Advances in Optical Biosensors and Sensors Using Nanoporous Anodic Alumina

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Abstract: This review paper focuses on recent progress in optical biosensors using self-ordered nanoporous anodic alumina. We present the fabrication of self-ordered nanoporous anodic alumina, surface functionalization, and optical sensor applications. We show that self-ordered nanoporous anodic alumina has good potential for use in the fabrication of antibody-based (immunosensor), aptamer-based (aptasensor), gene-based (genosensor), peptide-based, and enzyme-based optical biosensors. The fabricated optical biosensors presented high sensitivity and selectivity. In addition, we also showed that the performance of the biosensors and the self-ordered nanoporous anodic alumina can be used for assessing biomolecules, heavy ions, and gas molecules.

Keywords: nanoporous anodic alumina; optical biosensor; immunosensor; aptasensor; peptide-based biosensor; enzyme-based biosensor

1. Introduction

In recent years, three-dimensional nanostructures offer a new chance for researchers in the field of nanoscience to improve the performance of biodevices [1–3].

Self-ordered porous metal oxides (SOPMOs) are three-dimensional nanostructure platforms that are made with an electrochemical anodization method, such as tantalum [4–9], titanium [10–13], niobium [14–17], iron [18–20], stainless steel [21–23], silicon [24–27], aluminium [28–31], in acidic solutions. Among them, the self-ordered nanoporous aluminium oxide called nanoporous anodic alumina (NAA) has many advantages such as a durable platform, easy functional ability, high surface area, biocompatibility, and low-cost [32–42].

Several parameters should be optimized for the fabrication of NAA such as applied potential, temperature, and electrolyte. Both the inorganic acid (selenic acid [43,44], sulfuric acid [45,46], phosphoric acid [40,47]) and organic acid (oxalic acid [48–51], malonic acid [51,52], citric acid [53], etidronic acid [54,55], tartaric acid [56]) can be used as an electrolyte for the NAA fabrication.

Various applications have been reported for NAA such as biosensors [57–65], sensors [36,66–71], drug release [72–76], template-based nanowire, and nanotube fabrication [77–80]. Among the various sensors and biosensors that have been reported based on NAA, optical biosensors are the more interesting because of their remote sensing ability and properties such as being small and lightweight, having high sensitivity, and being immune to electromagnetic interference [81–86].

Up to now, several optical methods have been reported when using an NAA such as surface plasmon resonance (SPR) [87–95], interference localized surface plasmon resonance (ILSPR) [36,82,96–101], photoluminescence spectroscopy (PLS) [32,102–107], surface-enhanced Raman scattering (SERS) [84,108–114], and interferometric reflectance spectroscopy (IRS) [59,115–117]. Among them, IRS is a common optical method that has been applied as the basis for the NAA-based
biosensors and sensors. With this method, a white light beam is illuminate toincident the SOPMO, and Fabry–Pérot (FP) interferences are obtained from the two main interfaces: (1) the interface between the incident medium and the thin film constituted by the porous structure (interface-a) and (2) the interface between the porous thin film and the substrate (usually the remaining aluminum, interface-b). In a this FP interferometer, the incident beam splits at interface-a with one part reflected back and another part transmitted into the thin film. This transmitted part of the beam travels within the thin film until it reaches interface-b, where it is reflected back. The beam travels back again to interface-a where it is split again into a transmitted portion and reflected back into the thin film portion. This process repeats itself until no energy is remaining in the beam, and a number of beams are generated in the same direction as the first reflected beam. Two consecutive reflected beams have a difference in their optical paths that depends on the angle of incidence, the effective refractive index of the thin film and its thickness.

A charge-coupled device (CCD) spectrometer is used to collect and analyze these multiply reflected beams. The signal registered by the CCD depends on the optical path difference between two consecutively reflected beams. When the wavelength is such that the optical path difference is an integer number of wavelengths, the measured spectrum shows a maximum at that wavelength. On the other hand, if the optical path difference is one half of any odd integer, the spectrum shows a minimum. These maxima and minima in the spectrum are known as FP fringes. Figure 1 shows the schematic of the IRS detection system.

![Figure 1. Schematic representation of the interferometric reflectance spectroscopy (IRS) detection system.](image-url)

The effective refractive index is a function of the different refractive indices composing the thin film: the oxide, the filling medium, and the attached molecules. When the pores are filled with the liquid medium and different molecules attach to the pore surfaces, the effective refractive index changes, causing a resulting shift in the FP fringes. The effective refractive index is a function of the different refractive indices composing the thin film: the oxide, the filling medium, and the attached molecules. When the pores are filled with the liquid medium and different molecules attach to the pore surfaces, such effective refractive indices changes which causes a resulting shift in the FP fringes. The amount of change depends on the concentration of the analyte in the sample.

In the next sections, first, we explain the electrochemical fabrication and functionalization of NAA. Then, we address some of the most recent papers that prove that various optical biosensors and sensors are made using NAA such as immunosensors, aptasensors, enzyme-based sensors, gas sensors,
small biomolecule sensors, and ion sensors. We also discuss their structure, surface functionalization, method of detection, and performance parameters such as sensitivity, range of linearity, and limit of detection.

2. Fabrication and Functionalization of NAA

The electrochemical fabrication of NAA is achieved following a two-step anodization method in electrolyte solution under stirring conditions at low temperature (5 °C) [25–28]. To fabricate NAA, first, an aluminum sample should be electropolished in an ethanol solution containing perchloric acid (25%) to remove most of the irregularities on the Al surface. After that, the electropolished Al is immersed in an organic or inorganic electrolyte solution for the first step of the anodization process. During the first step, the Al sample corrodes and converts to disordered nanoporous anodic alumina. In order to obtain a porous thin film with good enough properties, the disordered nanoporous alumina is immersed in a phosphoric acid solution containing chromic acid to remove the porous aluminum oxide matrix leaving the aluminum surface with a highly ordered array of nano-concaves on its surface. According to reports, by anodizing Al with highly ordered nano-concaves in the same condition that is applied in the first step (second step), a highly ordered nanoporous alumina will be fabricated. The nano-concaves on AL play as the pore nucleation sites during the second step anodization. Finally, the pore widening process should be done in a phosphoric acid solution to increase the pore size. The schematic fabrication of NAA is shown in Figure 2.

![Figure 2. Schematic representation of the different steps to the fabrication of nanoporous anodic alumina (NAA).](image)

The effect of the pore widening time on the pore diameters of NAA can be studied using scanning electron microscopy (SEM) techniques. Figure 3 shows SEM images of the NAA fabricated in 0.3 M oxalic acid as electrolyte solution at 40 V at various pore widening times. As can be seen, the pore diameters of the NAA increased by increasing the widening time (A–D). Also, some cross-sectional images of thee NAA are shown in Figure 3E,F. In all the cases it can be seen the uniformity of the cylindrical nanopores. By considering the top views and cross-sectional view of an NAA, it can be concluded that an NAA is a three-dimensional nanoplatfrom.
Figure 3. SEM images of an NAA: top views (A–D) and cross-sectional views (E,F).

Since NAA does not have any functional group to interact with its biomolecules, it should first be functionalized. For this purpose, NAA was dipped into a 3.0 M hydrogen peroxide solution (H$_2$O$_2$) (T = 70 °C) for 1 h to generate a hydroxide (−OH) group. The generated −OH group can interact with the silane coupling agents, such as 3-aminopropyltriethoxysilane (3-APTS) [118] 3-mercaptopropyl-tirethoxysilane (3-MPTES) [119], 3-glycidoxypropyltrimethoxysilane [120], and 3-isocyanatopropyl triethoxy (3-ISCN) [121], inducing the functional group on the pores of the NAA. Then, the silanized NAA can interact with various biomolecules such as antibodies [60], amino-terminal aptamers [122,123], enzymes [105], and peptides [124,125].
3. Biosensors (Biorecognizer-Based Sensors)

A biosensor consists of a bio-recognizer and a transducer that detects an analyte quantitatively and selectively. The bio-recognizers binds or reacts with an analyte specifically, causing a biological event. After that, the biological event is converted by a transducer into a diagnostic signal such as an optical signal [126].

3.1. Immunosensor

An immunosensor is a highly affinity-based bioanalytical device that works based on interactions between an immobilized antibody (bio-recognizers) on a transducer and an analyte in the solution [127]. Kumeria et al. showed that their microchip IRS-based immunosensor (NAA-biotinylated anti-epithelial cell adhesion molecule antibody (anti-EpCAM)) can be used for sensing circulating tumor cells (CTCs) within 5 min [128]. To fabricate the immunosensor, they first coated the surface of the NAA with a thin layer of gold (NAA-Au). Then, 11-mercaptoundecanoic acid (MUA) was self-immobilized on the surface of the NAA-Au. Afterward, the carboxylic acid group of the MUA was activated with EDC/NHS to interact with streptavidin. Finally, the biotinylated anti-EpCAM antibodies were immobilized on the surface of the NAA-Au-streptavidin based on the avidin–biotin interaction.

As the CTC cell solution was injected to measure cells, they interacted with anti-EpCAM antibodies on the surface of NAA-Au. This interaction caused a redshift in the wavelength of the reflected light to the CCD detector. The amount of redshift depended on the CTC concentration in the sample. The proposed biosensor could detect the cancer cell in the concentration range of $1 \times 10^3$–$1 \times 10^5$ cells/mL and with a detection limit of <1000 cells/mL. The schematic representation for the fabrication of the NAA-anti-EpCAM antibody and the sensing mechanism is shown in Figure 4.

![Figure 4. Schematic representation of the fabrication of the nanoporous anodic alumina-anti-epithelial cell adhesion molecule antibody (NAA-anti-EpCAM) for the determination of CTCs using interferometric reflectance spectroscopy (IRS).]
In another work, Lee et al. studied the fabrication of a biosensor to detect serum amyloid A1 (SAA1) as a lung cancer-specific biomarker [98]. To detect this biomarker, the surface of the NAA was coated with Ni/Au, and then they immobilized the related antibody on it. After the injection of the SAA1 solution for measuring cells, the wavelength of the reflected light redshifted, indicating the interaction of SAA1 antigen with the immobilized antibody on the top of the NAA-Au. The results showed that the fabricated immunosensor could be used from 1 fg/mL to 1 µg/mL. The LOD was found to be 100 ag/mL.

They also showed that the same strategy can be used for the determination of C-reactive protein (CRP) [129]. The linear response of the biosensor ranged from 0.1 fg/mL to 1 µg/mL, and the LOD was 100 ag/mL.

In more recent work, an IRS-based immunosensor for the determination of tumor necrosis factor alpha (α-TNF) was reported by G. Rajeev et al. [121]. The first step was the fabrication and functionalization of the NAA with 3-isocyanatopropyl triethoxy silane. The 3-isocyanatopropyl function group can interact with the primary amine of antibody, immobilizing it on the NAA. After that, they injected various concentrations of α-TNF. The results demonstrated that the wavelength of the reflected light to the CCD redshifted as the concentration of TNF increased in the sample. When α-TNF interacted with the immobilized antibody, the effective refractive index of the NAA increased, and this phenomenon caused a shift to a longer wavelength and, consequently, a shift in the effective optical thickness (EOT) according to the Fabry–Perot equation: \( m \lambda = 2 n L = \text{EOT} \). In this equation, \( \lambda \) is the wavelength of the maximum constructive interference of order \( m \), \( n \) is the effective refractive index of the porous film and its contents, and \( L \) is the geometric thickness of the porous thin film. To calculate the amount of change in the EOT, they applied a fast Fourier transformation to the recorded interference spectra. The results showed that the change in the EOT of NAA could be used as a signal for the measurement of α-TNF in the concentration of 100 to 1500 ng/mL. The calculated LOD was 0.13 µg/mL. The schematic representation for the fabrication of the NAA-anti-TNF and the sensing mechanism is shown in Figure 5.

![Figure 5. Schematic representation of the fabrication of the nanoporous anodic alumina-tumor necrosis factor alpha antibody (NAA-TNF) for the determination of tumor necrosis factor alpha using interferometric reflectance spectroscopy.](image-url)
3.2. Aptasensor

Aptasensors are a type of biosensors that use the synthesized nucleic acid sequences named aptamer as a detection element. [130] The size of the aptamer is smaller than the antibody; therefore, in the same surface area, the amount of the immobilized detection element in the aptasensor is higher than the immunosensor and, subsequently, the linear response range of aptasensors, in the most of the times are wider than immunosensor [131]. In addition, instead of immunosensors that can be used for sensing the limited number of targets, aptasensors can be used for a large number of targets from cancer cells to ions. In this section, some of the more interesting aptasensors that have been made using NAA are collected.

Silicon nanoporous substrates are common materials that have been used for molecular gate-based aptasensing purposes [132]. In these kinds of sensing strategies, silicon nanoporous particles should be first loaded with dye molecules such as rhodamine B. Then, the entrance of the pores of the silicon nanoporous particles are functionalized with a silane coupling agent, such as (3-isocyanatopropyl)triethoxysilane, to attach the short single-stranded DNA sequences (normally from 4 to 12 oligonucleotide sequences). The first oligonucleotide sequences and last oligonucleotide sequences of the aptamer probe can interact complementary to the single-stranded DNA, immobilizing the aptamer probe, therefore aptamer. To explain it more clearly, in this sensing strategy, nanoporous particles and an aptamer play like a pot and a lid, respectively, and inside the pot (nanoporous particles) is filled with dye. If any analyte can interact with the aptamer probe selectively, so, the dye will release on nanoporous particles and then the color of the solution will change. If the length of the aptamer probe is smaller than the pore diameter of the nanoporous substrate, the dye molecule will not remain inside the porous and will release into solution. Hence, the length of the aptamer probe must be long enough to cover the pore diameter of the nanoporous substrate. Since the length of the aptamer is small (up to 10 nm), meso-sized porous substrates can be used for molecular gate-based sensing. Among the different nanoporous materials, NAA is a good candidate.

Nanoporous anodic alumina (NAA) fabricated in a sulphuric acid electrolyte can have a porous structure with an average pore size of 8 nm, making it a good candidate for molecular gate-based sensing. In this context Martínez et al. used NAA and molecular gates for the detection of cocaine using rhodamine B and the photoluminescence spectroscopy (PLS) method as a molecular probe and a sensing device, respectively [129]. The sequence of short single-stranded DNA (i.e., 5′-AAAAAACCCCCC-3′) was immobilized in the entrance of the nanopore using (3-isocyanatopropyl) triethoxysilane, and the sequence of the aptamer probe of cocaine was TTTTGG GGGGGG GAG ACA AGG AAA ATC CTT CAA TGA AGT GGG TCT CCA GGGGGGGTTT-3.

As demonstrated, the first oligonucleotide sequences and last oligonucleotide sequences of an aptamer probe interacted complementarily to single-stranded DNA, immobilizing the aptamer probe in the entrance of the pores of NAA. As the aptamer probe interacted with the cocaine, it removed from the NAA, and then rhodamine B was released into the solution. The amount of the released rhodamine B increased with the increase in the concentration of cocaine. The signal of the released rhodamine B was detected by the fluorescence spectrometer. The results showed that the proposed method could detect cocaine concentrations of 0.5 µM to 1 mM. The LOD was 0.5 µM and the sensor had a higher affinity to cocaine than morphine and heroin. The schematic representation for the fabrication of the aptasensor is shown in Figure 6.
According to previous reports, the amino-functional group can interact with streptavidin. To immobilize the aptamer probe, they used a biotinylated aptamer probe to interact with immobilized streptavidin. The results showed that the wavelength of the reflected light from the proposed aptasensor to the CCD shifted to a high wavelength (redshift) in the presence of TB, indicating the change in the effective optical index of NAA after the incubation of the TB with aptamer. The proposed sensor can be used for sensing TB in a concentration range of 0.54–2.70 µM. The experimental detection limit was 7.2 nM.

Recently, a novel strategy for the quantitative measurement of a target using the IRS technique was reported by Tabrizi et al. [130]. The sensitivity of the aptasensor would be higher if the intensity of the reflected light could be changed during the sensing process instead of the wavelength of the reflected light.

For this purpose, an IRS-based biosensor was fabricated using NAA and functionalized with 3-APTS. A guanine-rich aptamer was used as a probe and methylene blue as a photo-probe. Amino-terminal guanine-rich aptamers were immobilized on the NAA-NH$_2$ using glutaraldehyde (Glu) as the cross-linking agent. Following this, methylene blue (MB) was intercalated in the aptamer and generated the first MB/G-quadruplex complex-based IRS biosensor. A guanine-rich aptamer can fold into a G-quadruplex structure in the presence of K$^+$ ion. The G-quadruplex has a very high binding ability towards MB. Besides, an MB that has a high absorption coefficient ($\varepsilon = 95000 \text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$) can absorb light in the range of 550–725 nm. Therefore, the intensity of the reflected light from the MB/G-quadruplex complex-based IRS biosensor would be low compared with a common aptamer-based IRS biosensor.

Amyloid β (Aβ) oligomers were selected as a model analyte. To measure the Aβ oligomers' concentration, the first step was to record the signal of the MB/G-quadruplex complex-based IRS
biosensor in the absence of analyte. After the injection of the Aβ oligomers solution, the intensity of the reflected light to the CCD detector increased because of the release of MB on the MB/quadruplex complex. The decrease in light intensity led to a decrease in the peak area in the range of 450–1050 nm.

In this study, the slope of the calibration curve obtained with this method was compared with the slope of the calibration curve obtained with a common method based on the change in the EOT. The results indicated that not only was the slope of the plot obtained with the new method higher, but also the linear range of plot was wider. Consequently, the analytical performance of the sensor would be better if the Δpeak area, as a signal, was used to detect the analyte. The sensor showed a determination of Aβ oligomers in the range of 0.5 to 50.0 µg × mL⁻¹, and the LOD was 0.02 µg/mL. The proposed sensor has two advantages: first, the sensitivity of the sensor-based IRS was improved; and second, the data processing step for obtaining the signal was simpler. The schematic representation for the fabrication of the NAA-Aptamer/MB and the sensing mechanism is shown in Figure 7.

![Schematic representation of the fabrication of the NAA-Aptamer/MB and the sensing mechanism](image)

**Figure 7.** Schematic representation of the fabrication of the NAA-aptamer/MB for the determination of Aβ oligomers using IRS.

DNAzyme is an artificial enzyme that is made of hemin and aptamer [133]. Like peroxidase enzyme, DNAzyme that has hemin cofactor can oxidase ABTS²⁻ ion to ABTS⁻ (Figure 8).

![The mechanism of oxidation of ABTS²⁻ by DNAzyme](image)

**Figure 8.** The mechanism of oxidation of ABTS²⁻ by DNAzyme.

Recently, a DNAzyme-based IRS biosensor to detect Pb²⁺ ion concentration in the nanomolar range was reported [134]. In this work, NAA was fabricated using a two-step anodization process, silanizing the NAA pores with 3-APTS, and followed by immobilizing the glutaraldehyde cross-linker. Therefore, they put the functionalized nanosubstrate into an amino-terminal G-rich aptamer solution containing
hemin and potassium ions. According to the previous reports [96,135], the G-rich aptamer has been folded into a G-quadruplex structure named the K\(^+\)-stabilized G-quadruplex. The K\(^+\)-stabilized G-quadruplex shows a high binding ability towards the hemin cofactor. Therefore, in the presence of the G-rich aptamer, K\(^+\) ion, and hemin, an artificial peroxidase enzyme-based biosensor was fabricated. Peroxidase enzymes can oxidize 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution in the presence of hydrogen peroxide, generating a green colored ABTS\(^-\) anion radical solution which can absorb light in the 45–850 nm wavelength range. The idea is to record the artificial enzyme-based biosensor K\(^+\)-stabilized hemin-G-quadruplex DNAzyme using the IRS method. The results showed that the intensity of the reflected light from the biosensor to the CCD detector decreased when hydrogen peroxide was injected into the solution. It proved that the DNAzyme-based IRS biosensor worked properly. The generated green colored ABTS\(^-\) anion radical solution absorbed the white light and, therefore, the intensity of the reflected light from the biosensor to the CCD decreased. However, in the presence of Pb\(^{2+}\) ions, the catalytic property of the proposed biosensor decreased. As they added Pb\(^{2+}\) ions into the solution, the intensity of the reflected light to the CCD detector increased. In the presence of Pb\(^{2+}\) ion, the K\(^+\)-stabilized hemin-G-quadruplex DNAzyme re-designed to the Pb\(^{2+}\)-stabilized G-quadruplex. During this process hemin, the cofactor left the structure and washed away. Therefore, compared to the K\(^+\)-stabilized hemin-G-quadruplex DNAzyme, the Pb\(^{2+}\)-stabilized G-quadruplex did not show any peroxidase-like activity to oxide ABTS. Hence, the signal intensity increased. The results demonstrated that the fabricated biosensor can be used for the determination of Pb\(^{2+}\) ion in the nanomolar to the micromolar range (50–3200 nM). The low detection limit was calculated at 12 nM. The sensitivity of the proposed IRS-based sensor was higher than previous IRS-based sensors that have been used for heavy metal sensing [136–141]. It was also investigated the selectivity of the Pb\(^{2+}\) ion sensor towards interfering ions, such as Zn\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Bi\(^{3+}\), Sn\(^{2+}\), Co\(^{2+}\), and Hg\(^{2+}\), in the presence of SCN\(^-\) ions as a masking agent and also as a biosensor for the determination of Pb\(^{2+}\) ions in seawater. The proposed IRS biosensor presented a good selectivity, and the obtained recoveries were in the range of 93.8–110.2%. To sum up, the prepared biosensor exhibited high sensitivity, good selectivity, and applicability to Pb\(^{2+}\) ion in real samples. The schematic representation for the fabrication of the NAA-DNAzyme and the sensing mechanism is shown in Figure 9.

**Figure 9.** Schematic illustration of the NAA-DNAzyme sensor for the determination of Pb\(^{2+}\) ions using IRS.
3.3. Genosensor

Like an aptasenor, a genosensor uses synthesized nucleic acid sequences as a biological recognition element. But they are applied for sensing the RNA or DNA of targets such as bacteria [142] and viruses [143].

Candida albicans is one of the most common infectious fungi that can live inside the human body. Ribes et al. presented a molecular gate-based sensing method for the determination of Candida albicans-specific DNA fragments [144]. The proposed genosensor could detect Candida albicans within 20 min in the range of \(7\times10^2\) CFU/mL. The LOD was 8 CFU/mL.

In another recent paper, Tabrizi et al. designed a novel IRS-based genosensor for sensing Salmonella-specific DNA fragments as a model for DNA targets [145]. To fabricate the genosensor, first they immobilized the amino-terminal DNA probe inside the pores of the NAA using Glu as a crosslinker. Next, a known concentration of Salmonella-specific DNA fragment was added into the flow cell. After washing with deionized water, methylene blue solution was added into the flow cell. Finally, the fabricated aptasensor (NAA-Aptamer-MB) was washed several times to remove all the loosely attached MB. According to previous reports [146,147], methylene blue can intercalate between cytosine and a guanine bond, immobilizing on the genosensor. The intercalated methylene blue then absorbs the illuminating white light to the genosensor. Therefore, the intensity of the reflected light from genosensor to the CCD detector decreased. The decrease in the intensity of the reflected biosensor that was considered a signal had a logarithmic relationship within the range of 0.25–50.0 nM. The limit of detection was found to be 0.01 nM. A schematic representation for the fabrication of the genosensor is shown in Figure 10.

![Figure 10. Schematic representation of the genosensor for the determination of a Salmonella-specific DNA fragment using IRS.](image)

3.4. Peptide-Based Biosensor

A peptide-based biosensor is a type of biosensor where the synthesized peptide fragments or natural proteins, as biorecognition elements, are used as bio-recognizers to interact with the target. These kinds of sensors can be used for various targets [148].

Nemati et al. reported an IRS-based biosensor using a gelatin-modified NAA for the determination of trypsin enzyme as a protease enzyme [124]. Gelatin is a polypeptide that trypsin can cleave to
in small fragments at the carboxyl-terminal side of lysine and arginine residues. To fabricate the biosensor, 3-APTS and glutaraldehyde were used to immobilize gelatine on the pore of the NAA. The measurements showed that the length of the reflected light shifted to a high wavelength, as gelatin immobilized on the pore of the NAA. However, as enzyme trypsin was injected into the measuring cell, the wavelength of the reflected light blueshifted, indicating the cleavage of the gelatine and release the small peptide fragments into solution. The results demonstrated that the fabricated sensor can be used for the determination of trypsin enzymes in a concentration range of 0.0125–1 mg/mL and with 0.025 mg/mL. This result indicates that the sensitivity of the biosensor is still low for the determination of trypsin in real samples and further developments are needed.

Another interesting example is the development of an IRS biosensor for sensing a protease enzyme named cathepsin B (Cat B) [149]. For this purpose, the NAA surface was modified with 3-APTS and human serum albumin labeled with thionin (HSA-TH). The HSA-TH played the main role in the proposed biosensor. Because the absorption coefficient of TH is so high \( (5.85 \times 10^4 \text{ dm}^3\text{ mol}^{-1}\text{ cm}^{-1}) \) and can absorb light in the range 510–640 nm. Therefore, the immobilized TH adsorbed the light and the intensity of the reflected light from biosensor (NAA-HSA-TH) to the CCD detector was low in the absence of Cat B. In the presence of Cat B, peptides like HSA-TH cleaved to small peptide fragments. As the TH-peptide fragment washed away from the measuring cell, the intensity of the reflected light to the CCD detector increased. A schematic diagram of the sensing process is shown in Figure 11.

![Figure 11. Schematic representation for the sensing mechanisms of cathepsin B (Cat B).](image)

The proposed sensor can be used for the measurement of Cat B in the range of 0.5–64.0 nM. The limit of the detection of the biosensor was 0.08 nM. The good selectivity towards Cat B was also demonstrated by analyzing the interfering effect of some biomolecules such as proteases trypsin enzyme, urokinase enzyme, urea, glucose, and dopamine. The schematic representation for the fabrication of the NAA-HSA-TH and the sensing mechanism is shown in Figure 12.

### 3.5. Enzyme-Based Biosensor

An enzyme-based biosensor was considered the first biosensor that was fabricated. In these types of biosensors, an enzyme is immobilized on the surface of the transducer. An enzyme is a bio-recognizer that can catalyze a target selectively [150].
A highly sensitive enzyme-based biosensor was proposed for IRS sensing of cytochrome c (Cyt C) by immobilizing trypsin (Tryp) enzyme on NAA [151]. To fabricate the enzyme-based biosensor, it was silanized NAA with 3-APTS (NAA-NH$_2$). Then, the NAA-NH$_2$ was immersed in a glutaraldehyde solution. After that, the Tryp enzyme solution was dropped on it to immobilize the Tryp on the pores of the NAA. According to previous reports, the Tryp enzyme can cleave Cyt C to the short peptide [152] fragments, and some of those fragments have a hemin cofactor. The structure of hemin–peptide fragments which are generated by Tryp is as follows:

$$\text{Val-Glu(NH$_2$)-Lys-Cy-Ala-Glu(NH$_2$)-Cy-His-Thr-Val-Glu}$$

and

$$\text{Cy-Ala-Glu(NH$_2$)-Cy-His-Thr-Val-Glu-Lys}$$

In the presence of hydrogen peroxide, the heme–peptide fragments oxidized 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to a green colored ABTS$^-$ anion radicals [153]. The generated green colored ABTS$^-$ anion radical solution adsorbed the light. Hence, the intensity of the reflected light from NAA to the CCD decreased. The results revealed that the biosensor can be used for sensing Cyt C in the range of 1–100 nM, and the limit of detection is 0.5 nM. In addition, the analytical performance of the biosensor in real samples exhibited high selectivity, sensitivity, and good stability. The recovery of the analysis was 97.6%. A schematic representation of the design of the NAA-Try biosensor and the sensing mechanism is shown in Figure 13.
Another example of an enzyme- and IRS-based sensor is the urea biosensor [154]. The authors showed that the change in the intensity of the reflected light can be used for the determination of small molecules such as urea. In particular, fluorescein 5(6)-isothiocyanate (FLITC) attached to the urease enzyme could be used for the measurement of urea in the real sample. As per previous reports, in a high pH of the solution, FLTC can adsorb a greater intensity of light and, therefore, a limited fluorescence light of it would be high in compensation in high pH solution. Therefore, FLITC is considered a pH-sensitive photo probe.

As a consequence, a urease enzyme was immobilized on NAA and then attached to FLITC on an enzyme. Since the isothiocyanate functional group can interact with –NH₂, –SH, and OH groups, therefore FLITC can attach to enzymes that have a massive number of these groups. During the catalytic process of urea by urease enzymes, ammonia is generated [155]. As ammonia is considered a base, the pH of the solution will increase too. Consequently, the absorption light property of the attached FLITC on a biosensor (NAA–Urease–FLTC) will change. Because of that, the intensity of the reflected light from the NAA–urease–FLTC sensor to the CCD detector decreases. The proposed biosensor can be used for sensing urea in the range of 0.12 to 3.0 mM with a limit of detection of 0.06 mM.

The analysis of the results also demonstrated that the catalytic activity of the urease enzyme in the presence of trypsin as the protease enzyme would change [156]. In the presence of trypsin, the activity of urease decreased and the amount of the catalyzed urea to hydroxide ion and carbon dioxide decreased. Since the amount of the generated hydroxide ion decreased, the pH solution of the solution did not increase. In this condition, the FLITC did not absorb more light and, consequently, the CCD detector detected more light. The proposed biosensor exhibited a good response to a concentration of trypsin in the range of 1.0–6.0 µg/mL. The limit of detection (LOD) for trypsin was 0.23 µg/mL, respectively. The Michaelis-Menten constant (Kₘ) was calculated to be 0.078 mM for urea. The half-maximal inhibitory concentration of trypsin (IC₅₀) for the proposed biosensor was 6.2 µg/mL. The proposed biosensor exhibited good selectivity, linear range responsibility, and stability. The schematic representation for the fabrication of the NAA–Urease–FLITC and the sensing mechanism is shown in Figure 14.
4. Sensors

4.1. Gas Sensor

Kumeria et al. reported the use of NAA in an IRS-based gas sensor for the determination of hydrogen sulfide (H$_2$S) and hydrogen (H$_2$) gas [115]. The authors proved that if the surface of the NAA was coated with gold and platinum using a sputtering method, the fabricated sensor could be used for H$_2$S and H$_2$ sensing respectively. The optimum pore size and thickness of the porous layer were 30 nm and 4 µm, respectively. The fabricated sensor could detect the concentration of gas in the air up to 2%.

4.2. Non-Biorecognizer-Based Sensor

Teramae et al. designed and fabricated NAA to detect bovine serum albumin (BSA) by using nanoporous optical waveguide (NPWG) spectroscopy [86]. In the NPWG, like the conventional surface plasmon resonance (SPR), the changes in reflection spectra of the SOPMO film are measured in the Kretschmann configuration. The authors engineered two kinds of NAA substrate. The thickness and pore size of the substrate A was 220 nm and 39 nm, respectively, and its porosity was 34%. The thickness and pore size of the substrate B was 670 nm and 33 nm, respectively, and its porosity was 45%. According to the results, the sensor response of the NAA film B that had more porosity was about seven times larger than NAA film A due to the fact of its large adsorption capacity. The results confirmed that the wavelength of the recorded signal redshifted in the presence of BSA. The fabricated sensor could detect BSA concentration from 60 nM to 60 µM and the limit of detection is was 5.7 pg/mm$^2$. The schematic representation for the NPWG sensing of BSA is shown in Figure 15.
In another article, Chen et al. reported an IRS-based sensor for the determination of vitamin C [157]. To fabricate the sensor, first, the NAA was functionalized with 3-APTS, and then glutaraldehyde was immobilized on the NAA pores. The sensing was obtained when the vitamin C interacted with glutaraldehyde, changing the EOT of the NAA. The fabricated sensor showed a detected concentration of vitamin C ranging from 0.125 µM to 0.5 µM and a limit of detection of 20 nM.

4.3. Ion Sensor

Kumeria et al. presented a nanoporous interferometric sensor for label-free detection of gold (III) [138]. Nanoporous anodic alumina (NAA) was used as a nanoporous material and was functionalized with 3-mercaptopropyl-triethoxysilane. The selectivity of gold (III) ion was evaluated in the presence of some heavy metals such as Fe^{3+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Ag^{+}, and Pb^{2+} ions. The fabricated sensor detected the concentration of Au^{3+} (III) ion in the range of 0.1–80 µM with a lower detection limit of 0.1 µM. The interaction of the thiol (–SH) group of 3-MPTES with Au^{3+} (III) ion played a key role in this sensor. According to their report, the experimental data were fitted with Langmuir isotherm model binding better than Freundlich isotherm, suggesting absorption of Au^{3+} ions on NAA-3-MPTES.

Furthermore, they also reported that the immobilized 3-mercaptopropyl-tirethoxysilane on the NAA with rugate structure could be used for the determination of Hg^{2+} ion in the range of 1–100 µM [136]. The schematic representation for the fabrication of the NAA-3-MPTES and the sensing mechanism of Au^{3+} or Hg^{2+} ions is shown in Figure 16.
Table 1. The analytical performance of the optical biosensors and sensors by using NAA.

| Sensor                        | Type of Recognizers | Analyte            | Method                          | Linear Range | LOD       | Ref.   |
|-------------------------------|---------------------|--------------------|---------------------------------|--------------|-----------|--------|
| Nanoporous anodic alumina - Human serum albumin-Thionine | Labeled Peptide (Human serum albumin-Thionine) | Cathepsin B | Interferometric reflectance spectroscopy | 0.5-64.0 nM | 0.08 nM | [137] |
| Nanoporous anodic alumina - Urease-Fluorescein 5(6)-isothiocyanate | Labeled Enzyme (Urease-Fluorescein 5(6)-isothiocyanate) | Trypsin | Interferometric reflectance spectroscopy | 0.25-20 µg/mL | 0.06 µg/mL | [156] |
| Nanoporous anodic alumina - Gelatin | Peptide (Gelatin) | Trypsin | Interferometric reflectance spectroscopy | 1-7 mg/mL | 1 mg/mL | [124] |
| Nanoporous anodic alumina - Trypsin | Enzyme (Trypsin) | Cytochrome c | Interferometric reflectance spectroscopy | 1-100 nM | 0.5 nM | [131] |

5. Conclusions

This review article highlights recent advances in optical biosensors based on nanoporous anodic alumina. Detailed information was presented about the fabrication, structure, surface modification, and biosensing properties of nanoporous anodic alumina. We also provided fundamental aspects of optical techniques, such as interferometric reflectance spectroscopy, surface plasmon resonance, and photoluminescence spectroscopy, in combination with NAA platforms and discussed some relevant examples of optical NAA-based immunosensors, aptasensors, peptide-based biosensors, and genesensors.

In general, the optical sensors and biosensors based on NAA and new sensing strategies, such as plasmonic and interferometric spectroscopy, offer high sensitivity and low detection limits and present interesting features such as being remote, cheap, and integratable into lab-on-chip systems. The NAA-based sensors and biosensors have a high potential for the determination of a wide range of analyte from big bio-components, such as cancer cells, to small ones such as glucose.

Advances and developments in NAA optical structures and the versatility to modify the surface of NAA for new functionalities with specific selectivity will provide a new generation of NAA-based sensing systems with better performance and their use as a point-of-care testing devices. Finally, Table 1 summarizes an updated list of reported NAA-based optical biosensors and sensors.
Table 1. Cont.

| Sensor | Type of Recognizers | Analyte | Method | Linear Range | LOD | Ref. |
|--------|---------------------|---------|--------|--------------|-----|------|
| Nanoporous anodic alumina - ssDNA | Short chains of nucleotides (ssDNA) | Salmonella-specific DNA fragment | Interferometric reflectance spectroscopy | 0.25-50 nM | 0.01 nM | [145] |
| Nanoporous anodic alumina - Aptamer ᵃ | Short chains of nucleotides | Thrombin | Interferometric reflectance spectroscopy | 0.54-2.70 nM | 7.2 nM | [123] |
| Nanoporous anodic alumina - Aptamer ᵃ | Short chains of nucleotides | Amyloid β oligomers | Interferometric reflectance spectroscopy | 0.5-50 μg/mL | 0.02 μg/mL | [122] |
| Nanoporous anodic alumina - Anti-Tumor necrosis factor alpha Antibody (Anti-Tumor necrosis factor alpha) | Tumor necrosis factor alpha | Immunofluorescence | Interferometric reflectance spectroscopy | 100-1500 ng/mL | 100 ng/mL | [121] |
| Nanoporous anodic alumina - Anti-Epithelial cell adhesion molecule Antibody | Antibody (Anti-Epithelial cell adhesion molecule antibody) | Circulating tumor cells | Interferometric reflectance spectroscopy | 103-105 | >100 | [128] |
| Nanoporous anodic alumina - Anti-human immunoglobulin G Antibody | Antibody (Anti-human immunoglobulin G) | Human immunoglobulin G | Interferometric reflectance spectroscopy | 10-100 μg/mL | 1 μg/mL | [158] |
| Nanoporous anodic alumina - Streptavidin Peptide (Streptavidin) | Biotinylated thrombin | Interferometric reflectance spectroscopy | 10-100 μg/mL | 10 μg/mL | [159] |
| Nanoporous anodic alumina - Bovine serum albumin | Small molecule (Glutaraldehyde) | Vitamin C | Interferometric reflectance spectroscopy | 0.125-0.5 μM | 20 nM | [157] |
| Nanoporous anodic alumina | Small molecule (Glutaraldehyde) | Copper (II) ion | Interferometric reflectance spectroscopy | 0.0125-1 M | 0.0125 | [161] |
| Nanoporous anodic alumina | Glutathione-S-transferase | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.01-1.2 M | 100 mM | [162] |
| Nanoporous anodic alumina - ssDNAsal | Short chains of nucleotides | Salmonella-specific DNA fragment | Interferometric reflectance spectroscopy | 0.005-0.1 M | 5 mM | [162] |
| Nanoporous anodic alumina | L-cysteine | Interferometric reflectance spectroscopy | 0.005-0.1 M | 5 mM | [162] |
| Nanoporous anodic alumina | Glutathione-S-transferase | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.01-1.2 M | 10 nM | [163] |
| Nanoporous anodic alumina - 3-Mercaptopropyl-trimethoxysilane | Small molecule | Mercury(II) ion | Interferometric reflectance spectroscopy | 1-100 μM | 1 μM | [136] |
| Nanoporous anodic alumina - Glutaraldehyde | Glutaraldehyde | Copper (II) ion | Interferometric reflectance spectroscopy | 1-100 μg/mL | 0.007 μg/mL (7 ppb) | [137] |
| Nanoporous anodic alumina | Small molecule (Glutaraldehyde) | Glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.1-0.5 μM | 0.1 μM | [138] |
| Nanoporous anodic alumina - ssDNAsal | Short chains of nucleotides | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 50-3200 nM | 12 nM | [134] |
| Nanoporous anodic alumina - ssDNAsal | Short chains of nucleotides (ssDNAsal) | Salmonella-specific DNA fragment | Interferometric reflectance spectroscopy | 1-100 μg/mL | 10 μg/mL | [134] |
| Nanoporous anodic alumina | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |
| Nanoporous anodic alumina | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |
| Nanoporous anodic alumina - ssDNAsal | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |
| Nanoporous anodic alumina | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |
| Nanoporous anodic alumina | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |
| Nanoporous anodic alumina | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |
| Nanoporous anodic alumina | ssDNAsal | Human glutathione-S-transferase | Interferometric reflectance spectroscopy | 0.02-1 ng/mL | 0.02 ng/mL | [165] |

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**Acronyms**

| Acronym | Description |
|---------|-------------|
| SOPMO   | Self-ordered porous metal oxides |
| Al      | Aluminum    |
| NAA     | Nanoporous anodic alumina |
| 3-APTS  | 3-Aminopropyltriethoxysilane |
| 3-MPTES | 3-Mercaptopropyl-tirethoxysilane |
| 3-ISCN  | 3-Isocyanatopropyl triethoxy |
| PLS     | photoluminescence spectroscopy |
| IRS     | Interferometric reflectance spectroscopy |
| SERS    | Surface-enhanced Raman scattering |
| CCD     | Charge-coupled device |
| α-TNF   | Tumor necrosis factor alpha |
| anti-EpCAM | Anti- Epithelial cell adhesion molecule antibody |
| CTC     | Circulating tumor cells |
| Glu     | Glutaraldehyde |
| MB      | Methylene blue |
| TB      | Thrombin |
| EOT     | Effective optical thickness |
| Aβ      | Amyloid β |
| ABTS    | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) |
| CFU     | Colony-forming unit |
| Cat B   | Cathepsin B |
| Cyt C   | Cytochrome c |
| FLITC   | Fluorescein 5(6)-isothiocyanate |
| Kₘ      | Michaelis-Menten constant |
| H₂S     | Hydrogen sulphide |
| H₂      | Hydrogen |
| PEI     | Polyethylenimine |
| Rh B    | Rhodamine B |
| OWG     | Optical waveguide |

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