

# Chapter 3

## Surface Nano-patterning of Polymers for Mass-Sensitive Biodetection

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### Abbreviations

AIDS	Acquired immune deficiency syndrome
AFM	Atomic force microscopy
BLV	Bovine leukemia virus
BAW	Bulk acoustic wave
BG	Blood group
BSA	Bovine serum albumin
DVB	Divinyl benzene
ELISA	Enzyme-linked immuno sorbent assay
ESA	Electrostatic self assembly
FBAR	Film bulk acoustic resonators
FMDV	Foot and mouth disease viruses
HPLC	High performance liquid chromatography
HRV	Human rhinovirus
HSA	Human serum albumin
HRP	Horseradish peroxidase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IET	Indole-3-ethanol
LBL	Layer-by-layer
MAA	meth acrylic acid

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MIP	Molecular imprinted polymer
PET	Poly ethylene terephthalate
PCR	Polymerized chain reaction
QCM	Quartz crystal microbalance
RBC	Red blood cells
SAW	Surface acoustic wave
STW	Shear transverse wave
SAM	Self-assembled monolayers
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
SARS	Severe acute respiratory syndrome
TMV	Tobacco mosaic virus

### 3.1 Introduction

Detection of biological species such as enzymes, microorganisms, proteins, DNA, etc., has gained substantial importance in various fields, which include health care, industrial and environmental analysis, and biotechnology and process control. Currently, most of the detection systems used to monitor binding of biological molecules on sensor surface need some fluorescent or enzymatic labeling. This labeling step charges additional cost and time to the bioanalysis. The other concerned problem regarding this type of analysis is that, in some cases, the labeling reagent itself interferes with the analyte molecule, which then leads to false measurements. This makes the analysis more complex and less reliable. Considering the cost, time consumption, and reliability of the measuring system, this strategy has some disadvantages in monitoring the binding of biological species at the surfaces.

There is a growing interest in designing modern bio-recognition systems because of the rapid developments in this field. To obtain the desired information about the analyte molecule with certain degree of confidence, we have to develop suitable sensitive, selective, fast, inexpensive, and label-free detection systems. This problem needs to be addressed mainly in two parts: The first part is associated with the design of selective antibody surfaces that are capable of interacting only specifically with analyte molecules in complex matrices; and the second part is related with the design of transducer that can generate signals more sensitively for very low concentrations of analytes. Mergence of these two strategies can produce innovative detection systems, which can perform monitoring of different bioanalytes with appropriate selectivity and sensitivity.

Molecular imprinted polymers (MIPs) along with acoustic or mass-sensitive devices provide a highly favorable route to carry out various types of bioanalysis. Although there are some other surface structuring techniques that have some

applications in mass-sensitive biodetections, molecular imprinting has high reputation in this regard. The imprinted polymers are of rigid and robustness nature, which is not damaged by exposing them into extreme conditions, and, in contrast, acoustic devices give a direct change in frequency corresponding to the bounded mass on their surface. Such detection systems have gained substantial importance during the last decade because of their widespread applications in different fields such as degradation analysis, compost monitoring, enantio-selective sensors, and environmental monitoring. To understand how to design highly selective surfaces for bioanalytes and for their detection by mass-sensitive devices, one should follow the concept of molecular imprinting along with other surface crafting schemes and fundamental principles of acoustic devices. In this chapter, coming sections will focus on these issues and discuss their potential applications in bioanalytics.

## **3.2 Surface Structuring Strategies**

### ***3.2.1 Natural Antibodies: A Direct Tool for Biodetection***

Crafting of innovative materials for selective detection of different species is clearly a key assignment in bioanalytics, and especially their surface structuring is even more a challenging task at micro and nanoscale. The issue can be resolved by considering natural antibodies [1] as a direct tool for monitoring immunochemical reactions for detection purposes. One of the major advantages of this scheme is that natural antibodies provide sufficient degree of specificity for desired target molecule in complex mixtures. These materials can be immobilized on an appropriate transducer and be used to design biosensors [2, 3]. A very recent example of this strategy has been explained in a review article in which a thin layer of enzymes is immobilized on an electrochemical probe. This sort of detection systems has shown good results regarding sensitivity and selectivity. The major drawback of this technique is the lack of ruggedness and long-term stability of the designed surface, which limits their applications while crafting materials that can face severe conditions and retain their properties for longer period of time. The other concerned problem regarding these materials is that the interaction between antibody and antigen is usually very strong, which significantly reduces the reversibility and thus restricts their reuse.

### ***3.2.2 Layer-By-Layer Approach***

One of the modern trends in material designing to synthesize very thin films of layer heights ranging from one micrometer to several nanometers is set by the layer-by-layer (LBL) or electrostatic self assembly (ESA) methods. It was first introduced by Decher

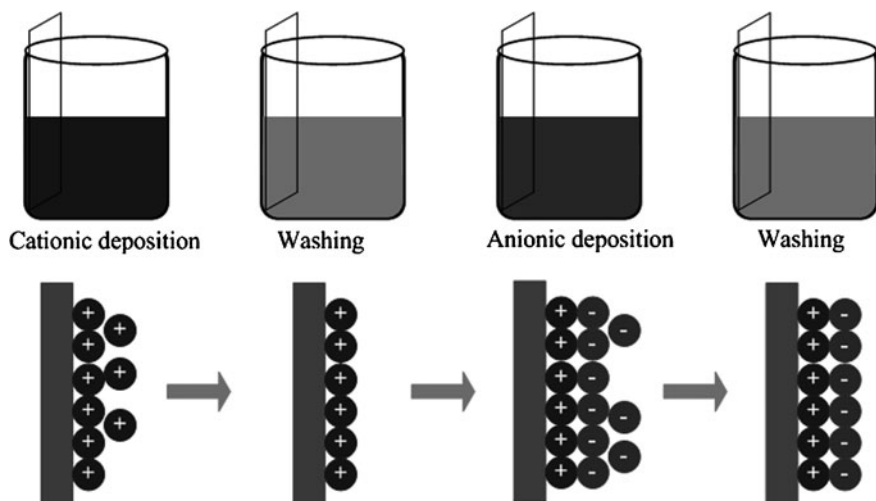


Fig. 3.1 Layer-by-layer assembling method

et al. [4] in 1992 during the synthesis of controlled thin films [5]. The synthetic procedure for developing LBL assembly is relatively easy and convenient. A schematic diagram for the preparation of LBL thin films is shown in Fig. 3.1.

This is a four-step procedure as shown in the figure, which is continuously repeated until the required numbers of layers are generated. This technique is quite new but still promising to craft the different materials to get the desired properties such as chemical, mechanical, thermal, and biocompatibility having sufficient degree of roughness. The synthesized LBL assemblies are of robust nature, which can effectively perform in severing conditions. The stimulating feature about these materials is that the layer-to-layer attractions are not restricted to electrostatic forces but assemblies can also be formed by hydrogen bonding [6], covalent bonding [7, 8], and some hydrophobic interactions [9, 10] as well. LBL assembly methods are very advantageous to organize the surface structures at nanometer scale and to incorporate the desired biomolecules. The versatility of this strategy enables us to launch any kind of charge species such as proteins [11–14], polypeptides [15], DNA [16], viruses [17], antibodies [18], and various other biological compounds in thin films preparation. The introduction of different biological species in LBL assembly generates very sensitive biological receptors having tunable selectivity. The induced functionalities in thin films truly favor the production of biorecognition materials. These materials can also be patterned by fabricating different components such as nanoparticles [19], nanotubes [20], and some other nanoplates [21–22]. This is of course of great interest in designing the biorecognition [23] of thin films at nanoscale [24], which can be deposited on a suitable transducer to conduct various bioanalyses.

Besides many significant advantages of LBL technique, there are certain limitations. The major drawback of this method is that it requires quite a long deposition

time of layer formation, which makes synthetic procedure too lengthy. Moreover, very high concentrations of deposition reactants are needed in order to coat appropriate layer material. Certain LBL assembly formation steps are very complex, which needs special masking and is time consuming. Although the binding of LBL receptors are of selective nature, sometime it is nonspecific due to exposition of the whole substrate surface to reactant solutions.

The practical examples of LBL assemblies in mass-sensitive biodetection applications are still infancy but, nevertheless, talented enough to meet the challenges of modern bioanalytics.

### ***3.2.3 Host–Guest Interactions***

Supramolecular chemistry provides an alternative synthetic route to design these materials, according to the technical needs. In these artificial materials, intramolecular and intermolecular noncovalent forces operate between the analyte and the host molecule. This strategy has been proven to be very successful in generating selective recognition systems for biomolecules [25]. It has solved the problem of reversibility and reusability when compared with the natural antibodies. The major drawback of this scheme is that the synthetic procedure is complicated and relatively time consuming. It is also a tedious task to find out the optimal interaction conditions between host and guest molecule. Therefore, to design an optimal recognitions system with minimum effort and technical expertise, we have to consider other strategies.

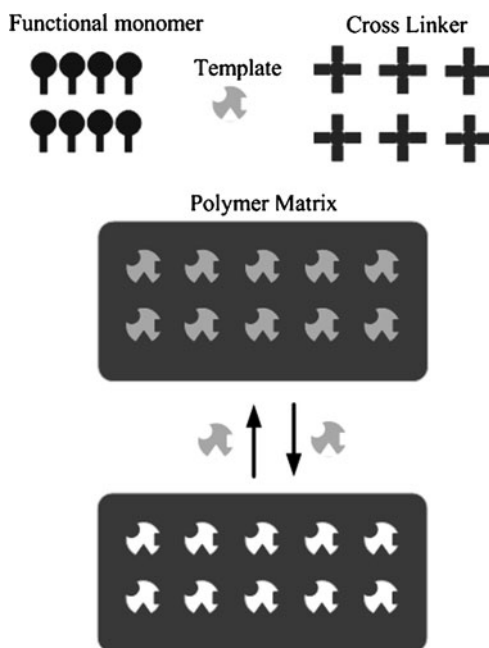
### ***3.2.4 Molecular Imprinting: A Novel Approach for Developing Surface Recognition***

The problem can be solved by introducing artificial recognition layers modified according to the shape and size of bioanalyte. These artificial recognition materials offer considerable flexibility in terms of tailoring the interaction sites in polymer surfaces. MIPs [26] promise to solve all the concerned problems and meet the desired needs in designing of novel materials. The most exciting aspect of molecular imprinting is that it provides molecular recognition to polymer matrix, which generates selectivity for very similar class of biomolecules. This technique was first discovered by the groups of Kiefer [27] and Wulff [28] independently in 1972, during the synthesis of organic polymers. Initial applications of molecular imprinting were found in separation processes and since after that the scope of this technique was extended to other fields such as chemical and biosensors [29], artificial biorecognition surfaces, and artificial synthetic antibodies.

### 3.2.4.1 Principle of Molecular Imprinting

The principle of molecular imprinting can be better understood from the following example in Fig. 3.2 where the model compound as template (i.e., usually analyte molecule) is introduced in polymer mixture along with monomers and cross linker. The required degree of polymerization is achieved by carefully controlling reaction conditions. During polymerization reaction, the polymer chains are self organized around the template compound, which are fixed at the end of reaction. The template or analyte molecules are finally removed from polymer matrix by heating or washing methods, which leaves behind geometrically adapted cavities for analyte reinclusion.

Generally imprinting procedure can be accomplished as a three-step process: the first step is the prearrangement of template, monomer, and cross linker; second is the engulfing of polymer chains around template; and the third is the extraction of the template molecules. In fact imprinted polymer possesses the memory of removed analyte molecule and can recognize and extract it selectively from a complex mixture of closely related compounds. The geometrical shape, size, and configuration of cavities can be improved for optimal template interaction by modifying the reaction conditions [30, 31] such as pressure, temperature, amount of cross linker and monomer to template proportion [32]. The amount of cross linker is very important as the higher degree of cross linking ensures the generation of cavities of very fine shape for optimal selectivity. Some post-imprinting modifications can also be employed to further improve the cavities shapes for reinclusion. The basic



**Fig. 3.2** Principle of molecular imprinting

requirements to conduct imprinting process are the high cross linking of polymer and the noninterfering nature of template with polymer matrix. Molecular imprinting can be classified into two types, according to the interaction of monomers with template molecules (i.e., covalent and noncovalent imprinting).

#### 3.2.4.2 Noncovalent and Covalent Molecular Imprinting

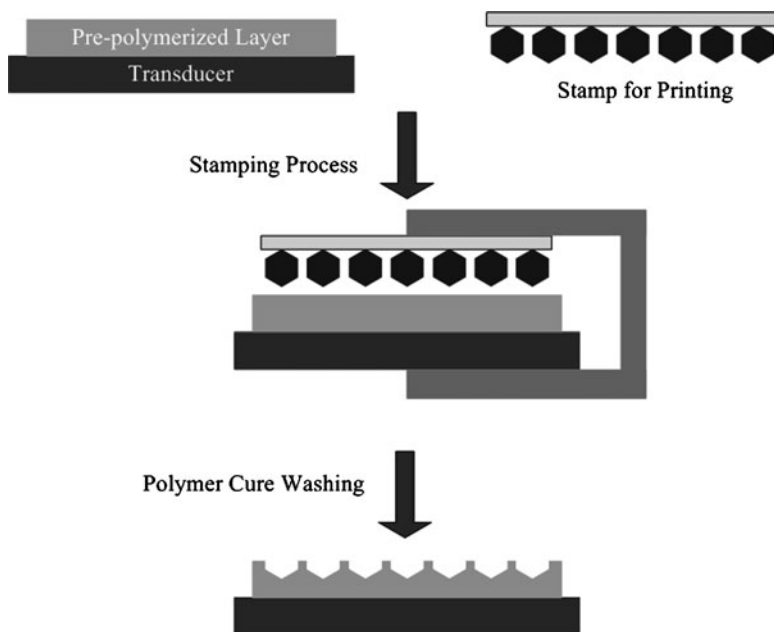
Noncovalent imprinting was first introduced by Mosbach [33], where the template molecules develop affinity with function groups of monomer through noncovalent associations such as hydrogen bonding, dipole–dipole interactions, Van der Waals forces, etc. prior to polymerization. The removal of template molecules takes place very fast as it does not require any bond rupture. This is a fast and straightforward way to design recognition materials for different detection purposes or developing chemical sensors. The only requirement for this process as earlier mentioned is that the template should not interfere with polymer, so there are no more restrictions for analyte size and shape. The only drawback of this technique is that it cannot be applied to those template molecules that do not have any functional group to interact with polymer system, which limits the construction of binding sites for analyte interactions. This problem is solved by later technique in which the model compound is covalently linked with polymer chain. After polymerization, the template molecules are removed by bond rupturing between template and polymer. The imprinted polymer can rebind the analyte molecules reversibly, and the selectivity is achieved by reactive groups in polymer matrix. Covalent imprinting is no doubt a practical method to various templates not having functional group but is limited due to the small number of useful applications of reversible covalent bonding.

A hybrid imprinting technique was developed by Whitecombe [34] in 1995 in which template molecule is covalently bonded to polymer matrix, whereas after its removal reversible and noncovalent interactions take place. Tepper [35] has adopted a different imprinting approach in which the polymerization was carried out on transducer surface in solid phase rather than in solution form in the presence of alkaline vapors that contribute to generate imprinted sites for analyte reinclusion.

The sensitivity achieved by MIPs is accessed by number of imprinting sites available for analyte interactions (i.e., more the imprinted sites available higher will be the sensitivity). Nanoporous polymers possess more imprinted sites when compared with microporous polymers. The other important aspect regarding the sensitivity is the distribution of imprinted sites in polymer layer prior to use for detection purposes. The size of analyte molecules governs the imprinting strategy whether *bulk imprinting* [36] or *surface imprinting* is suitable [37]. In bulk imprinting (as shown in Fig. 3.2), the template molecule is added along with monomer and cross linker at the start of reaction and after polymerization is removed. This strategy is useful for relatively smaller analyte molecules having molecular mass  $<500 \text{ g mol}^{-1}$  but while considering the larger analytes such as biomolecules, the bulk imprinting is not always complimentary. Although number of interaction sites in bulk imprinting are high

in comparison with surface imprinting, keeping in mind the larger size of biomolecules, incomplete reversibility, relatively longer diffusion pathways, and longer time for measurement make their detection highly unfavorable. Therefore, for larger biomolecules, surface imprinting, as shown in Fig. 3.3, is proposed where template molecules are directly imprinted on the prepolymerized surface by stamping method [38] with a little force. Thus, the patterned polymer surface possesses the dimensions from one to several hundred nanometers that can exclusively extract target molecule from the complex mixture.

Molecular imprinting provides a straightforward, versatile, and unproblematic way to synthesize selective coating materials for the detection of various biospecies. The most beneficial aspect of this scheme is that it is not limited to a certain class of compounds unlike the natural antibodies detection systems. The other significant feature of these materials is that they exhibit long-term stability and do not undergo degradation over the course of time, which makes use of these materials for extended period of time. Reversibility of surface imprinted materials makes reusable these materials for several analyses, which reduces the cost. The synthetic route of imprinting procedure is relatively easy as compared to the scheme followed for host–guest interactions such as in the case of cyclodextrines, paracyclophanes, and calixarenes. Considering the versatility in synthetic approach, ruggedness of designed imprinted material to severe conditions, flexibility regarding choice of analyte, and long-term stability make these materials superior for rapid, inexpensive, and selective detections of various bioanalytes over other strategies.



**Fig. 3.3** Surface imprinting strategy by using analyte stamping



In order to make use of surface strategies for the detection of biospecies, first we have to understand the fundamental principles involved in transducers (i.e., acoustic or mass-sensitive devices). There are different types of these devices, which are employed in different working environments, according to their specific job to get desired information with minimum error. Thus, it is very important to select a right device for the dedicated task. In the coming section, we will focus on the basic principles of these devices and their operating modes in diverse mediums to get an optimized detection signal of different analytes.

### 3.3 Basic Principle and Theory of Mass-Sensitive Transducers

The fundamental principle involved in acoustic or mass-sensitive devices can be explained by piezoelectric effect, which was first discovered in 1880 [39] by two brothers Pierre Curie and Jacques Curie, in some crystalline materials such as quartz, rockchille salt, and ceramic. They had observed that if stress is applied on such crystalline materials in certain dimensions, it separates the negative and positive charges from their center creating a dipole, which leads to the generation of electrical voltage. The same is true if we apply an electrical voltage to such materials, it causes mechanical deformation in their shape. Former phenomenon is called piezoelectric effect, while the later one is known as inverse piezoelectric effect. The common examples of such materials that possess piezoelectric character are cane sugar, Rochelle salt, berlinite ( $\text{AlPO}_4$ ), and quartz, which are natural materials, while synthetic examples are gallium orthophosphate ( $\text{GaPO}_4$ ), langasite ( $\text{La}_3\text{Ga}_5\text{SiO}_{14}$ ), and lithium tantalate ( $\text{LiTaO}_3$ ). The selection of these materials depends upon their application in a typical medium and working environment: for example, gallium orthophosphate has high temperature coefficient than quartz and can be employed in working environment having temperature up to  $900^\circ\text{C}$ .

Quartz crystals are well-known acoustic device, which are widely used as mass-sensitive transducers for different sensing purposes. They are capable of measuring the mass changes in nanogram range. To have the desired properties of piezoelectric material, the crystal plate is cut at a specific angle, which is AT-cut ( $35^\circ 15'$ ) and BT-cut ( $49^\circ$ ). The AT-cut quartz is very famous and most frequently used as mass-sensitive transducer, as it has remarkable temperature and frequency properties.

Acoustic devices are considered as mass-sensitive devices because when a certain mass is loaded on an oscillating device, there is a corresponding change in the frequency of these devices. This phenomenon was first reported by Sauerbrey in 1959 [40] in his famous article. Sauerbrey has developed a relationship of frequency change in acoustic resonators upon mass deposition considering all the physical parameters of crystal material. The relationship is best described by the following equation.

$$\Delta f = -f_0^2 \frac{2}{A_{cr}(\rho_m C_q)^{\frac{1}{2}}} \Delta m,$$

In this relationship, the symbols represent the following:

$\Delta f$  is the frequency change,

$f_0$  is the resonant frequency,

$\Delta m$  is the change in mass,

$A_{cr}$  is the active piezoelectric area of quartz (usually electrode area),

$C_q$  is the shear modulus of quartz crystal, and

$\rho_m$  is the density of quartz.

This equation clearly indicates that the frequency change is directly related to the mass load on the surface of substrate. This equation is designed for typical gas phase environment. If we shift from gaseous phase to liquid phase, the equation is modified as described, considering the certain physical parameters associated with liquids such as viscosity and density.

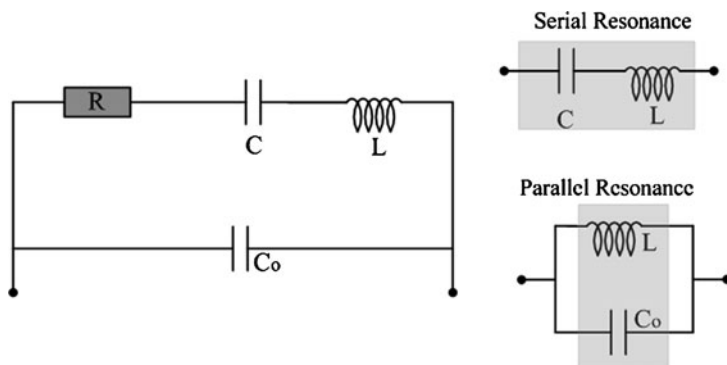
The other important information revealed from Sauerbrey equation is that the frequency change also depends upon the square of fundamental resonance frequency of device. It means by doubling the fundamental frequency of the device, the frequency change would be four times. This is very important in the design of device because extremely low concentrations of analyte can be determined by simply selecting a device of highest available frequency.

The frequency of a piezoelectric material is mainly determined by its thickness. The characteristic frequency of these materials is given by the following relationship.

$$f = \frac{C}{2d}.$$

In this relation,  $C$  is the velocity of sound in the material and  $d$  is the thickness of the sheet. The quartz sheet undergoes resonance oscillations when connected with circuit oscillator, where an electrical and mechanical oscillation comes close to the fundamental frequency of quartz. An equivalence circuit for quartz resonator is designed to understand the oscillation phenomena and to calculate the quality factor ( $Q$ ) of oscillations or dissipation ( $D$ ) (i.e., mass accumulation on quartz surface and visco elastic behavior of deposited polymer). This equivalence circuit is shown in Fig. 3.4.

The oscillations of resonating quartz having two electrodes can be compared with this circuit in such a way that  $L$  stands for deposited mass,  $C$  shows elastic behavior,  $R$  represents the damping loss, and  $C_o$  shows the capacity between two electrodes. The impedance is calculated by taking the ratio of applied electrical voltage and the current flowing through it. This value of impedance gives information about the quality of oscillations and explain about visco elastic nature of deposited layer on quartz surface. The different resonating modes of a 10 MHz quartz wafer (i.e., serial resonance and parallel resonance) can be compared with this circuit along with the phase shift. Generally, acoustic devices can be classified



**Fig. 3.4** Equivalent circuit to QCM resonator

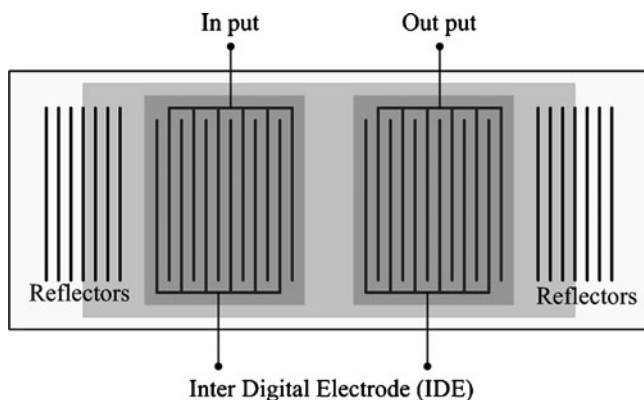
in two main types depending upon the nature of wave propagation mode (i.e., bulk acoustic wave (BAW) devices as discussed earlier and surface acoustic wave (SAW) devices, which is explained later).

### 3.3.1 Surface Acoustic Wave Resonators

As from the name, it is obvious these are the devices in which oscillation phenomena is related to the surface of the material. Rayleigh [41] had discovered the phenomena of surface waves and explained their propagation mode in 1885, since then they are known Rayleigh waves. SAWs have both components shear vertical and shear horizontal, which can couple with the surface of substrate and propagate in a nondispersive manner. The coupling of these components determines the velocity and amplitude of the waves that travel along the surface, and hence, make these devices a useful tool for mass sensing purposes. A typical setup for SAW resonator has been shown in Fig. 3.5. SAW devices are typically made of ST-cut quartz crystal on which comb-shaped electrodes are generated by lift off process. The distance between these comb-shaped electrodes determines the wavelength, which leads to a distinct resonance frequency of SAW devices. Although the maximum frequency achieved for SAW devices is 2.5 GHz, the analytically reported frequency for chemical sensors is about 1 GHz [42]. Such a high resonating frequency of these devices make themselves exceptionally valuable for detecting very low mass of analytes (e.g., for 430 MHz device mass changes up to 1 pg can be detected).

Apart from their successful sensor applications at higher frequencies, there are some limitations about their use in liquid phase. SAW resonators are influenced by the viscosity of surrounding liquid, which increases the damping of device significantly such that it becomes almost impossible to conduct measurements in liquid phase.

This problem can be solved by introducing shear transverse wave (STW) resonators [43, 44]. STW resonators are made of lithium tantalate ( $\text{LiTaO}_3$ ) in contrast to SAW devices, which are made of quartz substrate. The high dielectric



**Fig. 3.5** A typical design of SAW device

constant of  $\text{LiTaO}_3$  (i.e., (40)) is somehow similar to that of water, which is (80). This makes the STW devices operational in highly polar mediums avoiding any short circuiting. The other modification made in these devices is that the ST-cutting angle of crystal material is rotated in such a way that it demonstrates a complete shear horizontal wave that dissipates a very small amount of energy when subjected to liquid phase analysis. STW devices have established themselves a highly useful tool for various analysis particularly where the analyte concentration is too low to generate a reasonable signal.

### 3.4 Mass-Sensitive Detection of Bioanalytes by Tailored Surfaces

Different surface modifications techniques for the sensitive and selective detection of various biospecies have already been discussed including natural antibodies, host-guest biointeractions, LBL strategy, and surface imprinting. Among them, surface imprinting seems to be very attractive to get the desired specificity especially for bioanalytes. On the other hand, the basic principle and theory of mass-sensitive transducers have also been explained in previous section. The next headings will focus on the combination of surface modification techniques along with mass-sensitive transducers to design suitable biodetection systems.

#### 3.4.1 *Microorganisms Recognition System*

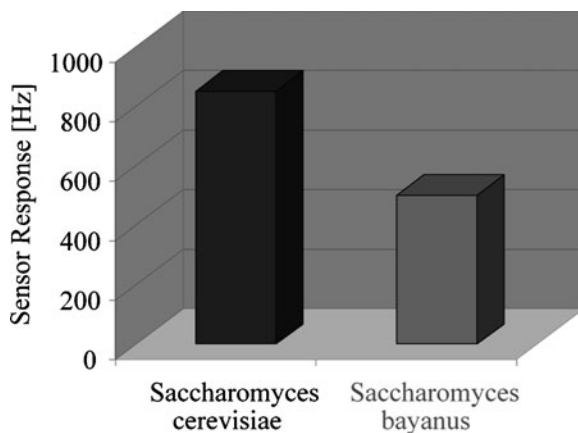
Microorganisms have key role in almost every field of human life and particularly toward the environment. The microbial activities have significant applications in different areas such as genetic engineering, food processing, photo synthesis,

biotechnology, bioremediation, and many more. Besides the numerous useful applications of microorganisms, there are some serious threats to health as some of them are source of infectious diseases. Classical methods for the analysis of dangerous microorganisms require very long time, which restricts the rapid determination. It is the beauty of molecular imprinting that design sophisticated sensitive surfaces for the quick detection of different microorganisms such as yeast, bacteria, different blood groups, viruses, and others. Some examples of mass-sensitive detection of unicellular and multicellular species have been discussed further.

#### 3.4.1.1 Yeast Cells Detection

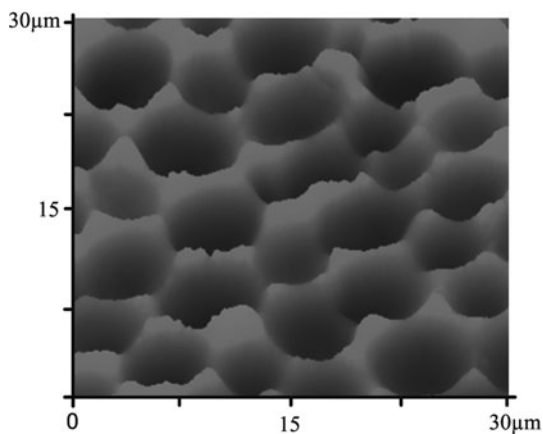
Yeast [45] are very important microorganisms that have several applications in biotechnology, among them fermentation of sugar is the most widely known. Basically, these are unicellular eukaryotic microbes having the length from 1 to 9  $\mu\text{m}$  and diameter of 1–5  $\mu\text{m}$ . Production of different wines through fermentation process, bread baking, and synthesis of ethanol at industrial scale are the well-established applications of yeasts. *Saccharomyces cerevisiae* [46] are the most widely used yeast species that have been extensively examined in microbiology laboratories because they are considered as the model microorganism [47] in the development of useful biosensors and to study certain biological phenomena. In different microbiology laboratories and other production plants, their growth in cultured medium is controlled at defined conditions. The growth and reproduction of yeast cells largely depends upon the composition of prepared medium, humidity, and temperature that demands constant surveillance. It has been noted that during the phase of production, concentrations of different reactants are changing with the passage of time that makes the mixture more complex. Already established methods for the monitoring of yeast cells in different production processes are based on indirect measurements or some labeling techniques. The online monitoring in fermentation plants at larger scale has always been of fundamental importance for process control. This can be achieved by employing mass-sensitive transducers such as quartz crystal microbalances (QCM) having patterned coatings on them for selective detection of yeast cells.

Highly sensitive and selective coatings can be prepared by surface imprinting strategy, which has already been explained. The selection of polymer for surface imprinting is also very important as the cavities generated by yeast imprints should retain the structural characteristics in terms of size and dimension after the interaction of analytes with surface. Polyurethane is a robust polymer that has been used widely for imprinting purposes. This polymer is synthesized using higher ratios of cross linker at defined reaction conditions. The image of *S. cerevisiae* is casted on polymer layer by pressing glass stamp to make highly packed and flat imprinted surface. The QCM measurement performed at suitable conditions on such surface shows remarkable sensor effect. While considering cross sensitive, it has been noticed that designed imprinted polymer surface shows highest frequency shift for the target molecule when compared with other similar yeast strands [48] as shown in Fig. 3.6.



**Fig. 3.6** Cross sensitivity measurements on *Saccharomyces cerevisiae* imprinted polyurethane surface to an analogous yeast strain (i.e., *Saccharomyces bayanus*), adapted from [48]

**Fig. 3.7** AFM image of yeast imprints on polyurethane surface



Although some of them have a size smaller than the cavity, they still do not have much impression on the same surface, which indicates the selective nature of the imprinted polymer. This reveals that the surface structure is patterned down to molecular/nanolevel, whereas the cavity shape and whole dimensions are optimized to the target analyte. An AFM image of imprinted surfaces has been shown in Fig. 3.7.

This measurement scheme does not stop here but it further investigates to monitor the growth stages [49] of yeast cells in order to study the cell behavior, which is of great interest in different pharmaceutical industrial applications, drug release, and many other biological processes. Especially in food processing industries, molecularly imprinted polyurethane surfaces of suitable layer heights can be used to inspect the changes taking place in surroundings such as concentration of nutrients,

humidity effect, and variable pH. This is indeed a significant achievement by biomimetic surfaces when realizing the concept of process control [49] for online monitoring purposes. Recent developments shows that individual growth stages of yeast cells can also be monitored simultaneously by employing a multichannel QCM [50] following the similar synthetic strategy.

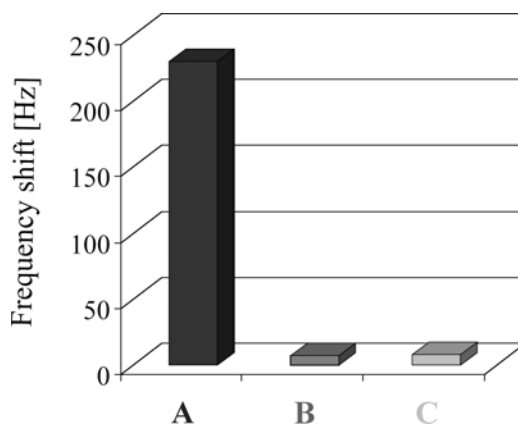
In comparison with classical methods for yeast detections, surface imprinted polymers along with mass-sensitive transducers are highly favorable in many regards. As already stated that classical methods are focused on indirect measuring techniques that are not much reliable in complex matrix where different chemical and physical parameters are varying continuously. The side-by-side selective detection of yeast cells at different growth stages is made possible by surface modified coatings on QCMs, but other methods lack this advance feature, and thus cannot be applied for online monitoring. Mass-sensitive devices having tailor-made surfaces have established themselves as promising tool to study cell properties and behavior in various biotechnology applications.

#### 3.4.1.2 Bacterial Detection with Synthetic Receptors

The detection of different pathogenic bacteria is of great concern in microbiology and modern clinical analysis owing to the public health and safety. The contamination of harmful microbes in drinking water and food can lead to severe diseases. For example, *Escherichia coli* (*E. coli*) are gram-negative pathogenic [51] bacteria, which causes contamination in food and beverages. Although not all of *E. coli* are toxic for human beings, still some of them are a potential hazard to public health. Among the dangerous species, *E. coli* O157:H7 is the most fatal one, which can cause severe diseases such as diarrhea, kidney failure, and even death in certain cases. In the United States, there are about 20,000 cases [52] related to different diseases spread by *E. coli* annually. Therefore, accurate, quick, cost-effective, and direct determination methods are needed in order to meet the requirements of environmental monitoring and other diagnostic purposes. The development of such detection systems are highly desirable in various industrial applications particularly related to food stuff [53]. Already established methods for bacterial detection are related to culturing, microscopy, and some other biochemical test. These methods are unfit for rapid analysis and particularly inappropriate for screening purposes, where prompt decision has to be made. The latest technology such as DNA chips has solved this problem to make faster analysis and reach toward conclusion. For a very sensitive and sophisticated detection of *E. coli*, polymerized chain reaction (PCR) [54, 55] technique is also very useful. Mass-sensitive devices such as QCM [56, 57] and surface plasmon resonance (SPR) [58] are also used for the characterization of PCR products. These detection methods are time consuming and not widely adoptable because of the high expertise required for handling. Some other sensing schemes based on electrochemical impedance [59, 60], chemiluminescence [61], SPR [62–64], and optical absorbance spectroscopy [65] are also reported in literature for *E. coli* O157:H7 detection. The direct

use of mass-sensitive devices as transducer for *E. coli* detection [66, 67] is not very general but still good enough to give a direct change in the frequency corresponding to the binding mass of analyte.

The idea of using functionalized surfaces with acoustic or mass-sensitive devices seems to be promising for developing a sensitive, selective, rapid, and relatively inexpensive detection system. In literature, there are different surface techniques that have been used with mass-sensitive transducers for detection of different types of bacteria. During the last decade, different groups tried to develop various biosensors based on natural antibodies for the detection of *E. coli* using different transducers. Most of them had applied specific antibodies immobilized on gold electrode of piezoelectric device such as quartz crystal microbalance. The strategy is very attractive in terms of getting selectivity as natural antibodies specifically bound to particular bacteria with sufficient sensitivity. Zhihong [68] has developed interestingly an unusual strategy for mass-sensitive bacterial detection. He has exploited the carbohydrates and protein recognition interactions for a very sensitive and specific *E. coli* detection. This method is not purely synthetic but in fact a hybrid approach by designing suitable mannose surfaces for a rigid and strong binding of *E. coli*. It has been noticed that the detection of *E. coli* on lectin modified mannose surface has much higher sensitivity about  $10^4$  times better than mannose alone detection. This in terms of sensitivity is quite good as it improves the detection limit. As the binding of bacteria is strong that does not increase damping drastically, which is good for smooth QCM measurements without too much loss. The sensor specificity was also characterized by exposing different electrodes coated with different chemical layers to known concentration (i.e.,  $7.5 \times 10^7$  cells/mL of *E. coli* W1485). The frequency shift for different layers, that is, lectin-modified mannose layer (A) and recombinant antibody (210E scFv-cys-) treated electrode QCM surface (B) was determined. The control antigen test was also conducted by adding *Staphylococcus aureus* (C), and the results have been shown in Fig. 3.8. The lectin-modified sensor layer exhibits highest sensor response



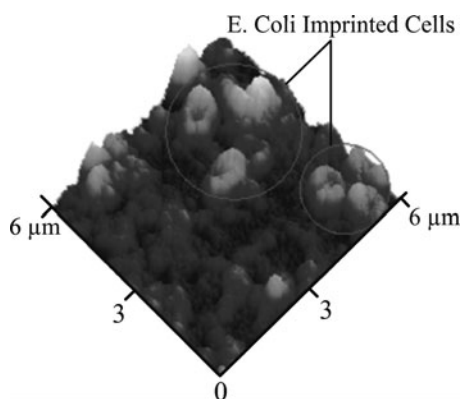
**Fig. 3.8** Evaluation of sensor specificity by exposing three different layer materials coated on gold electrodes a solution of *E. coli* W1485 (adapted from [68])



for *E. coli* W1485 in comparison with other layer and control antigen, which indicates the enhanced sensor specificity.

Recently, a new strategy has been introduced where gold nanoparticles [69] are immobilized on thiol modified QCM electrode to develop piezoelectric sensor for real time detection of *E. coli* O157:H7 detection.

The main problem is related to the low stability of antibodies deposited on the transducer surface. These antibodies cannot perform for a longer period of time as they tend to deteriorate with the passage of time and so cannot be stored for long-term use. Some antibody coatings are good for sensing purpose but most of them are of a flexible nature that can cause severe damping during mass-sensitive measurements, which leads to an unreliable data both in terms of qualitatively and quantitatively. Moreover, some natural antibodies once bound to microorganisms then afterward they are reluctant to release them, which reduce the reversibility of mass-sensitive measurements. The solution of these problems can be made by following a different approach in which artificial recognition materials are used. Surface imprinted layers can be used as artificial antibodies [70] for bacterial detection more effectively when compared with natural antibodies. Polyurethane layers that are robust and rigid in nature have been utilized for surface imprinting of *E. coli*. The imprinting is done on prepolymerized surface of polyurethane with a suitable stamp made of densely packed cells. The AFM image of *E. coli* imprinted polyurethane surface has been shown in Fig. 3.9. These layers coated on QCM exhibit significant sensor response to *E. coli*. Although there was anti-Sauerbrey effect, the reported sensitivity and the reversibility of the measurement is quite good for *E. coli* detection. Surface studies of imprinted polyurethane layers reveal that excess of phenolic groups hindered the covalent linkage with polymer surface. This indeed is very useful to improve the imprinting effects for bacteria sensing and to develop a completely reversible detection system.



**Fig. 3.9** *E. coli* imprinting on surface imprinted polymer surface

### 3.4.1.3 Virus Sensing by Nano-structured Polymer Surfaces

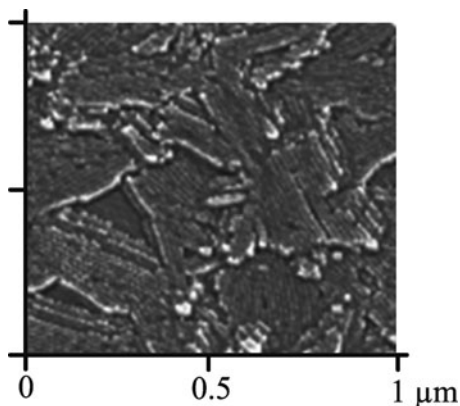
Molecular imprinting has introduced a new aspect in material science for designing sensor surfaces for exceptionally small entities. This becomes highly useful for the recognition of viruses, which have size in the range of some 100 nm. Their extremely small size makes their detection virtually impossible on normal optical devices. Viruses have serious health effects on human life as they can cause various diseases such as common cold, influenza, chicken pox, and some severe ones are acquired immune deficiency syndrome (AIDS), severe acute respiratory syndrome (SARS), hepatitis, etc. Enzyme-linked immuno sorbent assay (ELISA) [71] and PCR [72] are highly adopted methods for determining the exact stage of viral disease but their cost is too high and they need highly trained personnel to operate. Some inexpensive strips are available in the market for screening purposes for hepatitis C virus and HIV detection [73] in blood. This screening technology is of low cost and gives quick results, which are very suitable for rapid detection but these strips are limited to certain viruses and not generally available for a broad variety. At the moment, there are not much rapid and online analysis methods available for the viral detection, which have low cost, easily operable, and can be applied to a wider range of these species. This is a highly demanding task in which one has to make quick decisions about fatal viral diseases and reach a conclusion as some viral diseases are incurable and require immediate diagnosis. So, express detection systems need to be developed for fast and accurate analysis of viruses that have relatively low cost.

Tobacco mosaic virus (TMV) is the first discovered species of this class, which has the potential to damage the leaves of different plants particularly of tobacco. The structure of TMV [74] is surprisingly rigid and it can survive in a very broad range of pH 3.5–9.0 and can tolerate the temperature up to 90°C. AFM picture shows that TMV rods arrange themselves linearly over designed surface. It has been reported that the immobilization of TMV should be done on nonpolar surfaces, which prevent any structural distortion due to virus surface interaction [75] (Fig. 3.10).

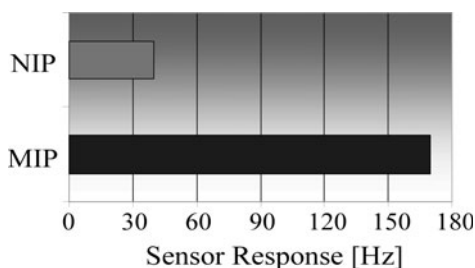
Surface imprinted polyacrylic [76] acid has been applied successfully for casting TMV image. Mass-sensitive measurement performed on 10 MHz QCM shows excellent response for imprinted channel, whereas nonimprinted exhibits very small effect, which is due to unspecific binding. The frequency shift for MIP and NIP has been demonstrated in Fig. 3.11. The surface modified polyurethane layers [74] offer better sensitivity for TMV than polyacrylic acid, which is very valuable to detect lower concentrations and ultimately avoid production losses.

Although TMV have not been considered widely as a model for designing virus sensitive layers, still it has found some important functions in other areas. An exciting application of TMV imprints has been reported by Kenz et al. [77] in which they have used this virus as template to generate highly selective nano-structured inorganic matrices for binding metal ions. This not only offers a true nano patterning of surfaces but also induces recognition properties to design functionalized materials.

**Fig. 3.10** AFM image of TMV aggregation on mica surface



**Fig. 3.11** Sensor response of TMV imprinted and nonimprinted polymer layers, respectively (adapted from [76])

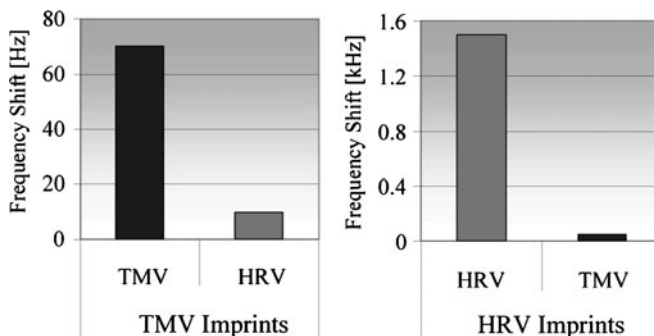


Similarly, another interesting example of TMV imprints has been reported by Bolisay et al. [78] in which they have used this virus to improve the binding affinities for itself against the other non-target virus. Although the detection was not made on acoustic devices, still this synthetic approach is useful to avoid nonspecific binding and is a step toward improving imprinting factor for TMV. Surface patterning can also be used to differentiate the different viruses of same group (e.g., human rhinovirus, HRV), which is an infectious virus that causes common cold. This class of virus has a very small diameter of about 18 nm but has a length of 300 nm for surface patterning of polymers. The study [79] has been conducted on different types of rhinoviruses (i.e., HRV1A, HRV2, and HRV14) to examine cross sensitivity. The reported results in Table 3.1 show that polymer surface imprinted with one specie prefer to bind selectively the very same virus over the others. In other words, HRV1A imprinted polymer surface exhibit comparatively higher frequency shift for HRV1A than the others.

This demonstrates that imprinted structure of polymer surface is competent enough to recognize the target virus among similar species at nanometer scale. The imprinting of HRV along with other viruses of same class and their selective detection on QCM is a model example of intragroup selectivity.

**Table 3.1** Comparison of relative sensor responses of various HRV imprinted polymers toward similar species (adapted from [79])

Imprinted HRV virus	Relative sensor effect [%]		
	HRV1A	HRV2	HRV14
HRV1A	100	15	17
HRV2	20	100	19
HRV14	44	37	100



**Fig. 3.12** Differential measurements conducted on TMV and HRV imprinted polymer surfaces and the respective frequency shift has been compared (adapted from [75])

The study can also be extended to attain intergroup selectivity pattern among different classes of viruses. Suitable imprinting procedures can also be employed to distinguish between two different groups of viruses, using acoustic devices. The detection system [74] has been designed for selectively determining TMV and HRV in controlled conditions. These viruses have entirely different structural dimensions, for example, TMV has cylindrical shape whereas HRV is found mostly in icosahedral form. Mass-sensitive measurements performed on premeditated polymer surfaces show excellent selectivity pattern as shown in Fig. 3.12 for the respective imprinted virus.

This detection scheme is very useful as it gives rapid results about different virus species including cross sensitivity studies in comparison with other modern techniques such as PCR. Recently, an outstanding advancement has been made in this regard where a suitable MIP chip [80] is designed for continuous monitoring of viral contamination. This development has introduced an innovative sensor platform for screening diverse biological complex mixtures and for micro total analysis system ( $\mu$ TAS).

These artificial receptors [79] are used to design more selective matrices for different pico viruses (i.e., HRV, foot and mouth disease viruses, FMDV). Although FMDV rarely infects humans however, it has strong impact on animals which is dangerous for agriculture industry and livestock. Molecular imprinting promise to achieve desired intergroup selectivity between HRV and FMDV. Mass-sensitive measurements performed on HRV stamped polyurethane layers

demonstrate substantial frequency change for the imprinted picovirus, whereas the same surface shows very little effect for FMDV.

One interesting strategy has been developed in which the whole analyte is not used as a template for the detection of biological analytes. This strategy is named as epitope approach [81] in which a certain protein sequence is used as template that acts as antigen for the detection of target microorganism. The method is somewhat inspired from natural antibodies where only a small part of antigen interacts (e.g., instead of whole protein molecule only amino acid sequence is detected). Although there are not too many applications of this approach in literature for biological detection, still this scheme can be introduced where practically whole structure cannot be used as a template. One successful example of this strategy is reported in Dengue virus [82] detection, which is an infectious pathogen virus that has been a serious threat to human life. A similar approach as epitope has been used to design biological sensor for bovine leukemia virus [83] (BLV).

The overall studies conducted on surface imprinted polymers along with mass-sensitive devices for viral detection open a new dimension for rapid, inexpensive, selective, and precise analysis methods, which is not provided by other analytical tools.

### ***3.4.2 Protein Imprinting and Their Mass-Sensitive Detection***

Proteins are highly significant macromolecules that possess very complex structure, and their selective recognition is also very important in biochemistry. Molecular imprinting has widely adopted technique to generate suitable polymer systems for protein sensing. In recent years, a number of review articles are presented for the imprinting of proteins [84–90] in which different strategies such as bulk imprinting, surface imprinting, epitope approach, and hydro gels are used. The protein imprinted surfaces are characterized by different techniques such as AFM and scanning electron microscopy (SEM). The images obtained from these methods reveal that the artificially imprinted protein materials have nano-patterned structures that possess specificity for template protein, which can be confirmed by other methods. It has been noted that the imprinted surface retains the utmost amount of that protein molecules, which were being used as model template while the nonspecific binding were very less when exposed to other protein solutions.

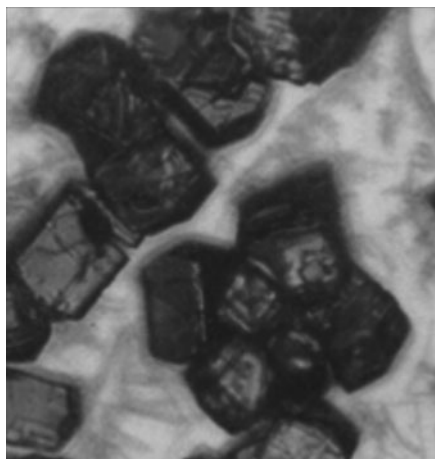
Despite the numerous applications of molecular imprinting for proteins and other macromolecules, their detection by mass-sensitive devices is not fully explored. Zahang et al. [91] has reported the piezoelectric detection of proteins using molecularly imprinted self-assembled thin films. They have selected human serum albumin (HSA) (i.e., a major plasma protein taken from urine) as a template and used silane sol-gel layers for imprinting. The inherent features of sol-gel technology provide a better platform for successful imprinting of biomolecules without modifying their original functionality. The imprinted sol-gel films were examined by SEM to observe the conductivity difference between coated and uncoated quartz crystal gold electrode. They have found that the impedance of coated electrode increases with increasing

layer height. Authors have also studied the other parameters such as temperature, effect of different salts and solvents on binding capacity, and concentration effect to develop an optimized detection system for HAS. The thin sol-gel films have shown suitable selectivity pattern in the presence of bovine serum albumin (BSA), horseradish peroxidase (HRP), and trypsin. The detection procedure of HAS was not fully directly based on mass change by quartz crystal, but rather based on electrochemical impedance, which is good enough for selective protein detection.

Surface imprinted polymers [92] are very effective for selective recognition of different proteins induction for crystallization. An example of such approach has been reported where methacrylic acid along with divinyl benzene (DVB) was polymerized at suitable conditions, and crystallized lysozyme was stamped for surface imprinting. Mass-sensitive measurements conducted on dual electrode QCM show that the imprinted channel shows significant frequency shift, while the nonimprinted channel remain unaffected. The growing of lysozyme crystals on imprinted polymer surface have been demonstrated by AFM image shown in Fig. 3.13.

Another very interesting example of trypsin imprinting [93] and its detection on QCM has been reported where template has been used in three different forms: amorphous, crystalline, and solubilized. Although the surface imprinting is possible with all three template models, experimental results suggest that the solubilized form of trypsin is highly appreciable concerning sensitivity point of view. These data obtained by exposing a set of different concentrations to three differently imprinted forms of trypsin have been presented in Table 3.2.

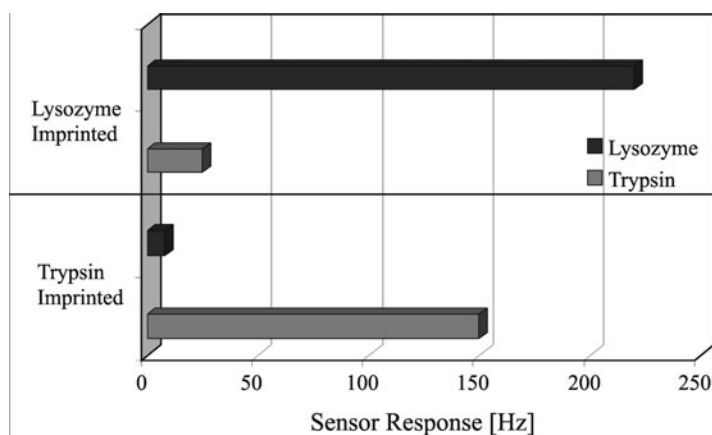
Trypsin imprinted surface was exposed to different other enzymes such as lysozyme and pepsin, and their relative mass change is noted. An excellent selectivity pattern is observed where the mass change is higher for trypsin than that for lysozyme and pepsin. This scheme was extended for lysozyme imprinting, and then a differential measurement was carried out for trypsin and lysozyme. It was evident from QCM results that surfaces imprinted with lysozyme and trypsin exhibit higher frequency response toward their original imprints in the presence of other (Fig. 3.14).



**Fig. 3.13** AFM image of lysozyme crystallization on MIP surface

**Table 3.2** Comparison of three differently imprinted polymer surfaces by calculating respective frequency shifts on various concentrations (adapted from [93])

Concentration of trypsin ( $\mu\text{g/mL}$ )	Sensor response [Hz] for three different template forms of trypsin		
	Lypophilized form	Crystalline form	Solubilized form
100	60	90	310
500	80	180	400
1,000	170	250	550
2,000	275	300	850

**Fig. 3.14** Cross sensitivity comparison by differential mass-sensitive measurements on trypsin and lysozyme imprinted surfaces (adapted from [93])

In all the mass-sensitive measurements, the response of nonimprinted channel was almost negligible to different enzymes. The effect of pH on trypsin detection was also monitored by performing the measurement in different conditions. The frequency shift for trypsin imprinted surface was calculated for three different pH values, 5, 7, and 9.2 and found to be highest at a pH value 7. This could be explained in a way that the varying conformations of enzymes and the columbic interactions between surface and analyte seriously distort the imprinted surface structure. The most remarkable feature of this sensing technique is that the detection limit of 100 ng/mL can be achieved.

Nicholas et al. [94] has also reported protein imprinting and their very sensitive and specific detection by QCM. A very interesting approach about protein imprinting has been presented by John Rick and Tse-Chuan Chou in which they have used single and double protein molecules and observed protein–protein interactions [95] by mass-sensitive devices. They have used lysozyme and cytochrome *c* as individual templates

and together in different molar ratios for imprinting using poly-aminophenylboronic acid as a monomer. Experimental results have revealed that the lysozyme imprinted layer shows fairly selective binding of the own template, while cytochrome *c* imprinted layer does not exhibit recognition properties. Regarding the double imprinted protein structure, both species were used as templates in different molar ratios and their response toward different proteins was monitored. It has been found that when lysozyme and cytochrome *c* were used in equal molar ratios for imprinting, the layer has shown maximum frequency change for the imprinted proteins. These observations provide a new route for multiple imprinting procedures for studying the interactions between same biomolecules on quartz surface.

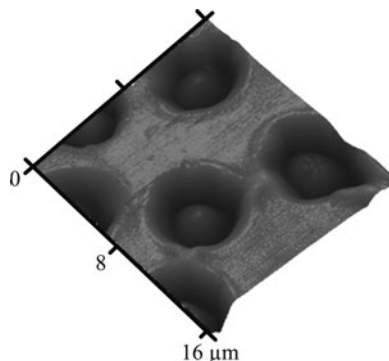
### 3.4.3 *Artificial Receptors for Erythrocytes*

Erythrocytes are basically red blood cells (RBC) that have hemoglobin and act as transporter for oxygen in blood. Erythrocytes are very flexible cells shaped like disks, having a diameter of approximately 7  $\mu\text{m}$ , and a thickness of 2  $\mu\text{m}$ . In previous examples, we already have knowledge of molecular imprinting for generating sensitive and selective materials for recognizing various microorganisms including bacteria and viruses. As erythrocytes have four different blood groups, A, B, O, and AB, for which molecular imprinting [96] can be handy to generate selective polymer layers capable of recognizing the respective blood group. The three blood groups, A, B, and AB, are recognized by the antigen on their surfaces and composed of oligosaccharides having six sugar molecules excluding blood group O, which has one less. The difference between blood group A and B is by only sixth sugar molecule at terminal position, whereas in the case of A, it is N-acetyl-D-galactosamine, and D-galactoside for blood group B. Molecular imprinting provides a straightforward approach to design suitable recognition materials for the detection of different blood groups on acoustic resonators. The blood groups A, B, AB, and O can be used as template model for imprinting on highly cross linked polyurethane layer. Typical polyurethane is composed of diphenyl diisocyanate as monomer, whereas bis phenol A and phloroglucinol are used as cross linkers. These cross linkers have excess of hydroxyl groups that play a very important role in imprinting as they offer suitable hydrogen bonding to the sugars present on blood group surface. Although the geometrical shapes of all these blood groups are almost identical, the predefined interaction of cells through hydrogen bonds with polyurethane surface offers suitable recognition. AFM image of imprinted erythrocytes on polymer surface have been shown in Fig. 3.15.

Different blood groups A, B, AB, and O have been used as templates [97] for surface imprinting by stamping method. The modified polyurethane surface was exposed to equal concentrations of different blood group samples. Mass-sensitive measurements reveal that the polyurethane surface imprinted with certain blood group (BG) shows utmost mass loads, particularly for BG that was used as template



**Fig. 3.15** AFM image of erythrocytes imprints



**Table 3.3** Relative sensor response of different BG imprinted polymers by differential QCM measurements (adapted from [97])

Imprinted blood groups (BG)	Relative sensor effect [%]			
	BG A	BG B	BG AB	BG O
BG A	100	37	62	57
BG B	45	100	50	70
BG AB	85	70	100	40
BG O	65	85	29	100

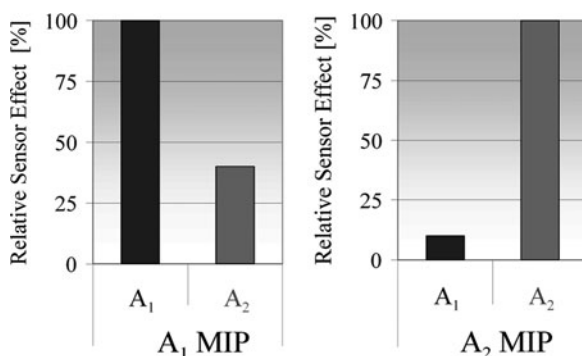
in surface imprinting procedures. Generally, in each case, the relative sensor response of a polyurethane layer is highest for its own template. These data obtained from mass-sensitive measurements have been presented in Table 3.3.

These findings show that these studies can be extended to real-life samples. Despite the fact that the structural dimensions of erythrocytes are in micrometer range, the recognition of antigens present on their interface ensures the nano structuring pattern on polymer surface.

The conventional methods for blood group detection are based on chemical reactions with specific antigens that form precipitates, whereas the latest methods give a change in the reflectance of laser beam when the surface is exposed to the samples. On the other hand, molecular imprinting in combination with mass-sensitive devices offers much cheaper and rapid detection scheme for different blood group samples.

MIPs can be designed in a more sophisticated way to differentiate the subblood groups (i.e., A1 and A2) by mass-sensitive transducers. Recently [98], polyvinyl pyrrolidone has successfully been used for surface imprinting of A1 and A2 and were put under mass-sensitive measurements. Experimental results showed that the MIP layer gives larger signal for that subgroup, which was used for imprinting. For example, the mass effect for A1 imprinted layer is higher by a factor of approximately three when compared with A2 (Fig. 3.16).

The results obtained from mass-sensitive measurements show remarkable selectivity as they have similar geometrical dimensions and belong to the same ABO



**Fig. 3.16** Comparison of relative sensor responses of A1 and A2 imprinted polymers (adapted from [98])

system. They differ from each other by the amount of antigen density on their surface as in the case of A1 it is  $0.81\text{--}1.17 \times 10^6$  A-antigens and for A2 it is  $2.4\text{--}2.9 \times 10^5$ . It is the beauty of molecular imprinting that provides patterned surfaces for selective incorporation of antigens. This study also reveals that MIP surfaces does not interact with analyte partially but shows affiliation with the whole cell to get complete information about surface density. These results are very appreciable and provide a complete understanding about the interactions of whole cell with modified polyvinyl pyrrolidone surfaces.

A slightly different synthetic strategy has been proposed for monitoring [99] of different blood groups and immunohematological reactions, using mass-sensitive devices (i.e., QCM) as transducers.

### 3.4.4 Hormones Detection

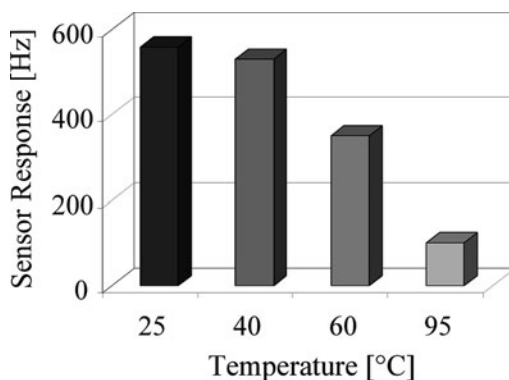
Hormones are basically chemical envoys in the body that carry the commands from one part to the other and are released by body cells in response to certain metabolic reactions. The excess or deficiency of any hormone in the body is called hormone disorder, which can lead to severe problems in functioning of cells. Different hormones in human body can be detected by examining blood, urine, and saliva samples, according to the desired hormone test. Among different hormones, insulin is very important, which takes the glucose from blood and stocks it up as glycogen in the liver and other cells and does not allow the use of body fat as energy resource. The disorder of insulin can cause serious diseases to the body such as diabetes, insulinoma, and others. In market, synthetic insulin can be produced for diabetic patients by fermentation process governed by yeast and bacteria. So, the detection of insulin is very important not only in human body to control metabolic reactions but also highly desirable in commercial point of view. The application of artificial materials for mass-sensitive detection of insulin and other hormones is very rare. The versatility of

surface imprinting makes promise to design suitable sensor materials for insulin detection [100] with a broad concentration range. Highly cross-linked polyurethane was prepared and coated on suitable mass-sensitive transducer (i.e., QCM) having 10 MHz fundamental resonance frequency. As mentioned already, solution phase stamping procedure was followed for imprinting of insulin on polyurethane surface. A series of different concentration solutions of insulin ranging from 1  $\mu\text{g/mL}$  to 7  $\text{mg/mL}$  has been exposed to imprinted channel and corresponding frequency change was monitored. It has been observed that the sensor response for different insulin solutions was linear for such a broad range, which suggests that this system can be used for quantitative determination of insulin in pharmaceutical formulations (i.e., 40 IU or 100 IU). IU stands for international unit for pharmacology activity that is 35  $\mu\text{g/mL}$  of insulin.

Interestingly, different experiments have shown that the sensor response for insulin does not depend upon the layer height of the polymer on quartz, which is very surprising while considering surface imprinted layers. The sensor signal was reversible and reproducible for several measurements performed on different layer heights of polyurethane.

The temperature effect on insulin has also been monitored to examine its behavior by performing mass-sensitive measurements at different temperatures (i.e., 25, 40, 60, and 95°C). At all these temperatures, same concentration of insulin (i.e., 1  $\text{mg/mL}$ ) was used for analysis. It has been observed that the frequency shift is highest for that measurement, which was conducted at 25°C and then gradually decreases until at 95°C where it is lowest. Although the concentrations are same, the effect is different at different temperatures. This is probably due to the denaturing of insulin in which the structural features and functionality is lost, which ultimately cuts down the surface interactions between the analyte and the polymer layer. It signifies that by selecting proper storage temperature, the shelf life of insulin drugs can be enhanced. These findings are very significant for monitoring the quality of insulin products in pharmaceutical industry (Fig. 3.17).

Another strategy has also been proposed by Satish et al. [101], where a sandwich layer material has been crafted and coated on QCM for insulin detection. They



**Fig. 3.17** Temperature effect on insulin sensor (adapted from [100])

have sandwiched an antigen between two specific antibodies and used it as sensing element. The lower antibody layer is deposited on quartz surface, while the upper antibody layer is used for insulin recognition and in between these layers an antigen is pressed. This layer material is exposed to different insulin concentrations and corresponding frequency shifts were noted. Although the lowest detection limit was achieved (i.e., 1 ng/mL), the sensor signal does not remain linear after 10  $\mu\text{g/mL}$  of insulin, which is not favorable for commercial use. The effect of temperature on mass-sensitive measurement has not been discussed. The synthetic scheme is also very complex for layer structuring that requires relatively more care, and more importantly low stability of natural antibodies restrict their use for long-term analysis.

Catecholamine is a very important class of hormones, which acts as neurotransmitters in the body and is released in response to certain psychological reactions. Major catecholamines are dopamine, norepinephrine, and epinephrine, which are excreted in urine. Disorder in this hormone can lead to serious complications in the central nervous system. The detection of these hormones can be made by fluorescent technique and other methods such as ion exchange chromatography and high performance liquid chromatography (HPLC), where different catecholamines are isolated and characterized from urine samples. Suitable sensor materials can be generated through molecular imprinting for selective detection of catecholamines in a complex urine solution. Tzong et al. [102] designed such materials by imprinting dopamine and developed a suitable sensor system using QCM as transducer. Authors have synthesized dopamine MIP powder and examined their binding properties at different pH values by HPLC studies. Dopamine MIP films were also generated by following a different reaction route and the effect of pH was also monitored on rebinding capacity of these films. These studies reveal that dopamine imprinted film preferably binds dopamine when compared with norepinephrine and epinephrine. Similarly other catecholamine imprinted films (i.e., norepinephrine and epinephrine) specifically bind the original imprint molecule as shown in Table 3.4. Dopamine MIP layer coated on 9 MHz quartz exhibits maximum frequency shift for dopamine itself, while for norepinephrine and epinephrine it is negligible. The results are summarized in Table 3.4. These studies are valuable and encouraging in designing a suitable detection model for catecholamines.

MIPs with acoustic devices have also been employed for detection of plant hormones, for example, indole-3-acetic acid (IAA) plays a vital role in plant development. Akimitsu and Toshifumi [103] had designed molecularly imprinted

**Table 3.4** Summarized results of different MIP hormones to monitor the rebinding quantity on from analyte solutions (adapted from [102])

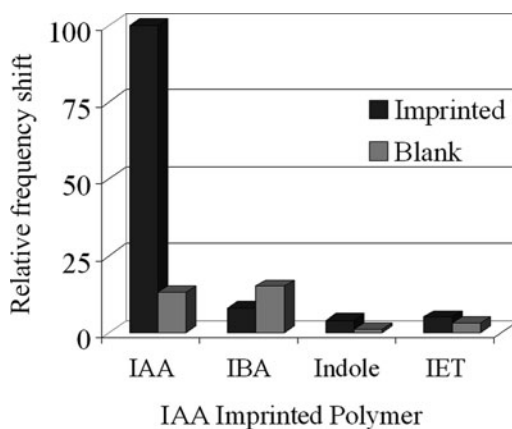
MIP hormones	Rebinding quantity of analyte solutions [ $\mu\text{mol}$ ]		
	Dopamine	Norepinephrine	Epinephrine
Dopamine	0.17	0.05	0.03
Norepinephrine	0.01	0.08	0.02
Epinephrine	0.01	0.02	0.05

meth acrylic acid (MAA) polymer for selective recognition of IAA, using 9 MHz QCM to perform mass-sensitive measurement. A series of different concentrations of IAA ranging from 10 to 200 nM has been used on imprinted and the nonimprinted QCM. The change in the frequency is linear in the defined range upon mass loading of IAA. This experiment was performed on three different QCMs to observe the relative variation in the frequency shifts on same concentrations of IAA. The reported results show a coefficient of variation for three different sensors 5.3%, 6.1%, and 8.8%, respectively, which means that there is no significant difference in their performance. This is an evidence for a sensor system to generate reproducible results. The selectivity of this sensor has also been monitored by performing measurements with indole-3-butyric acid (IBA), indole, and indole-3-ethanol (IET) of same concentrations. Sensor results clearly demonstrate that the frequency change for IAA is maximum, while for IBA, indole, and IET, the response is quite low, which shows the selective nature of designed sensor material. The reason of highly selective nature of imprinted MAA polymer is could be due to the suitable electrostatic interactions between *N,N*-dimethylamino group from monomer to carboxylic group of IAA (Fig. 3.18).

Synthetic receptors derived from molecular imprinting possess desired degree of selectivity in comparison with other schemes. In combination with mass-sensitive devices, molecular imprinting is very advantageous for developing suitable sensor systems for hormones detection. Despite of the fact that MIP applications for hormones detection are very few but still promising to replace conventional methods for their detection.

### 3.4.5 Bilirubin Detection by Synthetic Receptors

Molecularly imprinted polymers have been proved very effective for bilirubin detection via mass-sensitive devices. Bilirubin is a yellow color waste material



**Fig. 3.18** Relative frequency shifts for other similar hormones on IAA imprinted polymer surface (adapted from [103])

that is produced from the breakdown of hemoglobin molecules of RBC. Bilirubin tests are often conducted to examine its production and excretion from body, which ultimately refers to the working of the liver cells. Elevated amounts of bilirubin in the body indicate the improper functioning of liver, which causes hepatic diseases and in severe cases permanent damage to the brain or even results in death [104]. Various voltammetric [105] and fluorometric [106] methods have been reported for bilirubin analysis with substantial sensitivity. Conventional method for bilirubin detection is based on diazo reaction, where azobilirubin is produced by the condensation reaction of diazotized sulfanilic with bilirubin.

Authors [107] have successfully prepared bilirubin imprinted polymer, using 4-vinyl pyrrolidone as functional monomer along with DVB as cross linker. The polymerization reaction was initiated by benzophenone and carried out under UV light. The surface of gold electrodes on QCM is properly cleaned and is modified by treating it with thiols before coating MIP layer. Different topographical images of the coated film had been recorded by SEM that exhibit a uniform surface structure of bilirubin MIP. Mass-sensitive measurements performed on 9 MHz QCM exhibit suitable sensor response for defined amounts of bilirubin samples. The effect of pH on bilirubin detection was also examined by performing mass-sensitive measurements at different pH values ranging from 5.4 to 11.5. It has been found that at higher pH value (i.e., 11.5), the frequency shift is also higher. The bilirubin MIP surface was exposed to a set of different concentrations (i.e.) from 0.45 to 11.0 mg/dL of bilirubin and found that the sensor response is linear in this range. Biliverdin, which is a similar compound to bilirubin, was selected as a model to examine the selectivity of bilirubin MIP. The sensor signal for bilirubin is much higher when compared with biliverdin, which shows that bilirubin imprinted surface preferably binds the own template compared with other analogous species.

MIP coated on QCM has proven itself as an effective tool for clinical analysis of bilirubin, which offers a reliable, simple, and fairly cheap detection system in comparison with other methods.

### 3.4.6 *Miscellaneous Examples*

Molecular imprinting approach can also be extended for another very important class of materials (i.e., nanoparticles) to generate more sophisticated surfaces for sensing principles. Kristina et al. [108] prepared imprinted nanoparticles and introduced them in to a thin poly ethylene terephthalate (PET) layer coated on quartz wafer. The AFM study of imprinted nanoparticles shows a fine and uniform distribution that has potential to recognize pair (*R*)- and (*S*)-propranolol in aqueous buffer. Propranolol is a nonselective beta blocker drug that is very useful for hypertension treatment. The imprinted nanoparticles show a definite degree of chiral selectivity for enantiomeric pair of (*R*)- and (*S*)- propranolol. This indeed is a positive step toward designing enantiomeric biosensor materials and can only be achieved by careful controlling of the morphology of nanoparticles.

The applications of molecular imprinting with mass-sensitive devices such as QCM are very limited for DNA identification. Recently, a new biosensor has been designed for DNA recognition [109], based on molecularly imprinted polymers coated on QCM. Authors had synthesized thymine-MIP by using methacryloylamido, adenine, thymine (MA-Ade-thymine) as functional monomer along with EDMA as cross linker with AIBN initiator. The nonimprinted polymer was prepared in a similar fashion excluding thymine. They had modified the gold electrode surface of QCM before coating thymine-MIP layer. The surface of bare electrode, thiol-treated gold surface, and thymine layer was characterized by AFM on wave mode. MIP generates very suitable tailor-made cavities for thymine interactions through hydrogen bonding with functional groups of polymer. Mass-sensitive measurements performed on imprinted and nonimprinted channels exhibit a substantial difference in frequency shifts for a defined concentration range. The selectivity of MIP layer was also examined by calculating the sensor response for same concentrations of thymine, uracil, poly(dT) (ssDNA), and poly(U) (ssRNA). The sensor signal for thymine at all concentration points was higher in comparison with others.

Some other strategies are also reported where instead of a synthetic polymer, gold nanoparticles act as sensing material [110] for mass-sensitive detection of DNA molecules. Although these materials are very rarely used for biorecognition, they have very good sensitivity (e.g.,  $10^{-14}$  M and  $10^{-16}$  mol/L). Another more advanced design has been reported [111] where  $3 \times 3$  QCM matrix is assembled on which specific antibody is coated for DNA sensing. This detection mechanism is very attractive as it provides a suitable platform for online monitoring of various biological analytes. Colloidal gold nanoparticles have also been reported [112] to improve the gold-coated QCM surface. These gold particles possess high surface area and enhanced interaction sites that exhibit appreciable sensor signal for target DNA strands. Despite the fact that these schemes do not hold any modified polymer interface on quartz surface, they are good enough for designing DNA sensing systems.

The LBL technique also contributes to the detection of various bioanalytes but along with mass-sensitive devices, their applications are relatively limited. A LBL assembling of liposomes and protein membrane [113] was made on QCM surface for biological recognition. The sensitive layer was characterized by AFM and put under detection of nonylphenols where it had showed satisfactory results in low ppm concentration range. Some other examples are also reported where LBL films with QCM have proven very handy for sensitive detection of DNA strains. A very exciting example of dengue virus detection has been reported using LBL hybridized gold nanoparticles [114] as sensing element. These modified nanoparticles provide high sensitivity through QCM for the detection of dengue virus. In comparison with other polymer surfaces, nanoparticles provide more binding sites that has affinity for target analyte. SEM images give a clear picture of LBL assembling of gold nanoparticles on quartz surface. The modified oligonucleotide gold particles are not only sensitive for viral detection but also very specific for target specie. Along with mass-sensitive devices, they have proven themselves to be very successful for diagnostic purposes in real blood samples of dengue viral infections. Almost

more than hundred countries had suffered from this virus and very high rate of causalities have been reported in different regions. Such detection methods are very encouraging in developing more effective systems that are low cost and suitable for online surveillance.

Multi-sensor strategy is very exciting and innovative regarding the detection of diverse bioanalytes on a single platform. In this way, we would be able to immobilize different MIP on a single QCM that would be competent to bind specifically target analytes. One such approach has already been mentioned.

### 3.5 Conclusion and Future Outlook

Considering the material designing for biosensors, molecular imprinting has shown potential for recognition of various bioanalytes including microorganisms such as yeast, bacteria, viruses, and others such as proteins, enzymes, erythrocytes, etc. The tailor-made structures of imprinted polymers provide enhanced selectivity for target analyte molecules at nanometer scale where the size, shape, and dimensions of imprinted cavities are optimized for a desired biomolecule. The most significant aspect is that molecular imprinting is virtually applicable to almost all biological species unless template does not chemically react with polymer matrix. The synthetic procedure of imprinting is much more convenient when compare with other methods that are more complex, time consuming, and relatively need expansive chemical reagents. As we have learnt from the previous study that surface imprinted polymers have very broad spectrum for biosensors when compared with other material crafting technique. Unlike natural antibodies, MIPs bind analyte molecules reversibly through weak attraction forces and thus makes reusability of these materials that can be used for several analysis. The other material designing strategies such as natural antibodies, LBL, and host-guest interactions have some contributions in developing biorecognition materials but not exploited for commercial use of biosensors. The other part of biosensors concerning transducers is accomplished by acoustic or mass-sensitive devices such as QCM, which furnishes high sensitivity to designed biosensors. These devices are capable of sensing the mass of analyte in nanogram or even in picogram range (i.e., SAW devices, which are very valuable while dealing with extremely low concentrations).

The sensitive and precise detection of different bioanalytes can be enhanced in two ways: first, optimizing the synthetic procedures for imprinting, and second by tuning mass-sensitive devices for achieving best possible sensitivity. The first part of sensor design deals with imprinting technique where the analyte interactions with MIP surface need to be made better adjusting monomer cross linker ratio, establishing proper reaction conditions such as favorable solvent, temperature, and heating time. The careful monitoring of different reaction parameters not only provides maximum binding sites but also constructs template-oriented cavities for selective detection. Another very important aspect (i.e., response time) can be



improved by controlling the surface structures of MIPs so that the analyte molecule readily be attached or detached without consuming much time. These modifications in synthetic receptors enhance the sensitivity and permit only target analyte to be bounded specifically with MIP surface. Concerning the reusability of the sensor materials, the template MIP interaction should be entirely reversible that can only be achieved by developing noncovalent interface.

The other part is concerned with the design of transducers. At the moment, QCMs having frequency 5–20 MHz are used widely as mass-sensitive transducers. The fundamental resonance frequency depends on thickness of quartz sheet, which is a key parameter to amplify the mass sensing capabilities of acoustic devices. The sensitivity can be made better by producing more thin QCMs, which is not feasible from a mechanical point of view as these devices would turn out to be very fragile. This restricts the use of acoustic devices to extend the detection limits of acoustic biosensors. An alternative can be used to overcome this deficiency of acoustic resonators by introducing film bulk acoustic resonators (FBAR). These devices operate in the GHz range, which is higher by a factor of  $10^3$  than typical QCM resonance frequency. They not only offer enhanced sensitivity but also exhibit fine linearity, small and compact size, and less damping loss in viscoelastic medium.

The overall objective of this study is to demonstrate patterned polymer surfaces for selective recognition of different bioanalytes through acoustic resonators, thus providing a suitable, economic detection system to improve public health and ensure a safe environment.

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