Macromolecular Imprinting for Improved Health Security

Piyush Sindhu Sharma, Zofia Iskierko, Francis D'Souza and Wlodzimierz Kutner

Abstract There is a growing demand for rapid and reliable methods of determination of microorganism contamination of waters and food products to ensure quality assurance and to improve the health care system in general. Majority of the available methods for determination of microorganisms in foods are time consuming and expensive. In recent years, different approaches have been attempted to develop alternative procedures for determination of microorganisms. In the present chapter, we summarize the recent achievements in the development of synthetic recognition systems based devices for monitoring the presence of microorganisms, such as bacteria, bacteriophages, and viruses, in waters and food products. Molecular imprinting has been most successful in devising relevant synthetic receptors. Application of these recognition systems for determination of microorganisms is herein described in detail.

Keywords Molecularly imprinted polymer · Microorganism · Bacteria · Virus · Bacteriophage · Health security · Chemosensor

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List of abbreviations

ASPV	Apple stem pitting virus
CV	Cyclic voltammetry
cfu	Colony forming unit
DMSO	Dimethylsulfoxide
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
μIDC	Micro interdigited capacitor
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FESEM	Field emission scanning electron microscopy
LOD	Limit of detection
LOQ	Limit of quantitation
MIP	Molecularly imprinted polymer
MIPPy	Molecularly imprinted polypyrrole
OPPy	Overoxidized polypyrrole
PM	Piezoelectric microgravimetry
PMA	Poly(methacrylic acid)
PPy	Polypyrrole
PSS	Poly(styrene sulfonate)
PVC	Poly(vinyl chloride)
PVP	Polyvinylpirrolidone
SG	Sol-gel
SEM	Scanning electron microscopy
SiNP	Silica nanoparticle
SPR	Surface plasmon resonance
SRB	Sulfate-reducing bacteria
TMV	Tobacco mosaic virus
TYMV	Turnip yellow mosaic virus
VLP	Virus like particle

1 Introduction

With the increase of people awareness over the past few decades, the world demand for clean drinking water and healthy food supply have increased enormously. The domestic (sewage, solid waste disposal, low hygienic living conditions, etc.) and industrial pollutants have increased the risk of contamination of food products and water sources with microorganisms. Microorganism, such as the *E. coli* bacteria, lives in intestine of warm-blooded animals and humans. The presence of *E. coli* in water is a strong indication of sewage or animal waste contamination [1]. Together

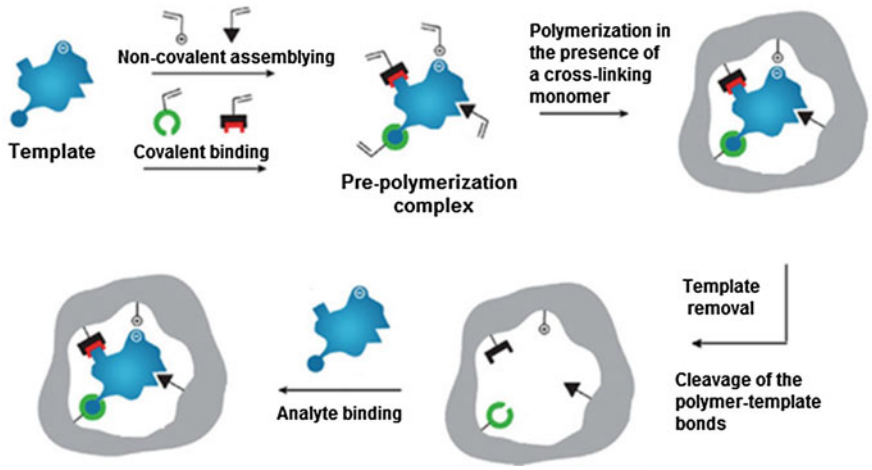
with other species of bacteria, e.g., *V. cholera*, these microorganisms are sources of serious waterborne diseases.

Different analytical methods are available for continuous monitoring of microorganism contaminants in water and food products [1], however, most of them use procedures involving culturing of these microorganisms. Unfortunately, this culturing is time consuming, usually taking 24–48 h and rather expensive.

Importantly, short determination time and easy operation with minimum interference of close analogs of analytes are two of many important requirements for successful application of chemosensors in a real world analysis of contaminants. For that, chemosensors using electrochemical [2] and electrical [3] transductions offer easy operation towards determination of target analytes. Additionally, possibility of miniaturization, ease of use, and a low cost make these determinations promising. Other than these, several transductions are used to develop sensing platforms for on-line monitoring of microorganisms. These include piezoelectric microgravimetry (PM) [4, 5] and surface plasmon resonance (SPR) [6, 7]. Unfortunately, selectivity of chemosensors using these transductions without any recognition is low.

To incur desired selectivity in chemosensing, surface of transducers is modified mostly with polymer based recognition units [8, 9]. Typically, these units, fabricated in the form of thin films, are deposited directly on the transducer surface. Detectability and selectivity of these polymer-based chemosensors are appreciable.

Selective receptors using target analytes as templates have become more and more popular [9–13]. These receptors often reveal stability constants of formation of complexes with analytes similar to those of biological receptors [14]. Generally, the procedure of molecular imprinting involves polymerization of functional monomers with cross-linking monomers in the presence of a template, which is most often the analyte itself (Scheme 1). Subsequent removal of the template leaves in the resulting molecularly imprinted polymer (MIP) molecular cavities complementary in their



Scheme 1 Consecutive steps of molecular imprinting (Adapted from [13].)

size, shape, and orientation of recognition sites to those of the template molecule. This imprinting generates shape-selective molecular cavities, which allow discriminating between close structural analogues of the analytes.

Three main strategies were developed to design molecular cavities in MIPs, namely, (i) covalent, (ii) non-covalent, and (iii) semi-covalent imprinting [15].

In covalent imprinting, templates are covalently bound to functional monomers during MIP preparation [16]. After polymerization, the template is removed by bond cleavage and the recognition sites of the molecular cavities left are capable of binding the target analyte molecule by re-establishing the same covalent bonds. Unfortunately, this imprinting involves derivatization of the template molecule, which adds an additional preparation step.

In non-covalent imprinting [17–19], interactions between functional monomers and the template during polymerization, including hydrogen bonding, ion-pairing as well as dipole-dipole and hydrophobic interactions, are reproduced in subsequent analyte binding by the template-free molecularly imprinted cavities of an MIP. Due to its simplicity, this strategy is most commonly used.

Semi-covalent imprinting combines advantages of both covalent and non-covalent imprinting [20–22]. In this strategy, initially, a template is trapped in the polymer matrix by covalent bonds. Then, after template removal, the analyte is bound in a molecular cavity through non-covalent interactions.

Application of molecular imprinting for devising recognition units of chemical sensors [10] and stationary phase materials for solid-phase extraction (SPE) [23–25] appears promising for MIP commercialization. Now, MIP based SPE sorption materials for extraction of traces of toxins, such as patulin, aminoglycosides, and bisphenol A, are commercially available. Unfortunately, despite variety of selective chemosensors devised in last decades [9, 10, 15], no chemosensor based on MIP is available on the market yet. Although sensing results presented in all reports cited above are very promising, the chemosensors described seek much improvement before commercialization. Undoubtedly, we will see first commercial chemosensing devices based on MIPs in the nearest future.

For on-line measuring, MIPs devised using non-covalent imprinting for chemosensing appeared efficient in comparison to MIPs using covalent imprinting [8, 17–19, 26–29]. In the earlier approaches, as mentioned above, an analyte binds in molecular cavities through weak and easy to disrupt interactions. Mostly, an excess of a carrier solution is sufficient to accomplish this latter task under flow-injection analysis (FIA) conditions [8, 17–19, 26–29]. However, in later approaches, there was a need to break strong covalent bonds between an analyte and a molecular cavity, which required different reaction conditions than those of subsequent recognition.

Until now, several reports claimed obtaining repeatable responses of MIP chemosensors devised using non-covalent imprinting, operating under FIA conditions [10, 17, 18, 26, 28, 29]. Advantageously, these chemosensors were inexpensive and could work under harsh conditions. These are just only few important advantages supporting suitability of the MIP based chemosensing systems for on-line analyte detection.

Table 1 Analytical parameters of MIPs devised for determination of bacteria

Bacteria species	Functional monomer/cross-linker	Method of imprinting	Template extraction with	Determination or recognition technique	Comment	Ref.
Eduscho grande	Acrylic acid/divinylbenzene	Stamping	Hot water	PM	LOD = 10^4 cells mL^{-1}	[40]
S. cerevisiae	Polyurethane	Stamping	Hot water	PM		[40]
S. cerevisiae	Polyurethane	Stamping	Hot water	PM	–	[41]
S. cerevisiae	Polyurethane Titanium ethylate/ (3-aminopropyl) methyldiethoxysilane	Stamping Stamping	Hot water Hot water	PM PM	LOQ = 10^4–10^9 cells mL^{-1}	[42]
E. coli MRE-600	Acrylamide/*N*,*N*′-methylenebis acrylamide	Bulk imprinting	Lysozyme enzyme/50 mM SDS/50 mM Tris-Cl pH = 8.5	Electrophoresis	Difference in migration time was observed for templated bacteria and other bacterial strains.	[36]
Bacillus thuringiensis kurstaki	Polyamide	Lithography	12 M HCl: methanol (50: 50, *v*: *v*)	Fluorescence	MIP and NIP captured 39 % and 13 % of spores, respectively.	[43]
Bacillus subtilis	Pyrrole	Electropolymerization	DMSO	EIS	LOQ = 10^4–10^7 cfu mL^{-1}	[44]
Deinococcus radiodurans *E. coli* *B. subtilis* *S. natans*	Tetraethoxysilane	Bulk imprinting	Ethanol (96 %)	Fluorescence	LOQ = 10^7–10^9 cfu mL^{-1}	[39]
Pseudomonas aeruginosa	Pyrrole	Electropolymerization	–	Fluorescence	–	[45]
E. coli	Tetraethoxysilane	Bulk imprinting	Ethanol	PM	LOD = 10^2 cfu mL^{-1}	[46]
Sulfate reducing bacteria	Chitosan-reduced graphene	Electropolymerization	Acetone	EIS	LOQ = 10^4–10^8 cfu mL^{-1}	[47]
E. coli *P. aeruginosa* *B. subtilis* *S. cerevisiae*	Pyrrole	Electropolymerization	Electrochemical overoxidation	PM	–	[48]

cfu, colony forming unit; DMSO, dimethylsulfoxide; EIS, electrochemical impedance spectroscopy; LOD, limit of detection; LOQ, limit of quantification; PM, piezoelectric microgravimetry; SDS, sodium dodecyl sulfate.

Table 2 Analytical parameters of MIPs devised for determination of viruses

Virus species	Functional monomer/cross-linker	Method of imprinting	Template extraction with	Determination or recognition technique	Comment	Ref.
TMV	Methacrylic acid/styrene, divinylbenzene	Stamping	Hot water/ 2 % SDS	PM	Only one concentration of virus was measured	[49]
	Polyallylamine hydrochloride	Bulk imprinting	Ethanol (70 %, 24 h)/ 1 mM NaCl (1 h, 100 °C)/ 1 M NaCl (100 °C)/ 3 days in water	UV-vis spectroscopy	MIP binding = 8.8 mg/g, NIP binding = 4.2 mg/g	[34]
	Polyallylamine hydrochloride	Bulk imprinting	1 M NaOH	Fluorescence spectroscopy	MIP binding = 2.73 mg/g, NIP binding = 1.18 mg/g	[30]
	Methacrylic acid/*N*,*N*′-(1,2-dihydroxyethylene) bis-acrylamide/1-vinyl-2-pyrrolidinone	Stamping	–	EIS	–	[50]
	Azobenzene bearing acrylate polymer	–	1 % SDS (24 h)	Chemiluminescence	–	[51]
Dengue	Acrylic acid, acrylamide/*N*-benzylacrylamide	Bulk imprinting	20 mM phosphate buffer (pH = 4)	PM	–	[52]
Influenza A H1N3	Acrylamide, methacrylic acid, methylmethacrylate/*N*,*N*′-(1,2-dihydroxyethylene) bisacrylamide	Stamping	10 % HCl (45 °C, 3 h)	PM	up to 2.5×10^7 particles/mL	[53]
Influenza H5N1	Acrylamide, methacrylic acid/methylmethacrylate	Bulk imprinting	10 % HCl (45 °C, 3 h)	PM	–	[54]

(continued)

Table 2 (continued)

Virus species	Functional monomer/cross-linker	Method of imprinting	Template extraction with	Determination or recognition technique	Comment	Ref.
Human norovirus VLPs	Tetraethylorthosilicate/aminopropyltriethoxysilane	Surface imprinting	0.1 M HCl (with 0.01 % Triton-X)	Indirect ELISA	–	[55]
Apple stem pitting	Acrylamide, *N*-isopropyacrylamide/ *N*,*N*′-methylene bisacrylamide	Bulk imprinting	Ethanol/NaOH	Diffraction grating	LOD = 10 ng mL^{-1}	[38]
Bacteriophage MS2	*N*-isopropylacrylamide, acrylic acid, *N*,*N*′-methylene-bis-(acrylamide), *N*-tetrabutyl acrylamide, *N*-(3-aminopropyl) methacrylamide hydrochloride	Surface imprinting	Water (60 °C)	SPR	LOQ = 0.33–27 pmol	[56]

EIS, electrochemical impedance spectroscopy; ELISA, enzyme-linked immunosorbent assay; PM, piezoelectric microgravimetry; SPR, surface plasmon resonance; TMV, tobacco mosaic virus; VLP, virus-like particle.

The present chapter critically summarizes the available information on the MIP based materials devised so far for microorganism determination in waters and food products (Tables 1 and 2). Moreover, we highlight here with some representative examples of imprinting of microorganisms using different strategies (Tables 1 and 2).

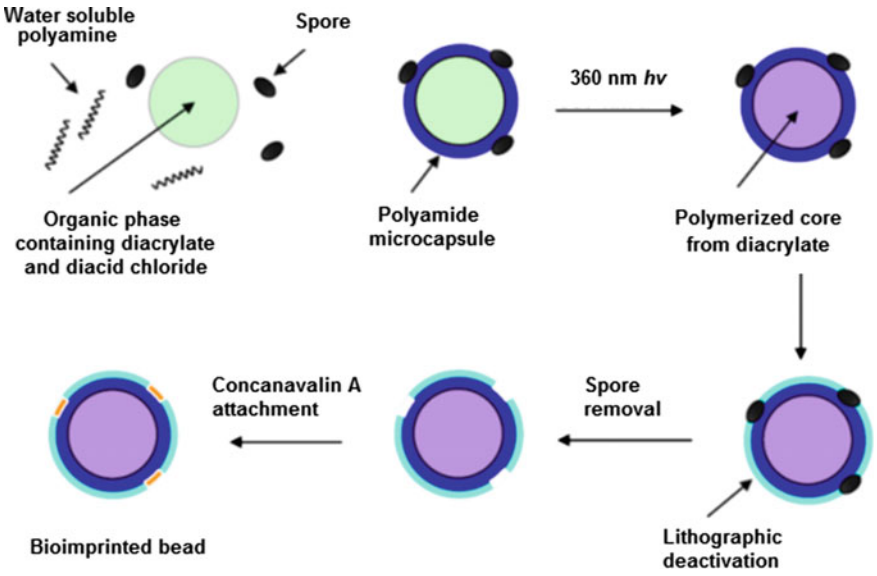
2 Microorganism Imprinting

In comparison to relatively easy imprinting of small molecules, molecular imprinting of whole microorganisms is challenging. Major difficulty in the latter imprinting is a huge size of microorganisms. Therefore, this size restricts their removal from the molecular cavities formed. Then, during recognition, this huge size makes the microorganism diffusion within the polymer matrix slow resulting in a long response time of chemosensors. To cope with these challenges, several strategies have so far been developed. They aim at optimizing MIP preparation procedures and MIP matrix composition to improve the imprinting. For instance, MIPs were prepared with different degree of cross-linking [30] or in a form of nanoparticles with the well-defined surface [31] for easy diffusion of the microorganism analyte to and from molecular cavities. Moreover, on the large surface of a microorganism, density of possibly accessible recognition sites is high. This high density requires either high number of mono-functional monomer molecules or functional monomers with many functionalities for establishing non-covalent interaction equilibria.

The earliest successful attempt of bioanalyte imprinting engaged emulsion polymerization resulting in polyamide beads templated with bacteria on the interface [32, 33] (Scheme 2). On the surface of the resulted beads, there were anisotropic patches of addressable functionalities of exactly the same size and shape as those of the microorganism template. To decrease non-specific binding, the exposed surface was derivatized with diisocyanato-terminated perfluoropolyether. Interestingly, this lithographic procedure did not use any specialized reagents or instrumentation. The SEM imaging of these beads after template removal showed the presence of 100–200 nm deep indentations (Scheme 2). The MIP material obtained based on this procedure was suitable for selective removal of bacteria cells from a test solution.

3 Imprinting of Microorganisms in Bulk Polymer

Another early approach described microorganism imprinting in hydrogel monoliths by bulk polymerization of monomer mixtures containing the microorganism template and a small amount of solvent acting as the porogen [30, 34–36]. Hydrogel is a three-dimensional insoluble polymer network, which can be homogeneously dispersed in water. High hydrophilicity of a hydrogel allows stronger biomolecule accommodating [37]. Moreover, hydrogels are biocompatible. For their preparation,



Scheme 2 Basic steps of the lithographic strategy of preparation of bacteria imprinted polyamide beads (Adapted from [32].)

polyallylamine was mostly used as the functional monomer [34, 35] (Table 2). The resulting imprinted polymeric hydrogel monolith was cut in order to produce sufficiently small-size particles with a reasonable fraction of accessible molecularly imprinted sites. Hydrogels of this type were designed to work as "sponges" capable of selective removal of pathogenic microorganisms from blood [34, 35].

For preparation of homogeneous molecularly imprinted cavities, virus aggregation in a pre-polymerization solution was prevented by using an optimized amount of functional monomers. In one example, an MIP hydrogel prepared for recognition of the tobacco mosaic virus (TMV) showed 2.1-fold higher affinity to TMV than the non-imprinted polymeric hydrogel did [34] (Table 2). This MIP hydrogel was suitable for separation purposes but it was not suitable for more advanced applications, such as that of serving as the recognition unit of a chemosensor.

Recently, a hydrogel MIP based chemosensor was fabricated for apple stem pitting virus (ASPV) [38] (Table 2). To provide additional selectivity to the molecular cavities imprinted, a polymerizable aptamer was devised and used as the functional monomer for ASPV imprinting. In the presence of the ASPV template, this monomer was copolymerized with additional functional monomers (*N*-isopropylacrylamide, acrylamide, and *N*,*N*′-methylene bisacrylamide) as well as the cross-linking monomer (*N*,*N*,*N*′,*N*′-tetramethylethylendiamine) to form imprinted cavities featuring multiple recognition sites. This hydrogel shrank in the presence of ASPV. This shrinking linearly depended on the virus concentration in the range of 1.0×10^{-2} to 1.0 μg mL^{-1}.

Other than polyallylamine, several reports described the use of the acrylic acid and acrylamide derivative based functional monomers for imprinting the bacteria and virus microorganisms [50, 53, 54, 56, 57] (Tables 1 and 2). Instead of preparing an MIP in the bulk form, in some reports, the pre-polymerization solution was drop- or spin-coated on surfaces of different transducers to prepare an MIP in the form of a thin film [54]. This film served the role of a recognition unit of a chemosensor. Towards that, the influenza A virus was successfully imprinted [53]. Binding this virus by molecular cavities of the resulting MIP was recognized with piezoelectric microgravimetry (Table 2). This MIP well discriminated between virus subtypes. In this approach to preparation of the chemosensor recognition unit, thickness of the MIP film was an important criterion. That is, removal of the template from a thin MIP film was, as expected, easier than from a thick MIP film.

Thin films of organically modified silica produced by a sol-gel method were imprinted with whole cells of different microorganisms in order to devise an alternative matrix for imprinting [39, 46] (Fig. 1). This sol-gel method for

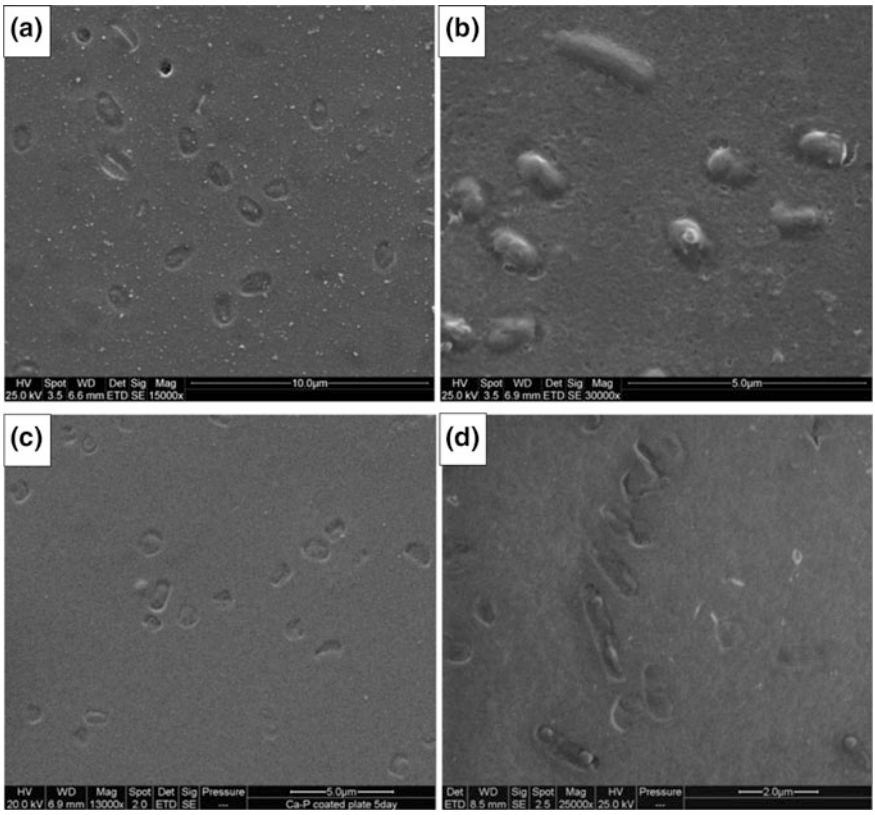
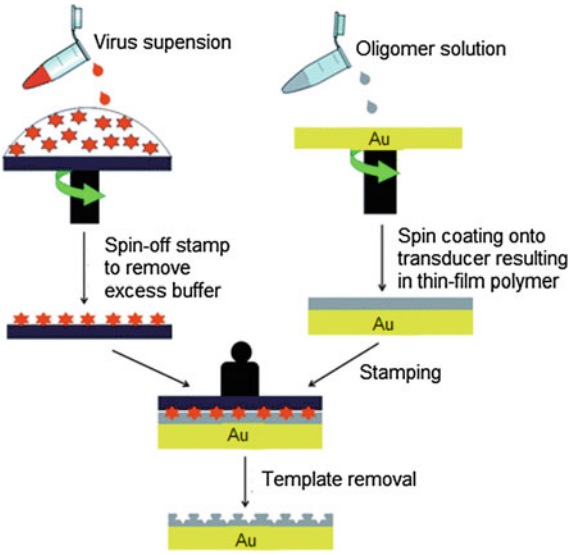


Fig. 1 SEM images of the bacteria-imprinted sol-gel films after removal of bacteria templates (**a**) *B. subtilis*, (**c**) *E. coli* as well as the same films (**b**) and (**d**), respectively, after 30-min exposure to the respective bacteria suspensions (Adapted from [39].)

polycondensation of silanes, used ambient conditions in comparison to those mentioned above of acrylate polymerization. Worth mentioning, an acrylate based functional monomer requires UV light or heat (~60 °C) for polymerization. However, these conditions are destructive for microorganisms. Therefore, several microorganisms including *D. radiodurans*, *E. coli*, *S. natans*, and *B. subtilis* [39, 46] were imprinted in sol-gel matrices (Fig. 1). After removal of the entrapped bacteria template, membrane components of these bacteria were left entrapped in the molecular cavities of the film [39]. These components provided selectivity to the imprinted matrix.

4 Microorganism Imprinting on a Polymer Surface

An interesting strategy involved "stamping technique" to form a polymer film deposited on a transducer surface with well-organized molecular cavities for bulky macromolecules [40–42, 49, 53, 57, 58]. For that, microorganisms were first drop- or spin-coated on glass microscope slides to prepare molecular stamps (Scheme 3). Then, theses stamps were pressed against the pre-polymerized solution coated transducer surfaces. Constant pressing force applied to the resulting sandwich of the stamp-template-sensor film transducer provided good mechanical contact between the polymer and the cell walls of the microorganisms (Scheme 3) [59]. Most often, polyurethane matrix was used for this preparation [40, 41, 53]. Typically, polyurethanes cure overnight under ambient conditions. The microorganism stamp was reasonably easy to remove with hot water leaving behind well-arranged molecular



Scheme 3 Steps of the stamping technique for microorganism imprinting (Adapted from [53].)

cavities. For improving operation performance, the polymer composition was optimized to increase reversible interactions between the imprinted cavities and the microorganisms. For that, a phenolic functionality was added to the highly cross-linked polymer matrix. Besides, the phenolic group afforded wetting to the polymer surface.

Similarly, microorganism stamps were prepared with silanes. They were produced by the sol-gel method [42]. The resulting relatively rigid matrix was suitable for fabrication of materials to be used in an on-line measuring system. The sol-gel method provided robust MIP films efficiently operating under harsh conditions. Importantly, high affinity towards microorganisms of the imprinted sol-gel films was governed not only by morphology of the imprinted cavity (rod, coccus, or tetrad) but also by binding functional groups present on the outer surface of the bacteria cell. These groups guided orientation of recognizing functionalities in the course of formation of these cavities. In effect, the resulting MIP film was able to discriminate between different species of the bacteria tested despite their similar shape.

The stamping technique was used to form imprints of TMV and human Rhinovirus serotype 2 in a microfluidic device [50] (Fig. 2). This devise contained microinterdigitated capacitor (μIDC) strips coated by MIP films for TMV detection by contact-less dielectric measurements. The virus stamps were prepared on a polyvinylpyrrolidone, PVP, and poly(methacrylic acid), PMA, copolymer.

The results obtained with the surface stamping technique were promising, therefore prompting the necessity of formation of homogenous molecularly imprinted cavities on the polymer surface. For that, an alternative strategy was then developed. This strategy consisted of three distinct steps, which included (i) immobilization of the microorganism template on the polymer surface, (ii) equilibration of the template-modified surface with a mixture of functional monomers, (iii) polymerization to grow a polymer coat around the template, and (iv) removal of the immobilized template to empty out the imprinted cavities. However, this strategy needed careful optimization of thickness of the polymer coat. This was because an overgrowth of the polymer could end up in permanent entrapment of the template in the polymer matrix.

A few recent reports described surface imprinting of turnip yellow mosaic virus (TYMV) [60] and human norovirus [55] with organosilanes. For that, the TYMV was covalently immobilized with glutaraldehyde on silica nanoparticles (SiNPs) [60] (Scheme 4). Then, a silsesquioxane film was grown on top of this virus modified SiNPs. The silsesquioxane molecules containing the $-OH$ and $-NH_2$ functional groups self-assembled on the virus surface, and then they were polycondensated. The resulting film growth was followed by the field emission scanning electron microscopy (FE SEM) measurements. The SEM image revealed the presence of open cavities with an average diameter of 20 nm on the surface of SiNPs (Scheme 4). The binding assays demonstrated that 84 % of the TYMV template was bound to SiNPs while only 10 % of the non-templated tomato bushy stunt virus was bound to these particles [60]. This result confirmed selectivity of the SiNPs surface imprinted with TYMV.

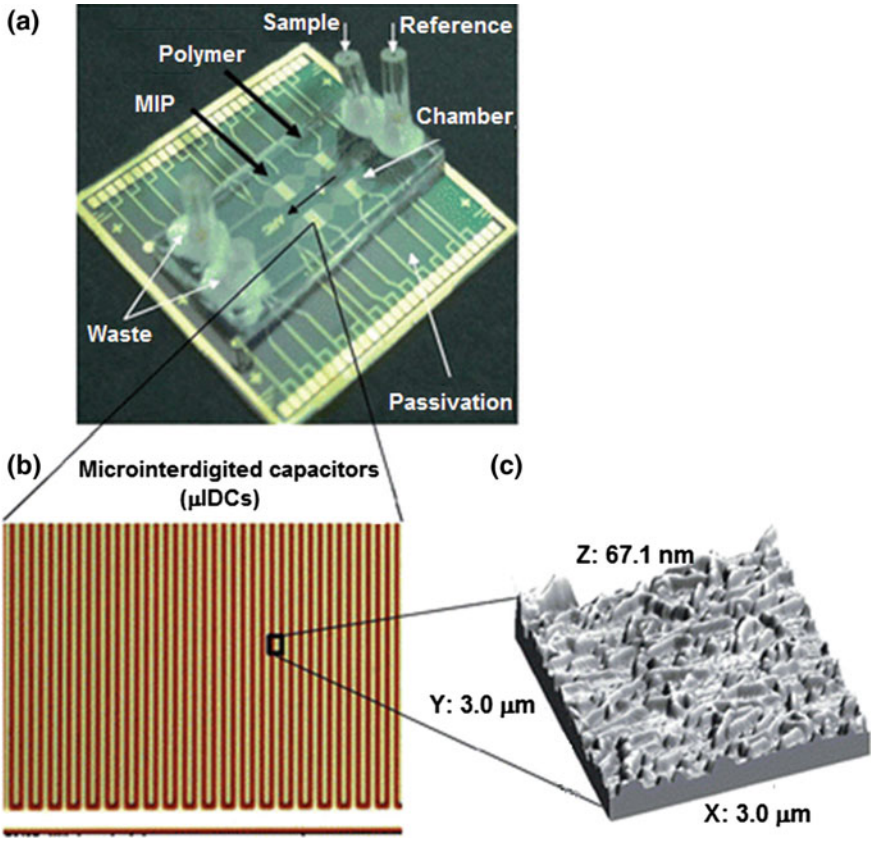
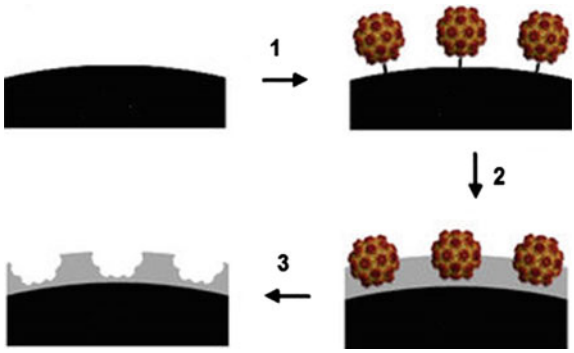


Fig. 2 **a** The picture of the microfluidic biochip composed of a glass support with contact-less dielectric microsenors, **b** the photo of the high-density microinterdigitated capacitor (μIDCs) of 5-μm width Au strips and 1000-μm length separated by 5 μm, **c** the AFM image of the TMV virus stamp used to imprint the poly(methacrylic acid), PMA, and polyvinylpyrrolidone, PVP, copolymer (Adapted from [50].)

Quite similar strategy was adopted to produce monoclonal MIPs in the form of nanoparticles [56]. For synthesis of these MIPs, MS2 bacteriophage was employed as the template. Nanoparticles of these MIPs were prepared using a new automated solid-phase method. That is, the MS2 template was immobilized on the surface of glass beads for imprinting. Then, acrylamide based functional monomers were used to imprint this surface immobilized bacteriophage. A dynamic light scattering (DLS) measurement confirmed the success of the synthesis of nanoparticles with the size of 200–230 nm. These nanoparticles were then immobilized on SPR chips for devising a chemosensor for the MS2 bacteriophage (Table 2). The MIP nanoparticles fabricated that way were able to bind the MS2 bacteriophage reversibly. The mean affinity of the MIP to the MS2 bacteriophage was at the nM level. The limit of detection of the resulting chemosensor was 5×10^6 plaque forming unit mL^{-1}.



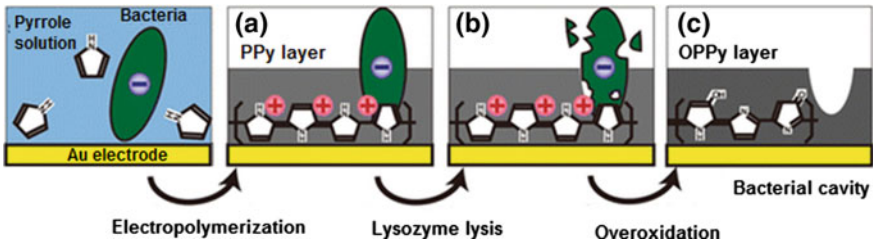
Scheme 4 Consecutive steps of preparation of MIP surface imprinted with turnip yellow mosaic virus (TYMV). *Step 1*, covalent immobilization of the TYMV template on the surface of SiNPs (in black). *Step 2*, addition of silane to build the recognition film (*in grey*). *Step 3*, removal of the immobilized template to empty out the imprinted cavities (Adapted from [60].)

5 Microorganism Imprinting in Conducting Polymers

The first and most important requirement to construct a sensitive chemosensor is to have a close proximity of the recognition unit to the transducer surface for precise measurement of small changes in physico-chemical parameters of this unit. Therefore, conducting conjugated polymers are still more frequently used ones to construct these units [8, 61]. A single-step preparation procedure and high conductivity are desirable properties of these polymers making them suitable for that purpose. Conducting polymer derivatives are biocompatible and, therefore, widely applied for immobilization of enzymes and DNAs for biomolecule chemosensing [62, 63]. Moreover, these derivatives are extensively used in staining cytoplasm of living cells for generating stable fluorescence without cell damaging [64, 65].

Other than acrylic and sol-gel based functional monomers, monomers producing conducting polymers are widely used for imprinting because of the above indicated advantages of these polymers [45, 48, 66]. The number of reports on chemical sensors with conducting MIP films used as recognition units is growing enormously [8–10]. Initial examples described the use of the polypyrrole (PPy) conducting polymer for imprinting of ions [67, 68]. Those studies concluded that ion dopants were incorporated in this conducting polymer to compensate for a cationic charge incurred by the polymer backbone [67, 68]. This feature was then utilized for selective recognition of some inorganic anions [67, 68].

Similarly, efforts were undertaken to imprint microorganisms in conducting polymers [44, 45, 48, 66, 69]. Several reports revealed successful entrapment of bacteria in PPy films without losing their viability [45, 48, 66]. The pK_a values of the *B. Subtilis* bacteria species used as the template for imprinting were 4.8, 6.9, and 9.4, corresponding to the carboxy, phosphate, and hydroxy functional surface groups, respectively. These values confirmed that anionic groups dominate on the



Scheme 5 Consecutive steps of entrapment of the bacteria template in a conducting polymer during polymerization; OPPy stands for overoxidized polypyrrole (Adapted from [48].)

microorganism surface in neutral solutions. Therefore, direct insertion of bacteria in a positively charged conducting polymer was possible (Scheme 5). Further fluorescence microscopy imaging proved entrapment of living bacteria in the polymer matrix [66].

Application of conducting polymers for microorganism imprinting solved one of the most important MIP issues, i.e., the problem of incomplete removal of templates from molecularly imprinted cavities. That is, the microorganism templates were electrostatically ejected from the conducting polymer matrix simply by electrochemical overoxidation [48]. Moreover, verification of template removal in many instances was quite easy [8, 10]. Importantly, this overoxidation treatment preserved the cavity shape in the polymer matrix. Toward that, the PM [48] and CV [44] based chemosensors were prepared for determination of the *E. coli* [48] and *Bacillus subtilis* [44] spores, respectively.

Now, deposition of conducting MIP films by electropolymerization is the most common procedure suitable for these films directly growing on the surface of transducers [10]. Therefore, this procedure was further extended to deposit biocompatible chitosan for imprinting of the sulfate-reducing bacteria, SRB, (Fig. 3) [47] (Table 1). Faradic impedance measurements in the presence of the $Fe(CN)_6^{4-/3-}$

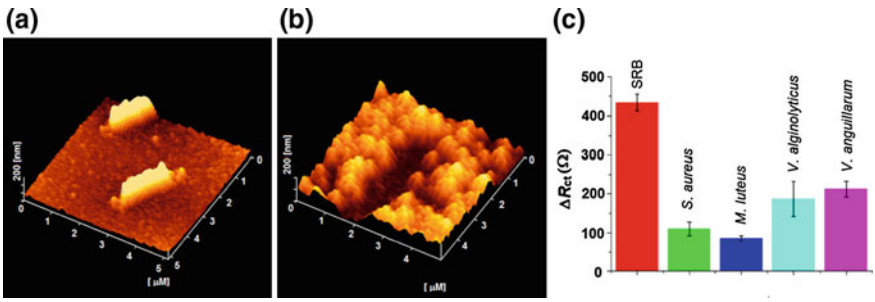


Fig. 3 AFM images of the polymer film imprinted with sulfate-reducing bacteria, SRB **a** before and **b** after the SRB template removal, **c** the change in the charge transfer resistance of the impedimetric chemosensor before and after immobilization of different bacteria species (Adapted from [47].)

redox probe revealed that the charge transfer resistance of the MIP film increased because of binding of SRB in molecular cavities of the MIP. Moreover, the chemosensor was selective with respect to different bacteria species including *S. aureus*, *M. luteus*, *V. alginolyticus*, and *V. anguillarum*.

6 Conclusions

After decades of improvement, several contemporary analytical devices have revealed the capability of on-line detection of the microorganism contamination of foods, waters, and environmental samples. To a large extent, application of synthetic polymers as recognition units in these devices has provided the much needed selectivity with respect to microorganism determination. Selective cavities generated in these polymers can distinguish different species of bacteria and viruses. Additionally, development of different procedures of MIP synthesis has allowed improving the MIP performance. Tables 1 and 2 summarize figures of merit of majority of the procedures developed to-date in this active area of research.

Undoubtedly, microorganisms are entrapped in the imprinted polymer matrices as templates. However, subsequent removal of these templates is still challenging, especially from the polymer bulk. This removal is slow and requires application of rather drastic conditions (Tables 1 and 2). Application of these conditions limits the lifetime of MIPs. In contrast, template removal from a surface imprinted polymer is easier and much faster (Table 1). Therefore, application of surface imprinting for devising chemosensors for determination of microorganisms is increasing. These chemosensors appeared to be more robust than the relevant biosensors and chemosensing less time consuming than the ELISA test.

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