% (w:v) | Total monomer concentration: 5% (w:v) |
|-------------|--------------------------------------|---------------------------------------|--------------------------------------|
|             | 35kDa PEG | 100kDa PEG | 300kDa PEG | 35kDa PEG | 100kDa PEG | 300kDa PEG | 35kDa PEG | 100kDa PEG | 300kDa PEG |
| Bis-acrylamide | 45.2 ±0.3 | 40.2 ±0.3 | 37.0 ±0.7 | 62.9 ±0.8 | 56.0 ±0.6 | 52.0 ±0.2 | 45.4 ±0.2 | 39.8 ±0.5 | 36.2 ±0.6 |
| PEGDA       | 50.7 ±0.4 | 45.3 ±0.8 | 42.5 ±0.7 | 67.9 ±0.7 | 59.1 ±0.7 | 55.7 ±0.5 | 47.8 ±0.6 | 40.3 ±1.1 | 34.4 ±0.9 |

Biosensing using LWs. The flow cell mounted on top of the LW had two channels; one of which served as a sample channel and the other as a reference channel. The amine groups in the acrylamide/APMA waveguide region under the sample channel were reacted with 2.5 mg/ml biotin-PEG-NHS prepared in 100 mM phosphate buffer, pH 8.0 for 20 min, while the region under the reference channel was just flushed with buffer. Streptavidin and PAB were mixed in 1:1 molar ratio in 100 mM phosphate buffer, pH 8.0 and allowed to bind for 10 min, and then the solution was injected in both the sample and reference channels. The streptavidin-PAB complex bound to the biotin in the region of the LW underneath the sample channel but did not bind to the region of the LW underneath the reference channel. Different concentrations of IgG solutions prepared in 100 mM phosphate buffer, pH 8.0 were introduced in the sample and reference channels, and the $\Delta \theta_R$ was monitored in real-time. The $\Delta \theta_R$ for the regions of the LWs corresponding to the reference channel were subtracted from the $\Delta \theta_R$ for the
regions of the LWs corresponding to the sensor channel. The differential measurements between the sensor and reference channels eliminated the effect of temperature variations, which affected the two channels equally, while studying the binding of IgG to PAB (see Figure 5 (a)).

![Figure 5](image)

**Figure 5.** (a) Plot of difference in shifts in resonance angles between sensor and reference channels versus time as different concentrations of IgG solutions were flowed on the LW (inset shows immobilisation strategy used, and different traces are the response across the width of the sample channel) and (b) binding curve for IgG

Figure 5(a) shows the response of a sensor to different concentrations of IgG/5. Based on this, the limit of detection for IgG is 3.69±0.37 nM. This is derived from three times the standard deviation of the noise on each trace at equilibrium for buffer and 50 nM IgG and assumes that at this concentration we are in the substantially linear region of the binding curve well below the K_d for the protein A – IgG complex. Figure 5(b) gives the binding curves for IgG for two different LW devices (i.e. LW 1 and LW 2), normalised to the resonance angle shift for streptavidin for that device. This was done to remove the effect of immobilising slightly different amounts of streptavidin on the two devices. Sigmoidal fits to this data gave K_d values of 375±58 and 523±48 nM for LW1 and LW2 respectively. These are significantly different, indicating that there is some device to device variability. It is unclear if this relates to hydrogel fabrication or immobilisation strategy. Because sheep IgG was used for this study, the K_d values obtained were considerably higher than those for human IgG (K_d between 7 and 34 nM according to literature (16, 17))

4. Conclusions
We have shown that acrylamide copolymerised with N-(3-aminopropyl)methacrylamide (APMA) hydrogels can act as leaky waveguides (LWs) with low refractive index contrast between the waveguide and cover layers. This in turn allows the LW resonances to be observed directly without the use of visualisation methods such as metal layers, dyes and gratings that increase the complexity of fabrication and can introduce unwanted effects such as increased non-specific adsorption. We have developed both qualitative and quantitative explanations for this direct observation of LW resonances and used the quantitative method to extract the thickness and refractive index increment of the waveguide.

The porosity of these LW devices was studied using glycerol as a low molecular mass probe and PEGs of 35, 100 and 300 kDa molecular mass to stand in for larger biomolecules. It was found that the porosity was highest at 4.5% w:v total monomer concentration and when using higher concentrations of APS and TEMED to initiate polymerisation. Porosity was also significantly increased when using a longer crosslinker (PEGDA Mn 700) compared to bis-acrylamide. In future, these results will allow more porous hydrogels to be synthesized.
Finally, it was shown that these hydrogel LWs could be used for biosensing by incorporating biotin groups into the waveguide using biotin-PEG-NHS as a linker that reacted with the free amines in the hydrogel. These biotin groups could in turn be reacted with a streptavidin-biotin-protein A complex that could then bind sheep IgG. Binding curves for different LW devices were obtained and the Kd for the protein A-IgG complex obtained. As expected, these were considerably higher than literature values for protein A-human IgG. These results have shown that diffraction-based LWs using synthetic hydrogels can act as biosensors, and future work will extend them to clinically relevant analytes.

5. References
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