Label—Free Integrated Optical Biosensors for Multiplexed Analysis

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Abstract | The need for real time, fast and frequent monitoring in healthcare, pharmaceuticals research, environmental monitoring, the food industry, and homeland security has been rising, leading to extensive research in the development of Point of Care (POC) devices for diagnostics. POC devices need to be handy, fast, robust, highly sensitive to sensing analytes, selective against other interferents, and accurate. Since, in the real world scenario, the analytes are typically complex and useful analysis depends on sensing multiple parameters, multianalyte sensing has emerged to help users arrive at concrete conclusions or inferences. Research has been conducted for exploiting a plethora of transduction phenomena, such as electrochemical, impedometric, piezoelectric, magnetic, optical, *etc.* Of these, optical methods of sensing have the upper hand due to their resistance to electromagnetic interference, fast response, ease of parallel analyte sensing, low Limit of Detection (LOD), and, most importantly, easy translation of well-established techniques to *in silico* devices.

Label-free biosensing provides the advantage of simplicity and cost reduction by avoiding complex steps prior to sensing by monitoring analytes in their unmodified state i.e. detecting analytes without any alteration. Further, sensing in ultra-small quantities has been possible due to the concurrent development of MEMS technology. All this has led to the development of Lab on Chip (LOC) devices which perform various separation, and detection, operation and analysis on small chips and are cost effective due to bulk fabrication. LOC devices integrating label—free optical sensing on small chips also give rise to the possibility of multi-analyte assays using small test samples. These facilitate on-site deployment of these devices for different applications. This review will present various optical waveguide-based devices, including those based on optical absorbance, evanescent wave absorbance and surface plasmon resonance. Various configurations of such devices that aid multianalyte on-chip assays will be explored.

Keywords: *Integrated, Label free, Lab-on-chip, Multiplex, Optical Biosensor.*

1 Introduction

The need for the detection of various physical quantities like temperature, pressure, electromagnetic waves, light, current, *etc.* has led to the development of various techniques in the field of physical sensors. Over the past century there has been significant progress in the development of chemical sensors as well. As may be easily surmised, physical and chemical sensors are distinguished on the basis of the measurand. Both types of sensors

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employ unique transduction phenomena in order to perform measurements. Biosensors on the other hand are sensors that employ biological recognition molecules and leverage on the physical/chemical transduction schemes of physical/chemical sensors, in order to qualitatively or quantitatively assess the measurand. Biosensors have gained tremendous importance ever since the first demonstration by Clark and Lyons[1] in 1962. They have the ability to detect analytes rapidly and precisely by using various biological molecules or biologically derived molecules like DNA,[2] peptides,[3] cell structures,[4] enzymes,[5] antibodies,[6] *etc.* as recognition elements, integrated or associated closely with different transducers. Based on the transduction principle used for sensing, biosensors may be grossly classified into electrochemical,[7] optical[8] and mechanical.[9]

Amongst the various types of biosensors, optical biosensors have emerged as the most popular. Most likely this is due to the fact that optical transduction is immune to electromagnetic interferences and mechanical vibrations. Further, quite a few of in vitro chemical and biological assays which had been developed earlier exhibit a change in optical properties on reaction. The translation of these chemical/biological assays to a biosensor format, so that minute changes in optical properties are detected, was a logical progression in terms of development. Optical biosensors, like other biosensors, can offer possibilities of multiplexing, real time measurements, low LOD, *etc.* Conventional optical biosensors relied on macro-scale instruments and devices; however, with the continuous development in microfabrication and micromachining technologies necessitated by ever-shrinking electronic devices, the possibility of shrinking the size of optical biosensor devices as well emerges. This has, in turn, reduced the volume

requirement of reagents, and analytes and brought forth the possibility of making multiple measurements using the same physical device or devices arranged within a few microns of each other.

In the early days of optical biosensor development, a large number of sensors and sensing systems relied on labeled assays or tagged assays. In these assays, typically, a capture or recognition event by a biomolecule was followed by a signal enhancement/amplification step using a probe molecule "tagged" or "labeled" with an enzyme, fluorescent marker, radioisotope, *etc.* In some cases, the probe molecule itself exhibited fluorescence or other signal amplification behavior. However, these assays obviously had a disadvantage in terms of the complexity of the measurement process. Along with the development in transduction technologies and science, it became possible to detect a change in optical properties without the application of a tag. Thus, in parallel with the development of optical microsensors, researchers also targeted the development of label free assays, which can significantly reduce the cost and complexity of the measurement in the hands of the user. The high degree of interest is developing label-free sensing technologies has resulted in a large number of publications in this domain (Figure 1). The increase in the number of publications which deal with the development of "label free biosensors" has been 50-fold over the last decade. Further, in 2011, amongst the publications related to sensors, about 53% dealt with optical sensors (Figure 2).

In recent years, optical biosensors have found a wide range of application in various fields like proteomics,[10] biomedical research,[11] biological interaction: to measure both kinetic and thermodynamic parameters of the interaction,[12] pharmaceuticals/drug discovery,[13] clinical diagnostics,[14] environmental
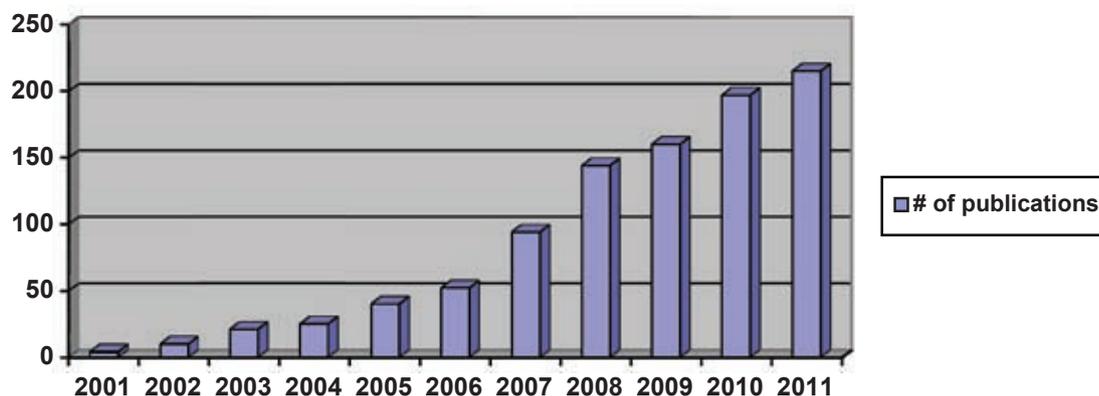

Figure 1: Number of publications involving the concept of "Label free biosensors".
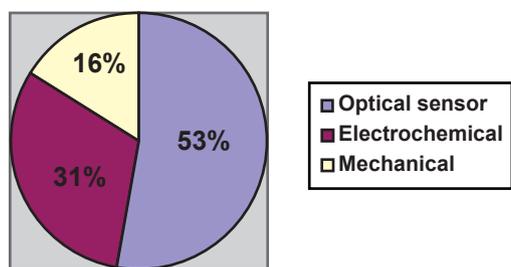*Source:* ISI Web of Knowledge.

Figure 2: Publications on different types of sensors in the year 2011.
Source: ISI Web of Knowledge.

monitoring,[15] combinatorial libraries,[16] food safety,[17] security,[18] *etc.*

This review aims at exploring the translation of large, macro-scale devices to miniaturized devices fully capable of analyzing a sample for more than one biological/chemical measurand. To this order, the review will initially discuss the various methods, phenomena and instruments adopted for developing optical biosensors. Thereafter, the research performed towards the integration of such devices on microfabricated chips and the creation of multiple sensors, contiguous to each other by various research groups will be discussed.

2 Biosensors, an Introduction

Biosensors are sensors that utilize a biologically derived species (molecule, cell, *etc.*) in close proximity to or contiguous to a physical or chemical transduction mechanism in order to create a reagentless (between the biological species and the transducer) system for the detection of an analyte. Biosensors can be classified according to the type of transduction mechanism, the transduction platform, the recognition mechanism or the analyte to be detected. Optical biosensors, a type classified on the basis of the transduction platform/mechanism, form an elite group of biosensors, which have been widely researched and commercialized. It is still one of the most hotly researched areas.

The scheme of a typical biosensor is given in Figure 3 and the gross mechanism of the functioning of a biosensor in Figure 4.

As can be seen from Figure 4, if the sensing action, or a measurable output signal, can be derived at step 3, reporter molecules, tagged with "labels", are not required, thereby eliminating a step in the measurement process and reducing complexity and the time consumed in measurement. Such measurements are termed "label free" and are at present one of the primary foci in biosensor research.

Biosensors are classified as labeled if the sensing element (or secondary sensing element, as in the case of sandwiched assays)[19] is tagged with radioisotopes,[20] fluorescent dyes,[21] semiconductor quantum dots,[22] *etc.* or as label-free if the sensing element is not tagged. Labeled biosensors have been used for quite a long time as they provide low LOD, ease of availability of instruments for sensing and optimized/standardized labeling procedures. However all these advantages of labeled sensing are not able to overcome the effect of problems faced due to labeling in various applications.[23,24] The labeling procedure is time-consuming; due to the addition of extra steps, labeling has a higher operation cost. As additional reagents are required, labeling cannot be used for real time monitoring of analyte binding: and can interfere with analyte binding. Labelling also poses a difficulty in multiplex analysis due to the need of different labels leading to further complex procedures. Also, different tags have their inherent problems such as: 1) In the case of radioisotopes: short lifetime, high cost, generation of a large quantity of hazardous contaminants and the need for sophisticated hot labs and 2) In the case of fluorophores: excitation and emission efficiency is reduced with usage by photobleaching and loss of kinetic information as the assay is read at the end to avoid interference by light[25] Also quenching and self-quenching reduces the efficacy of tags in a stochastic way. Also some of the tags are not compatible with live cells, so the same sample cannot be studied again.

These problems, along with the need for inexpensive, user friendly, POC devices, have led people in industry and research institutions to move towards their counterpart: label-free sensing.[26] Label-free sensing is relatively less expensive and reduces testing time. The sensitivity of label-free sensors was a concern; however, with advancements in transducer technology this problem has been largely overcome. These sensors also perform real time studies as they measure the inherent property of the analyte to be detected. This reduces the experimental uncertainty caused due to interference by the label. Based on these advantages, label-free sensing leveraging on transduction techniques like Surface Plasmon Resonance (SPR), Interferometer, *etc.,* has found applications in various fields.

Label-free sensing can be classified as direct or indirect. In direct assays, the response is directly proportional to the amount of analyte present. Direct label free sensing is a simple, one step, and reagentless operation.[27] However, direct assays may not always be ideally suited for a particular detection. In such cases, an indirect assay is used, where the binding reaction between a capture molecule immobilized on the sensor surface

Labeled biosensors: are biosensors using tags/labels along with transduction techniques to improve efficacy of detection.

Label-free biosensors: are biosensors using transduction principles for the direct detection of analytes.
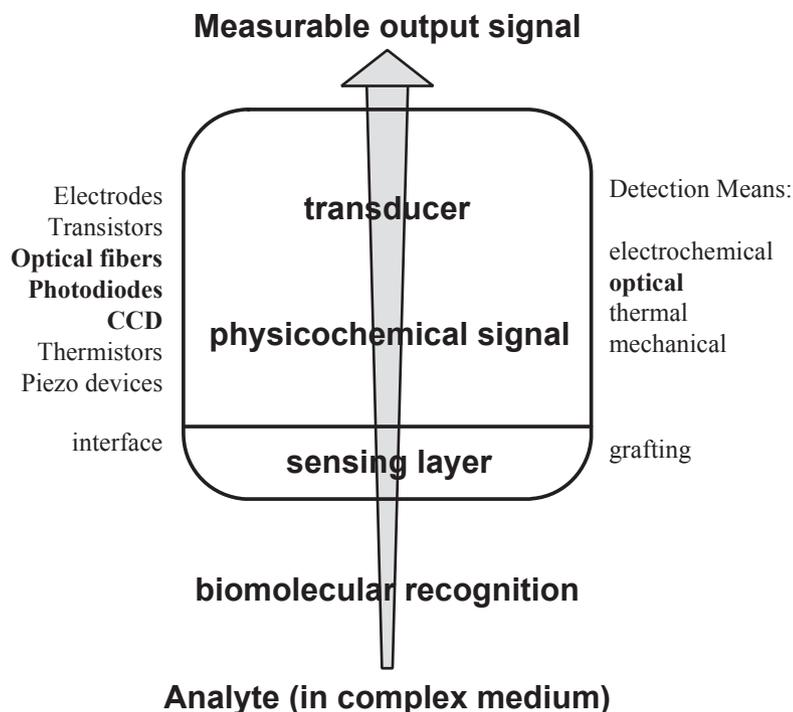
Measurable output signal

Electrodes
Transistors
Optical fibers
Photodiodes
CCD
Thermistors
Piezo devices

transducer

physicochemical signal

interface

sensing layer

Detection Means:

electrochemical
optical
thermal
mechanical

grafting

biomolecular recognition

Analyte (in complex medium)

Figure 3: Schematic of a biosensor.

Step 1.
Capture molecules
immobilized on
sensor surface

Step 2.
Reaction of analyte
species with capture
molecules from a
complex mixture

Step 3.
Direct assay

Step 4.
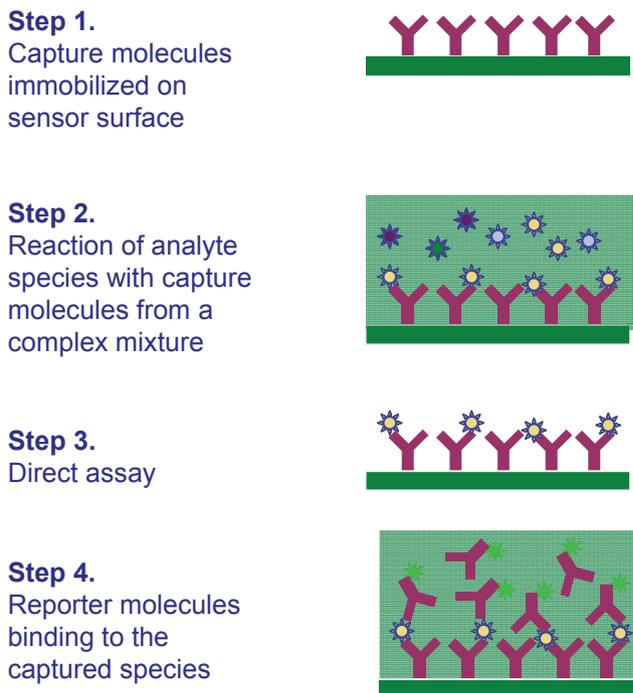Reporter molecules
binding to the
captured species

Figure 4: Direct assay and labeled assay.

and a pre-assigned complementary molecule (or species) is inhibited by the presence of the target measurand.

As mentioned earlier, optical sensing has gained tremendous importance due to its inherent advantages over other transduction mechanisms. Optical sensors can work along with both labeled and label free formats. Label free optical sensing usually induces a change in the amplitude, phase, polarization, or frequency of input light in response

to a physical or chemical change produced at the sensing surface. These changes in the light signal are usually related to small changes in the refractive index or absorbance due to the presence of the analyte to be detected in the proximity of the sensor. Different combination of materials and their concentrations may have a similar change in the refractive index leading to possible ambiguity in the case of complex analytes. Therefore, to make the sensing process specific/selective, a receptor molecule, which is specific for a particular analyte, has to be immobilized on the sensor surface. The binding of biomolecules will cause a change in the optical properties at the surface of the sensor and thus can be calibrated to predict the concentration.

One of the primary issues that researchers in the field of optical biosensors face, is the fact that simple optical transmission (or absorption) based detection systems, where light passes through the analyte volume, are frequently plagued by inaccuracies due to multiple interfering species in the analyte volume. Therefore the issue of light coupling into the system assumes crucial importance. The key to this lies in the use of the interaction of the evanescent wave with the biomolecule of interest. In this regard, a short discussion on the origin and properties of the evanescent wave may not be out of order.

When light is coupled into a material and the ray of light strikes at angle θ_i at the interface of two non-absorbing structures having refractive indices n_i and n_r ($n_i > n_r$), some part of the light is refracted and the remaining is reflected back as seen in Figure 5a. At the critical angle θ_c light is propagated at the surface as seen in Figure 5b. When incident angle $\theta_i > \theta_c$ light is reflected back totally as seen in Figure 5c. This phenomenon is

known as total internal reflection (TIR) and the wave is termed a 'guided wave'. In such cases, at the junction, there is no net transfer of energy. However, the boundary condition cannot be zero. This leads to the formation of an evanescent wave, whose power decreases along with the distance from the surface.

The evanescent wave is formed at the surface of all the waveguides and can be exploited for use in biosensors. It is also particularly advantageous since the light in this case never actually passes through the analyte volume and is therefore relatively uncorrupted by interfering species. We will illustrate the concept of evanescent wave (EW) based sensing using optical fiber sensors, while explaining/mentioning the EW based sensing that has been successfully exploited in many other formats.

2.1 Evanescent wave-based sensing on optical fibers

The structure of an optical fiber is shown in Figure 6. The region in which light is coupled and hits the junction of two surfaces is termed as the 'core', whose refractive index is greater than the cladding. Since light is coupled in such a fashion that the angle of incidence at the core-cladding interface is more than the critical angle, light propagates, virtually undiminished through an optical fiber. This has led to a revolution in communication technologies in the last fifty years. A careful analysis of the core-cladding interface reveals an evanescent field as the tail of the bell shaped field of the guided wave. The penetration and absorption of the evanescent wave is affected by the relative refractive indices of the core and its surroundings, the wavelength of light, *etc.* If the jacket and cladding are removed, leaving only the

Waveguides: are structures guiding electromagnetic waves using the principle of Total internal reflection.
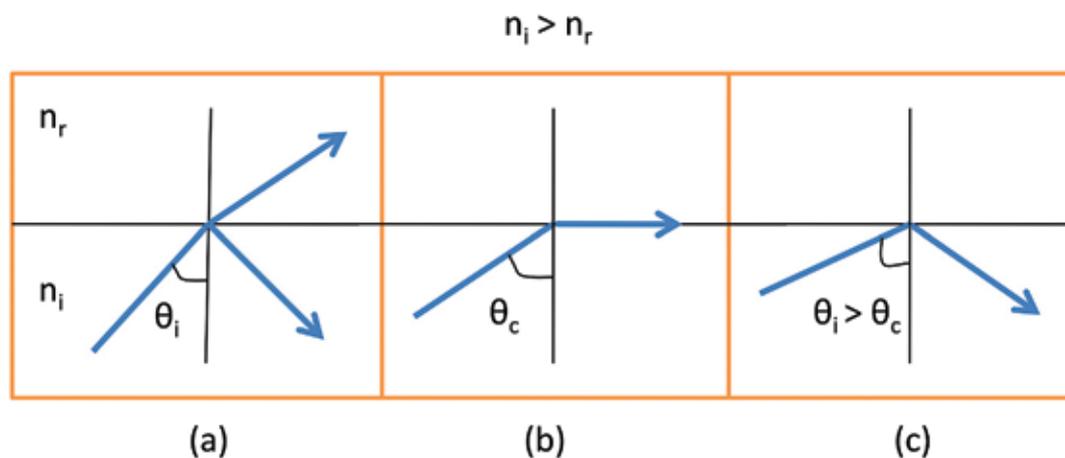
$$n_i > n_r$$

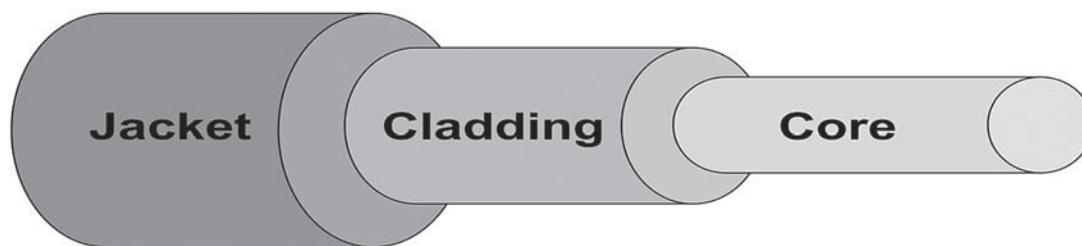**Figure 5:** Illustrations of total internal reflection.

Figure 6: Basic structure of optical fiber.

core, the evanescent wave can be seen penetrating into the medium surrounding the core. In case biological molecules are immobilized on the surface of the core, the evanescent wave will be affected not only by such molecules but also target molecules that may bind to the immobilized capture molecules.

Of particular interest is the fact that the penetration depth (d_p) of the evanescent wave (defined as the distance at which the magnitude of the electric field at the surface decays to its '1/e' value) and given by Eq. (1),[28] is very small, effectively of the order of a few hundred nanometers for straight fibers. Therefore, interfering species beyond this distance hardly affect the field of the propagating light.

$$d_p = \frac{\lambda}{2\pi \cdot n_{co} \sqrt{\left(\sin^2 \theta - \frac{n_{cd}^2}{n_{co}^2} \right)}} \qquad (1)$$

SPR: is a resonant oscillation of valence electrons in a metal due to the transfer of energy of incident light at resonance condition, which is dependent on the refractive index, wavelength, incident angle and surface material.

where λ is the wavelength of the incident light, θ is the angle of incidence, n_{co} and n_{cd} are the refractive index of the core and cladding, respectively.

Straight and cylindrical optical fibers provide good sensitivity and low loss. However, due to the low effective depth of penetration of the evanescent wave they are not sensitive towards target species which have a size beyond a few tens of nanometers. So, it is difficult to detect cells, which usually have sizes ranging beyond a few hundred nanometers. It has been theoretically proven, and practically demonstrated, that changing the design or geometry of optical fiber probes can increase the depth of penetration of the evanescent waves.[29] U-bend and tapered probes have been used by many researchers in order to increase the sensitivity of fiber probes.[30,31]

Bharadwaj *et al.,* 2011 had demonstrated evanescent wave absorbance based sensing using U-bent optical fibers for label-free detection of E. *coli* by measuring change in absorbance at 280 nm. A schematic depicting the relative advantage of a bent fiber over a straight fiber, with respect to evanescent power and depth at the sensing surface, is shown in Figure 7.[32] For the

purpose of sensing, the U-bend part is decladded in the bend region. The depth of penetration of the evanescent wave in the bend part increases to an extent that the evanescent wave can effectively be affected by bacteria cells. The binding of *E. coli* cells to the antibodies immobilized on the surface increases the absorption of the electromagnetic wave (UV light) propagating through the fiber, due to the absorption of 280 nm wavelength of light by proteins in and on the bacterial cell.

The evanescent wave is utilized for sensing in many other devices due to the advantages mentioned earlier. Some of the most prominent ones of these are those which utilize the interaction of the evanescent field with the surface plasmons in a metal-dielectric interface.

2.2 *Surface Plasmon Resonance (SPR) based devices*

Extensive research has gone into the field of SPR biosensing, right from the time it was first demonstrated for such a purpose by Liedberg *et al.,* in 1983.[33] At present, a large number of papers discussing optical biosensing are based on the principle of SPR. A surface plasmon wave (SPW) is a charge density oscillation occurring due to the interaction of light photons with free electrons at a metal dielectric interface. At specific resonant wavelengths, the energy of the incident photons is transferred to the free electrons on the metal surface, leading to the phenomenon of Surface Plasmon Resonance. The power of the surface plasmon wave decreases exponentially in the direction perpendicular to the surface. The wavelength of light at which SPR occurs depends critically on the refractive indices of the dielectrics on the two sides of the metal and the metal itself. Assuming that the medium on one side of the metal film and the film itself are fixed for coupling the light photons in, the primary determinant of the resonance frequency will then be the refractive index of the medium on the other side of the metal film and the angle (greater than the critical angle) of incidence of the light photons. On the other hand, if the wavelength of light being incident on the interface
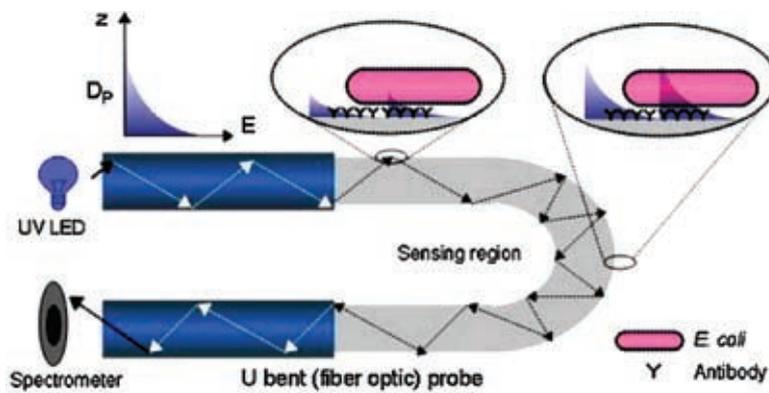
Figure 7: Enhancement of penetration depth 'D$_P$' in a bend fiber probe, enabling evanescent field overlap for the detection of *E. coli*.[32]
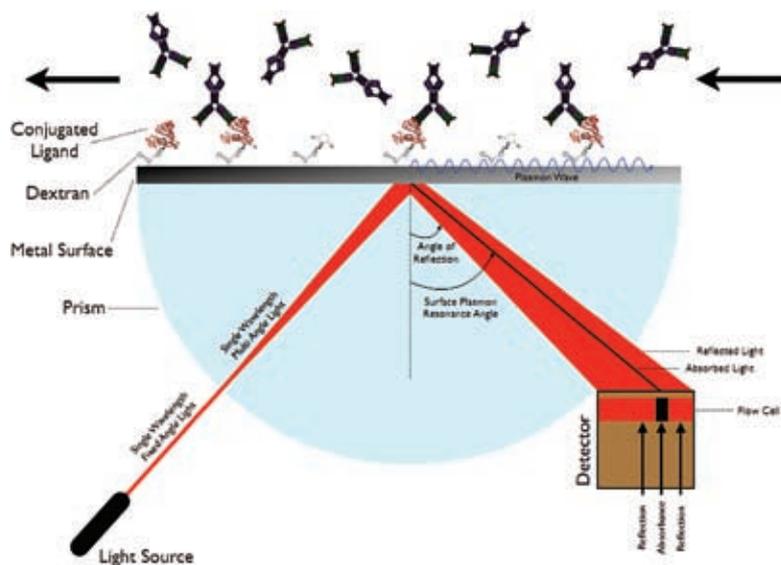

Figure 8: Schematic of SPR sensing.[37]

is fixed, then the angle at which resonance occurs will be determined by the refractive index of the aforementioned medium. Both of these are very sensitive to minute changes in RI and thus can be utilized to study the biomolecular binding that affects the refractive index of the medium near the interface. SPR devices conform to three common configurations, namely, the Prism coupled based SPR system,[34] the waveguide based SPR system,[35] and the Grating coupler based SPR system.[36] The Prism coupled based SPR system is the most commonly used configuration. A schematic of SPR sensing using prism coupled is shown in Figure 8.

2.3 Localized Surface Plasmon Resonance (LSPR) based sensors

Localized surface plasmons are charge density oscillations confined to around metal nanoparticles or nanostructures[38] as shown in Figure 9. This phenomenon, like SPR, is created due to light of a particular wavelength incident on an assemblage of dispersed nanoparticles or distinct nanostructures. The wavelength of light at which LSPR is observed therefore depends on the material, the size and shape of the nanoparticles or structures as well as the refractive index of the medium immediately around them. Like evanescent waves, the intensity of the oscillations decays along with the distance from the surface of the nanoparticles. LSPR based sensors have been in existence for a very long time and they have been used in various formats starting from dispersed nanoparticles in solution, to color changing strips and, more recently, nanoparticles immobilized on optical fibers[6] or waveguides.[39] More details regarding the use of such nanostructures and films can be found in the review written by Satija *et al*.[40]

LSPR: is surface plasmons generated in the vicinity of nanoparticles whose dimensions are less than the wavelength of incident light.
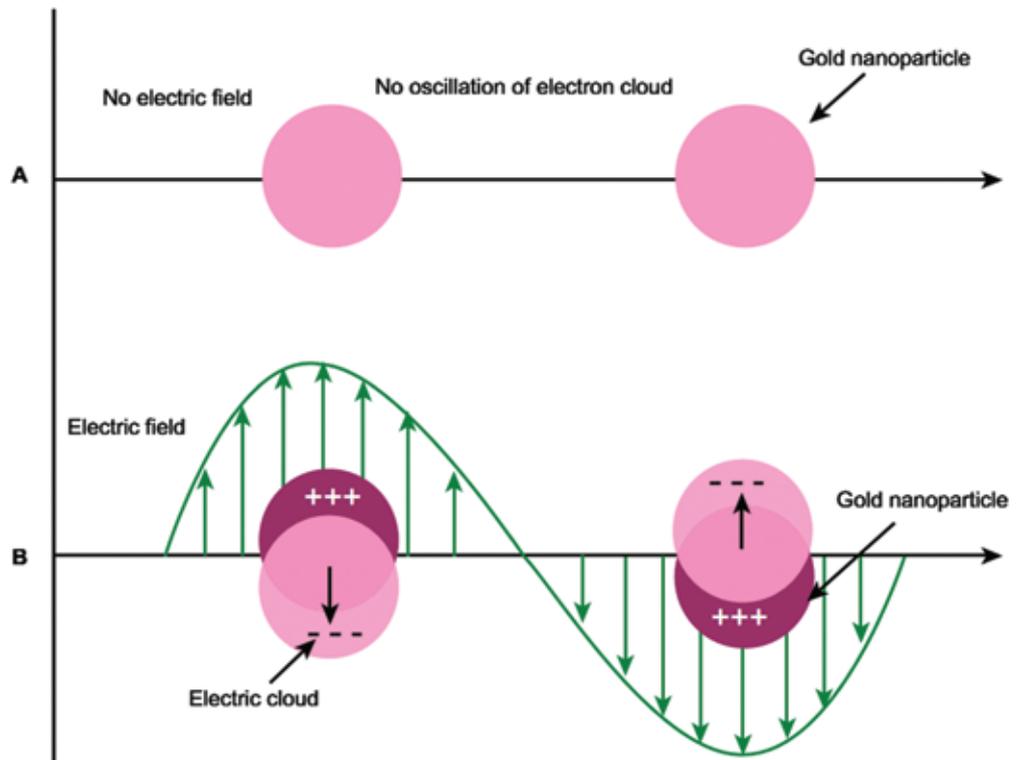
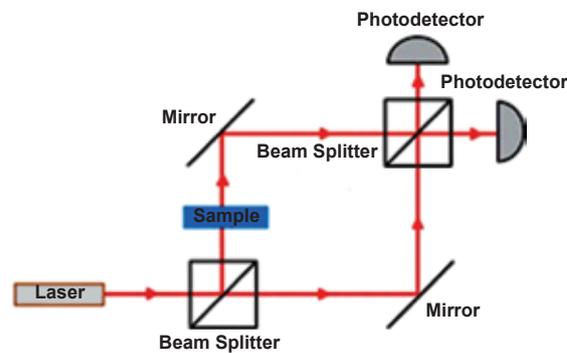**Figure 9:** Schematic of LSPR generation in the vicinity of a nanoparticle.[40]

Figure 10: Free space schematic of a Mach–Zehnder interferometer.[41]

2.4 *Interferometry-based devices*

The change in refractive index due to the binding of an analyte has also been exploited in interferometry based devices. These devices depend on the physical phenomenon that a refractive index change in a medium participating in the transmission of light leads to a change of phase in the transmitted electromagnetic wave. This, if allowed to interact with a reference wave (which is not subject to a refractive index change in its path), will produce an interference pattern. The pattern depends obviously, amongst other things, on the change in RI, which makes it amenable to be used

as a biosensor. The simplest configuration of an interferometry-based device is the Mach–Zehnder interferometer (Figure 10), where a light beam is split into two parts which pass through reference and sensing waveguides and finally meet at a junction where they interfere and produce a characteristic pattern on a linear array optical detector.

The phenomenon of interference has also been used in a slightly different format where rays reflected from the interface between various layers in a composite layered structure interfere and produce a pattern. It is quite obvious that the change in refractive index of one of the layers

(most frequently the uppermost one) will shift the interference pattern. This technique has been named Reflectometric interference spectroscopy (RIfS). White light is partially reflected and transmitted at the interface of thin transparent layers with negligible absorption. If the optical pathlength is smaller than the coherence wavelength of incident light, then the different reflected partial waves of the incident wave interfere as seen in Figure 11. This leads to the formation of an interference pattern (constructive or destructive) which is dependent on the wavelength, incident angle, optical thickness (optical thickness is defined as product of physical thickness and the refractive index of the layer) and the refractive index of the uppermost medium. The binding of an analyte molecule or particle to the sensor surface causes a shift of the interference pattern in the wavelength axis.

2.5 *Ellipsometry based devices*

As mentioned earlier, the primary optical changes that can occur with a receptor target interaction is a change in the refractive index of the nano or micro environment and a change in the optical absorption or extinction coefficient. A conventional and age-old technique called Ellipsometry, which exploits these changes has been adopted for use by many researchers for biosensing applications.[43–45] Ellipsometry is based on the alteration in the polarization state of the reflected light due to changes in the dielectric property or refractive index of a sample surface. The foundation for this was laid by P. Drude in the late 1880s in a series of papers.[46] Drude used the reflection of polarized light for measuring the optical constants of metals. With the development of semiconductor microelectronics, and the need for measuring thickness in microns, this technique became very popular and is still used routinely in microfabrication facilities.

Measurements using ellipsometry are based on measuring the optical constants of the material which depend on the refractive index and extinction coefficients. Figure 12 shows that the schematic of measurement, where linearly polarized light on reflection from a surface, becomes elliptically polarized. The eccentricity and the orientation of the axis of the ellipse are dependent on the angle of incidence and optical properties of the surface. Ellipsometric
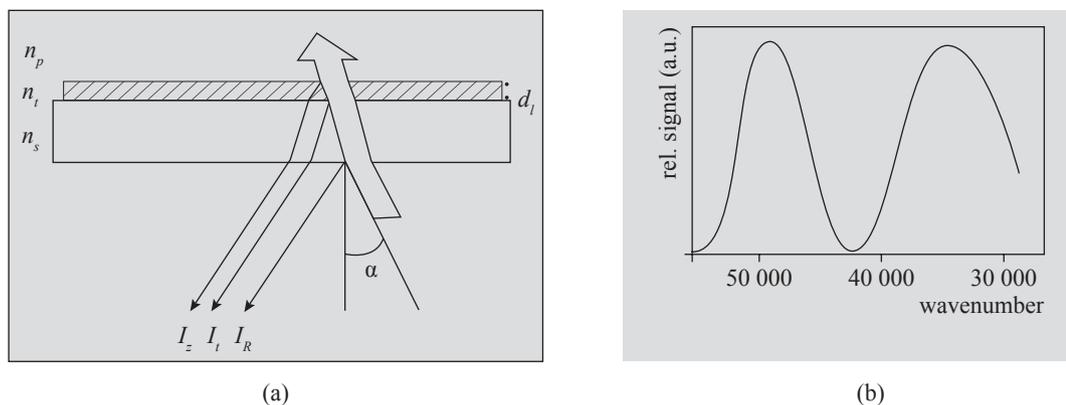

(a)

(b)

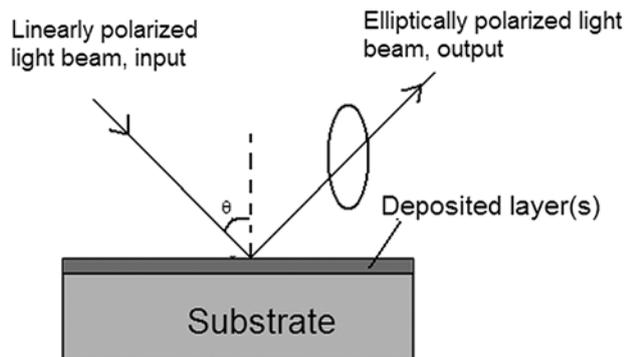**Figure 11:** Principle of RIfS sensing (a) Schematic; (b) Corresponding interference pattern.[42]

Figure 12: Schematic of principle of measurements using ellipsometry.

parameters i.e. the amplitude parameter (ψ) and phase parameter (Δ) derived from the output allows modeling the reflection coefficients which depend on the optical properties of the material, along with optical parameters like wavelength and angle of incidence of the input light. The two parameter measurements increase the accuracy and thereby the usability of ellipsometric measurements for the characterization of thin films.

One of the main limitations of conventional ellipsometry is that the light beam passes through the medium under study. This affects the use of this technique in real biosensing applications, as species other than the target may affect the output. Further, the sensitivity may also depend on the nature of the solid-liquid interface and be lower than solid-solid interfaces. Conventional ellipsometry has poor sensitivity for different solid liquid interfaces. To overcome the disadvantages, a modification of conventional ellipsometry known as Total Internal Reflection Ellipsometry (TIRE) or surface plasmon enhanced ellipsometry[47] has been developed.

It was reported that phase modulated ellipsometry can detect different phase transitions in the monolayers of surfactants on water.[48] This was backed by Kim et al., 1989[49] by using ellipsometry based on TIR, as in the SPR devices of the Kretschmann configuration, combined with ellipsometry of the reflected light. The schematic of TIRE is shown in Figure 13. The setup is similar to conventional ellipsometry, except for the addition of a prism along with the glass slide coated with a thin metal film. Polarized light enters the prism, undergoes TIR at the glass metal interface, and causes SPR at the metal surface when resonance conditions are fulfilled. SPR causes minima in the reflectivity vs. angle plot. A change in the surface characteristics of the metal layer (due to biological or chemical interactions) changes not only the position of the minima but also the phase of the reflected light. The specialty of TIRE is that it has the advantages of both SPR i.e. the light beam does not pass through the sample medium and is highly sensitive to refractive index coupling during weak coupling[50] and Ellipsometry i.e. it detects both amplitude variations and phase variations. It has been reported that TIRE is 10 times better than conventional SPR.[51]

2.6 Ring resonator

Ring resonators are based on a concept analogous to "whispering galleries". In these devices, there is a change in resonant wavelength due to a change in refractive index in the proximity of the resonating structure. In the setup of a Ring Resonator, a bus waveguide is kept adjacent to a circular (microring) waveguide structure (Figure 14) to couple light into the circular waveguide. Light of a particular wavelength will be coupled from the bus waveguide to the ring if it satisfies the resonant conditions given by Eq. (2):

$$n_{eff}L = m\lambda \qquad (2)$$

where n_{eff} is the effective refractive index of the ring which depends on immobilized biomolecules, analytes near the surface and bulk refractive index; L is the circumference of the ring; m is an integer; and λ is the wavelength. Ring resonator-based sensing is dependent on the quality factor. If the quality factor is high, then the resonance peaks become narrow, which facilitates the detection of
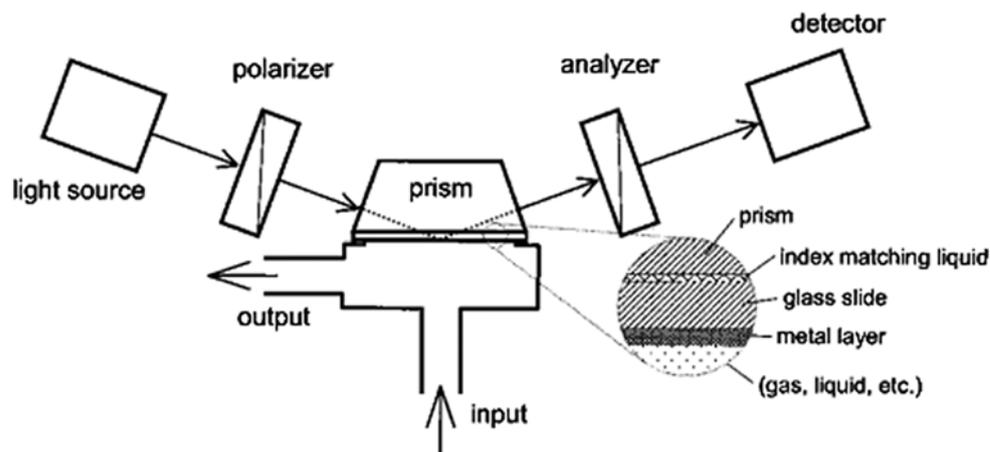

Figure 13: Schematic of TIRE. Magnification shows different stacks of layers between the flow cell and prism.[51]
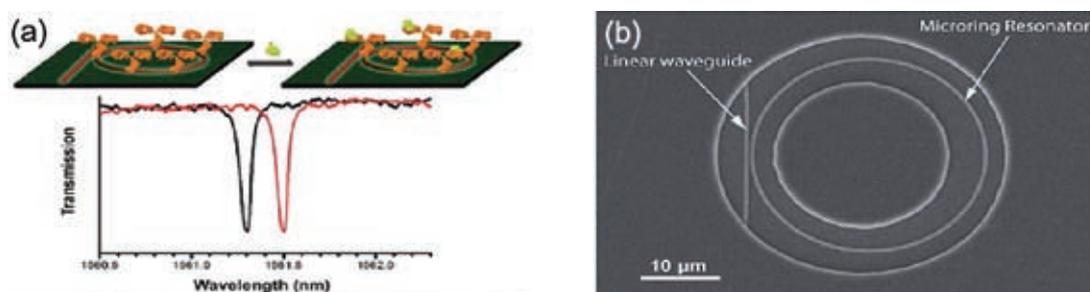
Figure 14: Micro Ring Resonator: (a) Working; (b) Top view of microring with adjacent waveguide.[41]

small shifts, thereby increasing spectral resolution. When the biomolecular capture agent is immobilized on the ring, a particular resonant peak is observed based on the effective refractive index. If there is a binding of the analyte with the capture agent, there is a change of the effective refractive index which changes the resonant condition and thereby causes a shift in the resonant wavelength as shown in Figure 14(a).

Most of the devices described in the preceding sections were developed relying on either large external instrumentation systems and/or large sensor devices. This makes them difficult to use in point-of-care applications and in detecting multiple analytes from a small sample volume. However, with the development of microfabrication, MEMS and MOEMS technologies, it is entirely possible to integrate these devices on a chip with minimal external instrumentation. Further, in the process of integration and miniaturization, it is also entirely possible to put multiple sensing heads on the same chip and essentially detect more than one target analyte in a sample. The following sections describe research and development efforts from laboratories worldwide in creating integrated and multiplexed sensing systems based on the principles of the devices described earlier.

3 Integration and Miniaturization

The emergence and prevalence of a large number of infective and pathological diseases, and the discovery of biological markers identifying the diseases and sometimes the causative organisms, coupled to the large population in India and other emerging economies, has brought about the requirement of high scale integration and multiplexing in biosensors and devices. This also has led to the possibility of Point of Care (POC) devices, which can perform the detection/testing decentralized either at the physician's office or at a patients place.[52,53] The first step towards such devices is the miniaturization of existing devices to perform sampling, detection, analyzing, *etc.* on a chip.

This section discusses some such efforts which can lead to multiplexed sensor applications which are discussed in a future section.

3.1 *Evanescent wave absorbance based sensing using miniaturized optical waveguides*

The requirement for on-chip optical communication has led to the development of on-chip optical waveguides. The structure of these waveguides is similar to optical fibers as seen in Figure 15. There is a high refractive index core sandwiched between two layers of low refractive index. The bottom layer is typically the substrate (silicon or an oxidized layer of silicon) and the top layer or "cover", is a deposited layer with a low refractive index, typically sputtered oxide or polymers. In the case of sensing applications, the cover is typically modified either physically or chemically. In the latter case, the cover layer is modified for selectivity towards a target by attaching receptor molecules. As in optical fibers, the evanescent wave generated at the interface is used for sensing. One of the main advantages of integrated waveguides is the ease of fabricating different probe geometries. Further, in the case of integrated waveguides, there is control over the dimensions of the waveguides down to sub-micron levels using fabrication technologies like E-beam lithography,[54] LIGA,[55] *etc.*

Prabhakar and Mukherji, 2010 have reported the fabrication of integrated polymer waveguides coupled with microfluidic channels using an SU-8 polymer. They have also reported the optimization of probe geometry and the dimension waveguides for higher sensitivity. U-shaped waveguides, along with the fiber couplers and microchannels were fabricated using single step SU-8 patterning. Figure 16(a) shows the image of a mask design for fabrication. The serpentine design helps in fabricating many waveguides together on the chip. The close-up in Figure 16(b) shows the microchannel having the U-bend area as one of the wall. Light from the source was coupled to the waveguide
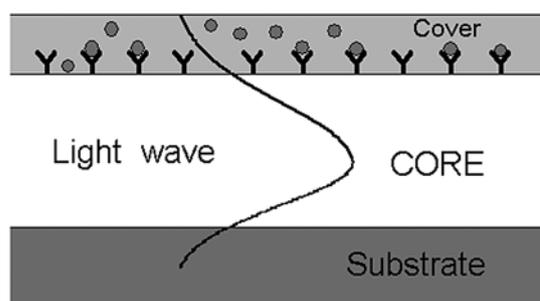
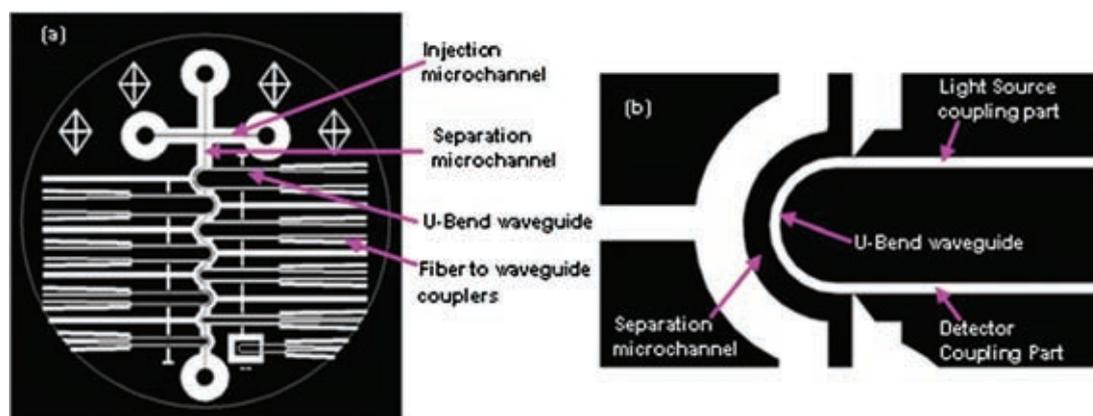**Figure 15:** Evanescent field propagating in a waveguide.

Figure 16: (a) Mask design of the U-bend waveguide; (b) Closet of a U-bend waveguide.[56]

using an intermediate optical fiber that is inserted into the waveguide-fiber coupler structure. The sensitivity of the waveguide was tested using different concentrations of Methylene blue and sucrose solutions.[56]

3.2 Miniaturized SPR based biosensors

The possibility of exploring the SPR phenomenon using optical fibers has been looked into by many research groups around the world for quite a while, more so since optical fiber based SPR sensors may be able to dispense with the prism for the coupling optics. The primitive fiber optic-based SPR sensors had tips coated with a metal layer,[57] side coated,[58] cladding partly removed and metal deposited,[59] metal on taper,[60] *etc*. A particularly interesting device has been demonstrated by Kurihara *et al*., 2004, who fabricated a fiber optic-based SPR microsensor by using selective chemical *etch*ing (SCE) technology.[61] A microprism was created by selective chemical *etch*ing on the flat end of an optical fiber and a metal layer was sputtered on it. Figure 17(a) shows a micrograph of a microprism on an optical fiber. Figure 17(b) shows a further close up of the microprism having a base of approximately 1μm. Figure 17(c)

shows a schematic of the generation of surface plasmons on top of the metal coated microprism. When linearly polarized light is incident at the metal surface, surface plasmon waves are generated. These surface plasmons are dependent on the refractive index of the medium surrounding the SPR probe, the wavelength of light incident on the metal surface and the angle of the prism cone. Light reflected from the metallic surface of the microprism acts as an SPR signal. Figure 17(d) shows the optical system used to measure the shift in resonance spectra. Light from the optical source falls on the linear polarizer and through a beam splitter enters the single-mode optical fiber. The reflected light propagates through the fiber to the beam splitter and is directed towards the analyzer. The polarizers help separate the SPR signal from the input laser light. The cone angle can be modulated to define the refractive index range to which the SPR is sensitive. It has been reported that SPR in near infrared regions (NIR) shows more than thirteen times improvement in sensitivity.[62]

The integration of the SPR phenomenon with optical fibers to reduce reagent and analyte volume requirements as well as to provide the possibility of multi-analyte sensing has also been attempted
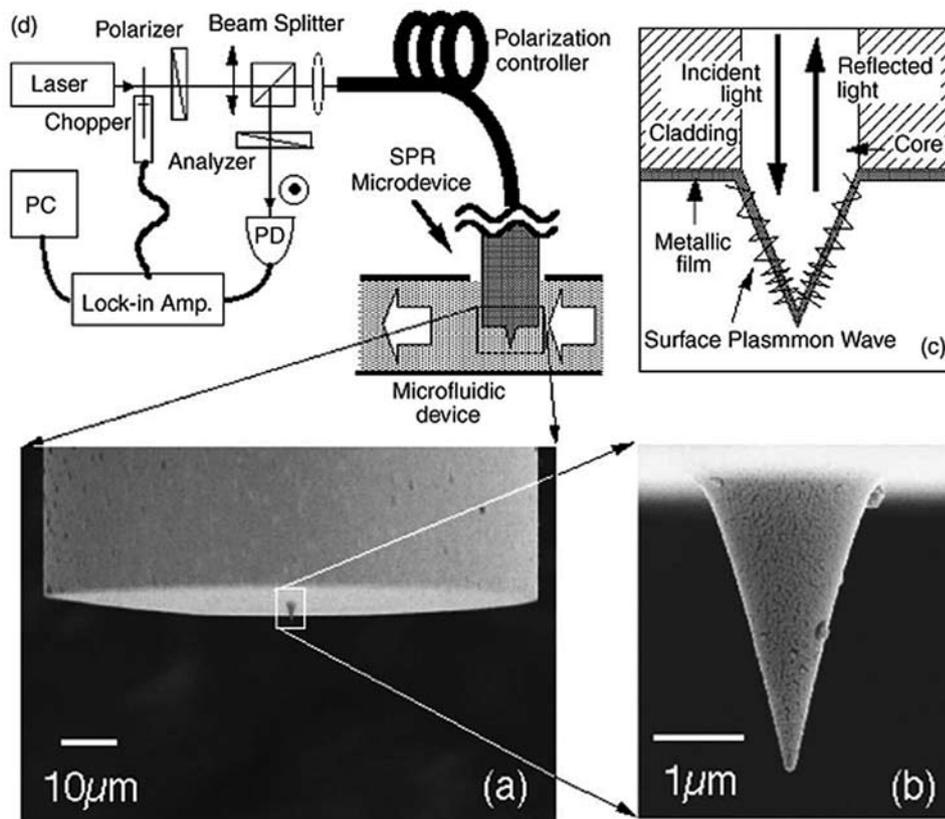
Figure 17: (a) Microprism fabricated using SCE; (b) Close up of microprism; (c) Generation of surface plasmon wave on the surface of metallic film on top of microprism surface; (d) Schematic of complete device.[61]
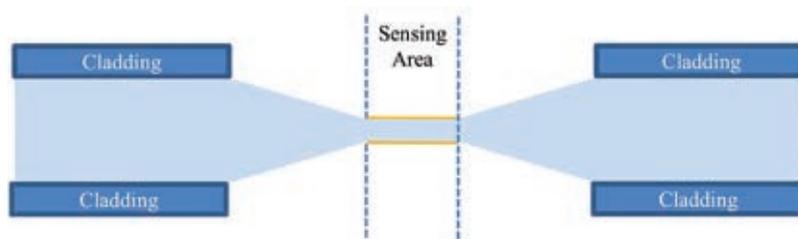

Figure 18: Cross-sectional drawing of a biconical tapered waveguide.

with tapered optical probes. The drawing of the design is shown in Figure 18, which is essentially a bi-conical taper with a straight neck used as the sensing region. This has shown better sensitivity in comparison to straight optical fiber-SPR probes. However, the taper ratio requires optimization since there is a maxima in terms of sensitivity.[31] This design has been modified by the introduction of a layer of Teflon between the dielectric core and the metal surface.[63] This caused the formation of two SP waves on the two interfaces of the metal providing a sensitivity 15 times better than the conventional uniform core fiber. A further level of

miniaturization and integration in terms of using metal-coated tapers for optical fiber-SPR has been demonstrated by Srivastava and Gupta, 2011.[64] In this case, there are multiple tapers in series with taper periods short enough to increase sensitivity. The waveguide is within the flow cell so as to be used for sensing applications. It was observed that with a decrease in the taper period, the shift in spectra increases but the accuracy decreases due to the broadening of the spectra.

Although the reduction of the SPR devices to optical fiber based ones from the prism and glass slide based ones represent a considerable degree

of miniaturization and possible integration, there are many issues that plague fiber based sensors and make their possible use in POC devices doubtful. The principal amongst these relates to the diameter of the fiber. Since the sensitivity is expected to be higher with lower diameter fibers, the sensors should be made of very thin fibers. However, that adds to problems due to fragility and difficulties faced during alignment for light coupling. These problems can be reduced by adopting the same principles and designing on-chip waveguides, which can be easily fabricated with small dimensions, fewer dimensional errors and suitable coupler structures.

Different bulk components have been integrated on chips to miniaturize the setup and reduce loss due to external coupling.[65,66] However, sensitivity and practicality issues have sometimes dictated only partial integration of the device, viz. integration of a select few of the individual components.

There is an elaborate review by Hoa *et al.*, 2007 giving insights about the change caused by integration on SPR.[67] The components involved in the basic SPR setup are the light source, optics like prisms and focusing lenses, a microfluidic system for controlling sample flow, a detector and supporting electronics and data analysis software to interpret the result. Also, the surface chemistry has an impact on the sensitivity of SPR. Light sources are shifting towards low power, stable, compact LED[68] and sophisticated laser sources.[69] One of the first integrated SPR sensors was Spreeta by Texas Instruments in 1996 which is currently owned by Sensata Technologies.[70] The Spreeta SPR is shown in Figure 19. The novelty in this miniaturized setup is that the source, detector and electronics are encapsulated in the optical covering thus making it compact.

The light path is from a AlGaAs (Aluminum Gallium Arsenide) LED source, followed by a polarizer. The polarized light then falls on the SPR chip with a limited range of incidence angles and the output light falls on the mirror, which reflects it to a sensitive linear photodiode array. Each pixel of the detector generates an output corresponding to a small range of incident angles All the components are enclosed by shielding to avoid stray/ambient light interference. Also, the housing includes a chip to monitor LED intensity variations. The output is a measure of the refractive index which is generated by the signal processor. The setup also consists of a temperature sensor remove the effect of temperature variations and to measure accurately the refractive index. The Spreeta setup has been modified and used for biosensing applications.[72]

4 Multiplexed Biosensor Applications

As a consequence of the intrinsic complexity of biochemical pathways (usually changed in disease states),[73] it has become established that multiplexed analysis (compared to conventional single-parameter assays) can offer a better biomolecular perspective of the onset of disease and its advance. A high throughput analysis of interactions also helps in understanding factors associated with it in real time. Both of these i.e. multiple analyte detection and high throughput screening drive the multiparameter technology.[74]
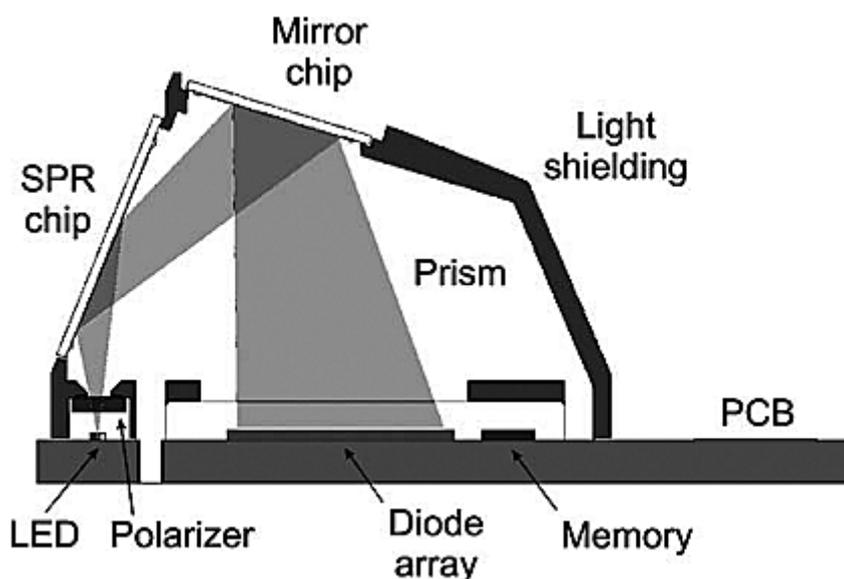

Figure 19: Cross-section of Spreeta 2000, indicating the light path and position of various components.[71]

4.1 Multiplexing using different label free optical sensing techniques

As mentioned earlier, one of the most promising technologies for multiplex/multianalyte detection is SPR. This is being performed in various formats in which multiple channels or micro arrays are created on a sensing surface and detection is performed using SPR microscopy or imaging SPR (iSPR). iSPR technology has ushered in microassays for high throughput analysis by the simultaneous screening of the analytes under consideration.

In the early stages of development, a multichannel SPR sensor consisted of a light source capable of exciting surface plasmons in multiple areas of a sensor chip and a detector system capable of detecting reflected light resolved into different co-ordinates of the same detector.[75] Later developments included detection of reflections on separate detectors;[76] and wavelength division multiplexing to increase the number of sensing channels by applying different wavelengths at different points.[77,78]

4.2 SPR Imaging (iSPR)

Homola et al., 2005 developed a multichannel SPR sensor based on wavelength division multiplexing of channels to detect multiple analytes.[79] Boozer et al., 2006 used this multichannel biosensor and applied their reported proof of concept for multiplexing by simultaneously detecting three fertility hormones: the human chorionic gonadotropin, the human luteinizing hormone, and the follicle stimulating hormone on the principle of SPR.[80] The iSPR apparatus consists of a p-polarized light source connected with a beam expander and a CCD detector to detect reflected light from the surface under consideration. The image is corrected from the background noise and is analyzed to extract binding curves as seen in Figure 20.

Huang and Chen, 2006 applied the technique of SPR imaging to protein arrays. They detected the presence of 4 proteins: bovine serum albumin (BSA), poly-l-lysine (PL), casein and lactate dehydrogenase (LDG) and also saw the change in the SPR signal during the denaturation of proteins.[81]

Dong et al., 2008 demonstrated the application of the SPR imaging technique for multiplexed analysis of Human immunoglobulin G–antibody interactions and they achieved an LOD of 6.7 nM IgG.[82]

4.3 SPR

Piliarik et al., 2005[83] demonstrated an SPR sensor generating a high contrast SPR image by merging iSPR with a polarization contrast and a multilayer SPR structure. They fabricated a chip with 216 sensing spots or 216 surface plasmon active areas with two types of multilayer structures, which exhibited diametrically opposite effects to the change in the refractive index of the sample. These spots were paired up forming a total of 108 channels. Light reflected from the uncoated areas of the chip surface was removed using a polarizer. This led to a high contrast SPR image and minimized cross talk between sensing spots. The sensitivity was increased and immunity towards input light intensity fluctuations was achieved by taking the ratio of the intensities of light reflected from the two different types of spots in a pair. The Refractive index resolution was measured as 3×10^{-6} refractive index unit (RIU). Initially, this novel design suffered from low reproducibility and reduced sensitivity which was addressed by using the microspotting method for immobilizing oligonucleotide probes which created 80% more binding sites and gave an LOD of 100 pM. The sensitivity was further improved (refractive index resolution $\sim2–3.5 \times 10^{-7}$) in 2008 by adding a self-referencing feature to the sensor system. In this design, two mirrors, formed at the bottom of the prism were used to provide a dynamic reference for the dark current correction of the detector and to provide dynamic referencing for fluctuations in the intensity of incident light (Figure 21).

On adding microspotting to this sensor, Piliarik et al., in 2009, could achieve a refractive index resolution of 2×10^{-7} RIU and a pathogen LOD of 100 pM.[85] Later in 2010, they went on to demonstrate high-throughput screening of protein biomarkers in diluted blood plasma. A high density protein array of 120 sensing spots was made by the microspotting method by immobilizing antibodies

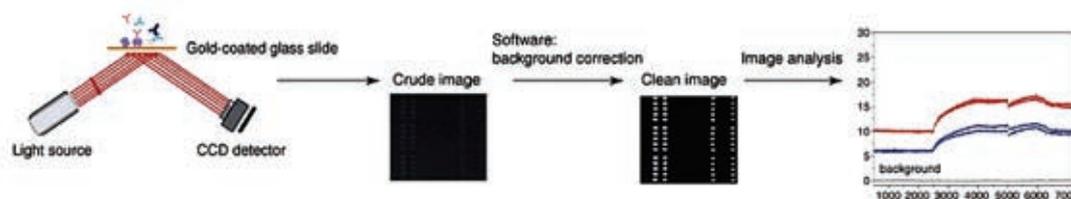**Multianalyte detection:** Detection of more than one analyte simultaneously.

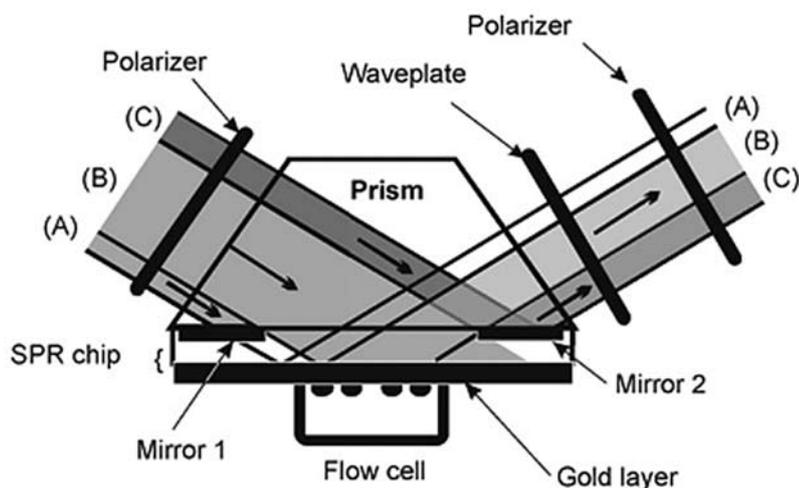**Figure 20:** A schematic representation of SPR imaging.[80]

Figure 21: SPR imaging with self-referencing and polarization contrast. Mirror 1 blocks part (A) of incident light and thus the detectors corresponding to that part of the sensor surface provide a reference for the dark current of the detectors since no light is reflected on to the detectors. Mirror 2 reflects part (C) of incident light to the detector for referencing against light source fluctuations.[84]

on the sensing spots and the remaining area was blocked by covalently immobilizing BSA to avoid non-specific adsorption on the surface. Using gaskets made up of adhesive material, they divided the SPR chip into 5 channels in an acrylic flow cell. Each channel had two pairs of 12 sensing spots, one spot for reading and a second spot for reference. They detected multiple cancer biomarkers like human chorionic gonadotropin (hCG) and an activated leukocyte cell adhesion molecule (ALCAM) with an LOD as low as 45 ng/mL for ALCAM and 100 ng/mL for hCG in blood samples.[86]

One of the major issues that any biosensor faces is the fact that the target molecules may not come close enough to the sensing surface to be captured. In this regard, Krishnamoorthy et al., 2009[87] demonstrated the use of electro-osmotic flow (EOF) for sample transport and electrokinetic focusing (EKF) for guiding the samples to specific array locations in iSPR. The channels were fabricated of PDMS by using replica molding[88] which was attached to the supporting substrate by a stamp and stick bonding technique.[89] The gold array was patterned on a glass chip by electron beam evaporation. The device was further improved[90] by forming 12 channels with 12 gold islands each (Figure 22). Limitations in iSPR camera dimensions allowed measurement of only 9 analytes (Anti-BSA, Anti-HSA, Anti-HIgG, Anti-neomycin, Anti-gentamycin, Fab specific AHIgG, F(ab)$_2$ specific AHIgG, Fc specific AHIgG and Anti-β2M) along with a reference channel. The system had the ability to check reproducibility without repeating experiments as

each channel measured the sample at 12 spots. The device suffered from problems of steric hindrance, due to the presence of capture ligands that were larger than the analyte molecules and target molecules sticking to the surface of PDMS.

Multiple analyte detection has also been achieved by Wavelength Division Multiplexing (WDM) of channels.[91] A special ATR prism coupler has been used which produces two SPR dips at region A and Region B as shown in Figure 23. Refractive Index resolutions of 1.3×10^{-6} RIU were observed in region A and 7×10^{-7} RIU in region B.

SPR-based sensors for multiple analytes have also been developed using diffraction gratings using a configuration termed a surface plasmon resonance coupler and disperser (SPRCD).[92–94] In SPRCD, as light is incident on a gold coated diffraction grating, a portion of the light in second order diffraction excites the surface plasmon wave and light diffracted in the first order forms the wavelength spectrum. The light is finally reflected off a toroidal mirror onto a CMOS detector array (Figure 24). This eliminates the need of an external spectrophotometer.

Diffraction gratings were fabricated using the holographic method.[95] A master grating was developed using the interference pattern of laser sources in an optical setup. The generated sinusoidal pattern on photoresist acts as a master for soft lithography-based grating fabrication.[96] Polydimethylsiloxane (PDMS) poured on the master grating was heat cured and then peeled off from the master grating as shown in Figure 25. This PDMS
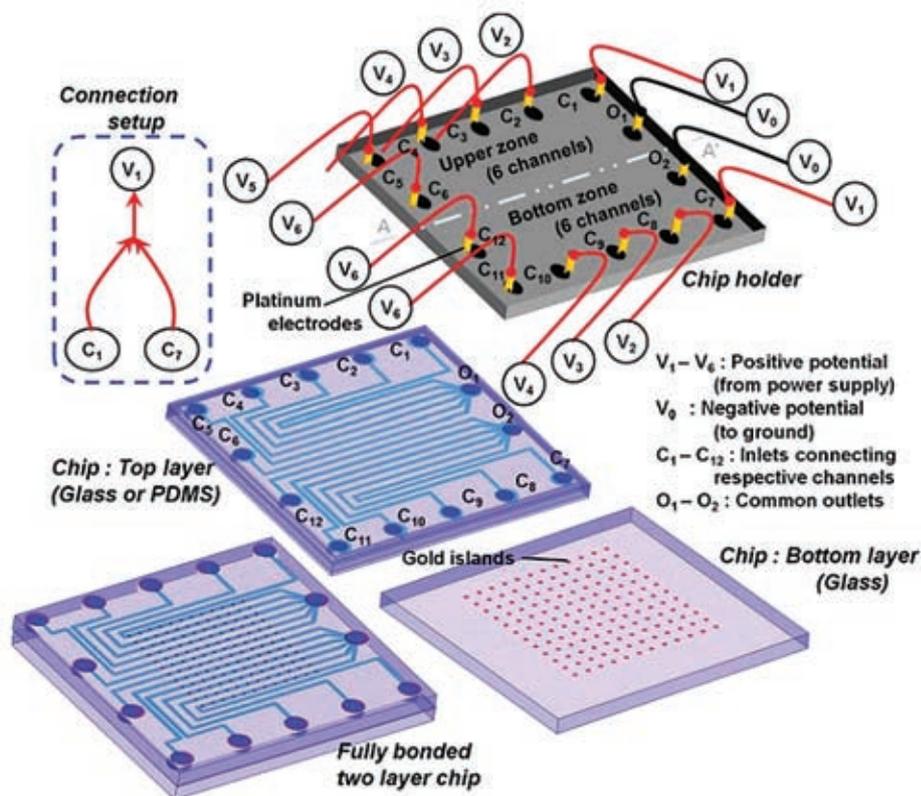
Figure 22: Illustration of a PDMS\Glass chip having 12 channels on a PDMS surface and 12 gold islands on glass, platinum electrodes were connected to the supply.[90]
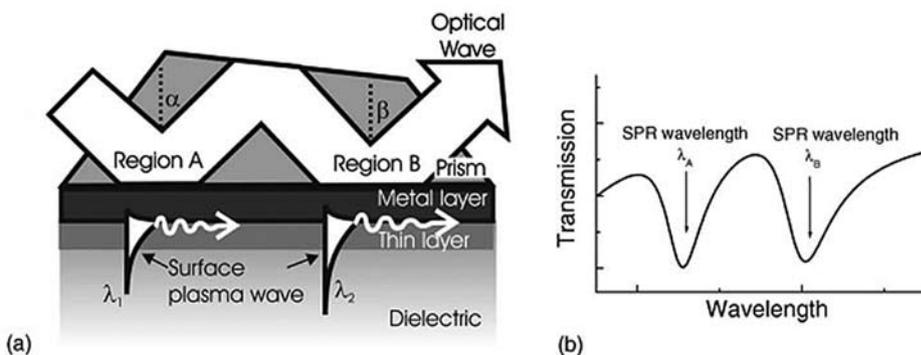

Figure 23: (a) Concept of WDM using a special prism and (b) Two SPR dips at region A and B.[91]

structure acted as the elastomer stamp. This stamp was then placed on top of a UV-curable polymer which was spun on the substrate. The polymer was exposed and then the stamp was removed. A film of gold was deposited on the replica obtained from the PDMS stamp.[97] This formed the grating used in the SPRCD structure. The SPRCD cartridge was made by clamping a 6 channel gasket to an SPRCD chip (Figure 26). The LOD observed were 0.30 μgL^{-1} for enrofloxacin (ERFX), 0.29 μgL^{-1}

for sulfapyridine (SPY) and 0.26 μgL^{-1} for chloramphenicol (CAP).

Akowuah *et al.*, 2010 did a theoretical study on dual channel planar waveguide SPR.[98] The channels had gold and silver SPR active areas, and showed distinct SPR dips when immersed in liquids of different refractive indices. Multianalyte sensing has also been achieved using ingeniously designed flow systems. Springer *et al.*, 2010 demonstrated a multichannel (4 channel) SPR sensor
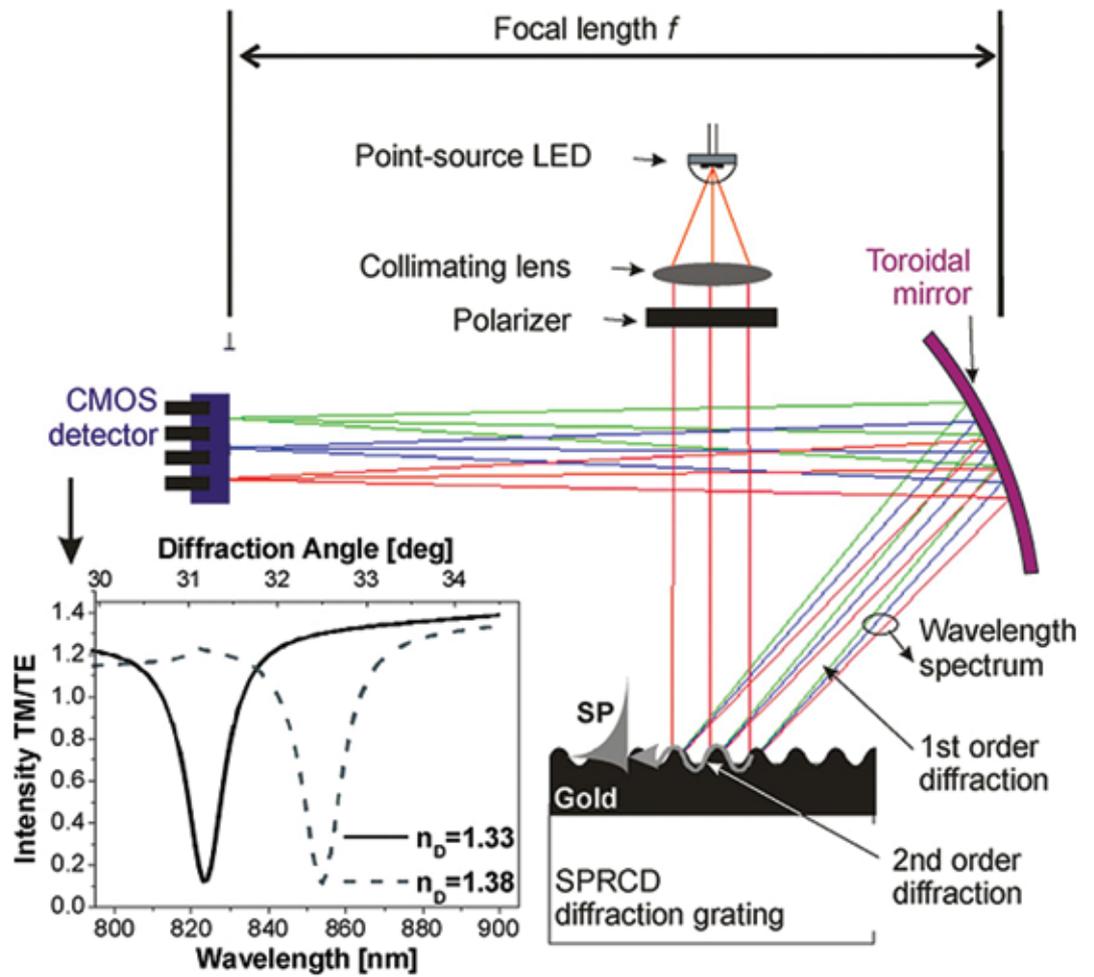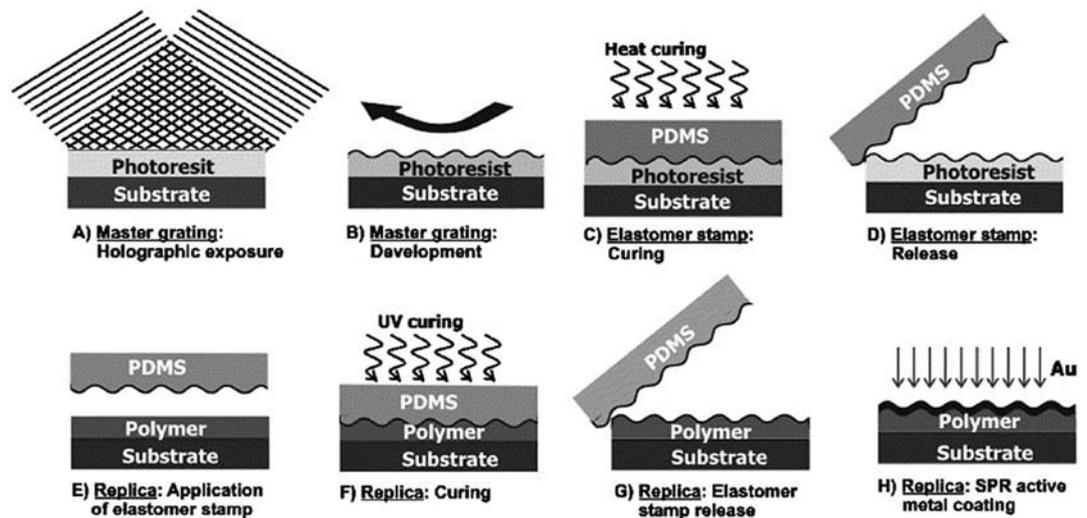
Figure 24: Concept of SPRCD.[94]

Figure 25: Fabrication steps of diffraction grating.[97]

detecting nucleic acids with an LOD of 3 fM in 4 min. As shown in Figure 27 the flow system has two states and in each state a different sample is allowed to flow over the sensing region.[99]

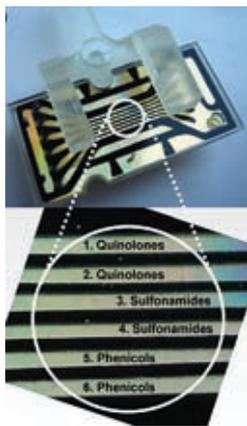Olkhov and Shaw, 2010 used microarray imaging to detect 4 proteins and to analyse

Figure 26: Image of an SPRCD cartridge for detection of antibiotics.[92]

antigen-antibody interaction. They used an inkjet printer to print an array of gold nanoparticle seeds on the surface. Thereafter, the seeds were grown to the required size and functionalized with the aid of an inkjet printer.[100] The LOD achieved was 250 ng/ml.[101] The capability of multiplexed SPR sensing for multianalyte detection was significantly extended when Ouellet *et al.*, 2010 came up with a microarray design using iSPR technology.[102] Utilizing microfluidics enhanced fabrication, a sensing platform was formed that could detect 264 analytes at a time. A PDMS layer was fabricated using soft lithography.[103] Up to 6 different concentrations of an analyte could be made on a chip, by a series of micromixers which diluted the analyte concentrations. Micro-pumps aided delivering the analytes to specific chambers and digital control helped in selecting particular chambers to be monitored. The details of the design are shown in Figure 28. There are six columns of 11 groups, each having four chambers. Row multiplexers were used to select a group of 4 chamber based binary control inputs. Samples
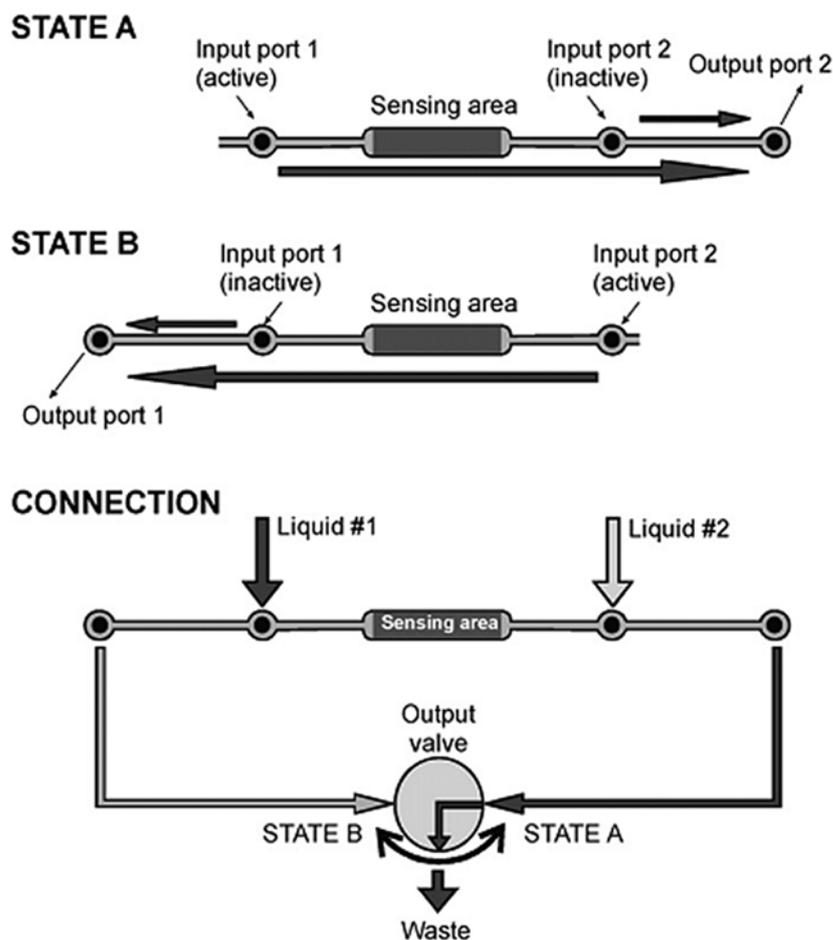

Figure 27: Schematic shows connections of sensing area to ports in different working states.[99]
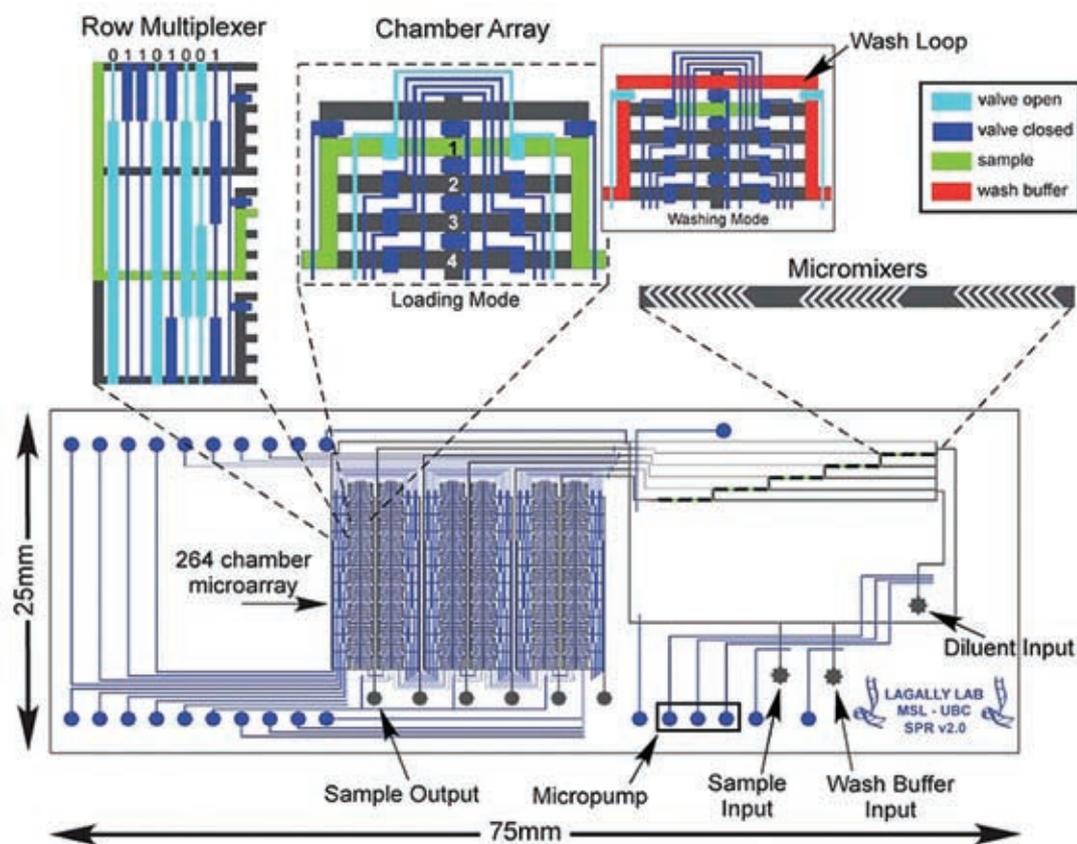
Figure 28: Schematic of microfluidic array.[102]

were loaded on to particular groups using an arrangement of valves which directed the sample appropriately (for example, in the Figure 28, green indicates that the valve of chamber 1 is open). Each sample loading was followed by a wash loop that removed remnant samples in the microfluidic path.[102]

In the area of multichannel SPR detection, a modified Polymerase Chain Reaction(PCR)-SPR setup was used to simultaneously detect pathogens (Pseudomonas aeruginosa, Staphylococcus aureus, Clostridium tetani and Clostridium perfringens) using a single stranded DNA (ssDNA) amplification scheme.[104] The complete scheme is shown in Figure 29. Pathogen specific complementary DNA probes were immobilized in line at a sufficient distance from each other on the gold film. The SPR setup was used in the Kretschmann configuration. The DNA of the bacteria was extracted and added to PCR for amplification. These PCR output was used as the input sample for SPR. The sample was recirculated in order to increase sensitivity.[105]

To increase resolution in multichannel sensing, a spectral/wavelength interrogation technique has also been explored. Depending on the capabilities of the recording spectrophotometer,

spectral interrogation can have better resolution. However, for sensing multiple channels/spots, a corresponding number of spectrophotometers and collimator pairs are required.[91,106] One of the possible cost-effective alternative methods is to use a programmable aperture for a spectrophotometer, described in a technique called spatial light modulation(SLM). The step by step process to collect output using SLM is shown in Figure 30. SLM has two states for its pixels: open i.e. transparent and closed i.e. opaque. Initially SLM pixels are set open. The active area of SLM is limited to the area of the aperture. Then regions of interest are identified and assigned to numbered channels. To remove background noise, SLM is closed and the background signal reference is recorded. Then one at a time, all the Region of Interest (ROI) areas are opened and signals are collected. The background signal is subtracted from the signals and high resolution SPR spectra are obtained. Sutapun *et al.*, 2008 demonstrated a prototype 5-channel setup made of PDMS by using the aluminum molding technique.[107] As may be obvious, the ability to measure the kinetics of reactions is limited by the rate at which all the ROIs can be cycled through. This technique, while introducing
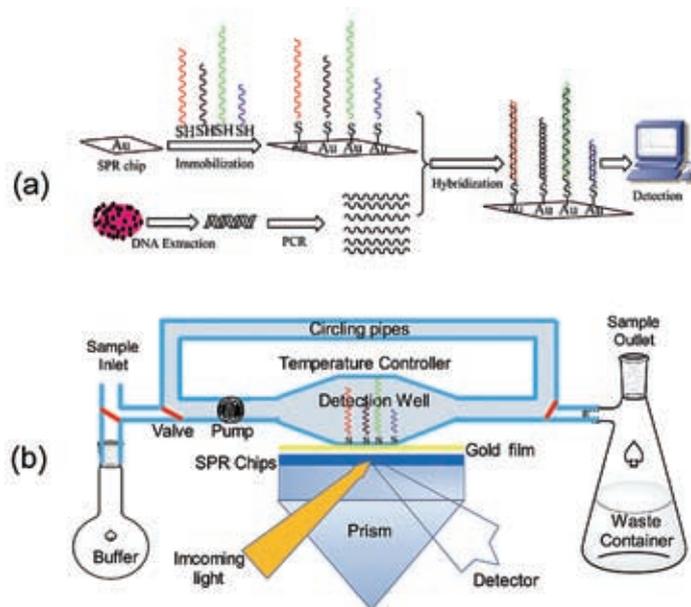
Figure 29: (a) Complete step from immobilization to hybridization and detection; (b) Modified SPR setup.[104]
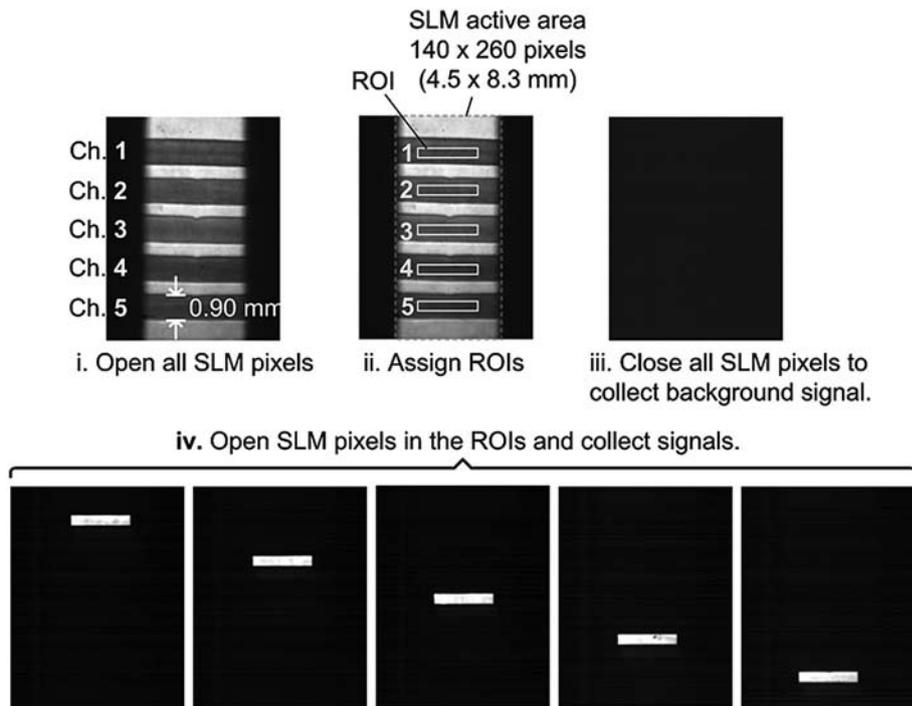
Figure 30: Operating procedures for collecting signal using SLM techniques.[107]

the possibility of using spectral interrogation in conjunction with a multichannel SPR setup, also provides the flexibility of obtaining data from array spots of any size and at any position on the substrate[108] limited only by the SLM spatial resolution. Therefore, many different methods like micro spotting, inkjet printing, *etc.* can be explored for creating arrays.

The coupling of light using SPR substrates has also been achieved using diffraction gratings. Thirstrup *et al.*, in 2004 presented a novel design using polymeric diffractive optical coupling

elements (DOCE) in conjunction with SPR sensors for multiplexing and ease of miniaturization. An injection-molded COC polymer chip integrated with two DOCEs replaced traditional prism and focusing optics for coupling light in and out of the SPR substrates (Figure 31(a)). The input light was collimated and projected perpendicular to the input DOCE surface. Because of mirror symmetry of the DOCE elements, the input light is focused to a point at the centre of the sensor element at different angles as can be seen in Figure 31(b). At the output side reflected light is converted to a parallel beam incident on different positions of the detector array. The gratings were fabricated using a holographic writing technique[109] involving two different lasers at two different angles to expose a photoresist to get the desired structure of the grating. A metal master is made by sputtering and galvanizing nickel on the photoresist. This metal master is used to make the polymer chip by injection molding and finally a gold layer is sputtered on the chip for sensing. The obvious advantage of this scheme lies in its possible parallelization.[110] In a parallel effort towards integrating diffractive optics to couple light in an SPR system, Chien et al., in 2009 designed an angular interrogation scheme based multichannel SPR chip using a focusing diffractive optical element (FDOE).[66]

In an extension to phase measurement schemes in SPR configurations,[111,112] Halpern et al., 2011 demonstrated phase measurements in a micro-
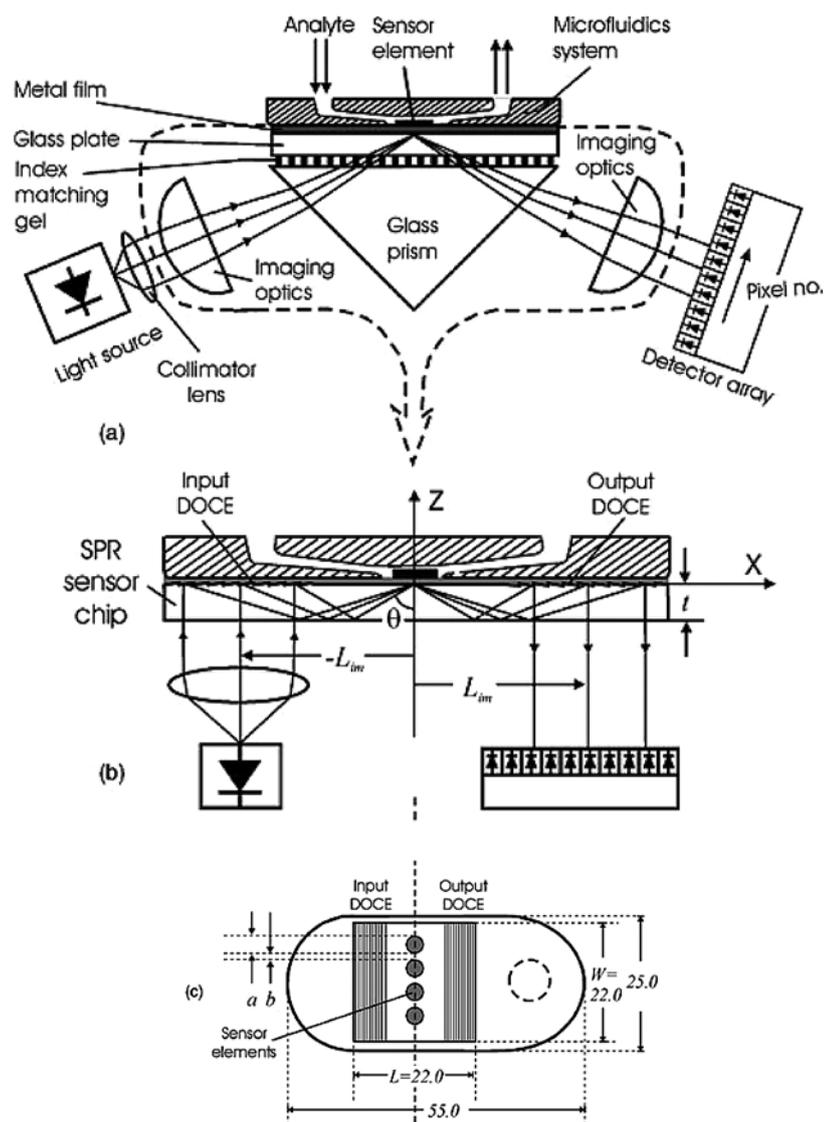

Figure 31: (a) Kretschmann configuration; (b) Modified SPR setup using DOCE; (c) Top view of the polymer chip along with dimensions in mm.[110]
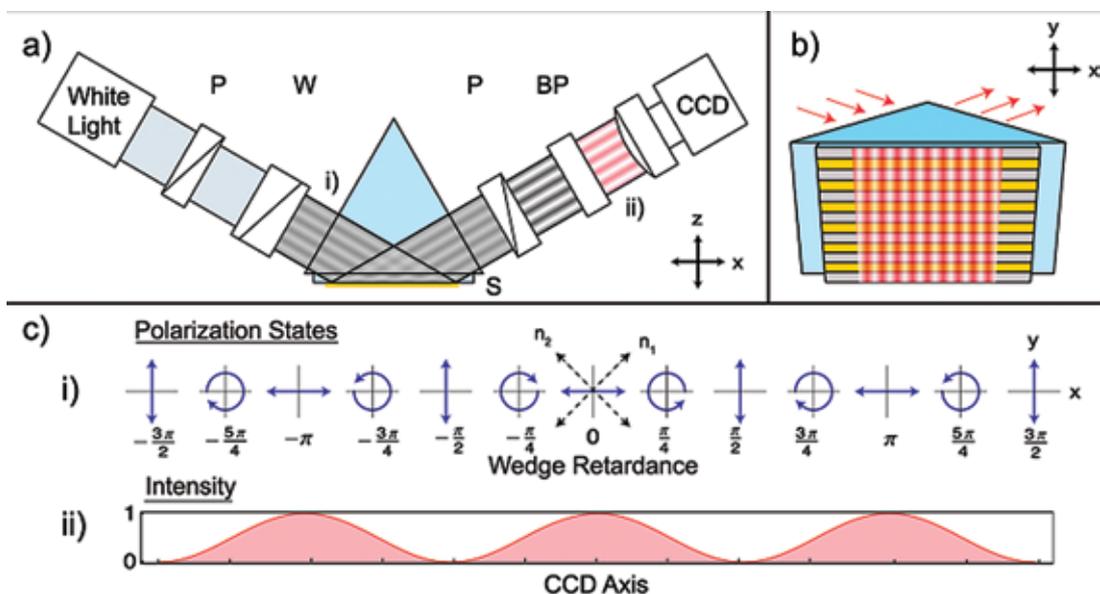
Figure 32: (a) Schematic of SPR-PI showing all the components; (b) Interference Pattern on the sensor surface; (c) Polarization states and Intensity plot.[113]

array format by using an SPR- Phase Imaging (SPR-PI) technique (Figure 32), that has the potential to increase the throughput of such measurements. The input light from the source is polarized and is passed through a wedge depolarizer. This creates a spatially distributed periodic polarization pattern on the sensing surface as seen in Figure 32(b). The reflected light passes through an output polarizer which creates intensity fringes related to a phase shift which, in turn, passes through a narrow band pass filter which generates a contrast. Finally, the pattern is detected by CCD.[113]

Biacore® was the first to commercialize an SPR apparatus in 1990 and since then many other SPR devices, based on various configurations and formats, have come in to the market. Since this is a very powerful technique that has captured the imagination of many researchers involved in proteomics research, various research groups have published results on the basis of data recorded from one machine or the other.[114] One example of this is the ProteON XPR36, which has been used by various researchers for multiplexed analysis. Situ *et al.*, 2010 designed a system with 4 x 4 arrays for label free detection using the SPR technique to detect 16 analytes simultaneously.[115] Abdiche *et al.*, 2011 expanded this instrument for 36 ligands and claimed it to be better than Biacore 2000 in terms of the time required for the same application.[116] Nahshol *et al.*, 2008 demonstrated the use of ProteON XPR36 to analyze parallel binding kinematics using multiple microfluidic channels.[117]

4.4 LSPR

As discussed earlier metallic nanostructures have been used for LSPR based sensing for a long time. With the development of various techniques, compatible with standard microfabrication tools, to create such nanostructures on a chip, this area of research has gained significant momentum. It is quite evident that multiplexed sensing systems can be developed using microfabrication coupled with nanostructure formation. Since the characteristics of the LSPR response depend on the size, shape and composition of the nanostructures, various methods have been adopted to create such structures on a chip.

Prabhakar and Mukherji[118] demonstrated a C-shaped waveguide coated with gold nanoparticles (GNP) using the single step lithographic processing used by them earlier.[56] The LSPR sensor had a sensitivity to RI changes better than the bare waveguide evanescent wave absorption sensor developed by them earlier.[39] The schematic of the waveguide used in the optical setup is shown in Figure 33. Light enters the waveguide through an optical fiber and propagates by TIR in the bend region. The evanescent wave generated at the interface of the waveguide interacts with immobilized GNP. The energy of the evanescent wave produces oscillations in the metal nanoparticles leading to localized surface plasmons. The sample under study flows through the microchannel which interfaces the bend region causing a change in the RI of the medium and thereby a change in the evanescent absorption characteristic.
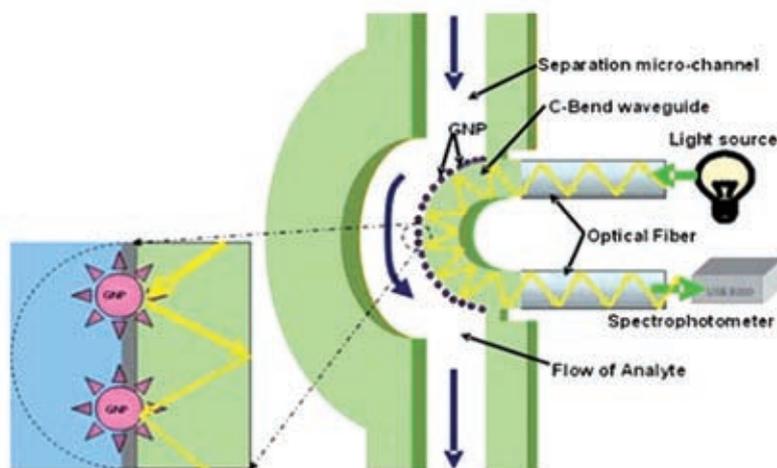
Figure 33: C-bend waveguide coated with GNP for LSPR based detection. The closet on the left shows the generation of surface plasmons due to the interaction of an evanescent wave and GNP.[118]
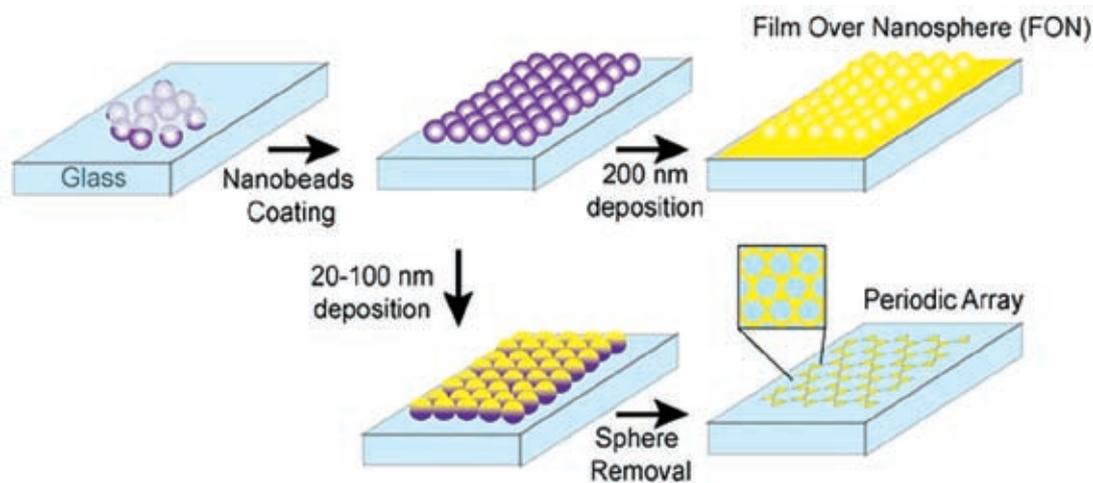

Figure 34: Steps in Nanosphere Lithography (NSL) to fabricate periodic nanoarrays.[121]

As size, arrangement, shape and material properties like the dielectric of nanoparticles are important in determining LSPR wavelength, a controlled synthesis of nanoparticles and a controlled arrangement of nanoparticles in the sensing region is important for the efficient use of LSPR. Many synthesis and fabrication methods for nanoparticles, nanostructures and nanoarrays are now available. We will look at some of the methods used for the fabrication of nanoparticle arrays.

Nanosphere Lithography (NSL) is one of the most common methods for nanoarray fabrication. The process involves an assembly of nanospheres made of polymers or some other material which can be dissolved or lifted off from the surface easily after being subjected to deposition of a metal by sputtering or vapor deposition methods. A schematic showing the steps for NSL is shown in

Figure 34. The substrate is cleaned and chemically modified (if required), so that the spheres can freely move and assemble on the surface. The spheres, along with a suitable dispersing medium, are drop-coated on the substrate. As the dispersing medium slowly evaporates, the nanospheres come closer and form a hexagonal close pack (HCP) structure on the substrate surface. This HCP is commonly termed a nanosphere mask. Once the self assembly of the nanospheres is complete, a metal layer of desired thickness is deposited on top of the microsphere mask. This layer is commonly termed metal film on nanosphere (FON). This plasmonic layer can be used as a stable substrate in various applications like SERS.[119] For fabricating LSPR devices, the substrate is then sonicated in a solvent to dissolve away the nanospheres and along with them remove the metal
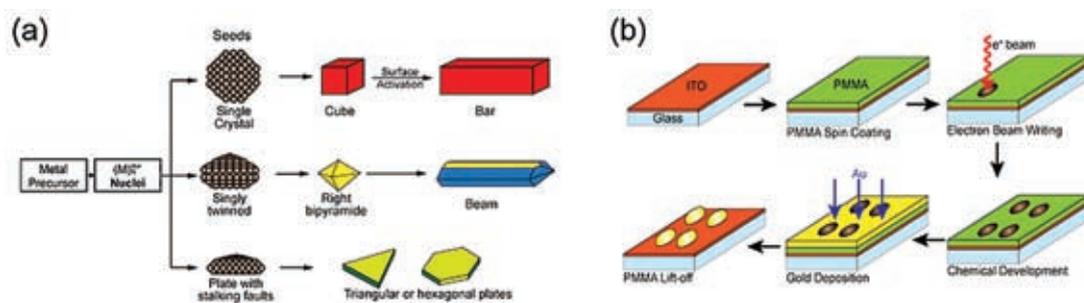
Figure 35: Techniques for fabrication of nano structures; (a) Chemical synthesis; (b) General steps for EBL.[121]

deposited on the surface of the spheres, leaving an array of triangular nanostructures on the surface of the substrate.[120]

To fabricate nanowells, the process is slightly different. Instead of depositing metal on the HCP, the substrate and the assembled HCP on it is processed in a reactive ion etcher (RIE) to get the desired nanowell depth (Obviously the nanospheres in this case have to be resistant to reactive ion etching). This creates an array of pits in the areas between the spheres. Then, instead of depositing metal film, the structure is sonicated to remove the nanosphere giving an array of pits and a metal layer is deposited to get a metal surface with an array of nanowells.[122] For getting the desired shape and structure using NSL, modifications can be performed at various steps of the process. NSL is rapid and cheap for fabricating many structures at the same time. However, since it is critically dependent on the shape and self-assembly properties of the nanospheres, it may have some defects like vacancies at some spots, polydispersity, line defects (slip dislocations), etc.[123]

Electron-beam Lithography is another popular technique for fabricating arrays of nanostructures on substrates. The main advantage of this technique over NSL is that it can fabricate nanoparticles of any shape and size with high accuracy. An electron sensitive resist is spin-coated on top of the substrate. The structure to be patterned is programmed into the system, and based on that, an electron beam scans over the surface to "write" the desired pattern and expose the resist. The remaining structure is removed and a metal layer is deposited. This has been used to fabricate nanoparticles of different shapes and sizes.[123] The fabrication steps are shown in Figure 35(b).

There are many other fabrication techniques like focused ion beam (FIB) milling,[124] dip-pen lithography,[125] etc. which provide control over the size and shape of the structures. Chemical synthesis methods (Figure 35a) have also been used for making nanoparticles on a substrate but they may

also exhibit polydispersity of nanoparticle size which has to be taken into account and reduced by optimizing the process.

Many conventional and novel fabrication techniques for nanopatterning are explained in a review by Stewart et al., 2008 which was followed by another review explaining nanofabrication techniques based on soft-lithography, along with an analysis of the effect of different dimensions on the spectra obtained.[126] All the techniques provide flexibility to modulate the shape, size and distribution of nanostructures, thereby selecting a working range of LSPR from the visible to the infrared region.[127,128]

Zhu and Zhou, 2011 fabricated an array of pentagram or star-like structures for application in an LSPR-based nanobiosensor.[129] They did the theoretical modeling of the device to design its structure and also to find out field distribution over its surface. Their calculation indicates it has high potential for applicability as a substrate for an LSPR based biosensor. Nanofabrication was done using the FIB technique by sputtering silver over the substrate milling out pentagram arrays.

Another excellent LSPR array of nano-cones were designed by Wu et al., 2010[130] using nanoimprint lithography (Figure 36). The silicon master was made using the Bosch RIE process.[131] Different echants with different *etch* rates for Silicon were used with deep RIE to create silicon cones on the wafer. Using UV-Nano Imprint Lithography an array of conical nanoholes was created on a polymer substrate. This nanohole array was used to create an array of nanocones on a different polymer substrate and a layer of gold was deposited to create gold-coated polymer cones.

The main aim of the iSPR technique was to take SPR sensing to high throughput parallelized sensing. As we saw, iSPR has been used in the Kretschmann configuration. One of the major limitations of this configuration is the use of a prism in the setup since it reduces the field of view, limits the scope of miniaturization and
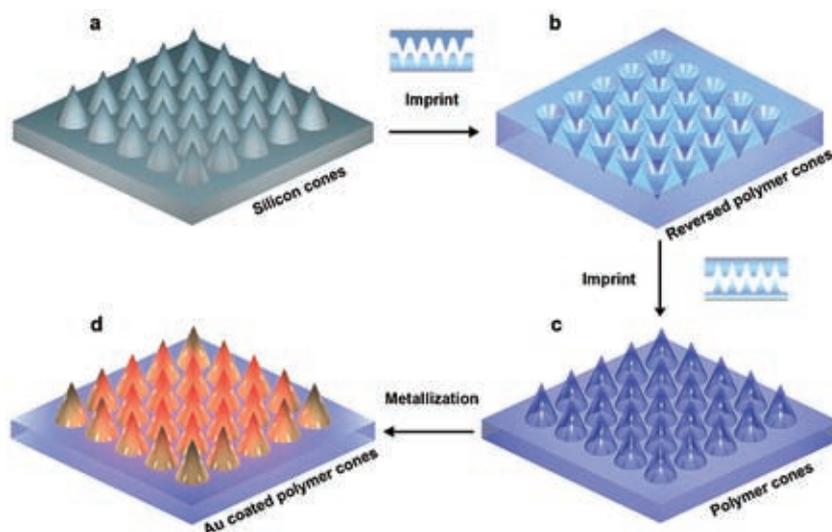
Figure 36: Steps for fabricating 3D cones using nanoimprint lithography; (a) Silicon master; (b) Reverse polymer cones made after imprinting into the polymer layer; (c) Polymer cones made from reversed polymer cones; (d) Gold deposited on top of the cones.[130]
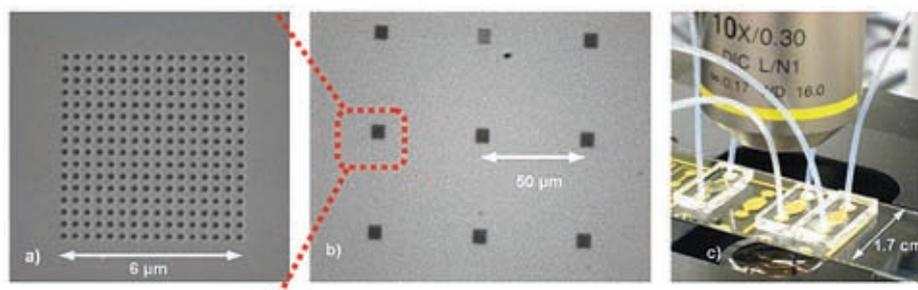

Figure 37: (a) Scanning electron microscopy (SEM) image of a 16 x 16 nanoarray; (b) Bright-field microscope image of a part of 5 x 3 microarray; (c) Microfluidic flow cell along with tubing.[132]

requires optics with low numerical apertures.[132] Research by Ebbeson *et al.*, 1998[133] and Martin-Moreno *et al.*, 2001[134] about enhanced optical transmission (EOT) in a periodic array of metal nanohole structures led to the use of nanohole arrays with SPR. They reported that surface plasmons on the top and bottom of the gold film are coupled through nanoholes at specific wavelengths. The wavelength is dependent on the refractive index of the sample and its surroundings, and also on the dimension and periodicity of the array. Basically, the nanoarrays act as couplers which convert light to surface plasmons on one side and surface plasmons to light on the opposite side. This property makes nanohole arrays good substrates for SPR sensing without the need of a prism by transforming the setup to utilize simple collinear optics. This was applied to biosensing by testing it in a transmission mode for monitoring biomolecule adsorption. 11-mercaptoundecanoic acid

(MUA), along with Bovine serum albumin (BSA) adsorption was successfully detected by Brolo *et al.*, 2004.[135]

Stark *et al.,* 2005 designed a nanohole array and sensing was based on monitoring changes in the intensity of EOT. They demonstrated high refractive index resolution (9.4×10^{-8} RIU) in the setup, which is better than conventional SPR and EOT sensing using wavelength interrogation.[137] Ji *et al.,* 2008 further developed 25 nanoarrays for multiplexed detection based on intensity sensing. On this nanoarray, 25 separate binding curves between glutathione S-transferase (GST) and anti-GST were measured in real time to demonstrate multiplexing.[137] This method was found to be suitable for multiple analyte measurements than the previously reported method by De Leebeeck *et al.*[136]

Lesuffleur *et al.*, (2008) demonstrated the potential of nanohole arrays based on the phenomena of EOT for high throughput scanning

SPR imaging.[132] They used a He-Ne laser source to improve detection sensitivity. They fabricated a 16 × 16 nanoarray using a focused ion beam technique on a gold-chrome coated glass substrate. Each nanohole had a diameter of 200 nm with a periodicity of 380 nm to 460 nm. A 5 × 3 microarray of such nanoarrays, each spaced at a distance of 50 µm from each other, was fabricated (Figure 37). A flow cell was fabricated using the soft lithography technique. A master mold was made using SU-8 and the pattern of 100 µm deep channels was transferred to PDMS. The detection setup had a He-Ne laser illuminating from the bottom of the chip. The optics was simplified due to the absence of a prism and the generation of surface plasmon waves directly from the nanoarrays. The transmitted light was detected using a 10x objective lens and imaged using a CCD in line on the opposite side of the chip. They optimized the array periodicity by measuring the kinetics of self-assembling monolayers at different periodicities. Im et al., (2009) demonstrated the applicability of the same setup for parallel measurements of molecular binding kinetics, by integrating a six-channel microfluidic chip to the SPR setup. The nanohole size was reduced to 150 nm from 200 nm to accommodate more nanoholes on a single chip.[138]

Yang et al., (2009) demonstrated multiplexed analysis using nanohole arrays which acted as a nanoplasmonic probing platform that exploited EOT.[139] They had already used the platform to detect single molecules (anti GST, LOD: 10 nM) and analysis of biorecognition events.[140] They showed multiplexing by fabricating 20 nanoarrays having different dimensions and periodicity, which consequently led to different nanostructures having different optical behaviors. They demonstrated analyte detection by passing NaCl, Coomassie blue, bovine serum albumin, and liposome solutions over the nanostructure surface. However the system developed had low refractive index resolution, in the range of 10^{-4} RIU, which is less than the conventional SPR.

Endo et al., (2006) developed a multiarray optical nanochip based on the principle of localized surface plasmon for high throughput screening. The multi array provided 300 nanospots with a core shell nanoparticle layer. In this case the LSPR nano chip was constructed by evaporating gold on a glass substrate and allowing a SAM of silica nanoparticles on the gold surface by modifying the surface chemistry. On top of the silica nanoparticle monolayer, gold was again evaporated. Thus, the silica nanoparticles act as a core and the top and bottom gold layers act as a shell (Figure 38). Light
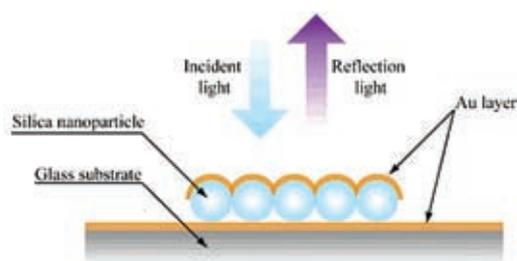

Figure 38: Core shell configuration for LSPR nanochip.[141]

was incident on the top of the surface through a fiber and reflected light having LSPR spectra was detected back by the fiber. Six different antibodies (Antibodies against IgA, IgD, IgG, IgM, CRP, and fibrinogen) were immobilized at this spot.[141] The antigen concentrations were determined by passing each sample through the complete array and it was quantified with respect to the peak absorption intensity of the LSPR spectra obtained. The detection LOD observed was 100 pg/mL.

Piliarik et al., in 2012 have published the design of an LSPR imaging based biosensor using a gold nanorod array.[142] They demonstrated similar sensitivity to LSPR imaging based sensors using a polarization contrast[111] by demonstrating detection at sub nanomolar concentrations. The Polarization contrast helps in taking advantage of the changes in both amplitude and phase of the reflected light. The polarized light was incident on the prism. The TIR at the prism metal interface causes an evanescent wave which excites the LSPR. When polarized light interacts with the surface, it becomes elliptically polarized on reflection from the surface. The parameters of the ellipse are dependent on the refractive index near the surface. This elliptically polarized light passes through a waveplate which causes a phase shift in TM and TE components. The output polarizer again linearly polarizes the reflected light. The enhanced output goes to CCD (Figure 39). A nanorod array was fabricated using e-beam lithography. They demonstrated the application of this array for oligonucleotide detection by detecting on an average one DNA per nanoparticle.

4.5 RIfS (Reflectometric Interference Spectroscopy) and SRIB (Spectral Reflectance Imaging Biosensor)

RIfS has found its application in High throughput scanning[42] and for performing binding studies.[143] Birkert and Gauglitz (2002) demonstrated RIfS for the development of a parallel affinity assay for thrombin inhibitors. Plastic microplates with 96 wells and 384 wells were used for sensing and were
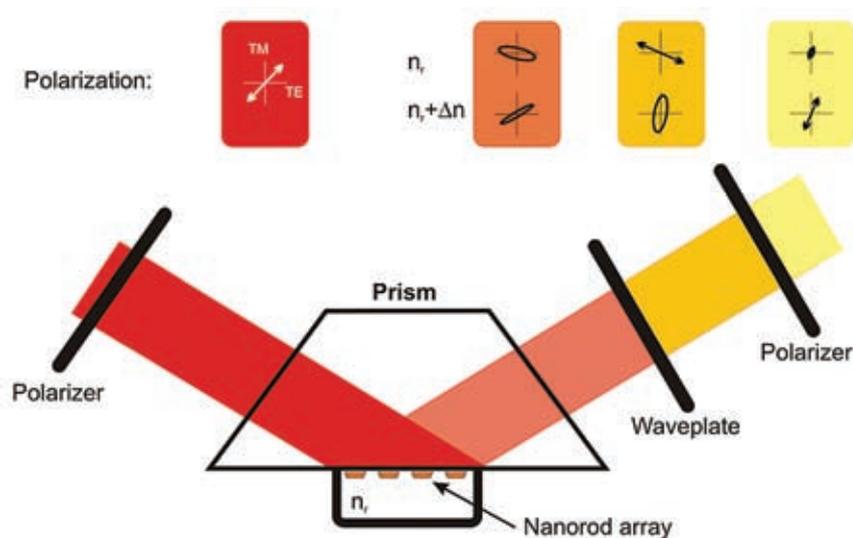
Figure 39: Schematic of an LSPR based optical biosensor using nanorod arrays along with polarization at different positions.[142]
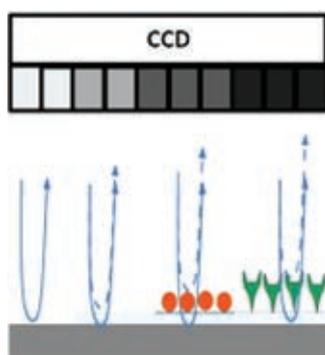

Figure 40: Principle of SRIB: Relative reflectance from different thicknesses can be seen as a change in intensity on CCD.[149]

glued to the transducer surface. The transducer surfaces were coated with biocompatible dextran. A thin layer of SiO_2 helps in the generation of waves for RIfS. The whole assembly was attached to a glass surface through which the sensor was "bottom illuminated" using a halogen source and a wheel monochromator. This light reflected by RIfS surfaces was detected using a CCD camera. The result was comparable to available fluorescent methods with no false negatives in the case of 96 well plates. This method can be applied to higher throughput screening like 1536-well plates.[144]

In 2008, Ozkumur et al., introduced a similar reflectance based interferometric detection method initially termed Spectral Reflectance Imaging Biosensor (SRIB) and recently renamed Interferometric Reflectance Imaging Sensor (IRIS) with a sensitivity comparable to an SPR sensor.

In this technique, in place of the white light source, a Laser/LED is used. SiO_2 is grown on a Si substrate for providing a platform for the attachment of molecular probes. The principle of spectroscopy is used to identify a change in optical thickness as seen in Figure 40. The light reflected from the substrate is detected by the CCD camera and the change in optical thickness is predicted based on interference patterns. They demonstrated the applicability of an SRIB by dynamically monitoring antigen antibody binding using 50 spots for each of four proteins consisting of BSA, human serum albumin, rabbit IgG, and protein G. An LOD of 19 ng/ml was achieved.[145] Later, they demonstrated a SRIB for the detection of DNA and proteins in microarray.[146]

The direct detection of DNA hybridization and single-nucleotide mismatches was achieved using SRIB technique. The results were excellent as mismatches were identified in more than 97% of the samples.[147] Recently, a review on the applicability of SRIB in different areas of research has been published.[148]

4.6 Integration and multiplexing in interferometry based devices

Compared to many other techniques, interferometry lends itself to integration on a chip, and thereby parallel detection schemes, more easily. Using standard microfabrication technology, waveguides can be fabricated so that they form the arms of an interferometer. One (or more) of the arms, works as the reference arm(s). Two configurations have mainly been used: the Mach Zehnder

interferometer and Young's Interferometer. In the former case, the input light, after passing through a common section of the waveguide is split into two parts using a "Y" junction, and after traversing through the sensing region of the waveguides, the light from the two arms is combined again using a similar junction (Figure 41). Since a sensing event leads to a phase change of the light in the sensing arm, the two waves interfere in a particular pattern.

In the case of Young's interferometer(YI) there is no Y-junction after passing through the sensing area, since the output light from reference and sensing arms interfere in free space and the pattern is picked up using a CCD detector. High sensitivity in the range of 10^{-6} to 10^{-8} RIU has been observed. Various materials like SiO_2, $Si_3 N_4$, polymers, *etc.* are being used for their fabrication. It is quite obvious that the degree of phase change will depend on the length of the sensing arm and the fact that only single mode waveguides can be used.

Interferometers have demonstrated high sensitivity and stability due to inbuilt referencing which has led researchers to apply interferometers for multianalyte sensing. Ymeti *et al.*, 2002 demonstrated the first multichannel YI based immunosensor.[150] They fabricated the YI using
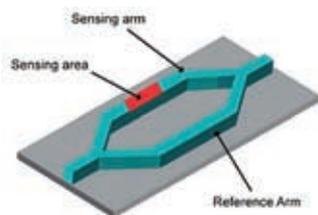

Figure 41: Mach–Zehnder interferometer integrated as waveguide.[41]

SiON technology and demonstrated a long term sensitivity of 2×10^{-7} RIU. They fabricated a 4 channel YI in such a way as to monitor the effects of the two channel pairs separately from the other. The distances between the channels were not the same. The Fast Fourier Transform output showed the presence of distinct peaks for different pairs of waveguides. This was modified later to a system which supported sensing from three channels, keeping the fourth as reference (Figure 42). Later, they fabricated 11 four channel YI on an optical chip and combined microfluidics to this multichannel YI.[151] A refractive index resolution of 6×10^{-8} RIU was observed which was quite high compared to other label free sensing methods. Further, the utilization of microfluidics made it possible to get a stable output in only 4 seconds which, compared to a standard time of 100 seconds, was very significant. They further detected different viruses using specific antibodies as shown in Figure 42.[152] The results showed the possibility of single virus detection and strengthened its feasibility for POC devices without extensive sample preparation steps.

Recently, Petrou *et al.*, (2010) fabricated a monolithic MZI array. They used a novel concept called Frequency-Resolved Mach-Zehnder Interferometry (FR-MZI) for detection. They integrated the light source and photodetectors on a chip, so as to avoid bulky optics, and thereby minimized the problem of light coupling. They developed an array of MZIs with individual light sources (LEDs) of different wavelengths and the output terminated at a single photodetector. A schematic depicting the MZI array is shown in Figure 43. Many sensors are connected in parallel for multiplexing. FR-MZI exploits the input frequency spectrum, offering high sensitivity robustness against phase ambiguities and loss of signal as seen in conventional single-wavelength MZI.[153]
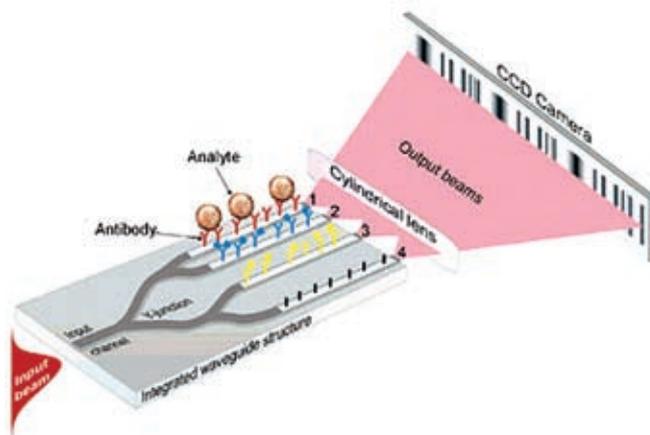

Figure 42: Representation of 4 channel YI showing different antibody immobilization at different channels.[152]
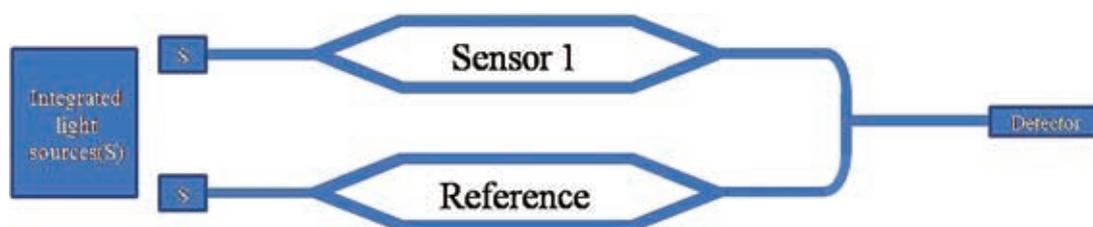
Figure 43: Representation of array of MZI for multiplexing. Adapted from Petrou *et al.,* 2010.

4.7 Ring resonators in integrated, multiplexed devices

Ring resonators have emerged as a class of compact sensors, by their ability to be integrated into small sizes without affecting the sensitivity of the device. This ability of integration and detection sensitivity down to single molecules has attracted the attention of many researchers towards the application of ring resonators in multiplexed sensing. The high sensitivity is achieved by the fact that the degree of interaction of light and the sample is not limited by the physical length of the sensing waveguide, but rather by the number of revolutions of light in the resonator. Further, ring resonators can be fabricated using different materials and microfabrication techniques that allow precise control of sizes. Etching and lithography techniques have helped make the surfaces smooth in order to increase the quality factor of ring resonators.

The initial application of microring resonators as optical biosensors was demonstrated by Vollmer *et al.,* in 2002 by detecting proteins.[154] This was closely followed by Ligler *et al.,*[155] who showed the possibility of multi analyte sensing by multiplexing 6 capillaries in a capillary based biosensor. Vollmer *et al.*, in 2003, demonstrated the application of multiplexed ring resonators to DNA detection using two microspheres. The spheres had unique resonant wavelengths so light could be coupled using a single waveguide and different oligonucleotides could be detected.[156] White *et al.,* (2006) fabricated and demonstrated the integration of 5 ring resonators together by using anti-resonant reflecting optical waveguide (ARROW) waveguide for coupling light and having a Liquid Core Ring Resonator (LCORR) for multiplex sensing.[157] Wang *et al.*, 2009 applied ring resonators to detect real time changes in the physical traits of live mammalian cells during cell adhesion and growth. They functionalized the ring resonator surface with an endothelial cell line (MS1) to detect the presence of two toxic chemicals, viz. sodium pentachlorophenate and aldicarb, in a solution which can be used for sensing toxic chemicals in water.[4] This demonstrated the applicability of ring resonator based sensing to biophysical studies and to screening toxic chemicals.

Washburn *et al.,* (2009) demonstrated microring resonators for the detection of cancer biomarkers in a complex medium like serum.[158] Later in 2010, they took forward the concept to multiplexed sensing by demonstrating the applicability of label-free ring resonator biosensors for accurate immunoassays by detecting clinically relevant protein biomarkers. They simultaneously detected 5 protein biomarkers *i.e.* prostate specific antigen (PSA), α-fetoprotein (AFP), carcino-embryonic antigen (CEA), tumor necrosis factor-α (TNF-α), and interleukin-8 (IL-8) at a concentration of 10 ng/ml.[159]

Iqbal *et al.*, 2010 presented a platform for multiple analyte sensing having an array of 32 ring resonators (8-controls and 24-sensing). They demonstrated multiplexing by using two DNA probes in a complex medium. They achieved a refractive index resolution of 7.6×10^{-7} and an LOD of 60 fM which was better than most other label-free sensor systems. A raster scan was performed to couple light to the input and detect it from the output side. The system used mirrors to detect light from different ring resonators on the array.[160] The system appeared to be functional, except for the fact that it requires complex optical and mechanical components for proper functioning.

A novel system was demonstrated by Carlborg *et al.,* in 2010 by integrating ring resonators with microfluidics for the detection of multiple analytes using multiple ring resonators on a single chip. They fabricated 7 ring resonators on the chip. Out of them, one was used for referencing to compensate for temperature variations.[161] Six microfluidic channels were fabricated using PDMS. Light was coupled through a grating coupler and split by a splitter into 8 channels (6-sensing and 2-referencing). The output was detected simultaneously by an array of photodiodes. The cartridge was assembled in layers as shown in Figure 44. The LOD of the sensing system was 5×10^{-6} RIU, which was better than many previously reported Silicon nitride ring resonators. The system was robust due to the easy coupling of light, the adhesive bonding method and reference channels for the compensation of laser intensity or temperature fluctuations.
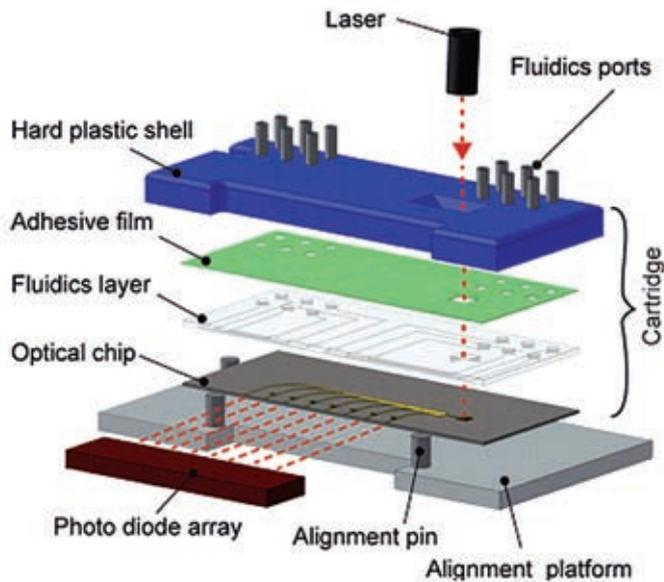
Figure 44: Different parts of the sensing system. At the base is the alignment platform and, above the platform is the optical chip consisting of ring resonators. The optical chip is covered with a hard plastic shell containing the microfluidics channels.[161]

(a)   **(b)**
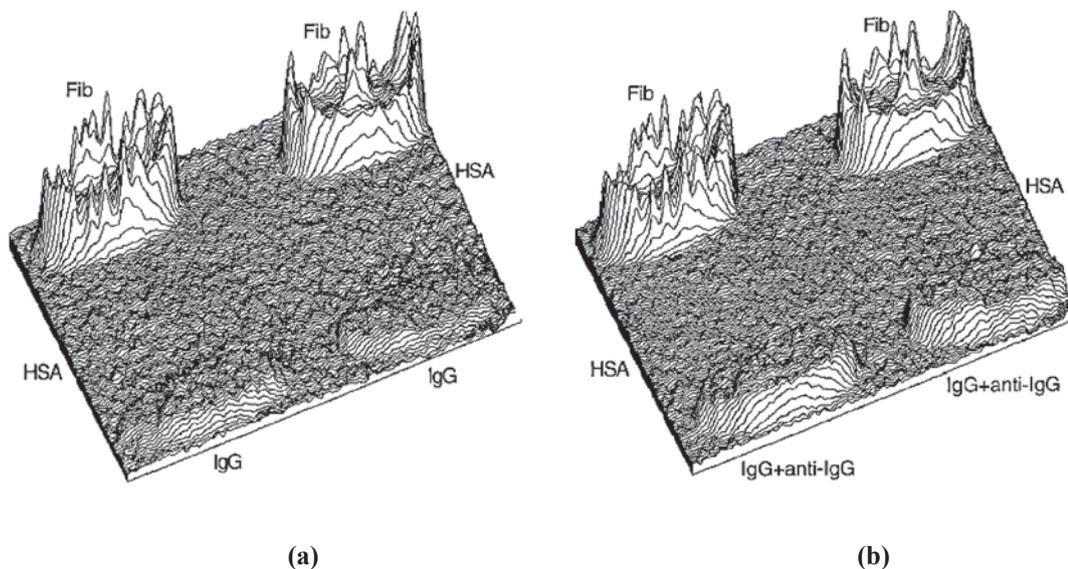
Figure 45: (a) Thickness of the substrate before incubation; (b) Thickness of the substrate after incubation of anti-IgG.[163]

4.8 *Ellipsometry*

Ellipsometry can also be used in multiplexed and parallelized assays. The instrumentation with the required condition for a proper error-free result in imaging ellipsometry is well explained by Jin *et al.*, 1996.[162] Since a simple TIRE-based technique is unlikely to be successful where multiple analytes have to be analyzed in parallel, researchers have developed Imaging Ellipsometry systems to overcome this limitation.

One of the first applications of biosensing using imaging ellipsometry was demonstrated for the detection of antigen-antibody complexes.[163] A silicon surface was taken and three types of antigens (Fibrinogen (Fib), Human serum albumin (HSA) and human immunoglobulin G (IgG)) were attached to the surface. Three rows of antigens were formed, each having two spots of the same antigen and about 3 mm in diameter. Various incubating steps were performed to

avoid nonspecific absorption. The ellipsometric image was taken before incubating in the antibody solution and the thickness profile was measured as shown in Figure 45a. After incubating in an Anti-IgG solution, the image was again taken as shown in Figure 45b. The difference in thickness is visible only at the row which contains IgG. The thickness change there is about 80%, compared to a change of less than 15% at other spots. Similar results were achieved with other antigen-antibody pairs.

Microfluidic array reactors are one of the major components of an ellipsometry setup. Using modern microfluidics techniques, many polymer-based flow cells are being fabricated which can increase the throughput of the technique.[11] The surface chemistry for interaction with the analyte or sample under study is also significant for increasing the sensitivity of the biosensor and for its prolonged use.

4.9 *Miscellaneous*

Many other unique and potentially powerful label-free optical detection schemes have been demonstrated for multiplexing. Cunningham *et al.*, 2002 demonstrated the applicability of narrow bandwidth guided mode resonant filters (GMRF) as biosensors.[164] They used this biosensor to perform a protein-protein affinity assay using a 96 well microplate. The setup was connected to the bottom of the microplate (Figure 46). White light was incident on the grating formed by the microwells and a shift in wavelength of the narrow band of resonant light which was dependent on the biomolecular interaction was detected.[165]

Another fascinating design for multiplex biosensing was developed by Yan *et al.,* in 2009 using the principle of local evanescent array coupling (LEAC) (Figure 47). When a layer of biofilm is formed on the surface it causes a shift in a localized evanescent field. This shift of an evanescent field
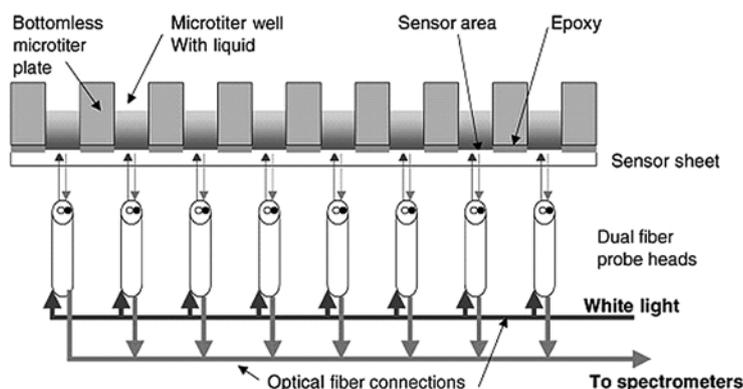

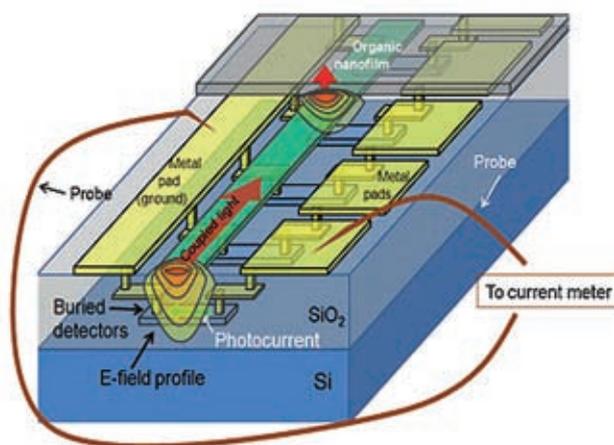**Figure 46:** Guided mode resonance filter based biosensors.[166]

Figure 47: Schematic of LEAC biosensor.[167]

in the vertical direction was detected by an array of buried photodetectors. As it is localized field redistribution, many different analytes could be detected at adjacent locations as the corresponding photodetector produces a current depending on the concentration of the binding analyte.[167]

There are many other techniques which are being used to develop biosensor arrays, including photonic crystal[168,169] arrays, and the amount of research in this area is evident from the fact that every alternate journal paper produced on biosensors deals with optical biosensors. In order to keep the length of this article within reasonable limits, those articles are not being discussed here. Readers of this article can find details about such techniques elsewhere, as in a recent review on label free photonic crystal biosensors.[170,171]

5 The Future of Optical Techniques in Multiplexed, Parallelized, High Throughput Biosensing

The developments in the biosensing domain are being driven by two requirements: (1) The need for point of care (POC) diagnostics,[52,172] (2) The need for high throughput laboratory based testing for a large number of samples and multiple target biomarkers.[173] The discovery of biomarkers as definitive diagnostics for various pathological and infective diseases is the causative factor for such requirements. As we have seen in the previous sections, the inherent advantages of optoelectronic sensing for biological markers have resulted in researchers and commercial organizations around the world competing to invent the most robust, sensitive and versatile biosensors. The possible impact of these sensors is tremendous, and the medical, public health and scientific advancements that may result from the development of appropriate biosensors is only to be imagined. The need for POC has been increasing and its applicability in different domains has been explored. In this context it is critical to have an idea of the latest research which is pushing the boundaries of sensing technologies to better sensitivities, higher specificities and hitherto unseen accuracies.

One of the obvious impacts would be felt in the area of software technology, which has been largely unexplored in this review article. A completely separate and equally long article can be written reviewing the impact of software on the parallelized biosensing domain. The impact starts right at the very early stages of development (in terms of simulation), continues through the control systems used in the driving electronics (FPGAs, *etc.*) and ends with the processing of acquired data to produce meaningful results, understandable to ordinary human beings. In particular, with the help of optical simulation software, one can explore different designs for various configurations coming up with an optimized design and a prediction of expected results given an input. This translates to significant savings in terms of time and cost since various mistakes can be avoided without actually fabricating or experimenting using an actual device (e.g. Wan *et al.*, 2012[174]).

It must be quite evident by now that the writers of this review consider SPR and LSPR techniques to be the most promising in terms of accuracy and integration in the short term. This is buttressed by the fact that quite a large number of research papers and reviews[175] have appeared in the last couple of years extending the boundaries of this sensing technique. Magneto Optic surface plasmon resonance that (MOSPR) technique is being developed and is based on increasing sensitivity by magneto plasmonic modulation.[176] This involves the excitation of surface plasmons along with a transverse magneto-optic Kerr effect (TMOKE) which only happens due to the presence of a magnetic field along the direction of the metal layers and perpendicular to the direction of the surface plasmon vector. This simultaneous excitement increases sensitivity.[177]

In terms of LSPR, micro and nano-scaled structures like triangles and microholes are being exploited for short range SPR demonstrating the same sensitivity compared to surface-propagating plasmons and better than nanoparticle based surface plasmons.[178–180] Nanotubes,[181] cones,[130] and, structures like dimers[182] and trimers[128] have shown a novel plasmonic property which can be suitably tuned and used for sensing applications.

Another important aspect of sensor development involves surface chemistry which can have a profound effect on the lowering of the limit of detection. New techniques like the DNA-RNA antibody assay[183] and novel functionalization techniques[184] are examples of scientific endeavor in this direction. The issue of surface functionalization is closely related to surface fabrication which now leverages heavily on the development of novel microfabrication techniques like soluble substrate technology for producing structures like 'graticles' which can be directly used as surfaces for SPR imaging.[185] Other techniques including Inclined nanolithography,[186] localized oblique angle deposition,[187] near field guided nanopatterning,[188] templated techniques[189] electrochemical deposition by tapping mode atomic force microscopy[190] are being explored to create better surfaces on substrates for SPR based sensing. Many groups are also looking in to functionalization by dendrimers to increase the surface area for sensing.[191]

Yet another important development is where various detection techniques are being merged to provide higher accuracy/sensitivity/specificity. Merging angle interrogation and wavelength interrogation to take advantage of the benefits of high sensitivity and simple geometry[192] is a simple example of this development. A more complex example involves the merging of SPR and mass spectroscopy. This utilizes the advantages of real time, kinematics study and the quantification of biomolecules by SPR and the elucidation of structural features by Mass Spectrometry (MS). It has found application in fields like proteome analysis[193] and miniaturized diagnostics.[194] They have been applied in microarray formats for multiplexed applications to meet the demand of upcoming "-omics" revolution.

It may be envisaged that in the near future POC diagnostics will attain fM sensitivity and proper sample preparation will be a key factor in accurate diagnostics. In this domain, further developments in microfluidics will play a key role, and so will sampling technologies. One of the major roles of microfluidics (simulation and hardware) will be in "focusing" the target analytes onto the sensing surfaces thereby increasing molecular capture rates.

Finally, in summary, in the view of the authors of this review, the possible domain of optical biosensing, both in terms of single analyte or multi-analyte sensing, is only explored partially and has ample scope for researchers to engage in research and development for cheaper, more sensitive, more specific and more accurate sensors.

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