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Abstract

The present review summarizes the molecular imprinting approach to bacteria electrochemical sensing developed over the last five years. Designing artificial molecularly imprinted polymer (MIP) and aptamer receptors for bacteria sensing is more challenging than for analytes of small and even large molecules like proteins. This herein-considered challenge arises from the bacteria's large size and numerous functional groups in bacteria cell walls for which selective complementary sites imprinted in a polymer are puzzling to generate. Moreover, the morphological characterization and recognition mechanism of the MIPs is discussed here in detail.

Keywords

Conducting polymer; Molecularly imprinted polymer; Non-conducting polymer; Pathogenic bacteria imprinting

Abbreviations

AFM, atomic force microscopy; AuNP, gold nanoparticle; ELISA, enzyme-linked immunological assay; MIP, molecularly imprinted polymer; PF-QNM, peak force quantitative nanomechanics; PDMS, polydimethylsiloxane; PCR, polymerase chain reaction; POC, point-of-care (device); SELEX, systematic evolution of exponential enrichment; SEM, scanning electron microscopy

Introduction

Bacteria are live pathogens capable of transmitting and spreading infections among humans and animals. Infectious diseases are unlike conventional diseases. They spread fast through the air, water, and direct contact with infected individuals. Therefore, their determination must be fast to stop the spreading of disease. Namely, *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, *Bacillus cereus*, and *Pseudomonas aeruginosa* are bacteria that fall in the high and moderate risk of infection category [1]. For instance, *Pseudomonas aeruginosa* can make a person seriously ill within 24 h after infection if not adequately treated [2].

The diagnostic facility must quickly and accurately identify microbial agents associated with infectious diseases. Early-stage detection of some high-risk bacterial infections would avoid lengthy treatments and possible death risks.

Microbiology laboratories have been developing suitable procedures to meet this requirement. However, there are several issues with the existing laboratory-based procedures. Culture-based methods require sample preparation, plating in suitable media, and waiting for appropriate incubation time to get visible colonies. For incubation, these tests require 48 to 72 h [3]. Therefore, they are time-consuming and laborious [4, 5]. Besides calorimetry, polymerase chain reaction (PCR) and enzyme-linked immunological assay (ELISA) techniques are developed [3]. These techniques provide the desired selectivity in complex sample matrices. Still, they demand trained operators, costly chemicals, and instruments. Moreover, they involve multistep sample preparation [6].

Comparatively, electrochemical sensors are more suitable for field analysis [7]. Now, handheld commercial potentiostats are available, which are much cheaper than corresponding

desktop instruments. Combining the electrochemical transduction of an analytical signal with artificial recognition appears appealing toward the selective determination of different bacterial species [7, 8].

Molecular imprinting is a procedure that generates in a polymer matrix artificial receptors complementary in shape, size, and orientation to the binding sites of the target analyte molecules [9]. Usually, molecularly imprinted polymers (MIPs) are prepared via copolymerization of functional and cross-linker monomers in the presence of the target analyte, first serving as the template. Removal of the template from the MIP results in vacating of molecular cavities capable of recognizing target analyte molecules selectively. The imprinting procedure is now sufficiently developed to incorporate MIPs into lab-on-a-chip [10, 11] and point-of-care technologies [12, 13]. MIPs are synthetic alternatives to biorecognition systems, e.g., enzymes and antibodies [14]. Their potential to overcome the disadvantages of natural recognition systems, including a high cost, low stability, and susceptibility to surrounding changes, like pH or temperature changes, is recognized [9, 14-16].

Designing artificial receptors for bacteria is more challenging than for small-molecules and even large-molecules, e.g., proteins [15, 16]. This challenge arises from the bacteria's large size and the numerous functional groups in bacteria cell walls for which selective complementary imprinted sites are difficult to generate [14]. Functional monomers should strongly interact with the bacteria's walls for successful imprinting. However, the composition and morphology of these walls can vary considerably, e.g., in the case of Gram-positive and Gram-negative bacteria or depending on the environmental conditions for the same bacteria. The present review critically summarizes strategies developed over the last five years for bacteria determination via

molecular imprinting. Moreover, the MIP morphological characterization and bacteria recognition mechanism are discussed in detail.

Bacteria imprinting at interfaces

Bacteria were first successfully imprinted in 1996 [17]. Gram-positive bacteria, *Listeria monocytogenes*, and *S. aureus* bacteria were imprinted over polymer beads by emulsion polymerization. This imprinting method was also explored for the imprinting of small molecules [18]. However, more reports later described the imprinting of bacteria by Pickering emulsion polymerization [19-21]. This polymerization led to MIP beads with well-controlled hierarchical structures. The interfacial imprinting was carried out in particle-stabilized oil-in-water emulsions, where the bacteria were present on the surface of nanoparticles during the polymerization of the monomer phase. The oil phase contained cross-linking monomers, and the initiator was in an emulsion. After mixing the oil phase with an aqueous suspension of the bacteria-monomer complex, a stable emulsion was produced by shaking the mixture vigorously. After polymerization, the (bacteria template)-modified nanoparticles were removed from the new spherical particles to leave tiny indentations decorated with molecularly imprinted cavities.

The pre-polymerization complexes were prepared with a monomer, including cationic chitosan [19, 21], that interacted with the target bacteria surface groups during this imprinting. A well-controlled hierarchical structure containing large pores decorated with easily accessible molecular binding sites was synthesized. The binding experiments involving different bacteria strains provided strong evidence that bacteria recognition with the beads depended on the nature of the monomer used to prepare a pre-polymerization complex and the target bacteria. However, the beads' size exceeded 100 μm.

To solve the size issue, *Escherichia coli* O157:H7 was imprinted over 5-µm diameter spheres in an aqueous medium by the layer-by-layer approach (Figure 1a) [22]. Plastic beads coated with Au film served as the core. On top of this Au film, an aminothiophenol monolayer was assembled. Then, through electrostatic interactions, Nafion was coated over the assembled monolayer. Nafion-modified surface played an essential role in grafting imprinting shell-over-core particles. The MIP shell was prepared by chemical oxidation of pyrrole in an aqueous medium containing *E. coli* O157:H7. The presence of bacteria in spherical particles was clearly evidenced by fluorescence imaging (Figure 1b).

An interesting report describes MIP film preparation via sol-gel transition-derived methods using functionalized organosilane as the monomer and lipopolysaccharide from *Pseudomonas Aeruginosa* as the template over kaolin particles. This material was prepared for wastewater purification, particularly focusing on removing Gram-negative and Gram-positive bacteria [23].

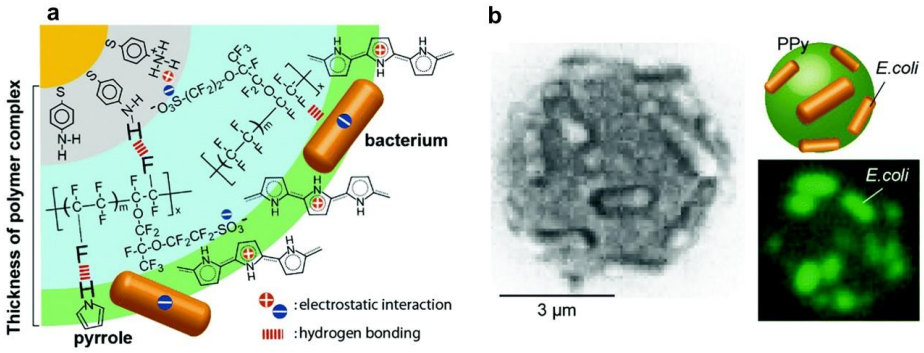


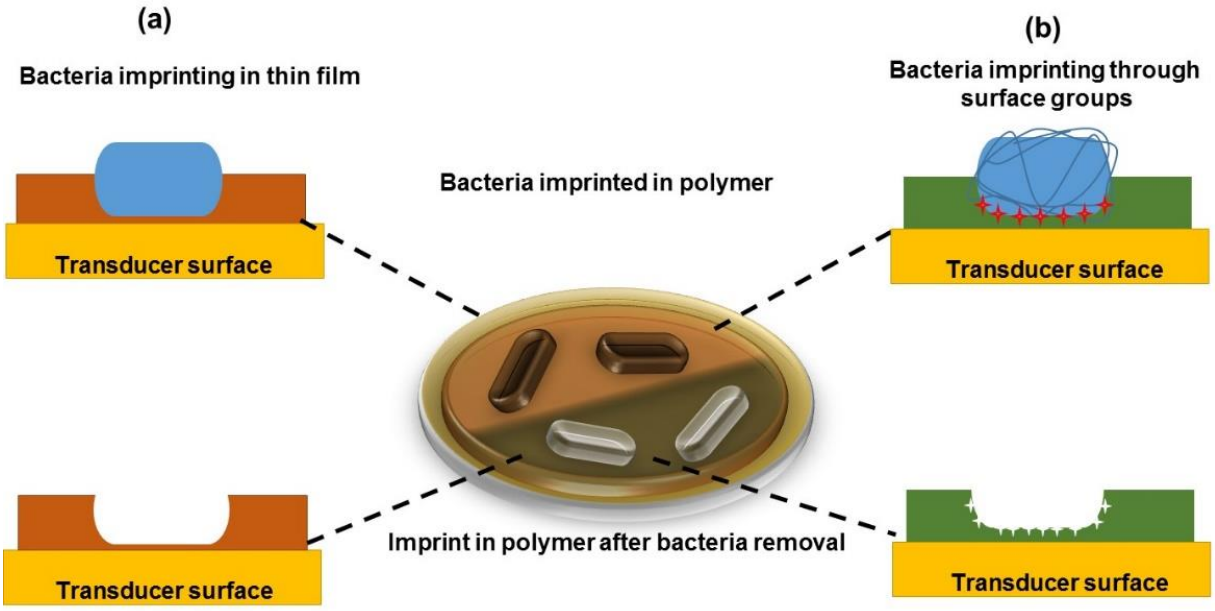
Figure 1. (a) Schematic illustration of the bacteria imprinting on the surface of the polypyrrole shell deposited on the Nafion/Au film-coated plastic microparticle core. (b) SEM and fluorescence images of the microsphere coated with the *E. coli*-doped polypyrrole. Adapted with permission from [22].

Bacteria imprinting in thin films

Bacteria imprinted in nanoparticles are not used in electrochemical sensor fabrication. Instead, imprinting in a thin polymer film is a much-explored approach. In this approach, the cavities imprinted for bacteria are generated on the surface of the polymer film deposited/coated over the transducer surface (Scheme 1a) [5, 24, 25]. Through this approach, imprints, complementary in shape and size to whole bacteria, were generated. Because of the large bacteria size, their immobilization before polymer deposition and careful tuning of polymer thickness is not mandatory (Scheme 1a). These steps are pivotal for small molecules and protein imprinting. This imprinting results in partial molecule encapsulation [24]. That makes bacteria surface imprinting different from the imprinting of small molecules and proteins.

Surface groups of bacteria play a crucial role in preparing selective cavities in MIP artificial receptors (Scheme 1b). Bacteria imprinting in a conducting polymer involves mostly one monomer for preparing selective cavities. For instance, a monomer containing the OH [26, 27], COOH [28], or boronic acid [24, 29, 30] moiety was used to introduce binding sites in cavities. The boronic acid group covalent binding of Gram-positive bacteria was exploited to differentiate it from Gram-negative bacteria because of the thick peptidoglycan cell wall. At the same time, polypyrrole (which contains N-H interaction sites) appeared efficient for providing desired selectivity [31, 32]. Besides, the imprinted polymer selectivity and binding performance increased if more than one functional monomer was used [33]. In one such report, an imprinted polymer for *E. coli* OP50 with 2.2(±0.4) µm thickness was prepared. The bacteria capture efficiency highly depended on the choice of the functional monomer. When the methacrylic acid was used as a functional monomer, the efficiency of the imprinted polymer film-coated wire was

the lowest (~40%). Adding acrylamide, methyl methacrylate, and *N*-vinylpyrrolidone as functional monomers significantly increased the bacteria capture efficiency to 76(±10)%, resulting in MIP formation. Further application of this polymer-modified wire has been predicted in the direct electrical detection of bacteria.The involvement of two or more functional monomers is commonly reported when bacteria are imprinted in a photo or thermally polymerizable monomers.



Scheme 1. Illustration of approaches used to prepare a bacteria-imprinted polymer film. (a) The bacteria-imprinted polymer film deposited by electropolymerization. (b) The bacteria-imprinted film prepared after engaging the surface groups of bacteria.

Drop coating [34], stamping [35-38], and electro-initiated deposition [29, 39-41] are the most common strategies to coat transducer surfaces with MIPs. Chemical immobilization through electrodeposition produced a much more stable film than those immobilized physically [42]. The recognition unit stability is an essential issue for POC device fabrication. Sometimes, a multiple-use system is required.

Bacteria presence and removal from imprints

The presence or templating of bacteria in the polymer is much easier to confirm than small molecules and proteins. Morphological characterization techniques, including scanning electron microscopy (SEM) [17, 21, 43, 44] and atomic force microscopy (AFM), are used to confirm bacteria presence in the polymer [35, 36]. The SEM micrometer-range resolution confirms the presence and absence of bacteria in the polymer.

Similarly, AFM, with its atomic scale resolution, allows the investigation of micro-sized patterning in the polymer. More advanced AFM techniques, e.g., peak force quantitative nanomechanics (PF-QNM), are now used to map adhesion properties [35, 36], as imprinting proposes that functional monomers interact with complementary groups present on the target species. After template removal, cavities containing recognizing sites complementary to the template functionalities in surface chemistry and shape are formed. That results in selective recognition. The PF-QNM was used to compare two imprinting techniques: stamping and Pickering emulsions polymerization. Both imprintings resulted in rod-shaped cavities on the imprinted polymer surfaces. However, the produced imprints distinctly differed from each other. Stamping imprinting led to cavities comprising smooth surfaces, reflecting the geometry of dried *E. coli* cells on the stamp. The cavities, produced via polymerization of *E. coli* stabilized o/w emulsions (Pickering emulsions), had rougher and globular structures because the adhesion properties inside the imprints differed from those outside [35].

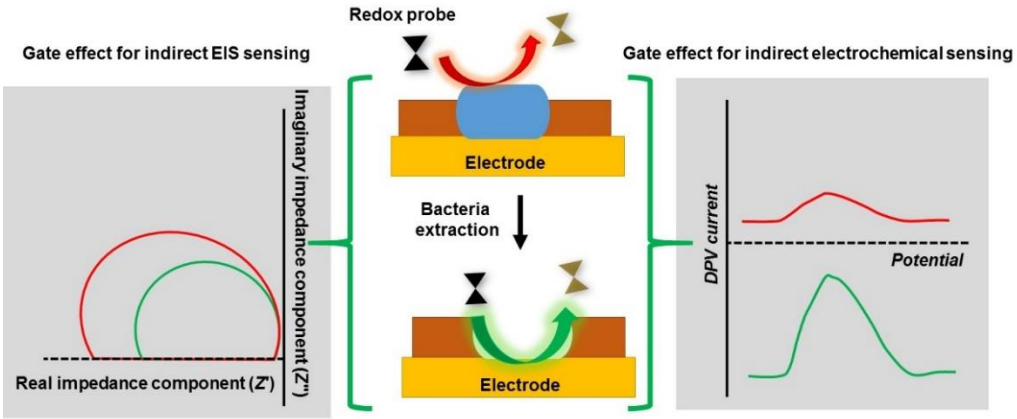
The presence and absence of bacteria templates in imprints were easily distinguished. However, the bacteria template removal from these imprints is not comparatively straightforward. Enzymes or suitable solution mixtures facilitated the digestion of the bacteria

templates through the exposed cell wall surface. Several reports adopted a multistep protocol for bacteria templates' removal from their imprints. Enzyme treatment, washing with surfactants, and the overoxidation of the MIP film were performed consecutively [24-26] or alone [28] to remove the bacteria. Moreover, multiple washing with ethanol-hydrochloric acid (3 M) (5:1, *v*/*v*) solution was reported [45]. This aggressive solution mixture was necessary because bacteria were imprinted in the bulk polymer. Interestingly, when bacteria were imprinted with (phenylboronic acid)-based monomer, a short-time washing with 20 mM fructose sufficed for their removal [29]. Removing bacteria from the PDMS stamps was easy; short sonication in water [46-48] or surfactant washing [49] successfully removed bacterial cells.

Bacteria electrochemical sensing

A redox probe is often added to the test solution for indirect electrochemical determination of the analyte with an MIP-based chemosensor. Initially, it was speculated that the target analyte molecules binding in molecular cavities caused MIP film swelling. This swelling would change the film permeability to redox probe molecules or ions, thus significantly increasing faradaic currents corresponding to oxidation or reduction of the redox probe. Therefore, this was called the "gate effect" [50]. This effect is much exploited in fabricating bacteria chemosensors [25, 28, 29, 42, 51]. However, a different sensing mechanism is proposed in this case. Bacteria accumulation in the MIP film pores blocks the redox probe molecules or ions flow through these films to the electrode surface (Scheme 3) [50]. That results in a decrease in redox probe faradaic current. In the bacteria's absence, unhindered redox probe diffusion results in a pronounced faradaic current. The decrease of current in the presence of bacteria in the polymer was successfully exploited in fabricating different chemosensors.

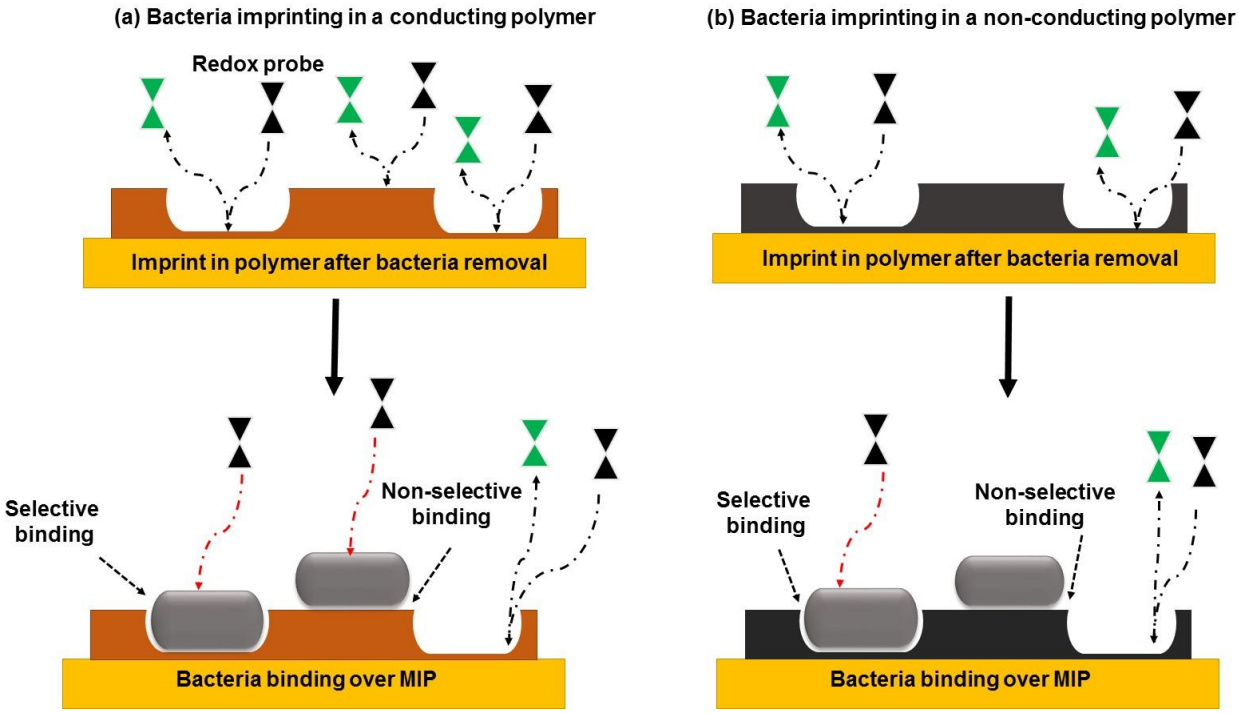
The imprinted polymer prepared and deposited by electropolymerization has the edge over polymers prepared and deposited differently for sensor fabrication for bacteria. Both conducting (pyrrole, thiophene, aniline derivative) [25, 28, 29, 40] and non-conducting (phenol) [26] polymers are involved in these preparations (Scheme 4). Non-conducting imprinted polymer matrices were prepared using products of sol-gel transition [5, 42] and polydimethylsiloxane (PDMS) [46]. Recently, examples of imprinting in a sol-gel-transition prepared matrix were summarized in a review article [42]. Even though the sol-gel transition generates a non-conducting matrix, applying this matrix in the fabrication of electrochemical sensors is growing [42].



Scheme 2. (Center) The "gate effect" mechanism showing physical pore blocking by bacteria that hinders redox probe diffusion through the MIP film to the electrode surface. (Left) The EIS and (right) DPV signal in (red curve) the presence and (green curve) absence of bacteria physical blocking of redox probe oxidation at an MIP film-coated electrode.

It is hard to infer whether a functional monomer, which produces conducting or non-conducting polymer, should be preferred for bacteria imprinting because comparative studies are unavailable. Considering sensitivity, conducting polymer-based MIP would be preferable as it can transduce every interaction of bacteria with cavities in the polymer (Scheme 3a). However,

at the same time, few such interactions can be non-selective (outside of imprints), resulting in a false analytical signal (Scheme 3a). In contrast, a non-conducting polymer-based MIP can be more selective because a redox probe can exchange charge with the electrode only owing to its diffusion through pores generated in the MIP after bacteria removal. This diffusion will only be blocked if bacteria are bound to its cavities (Scheme 3b). A report described highly selective *E. coli* imprinting in a non-conducting sol-gel matrix prepared by tetraethoxysilane condensation with (3-mercaptopropyl)trimethoxysilane [5]. However, not enough data were available to calculate the selectivity factor. Nevertheless, the conclusion follows from the response shown.



Scheme 3. Electrochemical signals originating from bacteria's physical blocking of the redox probes' charge exchange with the (a) conducting and (b) non-conducting MIP film-coated electrode.

The selectivity of the *Staphylococcus epidermidis* imprinted conducting polyaniline to *Staphylococcus epidermidis* was only two times higher than to *Deinococcus proteolyticus*, *E. coli*,

and *Streptococcus pneumonia* [29]. The involvement of aptamer is reported for fabricating selective MIP-containing sensing systems for *Staphylococcus aureus* [27, 52, 53]. Usually, aptamers are identified by cell-based systematic evolution of ligands by exponential enrichment (SELEX) [54]. The MIP film was deposited on gold nanoparticles at the iron(II, III) oxide particles (AuNPs@Fe_3O_4) coated electrode in the presence of aptamer-conjugated *S. aureus* by electropolymerization of the *o*-phenylenediamine monomer [52]. A similar approach used polydopamine to imprint *S. aureus* [53]. Because of the selective aptamer recognition unit, the chemosensor determined *S. aureus* even in complex milk samples at as low a concentration as 10 CFU mL^{-1} [27]. Moreover, it was possible to selectively measure the *S. aureus* concentration in a 10-fold diluted sample without pretreatment. This high sensitivity reaching was possible because of the synergy of aptamer and electroactive 6-(ferrocenyl)-hexanethiol co-functionalized gold nanoparticles as the signal probe.

Conclusions

The last five years' literature reveals that artificial receptors prepared with electropolymerizable monomers appeared to be the preferable choice to prepare "artificial receptors" for sensing bacteria. With this approach, in one step, encapsulation of bacteria in conducting or non-conducting polymer film is possible. These systems' sensitivity reaches 2 to 100 CFU mL^{-1} [25, 27-29]. However, multistep extraction is necessary to remove partially encapsulated bacteria without leaving any debris in imprinted cavities. For future applications, a mild procedure for bacteria removal must be developed. Aggressive extraction solutions can damage microfluidic or POC devices. Bacteria removal from MIPs in the stamping technique is easy and fast. However,

the cavities are formed by contacting substrate-immobilized dry cells, which do not necessarily have the same shape as water-swelled bacteria.

Although hundreds of reports described successful imprinting, prepared MIPs primarily determine non-pathogenic strains. There are few attempts to imprint pathogenic strain for fabricating sensors for their determination [27, 28, 55, 56]. Moreover, all reported bacteria determinations are indirect, involving an electrochemical response of a redox probe. Taking advantage of the electroactive nature of bacteria for their determination would be a significant step forward. The application of bacteria-imprinted microparticles in chemosensing requires further exploration.

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* of special interest

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