Cellular Analysis and Detection using Surface Plasmon Resonance (SPR) Techniques.

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Cellular Analysis and Detection Using Surface Plasmon Resonance Techniques

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CONTENTS

Surface plasmon resonance (SPR) has been steadily gaining popularity as a bioanalysis technique over the last 30 years. The growing availability of this technique, along with new technological advances, is making possible new experimental setups and applications beyond traditional molecular binding experiments. SPR provides label-free, real-time analysis of changes in refractive index near (~300 nm) a metal sensing surface. The real-time imaging capabilities of this technique allow observations of dynamic changes at the surface. The refractive index changes that occur when biomolecules attach near the surface allow binding kinetics to be determined. However, these measurement capabilities are also valuable for many cellular studies, such as physiological changes, interactions with surfaces, and sensing. In this Review, we summarize several studies involving analysis and detection of mammalian and bacterial cells that have been run with SPR. We also include the immobilization strategies that have been employed in these investigations. The examples were selected to highlight the emerging cellular analysis and sensing applications of SPR techniques.

SPR has become one of the primary methods for investigating and quantifying biomolecules and their interactions. This technique is routinely used to investigate interactions between biomolecules, such as protein−protein,[1−7] DNA−DNA,[8−11] DNA−protein,[12−16] RNA−DNA,[17,18] carbohydrate−protein,[19−23] and recently lipid−protein.[24] However, with the growing availability of these instruments, SPR is increasingly being used to investigate other biological systems, such as membranes, viruses, and cells. It is also gaining popularity for sensing applications.[25,26] Several excellent reviews of SPR-based

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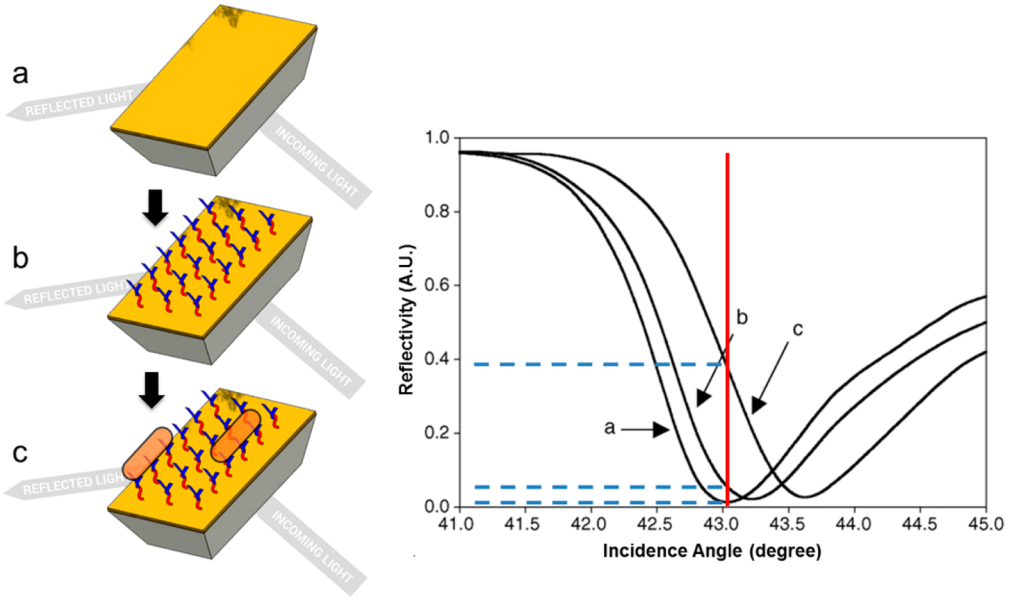

Figure 1. A schematic of a standard surface plasmon resonance (SPR) detection scheme and readout. (a) A resonance angle can be identified for incident light shining on a thin metal surface such that the light is converted to surface plasmons and very little light is reflected back from the surface. (b) When recognition elements, such as antibodies, are attached, the refractive index at the surface changes and the resonance angle shifts, causing an increase in the reflectivity for a fixed incident light angle. (c) The resonance angle shifts again when cells attach. The shift is proportional to the size and surface density of the attaching objects.

measurements and sensor applications are available.[27−30] This Review focuses solely on SPR experiments that involve cells. Far fewer SPR studies have been done so far with cells than other biological systems because of the additional handling requirements and potential instrument fouling issues. Mammalian cells, for example, can change the proteins they express when exposed to flow thus requiring special fluidic cells[31,32] and require CO_2 for growth studies. In contrast, bacterial cells grow under almost any conditions and can contaminate tubing and other components in the instrument, affecting other experiments. However, when the flow cells and chambers containing the samples are optimized and the instruments are carefully maintained, the quantitative information that this method provides, without the use of labels, is very valuable.

SPR instruments optically monitor changes that occur on certain metal sensor surfaces, such as gold, silver, or copper, while sample fluid flows past the surface. In SPR, the incident light is manipulated, by using a high-refractive index glass prism and adjusting the incident angle or wavelength, such that is converted into surface plasmons at the metal surface. The angle at which the light is absorbed is referred to as the resonance angle. When the refractive index of the material near the surface changes (e.g., through attachment of biomolecules), the surface plasmons can no longer form, and the light is instead reflected onto a detector. Surface plasmons are sensitive to changes of the local refractive index within approximately 300 nm of the sensor surface. The amount of surface coverage can be quantified by measuring the intensity of the reflected light or tracking shifts in the resonance angle, and typical detection limits are on the order of 10 pg/mm^2.[28,33,34] A more detailed explanation of the underlying physics of this measurement scheme is available elsewhere.[35,36] A schematic of a standard detection scheme and readout is shown in Figure 1.

This Review provides a brief but comprehensive summary of the use of SPR for cellular analysis and detection. It is separated into two main sections, mammalian cells and bacterial cells, which are further divided by the type of study or technology employed. Surface chemistry is one of the factors that play a critical role in determining the SPR sensor's characteristics, such as specificity, sensitivity, reproducibility, and limit of detection.[37] We therefore include also information about how the sensor surfaces were modified in the various experiments.

MAMMALIAN CELL ANALYSIS

Cellular Response. *Measuring Intracellular Activity.* One application of interest for SPR is the study of mammalian cell response to external chemical stimuli. In 2002, Hide et al. were one of the first groups to report that intracellular activity can be observed using SPR.[38] They used IgE-sensitized RBL-2H3 mast cells to detect dinitrophenol (DNP). A long-lasting SPR signal change, the intensity of which was dependent on the antigen concentration, was detected when the cells were exposed to DNP. Hide et al. hypothesized that the signal may have remained higher for an extensive amount of time, even after the ligand stimulation was removed, because of resulting biochemical reactions triggered by the binding. To test this theory, they first exposed the cells to chemicals known to inhibit cellular response to DNP, prior to exposing them to the antigen. One of the chemicals, genistein, prevented the SPR signal. The SPR signal was partially inhibited by phorbol 12-myristate 13-acetate and wortmannin. This study also tested degranulation, the release of antimicrobial molecules by the cells. β-Hexosaminidase release was measured using SPR and compared against colorimetric analysis. The controls for all of the experiments were cells that were not presensitized by anti-DNP IgE. The cells were cultured on the sensor surface, without surface modification, for one day prior to the experiments. This study established that SPR has the capability to detect biologically significant interactions between cells and reactive molecules. Their results demonstrated detection of not only simple binding kinetics between molecules and surface receptors on the cells but also reflected intracellular activity, such as the movement of Ca^{2+} ions. Figure 2 shows a

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typical SPR setup for measuring the physiological response of cells.

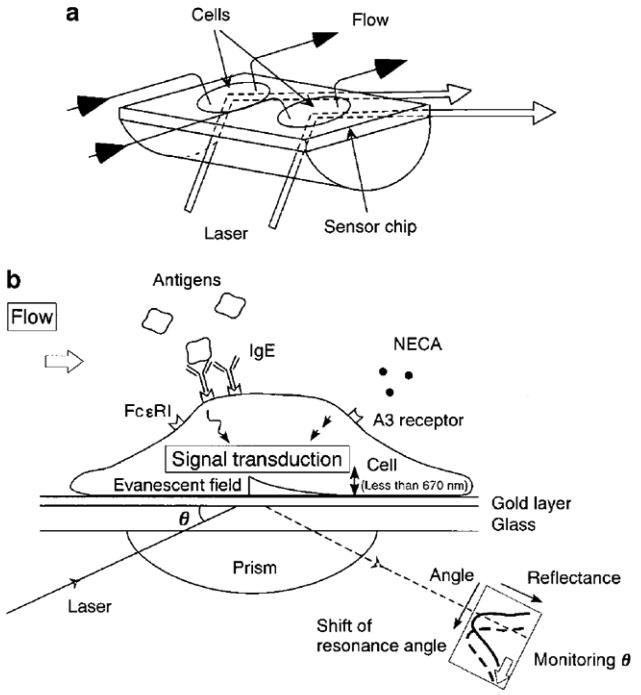

Figure 2. Principle of SPR-based analysis of living cell reactions. (a) Cells are typically cultured in two areas on a sensor chip, which are placed in a flow cell (thin black arrows with closed heads) in the SPR instrument. Laser beams (thick open arrows) are then irradiated from below the chip. (b) Principle of cell activation by ligands during SPR analysis. Antigens in a reaction medium flowing past the cell bind to receptors on the cell surface. The cell is attached to the gold layer coated on a glass plate. The laser beam is directed toward the cell and reflected by the gold coating onto a detector that measures the shift in resonance angle. The resonance angle is affected by the amount of substance in the evanescent field that extends a few hundred nanometers above the gold surface. Reproduced with permission from Hide, M.; Tsutsui, T.; Sato, H.; Nishimura, T.; Morimoto, K.; Yamamoto, S.;Yoshizato, K. *Anal. Biochem.* **2002**, *302*, 28−37 (ref 38). Copyright 2002 Elsevier.

Measuring Cell Adhesion and Motility. In 2006, Yanase et al. measured changes in cell adhesion area and motility on a sensor chip, in response to stimulants, by measuring shifts in the resonance angle using SPR.[39] They used RBL-2H3 rat mast cells and PAM212 mouse keratinocytes to study the structural changes in cell membranes when treated with epithelial growth factor (EGF) and DNP and cell motility inhibitors Mycalolide B and Toxin B. The cells were allowed to grow and attach overnight on the sensor chip prior to imaging. No surface prefunctionalization was used.

They observed changes in the area of cell adhesion and concluded, as in the Hide et al. study, that the changes in the cell membrane and cellular adhesion were insufficient to explain the observed SPR signal change. The results were validated using confocal microscopy. They found that the angle changed proportionally with the number of cells cultured on a sensor chip and that DNP and EGF induced large resonance angle changes in both types of cells. The motility inhibitors induced morphological changes but did not affect the SPR signal as much as the DNP and EGF.

Measuring Cell Growth and Size Changes. The Robelek Group studied the effectiveness of SPR for measuring volume changes in cells.[40,41] They attached two lines of renal epithelial cells, MDCK II and NRK, to sensor surfaces using glutaraldehyde. They exposed the cells to nonisotonic media to induce volume changes. By comparing their results against kinetic models, they determined that SPR could measure the integral, or volume, parameter as a sum of several cellular reactions and identified similar and different responses for the two cell types.

Upon hypertonic stimulation, the cells showed an increase of reflectivity that was concentration dependent. Hypotonic stimulation resulted in a decrease in signal. When the signal stabilized at a new reflectivity, they deduced that the cells had adapted to the new conditions in their environment. The cells in both tests returned to normal levels after an isotonic solution was added. The addition of $GdCl_3$ to the nonisotonic solutions decreased the amount of time necessary for the cells to stabilize as lanthanides affect cation transport channels in cells. The data show that cellular reactions caused by osmotic stress result in a shift in the cell layer's refractive index that can be measured with SPR and that the limit of detection was approximately 5 mOsm/kg, which is sufficiently sensitive for bioanalysis.

Stimulation of Human Embryonic Kidney Cells. In research done by Chabot et al., changes in the SPR signal were investigated when human embryonic kidney cells were stimulated by three specific types of chemical agents: lipopolysaccharide (an endotoxin), sodium azide (a chemical toxin), and thrombin (a physiological agonist).[42] These specific chemicals were chosen to examine the ability of SPR to measure different specific cellular activities. Lipopolysaccharides cause inflammatory response and have been shown to induce cell death by activating membrane receptors that result in cell rounding and membrane blebbing. Sodium azide inhibits cellular respiration. Thrombin affects cell membranes, which will test how SPR reacts to a change in the cell membrane integrity. The different chemicals each affect SPR by altering cellular morphology, which in turn changes the cell's effective refractive index at the interface between the cell membrane and the metal layer. Poly D-lysine was used to attach the cells in these experiments to the gold sensor surface.

Increasing lipopolysaccharide concentration resulted in a larger SPR response. The SPR measurements were cross-referenced with phase contrast microscopy to affirm morphological changes in the cells. Sodium azide caused a decrease in SPR reflectance as the cells shrank. Thrombin was also observed to cause cell contraction as the intercellular gaps increased upon application. Ultimately, SPR can be used as a real-time detection scheme to determine dose−response relationships in mammalian cells.

Identifying Intracellular Signaling Pathways. Lee et al. used SPR to characterize the response of human embryonic kidney (HEK-293) cells expressing the cell surface protein ODR-10 to identify intracellular events when these cells were exposed to diacetyl, an odorant molecule specific to ODR-10.[43] Exposure to diacetyl is known to open Ca^{2+} channels within the cell. SPR was used to detect the intracellular events that were caused by the binding of odorant molecules on the membrane/surface of the kidney cells. These ODR-10 modified cells were compared to a control experiment in which HEK-293 cells were not genetically altered to express the ODR-10 protein.

Poly D-lysine was used to induce cell adhesion to the gold surface. Poly D-lysine is an effective adhesive as it contains multiple positive charges. The rho-tag gene within the HEK cells

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was used to express ODR-10. Gel electrophoresis was then performed to affirm the production of ODR-10.

They measured the change in resonance angle with respect to time as the diacetyl was flowed over the cell surface. A maximum change in the resonance angle of the ODR-10 protein affected cells was observed within 150 s. This is when the team ceased diacetyl injection. The control HEK cell group showed no response to diacetyl on the SPR image. This change was proposed to be the caused in response to the intracellular change in Ca^{2+} ion concentration. This experiment successfully used SPR to identify odorant molecules specific to individual olfactory receptors in real-time without labeling.

Cell Immobilization and Release Strategies. In 2007, Yanase et al. investigated ways to (1) fix nonadherent cells on a gold surface and (2) recover adherent cells from culture dishes while preserving their functions for analysis with SPR.[44] Human basophils and B-lymphocytes were used in the adhesion studies, and RBL-2H3 cells were used to test removal protocols.

They tested three different ways to attach nonadherent living cells to a gold surface. They inserted a biocompatible anchor into the cell membranes, modified the gold surface with compounds containing a positively charged amino group that bound to the negatively charged cell membrane, and covalently attached the cells with peptide bonds formed between the cell and dithiobis[succinimydylpropionate] (DSP), a bifunctional linker. All three immobilization strategies worked; however, charge-based adhesion was the simplest and most effective. Of the aminoalkanethiol compounds tested, cysteamine was the most efficient.

Next, they tested four methods for removing cells from a culture plate while preserving desired functions for SPR analysis. First, they used a superhydrophilic dish where the cells are cultured while floating. Second, they cultured the cells on a temperature responsive polymer that melted at temperatures below 32 °C. Third, they used trypsin, an enzyme that breaks down proteins, to dissociate the cells from the culture plates. Finally, they vigorously pipetted at 4 °C on standard cell-culture dishes. As a control, cells were grown overnight directly on the sensor surface. The cells grown in the special culture plates and released using the low-temperature-melting polymer responded to external stimuli at the same amount as the control cells when measured with SPR. The response of the trypsin released and physically removed cells was significantly lower than that of the control cells, suggesting that the cells were damaged by these two methods. This study demonstrated that both adherent and nonadherent cells could be analyzed with SPR sensors by locating them on the surface of a SPR sensor chip.

Cancer Cells. *Measurement of VEGF Secretion from Cancer Cells.* Liu et al. examined the real time secretion of vascular endothelial growth factor (VEGF) using SPR.[4] VEGF is a widely studied protein biomarker produced by oxygen-hungry cancer cells to promote the growth of blood vessels. In a unique flow-cell configuration, Liu et al. cultured living cells on the ceiling of a customized polydimethylsiloxane (PDMS) SPR flow cell chamber, not on the sensor surface. SKOV-3 (human ovarian carcinoma) cells were attached to the channel ceiling with gelatin, prior to mounting it on the sensor. Monoclonal anti-VEGF was bound to the sensor surface to detect excretion of the protein. The antibodies were immobilized to the gold sensor surface using a base bifunctional linker terminated with carboxylic groups that was exposed to protein G solution, which captures the Fc portion of the antibodies and promotes proper orientation. Changes in the SPR angle as the cells grew indicated the presence of VEGF.

After proving cells could survive in the PDMS flow chamber, the flow cell was detached from the SPR instrument. The control experiments were repeated on an uncoated PDMS gasket and a tissue culture plate. They concluded that SPR is an effective tool to measure VEGF biomarker secretion by SKOV-3 carcinoma cells. In addition, their SPR flow cell chamber mimicked the *in vivo* microenvironment of the VEGF signaling pathway. This is important as it could lead to further studies that examine the cell signaling pathways in regards to drug development.

Detection of Carcinoma Cells. Hiragun et al. used a SPR system to examine how antigen stimulation and epidermal growth factor (EGF) affected changes Chinese hamster ovary (CHO) cells by monitoring changes in the angle of resonance (AR).[45] Further, human carcinoma cell lines were examined to determine if they have unique SPR signal patterns when exposed to stimuli. Cells were seeded directly on the sensing surface and cultured for 24 h prior to starting experiments. Solutions containing stimuli were flowed over the cells, and their response was measured continuously with SPR for up to 50 min.

They observed that CHO cells, expressing wild type EGFR, and HaCaT cells both showed a triphasic change in AR response to AGF where the AR initially increased, decreased, and then increased again over time. This led to the conclusion that SPR detected three distinct phases of cell activity. CHO cells expressing EGFR mutated at the ATP-binding domain caused minimal AR change in response to EGF. Next, the effect of wortmannin, a PI3K inhibitor, on the cell phases was investigated with SPR. They observed that inhibiting PI3K weakened the third phase of the AR shift. The pattern of AR change was independent of EGF concentration, leading to the conclusion that AR was a function of cell type and chemical stimulation. Finally, carcinoma cells exhibited different AR patterns than normal cells when exposed to EGF, demonstrating that SPR could potentially be used to detect and diagnose malignant tumors without the use of histological or immunological labels.

CatG Detection in Leukemia Patients. Cathepsin G (CatG) is a serine endopetidase that is stored in neutrophilic leukocytes, monocytes, and mast cells. It is involved in several physiological processes, but it is mainly associated with early immune response. Gorodkiewicz et al. developed an SPR sensor for measuring the amount of CatG in white blood cell samples using cathepsin G inhibitor 1 (CGI-1), which is a small molecule that binds selectively to CatG.[46]

A SAM was formed on the gold sensing surface using 1-octadecanethiol (ODM). A phosphate buffered solution containing CGI-1 was placed over the ODM and incubated for 24 h prior to the start of the experiment. Since leukocytes are overexpressed during leukemia, they tested blood samples from nine leukemia patients. They first centrifuged the samples to separate the leukocytes from the other blood cells. They then placed the solution containing the white blood cells onto the sensor and incubated it for 10 min. The supernatant from centrifuged saliva samples from healthy donors was also tested, and the CatG concentration in these samples was 10 to 100 times lower than in the leukemia patient samples. The release of CatG from the cells and saliva is not described.

Cell Analysis and Detection Using SPRi. SPR imaging (SPRi), also known as two-dimensional SPR (2D-SPR), obtains 2D images of local refractive index change on the surface of a thin gold film on a glass plate. With this approach, depending on the area being imaged, dozens of cells can be imaged simultaneously

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and each individual cell can be analyzed versus standard SPR, which obtains an average response for the entire sensing surface. The resolution of this SPRi is currently limited by the pixel size in the detection system used to collect the reflected light, which is currently approximately 10 μm^2.

Detection of Histamine Release in Basophils. Horii et al. tested the allergenic response of rat basophilic leukemia cells (RBL-2H3) with 2D-SPR.[47] The cells were presensitized with anti-DNP IgE, and the response was measured by the change in reflection intensity in the SPR signal. The SPR angle in regions containing cells was 52.2° and 50.8° for bare gold regions. The angle shifted to 52.4° where cells were located when antigen stimulation was introduced. RBL-2H3 cells undergo degranulation after exposure to an allergen. Typically, this response is measured by determining the amount of histamine released from the cells using HPLC or measuring enzyme activity. Observation of Ca^{2+} concentration changes during histamine release is more direct, but incorporation of a fluorescent indicator dyes into the cells is necessary. Comparing these methods against SPR imaging, they concluded that the direct SPR measurement was more sensitive. The signal change observed, as a result of cellular changes, after exposure to antigen was greater than detecting capture of the histamine using immobilized antibodies on the sensor surface without cells. This study demonstrated the use of 2D-SPR as a label-free and real-time monitoring tool for cellular studies and sensor applications.

Allergy Diagnosis in Humans. Yanase et al. expanded on their earlier work studying allergen response in basophilic cells.[48] They analyzed individual human basophils, isolated from patients, using SPRi.[49] Basophils were first isolated from blood using antibody conjugated magnetic beads. The cells were then immobilized on the sensing surface using antibasophil antibodies and stimulated with allergens. The antibodies were immobilized on the sensor surface using dithiobis[succinimidylpropionate] (DSP), which covalently bound with primary amines on the antibodies. Basophils suspended in 10 mM HEPES buffer were incubated on the functionalized sensor surface for 15 min; the surface was rinsed with buffer and imaged with the SPRi instrument. The responses of the individual captured cells to anti-IgE antibody were measured simultaneously, in real time, using SPRi. The responses among individual cells were similar. Next, basophils were exposed to a gamut of stimuli and inhibitors, and the responses were again recorded.

Basophils were then collected from four donors. Donor 1 had mild asthma, donor 2 pollen hypersensitivity, donor 3 atopic dermatitis (AD), and donor 4 had no known allergies. Their cells were mounted on the SPRi chip and exposed anti-IgE, mite antigen, sweat antigen, and cedar pollen antigen. Excellent correlation was obtained between donors and cellular responses measured with SPRi. Next, they tested six healthy donors and six donors with AD. The results showed that positive identification could be made by monitoring as few as five cells.

Distinguishing B- and T-Type Lymphocytes. Iribe et al. examined whether B- and T-type lymphocyte cells could be distinguished using SPR when antibodies bound to their surface.[50] While the cells are much larger than the 300 nm that the SPR field extends, the authors proposed that it would still be possible to detect changes via intracellular events that take place because of the surface antigen binding. B and T lymphocytes were collected from mouse spleens for this study as lymphocytes undergo an intracellular reaction with a minimal morphological change. The cells were grown on the sensor surface and analyzed using high-resolution 2D-SPR.

Anti-IgM and anti-CD19 were used for identifying B lymphocytes, and anti-CD3 were used for T lymphocytes. B-type cells responded to anti-IgM and anti-CD19 but not the anti-CD3. The spleen cells, which are a mixture of B and T cells, responded to both sets of antibodies by showing selectively increased SPR signal response. The study concluded that specific surface antigens could be used to identify individual B and T lymphocytes in a SPR imaging system without any labeling.

Suraniti et al. created an antibody-coated chip to selectively capture B- and T-type lymphocytes from a mixture.[51] They were able to count individual cells as they were selectively captured on the sensor surface over the course of 50 min. Since SPRi provides spatial resolution on the sensing surface, they patterned spots of three different antibodies on a single surface using electropolymerization. CD19, CD3, and IgG antibodies were first functionalized with *N*-hydroxysuccinimidyl 6-(pyrrol-yl)-caproate (NHS-pyrrole). The gold sensing chip was masked and patterned with a hydrophobic material. By selectively applying an electrical potential to unmasked regions on the chip, fluid droplets containing individual functionalized antibodies were polymerized on the chip surface to create an array of spots.

Solutions containing lymphocytes were flowed past the functionalized sensing surface, and attachment of cells was monitored continuously with SPRi. Injection of solutions containing only T-type lymphocytes resulted in cells attaching only on CD3 spots, and solutions with only B-type lymphocytes only showed binding events on CD19 spots, indicating that capture is selective. Solutions containing a mixture of both T-type and B-type cells resulted in cellular attachment at both CD3 and CD19 spots. IgG spots were negative controls, and negligible amounts of cells bound to those spots during all experiments, as expected.

Capture and Selective Release of Lymphocytes. Bombera et al. created a protein microarray using antibodies covalently coupled with DNA for selective capture and subsequent release of primary spleen cells.[52] A heterobifunctional linker was used to covalently connect antibodies with DNA strands. Anti-CD19 IgG was used to target B lymphocytes, and anti-CD90 IgG was used for capturing T lymphocytes. Each antibody was functionalized with a unique DNA sequence that could be cut with a unique restriction enzyme. The gold sensing surface was functionalized with a 1-dodecanethiol SAM. DNA probe sequences were attached to the SAM using electropolymerization to form 800 μm-diameter spots on the surface. The sensing surface was placed in the SPRi instrument, and intermediate DNA strands were hybridized with the immobilized DNA. The functionalized antibodies were then flowed over the surface to attach the intermediate DNA. Next, a mixture of T- and B-type lymphocytes was flowed past the immobilized antibody spots. *Pvu*II restriction enzymes were flowed through the system to cut one of the DNA sequences and release T-cells followed by *Eco*RI enzyme to release the B-cells. Figure 3 shows a general scheme of the construct assembled on the sensor surface and where the restrictions enzymes cut the DNA to release the cells. The entire process was monitored with SPRi, and the locations where individual cells attached and were released from were observed in real time.

BACTERIAL CELL ANALYSIS

The second half of this Review focuses on analyzing bacterial cells, using bacteria for sensing purposes, and detecting pathogenic species with SPR instruments. While SPR can provide significant new fundamental insights about bacteria, thus

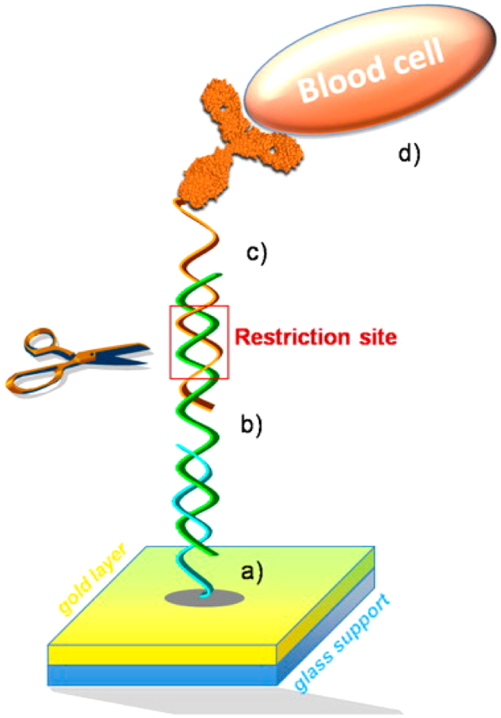

Figure 3. General scheme of the biochip assembly for cell capture and enzymatic release. (a) DNA probe sequence grafted onto a gold surface; (b) intermediate DNA strand containing a unique restriction site; (c) modified biorecognition element bridging the target to the solid support; (d) target cell. Reproduced with permission from Bombera, R.; Leroy, L.; Livache, T.; Roupioz, Y. *Biosens. Bioelectron.* **2012**, *33*, 10−16 (ref 52). Copyright 2012 Elsevier.

far research efforts have justifiably explored sensing applications. In order to decrease health risks and deaths and reduce economic losses due to pathogenic bacteria, there is a critical need for rapid, sensitive, and selective detection methods to sense the disease-causing organisms in food and beverages.[53]

Among all microorganisms, bacteria cause 91% of all foodborne illnesses in USA.[54,55] On the basis of the estimates from the Centers for Disease Control and Prevention (CDC), in the United States, foodborne pathogens cause roughly 79 million illnesses, 325,000 hospitalizations, and nearly 5000 deaths each year.[56] In addition, outbreaks of foodborne illnesses result in economic losses totaling several billions of dollars annually. According to the United States Department of Agriculture (USDA) Economic Research Service (ERS), medical costs and loss of productivity caused by five major pathogens, *Escherichia coli* O157:H7, non-O157 STEC (Shiga toxin producing *E. coli*), *Salmonella* (nontyphoidal serotypes only), *Listeria monocytogenes*, and *Campylobacter*, is $6.9 billion annually.[57,58] Various detection techniques have been employed for pathogenic bacterial detection applications,[59] such as conventional microbiological culture method,[60] polymerase chain reaction (PCR),[61−68] enzyme-linked immunosorbent assays (ELISA),[69,70] amperometric biosensors,[71−75] piezoelectric biosensors,[75−78] potentiometric biosensors,[79,80] bioluminescence,[81,82] fluorescent labeling,[83,84] and ultrasound.[85] All of these methods have concerns such as long detection time, enrichment requirements, labeling necessity, requirements of trained personnel, and high cost.[86,87] Therefore, there is a need for alternative rapid, low cost, and sensitive detection in complex samples. These needs can be fulfilled by surface plasmon resonance (SPR), which provides label-free, real-time, and quantitative detection.[88−90]

Surface Coatings and Cellular Adhesion. *Evaluation of Surface Coatings for Preventing Cell Adhesion.* Ostuni et al. examined the hypothesis that self-assembled monolayers (SAMs) that resist protein adsorption also resist the attachment of bacterial and mammalian cells to the surface.[91] Using SPR, the researchers were able to determine the qualities of inert SAMs (i.e., SAMs that adequately resist protein adsorption). Inert SAMs generally contain only hydrogen bond acceptors, have an overall neutral charge, and are polar. Using these characteristics, the experimenters developed six homologous single-component SAMs with structurally different terminal groups in order to test their effects on protein adsorption and cell adhesion. In the first set of experiments, adsorption of proteins, specifically fibrinogen and lysozyme, onto the SAMs was tested using SPR. Hexadecanethiolate (HDT) was used as a reference for this experiment, and the changes in reflectivity of the chosen SAMs were normalized into a ratio of refractive index change of the SAM vs refractive index change of HDT. None of the chosen SAMs resisted adsorption better than the most inert SAM known (triethylene glycol); however, the protein adsorption for both fibrinogen and lysozyme was sufficiently low for practical applications.

Subsequently, using the principle that the number of bacterial colonies grown on an agar plate is proportional to the number of colony forming units (CFU) recovered from the SAMs, bacterial adhesion to each of the single-component SAMs was tested. The species used in this test were *S. aureus* and *S. epidermidis*, as these species are responsible for 30−50% of infections on indwelling medical devices. After adhesion of these species to the SAM surfaces, agar plate cultures were made from each SAM coated surface for both bacterial strains. The results of the colony formation showed that bacterial adhesion had little to no correlation with protein adsorption and that bacterial adhesion must be related to other factors. These results go against the previously stated hypothesis. In addition, bovine capillary endothelial (BCE) mammalian cells were used to test mammalian cell adhesion to each SAM. After the cells in modified Eagle's medium were allowed to adhere to the SAM surfaces, the cells were fixed and counted. The results showed that mammalian cell adhesion also did not correlate with protein adsorption. The results of these experiments did not agree with the hypothesis; however, the SPR investigations illustrated the use of SPR for SAM testing and testing protein adsorption, allowing the researchers to quickly screen for the best possible SAMs for their experiment.

Study of Staphylococcus Species Binding to Fibronectin. In order to protect a wound, the body coats the injured area with proteins, such as fibronectin. When *S. aureus* binds to the fibronectin, this can lead to infection. Holmes et al. developed a method using SPR to demonstrate the role of fibronectin binding protein A (FbpA) in the attachment of *S. aureus* and identify the domain in fibronectin used as a binding site location for *S. aureus* and *S. epidermidis*.[92] SPR was used to determine experimentally the optimal concentrations, flow rates, and the rate of binding for FbpA, *S. aureus*, and *S. epidermidis* to fibronectin and its domain fragments. Fibronectin was purchased and purified, and fibronectin fragments were isolated. FbpA was purified from *S. aureus*.

The gold-plated surface was functionalized with fibronectin or fragments (30−100 μg/mL) in sodium acetate flowed over the surface for 2 to 7 min at 5 μL/min; the remaining surface was

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blocked with ethanolamine. During the experiments, the bacteria were again suspended and flowed over the surface at varying concentrations and flow rates. Controls were run using gelatin and polyclonal fibronectin antibodies. The experiments found that the binding of *S. aureus* to fibronectin required long contact times and the bonding kinetics were largely unaffected by the flow rate. The flow rate that elicited the highest response was 2 μL/min. Binding of the *S. aureus* to immobilized fibronectin had a limit of detection of 1×10^8 CFU/mL. They also found that FbpA and *S. aureus* bound with high affinity to both whole fibronectin and the 27 kDa fragment (containing the N-terminal of fibronectin), which suggests that FbpA is the protein in *S. aureus* responsible for binding and that *S. aureus* binds to the N-terminal of the fibronectin protein. Their high affinity is further illustrated by their unwillingness to dissociate. *S. epidermidis* bound to the C-terminal of fibronectin with a low affinity, requiring a concentration of 5×10^9 CFU/mL.

This study successfully pioneered the use of SPR in the detection of binding of whole bacterial cells. The use of intact bacteria and the lack of molecular labeling and protein modification highlight the value of SPR in the field of cell detection.

SPR Sensors Employing Bacterial Cells. *Pollutants Detected by Escherichia coli.* In the study done by Choi et al., changes in bacterial cells were monitored using SPR as they were exposed to environmental pollutants, such as phenol.[93] To optimize the attachment of cells to the surface, self-assembled synthetic oligopeptides were investigated. Two different cysteine-terminated oligopeptides were evaluated. The cysteine end of the oligopeptide attached to the gold sensor surface, while the other end, which terminated in Arg-Gly-Asp (RGD), immobilized the cells on the surface.

Two different designs of peptides were used for the immobilization of cells, one was a single stranded oligopeptide (C-$(RGD)_4$) and the other was a poly oligopeptide network consisting of four grafted branches of (C-$(RGD)_4$). The angle shift in SPR was monitored in each step during surface functionalization. The resonance angle for bare gold surface was determined with the instrument. Adsorption of single stranded oligopeptide (C-$(RGD)_4$) caused a shift in the resonance angle. Then, the resonance angle shifted significantly after immobilization of *E. coli* O157:H7 on the surface. The resonance shift caused by immobilization of *E. coli* was larger when the cells were attached to the grafted C-$(RGD)_4$ than to the straight C-$(RGD)_4$. The number of immobilized cells also increased with increasing concentrations of synthetic oligopeptide on the sensor surface. The concentration of immobilized bacteria plays a significant role in toxicity detection because a higher amount of immobilized bacteria creates more sensing elements on the surface, which leads to a larger shift in resonance angle and determines the limit of detection for the sensor. The researchers combined atomic force microscopy (AFM) with SPR to study surface topography as well as biological interactions on the surface. The AFM results from the surface before and after each immobilization step confirmed the attachment.

The live cells maintained their physical integrity during the experiments, while cells exposed to toxic compounds died and lost physical integrity, which caused the intracellular material to decrease and resulted in angular shifts in the plasmon curve. The researchers exploited this fact to detect the presence of toxic chemicals with SPR. Different concentrations of phenol were injected to the modified surface, and the angular shift in the plasmon curve was obtained for each concentration. The smallest detectable shift occurred for 5 ppm of phenol, which determined the limit of detection for this sensing system.

Escherichia coli Autodisplaying Z-domains as Sensors. In studies done by the Pyun Group, the efficacy of *E. coli* cell immobilization on different surfaces, the stability of the immobilized cells, and the sensitivity of the sensor for detection of C-reactive protein (CRP) was investigated.[94,95] *E. coli* with autodisplayed Z-domains, which orient antibodies on the cells surface, were immobilized on modified SPR sensor surfaces for molecular recognition.

The outer membrane of *E. coli* cells is negatively charged because of phosphate groups in the lipopolysaccharide layers, which allowed immobilization on the surface coated with a positively charged layer, such as poly-L-lysine, by charge interaction. The efficacy of immobilizing fluorescently labeled *E. coli* was determined by counting the immobilized cells on three different surfaces: bare gold, only poly-L-lysine coated, and parylene-H film functionalized with a poly-L-lysine coating. The results showed a greater number of cells immobilized on the surface coated with parylene-H film containing poly-L-lysine compared to the other two surfaces.

The stability of the coatings was studied by treating the surfaces with salt solutions at different concentrations, and the SPR response change was monitored for each surface. The results indicate much less change in the SPR response after sequential treatments of the gold surface coated with poly-L-lysine functionalized parylene-H film, indicating that this surface had greater stability than the others.

To measure the sensitivity of the sensor for detecting CRP, first anti-CRP antibodies were introduced onto the surface to react with auto displayed Z-domains on the *E. coli* cells, and then, the SPR response to different concentrations of CRP was monitored. The results show a higher sensitivity and lower detection limit (1 ng/mL) for the surface coated with poly-L-lysine functionalized parylene-H film compared to the other two surfaces.

SPR Sensors Detecting Bacterial Cells. *Optimizing Sample Preparation for Escherichia coli Detection.* Taylor et al. used SPR to detect *E. coli* O157:H7 that were processed in three different ways: untreated, heat killed then soaked into ethanol, and detergent lysed to determine which preparation method provided the lowest limit of detection.[59] In these experiments, the surface was functionalized with a mixed, self-assembled, monolayer of alkanethiols followed by immobilization of mouse anti-*E. coli* O157:H7 monoclonal antibody (mAb). After surface modification, SPR experiments were run to determine the detection range of each bacterial sample. In each experiment, different concentrations of bacteria were flowed over the surface. The resonance wavelength shift was detected with the device, and results show that for untreated bacteria, heat killed, and detergent lysed bacteria the limit of detection was 10^7, 10^6, and 10^5, respectively. Subsequently, additional mAb was flowed over the surface with captured bacteria to amplify the response. This additional step amplified the detection limit by an order of magnitude for all three samples.

The selectivity of this SPR sensing setup was tested with three different bacterial species: *E. coli* K12 serotype, *S. choleraesuis*, and *L. monocytogenes*. In this set of experiments, the nonspecific bacteria were flowed over the sensing surface and any shifts in the resonance wavelength were due to binding of nonspecific bacteria to antibodies on the surface. The results showed negligible resonance shift after running the nonspecific bacteria

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over the surface. Addition of the sandwich assay protocol after the nonspecific bacteria also did not shift the resonance angle.

Optimization of Surface Chemistry for Detection of Staphylococcus aureus. Subramanian et al. studied the effect of different surface chemistry for *Staphylococcus aureus* detection with SPR.[96] They argue against the use of the commonly used carboxymethyl dextran platform for bacterial capture, as it does not readily allow transport of cells, which are hundreds of nanometers in diameter, through the porous polymer. Dextran also extends beyond the SPR evanescent field, limiting the sensitivity of the setup even when cells attach to the polymer surface. Instead, Subramanian et al. examined tri- and hexa-ethylene glycol terminated monothiol and tri- and tetra-ethylene glycol terminated dithiol self-assembled monolayers (SAMs) to identify the best surface chemistry for the biosensor.

The ethylene glycol thiols bind to the surface via gold−thiol bonds as they are flowed through the sensor flow cell. After immobilization of the SAMs on the gold surface, the exposed ethylene glycol ends were activated by flowing *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) over the sensor chip. The activated SAMs formed covalent amide bonds with antibodies flowed over the surface. Unbound antibodies were removed from the surface by flowing 1% sodium dodecyl sulfate (SDS), and the remaining unbound NHS esters were blocked with ethanolamine.

Binding of *S. aereus* against anti-*S. aureus* antibodies was measured by increases in light reflectance in the SPR output as the resonance angle shifts during binding. Increasing the amount of immobilized bacteria on the surface resulted in a higher peak in the corresponding SPR signal. The results show that both the monothiols and dithiols work very well, but a slightly greater response was observed for surfaces functionalized with the monothiol SAM mixture. The sensitivity of the sensor was increased by using a sandwich assay detection strategy, where there is binding of secondary anti-*S. aureus* antibodies to the already immobilized *S. aureus* bacteria on the surface. This additional step lowered the sensor detection limit to 10^5 CFU/mL, which was 100 times better than direct detection alone. The specificity of the surface was tested using *E. coli* O157:H7.

Escherichia coli Detection Using a Lectin Functionalized Surface. Wang et al. modified the sensing surface of SPR biosensors with lectin, which was to act as a receptor to detect *E. coli* O157:H7.[97] Lectins are proteins and glycoproteins that selectively bind to mono- and oligosaccharides that are found on the surface of bacterial cells. To choose the best lectin for binding to *E. coli* O157:H7, lectins from five following species were evaluated: *Triticum vulgaris* (WGA), *Canavailia ensiformis* (Con A), *Ulex europaeus* (UEA), *Arachis hypogaea* (PNA), and *Maackia amurensis* (MAL). Each lectin was immobilized on a separate chip, and the amount of bacterial attachment to the modified surfaces was used as the selection criteria for choosing the best lectin for use as a receptor. A chip, commercially coated with a carboxylic acid terminated SAM, was used to immobilize the lectins. The lectins, which have exposed amine groups, were covalently bonded to the carboxylic acid groups using standard EDC/NHS chemistry. When *E. coli* O157:H7 binds to the immobilized lectin on the surface, an increase in the refractive index occurs, which is detected with SPR. SPR also determines kinetic binding parameters (K_a = K association, K_d = K dissociation), which allows the determination of K (affinity parameter), which determines how tightly bacteria bound to lectin. The highest K value was obtained for the WGA lection, with a detection limit of 3×10^3 CFU/mL. It was proposed that WGA binds with a higher affinity to bacteria better because of its biological structure, which provides more binding sites for cells to attach to than the other lectins. The specificity of the binding element for *E. coli* was tested with *Listeria monocytogenes*. Using the WGA lectin, they were able to detect *E. coli* O157:H7 in cucumber and ground meat with a LOD of 3×10^4 and 3×10^5 CFU/mL, respectively. The foodstuffs were homogenized, diluted in buffer, and filtered through filter paper prior to adding known quantities of bacteria to the samples.

Gold Nanoparticle Sensor Coating to Improve Escherichia coli Detection. The use of gold nanoparticles to improve detection sensitivity with SPR was studied by Baccar et al.[98] Several other surface modification methods, such as roughening and graphene coatings, are available for improving the sensitivity of SPR but have not yet been tested with cellular systems.[99] They synthesized 6−8 nm gold nanoparticles and functionalized them with an amine-thiol bifunctional linker. The sensing surface was functionalized with an acid-thiol SAM, activated with EDC/NHS, and exposed to the nanoparticles, which attached covalently via peptide bonds. The unreacted amine groups on the nanoparticles were activated with glutaraldehyde solution. The aldehydes that formed on the nanoparticles then reacted with amine groups on anti-*E. coli* antibodies. As a control experiment, they attached the same acid-thiol SAM to the sensor surface, but then after activation with EDC/NHS, the surface was directly exposed to the anti-*E. coli* antibodies.

SPR experiments were performed by flowing *E. coli* bacteria over the modified surfaces. The resonance angle shift was monitored with the device for all surface functionalization steps as well as subsequent bacterial attachment to the ligands. The results showed more bacterial attachment to surfaces functionalized with gold nanoparticles. The limit of detection for gold nanoparticle functionalized surfaces and direct antibody functionalized surfaces was 10^3 and 10^4 CFU/mL, respectively.

Identification of Pathogens using Bacteriophages. Arya et al. used SPR as a transduction technique to detect a specific strain of *Escherichia coli* bacteria, *E. coli* K12.[100] T4 bacteriophages, as specific receptors for the bacteria, were immobilized on the gold surface using a self-assembled monolayer of dithiobis-(succinimidyl propionate) (DTSP).

All steps of surface functionalization were monitored with SPR. It was shown that using a higher concentration of T4 bacteriophage solution increases the phage immobilization on the surface and subsequently results in a higher SPR response for the same concentration of bacteria. The bioassay platform was also shown to be specific to *E. coli* K12 as negligible changes in SPR signal were observed for the nonspecific bacteria strains, *E. coli* NP10 and NP30. A reproducibility experiment, which was done by injecting a regeneration solution after each injection (in order to reset the baseline signal), showed a stable platform for multiple experiments on the same chip. This SPR-based platform was able to detect K12 bacteria concentrations in the range between 7×10^2 and 7×10^8 CFU/mL.

Bacteriophage Sensor for MRSA. Tawil et al. built a custom SPR biosensor instrument to specifically detect *E. coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) in less than 20 min without further enrichment requirements.[101] They functionalized the sensor surfaces by immobilizing T4 and BP14 bacteriophages for detection of EC12 *E. coli* and SaA4MRSA. The sensor was used to detect different concentrations of bacteria with a detection limit of 10^3 CFU/mL. The specificity of the sensor for MRSA versus *E. coli* was studied. No SPR response

was observed upon *E. coli* injection, confirming the specificity of the sensor surface.

Vibrio cholerae Sensor. Jyoung et al. developed a sensor for detection of *Vibrio cholerae* O1 with SPR.[102] To control the orientation of capture antibodies on the sensor surface, the surface was coated with a layer of G protein. A SAM of 11-mercaptoundecanoic acid (11-MUA) was added prior to the G protein layer to help with protein adhesion. The formation of SAM of 11-MUA, protein G layer, and immobilization of monoclonal antibodies (MAbs) against *Vibrio cholerae* O1 was monitored with SPR spectroscopy, and the shift in SPR resonance angle increased in each step, as expected.

After surface modification with MAbs, different concentrations of *V. cholerae* were flowed past the sensor surface, and changes in the SPR resonance angle were monitored. The results show that increasing the concentration of *V. cholerae* increased the angle linearly. A detection range between 3.7×10^5 and 3.7×10^9 cells/mL was obtained, which is relevant for detecting *V. cholerae* in fecal samples. The specificity of the sensor for *V. cholerae* was investigated by using *E. coli* O157:H7 and *L. pneumophila* as nontarget samples. Very small shifts in SPR angle indicated that the immunosensor was sufficiently selective for detection of *V. cholerae*.

Escherichia coli O157:H7 Sensor. Subramanian et al. fabricated a SPR chip for direct detection of *E. coli* O157:H7.[53] They investigated multiple parameters within the surface functionalization protocol. First, they used a mixed alkanethiol self-assembled monolayer to create a reactive surface on the gold sensing surface. They then attached various concentrations of polyclonal antibodies to the surface to determine the optimal coverage density. The orientation of the antibodies was investigated by adding Protein G to the SAM prior to attaching monoclonal antibodies, which oriented the receptor portion of the antibodies away from the surface. The oriented monoclonal antibodies bound more of the cells than the randomly oriented polyclonal antibodies at any concentration.

To enhance the detection signal, they used a sandwich assay approach by passing secondary antibodies (anti-*E. coli* O157:H7) over the sensor surface after bacterial immobilization. The sensitivity of the sandwich assay was optimized by varying the concentrations of primary and secondary polyclonal antibodies. The results indicate that adding the secondary antibody binding improved the sensitivity by 1000 times over capture with only primary antibodies, and the limit of detection was found to be 10^3 CFU/mL for *E. coli* O157:H7 using the sandwich assay format. The specificity of the sensor against different concentrations of *Salmonella enteritidis* in the cocktail including *E. coli* O157:H7 (10^6 CFU/mL), *S. enteritidis* (10^6 CFU/mL), and *E. coli* O55 (10^9 CFU/mL) was also studied.

Escherichia coli Sensing in Water Samples. Dudak et al. developed a SPR-based immunosensor for enumeration of *E. coli* in water samples from rivers and *E. coli*-inoculated tap water.[103] The sensing surface was modified by immobilization of streptavidin followed by biotin conjugated polyclonal antibodies against *E. coli*. SPR response signal to each step of surface functionalization was monitored with the device. To show the sensitivity, different concentrations of *E. coli* in water samples were passed over the surface and the changes in the RU were determined by measuring the difference between the sensing signal and the baseline. The sensitivity was established to be comparable with other methods such as plate counting but required only 30 min, which is much less than the 24−48 h required for conventional methods. The specificity of the above sensor was tested against *E. aerogenes* and *E. dissolvens*, which are found in water contaminated with feces. The results showed a much lower response to these species than to the target bacteria.

Escherichia coli O157:H7 Detection in Food. Waswa et al. used a SPR-based biosensor to directly detect *E. coli* O157:H7 spiked into milk, apple juice, and ground beef food samples.[104] Pasteurized skimmed milk and apple juice was flowed directly over the sensing surface. The ground beef was first mixed with phosphate buffered saline (PBS) and homogenized. The clear supernatant was removed from the meat mixture and used for analysis. The gold surface of the sensor was modified with biotinylated Rabbit antisera containing polyclonal antibodies against the pathogen, and food samples spiked with *E. coli* O157:H7 in different concentrations were flowed over the sensor surface. The sensitivity of above sensor for bacterial detection was established to be 10^2−10^3 CFU/mL. The detection limit of the sensor was based on the lowest bacterial concentration that generated a response signal and was at least three standard deviations larger than the signal from a negative control in the spiked food samples. The sensitivity is comparable to other commonly employed detection techniques for food. Specificity of the sensor for *E. coli* O157:H7 was tested against genetically similar species, *Shigella* spp. and *E. coli* K12, added individually to food samples. The response of the sensor to nontarget pathogens was similar to the negative control in which there were no bacteria present.

Escherichia coli Detection in Spinach. Linman et al. developed a SPR-based biosensor for detection of *E. coli* bacteria in fresh spinach.[105] First, a SAM of MUA was formed on the sensing surface, and then, the MUA was activated by NHS-EDC, which was followed by immobilization of anti-*E. coli* antibodies. Different concentrations of *E. coli* in PBS and in samples extracted from spinach were passed over the sensor surface. To enhance the sensitivity, a sandwich assay was performed where anti-*E. coli* antibodies fused with horseradish peroxidase (HRP) were flowed over the surface to bind to already immobilized bacteria, followed by injection of undiluted tetramethylbenzidine (TMB) over the surface. The HRP reacts with the TMB to produce an insoluble blue product that significantly increases the amount of material on the sensor surface.

For *E. coli* samples in PBS, SPR results showed very little response upon binding of bacteria on the modified surface and the signal increased only a small amount after binding of the anti-*E. coli* HRP conjugated antibody on the bacteria surface. The authors claim that this is because bacteria are large (1−5 μm in diameter) compared to how far the evanescent field in SPR extends into solution (~300 nm).[106] The SPR signal increase upon injection of the TMB was 263% compared to samples without using HRP/TMB. The limit of detection of *E. coli* in PBS was calculated to be 6×10^3 CFU/mL. For *E. coli* samples in spinach, the results showed the same behavior but with a 150% signal increase upon injection of TMB, with a detection range from 10^4 to 10^6 CFU/mL. The calibration curves indicated a linear relationship with TMB enhancement in which the SPR response was directly proportional to the concentration of *E. coli*.

Salmonella paratyphi Sensor. *Salmonella* species are a major cause of infections and foodborne illnesses worldwide, and traditional methods of detecting the bacteria are costly, time-consuming, and require large samples. Oh et al. developed a SPR-based immunosensor for rapid detection of *Salmonella paratyphi*.[107] To increase the sensitivity, Protein G was directly coated on the gold surface. The thiol groups on the protein were functionalized using 2-iminothiolane to allow self-assembly of

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the protein on the sensors. The SPR resonance angle shift was monitored while coating the surface with Protein G, immobilizing anti-*S. paratyphi* MAbs, and subsequently capturing the target *S. paratyphi*. The SPR angle shift increased significantly upon adsorption of antibodies and subsequent capture of bacteria. The SPR angle increased linearly with increasing *S. paratyphi*. The lower detection limit of 10^2 CFU/mL was 4 orders of magnitude more sensitive than other common detection methods, such as ELISA.

The specificity of the sensor was determined by monitoring the cross reactivity between anti-*S. paratyphi* MAbs and *E. coli* O157:H7, *Yersinia* spp., and *Legionella* spp., which exist in contaminated water samples. The data showed that the SPR angle shift for binding with these nonspecific pathogens was much less that the shift due to specific binding between the antibody and *S. paratyphi*.

Salmonella typhimurium Detection in Meat. Lan et al. used SPR to detect the *Salmonella typhimurium* bacterium in a chicken carcass, a common carrier of the foodborne pathogen.[108] The *S. typhimurium* was purchased, cultured, and diluted in buffered peptone water (BPW), and the chickens were purchased and washed with PBS; that PBS wash was then diluted in BPW containing different amounts of bacteria for use in the experiment. The samples were passed over a sensing surface that was first coated with neutravidin and then functionalized with a biotinylated antibody against *Salmonella* common structural antigens (CSA-1) during SPR measurements. It was determined that the limit of detection of this process was 1×10^6 CFU/mL. Although the detection limit for the experiments by Lan et al. is not groundbreaking, the experiments show promise in the field of rapid, real-time detection of pathogenic bacteria, saving time and money for the food industry.

Salmonella enteritidis and Escherichia coli Sensing in Food. Waswa et al. used SPR to detect *Salmonella enteritidis* and *Escherichia coli*.[109] *S. enteritidis* and *E. coli* are common bacterial foodborne pathogens in the United States and abroad, causing approximately 1.7 million cases of illness annually in the United States. Therefore, a method of rapid detection of these pathogens is important for public health.

In this setup, the gold sensor surface was coated with carboxymethyl dextran, which provided a binding site for antibodies. For *Salmonella*, mouse polyclonal affinity-purified antiserum was used, and polyclonal rabbit antibodies for *E. coli* O26 were purchased. The surface was functionalized by covalently linking protein A to the carboxymethyl dextran layer with EDC/NHS coupling and binding the antibodies to the protein A. Only one antibody was bound per sensing surface. Experiments were performed using bacterial solution in buffer as well as skim milk spiked with varying concentrations of bacterial cells (from 10 to 10^6 CFU/mL, with a negative control of 0 CFU/mL).

For the bacterial solution tests, each species in various concentrations (from 10^2 to 10^7 CFU/mL) was injected into the SPR system with the sensor surface functionalized with each species' respective antibody. The change in the refractive index was recorded and plotted, and the results were normalized by expressing the refractive index change for each concentration as a ratio with respect to that of the same species at the maximum concentration (10^7 CFU/mL). The results of the tests were averaged by species and concentration, and the R^2 results indicate that the process was very sensitive for both *Salmonella* and *E. coli*. The bacteria could be easily washed off the surface with sodium hydroxide solution, allowing the same functionalized surface to be reused several times. Specificity was tested by cross-binding antibodies with increasing concentrations of nonbinding bacterial species.

Subsequently, tests were conducted with spiked skim milk containing bacterial concentrations ranging from 10^1 to 10^6 CFU/mL. The limit of detection was set to be three standard deviations higher than the signal of the negative control. The authors claim a limit of detection of 25 CFU/mL for *E. coli* and 23 CFU/mL for *Salmonella enteritidis*. The sensitivity of this assay shows promise for the fields of public health and pathogen detection.

Salmonella enteritidis and Listeria monocytogenes Sensor. In work from 2001, Koubová et al. created a SPR sensor detection of the bacterial pathogens *Salmonella enteritidis* and *Listeria monocytogenes*.[110] The bacteria were identified specifically through the attachment of antibodies to the sensor surface. The antibodies used in this study were a monoclonal antibody specific to the somatic antigen (O) serotype 9 surface lipopolysaccharide of *Salmonella* and rabbit anti-*Listeria* polyclonal antibodies.

The surface was functionalized in two distinct ways. In the first method, an antibody layer was adsorbed directly on the gold surface from citrate buffer (CB) at a pH of 4. Dextran sulfate sodium salt (DS) polyanions were electrostatically attached to the positively charged antibodies. A second layer of antibodies was then electrostatically adsorbed on the DS layer. 0.5% glutaraldehyde in CB was used to cross-link the coating, connecting the two layers of antibodies through covalent bonding. Phosphate buffered physiological saline (PBS) was used to wash out the DS and any antibody molecules that were not cross-linked (which were both negatively charged at a pH of 7.4). In the second functionalization method, a bovine serum albumin (BSA) layer was adsorbed on the gold surface from CB at a pH of 4. A 2% glutaraldehyde solution in CB was used to cross-link the amino groups of the BSA, and antibodies were bound to the resulting aldehyde groups on the BSA layer from PCB (a mixture of PBS and CB). Throughout both of these processes, the SPR system was used to monitor the presence and attachment of the various solutions and antibodies. The first method, which utilized a double layer of antibodies, elicited a larger response from the optical sensor in the SPR mechanism than did the BSA method.

The attachment of the bacterial antigens to the antibodies fixed on the sensor surface altered the wavelength of the reflected light, and this change in wavelength was used in the SPR process to detect the presence of these specific bacteria. The resulting data from the SPR wavelength detection process showed that this sensing scheme for bacterial detection and identification was able to detect down to 10^6 cells per milliliter of solution for both *Salmonella* and *Listeria* using the first functionalization method. This is on par with the results of the ELISA identification technique but is not yet sensitive enough for practical health applications. In addition, the flow rate in this study, unexpectedly, seemed to have a large influence on bacterial attachment and overall reflectivity, and this problem was not addressed.

Sensor to Distinguish between Salmonella typhimurium and Salmonella enteritidis. Barlen et al. developed a new technique to both detect and identify different serovars (specifically *typhimurium* and *enteritidis*) of *Salmonella* simultaneously using SPR.[87] The SPR system measured the change in refractive index of the light reflected caused by bacterial cell attachment to antibodies on the functionalized hydrophobic gold surface. The system used was cuvette-based; allowing the

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researchers to bypass the difficulties associated with fluid flow and use only 10 μL of sample.

The antibodies used in this experiment were composed of two categories. Polyclonal antibodies were used to first attach the bacterial cells to the surface, and O-specific antibodies (O:4 and O:9) were used for specific serovar identification. The first test was to determine the lower detection limit of each bacterium in separate buffer solutions (PBS). The surface was functionalized by binding polyclonal antibodies to a hydrophobic C18 alkylsilane functionalized gold surface. The bacteria were then captured. In order to determine the specific bacterium, O-specific antibodies for a certain serovar (O:4 for *typhimurium* and O:9 for *enteritidis*) were then allowed to attach to the bacteria, increasing the change in refractive index. The detection limits in buffer were 1.25×10^5 cells/mL for *typhimurium* and 2.50×10^8 cells/mL for *enteritidis*. The same test was performed with bacteria in spiked milk. The detection limit for *typhimurium* increased to 2.50×10^5 cells/mL and remained at 2.50×10^8 cells/mL for *enteritidis*. O-specific antibodies were cross-reacted with the opposite strain of *Salmonella* and with *E. coli*, and these tests determined that the polyclonal antibody was specific to *Salmonella* and the O-specific antibodies were specific to their respective serovars.

Subsequently, experiments were performed to test the detection of each serovar in a mixture of both serovars in spiked milk. Lipopolysaccharide (LPS) for both serovars of *Salmonella* was purchased for further studies on sequential identification in single-channel SPR. The O-specific detection signals were additive when both antibodies were added at the same time, indicating that simultaneous detection is possible. Multichannel analysis was used to detect the different serovars (one channel per serovar). Sequential detection in a single channel was also performed successfully; however, it was determined that, due to the small field size in the SPR system, the signal of the second O-specific interaction was reduced. Therefore, the serovar with the lowest detection signal must be tested first if the assay is to be performed sequentially in a single channel.

Bacterial Cell Detection with Surface Plasmon Resonance Imaging (SPRi). The commercialization of SPRi systems is allowing a significant expansion in the number of measurements that can be obtained in a single experiment. Multiplexed binding studies can be performed using a single sample, which provides a better comparison between receptors and/or coatings. Multichannel experiments can also be performed on a single sensing surface, significantly increasing testing throughput.

Acidovorax avenae Detection in Plant Samples. *Acidovorax avenae* subsp. *citrulli* (*Aac*) is a bacterium responsible for bacterial fruit blotch in watermelons and cantaloupes, a devastating crop disease that is transmitted through seed infection. In a series of experiments, Puttharugsa et al. tried to determine if surface plasmon resonance imaging (SPRi) is a viable option for the detection of *Aac* in naturally infected plants.[111] Antibodies fixed to the gold sensor surface selectively bound the *Aac* bacteria, and the home-built 7 fluid channel SPRi instrument interpreted the changes in light reflectivity to determine the presence of the specific bacteria at various concentrations. The antibodies used were monoclonal antibody mAb 11E5 (produced in mice and found to be very specific to *Aac*) and rabbit polyclonal antibody to membrane protein complex of *Aac* (rPAb-MPC).

The surface was functionalized by utilizing mixed self-assembled monolayers (SAMs) which consist of two thiols of different chain lengths. A mixture of 11-MUA, which contained a carboxyl group that bound to the monoclonal antibody, and 3-mercaptopropanol (3-MPOH), containing a hydroxyl group as a spacer, in a concentration ratio of 1:40 was used as an attachment point for the monoclonal antibody mAb 11E5. Casein was used to block uncoated areas and prevent nonspecific adsorption, increasing the specificity of the process for detecting *Aac*. In addition, a sandwich assay was created by adding rPAb-MPC to the bound *Aac*, which was found to reduce the amount of cells required for detection.

The SPRi system was first used to identify the optimal antibody surface concentration for the highest specific *Aac* binding. Through a multichannel experiment, both whole and broken cells were added to channels with varying amounts of bound antibody, and it was found that 10 μg/mL was the optimal concentration of antibodies for specific cell binding. Subsequent SPRi experiments determined that the limit of detection with the direct detection assay (only monoclonal antibody) was 10^6 cells/mL; with the polyclonal antibody added in the sandwich assay, the limit of detection dropped to 5×10^5 cells/mL. Although these processes do not match up to the level of detection of the ELISA process (5×10^4 cells/mL), the SPRi technique is sufficiently sensitive for infection detection for *Aac*. In addition, many attributes of the SPRi identification processes described in this paper set SPRi apart from its competition, including the ability to perform multiple cycles with the same mixed SAM (a wash with 10 mM glycine pH 2.0 washed off the *Aac* cells but left the SAM intact) and the performance of simultaneous multichannel analysis. Most importantly, the SPRi process was able to selectively detect *Aac* in a naturally infected plant.

On-Chip Culturing for Low-Level Bacterial Detection. Very recently, Bouguelia et al. used SPRi to detect very low levels of several bacterial species by adding a culturing step on the sensing chip.[112] They tested *Streptococcus pneumonia* in growth media, *E. coli* in spring water, and *Salmonella enterica* in raw cow milk and ground meat. To selectively detect the bacterial species, they used a series on antibodies, which were first functionalized using NHS-pyrrole and immobilized onto the sensing surface using electro-polymerization. The remaining unspotted surface was blocked with 1% (w/v) bovine serum albumin (BSA).

Two 500 μL reaction chambers were custom-made using polyether ether ketone (PEEK) plastic to fit over the patterned sensing chip. One chamber was filled with a blank control while solution in the second chamber contained bacteria. The samples were incubated at 37 °C and continuously monitored for approximately 24 h using SPRi. Bacterial attachment was detected within 3 to 10 h after the start of the experiment depending on the starting concentration of bacteria used in the samples. The lowest bacterial concentration detected using this approach was 2.8 ± 19.6 CFU/mL.

Monitoring of Biofilm Formation and Removal. The imaging capabilities of SPRi also expand the applications beyond binding and coating studies. Our group is exploiting these capabilities to visualize biofilm formation and removal.[113,114] We placed a custom-made polymer chamber over bare SPRi gold sensing surfaces. For biofilm formation, we filled the chambers with trypticase soy broth (TSB) solutions containing wild type *Pseudomonas aeruginosa* and PelA mutant cells. The PelA mutant *P. aeruginosa* are genetically modified so that they are not able to produce robust biofilms. We imaged the chambers for several hours and were able to observe both types of cells attaching to the sensor surface and the wild type cells producing biofilm. For biofilm removal, we allowed wild type *P. aeruginosa* to grow on the surface for several hours while continuously flowing fresh growth media through the chamber to produce a healthy biofilm on the sensor surface. After 17 h, we introduced 1% sodium

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dodecyl sulfate (SDS) to the flow and observed the decomposition of the biofilm (Figure 4).

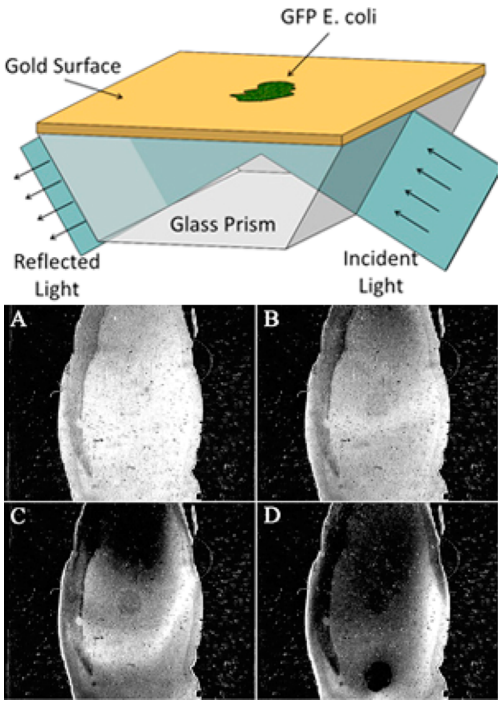

Figure 4. Surface plasmon resonance imaging (SPRi) of biofilm removal. After 17 h of biofilm formation with continuous flow of fresh media, the solution being introducing into the chamber was switched to 1% SDS. (A) At the start of SDS flow; (B) after 1 h; (C) 2 h; and (D) 3 h. Each image is 1 cm in width.

SUMMARY

The spectrum of cell analysis applications described in this Review illustrates the growing level of interest and activity in this area. The combination of quantitative and label-free analysis with SPR provides interesting and new opportunities for fundamental and applied research. The examples above demonstrate the ability to manipulate and characterize cells on surfaces with SPR, and this technology will further our understanding of cellular behavior in physiologically relevant contexts. We expect that much more engineering and customization effort will go into SPR instrument development in the coming years to expand analysis capabilities and couple SPR with other analytical techniques for both mammalian[115] and bacterial cells[116] and that the use of SPR for cellular analysis will increase exponentially as the instruments continue to decrease in price and become readily available. Meanwhile, efforts to detect cells in complex samples using SPR are progressing rapidly, and the focus will soon shift from "proof-of-concept" biosensors to commercially available functionalized sensing chips and specialized SPR systems for agricultural, diagnostic, and other point-of-care applications.

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Notes

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