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Review

Biosensors of bacterial cells

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ABSTRACT

Biosensors are devices which utilize both an electrical component (transducer) and a biological component to study an environment. They are typically used to examine biological structures, organisms and processes. The field of biosensors has now become so large and varied that the technology can often seem impenetrable. Yet the principles which underlie the technology are uncomplicated, even if the details of the mechanisms are elusive. In this review we confine our analysis to relatively current advancements in biosensors for the detection of whole bacterial cells. This includes biosensors which rely on an added labeled component and biosensors which do not have a labeled component and instead detect the binding event or bound structure on the transducer. Methods to concentrate the bacteria prior to biosensor analysis are also described. The variety of biosensor types and their actual and potential uses are described.

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Contents

1. Introduction

The detection and identification of bacterial pathogens remain a high priority goal. There are many applications, with detection of pathogens in clinical samples a task requiring especially rapid and accurate determinations. Rapid detection often means the difference between life and death for the patient. Detection of pathogens from environmental samples, such as water, food, and in air samples are also necessary to maintain public health. A rapid answer is always preferred because it allows faster decision-making on treatment or safety of the environment, while specificity is needed to inform appropriate clinical treatment or to accurately assess the danger to the public. The identification and quantification of bacteria has traditionally been performed using biochemical assays and growth on selective and differential agar media. These methodologies have worked well, however their procedures are time-consuming, often two to three days, and require skilled personnel to run the appropriate tests and interpret results. Current technology is at a point where biosensors can be used to detect pathogenic bacteria in a rapid and cost-effective manner. Yet technology alone will not solve problems if the cost is prohibitive.

A biosensor is a scientific instrument that incorporates a biomolecule in order to detect and possibly quantify an analyte. It will also have a transducer component, which is necessary to convert the biological event into a measureable signal. The signal is usually electrical in nature. The biomolecule is often a nucleic acid, an antibody or an enzyme, but the list of possible biomolecules is increasing as the number of sensing methods has increased. Lectins, cell surface receptors, and complex polysaccharides can be used to bind to biological targets. The field is now so large that specialized journals are devoted to the topic. A microbiology researcher will probably understand the biological component perfectly well, yet may have trouble understanding the transducer component. Throughout this review particular attention is given to the transducer technology.

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After isolation of bacteria, identification is now routinely performed using 16S rRNA sequencing. There are many biosensor methods that incorporate the rRNA sequence, and it would be appropriate to include nucleic acid-based biosensors in this review. However, except for a brief comparison of these valuable methods this review will be limited to biosensors of whole bacterial cells. Identification of whole bacterial cells is a good alternative to nucleic acid methods since they avoid the step of nucleic acid isolation and purification. There is also the issue of live versus dead bacteria in any sample. 16S rRNA collection does not discriminate between live and dead cells; plating on agar media will identify only living bacteria while missing the viable-but-not-culturable (VBNC) cells. VBNC cells are still important because they have the potential to recover and multiply. Relatively little is known about the VBNC state, although conditions for regrowth have been described (Coutard et al., 2007) and a resuscitation factor has been described (Ayrapetyan et al., 2014). Therefore they should be considered as potentially pathogenic (Besnard et al., 2002). A DNA hybridization method would identify the bacteria but not distinguish live and dead cells, while an RNA hybridization might suffice if the RNA species were carefully chosen. In dead cells the RNA degrades quickly.

Biosensors of whole bacterial cells may also detect dead cells, but typically depend on the structural integrity of cell surface biomolecules which may also degrade quickly after the cell dies. Ramamurthy et al. (2014) describe some of the targets which can be used to detect the VBNC cells. Even with the limitation to biosensors of whole bacterial cells this review should not be considered a comprehensive work. The field is vast and new ideas occur constantly. From the examples shown here much can be discovered about the latest methods of detecting biomolecules in general, and may stimulate additional innovative ideas.

2. Concentration and sequestration

A critical facet of cell detection is the isolation of the cells from the bulk phase. The bacteria of interest are a tiny fraction of the volume and are probably only a small fraction of the total bacterial population present in the sample. The chances of the bacterial cells contacting the transducer of the biosensor are thus very small if only a small volume is examined. Effectively, the same number of target cells must be present in a smaller volume to allow contact with the biosensor. This is also a major problem with a real-time, on-line biosensor, since it is unlikely that the sample could ever be concentrated in a high-volume flowing environment.

Reducing the volume might be accomplished through centrifugation or filtration of a sample. Resuspension of the collected particles in a smaller volume results in a higher concentration. However, there are alternatives that allow efficient collection of bacteria without the need for physical handling of the bulk fluid. The use of magnetic nanobeads has proven to be a popular and resourceful way to capture and concentrate the target bacterial cells. The recent review by Bohara and Pawar (2015) gives an extensive overview of the uses of magnetic nanoparticles. Conceptually, the simplest application is when magnetic particles are modified with an antibody and are mixed into a sample that may contain the bacteria of interest. The antibodies attach to the bacteria and the nanoparticles are then removed from the bulk phase using a magnet. A wash buffer is typically applied to remove any loosely bound material. This is the basis of immunoseparation (Wang et al., 2016).

The antibody sandwich assay, described in detail below, can take advantage of immunoseparation if a second antibody is used that has a detectable label. A good example is the use of magnetic nanoparticles to facilitate the discovery of *E. coli* O104:H4, an enterohemorrhagic strain (Luciani et al., 2016). It is often the case that enterohemorrhagic bacterial strains are found in milk which has not been effectively pasteurized or in ground beef which has not been sufficiently cooked. In this application the target was actually the lipopolysaccharide of the cell, defined by the O104 antigen. Here two monoclonal antibodies were used: one was attached to magnetic nanobeads and used to capture the antigen in a milk sample. This demonstrates that the antibodies need not be attached to a fixed surface and may be suspended in a liquid sample where exposure to the bacteria might be increased. After concentration the bacteria were enumerated using a simple selective media and colony counting. They did not take the idea to the next logical step and create a rapid assay.

The core of the magnetic nanoparticle must be a ferromagnetic element, such as iron, nickel, or cobalt. Iron compounds are very commonly used for this purpose. Colloidal suspensions are typically produced, with particle diameters in the range of 5–200 nm. A surface coating is recommended since iron will oxidize quickly. Ideally, the surface coating should protect the iron core from oxidation while increasing the particle dispersion in water. This last point is critical since clumping of particles will be counterproductive. If possible, the surface coating might be used to attach the next particle modification. For example, a coating that also allows easy attachment of antibodies is very useful. Some coatings are deposited as thin films such as gold or graphite, while other coatings are common polymers like polyethylene glycol (PEG), polyethylene imine (PEI) and polyvinyl alcohol (PVA). There are many surface coating applications which have been described, too many to adequately address here.

A common mechanism of detection is the attachment of the target bacteria using a biomolecule, after which they are concentrated near or at the transducer. In theory, any molecule that bacteria of interest are known to bind to can potentially be used as the biomolecule in a biosensor. Specificity of the interaction is obviously important as is the affinity of the target for the probe biomolecule. One of the more interesting applications involves the attachment of the antibiotic vancomycin to magnetic nanoparticles (Chung et al., 2011). Vancomycin binds to the small peptide in the peptidoglycan cell wall of actively growing cells. When vancomycin is attached to the nanoparticle it is able to bind to many Gram positive genera of bacteria in an environmental sample. Sequestration by magnet then concentrates the bacteria. The method selects for bacteria which have a certain marker, and if more than one genus or species has this marker they all have an opportunity to be detected.

Any biomolecule which recognizes or attaches to a bacterial cell can potentially be used in a biosensor. This includes specific recognition molecules on eukaryotic cell surfaces, which are often the targets for bacterial attachment prior to infection. Lectins, which bind carbohydrates, should work as well to bind bacterial surface structures. An obvious choice for a biomolecule is an antibody, the use of which will be described below. They combine specificity and sensitivity and so are ideal for identifying small quantities of specific markers. Production of antibodies, either monoclonal or polyclonal, is an advanced technology. Antibodies, usually IgG, are available commercially for many targets while businesses offer services to produce antibodies against targets on demand. There are established techniques to attach antibodies to a variety of surfaces such as plastic and glass (Shriver-Lake et al., 1997). As mentioned above, they can easily be attached to a magnetic nanoparticle and used to isolate the bacteria from a sample.

In practically any biosensor there is a possibility for non-specific binding of organic compounds. This is always an aspect that must be addressed in assembling a new method or investigating a new sample source. The issue of non-specific binding during an antibody-based assay is conceptually easy to understand. Many immune-based assays are based on two antibodies which both recognize an antigen, the so-called sandwich assay. The first antibody must be attached to a surface, whether it is an immobile surface as part of the entire biosensor or a nanoparticle that must be concentrated. The antibody captures the target antigen, such as a bacterial cell or cell component. Then the second antibody, usually bearing a convenient marker (e.g. fluorescent molecule, enzyme) attaches to the target as well, which will produce the signal of the biosensor. This describes a label-based biosensor. If the technique detects the actual event of antibody:antigen binding it

describes a non-label-based biosensor but still has an issue with non-specific binding.

Ideally, the first antibody will cover the surface completely, leaving enough room between antibodies for effective molecular interactions with the antigen. There is no way to guarantee this result but optimization experiments can produce reliable results. There is still unfilled space on the surface to which other materials might bind non-specifically, and therefore a blocking reagent should be used. While it is important that enough blocking reagent is used to coat the surface, too much blocking reagent runs the risk of interfering with the antibody itself. After the antigen binding step is completed the second antibody is added. A far smaller concentration of second antibody is used when compared to the first antibody. In this step it is particularly important that the antibody cannot bind non-specifically to the surface. If the antibody does bind it will bring with it the marker and a false positive signal will be detected ("noise"). Optimization of the individual steps will produce a biosensor that is both accurate and sensitive, with a high signal-to-noise ratio.

Blocking reagents are usually a combination of a common protein, such as non-fat dry milk or bovine serum albumin, and a non-ionic detergent, such as Tween-20 or Triton X-100. If there is any possibility that the assay antibodies will recognize epitopes on the protein blocking reagent then a different formula will obviously be needed. The detergent component disrupts ionic and hydrophobic bonds. Other methods have been developed that may have advantages for some workers (Charles et al., 2009; Liberelle et al., 2014). The individual steps of the assay are followed by washing steps which remove loosely bound material while maintaining the tightly bound antibody:antigen reactions. In these wash solutions the salt concentration is critical to optimization.

One of the biggest problems in detection is getting the bacteria to a stationary sensor. Consider a typical case of an antibody-based biosensor. Unless the antibody and bacterial cell actually meet the cells will not be captured. Lu and Jun (2012) constructed a nanowire that was suitable for exposure to *dielectrophoresis*. The construction of the nanowire was fairly conventional, with a gold-tungsten wire modified with an organic linker molecule. The linker is attached to streptavidin, which tightly binds to biotin which itself has been conjugated to an antibody (Fig. 1). In this way the antibody is secured to the probe tip without the loss of activity that direct attachment to a surface can sometimes occur. The only other problem is how to move the bacteria to the vicinity of the probe tip so that attachment can take place.

A dielectrophoresis field is created using an alternating current, resulting in a non-uniform electric field that places a force on the bacterial cells. This force drives the cells around the nanowire tip, greatly increasing the likelihood of antibody attachment. In this case the attached bacteria were detected after a second antibody conjugated with fluorescein was also attached. These were visualized through microscopy and a charge-coupled device to detect the fluorescence. Other markers could be substituted for the fluorescein, allowing a variety of detection methods such as described in this article.

Dielectrophoresis is often used with biosensors which employ microfluidics. Microfluidics offers a powerful method to analyze small representative samples in a rapid and sensitive manner, and with little energy demand (Bridle et al., 2014). This makes them attractive for field use, either as portable devices or at monitoring stations. The drawback is that very small samples are used to represent the larger volume under study, making the analysis dependent on a statistical model of the likelihood of detection. Any analytes in the small sample must be found because the few are representative of the total sample, and to do that means that each and every analyte must contact the detector. In addition, microfluidic environments use volumes that are so small that fluid movement is significantly different from bulk flow conditions; a simple continuous flow model is not realistic. This complicates the movement of bacteria through the microfluidic environment just when it is important that the bacteria in the fluid have a chance to contact the biosensor. Many methods have been described to achieve predictable particle movement in microfluidics, such as through magnetic forces (Darabi and Guo, 2016) or acoustic energy (Ohlsson et al., 2016).

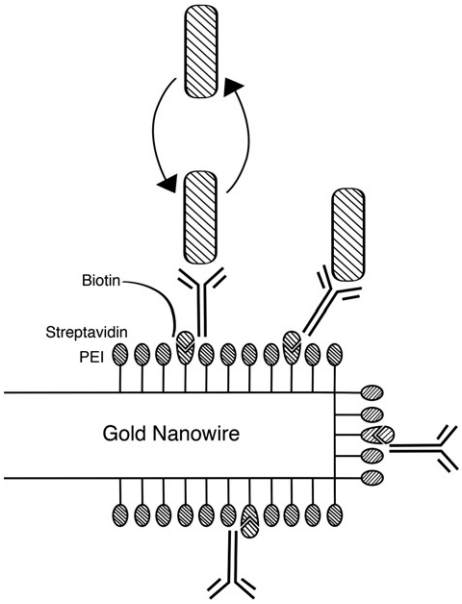

Fig. 1. An antibody is attached to a gold nanowire using streptavidin-biotin binding. A dielectrophoresis field is created using alternating current. This field creates a non-uniform field and causes the bacteria to polarize. The bacterial cell will follow the greatest electric field line which will cause the cell to interact with the antibody. PEI – polyethylene imine. Based on the work of Lu and Jun (2012).

One such example (Zhang and Radadia, 2016) used microfluidics to concentrate nanoparticles conjugated with antibodies that were used to capture bacteria. Their novel idea was to use boron-doped diamond electrodes for dielectrophoresis. Electrodes are often made of metals, such as gold with an overlay of silicon dioxide, because they are resistant to corrosion which can occur under the strenuous conditions of dielectrophoresis. For instance, electrolysis and joule heating are common occurrences. Conditions which will polarize the cells can include voltages as high as 240 kV/m (although note that the path itself is very short) and require extended times (over an hour) for the bacteria to contact the biosensor. A thin (1 μm) film of diamond covered with silicon dioxide is very resistant to degradation and can withstand extreme conditions. This allows detection at an electrode with great capture efficiency and selectivity. This assay actually captures and concentrates the nanobeads which have already captured the bacteria by attached antibodies. This step is followed by detection using impedance (see below). The greatest problem in this method is the risk of concentrating other organic compounds by non-specific binding. The attachment of other materials non-specifically is much the same problem as discussed previously for antibody:antigen interaction. Particles with an affinity for a surface may bind there, even if only loosely with weak bonds. In a sensitive instrument such as the one described here, even a little bound material will confound the assay. A washing step is needed to remove the weakly bound material. This recent report is still only a proof-of-principle investigation and so has been tested only in the laboratory. However, its success should inspire the next stage of development.

3. Labeled biosensors

A variety of antibody-based methods has been described, all of which take advantage of the high specificity of the antibody for an epitope. This is the basis of the Enzyme-Linked ImmunoSorbent Assay (ELISA), in which an antibody is used to detect an immobilized antigen (Aydin, 2015). An enzyme conjugated to the antibody demonstrates the presence of the antigen. This is an excellent laboratory tool but probably

not suitable for identifying specific bacteria in a complex medium like food or natural water supplies.

Many antibody-based biosensors rely on the previously mentioned "sandwich" protocol for detection in which an antibody is attached to a surface, followed by the target cell binding to the antibody. The next step is the application of a second antibody which binds the now-immobilized target. The complex is then able to be detected, often by a marker attached to the second antibody. The sandwich assay can be seen in the two configurations of Fig. 2, both of which use the same enzyme for detection but with different methods. A nucleic acid sandwich assay is shown in Fig. 2A. In this configuration a nucleic acid is used to bind 16S rRNA strands which were isolated from bacteria (Haile and Ryon, 2004; Halford et al., 2013; Leskela et al., 2005). The Halford assay used an oligonucleotide probe that is tethered to a gold electrode. The probe is long enough to hybridize with only a portion of the rRNA target. The rest of the rRNA strand is then able to hybridize with the second nucleic acid probe, this one conjugated to fluorescein. Choosing the hybridized region carefully is required to get a specific result. In a subsequent step an *anti*-fluorescein antibody conjugated to the enzyme horseradish peroxidase (HRP) is added. Effectively, if the right rRNA strand is available it allows capture of the HRP enzyme. This is an amperometric assay, a typical electrochemical biosensor. Enzyme activity is detected as a current that is induced when hydrogen peroxide and the substrate 3, 3′, 5, 5′ tetramethylbenzidine (TMB) are added. Oxidation of the TMB allows the flow of electrons from a gold electrode and thus a current as long as substrate is available. Current can be measured accurately and sensitively, leading to a signal that reveals the presence of the 16S rRNA target. If a microarray were to be modified with addressed nucleic acid probes on its surface, the signal would be predictive of a match at a specific address on a microarray (Hwang et al., 2012; Loy et al., 2002). The use of 16S sequences for the identification of bacterial species is now an established technology (Guschin et al., 1997) and the concept works for many related applications (Cheng et al., 1998). For identification of bacteria in a clinical setting this technology is especially important, because the cultivation method for identification is very slow and may require days for a definitive answer. Gram negative bacilli can be especially difficult. However, the preparation of nucleic acid samples and the use of a microarray procedure will only take a few hours at most. A procedure for detecting Gram negative bacilli has been described that is both comprehensive and methodical (Fujita et al., 2011).

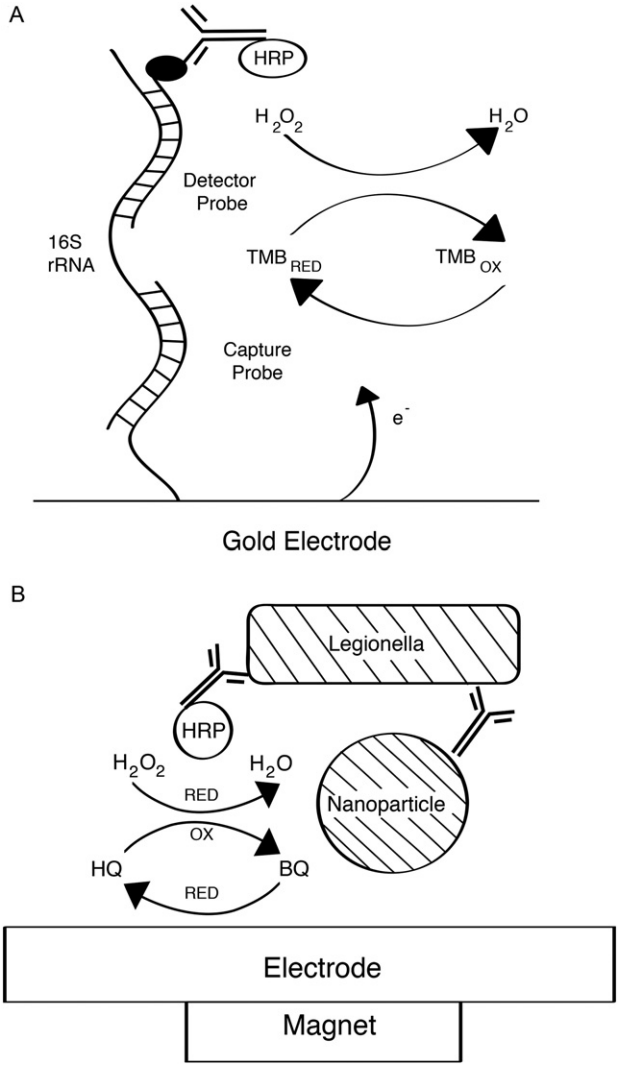

Fig. 2. A. Example of a sandwich assay using nucleic acids. The capture probe is attached to a surface and is able to bind the target nucleic acid (often 16S rRNA). Then a detector probe bearing the enzyme horseradish peroxidase (HRP) binds the target at a different sequence. The target is detected when the peroxidase acts on hydrogen peroxide, oxidizing the 3, 3′, 5, 5′ tetramethylbenzidine (TMB) substrate and creating a detectable current. Figure based on the work of Halford et al. (2013). B. Example of a sandwich assay for whole bacterial cells. The cell is bound to an antibody on a magnetic nanoparticle, which is removed from the bulk fluid using the magnet. A second antibody labeled with horseradish peroxidase is then able to attach to the cell. Addition of hydrogen peroxide causes hydroquinone (HQ) to be oxidized to benzoquinone (BQ), which creates a measurable current.
Figure based on the work of Martin et al. (2015).

The sandwich assay involving antibodies is analogous (Fig. 2B). The number of applications is vast, but a few specific examples should demonstrate the range of uses of the antibody sandwich configuration, even if the examples are confined to those which identify bacteria. A good example is provided by the work of Gholipour et al. (2014), who detected a bacterial protein from the pathogen *Legionella pneumophila*. What makes this technique of greater interest is that a urine sample was examined, in which the protein from a *Legionella* pneumonia was found. A simple boiling of the urine sample allowed the protein to be freed from the bacterial cells. Rat polyclonal antisera was used for the capture phase while rabbit polyclonal antisera was used in the detection phase. This was followed by a commercial *anti*-rabbit antibody conjugated with HRP, along with the hydrogen peroxide and 3, 3′, 5, 5′-tetramethylbenzidine substrates. A similar method was used to detect *Mycobacterium ulcerans* in a point-of-care setting (Dreyer et al., 2015). In this application a cell wall-bound protein was first captured by a mouse monoclonal antibody and then detected in a two-step procedure involving a rabbit monoclonal against the same protein target, followed by a commercial goat *anti*-rabbit antibody conjugated to HRP. In the future this procedure might be simplified by attaching HRP directly to the rabbit antibody. Another point-of-care application was described by Tuteja et al. (2007) when they created a dipstick test for *Vibrio cholerae* in fecal samples. The dipstick was made of a nitrocellulose membrane to which rabbit polyclonal antibodies were attached as capture antibodies. Detection was performed using mouse monoclonal antibodies, followed by a commercial rabbit anti-mouse antibody conjugated to HRP. Not only was this method useful for bedside use, it was also a good tool for water sample contamination.

HRP is a very useful enzyme because of its ability to oxidize substrates. In other iterations of this same idea, a colorimetric assay is created using HRP and an assay buffer composed of hydrogen peroxide, sodium acetate, and 3,3′,5,5′-tetramethylbenzidine (TMB). A positive result will turn blue. An ordinary microplate reader is used here and so this technique does not depend on unusually expensive equipment. This test is very rapid, although there is a potential problem with non-specific reactions due to protein overload in the assay. A variant of this approach also used HRP (Karoonuthaisiri et al., 2009). In this technique an antibody sandwich assay is utilized for the detection of *Salmonella* and *E. coli* O157:H7. The second antibody is conjugated to HRP, but here the signal is chemiluminescent and therefore light production is detected. The target for oxidation is the substrate luminol, and so light is produced and is detected by means of photographic film or

electronically through a charge-coupled device (CCD) camera. The former is quite inexpensive, while the latter requires a significant investment. However, electronic measurement reduces the assay time to four hours, which is suitable for commercial uses.

The technique is inexpensive and appears suitable for field use, but requires substantial optimization before it can be used reliably. It is not particularly sensitive, with a lower limit of detection for *E. coli* cells at 10^5 and for *Salmonella* at 10^6. A preconcentration step would be helpful for most samples. Their tests with contaminated milk samples suggest that the technique can be successfully commercialized. An obstacle remains in the issue of cross-reactivity of antibodies. In this assay, the antibodies displayed low, but measureable, cross-reactivity that must be considered in reporting results.

Liu et al. (2013) used an antibody sandwich assay to detect *Bordetella avium*, which is a pathogen of poultry. They started the assay with a microplate well coated with rabbit polyclonal antibodies raised against the bacteria. This was sufficient to trap the *Bordetella* cells. There was little concern over trapping other bacteria because the rabbits were not exposed to other pathogens and because the secondary antibody was a monoclonal and thus specific for *Bordetella avium* (raised against the strain's outer membrane protein). The monoclonal antibody was conjugated to horseradish peroxidase, allowing a clear color change in the presence of hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine. The assay was conceived as a rapid screen of poultry specimens in a farm setting. It has a sensitivity of 10^4 cells/ml and is highly specific for the pathogen. It shows great promise because the level is appropriate for detection of sick animals, but not so sensitive as to find low-level contaminants.

The work of Parma et al. (2012) is notable because it makes use of the IgY antibodies of chicken eggs. IgY is not a well-known class of antibodies but they are found in birds and reptiles and resemble IgG. Coming from such a different class of animal the antibodies are different enough to avoid cross-reactivity with many host molecules. They have been used successfully when detecting human antigens, but here are used as the capture antibody for enterohemorrhagic *E. coli* strains. The detection antibody was raised in rabbits and was conjugated to HRP. A similar method used antibodies attached to 96 well plates in which contaminants of ground beef were examined (Gehring et al., 2008). Here the detection antibody was conjugated to a fluorescent marker and read by a plate reader. The multiwall plate format is versatile, allowing either many food samples to be examined at once, or fewer samples to be examined for different bacterial contaminants.

Martin et al. (2015) used a magnetic nanoparticle which had been modified with poly-(dopamine). The addition of poly-(dopamine) was used because dopamine offers hydroxyl and amine groups. These groups are useful for interactions with other molecules and thus modifications of the shell of the magnetic nanoparticle. The first antibody recognized *Legionella* and was attached to the poly(dopamine) layer; excess binding sites were blocked using bovine serum albumin (BSA). Magnets were used to sequester and concentrate the nanoparticles. This step was followed by the second antibody of the sandwich, one which had been modified with horseradish peroxidase. The detection step in this assay is somewhat different from the previous example. The electrode here is a screen printed carbon electrode. These are inexpensive and commercially available. A sodium phosphate buffer containing hydroquinone is added; the nanoparticles could be held in place on the electrode by a magnet. Addition of hydrogen peroxide creates a current which is compared to a reference electrode. For Martin et al., their reported sensitivity with this method was 10^4 bacteria/ml even without a preconcentration step, with the possibility of only 10 bacteria/ml after concentration. This is an excellent example of a combination of different techniques to create a highly sensitive biosensor. While their published work described only a laboratory-based analysis of spiked water samples, the apparatus is uncomplicated and might easily be adapted for field use. It consists of the molecular reagents which should be prepared beforehand in the laboratory, a potentiometer to read results, and a power supply. The separation step is magnetic and so a simple magnet is required. The reagents are all stable at room temperature. There is an opportunity to create a valuable field-portable detector of specific bacterial species.

An electrochemical biosensor was created by Jayamohan et al. (2015). Magnetic nanoparticles were conjugated to the first antibody, which were used to bind to and concentrate *E. coli* O157:H7 using a conventional magnet. The concentrated nanoparticle/*E. coli* were then attached to polystyrene beads which had been modified with another *E. coli* specific antibody as well as poly-guanine oligonucleotides (20-mers). Essentially this creates another antibody sandwich assay (Fig. 3) that is similar to the work of Karoonuthaisiri et al. (2009), but the detection step is unique. The polyG oligonucleotide is able to hybridize with a complementary probe on a glassy carbon electrode. In the detection phase of the assay the guanine is oxidized, producing an electrochemical signal that is detected by the electrode. Here the method is *differential pulse voltammetry* (DPV). This method is used because it is a sensitive indicator of redox reactions, and in this case the guanine residues are oxidized. An acetate buffer containing tris (2,2′-bipyridyl)ruthenium (II) chloride hexahydrate is used in this assay. Guanine oxidation will create a current with the underlying modified glassy carbon electrode. The opportunity to attach many polyG signals on the polystyrene bead means that there is a substantial amplification of signal that will be detected by the electrode. In simulated wastewater samples spiked with *E. coli* they found a linear relationship between signal and cell count between 0 and 300 cells per ml. One major limitation here is the efficiency of bacterial capture by the immunomagnetic system, which was estimated at only 22% of the available cells. A pre-filtering step improved the yield to 47%. Another limitation is the cross-reactivity of the antibodies. They estimated that 0.4% of the signal was attributed to antibody cross-reactivity, which must be considered during the analysis of signals.

A related configuration has been described (Spehar-Deleze et al., 2016). In this study the capture antibody is attached to a gold electrode surface. After the target cell binds (here, *Francisella tularensis*), a second antibody is added which is conjugated to silicate nanoparticles containing ruthenium trisbipyridine (Ru(bpy)). The positively charged ruthenium can react with the negatively charged silicate and cause a chemiluminescent signal to be produced. The light is easily detected by a photomultiplier, revealing the presence of the bacteria. This

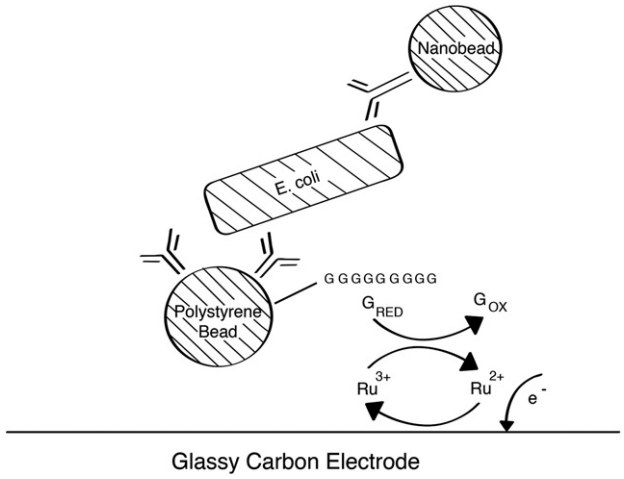

Fig. 3. A variation of the sandwich assay in which the first step is for magnetic nanoparticles conjugated to antibody are used to trap the bacterial target. The second antibody attaches a polystyrene bead which also holds poly-guanine sequences. These sequences will hybridize with poly-cytosine on the transducer. Detection results from the current created when trapped guanine is oxidized in the tris(2,2′-bipyridyl)ruthenium (II) chloride hexahydrate buffer.
Figure based on the work of Jayamohan et al. (2015).

technique has been reduced to a portable scale, making it an attractive possibility for field work.

An *immunochromatography* method has been described (Li et al., 2011) which should be familiar in design to most researchers. This method utilizes a nitrocellulose membrane as a carrier for the sample. Specific antibodies are attached to gold nanoparticles and these are infused in the nitrocellulose membrane (Fig. 4) in specific bands. A sputum sample is loaded at one end of the strip (on the sample pad) and moves through the medium by capillary action. At a specific point in the conjugating pad the sample encounters the gold nanoparticle conjugated to antibody, where antigen:antibody reaction can occur. For example, their work utilized *Pseudomonas aeruginosa* and a rabbit polyclonal antibody fraction. This complex continues to move and contacts a second antibody placed in a thin band across the paper in the nitrocellulose membrane. The second antibody is mouse *anti-Pseudomonas* monoclonal antibody and is immobilized, ensuring that the complex will remain at that location and produce a red color (i.e. a positive reaction). As an internal control, excess nanoparticle/first antibody complexes which did not contact bacteria will proceed through the paper and contact a Donkey anti-rabbit polyclonal mix which effectively traps the excess gold nanoparticles. Here another red line (control) will appear indicating that the test was successfully performed. This is essentially the same technique as used by a rapid pregnancy test, in which a hormone is recognized by an antibody and a color change is produced. In this case the use of whole bacteria as the target is performed. The method is also rapid, taking only a few minutes. Rapid tests are highly valued in the clinical world because the answers affect the course of treatment. Medical centers are familiar with problem pathogens for which treatment options are already established; knowing what the bacterial isolate is may mean that the most effective treatment is prescribed initially and not after empirical treatment has failed. In environmental or public health settings rapid tests are also valued. Detection of pathogens in a water supply allows effective treatment changes, limiting the amount of contaminated water that is distributed. Field researchers who have access to rapid tests may change their experimental procedure to account for new and unforeseen results.

Often these tests only indicate a positive/negative reaction, but the intensity of the red color is proportional to the number of specific bacterial targets in the sample. To determine the bacterial concentration a portable optical scanner is used which will give a numerical reading. The authors demonstrated the efficacy of the technique using *Pseudomonas aeruginosa* and *Staphylococcus aureus* tested together. Both were able to be detected because the configuration of the test strip was circular, allowing the sample to flow into different antibody zones. There should not be a problem in creating a test disc of this type with many more pathogens represented in the antibody field.

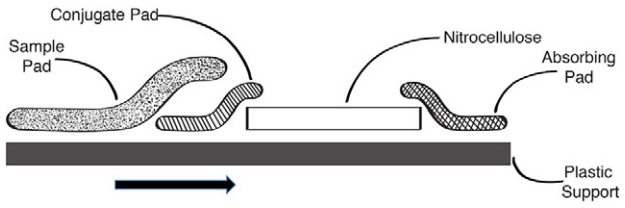

Fig. 4. Side view of a chromatographic biosensor. The well (not shown) in the sample pad receives the unknown sample. As it moves through the conjugate pad the bacteria will interact with antibodies and be borne along as a complex. These antibodies are conjugated to gold nanoparticles, which form the basis for detection. A second, immobilized antibody traps the bacteria at a specific point in the nitrocellulose and becomes visible as a color change. The arrow shows the direction of travel for the components.
Figure based on the work of Li et al. (2011).

4. Unlabeled biosensors

The weakness of the labeled biosensor is that a marker must be attached that identifies the binding of the target. Every additional step in an assay is one more step in which something can go wrong. Sensitivity may vary, and the detection method may have a limit that is too high for the specific task. These issues can often be improved using technological alterations, such as are described here.

There is another category of biosensor, this one defined as having unlabeled biomolecules. In these cases the act of analyte attachment to the biomolecule is sufficient to cause a detectable change in the biosensor. From a purely conceptual basis this is easily understandable; if you could visually detect an antibody you would then be able to notice the difference when that same antibody was attached to a bacterial cell. Obviously a visual detection is not possible, but any of a number of other detection methods might be used to detect the difference between them. The introduction of energy into the system and the change in energy as it encounters the target becomes the objective. In some cases a quantification is possible if the method is able to resolve the number of different events after binding of the targets. Massad-Ivanir et al. (2016) attached antibodies to a porous silicon surface and used them to capture *E. coli* cells from an aqueous mixture of bacterial species. Detection was by reflectivity from the surface, which was detected and quantified by a charged-coupled device (ccd camera).

As would be expected, the unlabeled biosensors make use of antibodies, although there is no reason other binding materials could not be used if they have high specificity for the target bacteria as well. There is versatility in the use of antibodies conjugated to magnetic nanoparticles. They can attach to the target bacteria and then sequester the target cells through the use of a magnetic field, after which they can be analyzed in a surprising variety of ways even without a labeled probe attached. Starting with this procedure, Wang et al. (2015) resuspended the sample in a redox probe solution and applied it to a screen-printed electrode. Held in place by a magnetic field, the sample was interrogated by *impedance*. That is, an applied alternating current with a voltage of 50 mV was measured at various points in the sample. Impedance is analogous to resistance in a direct current circuit, which is found as the ratio of voltage divided by current, giving resistance. Impedance is voltage divided by the current in an alternating current circuit. Impedance is represented as Z and has the units of ohms. Both magnitude and phase of impedance can be affected when a sample is present, and either can be measured as a signal. The impedance measurement by the biosensor will be affected by the presence of bacteria in the sample which bind to the attached antibodies. A negative control must be used since the nanoparticles with the conjugated antibody alone will affect the impedance as well. In this assay, the number of bacteria (*E. coli* O157:H7) were proportional to the impedance.

Impedance is also an excellent method to determine whether a thin surface coating has changed, perhaps by adding a target bacterium (Radke and Alocilja, 2005). This method utilizes a microelectrode which was created using a silicon base overlaid by an insulating layer and then topped with a closely spaced gold electrode array using photolithography. The antibodies are directly bound to the electrode array. In this configuration the sample is introduced and the target bacteria (here *E. coli* O157:H7) will attach to the antibodies. When the impedance is measured the change in impedance is proportional to the number of attached bacteria. Similar procedures have been described (Bekir et al., 2015; Yang and Bashir, 2008).

In another configuration (Basu et al., 2004) the biosensor was configured as a gold nanowire. A base aluminum layer was coated with porous aluminum oxide, through which channels were chemically carved and into which gold was added as a nanowire (approximately 10 nm diameter). After partially or totally dissolving away the aluminum oxide that acts as a form, gold nanowires were effectively created. Antibodies can be indirectly attached to gold surfaces using an organic linker molecule. Three configurations were investigated in this study: a simple

gold plate electrode, gold nanowires embedded in aluminum oxide, and gold nanowires without the aluminum oxide. Results demonstrated that the high surface area of the gold nanowires without aluminum oxide was the best choice, having the greatest sensitivity in terms of Z magnitude and frequency shift.

With this construction, a specific detector of *E. coli* O157:H7 was created. An antibody:antigen assay is essentially the configuration, with the antibody trapping the bacterial cell from a bulk fluid and the antibody:antigen complex detected. Here again, the presence of the complex can be determined by electrochemical impedance spectroscopy. The capacitance of the antibody-bacterial cell layer is determined to detect the difference when bacteria are attached.

The discovery that some molecules will change color after exposure to specific conditions created a novel class of biomolecule for unlabeled biosensor applications. That is, it describes a label-free *chromogenic* biosensor. The molecule diacetylene is notable for its two triple bonds (Fig. 5a). Polydiacetylene (PDA) molecules are polymers of some type of diacetylene molecules, often long chain fatty acids. For example, 10,12- pentacosadiynoic acid-aminobutyric acid (PCDA– ABA) is a commonly used PDA (Fig. 5b). The monomers are often used to create liposomes or lipid vesicles, as well as Langmuir-Blodgett films. The latter are thin films (perhaps with a thickness of three molecules) which can be transferred to a solid surface (Guan et al., 2015).

An unusual characteristic of certain PDA monomers is polymerization after exposure to such diverse stressors as heat, pH or even mechanical stress, yielding a repeating structure of double and triple bonds in the molecules (Fig. 5c). This produces a shift in color from blue to red (Carpick et al., 2000). The blue-to-red transition is usually an irreversible one, but modifications of the constituents have been shown to be reversible (Ahn et al., 2003). One of these modifications is shown in Fig. 5d.

A number of PDA biosensors have been developed for a variety of biomolecules, including antibodies and antigens, toxins, lipopolysaccharides and nucleic acids. By conjugating sialic acid to a PDA (Fig. 5e) a biosensor for influenza virus was created (Charych et al., 1993). The virus preferentially attaches to sialic acid on host cells, and here it attaches and causes the blue/red shift. Kim et al. (2008) took advantage of the trait that the particles also become fluorescent, an easily detected event. These workers created liposomes with diacetylene derivatives and then included phospholipids to enhance the speed of the reaction. The strategy for the use of these liposome vesicles is to tether them to a surface using avidin. The vesicles are modified using biotin, allowing the avidin-biotin reaction to hold the vesicle tightly in place. The biotin molecules are also used to hold antibodies which are conjugated to avidin. When the target bacteria attach to the antibody a stress is placed on the liposome and there is a shift from blue to red, with the accompanying fluorescence. In this case the very event of binding of a target cell to the antibody is sufficient for the production of fluorescence; no subsequent step is needed.

Surface acoustic wave (*SAW*) technology is now a commonly used method with many purposes. It is based on the propagation of an acoustic wave over the surface of an elastic medium. In the simplest format, an interdigital transducer (IDT) converts an electrical signal to an acoustic signal, the wave travels to another interdigital transducer and is there converted back to an electrical signal. Perturbations in the surface will change the nature of the wave in a predictable manner, revealing information about the surface constituents. In this case the transmission coefficient is measured, which describes what happens to a wave as it passes from one medium to another (i.e. it encounters a discontinuity). There will be some transmittance but also reflectance, leading to a change in wave amplitude and power.

SAW has been used extensively for the detection of analytes, most of which describe typical antibody:antigen reactions. The analyte can be supplied as a flow-through cell, as an immersion, or as a discrete sample (the so-called dip-and-dry). This last method may seem limited, but in fact the act of drying will concentrate the analytes and make them more available to the antibody, whereas the other methods preserve the bulk nature of the sample fluid. Adapting the technology for a method for whole cell detection was a natural development. Howe and Harding (2000) describe their use of SAW technology to identify both *E. coli* and *Legionella* in water samples. The thin surface consists of a quartz chip on which a silicon dioxide film is deposited (Fig. 6). The thin film is used to increase sensitivity as well as create insulation for

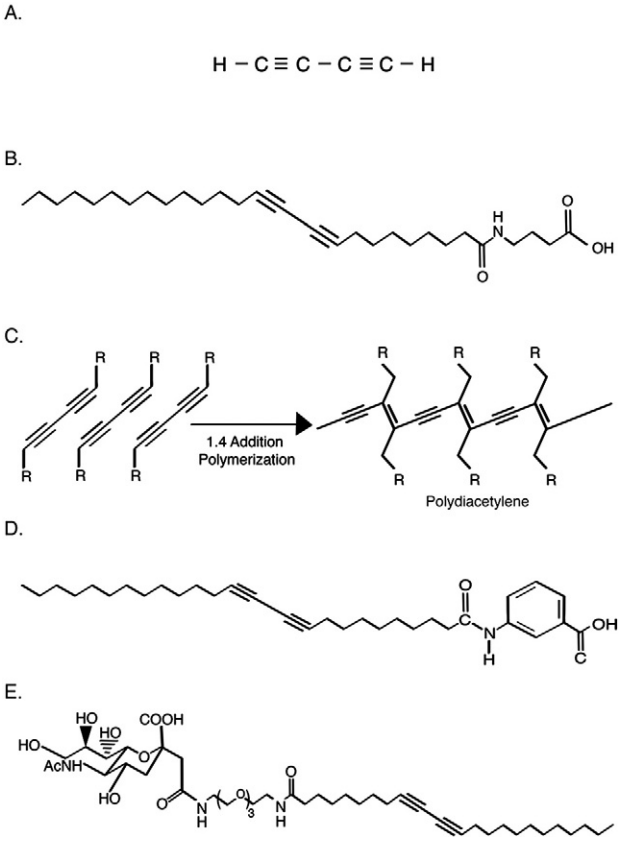

Fig. 5. Chemical structures of molecules important to diacetylene methods. A. Diacetylene. B. PCDA-ABA, a diacetylene derivative that can be used for thin films. C. Polymerization of diacetylene results in molecules with alternating double and triple bonds in a predictable pattern. D. A diacetylene derivative that has been used for reversible color changes. E. A diacetylene derivative with sialic acid attached. This molecule will selectively bind influenza virus.

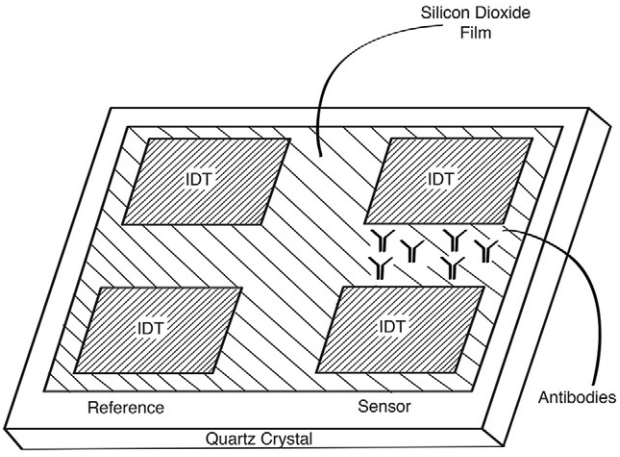

Fig. 6. Schematic of a quartz crystal microchip for the detection of bacteria. Two lanes allow for a sensor and reference analysis. The interdigitated transducers detect the signal of a surface acoustic wave, which is perturbed by the presence of the bacteria binding to the antibodies on the chip.
Figure based on the work of Howe and Harding (2000).

the IDTs. The other novel application in this study was their use of Love waves in the SAW. Love waves are designed to move in a perpendicular motion to the main direction of wave propagation. The amplitude of the motion decreases with depth of the sample, and for this reason are particularly good for measuring slight differences in the attached molecules/cells on the thin surface.

They tried two configurations of the surface under investigation. In one the specific antibody was attached to the thin surface, which facilitated the attachment of the bacterial cells. In the other configuration the bacterial cells were bound to the thin surface first, much like an ELISA, and then the antibodies were bound to the specific targets. Both configurations have advantages and disadvantages. The antibodies can be attached directly to this surface and should be very sensitive if full coverage of the surface is achieved. However, this might become unnecessarily expensive and the antibodies might not be useful for very long. In addition, the SAW would have to have sufficient resolution to identify individual bacteria attached to the antibodies. On the other hand, attaching the bacteria to the surface first means that a field of many bacterial species (the targets of the assay) will be the first field interrogated by the SAW. If the bacterial concentration is too high there might not be enough space to attach all of them, or a representative sampling of them. In this case the SAW must interrogate the surface before and after antibody attachment, and find the difference due to the antibody attachment. Conjugating the antibody with another marker may make this difference easier to detect. In this case the configuration with the cells attached first was found to be superior, with a limit of detection at 5×10^4 cells /ml. However, from a practical standpoint the antibody should be attached and used to detect the presence of the pathogenic bacteria.

SAW detectors can also use Rayleigh waves, which propagate longitudinal waves through the bulk fluid and can reveal some of the details of that structure (Renaudin et al., 2010). The use of shear horizontal (SH) waves is potentially superior to Rayleigh waves because they penetrate the solid surface to an extent and thus give a better three dimensional analysis of the layer. Berkenpas et al. (2006) used SH SAW to detect toxigenic *E. coli* species. To improve the technique they used a small volume chamber with fluid injection and an antibody-coated chamber. In many SAW devices a quartz crystal surface is used because of its piezoelectric qualities. That is, mechanical stress on the crystal will result in an electrical charge. However, quartz allows higher penetration, which is a drawback in a SAW device. Here they employ any of several langasite crystals. Langasites are composed of lanthanum and gallium along with other elements, such as found in langasite ($La_3Ga_5SiO_{14}$) and langatate ($La_3Ga_{5.5}Ta_{0.5}O_{14}$). These crystals allow the SAW to enter at an angle and propagation of the wave at a relatively shallow depth.

Two different methods used the same biomolecule to detect *Staphylococcus*. Balasubramanian et al. (2007) were able to detect *Staphylococcus aureus* using a lytic bacteriophage that is specific for *Staphylococcus*. The phage was immobilized on a detection platform and analyzed using *surface plasmon resonance* (SPR) to detect the attached bacterial cell. This is a good example of a biomolecule other than an antibody that is used in a biosensor. SPR is based on the rapid oscillations of electron density that occur when an incident light strikes a certain surface, such as a plasma or a metal. The result is the propagation of conduction electrons along a defined surface that is very sensitive to perturbations. In this case the bacteriophage 12,600 is attached to a gold surface with a thickness of 50 nm. On the side that is opposite the attached phage, a light emitting diode provides the incident light. The width of the gold surface is great enough to allow both a target and a reference channel to be examined simultaneously. Attachment of a *Staphylococcus* bacterial cell to the phage via the specific surface receptor is a detectable event by the sensitive SPR method.

Although many phages for genera are known, many more specific phages would need to be identified in order to make this technique effective for the detection of a broad array of pathogens. It is also notable that the specificity test was conducted with *Salmonella* rather than *Staphylococcus epidermidis*. The technique could be expected to identify the very common *S. epidermidis* as well as *S. aureus*, which limits the overall specificity of the test.

The other use of the phage 12,600 uses a *quartz crystal microbalance* (QCM) as the detector (Guntupalli et al., 2013). QCM is related to the SAW assays mentioned above in that they both use a piezoelectric crystal as a resonator. Changes in the resonance frequency due to an increase in mass are easily detected. In this case the phage is associated with a spheroid created by a water:chloroform mixture. The tiny spheroids are able to be detected by QCM, as well as the change brought about by attachment of the phage to the bacterial cell. This technique is an interfacial acoustic sensing method which is very practical for thin films, such as created at the spheroid interface. An alternating current causes the quartz crystal to oscillate at a specific resonant frequency. This frequency will change in a predictable manner when the mass on the surface changes.

A promising technology is whispering-gallery mode (WGM) *optical microcavity-based sensors*. The production and use of microcavities is itself a complex topic (Righini and Soria, 2016; Vahala, 2003). Microcavities are extremely small, often in the 10–100 μm diameter range but even smaller versions are possible. Silica microspheres of great precision in manufacture have been created, such that the surface has a smoothness within a few nanometers of diameter and thus become excellent resonators. The unusual name of WGM comes from the observation that certain architectural structures conducted whispered sounds very efficiently as they reflected along nodes in the structure. Light waves will behave similarly, and light which enters a highly reflective chamber will undergo total internal reflectance (resonant recirculation). That is, the light has a very high Q value, which indicates a resistance to damping or weakening of the signal.

WGM resonators can be found in several configurations, as long as they produce the resonant wave characteristic of the method. Spherical resonators are common and relatively easy to make. Circular ("donut-shaped") resonators are also frequently used (Sun and Fan, 2011). Ring shapes and various toroid shapes have been described, although the Q value is usually less. The incident light is carried by a waveguide which interacts with the resonator and becomes trapped in the resonator due to total internal reflection. Sharp peaks of resonance will appear which are dependent on the refractive index of the resonator and the medium in which it resides, and the radius of the resonator. As the light is reflected very efficiently around the interior of the chamber an evanescent field is created on the outside of the chamber. The evanescent field can interact with attached or associated molecules on the surface upon which the evanescent waves propagate. This will change the resonant frequency and the amplitude of response, both of which are measureable. It is the same principle as used for total internal reflectance microscopy, in which bacteria are detected by their scattering of energy from the evanescent waves.

If a substance binds to the surface the thickness of the device will increase, and the wave will adjust to the new thickness. This produces a resonance with a distinct difference from the previous version. Specifically, the wavelength will become longer. If you know the shift in wavelength and the radius of your device you can determine the increase in thickness. How sensitive the device is depends on the size (radius) of the resonator and the Q factor (Sun and Fan, 2011). A high Q value means the oscillations of the light around the resonator continue longer, while a low Q means significant damping and a faster loss of signal energy. For example, Vollmer et al. (2008) demonstrated the detection of single Influenza virus particles using a WGM device. This is a special case in that a specific capture antibody was not used; the virus, which is approximately 50 nm in size, sticks to the silica microsphere. Therefore it was not a test of functionality in real-world systems, although the sensitivity of the technique was demonstrated. This same group was also successful in detection of nucleic acid hybridization on the surface of a WGM biosensor (Vollmer et al., 2003) even to the point of identifying single basepair mismatches in the DNA strands.

WGM is label-free, and so sample preparation should be minimal and the time factor for detection is minimized. Anderson et al. (2015) used this method for the detection of *Helicobacter* species. However, while perfectly acceptable for a presence/absence assay, it is also non-specific. Methods to introduce specificity to the technology, such as using antibodies or other probes, seem like the next reasonable development in the detection of bacterial cells. Introduction of other techniques discussed in this review, such as dielectrophoresis to improve component interaction, could further improve the technique. The velocity of analyte diffusion is a major factor in the efficiency of the technique.

WGM biosensors are extraordinarily sensitive and produce a high signal-to-noise ratio but they also suffer from severe limitations (Foreman et al., 2014). One is that they are so sensitive that the binding of any material will easily be detected, even if it just loosely bound background material. Proper washing is essential and so this technique is more appropriate in a lab-on-a-chip setting in which microfluidics is employed. They are so sensitive that they are susceptible to thermal fluctuations within the resonator or minor fluctuations of the incident laser light. While good methods are described for creating the WGM components, the needed skill is not trivial. Slight variations in construction will produce unacceptable variation, as will the choice of coatings for attachment of biomolecules. These biosensors are not yet ready for field use.

5. Summary

The methods described here are reduced to the fundamental concepts of the technique. They should all be understood as having multiple steps in preparation of the biosensor, quality control and in actual use. To a great extent this explains why research in biosensors is so active; there are innumerable modifications which could be considered and incorporated into current designs, and a good combination of techniques may be particularly efficient. Reference has already been made to the use of magnetic nanoparticles to capture target bacteria, followed by detection using an enzymatic assay and a screen printed carbon electrode. Another example by Charlebois et al. (2010) used a Whispering Gallery Mode biosensor that had been modified by decoupling the microsphere from the device, yet amending the microspheres with a fluorophore. This increases the interactions between the microspheres (which would otherwise be fixed) and the analyte in the surrounding solution. All that is then required is for the microsphere to enter the evanescent field of the device. They were also able to create a true biosensor by coating a conventional microsphere with poly-lysine, giving it a net positive charge that was then able to attract spores of *Bacillus globigii*. Although the conditions were contrived for a proof-of-principle demonstration, the result was unambiguous and suggests where further research will lead. They extended this idea by incorporating quantum dots, a technology that also has been growing in applicability but was not further discussed here. As colloidal particles (<10 nm) they are free to move in suspension while their fluorescent properties give a spectrum of signals for detection.

Miniaturization is a definite trend in biosensors. Biosensors are characterized by employing relatively low volumes for analysis, yet if these volumes are characteristic of the bulk fluid then the biosensors do a credible job. The production of lab-on-a-chip biosensors will require miniaturization of components and reduction of power needs. This is true for remote sensing where power supplies are not readily available and for enclosed spaces where every attempt is needed to conserve space. For example, space exploration will require constant monitoring of the travelers' environment to prevent illness.

The number and uses of biosensors has continued to increase. The field attracts teams of researchers who understand the potential and limitations of both the transducers and the biomolecules. As technological improvements are made in the individual components of a biosensor, the biosensor itself will improve. As the cost declines they will find more extensive markets and gradually become the standard for bacterial detection in many industries, especially in clinical settings where speed is needed. Development of new biosensors may be hastened by demand in niche markets that require very specific detection capabilities and specific environmental conditions.

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References

Ahn, D.J., Chae, E., Lee, G.S., Shim, H., Chang, T., Ahn, K., Kim, J., 2003. Colorimetric reversibility of polydiacetylene supramolecules having enhanced hydrogen-bonding under thermal and pH stimuli. J. Am. Chem. Soc. 125, 8976–8977.

Anderson, M.E., O'Brien, E.C., Grayek, E.N., Hermansen, J.K., Hunt, H.K., 2015. The detection of *Helicobacter hepaticus* using whispering-gallery mode microcavity optical sensors. Biosensors 5, 562–576.

Aydin, S., 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides 72, 4–15.

Ayrapetyan, M., Williams, T.C., Olivera, J.D., 2014. Interspecific quorum sensing mediates the resuscitation of viable but nonculturable Vibrios. Appl. Environ. Microbiol. 80, 2478–2483.

Balasubramanian, S., Sorokulova, I.B., Vodyanoy, V.J., Simonian, A.L., 2007. Lytic phage as a specific and selective probe for detection of *Staphylococcus aureus* – a surface plasmon resonance spectroscopic study. Biosens. Bioelectron. 22, 948–955.

Basu, M., Seggerson, S., Henshaw, J., Jiang, J., del A Cordona, R., Lefave, C., Boyle, P.J., Miller, A., Pugia, M., Basu, S., 2004. Nano-biosensor development for bacterial detection during human kidney infection: use of glycoconjugate-specific antibody-bound gold nanowires arrays (GNWA). Glycoconj. J. 21, 487–496.

Bekir, K., Barhoumi, H., Braiek, M., Chrouda, A., Zine, N., Abid, N., Maaref, A., Bakhrouf, A., Ouada, H.B., Jaffrezic-Renault, N., Mansour, H.B., 2015. Electrochemical impedance immunosensor for rapid detection of stressed pathogenic *Staphylococcus aureus* bacteria. Environ. Sci. Pollut. Res. Int. 22, 15796–15803.

Berkenpas, E., Millard, P., Pereira da Cunha, M., 2006. Detection of *Escherichia coli* O157: H7 with langasite pure shear horizontal surface acoustic wave sensors. Biosens. Bioelectron. 21, 2255–2262.

Besnard, V., Federighi, M., Declerq, E., Jugiau, F., Cappelier, J.M., 2002. Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*. Vet. Res. 33, 359–370.

Bohara, R.A., Pawar, S.H., 2015. Innovative developments in bacterial detection with magnetic nanoparticles. Appl. Biochem. Biotechnol. 176, 1044–1058.

Bridle, H., Miller, B., Desmulliez, M.P., 2014. Application of microfluidics in waterborne pathogen monitoring: a review. Water Res. 55, 256–271.

Carpick, R.W., Mayer, T.M., Sasaki, D.Y., Burns, A.R., 2000. Spectroscopic ellipsometry and fluorescence study of thermochromism in an ultrathin poly(diacetylene) film: reversibility and transition kinetics. Langmuir 16, 4639–4647.

Charlebois, M., Paquet, A., Verret, L.S., Boissinot, K., Boissinot, M., Bergeron, M.G., Allen, C.N., 2010. Toward automatic label-free whispering gallery modes biodetection with a quantum dot-coated microsphere population. Nanoscale Res. Lett. 5, 524–532.

Charles, P.T., Stubbs, V.R., Soto, C.M., Martin, B.D., White, B.J., Taitt, C.R., 2009. Reduction of non-specific protein adsorption using poly(ethylene) glycol (PEG) modified polyacrylate hydrogels in immunoassays for staphylococcal enterotoxin B detection. Sensors 9, 645–655.

Charych, D.H., Nagy, J.O., Spevak, W., Bednarski, M.D., 1993. Direct colorimetric detection of a receptor-ligand interaction by a polymerized bilayer assembly. Science 261, 585–588.

Cheng, J., Waters, L.C., Fortina, P., Hvichia, G., Jacobson, S.C., Ramsey, J.M., Kricka, L.J., Wilding, P., 1998. Degenerate oligonucleotide primed-polymerase chain reaction and capillary electrophoretic analysis of human DNA on microchip-based devices. Anal. Biochem. 257, 101–106.

Chung, H.J., Reiner, T., Budin, G., Min, C., Liong, M., Issadore, D., Weissleder, R., 2011. Ubiquitous detection of gram positive bacteria with bioorthogonal magnetofluorescent nanoparticles. ACS Nano 5, 8834–8841.

Coutard, F., Crassous, P., Droguet, M., Gobin, E., Colwell, R.R., Pommepuy, M., Hervio-Heath, D., 2007. Recovery in culture of viable but nonculturable *Vibrio parahaemolyticus*: regrowth or resuscitation? ISME J. 1, 111–120.

Darabi, J., Guo, C., 2016. Continuous isolation of monocytes using a magnetophoretic-based microfluidic Chip. Biomed. Microdevices 18, 77.

Dreyer, A., Roltgen, K., Dangy, J.P., Ruf, M.T., Scherr, N., Bolz, M., Tobias, N.J., Moes, C., Vettiger, A., Stinear, T.P., Pluschke, G., 2015. Identification of the *Mycobacterium ulcerans* protein MUL_3720 as a promising target for the development of a diagnostic test for Buruli ulcer. PLoS Negl. Trop. Dis. 9 (2), e0003477. http://dx.doi.org/10.1371/journal.pntd.0003477 (2015 Feb 10).

Foreman, M.R., Jin, W., Vollmer, F., 2014. Optimizing detection limits in whispering gallery mode biosensing. Opt. Express 22, 5491–5511.

Fujita, S., Yosizaki, K., Ogushi, T., Uechi, K., Takemori, Y., Senda, Y., 2011. Rapid identification of gram-negative bacteria with and without CTX-M extended-spectrum beta-lactamse from positive blood culture bottles by PCR followed by microchip gel electrophoresis. J. Clin. Microbiol. 49, 1483–1488.

Gehring, A.G., Albin, D.M., Reed, S.A., Tu, S., Brewster, J.D., 2008. An antibody microarray, in multiwall plate format, for multiplex screening of foodborne pathogenic bacteria and biomolecules. Anal. Bioanal. Chem. 391, 497–506.
Gholipour, A., Moosavian, M., Makvandi, M., Galehdari, H., Alvandi, A., Mard, S.A., 2014. Development of an indirect sandwich ELISA for detection of urinary antigen, using *Legionella pneumophila* PAL protein. World J. Microbiol. Biotechnol. 30, 1463–1471.
Guan, W., Zhou, W., Lu, J., Lu, C., 2015. Luminescent films for chemo- and biosensing. Chem. Soc. Rev. 44, 6981–7009.
Guntupalli, R., Sorokulova, I., Olsen, E., Globa, L., Pustovyy, O., Vodyanoy, V., 2013. Biosensor for detection of antibiotic resistant staphylococcus bacteria. J. Vis. Exp. 75, e50474. http://dx.doi.org/10.3791/50474.
Guschin, D.Y., Mobarry, B.K., Proudnikov, D., Stahl, D.A., Rittmann, B.E., Mirzabekov, A.D., 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol. 63, 2397–2402.
Haile, Y., Ryon, J.J., 2004. Colorimetric microtitre plate hybridization assay for the detection of mycobacterium leprae 16S rRNA in clinical specimens. Lepr. Rev. 75, 40–49.
Halford, C., Gau, V., Churchill, B.M., Haake, D.A., 2013. Bacterial detection & identification using electrochemical sensors. J. Vis. Exp. 74, 1–8.
Howe, E., Harding, G., 2000. A comparison of protocols for the optimization of detection of bacteria using a surface acoustic wave (SAW) biosensor. Biosens. Bioelectron. 15, 641–649.
Hwang, B.H., Shin, H.H., Seo, J.H., Cha, H.J., 2012. Specific multiplex analysis of pathogens using a direct 16S rRNA hybridization in microarray system. Anal. Chem. 84, 4873–4879.
Jayamohan, H., Gale, B.K., Minson, B., Lambert, C.J., Gordon, N., Sant, H.J., 2015. Highly sensitive bacteria quantification using immunomagnetic separation and electrochemical detection of guanine-labeled secondary beads. Sensors 15, 12034–12052.
Karoonuthaisiri, N., Charlermroj, R., Uawisetwathana, U., Luxananil, P., Kirtikara, K., Gajanandana, O., 2009. Development of antibody array for simultaneous detection of foodborne pathogens. Biosens. Bioelectron. 24, 1641–1648.
Kim, K., Choi, H., Lee, G.S., Ahn, D.J., Oh, M., 2008. Effect of phospholipid insertion on arrayed polydiacetylene biosensors. Colloids Surf. B. Biointerfaces 66, 213–217.
Leskela, T., Tilsala-Timisjärvi, A., Kusnetsov, J., Neubauer, P., Breitenstein, A., 2005. Sensitive genus-specific detection of Legionella by a 16S rRNA based sandwich hybridization assay. J. Microbiol. Methods 62, 167–179.
Li, C., Vandenberg, K., Prabhulkar, S., Zhu, X., Schneper, L., Methee, K., Rosser, C.J., Almeide, E., 2011. Paper based point-of-care testing disc for multiplex whole cell bacteria analysis. Biosens. Bioelectron. 26, 4342–4348.
Liberelle, B., Fortier, C., De Crescenzo, G., 2014. Enhanced ELISA based on carboxymethylated dextran coatings. Methods Mol. Biol. 1172, 39–47.
Liu, G., Liang, M., Zuo, X., Zhao, X., Guo, F., Yang, S., Zhu, R., 2013. Monoclonal antibodies directed against the outer membrane protein of *Bordetella avium*. Monoclon. Antib. Immunodiagn. Immunother. 32, 295–300.
Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.H., Wagner, M., 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68, 5064–5081.
Lu, L., Jun, S., 2012. Evaluation of a microwave sensor functionalized to detect *Escherichia coli* bacterial cells. Biosens. Bioelectron. 36, 257–261.
Luciani, M., DiFebo, T., Zilli, K., DiGiannatale, E., Armillotta, G., Manna, L., Minelli, F., Tittarelli, M., Caprioli, A., 2016. Rapid detection and isolation of *Escherichia coli* O104:H4 from milk using monoclonal antibody-coated magnetic beads. Front. Microbiol. 7, 1–7.
Martin, M., Salazar, P., Jimenez, C., Lecuona, M., Ramos, M.J., Ode, J., Alcoba, J., Roche, R., Villalonga, R., Campuzano, S., Pingarron, J.M., Gonzalez-Mora, J.L., 2015. Rapid *Legionella pneumophila* determination based on a disposable core-shell Fe_3O_4@poly(dopamine) magnetic nanoparticles immunoplatform. Anal. Chim. Acta 887, 51–58.
Massad-Ivanir, N., Shtenberg, G., Raz, N., Gazenbeek, C., Budding, D., Bos, M.P., Segal, E., 2016. Porous silicon-based biosensors: towards real-time optical detection of target bacteria in the food industry. Sci Rep. 6:38099. http://dx.doi.org/10.1038/srep38099 (2016 Nov 30).
Ohlsson, P., Evander, M., Petersson, K., Mellhammar, L., Lehmusvuori, A., Karhunen, U., Soikkeli, M., Seppä, T., Tuunainen, E., Spangar, A., von Lode, P., Rantakokko-Jalava, K., Otto, G., Scheding, S., Soukka, T., Wittfooth, S., Laurell, T., 2016. Integrated acoustic separation, enrichment, and microchip polymerase chain reaction detection of bacteria from blood for rapid sepsis diagnostics. Anal. Chem. 88, 9403–9411.
Parma, Y.R., Chacana, P.A., Lucchesi, P.M.A., Roge, A., Grenobles Velandia, C.V., Kruger, A., Parma, A.E., Fernandez-Miyakawa, M.E., 2012. Detection of Shiga toxin-producing *Escherichia coli* by sandwich enzyme-linked immunosorbent assay using chicken egg yolk IgY antibodies. Front. Cell. Infect. Microbiol. 2, 1–8.
Radke, S.M., Alocilja, E.C., 2005. A high density microelectrode array biosensor for detection of *E. coli* O157:H7. Biosens. Bioelectron. 20, 1662–1667.
Ramamurthy, T., Ghosh, A., Pazhani, G.P., Shinoda, S., 2014. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Front. Public Health (31 July 2014 |). 10.3389/fpubh.2014.00103.
Renaudin, A., Chabot, V., Grondin, E., Aimez, V., Charette, P.G., 2010. Integrated active mixing and biosensing using surface acoustic waves (SAW) and surface plasmon resonance (SPR) on a common substrate. Lab Chip 10, 111–115.
Righini, G.C., Soria, S., 2016. Biosensing by WGM microspherical resonators. Sensors 16 (6). http://dx.doi.org/10.3390/s16060905 (pii: E905).
Shriver-Lake, L.C., Donner, B., Edelstein, R., Breslin, K., Bhatia, S.K., Ligler, F.S., 1997. Antibody immobilization using heterobifunctional crosslinkers. Biosens. Bioelectron. 12, 1101–1106.
Spehar-Deleze, A., Julich, S., Gransee, R., Tomaso, H., Dulay, S.B., O'Sullivan, C.K., 2016. Electrochemiluminescence (ECL) immunosensor for detection of *Francisella tularensis* on screen-printed gold electrode array. Anal. Bioanal. Chem. 408, 7147–7153.
Sun, Y., Fan, X., 2011. Optical ring resonators for biochemical and chemical sensing. Anal. Bioanal. Chem. 399, 205–211.
Tuteja, U., Kumar, S., Shukla, J., Kingston, J., Batra, H.V., 2007. Simultaneous direct detection of toxigenic and non-toxigenic Vibrio cholera from rectal swabs and environmental samples by sandwich ELISA. J. Med. Microbiol. 56, 1340–1345.
Vahala, K.J., 2003. Optical microcavities. Nature 424, 839–846.
Vollmer, F., Arnold, S., Braun, D., Teraoka, I., Libchaber, A., 2003. Multiplexed DNA quantification by spectroscopic shift of two microsphere cavities. Biophys. J. 85, 1974–1979.
Vollmer, F., Arnold, S., Keng, D., 2008. Single virus detection from the reactive shift of a whispering-gallery mode. Proc. Natl. Acad. Sci. U. S. A. 105, 20701–20704.
Wang, R., Lum, J., Callaway, Z., Lin, J., Bottje, W., Li, Y., 2015. A label-free impedance immunosensor using screen-printed interdigitated electrodes and magnetic nanobeads for the detection of *E. coli* O157:H7. Biosensors 5, 791–803.
Wang, Y., Chen, Q., Gan, C., Yan, B., Han, Y., Lin, J., 2016. A review on magnetophoretic immunoseparation. J. Nanosci. Nanotechnol. 16, 2152–2163.
Yang, L., Bashir, R., 2008. Electrical/electrochemical impedance for rapid detection of foodborne pathogenic bacteria. Biotechnol. Adv. 26, 135–150.
Zhang, W., Radadia, A.D., 2016. Toward a boron-doped ultrananocrystalline diamond elelctrode-based dielectrophoretic preconcentrator. Anal. Chem. 88, 2605–2613.