Chapter 13
Immunosensors

As we have learned from Chap. 1, biosensors utilize bioreceptors to capture and analyze specific biomolecules of interest. Antibodies, enzymes, and DNAs have been used commonly as bioreceptors; however, *antibodies* are the most common and widely studied. In addition, *antigens* can also be used as bioreceptors to capture and analyze specific antibodies. Biosensors that utilize antibodies or antigens as bioreceptors are called *immunosensors*.

Immunosensors are being widely investigated and developed for practical applications such as medical and veterinary diagnostics, food safety, and environmental monitoring. Because antibodies are very specific to proteins, viruses, bacteria, cells, etc., the sensitivity and specificity of immunosensors are much more superior to other types of biosensors.

We have learned three major transducers that are commonly used in biosensor applications: optical (Chaps. 8 and 9), electrochemical (Chap. 10), and piezoelectric (Chap. 11). Immunosensors are generally classified depending on the type of transducers: optical immunosensor, electrochemical immunosensor, and piezoelectric immunosensor.

13.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Immunosensors have become very popular recently, although the use of antibodies in biological assays has been and still is a very common practice in laboratory-based analyses. One particular assay protocol that has dominated over others is the enzyme-linked immunosorbent assay (ELISA).

We have already learned briefly about the working principle of ELISA in Chap. 1. Figure 13.1 is basically identical to the sandwich immunoassay with enzyme, described previously in Fig. 1.11. In ELISA, a solid support (microwells; described later) is pre-immobilized with antibodies. Any empty spaces are further immobilized with *passivating proteins*, usually bovine serum albumin (BSA), to prevent any nonspecific bindings. A target solution is added to this surface, and

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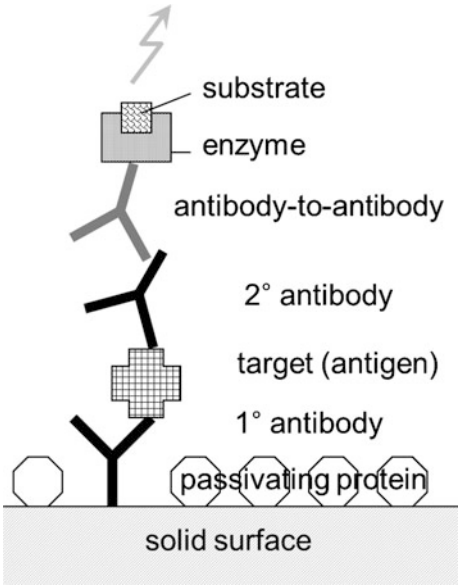

Fig. 13.1 Typical ELISA (identical to the portion of Fig. 1.11)

antibody-antigen binding occurs if a complementary antigen exists in the target solution. The surface is then rinsed; leaving only the bound target antigens on the surface. Antibody-antigen binding now occurs, but at this moment there is no way to tell whether binding has really occurred or not. Therefore, we need to add additional materials that will bind specifically to the target antigen as well as generate optical or electrochemical signals. To this end, the same antibody is added (called *secondary antibody* or 2° *antibody*) to the surface, which should specifically bind to the target molecule. As a result, the target molecule is sandwiched between two identical antibodies. In this sense, ELISA is often referred to as a *sandwich immunoassay*. The surface is rinsed again to remove excess secondary antibodies. After that, antibody-to-antibody is added that should specifically bind to the secondary antibody, which is typically pre-conjugated (through covalent binding) with an enzyme. To save time, secondary antibody is sometimes pre-conjugated with antibody-to-antibody-enzyme conjugate. Rinsing is then followed to remove any excess antibody-to-antibody-enzyme conjugates. Substrates to the enzyme are finally added, which is usually designed to undergo color change, typically from colorless to yellow or blue colorations. The more targets you have on the surface, the more coloration you should have from the surface.

Note that the above description is about detecting antigens using antibodies. Obviously, the reverse is also possible, i.e., detection of antibodies using antigens. In that case, the ELISA plate should be pre-immobilized with antigens rather than antibodies, and the secondary antigens must be covalently conjugated to an appropriate enzyme. A good example of antibody assay using antigen as bioreceptor is the detection of human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS). The amount of HIVs in the human body is typically very small that is hard to detect. The presence of HIVs in the human body triggers the generation of a substantial amount of antibodies against them (anti-HIV; unfortunately, these antibodies cannot eliminate HIVs), which are a lot easier to detect.

At this point, you may ask: "Why not directly tag the secondary antibody with an enzyme or a fluorescent dye?" The reason is that as there are so many different types of antibodies, it is impractical to tag all the different antibodies with an enzyme or a dye. It is better and more economical to tag the antibody-to-antibody than tagging the entire library of antibodies.

ELISA is typically performed in a plastic container that has multiple wells, typically 8 × 12, thus 96 wells. This container is called a *microwell plate* or simply *microplate* (Fig. 13.2). This setup allows the user to perform 96 independent assays at the same time. For example, the glucose assay performed in the previous chapter would preferably be performed using a microplate. Typical ELISA kits come with all necessary reagents along with a microplate that are pre-immobilized with the antibodies (and of course with passivating proteins). This antibody-immobilized microplate is specifically referred to as *ELISA plate*.

Solutions are added to each well of a microplate using a pipette. To speedup the assay, a multi-channel pipette can be used, where 8 pipettes are bundled together to dispense liquid at a single stroke. Since a standard microplate has 8 rows and 12 columns, a multi-channel pipette can feed the entire 96 wells in just 12 strokes.

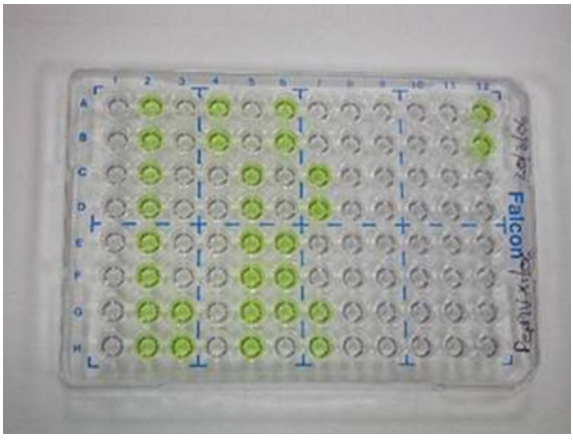

Fig. 13.2 A microplate

Fig. 13.3 A microplate reader. A microplate is placed onto the bottom right tray and inserted into the reader

Rinsing is also done using a pipette. One example of rinsing using a pipette is: (1) eliminate the liquid from a well through suction, (2) add a rinsing solution, usually a phosphate-buffered saline (PBS) with a small amount of detergent, (3) wait for a short period of time, and (4) eliminate the rinsing solution. As you can imagine, typical ELISA requires multiple steps of reagent addition and subsequent rinsing, which is highly labor-intensive.

In the earlier days, the light emission from enzyme-substrate binding was visually identified by the naked eye. The light intensity is often classified into several categories such as +++ (strong positive), ++ (very positive), + (positive), w+ (weak positive), and − (negative). Nowadays, of course, we have a sophisticated machine to quantify these light emissions, called the *microplate reader* (Fig. 13.3). The microplate reader is essentially a 2-D version of a spectrophotometer, or a scanner with specific light sources.

Many commercial ELISA kits are available on the worldwide market. We perform an ELISA exercise on *insulin* in this chapter, a hormone responsible for the control of glucose metabolism, and thus important in the diagnosis and prognosis of diabetes.

13.2 Antibodies

The choice of antibodies greatly affects the performance of ELISA and other immunosensors. Antibodies can be produced by injecting a specific antigen into animals, typically a mouse, rat, goat, rabbit, pig, or a horse, etc. The animal's immune system will try to recognize the shape of the antigen, and eventually generate a mixture of antibodies that recognizes different portions (called *epitopes*) of the antigen. The resulting "mixture" of antibodies is called polyclonal antibody

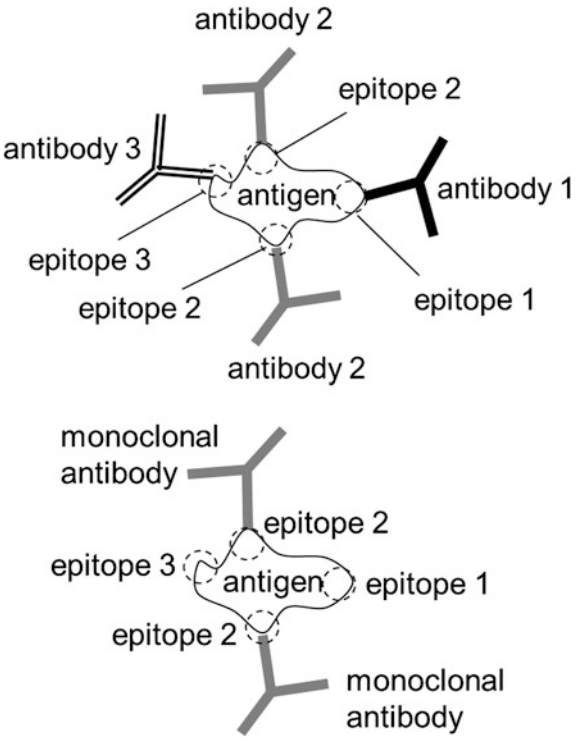

Fig. 13.4 Polyclonal antibodies bind to multiple types of epitopes of a single antigen (*top*). Monoclonal antibodies bind to a single type of epitopes of an antigen (*bottom*)

(pAb) (Fig. 13.4). Despite being a mixture, all of them are still able to recognize the same antigen.

In many cases, certain epitopes may exist over several different antigens. A good example is *Escherichia coli* and *Salmonella*. These two bacteria are somewhat similar to each other and share a certain amount of epitopes. Therefore, pAb to *E. coli* can also bind to *Salmonella*, albeit to a lesser extent, since only a fraction of epitopes are identical to each other. This problem is referred to as *cross-reactivity*. Therefore, there is a need to develop an antibody that recognizes only one type of epitope, called monoclonal antibody (mAb) (Fig. 13.4).

Monoclonal antibodies are first produced using spleen cells from mice. The *spleen* is found in all vertebrate animals and one of its functions is to generate antibodies. A specific antigen is injected into a mouse, and the spleen cells (specifically *B cells* that produce antibodies) are harvested. These spleen cells are then fused with *myeloma cells* (*B cell cancer*), resulting in hybrid cells (*hydridoma cell*). These cells do not die but proliferate virtually infinite number of times. A single cell line is chosen and cultured, which will produce only a single type of antibodies (i.e., mAb). Recently, other animal cells have also been used to produce mAb, most notably rabbits. These mAb are widely used in ELISA and other immunosensor applications whenever a higher specificity is required.

13.3 Antibody Fragments and Aptamers

Antibodies are *Y*-shaped and have two pairs of polypeptide chains: heavy chains and light chains (Fig. 13.5). The very top portions of both heavy and light chains vary depending on the target antigen that the antibody can recognize (called *hypervariable regions*) and the remainder are identical (called *constant regions*). In general, for ELISA and immunosensing applications, we do not need the constant regions. The top branch portion of antibodies, called *F(ab)$_2$ fragment*, or even smaller fragments such as single-chain antibody (scAb) fragment and single-chain variable antibody (scFv) fragment, can be used for ELISA and immunosensors (Fig. 13.5). Since these fragments are smaller than whole antibodies, they are potentially more stable, easier to immobilize on solid surfaces, and show better reactivity with the target antigen due to its smaller size. F(ab)$_2$ fragment can be made by cleaving a whole antibody with *papain enzyme*. The smaller fragments are often synthesized by *recombinant technology*. The recombinant technology has a lot of potential, since they are synthesized within bacteria by inserting a genetic sequence that will synthesize only one type of polypeptide (thus antibody fragment), which may replace the mAb. Currently the binding activity of these recombinant antibody fragments is inferior to that of mAb.

Antibodies have been used as a primary type of bioreceptors in bioanalytical methods and biosensor applications. There are, however, many obstacles in large-scale production of antibodies. They still require animals (polyclonal antibodies) or cell lines (mAb). If the antigens do not trigger the immune response,

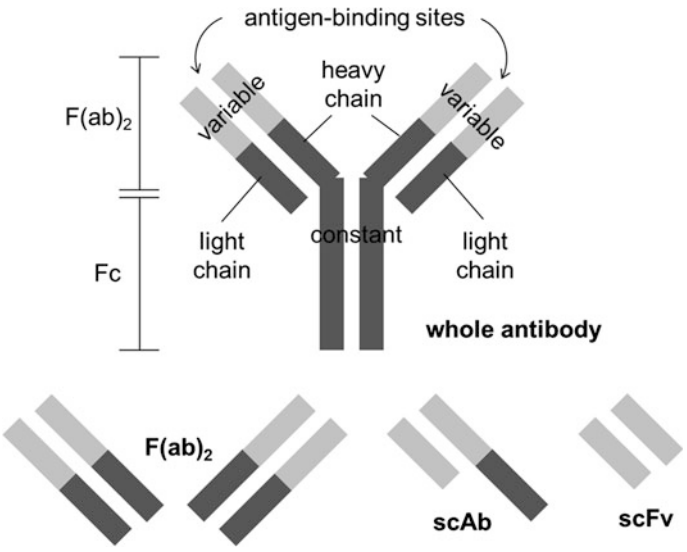

Fig. 13.5 A whole antibody molecule (*top*), showing heavy and light chains; variable and constant regions; F(ab)$_2$ and Fc portions. Antibody fragments (*bottom*), showing F(ab)$_2$, single-chain antibody (scAb) and signle-chain variable fragment (scFv)

there is no way to produce antibodies. Some scientists believe aptamers could serve as a good alternative bioreceptor to replace these limitations of antibodies.

Aptamers are essentially artificial nucleic acids (DNA or RNA) that recognize or bind to specific antigens, just like antibodies. Recall that antibodies are proteins, which are made from posttranslational modifications and folding of polypeptide chains. Aptamers are mostly single-chain nucleic acids, either single-stranded DNA (ssDNA) or RNA. (Note that the stable nucleic acids inside the nucleus of a cell are *double-stranded DNA* or *dsDNA*, which has a double-helical structure. RNA is inherently single-stranded.) These single-chain structure can bind partially to itself and/or fold just like proteins, resulting in a 3-D structure. Some single-chain nucleic acids can recognize and bind to a specific molecule, which are called aptamers.

Aptamers are isolated from complex libraries of artificial nucleic acids by an iterative process called systematic evolution of ligands by exponential enrichment (SELEX). A large number of genetic sequences are chosen and synthesized, including a randomized region. The amount of these nucleic acids are amplified through the process called polymerase chain reaction (PCR). The target antigen is added to the resulting mixture, and only a very small portion of candidate nucleic acids are able to bind to the target antigens. The resulting nucleic acid–antigen complex can be filtered, precipitated, or captured with gel, to choose the candidate molecule that possesses the best binding performance. The amount of chosen molecule is again amplified by PCR. The whole cycle is repeated more than 10 times to select only the best ones.

These aptamers can be used just like antibodies. Figure 13.6 illustrates how aptamers can be used for an ELISA-like process, called enzyme-linked oligonucleotide assay (ELONA), where two aptamers sandwich the target antigen. Aptamer-based biosensors are specifically called *aptasensor*. A hybrid of ELISA and ELONA is also possible. For example, an antibody can be immobilized on a solid surface (as a capture molecule) while an aptamer is used to sandwich the

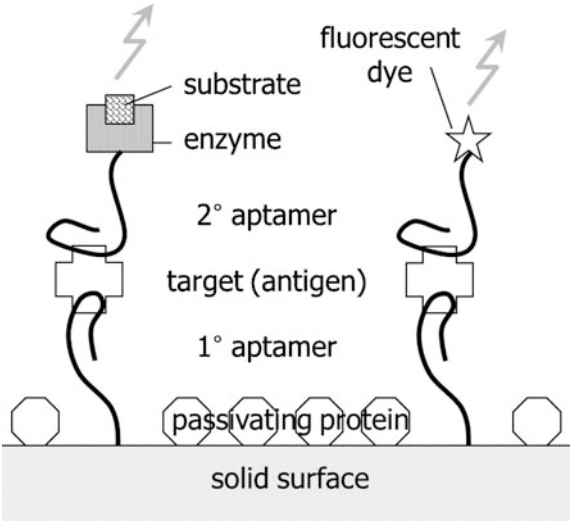

Fig. 13.6 Enzyme-linked oligonucleotide assay (ELONA) or sandwich aptamer assay

target molecule labeled with an enzyme or a fluorescent dye (as a detector molecule). Conversely, the reverse is also possible: aptamer as a capture molecule and antibody as a detector molecule.

Theoretically, we can produce aptamers for any target antigens, as they do not require any immune response of animals or cell lines. Moreover, there are virtually no batch-to-batch variations, which are commonly found in antibodies. The small size of aptamers (antibodies are relatively big, with molecular weight of 150,000) makes it less vulnerable to the denaturation caused by heat and/or long-term storage.

Unfortunately, the number of available aptamers that can readily be used for ELONA and aptasensors is very small at this moment, mainly due to the labor-intensive and time-consuming nature of the SELEX process. For this reason, the commercial market is still dominated with antibodies. However, it may be a matter of time for the aptamers to dominate this market, yet we do not know how long it will take.

13.4 Lateral-Flow Assay (LFA)

There is a simpler format of immunosensors that gained great popularity in the diagnostics market, called *lateral-flow immunochromatic assay* or simply lateral-flow assay (LFA). The most well-known example of LFA is the pregnancy test. The user applies a drop of urine sample to a cassette-type device or dips the strip into a urine sample. If two pink lines show up, you are pregnant; and if one pink line shows up, you are not pregnant. No line indicates the test failed (Fig. 13.7).

LFA is essentially a membrane that is pre-loaded with a couple of different antibodies. Liquid (urine or blood sample) flows through the membrane by osmotic action. There are two bands in the strip, as shown in Fig. 13.8, one with antibodies to the target (for pregnancy, the target is human chorionic gonadotropin or hCG), the other with antibodies to antibody (anti-IgG). Once the urine/blood sample (which may

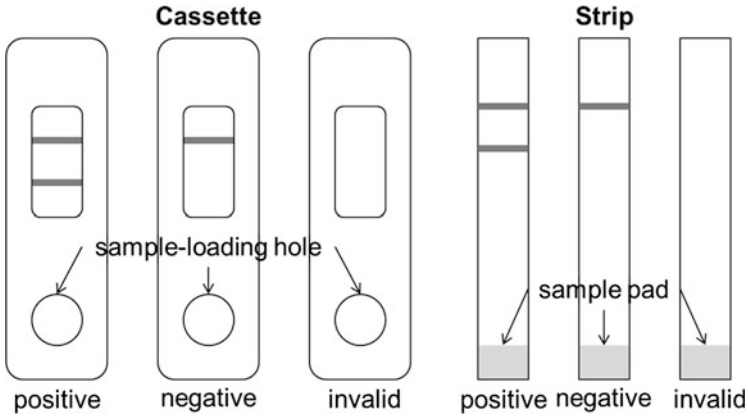

Fig. 13.7 Cassette-type and strip-type LFA

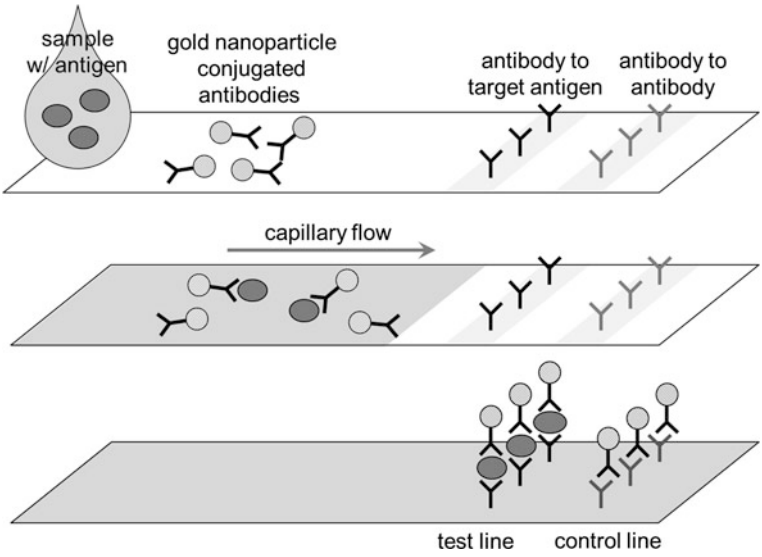

Fig. 13.8 Schematic of LFA

contain hCG) is applied to the inlet, the anti-hCG-gold nanoparticles (anti-hCG-AuNP; pre-loaded within the strip) may or may not bind to the target hCG. The liquid travels through the membrane by capillary action. When the liquid hits the test line, the hCG + anti-hCG-AuNP complex is captured there. The unbound, excess anti-hCG-AuNP continues to travel to the control line, which is eventually captured there.

Gold nanoparticles (*AuNPs*; will be further discussed in Chap. 15) absorb green light and some blue light, so it looks pink (Fig. 13.9). Therefore, two pink bands indicate the presence of target, one band the non-presence of target, and no band the failure of assay (mostly because of not enough sample volume to achieve capillary action).

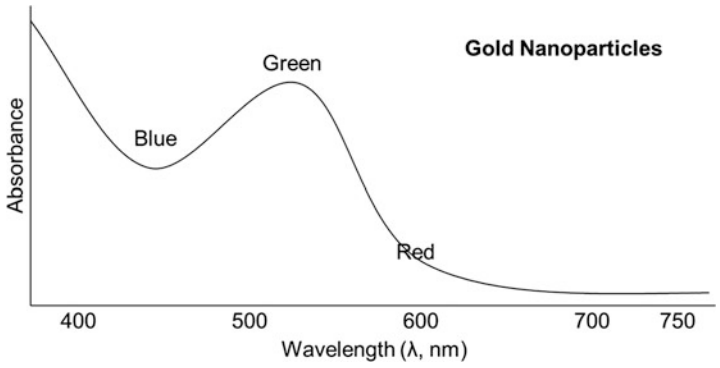

Fig. 13.9 Absorption spectrum of gold nanoparticles (AuNPs), indicating strong absorption in green, moderate absorption in blue, and almost no absorption in red. With white light, it will reflect most of red and some of blue, resulting in a pink coloration

LFA is an easy-to-use and inexpensive immunosensor, although there is an issue of reproducibility, as well as inferior sensitivity over ELISA (high detection limit; *detection limit* is the lowest concentration that can be detected with statistical significance). LFA itself does not provide quantitative readouts, but can be complemented using an optical reader similar to the microplate reader. Recently, the use of a smartphone camera is suggested to obtain quantitative readouts from LFAs.

13.5 Optical Immunosensors

The use of a microplate reader for ELISA is, in a way, a form of *optical immunosensor*. There have been numerous efforts in minimizing the labor required for ELISA, converting the ELISA kit into a user-friendly optical immunosensor. If only a single assay is required, the microplate can be replaced with a single test tube and the microplate reader with a pair of LED and PD (refer to Chap. 8 Laboratory Task 2).

The test tube may be further replaced with an optical fiber probe, where the antibodies are immobilized onto the surface of the exposed optical fiber core, again with some passivating proteins. The probe is then dipped into the target solution, followed by dipping and stirring in the rinsing solution (typically a buffer solution with some detergent). The probe is then dipped into a solution of secondary antibody/antibody-to-antibody conjugate, the latter of which is labeled with a fluorescent dye (Fig. 13.10).

The test tube can also be replaced with a microchannel, where the target solution continuously flows through the microchannel (flow injection analysis). The antibodies are immobilized onto the inner surface, at a specific location, of a microchannel. The continuous flow characteristics make the rinsing very effective.

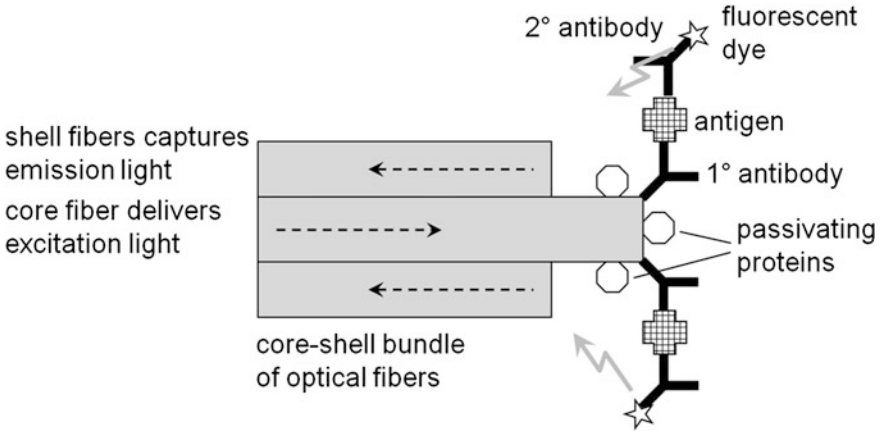

Fig. 13.10 Optical immunosensor using an optical fiber probe. Note that the antibody-to-antibody is omitted here for clarification purpose

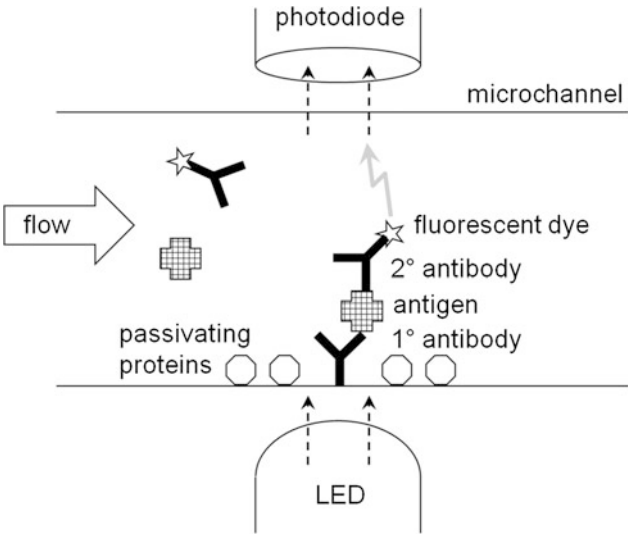

Fig. 13.11 Optical immunosensor using a microchannel

In fact, neither suction-dispensing cycle nor stirring is required, as the flow should remove any excess molecules from the inner surface of a microchannel. Light is irradiated to the location where the antibodies are immobilized; while the light sensor reads the signal from the other side of a microchannel (Fig. 13.11). This schematic is popular in lab-on-a-chip applications, which will be discussed in Chap. 14.

13.6 Surface Plasmon Resonance (SPR) Immunosensor

The above approach is still cumbersome as it requires secondary antibody and subsequently multiple rinsing steps. There is an alternative biosensor that requires neither secondary antibody nor fluorescent dye, called surface plasmon resonance (SPR) immunosensor.

SPR immunosensor is largely based on total internal reflection (TIR) that we have learned in Chap. 8. As shown in Figs. 13.12 and 13.13, light hits the metal–liquid interface, and it exhibits TIR due to the difference in refractive indices of metal and liquid. Light beams are irradiated at a range of incident angles and so are the reflected light beams. In SPR, there are two important modifications: (1) the incident light is polarized and (2) the surface is a thin metal film coating, usually gold. Polarization means that the light is oscillating only at a certain orientation, where normal light oscillates at various orientations. When light hits the gold

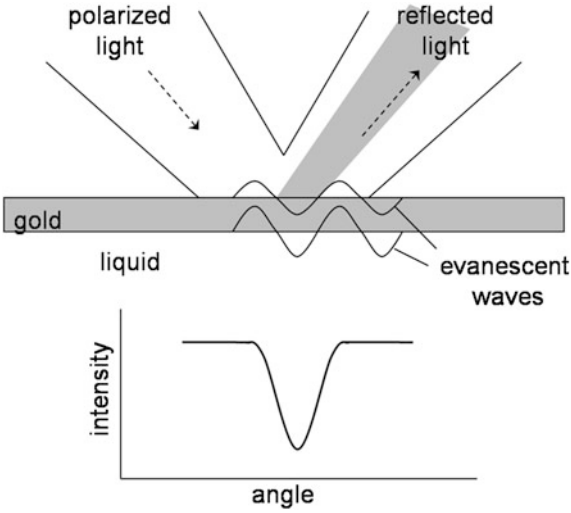

Fig. 13.12 Surface plasmon resonance (SPR)

surface, electrons and holes are created (just like photoresistor, photodiode, or phototransistor). Because the incoming photons are oscillating only at one orientation, the generation of electrons/holes (electric charges) will also be oscillated in one direction. This charge oscillation can propagate parallel to the gold film, called *evanescent wave*. Also this propagation is short-lived, thus it does affect the reflected light. For a certain angle of incident light, the incident light can be matched to this evanescent wave (resonated). If this resonance happens, the light intensity of reflected light at that angle will be greatly reduced (Fig. 13.12).

The surface evanescent wave is a function of refractive indices of gold and liquid. If some molecules adsorb to the surface of gold film, the overall refractive index of liquid near the gold film changes, as well as the resonance angle (Fig. 13.13). In a typical SPR apparatus, this change of resonance angle (with reference to a bare surface) is shown as its sensor signal. This sensor signal is roughly equivalent to the mass of the adsorbed molecules. However, exact determination of the adsorbed mass is difficult in SPR sensors, as the refractive indices and other optical properties are quite different from molecule to molecule.

In an SPR immunosensor, the gold surface is pre-immobilized with antibodies, and the SPR signal with these antibodies will serve as a baseline. The sensor chip is exposed to a flow channel to facilitate the introduction of target/reagent solutions and subsequent rinsing. The first commercial SPR-based biosensor was marketed by Biocore AB Corporation based in Sweden; and commonly used in many applications. Recently there have been many attempts to further miniaturize the entire system, especially miniaturizing the flow channel into a microchannel, thus lab-on-a-chip platform (refer to Chap. 14).

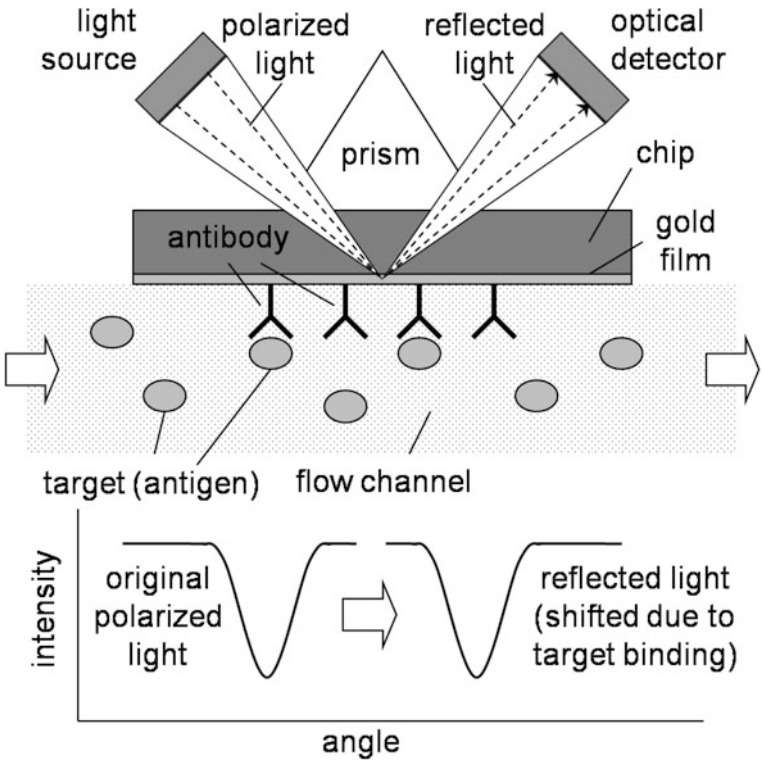

Fig. 13.13 SPR immunosensor

13.7 Electrochemical Immunosensors

As shown in Fig. 13.1, the enzyme-substrate binding typically involves oxidation reaction. This opens up an opportunity to detect ELISA in an electrochemical way (*electrochemical immunosensor*), as shown in Fig. 13.14. This oxidation itself, however, generates too small amount of electrical charges (electrons and holes). Therefore, we need a *mediator*, which can be repeatedly oxidized and reduced under an applied voltage. With the help of a mediator and an applied voltage, the oxidation and reduction reactions can make cycles, which will generate measurable change in electric current (thus *amperometric* detection). This concept is identical to the electrochemical glucose sensing that we have learned in Chap. 12.

The most common substrate/mediator pair used for ELISA and other types of electrochemical immunosensors is horseradish peroxidase (HRP) and tetramethylbenzidine (TMB). The substrate for HRP is hydrogen peroxide (H_2O_2) (Fig. 13.15).

Like electrochemical glucose sensors, typical electrochemical immunosensors involve the use of test strips, shown in Fig. 13.16. The sensor surface is pre-coated with appropriate antibodies (with passivating proteins), and a series of solutions

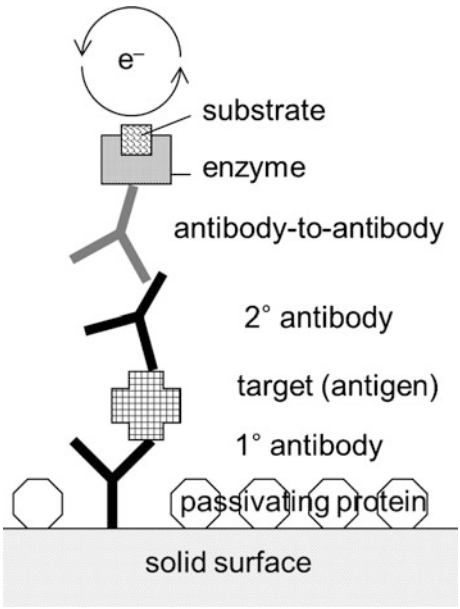

Fig. 13.14 Electrochemical ELISA (identical to the portion of Fig. 1.11)

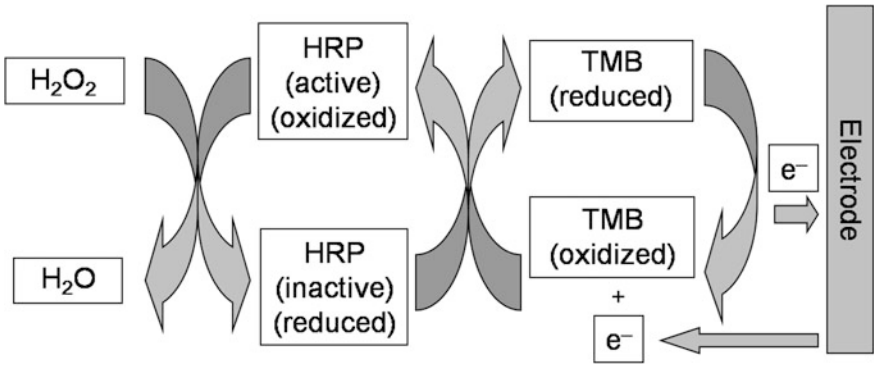

Fig. 13.15 Typical electrochemical detection in ELISA. HRP = horseradish peroxidase; TMB = tetramethylbenzidine

(target, secondary antibody, antibody-to-antibody-enzyme conjugate, and mediator/substrate) are added drop-by-drop onto the sensor surface with appropriate rinsing steps.

There have been attempts to embed an array of electrodes to a 96-well ELISA plate. This electrochemical ELISA plate is placed onto an electrochemical microplate reader, which is essentially a current meter that measures current changes from 96 wells simultaneously.

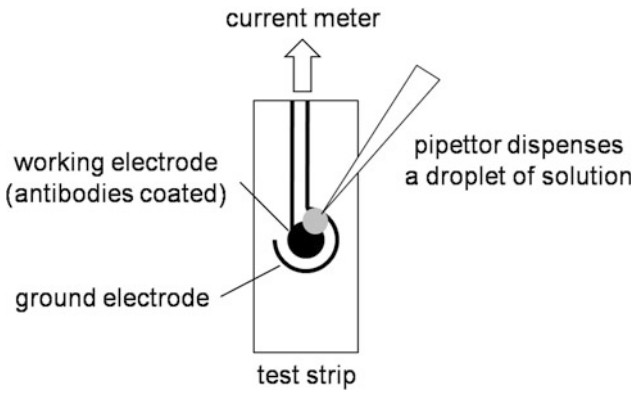

Fig. 13.16 A test strip for a typical electrochemical immunosensor

Question 13.1
Electrochemical immunosensors can also be implemented into a microchannel, just like the optical immunosensor. Design the electrode layout around the microchannel, using Figs. 13.1 and 13.11 as the starting point.

13.8 Impedance Immunosensors: Interdigitated Microelectrode (IME) Immunosensor

The SPR immunosensor is a label-free simpler version of optical immunosensors. It does not require secondary antibody, enzyme-substrate pair, or fluorescent dye. There is a similar version for electrochemical immunosensors, called *impedance immunosensors*.

Figure 13.17 shows a schematic of a typical impedance immunosensor. Antibodies are immobilized on a surface where two electrodes are patterned in a configuration called *interdigitated array*. When a big target like bacterium binds to the surface-bound antibodies, some of the target may be able to bridge the two electrodes, thus lowering the resistance. If electrode patterns are made very small (micro or even nanoelectrode), smaller targets such as viruses and proteins may be detectable.

To maintain the antibody-antigen binding and to prevent any possible oxidation/reduction, it is beneficial to apply alternating current (AC) rather than direct current (DC). We need to use an expanded version of resistance with AC power, called *impedance*. Impedance is described as a complex number (refer to Chap. 11):

$$Z(\text{impedance}) = R(\text{resistance}) + jX(\text{reactance}) \tag{13.1}$$

The magnitude of voltage oscillates at a certain frequency with AC power, and so is the current. The current-to-voltage ratio gives the impedance, where the real

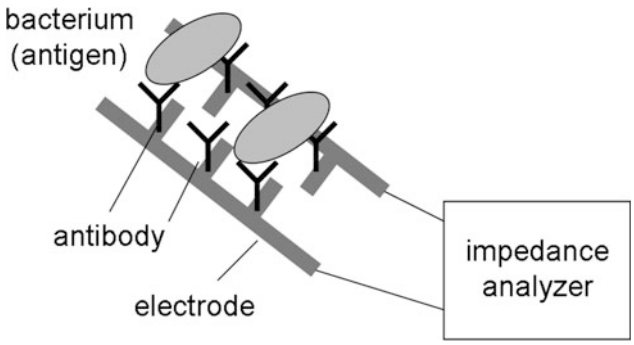

Fig. 13.17 An impedance-based immunosensor

part R (resistance) is related to the magnitude of oscillation while the imaginary part X (reactance) is related to the phase shift of oscillation. With DC power, there is no such oscillation and thus no phase shift, resulting in $X = 0$ and $Z = R$.

The interdigitated microelectrode (IME) can be also made as a test strip, similar to what is shown in Fig. 13.16. Significant steps of reagent additions and subsequent rinsing can be eliminated, thus making this assay a lot simpler and easier to use than any other electrochemical immunosensors. The only disadvantage of IME immunosensor is its complexity in microelectrode fabrication and subsequent cost associated with it. *IME immunosensors* have been successfully demonstrated for detecting pathogens in many different sample matrices, including *E. coli*, *Salmonella,* and avian influenza viruses.

Question 13.2
Again, the IME immunosensor can be implemented into a microchannel, as the optical immunosensor. Design the electrode layout around the microchannel, using Figs. 13.11 and 13.14 as the starting point. What is the benefit for incorporating the IME immunosensor in a microchannel rather than in a test strip?

13.9 Piezoelectric Immunosensors: QCM Immunosensor

Piezoelectric transducers can also be used for immunosensor applications, thus *piezoelectric immunosensors*. The quartz crystal microbalance (QCM) shown in Fig. 13.18 is the most popular type of piezoelectric transducer commonly used for immunosensing. (Refer to Chap. 11 for details).

Like SPR and IME immunosensors, *QCM immunosensor* is a label-free biosensor, i.e., it does not require secondary antibody, enzyme-substrate pair, or fluorescent dye. In addition, QCM immunosensor has the following advantages over SPR and IME immunosensors: (1) smaller and cheaper instrument than SPR immunosensor, potentially comparable to IME immunosensor; (2) simpler fabrication of sensor strips (QCM crystals) than the SPR and IME immunosensors.

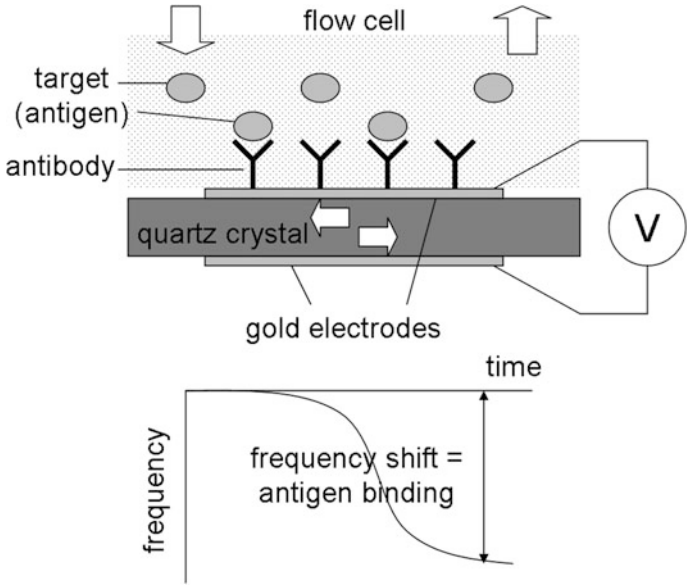

Fig. 13.18 A QCM immunosensor

Disadvantages of QCM immunosensor include: (1) the need for long incubation times with samples, typically in tens of minutes or more; (2) constant drifting of frequency (*f*) signal due to the energy accumulation of crystal oscillations (may be avoided by using QCM-D; refer to Sect. 11.5).

13.10 Immunosensing Kits Versus Handheld Immunosensors

All immunosensors described in this chapter require manual liquid handling, including reagent dispensing and subsequent rinsing using a pipettor. Several label-free immunosensors, SPR, IME, and QCM immunosensors, do reduce the amount of such labor, but they still require at least one step for each of reagent dispensing and subsequent rinsing. Many commercial immunosensors, therefore, are offered as kits, which include several bottles of reagents (some of them need to be refrigerated), disposable sensor strips or a microplate, and a transducer (a microplate reader, an SPR instrument, an impedance analyzer, or a QCM instrument). A pipette is not included in these kits but it is required. Due to the size and AC power requirement of these transducers, and the requirement of sample refrigeration, these assays must be conducted in a wet laboratory.

Nowadays, there have been attempts to convert this immunosensor kit into a handheld immunosensor device. The transducer is made as small as possible,

preferably with battery power. It is also equipped with its own microprocessor (for signal processing) and a small liquid crystal display (LCD) panel, with a small user interface (cursor keys and a couple of menu buttons). Reagents that require refrigeration, especially antibodies, are freeze-dried (or vacuum-dried) as powder, so that they can be stored at room temperature at least for a short term. The handheld device usually involves a microchannel to facilitate rinsing without necessarily using a pipette to rinse the surface.

There are other attempts to further automate the assays by incorporating more complicated shapes of microchannels, which enables facilitated mixing, separation, prolonged incubation, heating, etc. This area is called lab-on-a-chip, which will be discussed in Chap. 14.

13.11 Laboratory Task 1: Insulin ELISA Kit

In this task, you will need the following:

- Insulin ELISA kit (IS130D from Calbiotech Inc.)
- Deionized or distilled water
- Pipettes and pipet tips
- A microplate reader (or ELISA reader)
- A vortex mixer or a microplate shaker (optional)
- Absorbent paper towel

The insulin ELISA kit includes the following:

- A microplate, whose wells are coated with mAb to insulin
- Insulin standards
- Insulin enzyme conjugate
- Assay diluent
- TMB substrate
- Stop solution
- Wash solution concentrate

Specimen Collection

– The kit is originally intended for human serum, but we will test the kit using one of the insulin standard solutions. Obtain one of the insulin standard solutions created by the other team, without being informed of its concentration. This is your specimen.

Reagent Preparation

– Insulin enzyme conjugates come as a 20X concentrate. Use the assay diluent to dilute it to 1X, or in other words, 0.1 mL of the stock conjugate in 1.9 mL of assay diluent, which is sufficient for 20 wells.
– The wash solution also comes as 20X concentrate. Add 25 mL of wash stock to 475 mL of distilled and/or deionized water to make it 1X.

– There are six insulin standards in the kit, 0, 6.25, 12.5, 25, 50, and 100 μIU/mL. IU stands for international unit, an amount of substance based on biological activity of effect. The actual mass varies with substances. For insulin, 1 IU is equivalent to 45.5 μg. Therefore, the above concentrations correspond to 0, 0.28, 0.57, 1.1, 2.3, and 4.6 ng/mL.

Assay Procedure

– Pipette 25 μL of six insulin standards into appropriate wells.
– Pipette 25 μL of three identical urine samples into appropriate wells.
– Add 100 μL of 1X (i.e., diluted) insulin enzyme conjugate to all wells that contain standards and samples.
– Mix the solutions. You can place a sticker or a lid on top of the microplate to prevent leakage during mixing. You can then mix it manually with your pipette tip, or place the entire microplate onto a vortex mixer (for vigorous mixing) or microplate shaker (for gentle mixing).
– Incubate for 60 min at room temperature (18–26 °C).
– Remove liquids from all wells. Wash wells thrice with 300 μL of 1X (diluted) wash solution (Fig. 13.19). After the final wash, use an absorbent paper towel to remove all liquids from the wells.
– Add 100 μL of TMB substrate to all wells that contain standards and specimens.
– Incubate for 15 min at room temperature.
– Add 50 μL of stop solution to all wells that contain standards and specimens.

Visual Identification

– Visually identify the color intensity. Determine the standard that most closely matches the color intensity of your specimen. For example, if your specimen color is somewhere between 25 and 50 μIU/mL, your specimen's insulin concentration is estimated to be 37.5 μIU/mL.
– Repeat this for all three specimens. Calculate the average and standard deviation.

Microplate Reader

– Read absorbance on a microplate reader at 450 nm within 15 min after adding the stop solution.
– Construct a standard curve from six insulin standards and perform a linear regression (Fig. 13.20).
– Using this equation, determine the insulin concentrations of your three specimens. Calculate the average and standard deviation.

Question 13.3
Why do you have to construct a standard curve each time you make measurements?

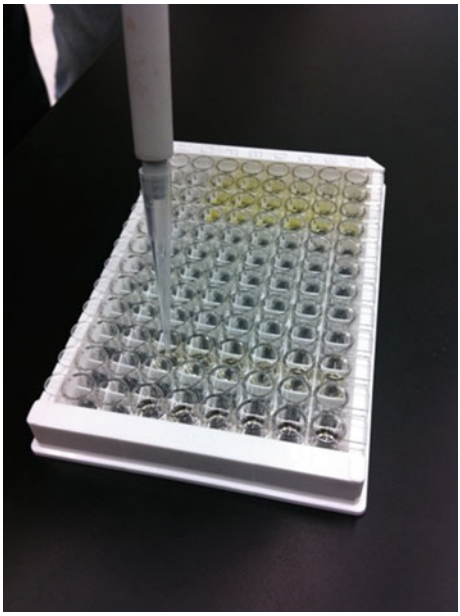

Fig. 13.19 Washing a well with a pipette

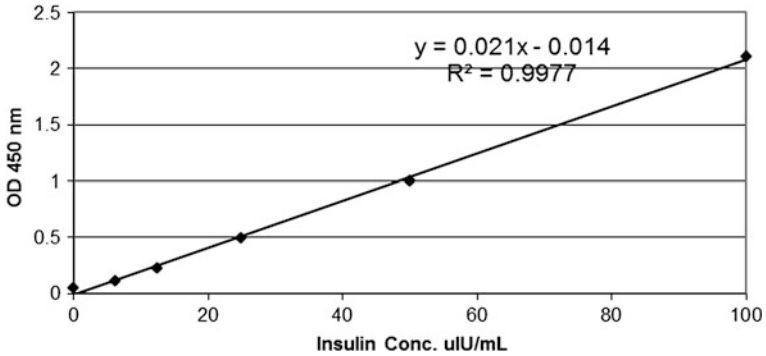

Fig. 13.20 An example of a standard curve from a microplate reader

13.12 Laboratory Task 2: Insulin ELISA Kit with Smartphone Camera

Today's *smartphones*, are mostly equipped with a high-resolution digital camera, a very bright white LED flash, and an operating system (either iOS or Android). Recently, there have been various attempts at using this smartphone as all-in-one

optical sensing platform, utilizing the flash as a light source, the camera as an optical detector, with the appropriate software (*application* or *app*, loaded in a smartphone) for data storage and processing.

How Digital Camera Stores Images

The resolution of a digital camera is typically represented by megapixels (MP), such as 5, 8, 10 MP, etc. For an 8 MP camera, the resulting size of a digital image is 3266 × 2450 pixels, which are roughly 8 million pixels. Each pixel is typically stored in 24-bit resolution. This 24-bit is further divided into three 8-bit groups, each corresponding to red (R), green (G), and blue (B) colors. Ideally, it would be great to store an entire visible spectrum (from 400 to 750 nm) per each pixel. However, the human eyes have only three types of *cone cells* that recognize only three different colors: red, green, and blue (*RGB*; called *trichromatic vision*), hence dividing into three basic colors is sufficient rather than storing an entire spectrum.

From Fig. 13.21, you can probably notice that the green and red spectra are very close to each other. In fact, some humans cannot see the difference between red and green colors: they see both red and green as a single color. This particular situation is called *color weakness* or *dichromatism*. Bear in mind that most vertebrate animals (e.g., dogs) have *dichromatic vision*. Scientists believe that humans have evolved from dichromatic vision to trichromatic vision, where red-green color differentiation is not substantial compared to green-blue color differentiation.

Simply take a photograph of the microplate using your smartphone, preferably with white background. Figure 13.22 shows the actual image. Using an appropriate software, preferably with ImageJ (free software available from U.S. National Institutes of Health, http://rsbweb.nih.gov/ij/) or Adobe Photoshop (commercial software), take 10 pixels from each well and record the RGB color codes. Note that

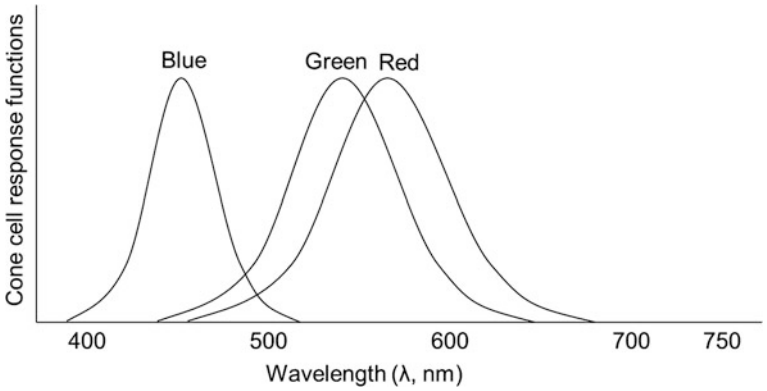

Fig. 13.21 Response functions curves for human cone cells

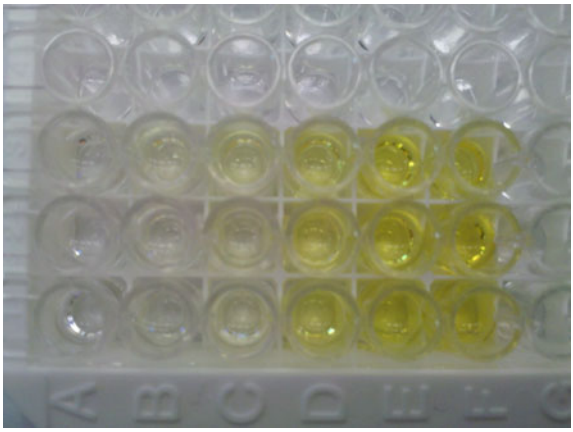

Fig. 13.22 A digital photograph of a microplate from Task 1

8-bit resolution enables the color code to change from 0 to 255 (2^8 = 256). The average color codes are shown in Fig. 13.23. You can notice that both red and green color intensities are relatively constant (around 126) and do not change over insulin concentration. However, blue color intensities do decrease as increasing insulin concentration. When red, green and blue intensities are about the same and

Insulin conc. μIU/mL	0	6.25	12.5	25	50	100
Red intensity	123	126	128	127	125	116
Green intensity	123	129	132	132	128	120
Blue intensity (I)	127	111	107	75	39	7
A = log (I_0 / I) [blue]	0	0.0585	0.0744	0.2287	0.5123	1.2587

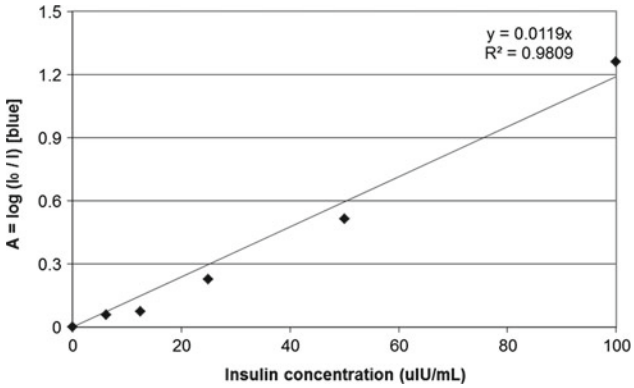

Fig. 13.23 Experimental data of Task 2: standard curve using a smartphone camera. The blue intensity with 0 μIU/mL insulin solution was used as I_0

sufficiently bright, the human eye would perceive it as white color (e.g., column A in Fig. 13.22). If blue intensity is significantly lower than red and green intensities, the human eye would perceive it as in-between color of red and green, which is yellow (e.g. columns E and F in Fig. 13.22). Therefore, we need to evaluate blue color intensities to quantify the yellowness of each microplate well. This corresponds very well with Task 1, where we measured the absorbance at 450 nm, which is blue color. Using only the blue intensities, we can calculate the absorbance.

The slopes of two different standard curves are: 0.021 (microplate reader) >0.012 (cell phone camera).

Question 13.4
If the smartphone camera processes each pixel at 36-bit (12-bit each for three basic colors), can the slope of standard curve with a cell phone camera be increased (i.e., becomes more sensitive)? Repeat this question if the cell phone camera processes each pixel at 24-bit grayscale.

13.13 Laboratory Task 3: Pregnancy Test (LFA) with Smartphone Camera

By far the most commercially successful LFA is the pregnancy test. These tests detect hCG, a glycoprotein hormone produced in high concentration almost exclusively during pregnancy. Thus, while a female who is not pregnant will have an hCG concentration of <5 mIU/mL in her urine, a female who is pregnant may have early concentrations as high as 500–8000 mIU/mL, and later-term concentrations above 50,000–100,000 mIU/mL. Much of this appears in the urine, which allows for simple and noninvasive pregnancy testing. The extremely high hCG concentration from the pregnant female's urine enabled the popularity and commercial success of pregnancy test. Other LFA's have followed, although most of them have not yet been successful compared to the pregnancy test.

While pregnancy tests are typically analyzed in a positive-or-negative ("yes-or-no") manner, it is actually possible to quantify the concentration of hCG in a sample based on the relative darkness of the positive "test band." In this task, we will be using pregnancy tests to examine three known concentrations of hCG suspended in water. Using a smartphone camera, we will take pictures of the bands and then quantify the concentrations based on the relative pixel intensities in these images. Similarly, we will complete one additional test with an unknown sample and determine its approximate hCG concentration based on the standard curve we develop from the three known samples.

In this task, you will need the following:

- Five pregnancy tests (LFA)
- hCG
- Five centrifuge tubes
- Pipettes and pipet tips
- A vortex mixer
- Smartphone

1. Prepare a stock solution of hCG in 2000 mIU/mL. Using this stock solution, prepare three 500 μL dilutions of hCG in concentrations of 0, 500, and 1000 mIU/mL. These will be used in forming your standard curve. Also prepare an hCG solution of unknown concentration (e.g., other than 500 and 1000 mIU/mL).
2. With one pregnancy test strip for each of the hCG suspensions, dip the end of the pregnancy test strip into each tube for approximately 3 s. Remove from the tube and set test down horizontally. Streaking may occasionally occur, particularly during the initial flow of the LFA, but your test results should still be valid.
3. Wait for 5–15 min for the band coloring to fully develop. Place the strips directly next to each other on the benchtop.
4. Using a smartphone, take a picture of the band colors from about 4–6 inches above the benchtop.
5. Ensure that your images adequately show the test and control bands for each strip. Flash is not necessary, but you may use it if you choose to (Fig. 13.24).
6. Load your image into an appropriate software (e.g., ImageJ). Move your mouse over the test line of one of the pregnancy test strips. For 5 separate points within the test line, record the blue pixel value. Pixel values are listed directly beneath the toolbar as R, G, B. Average these 5 values for each line. This will serve as your blue pixel intensity for each strip (Fig. 13.25).
7. With these blue intensity values, determine the red, green, and blue absorbance for each of the hCG concentrations.
8. Plot the red, green, and blue absorbance versus hCG concentration for the three known standard solutions, as well as for the unknown hCG solution. Determine the approximate hCG concentration of this unknown solution (Fig. 13.26).

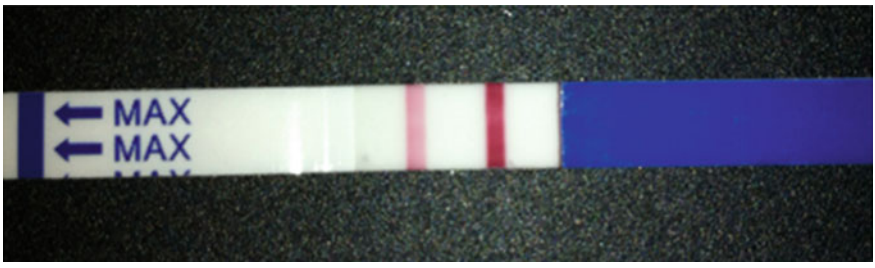

Fig. 13.24 Sample pregnancy test image

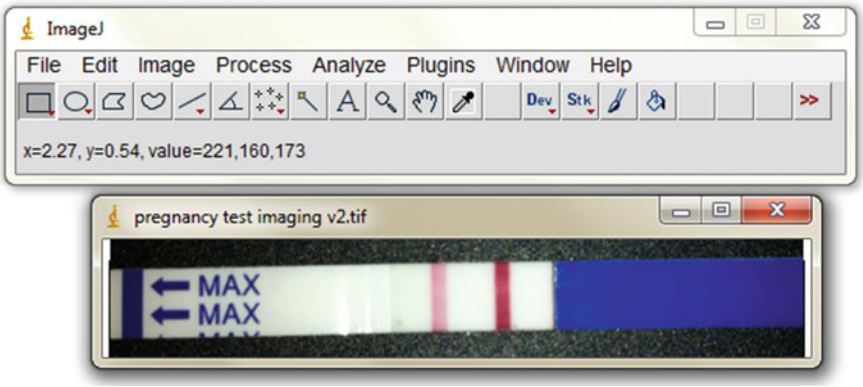

Fig. 13.25 Analyzing the test band pixel intensity within ImageJ

hCG conc. mIU/mL	0	500	1000
Red intensity	183.4	181.5	172.9
A = log (I_0 / I) [red]	0	0.00452	0.0256
Green intensity	186.7	174.1	154.5
A = log (I_0 / I) [green]	0	0.0303	0.0822
Blue intensity (I)	181.3	175.6	163.4
A = log (I_0 / I) [blue]	0	0.0139	0.0451

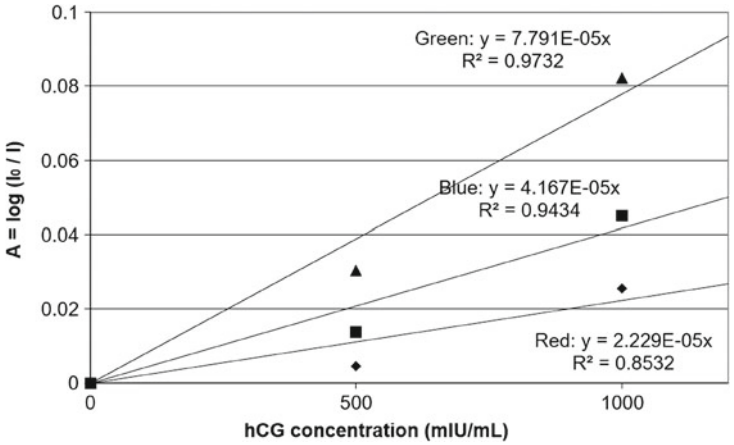

Fig. 13.26 Experimental data of Task 3: standard curve using a smartphone camera. The red, green, and blue intensities with 0 mIU/mL hCG solution was used as I_0

Question 13.5

Compare the standard curves with red, green, and blue pixel intensities. Which color shows the best result, and why? (Hint: use the absorption spectrum of gold nanoparticles—Fig. 13.9).

13.14 Further Study: DNA Sensors

Although antibodies are the most popular bioreceptors in biosensor applications, other types of bioreceptors can be used as well. Nucleic acids, such as DNAs and RNAs are also very popular in biosensor applications. These types of biosensors are typically called *DNA sensors.*

As shown in Fig. 13.27, a relatively short chain of single-stranded DNA (ssDNA) is used in place of a primary antibody. This ssDNA is called *capture probe*, which is complementary to the specific sequence of a desired (target) DNA. The capture probe is typically conjugated with biotin. The sensor surface is pre-immobilized with either *streptavidin* or its one-quarter version, *neutravidin*. Due to the strong binding of biotin-avidin, the capture probe makes the target DNA firmly bound on the surface.

To confirm this binding, a secondary ssDNA is needed, which is complementary to the specific sequence of the target DNA (but a different region). This ssDNA is called *detector probe*, and is analogous to the secondary antibody in immunosensors. The detector probe can be conjugated with a fluorescent dye, where any optical detection can confirm the presence of target DNA in a sample. Additionally, antibody to a fluorescent dye that is conjugated to an enzyme (for example, HRP) can be additionally added, where its presence can be detected in an electrochemical way.

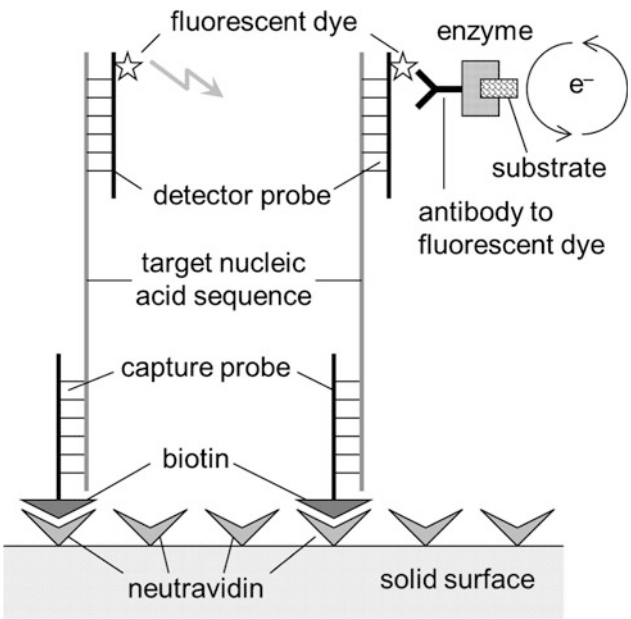

Fig. 13.27 DNA sensors. Identical to Fig. 1.14

Note that it is quite difficult to conjugate proteins such as antibodies and enzymes directly to detector probes. Short ssDNA probes are quite small and significantly smaller than those proteins, and their mobility and binding ability to the target DNA would be greatly affected if they are conjugated with such proteins. These ssDNA probes are almost always conjugated with small chemical compounds, mostly with *biotin* (it is essentially vitamin B_7) and/or fluorescein.

Question 13.6
Will it be possible to make label-free DNA sensors, using (a) SPR, (b) IME, and (c) QCM transducers? Will the transducer instrumentation and sensor strips remain the same as those of SPR, IME, or QCM immunosensors?

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