Surface plasmon resonance (SPR) biosensor for rapid detection of *Salmonella* and *Salmonella* infections

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Saikat Datta Mazumdar
aus Shillong, Indien

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Erstgutachter:	Prof. Dr. Michael Keusgen
Zweitgutachter:	Prof. Dr. Maike Petersen
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This work is dedicated to my Parents and Elders and inspired by my wife and our son Shyamanjan (Riddhi)

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List of Abbreviations

3-APTES	(3-aminopropyl)triethoxysilane
BSA	Bovine serum albumin
CCD	Charge coupled device
CDC	Centres for disease control
CFU	colony forming units
DCC	N, N′-Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl
g	gram(s)
h	hour(s)
HRP	horse raddish peroxidase
K_A	equilibrium association constant (M)
K_D	equilibrium dissociation constant (M^{-1})
KDO	3-deoxy-D-manno-octulosonic acid
L	liter(s)
LB	Luria-Bertani
LLD	Lower limit of detection
LPS	Lipopolysaccharide
M	mol L^{-1}
min	minute (s)
mL	millilitres
MTP	microtitre plate
MWCO	Molecular weight cut-off
NHS	N-Hydroxysuccinimide
sulfo-NHS	N-Hydroxysulfosucccinimide
OGC	optical grating coupler
PBS	Phosphate buffered saline
RT	room temperature
S.	*Salmonella*
SAM	self-assembled monolayers
SPR	Surface plasmon resonance
SPW	Surface plasmon wave
TMB	3,3′,5,5′-tetramethylbenzidine
w/v	weight/volume
w/w	weight/weight
WHO	World health organisation
WHOCC-Salm	WHO Collaborating Centre for Reference and Research on *Salmonella*

1 Introduction

1.1 Biosensors

A biosensor uses a biological system to measure a substance and differentiate this from other substances in a test sample. It is a measurement device that is composed of three components: a **biological component** (enzymes, nucleic acids, antibodies, bacteria, animal or vegetable tissues etc.) of appropriate specificity for the analyte (or the test material to be measured); a **transducer** to convert the recognition event into a suitable physical signal (electrical, optical etc.), and a **detection and recording system**, including analysis and processing, that is usually electrical or computer controlled (Gimzewski et al., 2005). The principle and function of biosensors is schematically represented in Fig. 1.1.

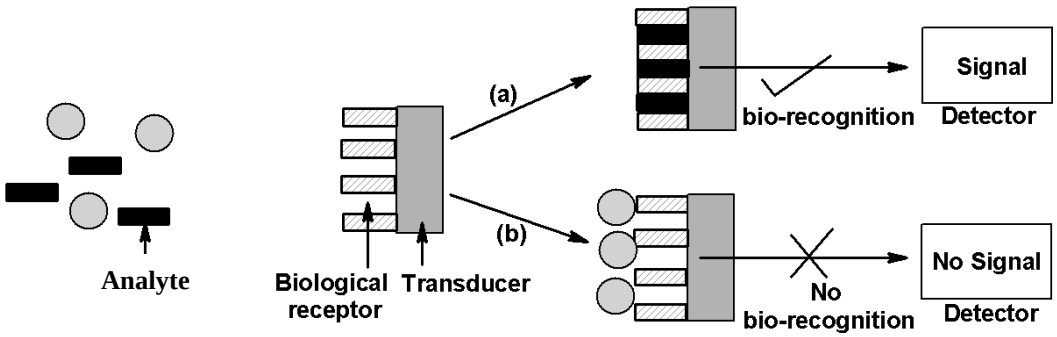

Fig. 1.1: Schematic representation of the principle and functioning of a biosensor showing the biological receptor, transducer and the detector. (a) Bio-recognition due to binding of the analyte (black rectangle) to its specific biological receptor is converted by the transducer into a physical signal, which is received and recorded by the detector (b) substances (grey circles) not recognised by the biological receptor do not result in any detection signal.

1.1.1 Classification of Biosensors

1.1.1.1 Electrochemical sensors

The basic principle of electrochemical sensors is that the electroactive analyte species is oxidised or reduced on the working electrode surface, which is subjected to some predefined pattern of fixed or varying potential. The change in electrical parameters resulting from this redox reaction, as a function of the type or concentration of analyte, is measured (Ahmed et al., 2008). Three options are

available, namely, amperometric, potentiometric and conductometric, each with their inherent advantages and disadvantages (Stradiotto et al., 2003). Electrochemical sensors have found wide application in food and medical applications (Ahmed et al., 2008). In electrochemical sensing, at present, the usual electrode configuration involves a three electrode arrangement: The working electrode, where the electron transfer reaction takes place, the reference electrode, which maintains a stable potential with respect to the working electrode and the auxiliary electrode, made of inert conducting material like Pt, graphite etc.; with some supporting electrolyte for the electrode to eliminate electromigration effects, decrease solution resistivity and maintain the ionic strength constant (Wang, 1994). Choosing the working electrode material is fundamental to the success of the assay. Development of microelectrodes (<2 mm dimension) has opened the horizon of in vivo and in vitro applicability of electrochemical sensor systems requiring only microliter volumes of analyte and reagent (Stradiotto et al., 2003). Screen-printed electrodes (Brunetti et al., 2000), which involves deposition of electrode material, mainly carbon and noble metals, on inert PVC or ceramic supports has been a breakthrough in the advancement of electrochemical biosensors. The use of materials like carbon nanotubes (CNT) and nanoparticles are being explored to enhance the functionality, sensitivity and applicability of electrochemical sensors (Ahmed et al., 2008).

1.1.1.1.1 Amperometric sensors

Amperometric biosensors mostly use reduction-oxidation (redox) enzyme systems. The redox enzyme is immobilised on the surface of the electrode. The electrode is held at a fixed potential, adjusted so that electrons arising from an oxidised substrate are transferred to the electrode (or vice versa for a reduction reaction), and this regenerates the active form of a cofactor for another redox cycle. The enzyme used determines the specificity of the reaction. As the rate of enzymatic reaction at a fixed temperature and pH is directly proportional to the substrate concentration, the current produced at the electrode is proportional to the rate of modification of the substrate by the enzyme. The most well-known amperometric sensor, which is also considered to have pioneered the biosensor revolution, is the amperometric sensor detecting glucose level in blood developed by Clark and Lyons (1962). This biosensor was used in continuous monitoring of glucose in cardiovascular surgery. Since the first glucose enzyme electrode based biosensor a lot of development (such as use of

artificial mediators) has taken place in the area of glucose biosensors, though the underlying principle is still amperometric detection (Wang, 2001).

1.1.1.1.2 Potentiometric sensors

According to the definition provided by IUPAC, potentiometric measurements involve the determination of the potential difference between either an indicator and a reference electrode or two reference electrodes separated by a permselective membrane, where there is no significant current flowing between them (Thévenot et., 1999). The transducer may be an ion-selective electrode (ISE), which is based on thin films or selective membranes as recognition elements. The most common potentiometric devices are pH electrodes; several other ion- (F^-, I^-, CN^-, Na^+, K^+, Ca^{2+}, NH_4^+) or gas- (CO_2, NH_3) selective electrodes are available. The first potentiometric biosensor demonstrated was for the detection of urea (Guilbault and Montalvo, 1969). The enzyme urease was immobilised in a layer of acrylamide polymer on the surface of a cationic electrode sensitive to ammonium ion. The substrate urea diffuses to the enzyme electrode and reacts with the immobilised enzyme to produce ammonium ion at the surface of the glass electrode. A thin film of cellophane was placed around the enzyme gel layer to prevent leaching of urease into the surrounding solution. Several potentiometric sensors use a transducer scheme based on potentiometric monitoring of local pH changes. An enzymatic pathway, which leads to change in hydrogen ion activity, can be applicable for this task. This principle has been used for the development of a potentiometric-penicillin-sensitive biosensor. The enzyme penicillase is used as the immobilised biorecognition element (Taylor et al., 1996). Two other categories of potientiometric sensors use coated-wire electrodes (CWES) and field effect transistors (FET) as transducers (Stradiotto et al., 2003).

1.1.1.1.3 Conductometric sensors

The principle of conductometric measurements is based on the detection of solution conductivity variations. Most of these devices are based on the fact that a large number of enzymatic reactions involve the production or consumption of charged species (Taylor et al., 1996). Many enzyme reactions, such as that of urease, and activities of many biological membrane receptors may be monitored by ion conductometric devices (Cullen et al., 1990). As the sensitivity of the measurement is hindered by the parallel conductance of the sample solution, usually a differential

measurement is performed between a sensor with enzyme and an identical one without enzyme. Such a device has also been used in monitoring of heavy metal ions and pesticides in water samples (Chouteau et al., 2005). The biorecognition element used in this case was the microalgae *Chlorella vulgaris.*

1.1.1.2 Nanomechanical sensors

1.1.1.2.1 Piezoelectric mass sensors

Mass detection sensors are among the most widely used microanalytical sensors. These methods rely in general on measuring the changes in vibrational resonat frequency of piezoelectric quartz oscillators that result from changes in mass on the oscillator's surface.

The most common form of such sensors is the quartz crystal microbalance (QCM) and the surface acoustic wave (SAW) device. The QCM device consists of a quartz crystal disk driven by electrodes on either face. The mass of analytes that bind to the sensor is measured as a change in the crystal's resonat frequency. This type of sensor is also known as a thickness-shear mode (TSM) device

In the SAW sensor an acoustic wave is created by applying an alternate voltage to a metallised, inter-digitated electrode plated onto one end of a thin piezoelectric planar substrate of the device.

1.1.1.2.2 Microcantilever Sensors

The principle of the cantilever biosensor is based on mechanical stresses produced in a sensor upon molecular binding. This stress bends the sensor mechanically and can easily be detected.

1.1.1.3 Optical sensors

Optical biosensors are powerful detection and analysis tools that has vast applications in biomedical research, healthcare, pharmaceuticals, environmental monitoring and fight against biological threats (Narayanaswamy and Wolfbeis, 2004). The main advantage of optical biosensors over electrochemical biosensors is that they are resistant to electromagnetic interference, capable of performing remote sensing, and can provide multiplexed detection within a single device. Generally, there are two broad detection protocols that can be implemented in optical biosensing: labeled (e.g., fluorescence-based detection) and label-free detection.

1.1.1.3.1 ***Optical biosensors using labels***

The most commonly used format of labelled optical biosensor uses fluorescence-based detection. In fluorescence-based detection, either target molecules or biorecognition molecules are labelled with fluorescent tags, such as dyes; the intensity of the fluorescence indicates the presence of the target molecules and the interaction strength between target and biorecognition molecules. While fluorescence-based detection is extremely sensitive, with the detection limit down to a single molecule (Moerner, 2007) it suffers from laborious labelling processes that may also interfere with the function of a biomolecule. Fluorescence based biosensors have been used in conjunction with fibre-optics to create what are called optrode-based fibre optic biosensors (Bio-optrode) and evanescent wave fibre optic biosensors.

Bio-optrodes are analytical devices incorporating optical fibers and biological recognition molecules. Optical fibers are small and flexible "wires" made out of glass or plastic that can transmit light signals with minimal loss over long distances. The light signals are generated by a sensing layer, which is usually composed of biorecognition molecules and dyes, coupled to the fiber end. Light is transmitted through the optical fibers to the sensing layer, where different optical phenomena such as absorption or luminescence are used to measure the interactions between the analyte and the sensing layer. Bio-optrodes can be used for remote analytical applications including clinical, environmental, and industrial process monitoring (Biran and Walt, 2002).

Evanescent wave fibre optic biosensors differ from the optrodes with respect to the nature of the light used. In case of optrodes the light shining out of the end of the optic fiber is used to generate a signal either at the distal face of the fiber or in the medium near the fiber´s end. On the other hand, evanescent wave sensors rely on the electromagnetic component of the reflected light at the surface of the fiber core to excite only the signal events localized at that surface. Thus, in case of the evanescent fibre optic sensors the penetration depth of the light into the surrounding medium is much more restricted, than for optrodes, while the surface area interrogated is much larger in comparison to optrodes of equal diameter. The result is that evanescent wave biosensors require immobilization of the biological recognition molecules onto the longitudinal surface of the optical fiber core (Biran and Walt, 2002).

Recently, the use of quantum dots as labelling agent has been used in the development of fibre optic based biosensors (Ayogai and Kudo, 2005). The quantum dots are semiconductor nano-crystals. Quantum dots have a number of unique properties that can be used in development of fluorescence and fluorescence resonance energy transfer (FRET) based biosensors (Algar and Krull, 2008).

1.1.1.3.2 ***Label-free optical biosensors***

The most commonly used optic biosensors fall into two broad categories based on the technology platform used. These are the evanescent wave biosensors and the interferometer-based biosensor. The different label free optical biosensors has been recently been reviewed by Fan et al. (2008). The most widely used evanescent wave optical biosensor is the surface plasmon resonance biosensor.

Evanescent wave biosensors

The operation of these biosensors is based on the optical properties of thin metal films with high refractive index deposited on the surface of a glass prism. Light coming from the glass is totally internally reflected from the metal surface, and at a certain angle of incidence the excitation of resonance in the film produces intensity and phase changes in the reflected beam. An evanescent field is also generated which travels in a direction perpendicular to the surface. The detection of biomolecular interaction is based on the fact that the resonance characteristic is very sensitive to changes of the refractive index in the evanescent field. The detailed description of the physics behind this phenomenon has been reviewed by Cush et al. (1993) and

Raether (1997). Two types of optical biosensors based on this principle are surface plasmon resonance (SPR) biosensor and the resonant mirror (RM) optical biosensor. As both of these biosensors are based on monitoring changes in refractive index occurring with each biomolecular interaction, no labeling of the analyte is required.

Surface plasmon resonance (SPR) biosensor was first demonstrated for biosensing by Liedberg et al. (1983). SPR sensors consist of a thin metallic layer (typically Au or Ag) of about 50 nm deposited directly onto a glass prism. The measurement principle is based on excitation of surface plasmons at the boundary between the metal film and the sensing layer. As a result, incident light is adsorbed, resulting in a decrease in the intensity of the reflected light. The angle of incidence of the exciting light is extremely sensitive to the refractive index occurring in the sensing film at the metal-film boundary. The fundamentals of SPR will be discussed in detail in section 1.1.2.1.

RM instruments are based on a waveguide structure (RM) (Cush et al. 1993), where the evanescent field results from the propagation of light along the waveguide. This waveguide is made from a metal oxide. Refractive index changes occurring due to biomolecular interactions on the sensing layer, at the surface of the waveguide, alter the conditions under which the light couples in and travels along the guide. Monitoring these changes with time forms the basis of the measurement (Kinning and Edwards, 2002). Unlike SPR devices, the system is looking for a peak of intensity, not a decrease.

1.1.2 Surface Plasmon Resonance (SPR)

1.1.2.1 Fundamentals of SPR

Surface plasmon resonance (SPR) biosensors belong to the category of evanescent wave biosensors. When a light beam passes from a material having a relatively high refractive index (e.g., a glass prism) into a material having a lower refractive index (e.g., water), the light is bent towards the plane of interface. Total internal reflection occurs when the angle, at which the light strikes the interface, is greater than the critical angle. The critical angle is defined as the angle of incidence, which provides an angle of refraction of 90-degrees. SPR is observed under conditions of attenuated total internal reflection (ATR), when the surface of the prism coated with so-called

"free electron metals" like gold is placed in contact with a dielectric material (buffer with sample). Surface plasmon waves (SPW) are present on the gold surface due to the free-oscillating electrons. The basic setup of exciting SPW consists of coupling a prism coated with a thin metal in contact with a dielectric (Fig. 1.2). The excitation of the SPW at the interface of non-magnetic metals such as gold and a dielectric is possible only using the transverse magnetic mode (TM) of plane-polarised light.

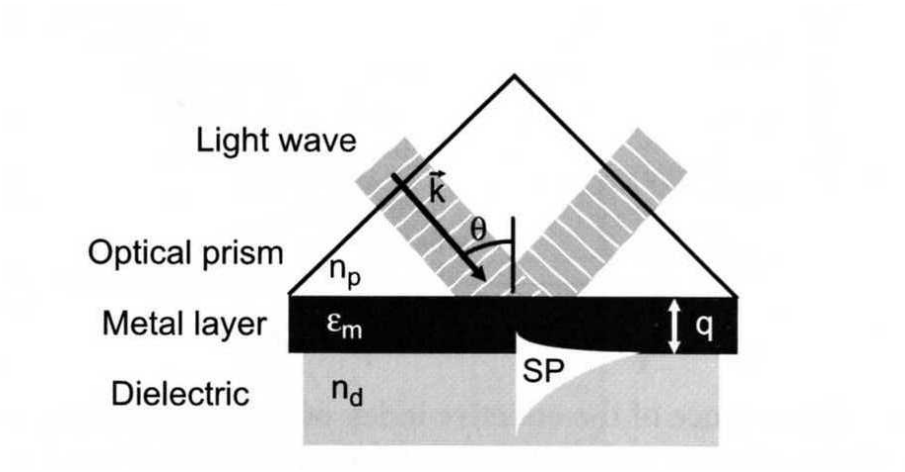

Fig. 1.2: Excitation of surface plasmons in the Kretschmann geometry of the attenuated total reflection (ATR) method (adapted from Homola, 2006).

When a light wave propagating in the prism is made incident on the metal film a part of the light is reflected back into the prism and a part propagates in the metal in the form of an inhomogeneous electromagnetic wave. This inhomogeneous wave decays exponentially in the direction perpendicular to the prism-metal interface and is therefore referred to as the evanescent wave. If the metal film is sufficiently thin (less than 100 nm), the evanescent wave penetrates through the metal film and couples with the surface plasmon at the outer boundary of the metal film.

At a particular angle of incidence, the propagation constant of the incident light matches with the propagation constant of the SPW, resulting in SPR. This angle, at which there is a minimum in the intensity of reflected light, is called the SPR angle.

The propagation constant is defined as the product of the effective refractive index (n_{eff}) times the vacuum wave number and is represented by the equation:

$$\beta = n_{eff} \frac{2\pi}{\lambda} \qquad (1.1)$$

Where n_{eff} is the effective refractive index and has the analogous meaning for light propagation in a waveguide.

The propagation constant of the surface plasmon, propagating along the metal film β^{SP} is influenced by the presence of the dielectric on the opposite side of the metal film and can be expressed as

$$\beta^{SP} = \beta^{SP_o} + \Delta\beta \qquad (1.2)$$

β^{SP} is the propagation constant of the surface plasmon wave and is defined by the equation:

$$\beta^{SP} = \frac{2\pi}{\lambda} n_p \sin\theta \qquad (1.3)$$

n_p = refractive index of the prism

θ = incident angle of the TM polarised light

β^{SP_o} = propagation constant of the surface plasmon wave in the absence of the prism and is defined by the equation:

$$\beta^{SP_o} = \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon_d \times \varepsilon_m}{\varepsilon_d + \varepsilon_m}} \qquad (1.4)$$

ε_d = permittivity of the dielectric

ε_m = permittivity of the metal

$\Delta\beta$ = term accounting for the finite thickness of the metal film and the presence of the prism

The propagation constant of the evanescent field is defined as the real part of the β^{SP} and is represented by the equation:

$$\beta^{EW} = \mathrm{Re}\left(\frac{2\pi}{\lambda}\sqrt{\frac{\varepsilon_d \times \varepsilon_m}{\varepsilon_d + \varepsilon_m}} + \Delta\beta\right) \qquad (1.5)$$

For surface plasmon resonance to occur the propagation constant of the surface plasmon (β^{SP}) should be equal to the propagation constant of the evanescent field. (β^{EW}).

Therefore from equations 1.3 and 1.5 :

$$\beta^{SP} = \frac{2\pi}{\lambda} n_p \sin\theta = \beta^{EW} = \mathrm{Re}\left(\frac{2\pi}{\lambda}\sqrt{\frac{\varepsilon_d \times \varepsilon_m}{\varepsilon_d + \varepsilon_m}} + \Delta\beta\right) \qquad (1.6)$$

In terms of the effective refractive index, this coupling condition (1.6) can be written as follows:

$$n_p \sin\theta = n_{ef}^{EW} = n_{ef}^{SP} = \mathrm{Re}\left(\sqrt{\frac{\varepsilon_d \times \varepsilon_m}{\varepsilon_d + \varepsilon_m}} + \Delta n_{ef}^{SP}\right) \qquad (1.7)$$

Where n_{ef}^{EW} is the effective refractive index of the evanescent wave

n_{ef}^{SP} is the effective refractive index of the surface plasmon, defined in equation (1.1)

The coupling condition is summarised in equation (1.3). According to this equation it is clear that for each wavelength and the corresponding effective refractive index, the matching condition is satisfied for a single angle (θ) of incidence, the SPR angle. This is illustrated in Fig. 1.3.

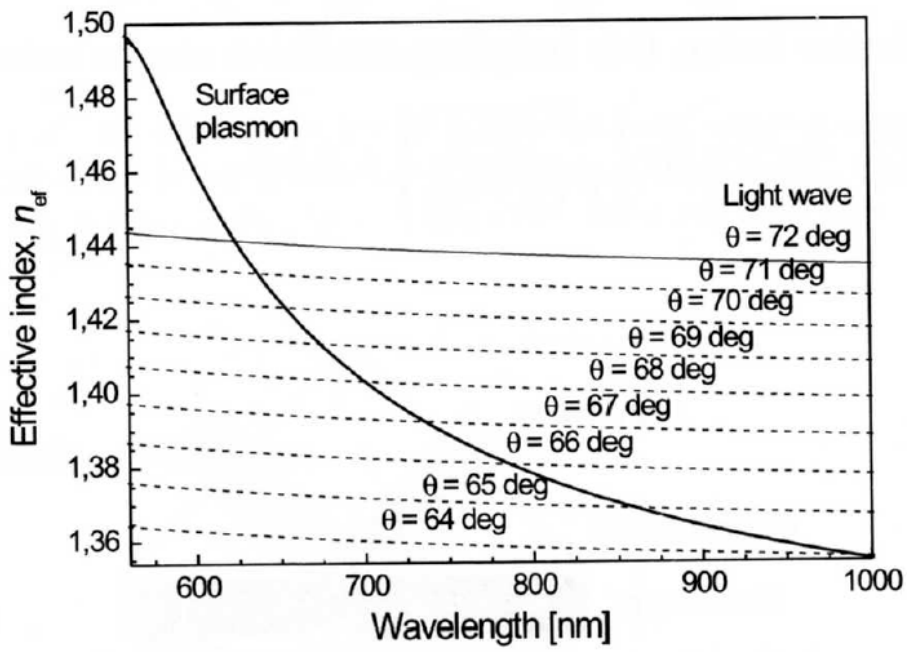

Fig. 1.3: Representative plot explaining the spectral dependence of the effective refractive index of a surface plasmon wave on the interface of gold-water, produced by a plane light wave incident on the gold film of an optical prism (BK 7 glass) at nine different angles of incidence (adapted from Homola, 2006).

This angle increases with decreasing wavelength. The SPR condition is also explained with respect to the effective refractive index as shown in equation (1.7).

Two main configurations are used in the ATR method. Namely, the Kretschmann geometry and the Otto geometry. In the Kretschmann geometry of the ATR method, a high refractive index prism with refractive index n_p is interfaced with a metal-dielectric waveguide consisting of a thin metal film with permittivity ε_m and thickness q, and a semi-infinite dielectric with a refractive index n_d ($n_d < n_p$) (Fig. 1.2).

In the Otto geometry, a high refractive index prism with refractive index n_p is interfaced with a dielectric-metal waveguide consisting of a thin dielectric film with refractive index n_d ($n_d < n_p$) and thickness q, and a semi-infinite metal with permittivity ε_m (Fig. 1.4). Of the two geometries presented here, the Kretschmann configuration is most commonly used, probably due to the ease of construction. In case of this work the Plasmonic® SPR device used works on the Kretschmann configuration. The details of the device are presented in section 3.2.5.

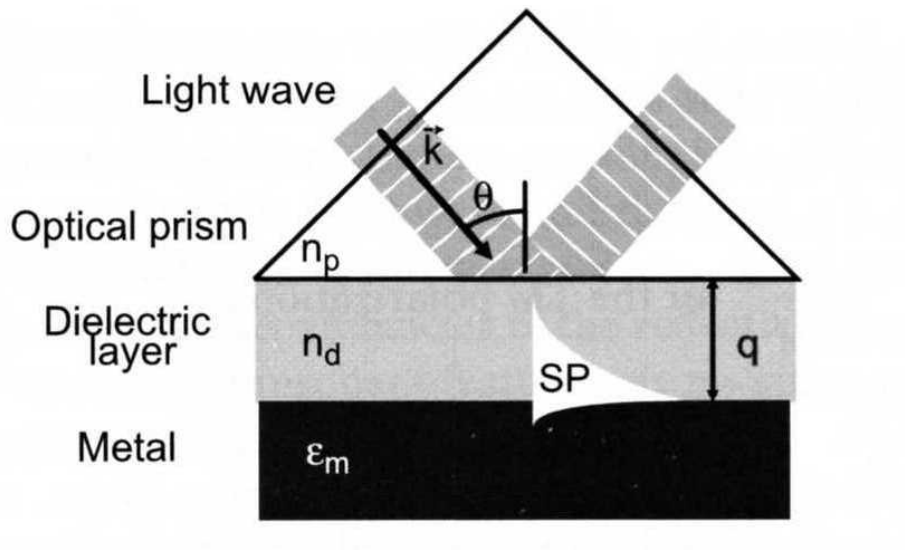

Fig. 1.4: Excitation of surface plasmons in the Otto geometry of the attenuated total reflection (ATR) method (adapted from: Homola, 2006).

As mentioned, the evanescent wave produced during SPR is an exponentially decaying wave and has a penetration depth of about 100-200 nm into the dielectric (Ekgasit et al., 2004). It is important to note that in case of SPR, when biomolecular interactions occur on the gold surface (within the range of the penetration depth of the evanescent field) there is a change in refractive index of the dielectric sample medium. This change in refractive index causes a change in the propagation constant of the SPW. Consequently, the angle at which SPR occurs also changes. This change is recorded as a shift in the SPR angle with time, resulting in SPR sensorgrams. For each SPR angle there is a minimum in the intensity of reflected light. This change in angle, corresponding to the minimum in reflected light, is directly proportional to the loading of biomolecules on the gold surface of the SPR prism (Keusgen, 2002).

1.1.2.2 Detection formats in SPR

In SPR assays the detection format is chosen based on the size of target analyte molecules, binding characteristics of available biomolecular recognition element, range of concentrations of analyte to be measured, and sample matrix (Tüdös et al., 2003). The following four formats are commonly used in SPR based assays:

(a) Direct detection:

In the direct detection mode (Fig. 1.5 a), the biorecognition element (e.g., antibody) is immobilised on the SPR sensor surface. Analyte in solution binds to the antibody, producing a refractive index change, which is detected by the SPR sensor. Direct

detection is usually preferred in applications, where direct binding of analyte of interest produces a sufficient change in the refractive index.

(b) Sandwich detection:

The specificity and limit of detection of the direct assay can be improved by using the sandwich detection format (Fig. 1.5 b), in which the sensor surface with captured analyte is incubated with a second detection antibody. Smaller analytes having molecular weight less than 5000 daltons often do not generate a sufficient change in the refractive index (Homola, 2008) and are therefore measured using either the competitive or the inhibition detection format.

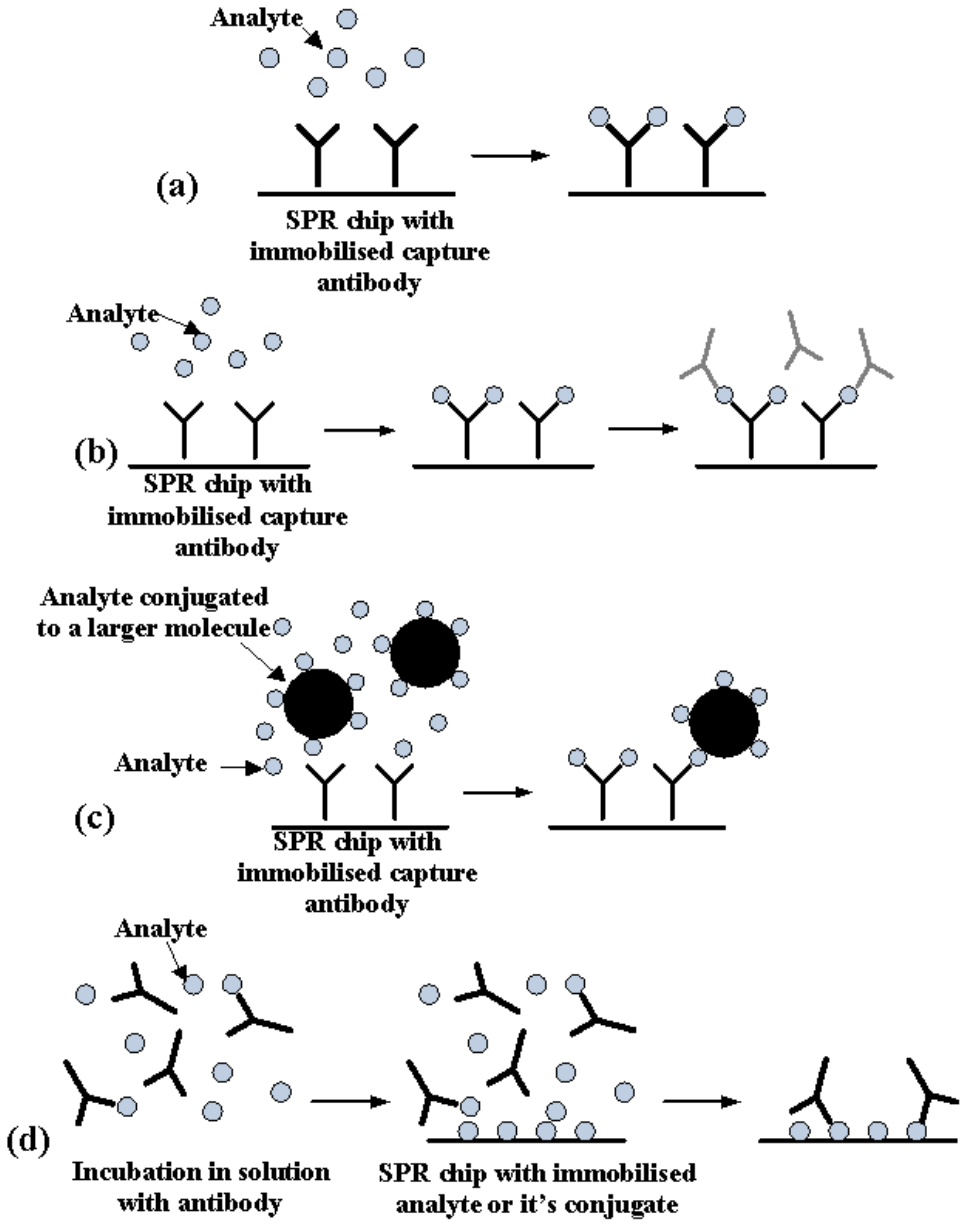

Fig. 1.5: Different detection formats used in SPR assays (a) direct detection (b) sandwich detection (c) competitive detection (d) inhibition detection.

(c) Competitive detection:

In the competitive detection format the sensing surface is coated with an antibody interacting with the analyte; when an analyte conjugated to a large molecule is added to the sample, the analyte and its conjugated analogue compete for a limited number of binding sites on the surface. The binding response is inversely proportional to the analyte concentration (Fig. 1.5 c).

(d) Inhibition detection:

In the inhibition detection format (Fig. 1.5 d) a fixed concentration of an antibody with affinity to analyte is mixed with a sample containing an unknown concentration of analyte. Then, the mixture is passed over a sensor surface to which the analyte or its analogue is immobilised. Antibodies, which do not bind to the anlyate in solution, are measured as they bind to the analyte molecules immobilised on the sensor surface. The binding response is inversely proportional to the concentration of analyte.

1.1.2.3 Biorecognition elements in surface plasmon resonance

In SPR biosensors, one of the interacting molecules is immobilised on the solid surface of the SPR sensor and the other is contained in a liquid sample. Which of the molecules is immobilised depends on the used detection format (section 1.1.2.2). In case of the direct, sandwich and competitive detection formats the molecule that needs to be immobilised is a biorecognition element. In the inhibition detection format, the immobilised molecules are the target molecules or their derivatives. The choice of appropriate biorecognition elements and immobilisation methods is of critical importance with direct impact on key performance characteristics of the sensor such as sensitivity, specificity, and limit of detection of the assay.

Biorecognition elements

Various kinds of biorecognition elements have been employed in affinity SPR biosensors. Antibodies remain by far the most frequently used biorecognition elements. They offer high affinity and specificity against target analyte. Moreover, antibodies against numerous target molecules are now commercially available. The structure and properties of antibodies is discussed in section 1.1.2.4. Recently, single-chain antibody fragments (scFvs) have been used as biorecognition elements (Dunne et al., 2005). Biotinylated scFv fragments expressed in yeast can be spotted

on streptavidin-coated sensor surfaces directly from cell supernatant without the need for purification (Scholler et al., 2006). Another type of biorecognition element that has been employed in SPR sensors are peptides. In comparison with antibodies, peptides, in general, are inexpensive, more stable, and easier to manipulate. However, peptides sometimes lack high affinity and specificity against the target. In SPR biosensors, peptides have been applied mainly for the detection of antibodies, for example, antibodies against hepatitis, herpes simplex virus type 1 and type 2, (Wittekindt et al., 2000) and Epstein-Barr virus (Vaisocherova et al., 2000), and also for the detection of heavy metals (Forzani et al., 2005). Recently, aptamers emerged as another promising type of biomolecular recognition element for SPR biosensors (Win et al., 2006; Wang et al., 2007). DNA or RNA aptamers are single-stranded oligonucleotide sequences, which can be produced to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms (Wang et al., 2007; Brody et al., 1999). It is important to mention here that purified LPS from bacteria has been used as biorecognition element in this work.

Immobilisation of biorecognition elements

In SPR biosensors, one of the interacting molecules (mostly the biorecognition element) is immobilised on the sensor surface. The surface chemistry has to be designed in such a way that it enables immobilisation of a sufficient number of biorecognition elements on the sensing surface while minimising the non-specific binding to the surface. In addition, biorecognition elements need to be immobilised on the sensor surface without affecting their biological activity. In principle, the molecules can be immobilised either on the surface or in a three-dimensional matrix. Although immobilisation on surfaces is more straightforward to perform, the number of accessible biorecognition elements is limited by a number of factors such as orientation on the surface and steric hindrance. Immobilisation in a three dimensional matrix is an approach to obtain more binding sites than immobilisation on the surface (Zhang, 2004). The most widely used three-dimensional matrix for immobilisation of molecules in a structured environment is the carboxymethylated dextran matrix (Lofas et al., 1995). For two-dimensional surface immobilisation of biorecognition elements on the sensing gold surface, self-assembled monolayers (SAMs) of alkanethiols or disulfides have been widely used (Knoll et al., 1997). To provide a desired surface concentration of biomolecular recognition elements and a

non-fouling background, mixed SAMs of long-chained alkanethiols terminated with a functional group for further attachment of biomolecular recognition elements and oligo(ethylene glycol) terminated shorter-chained alkanethiols for a non-fouling background have been developed (Jung et al., 2000, Nelson et al., 2001). The main approaches to immobilisation of molecules to the surface of SPR sensors are based on physical adsorption involving hydrophobic and electrostatic interactions (Koubova et al., 2001), covalent coupling (Lofas et al., 1995). Another approach is based on attachment of tagged molecules by a site-specific non-covalent interaction between the tag and an immobilised capture molecule via biotin-avidin (Busse et al., 2002) or histidine-chelated metal ion (Zhen et al., 2006) interaction or DNA hybridisation (Ladd et al., 2004). Recently, Rusmini et al., (2007) has provided a general overview of different immobilisation strategies.

However, biosensors based on enzymes or antibodies often suffer from poor immobilisation of the biological component. This leads to partial loss of function, sensitivity or lifetime of the sensor. Most immobilisation methods are based on unspecific cross-linking of functional groups of the protein, mostly amine or carboxyl functions (Keusgen, 2002). As all these methods are not site-directed they often result in a partial or complete loss of the function of the bio-molecule. In case of antibodies, the most common immobilisation procedures are based on capturing amino functions. This is rather crucial for the binding of antibodies because terminal amino functions are placed in the recognition area. This usually results in functional loss of antibodies. In addition, amine and carboxyl groups are known to be well distributed on the surface of the antibody. Thus, immobilisation procedures using these functional groups will randomly orient the antibody making the binding site unavailable to the antigen (Hermanson, 1996). In recent years, there has been a shift in focus to develop biosensors for the rapid detection of pathogens. SPR has been successfully used for the rapid detection of different pathogens (Lazcka et al., 2007). Different strategies for immobilisation of the capture antibody have been used in SPR-based immunoassays for detection of bacteria including *Salmonella* cells. Most of these strategies either use direct adsorption of the antibody on the gold surface (Koubova et al., 2001) or surfaces having standard alkylthiol chemistry (Jyoung et al., 2006; Subramanian., 2006; Perkins and Squirrell., 2000) or the carboxymethyl-dextran (Waswa et al., 2006; Bokken et al., 2003; Fratamico et al., 1998) surface for coupling of the capture antibody. Few authors have also reported the use of antibody-

binding proteins such as protein A (Waswa et al., 2006) or protein G (Oh et al., 2005) on these surfaces. The literature review presented here shows a lack of any comparative study on the different antibody immobilisation techniques, especially with respect to detection of bacteria.

1.1.2.4 Antibodies

As most of this work is based the use of a SPR sensor surface with immobilised capture antibodies, a brief understanding of the structure and properties of antibodies is reviewed in this section. Antibodies are host proteins produced as a response to the presence of foreign molecules in the body. They are produced primarily by the plasma cells, terminally differentiated cells of the B-lymphocyte lineage, and circulate throughout the blood and lymph where they are capable of binding to foreign antigens. The antigen antibody conjugates then are removed from circulation primarily through phagocytosis by macrophages. Antibodies are a large family of glycoproteins that share key structural and functional features. They are also referred to as immunoglobulins (Ig). Functionally they can be characterised by their ability to bind both to antigens and to specialised cells or proteins of the immune system. Structurally, antibodies are composed of one or more copies of a characteristic unit that can be visualised as forming a Y- shape. Each Y-shape contains four polypeptides – two identical copies of a polypeptide known as the heavy chain (approx.55 kDa) and two identical copies of a polypeptide called the light chain (approx. 25 kDa). The light and the heavy chain are held together by disulphide and other non-covalent bonds resulting in the Y-shaped molecule. There are two forms of light chains that may be found in antibodies. A single antibody will have light chains of either lambda (λ) or kappa (κ) variety, but not both types in the same molecule. An antibody's heavy chain determines the immunoglobulin class. A single antibody will possess only one type of heavy chain (designated as γ, μ, α, ε or δ). Thus, there are five major classes of antibody molecules in mammals, each identified by their heavy chain type, and designated as IgG, IgM, IgA, IgE, or IgD. Three classes (IgY, IgM and IgA) are known in avians. The characteristic of each of the five classes of antibody is summarised in Table 1.1.

Table 1.1: Classification and characteristics of antibodies (adapted from: Harlow and Lane, 1988)

Characteristics	IgG	IgM	IgA	IgE	IgD
Heavy chain	γ	μ	α	ε	δ
Light Chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Molecular formula	$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$	$(\alpha_2\kappa_2)_n$ or $(\alpha_2\lambda_2)_n$	$\varepsilon_2\kappa_2$ or $\varepsilon_2\lambda_2$	$\delta_2\kappa_2$ or $\delta_2\lambda_2$
Valency	2	10	2,4, or 6	2	2
Concentration in serum	8-16 mg mL^{-1}	0.5-2 mg mL^{-1}	1-4 mg mL^{-1}	10-400 ng mL^{-1}	0-0.4 mg mL^{-1}
Function	Secondary response	Primary response	Protects mucous membrane	Protects against allergens and parasitic infections	Appears when young B cells in the spleen are ready to be activated

For IgG molecules the intact molecular weight representing all four subunits is in the range 150 kDa to 160 kDa. IgG have three protein domains (Fig. 1.6). Two of the domains are identical and form the arms of the Y. Each arm contains a site that can bind to an antigen, making the IgG molecules bivalent. The proper orientation of the antigen-binding domain is the most important when deciding on the antibody immobilisation strategies in case of SPR-based biosensors. In addition it is important that the antigen binding activities of these domains are not destroyed during the immobilisation procedure. The third domain forms the base of the Y. When designing immobilisation strategies, attempt is made to immobilise the antibody using functional groups present in this region. The three domains may be separated from each other by cleavage with the protease papain. The two fragments that carry the antigen binding fragments are called Fab (**F**ragment **a**ntigen **b**inding) fragments. The third protein domain that is involved in immune regulation is termed the Fc (**F**ragment that **c**rystallises) region. The region between the Fab and Fc fragments is called the hinge region. The structure of an IgG molecule is presented in Fig. 1.6. The light chains are divided into two regions. The amino terminal half of all light chains from different antibodies is heterogeneous with respect to the amino acid sequence. This is region is know as the variable region of the light chain (V_L). The carboxy-terminal is known as the constant region (C_L) and there are two types of constant regions, which forms the basis of classification of the light chains into λ and

κ. Heavy chains of IgG contain one variable region (V_H) and three constant regions (CH^1, CH^2, CH^3).

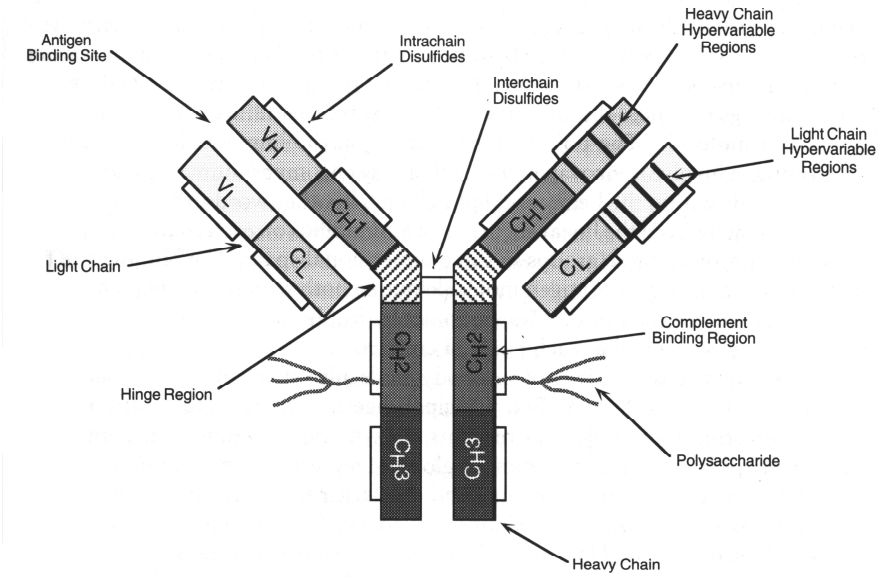

Fig. 1.6: Detailed structure of an immunoglobulin G (IgG) molecule (adapted from: Harmanson et al., 1996).

Protein A and Protein G have the unique property to bind specificallyto the Fc regions of antibodies. This property is used to orient antibodies on biosensor surfaces. The properties of these antibody-binding proteins are discussed in the next section. The heavy chains of immunoglobulin molecules are also glycosylated, typically in the CH^2 domain within the Fc region. These polyssacharides present in the Fc-region also form the basis of immobilisation of antibodies and is dealt with in detail in section 4.1.1.3.

1.1.2.5 Antibody binding proteins

Protein A

Protein A is a 42 kDa peptide that is a normal constituent of the cell wall of *Staphylococcus aureus*. Protein A has four potential binding sites for antibodies; however, only two of them can be used at one time. The binding site on the antibody molecule is found in the second and third constant regions of the heavy-chain polypeptide. Thus, there are at least two binding sites on each antibody molecule for protein A. Co-crystals of the Fc domain and protein A have been resolved upto 2.9 Å (Deisenhofer, 1981). There are extensive hydrophobic interactions between the

second and the third constant regions of the Fc domain. The affinities of protein A to antibodies obtained from several commonly used sources is summarised in Table 1.2. The usefulness of protein A in the study of antibodies and antibody-antigen interactions can be clearly explained by the three important characteristics of protein A. First, because the binding site to protein A on the antibody is found in the Fc region, the interaction with protein A does not change the ability of the antibody to combine with the antigen. Secondly, protein A can be easily renatured to its fully binding capacity from its denatured state (Harlow and Lane, 1988). Finally, even though the affinity of antibody to protein A is high, lowering the pH easily breaks the bond. This important characteristic of protein A has been used in purification of antibodies from crude preparations.

Table 1.2: Affinities of Protein A and Protein G to antibodies obtained from different species (adapted from: Harlow and Lane, 1988)

Species	Affinity for protein A	Affinity for protein G
Human	++++	++++
Horse	++	++++
Cow	++	++++
Pig	+++	+++
Sheep	±	++
Goat	-	++
Rabbit	++++	+++
Chicken	-	+
Hamster	+	++
Guinea pig	++++	++
Rat	±	++
Mouse	++	++

Protein G

Protein G is a 30 KDa to 35 KDa protein isolated from the cell wall of β-hemolytic streptococci of the C or G strains (Kronvall, 1973). The binding of protein G to IgG molecules occurs both through interaction of the Fc regions of the antibody as well as to the highly conserved CH^1 domain of the Fab region (Derrick et al., 1992). Those antibodies, which do not bind very well to protein A, bind very well to

protein G (Table 1.2). One disadvantage of protein G is that it contains a second binding site that will interact with albumin (Björck et al., 1987).

1.1.2.6 Biotin-avidin and biotin-streptavidin interactions

Another important biochemical interaction, which is used for the site-directed immobilisation of antibodies on the gold surface of the SPR chip, is the interaction between biotin and avidin or biotin and streptavidin. Here we briefly discuss the important properties of each of these components in context to the immobilisation of antibodies for biosensor applications.

Biotin

Biotin or vitamin H is a naturally occurring vitamin found in all living cells (Fig. 1.7). Only the bicyclic ring is required to be intact for the interaction with avidin; the carboxyl group on the valeric acid side chain is not involved and can be modified to generate biotinylation reagents used for conjugation with proteins. Since biotin is a small molecule, its conjugation to antibodies does not affect conformation, size, or functionality. Biotinylation reagents can be classified depending on their reactivity to diverse functional groups. The NHS ester of biotin is the most commonly used biotinylation reagent to target amine groups (Luo and Walt, 1989), whereas biotin hydrazide can be used to target either carbohydrates or carboxyl groups.

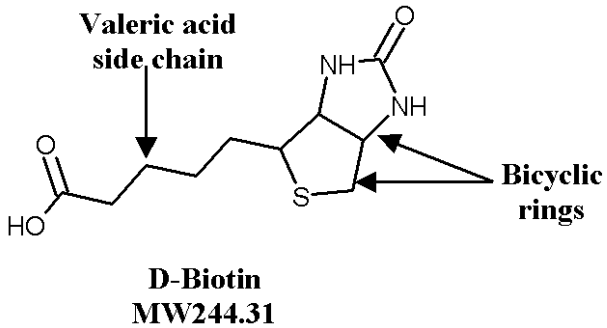

Fig. 1.7: Structure of D-Biotin showing the bicyclic rings and the valeric acid side chain.

Avidin

Avidin is a glycoprotein that is found in egg white and contains four identical subunits of 16,400 daltons each, giving an intact molecular weight of approximately 66 kDa (Green, 1975). Each subunit contains one binding site for biotin and one oligosaccharide modification (Asn-linked). The tetrameric protein is highly basic, having a pI of about 10. The biotin interaction with avidin is on of the strongest non-covalent affinities known, exhibiting a dissociation constant of about $1.3 \text{ X } 10^{-5}$ M. Tryptophan and lysine residues in each subunit are known to be involved in forming the binding pocket (Gitlin et al., 1987, 1988). The tetrameric native structure of avidin is resistant to denaturation under extreme chaotropic conditions. Even in 8 M urea or 3 M guanidine hydrochloride the protein maintains structural integrity and activity. When biotin is bound to avidin, the interaction promotes even greater stability to the complex. An avidin-biotin complex is resistant to breakdown in the presence of 8 M guanidine at pH 5.2. A minimum of 6-8 M guanidine at pH 1.5 is required for inducing complete dissociation of the biotin-avidin bond (Bodanszky and Bodanszky, 1970).

The only disadvantage in use of avidin is its tendency to bind non-specifically to components other than biotin due to its high pI and carbohydrate content. The strong positive charge on the protein causes ionic interactions with more negatively charged molecules, especially cell surfaces. In addition, carbohydrate-binding proteins on cells can interact with the polysaccharide chain of the avidin molecule to bind them in regions devoid of targeted biotinylated molecules. These non-specific interactions can lead to high background signals in some assays. Most of these problems can be avoided by using streptavidin.

Streptavidin

Streptavidin is another biotin binding protein isolated from *Streptomyces avidinii* that can overcome some of the non-specificities of avidin (Chaiet and Wolf, 1964). Similar to avidin streptavidin contains four subunits, each with a single biotin binding site. After some post-secretory modifications, the intact tetrameric protein has a molecular mass of about 60 KDa, slightly less than that of avidin (Bayer et al., 1989). The primary structure of streptavidin is however different from that of avidin inspite of both having the same number of binding sites and similar avidity for biotin. Streptavidin has a lower isoelectric point (pI 5-6) in comparison to the highly basic

pI of 10 for avidin. This difference in pI is attributed to the variation in amino acid sequence between them. Thus the reduced overall charge of streptavidin results in reduction of non-specific binding of the protein, due to ionic interactions with other molecules. Unlike avidin, streptavidin is not a glycoprotein and thus the possibility of binding to carbohydrate receptors is also eliminated. These properties of streptavidin result in better signal-to-noise ratios in assays using streptavidin-biotin interactions in comparison to avidin-biotin interactions.

The basis of carrying out immunoassays using biotin-avidin and biotin-streptavidin assays is the ability of biotin to be covalently linked to other targeting molecules, such as antibodies. Biotin derivatives may be prepared that contain reactive groups capable of coupling with particular functional groups on proteins, antibodies and other molecules. Such biotin modification of secondary molecules is called "biotinylation". Biotinylation results in formation covalent derivatives containing one or more biotin rings extending from the parent structure of the molecule. The biotin rings present on these molecules are used for immobilising the molecules to streptavidin. The basic design of a biotin-labelling compound is presented in Fig. 1.8.

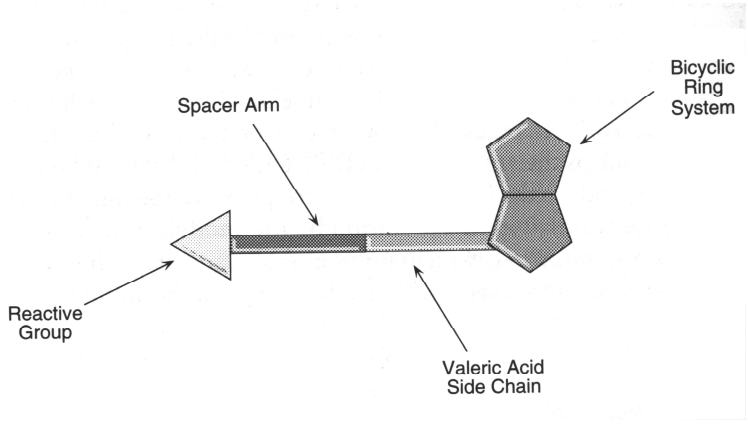

Fig. 1.8: Schematic explaining the basic design of a biotinylation reagent showing the bicyclic ring system and valeric acid side chain of D-biotin at one end of the reactive functional group. The reactive group in case of immunoassays is coupled to the antibody. Spacer groups may be included in the design to extend the biotin group away from the reactive group, thus ensuring better interaction capability with avidin or streptavidin (adapted from: Harmanson et al., 1996).

1.1.2.7 SPR–based biosensors for detection of bacteria

Surface plasmon resonance is a label-free optical detection technique, which has been successfully exploited as a technique for real time monitoring of both chemical and biological species (Homola, 2008). Using SPR technology, it is possible to detect

binding events to antibodies without additional labelling steps (Liedberg et al., 1995). The SPR-based assays besides having the advantages of being label-free and in real-time are also less time consuming (Lundstrom et al., 1994). Here we present a literature review of the work (available at the time when we had conceived the project in 2005) that had been carried out using SPR for the detection of different bacteria. This is important in understanding of the significance of the present work. Literature on bacterial detections using SPR beyond this period has been referred to in this work wherever applicable and they have been cited in the reference section.

Escherichia coli O157:H7 was first detected by SPR by Fratamico et al. (1998). The detection of *E. coli* O157:H7 was reported using monoclonal antibodies immobilised on a protein G-coated sensor surface (Oh et al., 2002). The sensor was demonstrated to be able to directly detect *E. coli* O157:H7 at concentrations of 10^4 cells mL^{-1}. Subsequently, the same authors demonstrated the ability of the same SPR device to detect *E. coli* O157:H7 down to 10^2 cells mL^{-1} by immobilisation of antibodies via a mixed SAM of alkanethiols. (Oh et al., 2003). Taylor et al. (2005) detected *E. coli* O157:H7 using a custom-built SPR sensor with wavelength modulation and examined the effect of various treatment methods on the sensor performance. A monoclonal antibody was immobilised on a mixed -COOH- and -OH-terminated SAM of alkanethiols via amine coupling chemistry. Detection of *E. coli* O157:H7 was performed in the sandwich detection format using a secondary polyclonal antibody. Detection limits for detergent-lysed bacteria, heat-killed bacteria, and untreated bacteria were determined to be 10^4, 10^5, and 10^6 cfu mL^{-1}, respectively. The detection of *E. coli* O157:H7, using a commercially available Spreeta® biosensor (Texas Instruments Co.) was reported by Meeusen et al. (2005). A biotinylated polyclonal antibody against *E. coli* O157:H7 was immobilised on the avidin-functionalised gold surface. The SPR biosensor was shown to be capable of detecting *E. coli* O157:H7 in cultures at levels down to 8.7 X 10^6 cfu mL^{-1}. Another SPR sensor for the detection of *E. coli* O157:H7 based on the Spreeta® sensor was reported by Su and Li (2005). In that work, polyclonal *E. coli* O157:H7 antibodies were immobilised via protein A adsorbed on the sensor surface. The sensor was demonstrated to be able to detect *E. coli* O157:H7 in an aqueous environment at levels down to 10^6 cells mL^{-1}. It is important to note here that none of the assays above were carried out in a food matrix.

Salmonella Enteritidis was detected using a custom-built SPR sensor with wavelength modulation by Koubova et al. (2001). In this work, a double layer of antibodies was physisorbed on a bare gold surface and cross-linked with gluteraldehyde. Direct detection of heat-killed, ethanol-soaked *S.* Enteritidis at a concentration of 10^6 cfu mL^{-1} was demonstrated. Bokken et al. (2003) demonstrated detection of *Salmonella* groups A, B, D, and E using the commercial SPR sensor Biacore®. Antibodies were immobilised on a carboxymethylated dextran layer via amine coupling chemistry, and detection of *Salmonella* was performed using the sandwich assay format. *Salmonella* were detectable at a concentration of 1.7×10^5 cfu mL^{-1}. Oh et al. (2004a) demonstrated the detection of *S.* Typhimurium using monoclonal antibodies immobilised via protein G attached to an alkanethiol SAM on the sensor surface. The limit of detection was 10^2 cfu mL^{-1}. An SPR sensor for the detection of *S.* Paratyphi was demonstrated by the same authors (Oh et al., 2004b). In this case attachment of monoclonal antibodies to the sensor surface was also achieved using protein G. Detection of *S.* Paratyphi was possible down to a concentration of 10^2 cfu mL^{-1}. SPR studies to evaluate compounds that may prevent *Salmonella* attachment to poultry skin and carcasses was carried out by Medina (2004). An investigation to evaluate binding of *S.* Enteritidis to collagen and inhibition of such binding by use of various food additives had been carried out using SPR (Miyamoto et al., 2003). Again, as in the case of detection of *E. coli* using SPR, all the SPR-based assays for the detection of *Salmonella* mentioned above were limited to buffer systems.

Listeria monocytogenes was detected by Koubova et al. (2001). The authors used heat killed bacteria and the lower limit of detection was 10^7 cfu mL^{-1}. Leonard et al. (2004) used the commercial SPR sensor Biacore 3000® and a competitive assay format to detect the *L. monocytogenes.* A polyclonal anti-goat antibody was immobilised on a carboxymethylated dextran layer using amine-coupling chemistry. Solutions of known concentrations of *L. monocytogenes* were incubated with rabbit anti-*Listeria* antibodies. Cells and bound antibodies were then centrifuged out of solution, and the unbound antibodies remaining in solution were detected by the SPR sensor. The limit of detection was determined to be 10^5 cells mL^{-1}.

Thus it is seen from the literature review presented that at the time when this work was started there was no literature available on the detection of bacteria using SPR in complex matrices or in food systems.

1.2 Salmonella

1.2.1 Taxonomy and nomenclature

Salmonella are Gram-negative, facultatively anaerobic, rod-shaped (0.7-1.5 x 2.0-5.0 µm), non-spore forming, mainly motile bacteria (few exceptions), which belong to the family of Enterobacteriaceae. *Salmonella* are the most common and well-known bacterial foodborne pathogens (Le Minor, 1984). They can metabolise a wide variety of organic substrates by both respiratory and fermentative pathways.

The genus *Salmonella* consists of only two species, *S. enterica*, and *S. bongori*. The species *S. enterica* is divided into six subspecies: *S. enterica* subsp. *enterica*, *S enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*. The species and subspecies differentiation are obtained based on various biochemical and genetic tests. Table 1.3 summarises the differential characteristics of *Salmonella* species and subspecies based on the various chemical and biochemical tests.

Table 1.3: Response observed for species and subspecies of genus *Salmonella* to different biochemical tests (adapted from Grimont and Weill, 2007)

Species	***S.* enterica**						***S.* bongori**
Subspecies	***enterica***	***salame***	***arizonae***	***diarizonae***	***houtenae***	***indica***	
β-galactosidase(ONGP test-2h)	-	-	+	+	-	**d**	+
Acid production from:							
lactose	-	-	-(75 %)	+(75 %)	-	**d**	-
sorbitol	+	+	+	+	+	-	+
dulcitol	+	+	-	-	-	**d**	+
mucate	+	+	+	-(70%)	-	+	+
salicin	-	-	-	-	+	-	-
galactouronate	-	+	-	+	+	+	+
Utilisation of:							
Malonate	-	+	+	+	-	-	-
L(+)-tartrate (d-tartrate)	+	-	-	-	-	-	-
Activity of enzymes:							
γ-glutamyltransferase	+(*)	+	-	+	+	+	+
β-glucuronidase	**d**	**d**	-	+	-	**d**	-
gelatinase	-	+	+	+	+	+	-
Lysis by phage O1	+	+	-	+	-	+	**d**

* = Typhimurium , Dublin -

- = 90 % or more negative reactions

+ = 90 % or more positive reactions

d = different reactions given by different serovars

The o-nitrophenol-β-galactopyranosidase test (ONPG) test is used to determine the presence or absence of the enzyme β -galactosidase in an organism. The presence of two enzymes, permease and β -galactosidase are required to demonstrate lactose fermentation. True lactose non-fermenters do not possess either of these enzymes. Late lactose fermenting organisms do not have permease but do possess β-galactosidase, which hydrolyses lactose to form galactose and glucose. ONPG is similar in structure to lactose. If β-galactosidase is present, the colourless ONPG is split into galactose and o-nitrophenol, a yellow compound. Acid production from lactose, sorbitol, dulcitol, mucate (salt of mucic acid), salicin and galactouronate are also monitored as part of the biochemical tests. The monitoring of the utilisation of malonate and L(+)-tartrate (d-tartrate) is another important biochemical test. The malonate test is carried out by growing the bacteria in malonate broth. Malonate broth contains ammonium sulfate, which is the sole source of nitrogen in the medium; sodium malonate is the sole source of carbon. Dipotassium phosphate and monopotassium phosphate provide buffering capability. Sodium chloride maintains the osmotic balance of the medium. Increased alkalinity resulting from malonate utilisation causes the indicator, bromthymol blue, to change colour from green to blue. Jordan´s tartrate agar is used for the tartrate test. *Salmonella,* which utilise tartrate, ferment the tartrate in the medium resulting in change of colour of the phenol red dye, present in the medium, to yellow. The presence or absence of certain enzymes is also used as a parameter to assign the subspecies. Nutrient gelatin is a differential medium that tests the ability of an organism to produce an exoenzyme, called gelatinase that hydrolyses gelatin. The organism growing on a nutrient gelatin will liquify the medium if it is gelatinase positive. In such a case the media does not solidify even on refrigeration. The other tests are the tests for the presence or absence of the enzymes γ-glutamyltransferase and β-glucuronidase tests (using p-nitro-phenyl-beta-D-glucuronide as substrate). The test for the ability of the *Salmonella* isolate to grow in presence of KCN is also utilised in the allocation of the species and subspecies.

1.2.1.1 Kauffmann-White-Le Minor scheme (formerly Kauffmann-White scheme)

Salmonella belonging to each subspecies are grouped into serogroups based on their somatic O-antigens. The O-antigen is a carbohydrate antigen that is the outermost component of lipopolysaccharide (LPS). The LPS is unique to Gram-negative bacteria and the structure of the LPS is discussed in detail in section 1.2.2. The O-antigen is a polymer of O-subunits; each O-subunit is typically composed of four to six sugars depending on the O-antigen. Variation in O-antigen results from variation in the sugar components of the O-subunit, the nature of the covalent bond between the sugars of the subunit, and variation in the nature of the linkage between the O-subunits that form the O-antigen polymer. Based on the type of common O-antigens present *Salmonella* are divided into O-serogroups, also called O-groups. Many of the common O-groups were originally designated by letters and are still commonly referred to by letters. The different *Salmonella* serogroups along with the common and associated O-antigens is presented in Table 1.4.

Salmonella are further assigned to serotypes or serovars based on a combination of their somatic O-and flagellar H-antigens. H-antigen is the filamentous portion of the bacterial flagella; H antigen is made up of protein subunits called flagellin. The ends of flagellin are conserved and give the flagella its characteristic filament structure. The antigenically variable portion of flagellin is the middle region, which is surface-exposed. *Salmonella* is unique among the enteric bacteria in that it can express two different flagellin antigens. Typically, this is coordinated so that only one antigen is expressed at time in a single bacterial cell. The two antigens are referred as Phase 1 and Phase 2. “Monophasic” isolates are those that express only a single flagellin type. The H-antigens of *Salmonella* are listed in Table 1.5.

Table 1.4: *Salmonella* O-serogroups and associated O-antigens (source: Grimont and Weill, 2007)

O-Group (number designation)	O-Group (letter designation)	Antigens present in all serotypes	Additional antigens that may be present in some serotypes
O:2	A	2,12	1
O:4	B	4, 12	1,5,27
O:7	C_1	6,7	14,[Vi]
O:8	*C_2. C_3	6,8	20
O:9	D_1	9,12	1,[Vi]
O:9,46	D_2	9,46	none
O:9,46,27	D_3	9,12,46,27	1
O:3,10	E1	3,10	{10}, {15}, {15, 34}
O:1,3,19	E4	1,3,19	{10}, {15}
O:11	F	11	none
O:13	G	13	1,22,23
O:6,14	H	6,14	[1], 1, [24], 24, [25], 25
O:16	I	16	none
O:17	J	17	none
O:18	K	18	6,14
O:21	L	21	none
O:28	M	28	none
O:30	N	30	none
O:35	O	35	none
O:38	P	38	none
O:39	Q	39	none
O:40	R	40	1,40
O:41	S	41	none
O:42	T	42	1
O:43	U	43	none
O:44	V	44	1
O:45	W	45	none
O:47	X	47	1
O:48	Y	48	none
O:50	Z	50	none
O:51		51	1
O:52		52	none
O:53		53	1
O:54		54	3,4,6,7,8,12,20,21{6,7,14},{54}
O:55		55	none
O:56		56	none
O:57		57	none
O:58		58	none
O:59		59	1
O:60		60	none
O:61		61	none
O:62		62	none
O:63		63	none
O:64		64	none
O:65		65	none
O:66		66	none
O:67		67	none

*Groups O:6,8 (C2) and O:8 (C3) which differed only by the presence or absence of factor O:6,and hence has recently been put together in a single group O:8.

Table 1.5: H (flagellar antigens) of *Salmonella* (source: CDC, 2004).

1 complex	Other antigens not part of the complex
1,2	a
1,5	b
1,6	c
1,7	d
1,2,5	e,h
1,2,7	i
1,5,7	k
1,6,7	(k)
EN complex	r
e,n,x	r,i
e,n,x,z15	y
e,n,z15	z
G complex	z6
f,g	z10
f.g,m,t	z29
f,g,s	z35
f,g,t	z36
g,m	z36,z38
g,m,p,s	z38
g,m,q	z39
g,m,s	z41
g,m,s,t	z42
g,m,t	z44
g,p	z47
g,p,s	z50
g,p,u	z52
g,q	z53
g,s,q	z54
g,s,t	z55
g,t	z56
g,z51	z57
g,z62	z60
g,z63	z61
g,z85	z64
m,p,t,u	z65
m,t	z67
L complex	z68
l,v	z69
l,w	z71
l,z13	z81
l,z13,z28	z83
l,z28	z87
Z4 complex	z88
z4,z23	
z4,z23,z32	
z4,z24	
z4,z32	

Some antigens are composed of multiple factors, which are separated by commas; for example, the second phase antigen of *S.* Typhimurium is composed of factors 1 and 2, which is represented as "1,2". Some of the H-antigens share common antigen factors. These antigens are clustered in five complexes, the 1, EN, G, L, and Z4 and complex (Table 1.5).

This combination of O- and H-antigens of each *Salmonella* serovar is referred to as the antigenic formula and forms the basis of the "Kauffmann-White scheme". Recently the WHO Collaborating Centre for Reference and Research on *Salmonella* (WHOCC-Salm, Institut Pasteur, Paris) has renamed the "Kauffmann-White"

scheme as “Kauffmann-White-Le Minor” scheme. The WHOCC-Salm maintains and updates this list of antigenic formulas of *Salmonella* serovars. The Kaufmann-White scheme was first published in 1934 and contained only 44 serovars. The latest update (January 1st, 2007) from WHOCC-Salm lists a total number of 2579 serovars of the genus *Salmonella*. Of which majority 2557 (99.1%) belong to the species S. *enterica*. Within the species *S. enterica* the subspices *enterica* accounts for 60% (1531) of the serovars (Table 1.6). According to this scheme the six subspecies of the species *S. enterica* are denoted by Roman numerals I, II, IIIa, IIIb, IV, and VI, respectively. The species *S. bongori* is designated by the Roman numeral V.

1.2.1.2 Designation of antigenic formula of *Salmonella* serotypes

As mentioned *Salmonella* serotypes are designated according to the conventions of the “Kauffmann-White” Scheme. All *Salmonella* serotypes can be designated by a antigenic formula. Additionally, subspecies I serotypes are given a name (e.g., Typhimurium, Enteritidis, Typhi, etc). Serovars belonging to *S. enterica* subsp. *enterica* are designated by a name usually related to the geographical location where the serovar was first isolated in addition to their antigenic formulae. This name is written in Roman letters (not italicised) and the first letter is a capital letter. Serovars belonging to other subspecies are designated by their antigenic formulae, following the subspecies name. (Tindall et al., 2005; Popoff et al., 2004).

The typical format for an antigenic formula is:

Subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen

Examples:

I 4,5,12:i:1,2 (*S. enterica* serotype Typhimurium or *Salmonella* Typhimurium)

I 9,12:g,m:- (*S. enterica* serotype Enteritidis or *Salmonella* Enteritidis)

II 47:b:1,5 (*S. enterica* serotype II 47:b:1,5 or *Salmonella* II 47:b:1,5)

IV 48:g,z51:- (*S. enterica* serotype IV 48:g,z51:- or *Salmonella* IV 48:g,z51:-)

IIIb 65:(k):z (*S. enterica* serotype IIIb 65:(k):z or *Salmonella* IIIb 65:(k):z)

Other conventions:

- Some O and H factors are variably present. This is indicated in the generic serotype formula by underline when the variable factor is known to be encoded on a bacteriophage (e.g., O factor <u>1</u>; only described for O antigens) or by square brackets (e.g., O factor [5] or H antigen [1,2]) when it is not. For

an individual isolate, if the variable factor is detected it is included in the formula without additional notation. If the variable factor is not detected, it is not listed in the formula.

- Some O and H factors are variably expressed. Weakly recognized antigens are indicated by parentheses; e.g., O antigen (6),14 or H antigen (k).In monophasic isolates, the absence of an H antigen is indicated by a minus sign (-) for the particular phase. Variants of serotypes that do not express all the recognized antigens characteristic of a particular serotype are not uncommon. This is a particular issue for subspecies I serotypes, where a serotype name cannot be designated without the detection of all the antigens specified in the Kauffmann-White scheme for that serotype. Isolates missing one or more antigens are designated by a formula. For example monophasic variants are variants of typically diphasic serotypes that lack the expression of either the flagellar Phase 1 or Phase 2 antigen; these are indicated by a minus sign ("-") in place of the missing phase; e.g., monophasic variants of *S.* Typhimurium that lack the second phase H antigen 1,2 are designated as *S.* I 4,5,12:i:- or *S.* I 4,12:i:-; monophasic variants of *S.* Typhimurium that lack the first phase H antigen i are designated as *S.* I 4,5,12:-:1,2 or *S.* I 4,12:-:1,2.
- Nonmotile variants express no H-antigens and are indicated by minus signs in both phases or by "nonmotile" in place of the H-antigens; e;g., *S.* I 4,5,12:nonmotile or *S.* I 4,5,12:-:-.
- Rough variants are isolates that do not express O-antigen. This is indicated by "Rough" in place of the O-antigen in the antigenic formula; e.g., I Rough:i:1,2.
- Mucoid variants express a capsule that prevents immunologic detection of the O-antigen. They are indicated by "Mucoid" in place of the O-antigen in the antigenic formula; e.g., I Mucoid:i:1,2.
- Rarely, isolates express a third H antigen that is noted by a colon followed by the antigen after the Phase 2 H antigen (e.g., S. II 9,12:g,m,[s],t:1,5,7:z42)

This nomenclature reflects the presently used *Salmonella* taxonomy (Brenner et al., 2000; Heyndrickx et al., 2005). The genus *Salmonella* encompasses a large taxonomic group having till date 2579 recognized serovars (Grimont and Weill, 2007.

1.2.1.3 Serotyping of *Salmonella*

The determination of the antigenic formula of isolated *Salmonella* is referred to as serotyping. As explained above, the determination of the antigenic formula involves identification of the O- and H-antigen combinations. Serological interactions between the *Salmonella* antigens and their respective antibodies form the basis of serotyping. The antibodies are usually raised against the purified form of the antigens in animals (mouse, goat or rabbit) using different antibody production techniques (Herrera-León S et al., 2007; Iankov et al., 2001). Serotyping is used to identify the organism beyond the level of subspecies and is the most important tool used in identification, taxonomic classification and monitoring and control of *Salmonella* and *Salmonella* outbreaks. The 2579 serotypes (Table 1.6) have been described on the basis of somatic (O), flagellar (H), and capsular (Vi) antigens (Grimont and Weill, 2007).

Table 1.6: *Salmonella* species, the subspecies, numbers of serotypes in each subspecies and their usual habitats.

***Salmonella* species and subspecies**	**No. of serotypes within species**	**Usual habitat**
S. enterica	2557	
S. enterica subsp. *enterica* (I)	1,531	Warm-blooded animals
S. enterica subsp. *salamae* (II)	505	Cold-blooded animals and cold environment
S. enterica subsp. *arizonae* (III a)	99	Cold-blooded animals and cold environment
S. enterica subsp. *diarizonae* (III b)	336	Cold-blooded animals and cold environment
S. enterica subsp. *houtenae*(IV)	73	Cold-blooded animals and cold environment
S. enterica subsp. *indica* (VI)	13	Cold-blooded animals and cold environment
S. bongori (V)	22	Cold-blooded animals and cold environment
Total	2,579	

Slide agglutination test (SAT)

The conventional and most commonly used method of serotyping is the slide agglutination test (SAT). The method of serotyping using the SAT involves mixing the antisera with the bacterial culture on a glass slide. Each antiserum is tested separately. After mixing the antisera and the bacterial culture, visual observation is carried out for either the presence or absence of agglutination. This observation is

carried out in front of a light source against a dark background. This evaluation is therefore subjective. O-antigens and H-antigens are detected in independent agglutination assays using antisera that react with groups of related antigens or a single antigen. In case of biphasic strains both H-antigens can sometimes be detected in a single culture. When only one H-antigen is detected, the isolate is inoculated in a phase reversal media, a semisolid media containing antisera to the H-antigen that has already been identified. Organisms expressing the previously detected H-antigen are immobilised in the media by the added antisera. Organisms expressing the second H-antigen are able to move and are isolated from the surface. The second H-antigen is then determined using the bacteria, which are motile on the phase inversion media.

1.2.2 *Salmonella* Lipopolysaccharide

1.2.2.1 Gram-negative and Gram-positive bacteria

Salmonella is a Gram-negative bacteria. The Gram stain broadly differentiates bacteria into Gram-positive and Gram-negative groups; a few organisms are consistently Gram-variable. Gram-positive bacteria are encased in a plasma membrane covered with a thick wall of peptidoglycan. Gram-negative bacteria are encased in a triple-layer. The outermost layer contains lipopolysaccharide (LPS). Most Gram-positive bacteria have a relatively thick (about 20 to 80 nm), continuous cell wall (often called the sacculus), which is composed largely of peptidoglycan (also known as mucopeptide or murein). Within this thick cell wall, other cell wall polymers (such as the teichoic acids, polysaccharides, and peptidoglycolipids) are covalently attached to the peptidoglycan. In contrast, the peptidoglycan layer in Gram-negative bacteria is thin (about 5 to 10 nm thick). Outside the peptidoglycan layer in the Gram-negative envelope is an outer membrane structure (about 7.5 to 10 nm thick). In most Gram-negative bacteria, this membrane structure is anchored non-covalently to lipoprotein molecules (Braun's lipoprotein), which, in turn, are covalently linked to the peptidoglycan. The LPS of the Gram-negative cell envelope forms part of the outer leaflet of the outer membrane structure.

The basic differences in surface structures (Fig. 1.9) of Gram-positive and Gram-negative bacteria explain the results of Gram staining. Both Gram-positive and

Gram-negative bacteria take up the same amounts of crystal violet and iodine. The crystal violet-iodine complex, however, is trapped inside the Gram-positive cell by the dehydration and reduced porosity of the thick cell wall as a result of the differential washing step with 95 % ethanol or other solvent mixtures. In contrast, the thin peptidoglycan layer and probable discontinuities at the membrane adhesion sites do not impede solvent extraction of the crystal violet-iodine complex from the Gram-negative cell. The above mechanism of the Gram stain based on the structural differences between the two groups has been confirmed by sophisticated methods of electron microscopy (Beveridge and Davies, 1983).

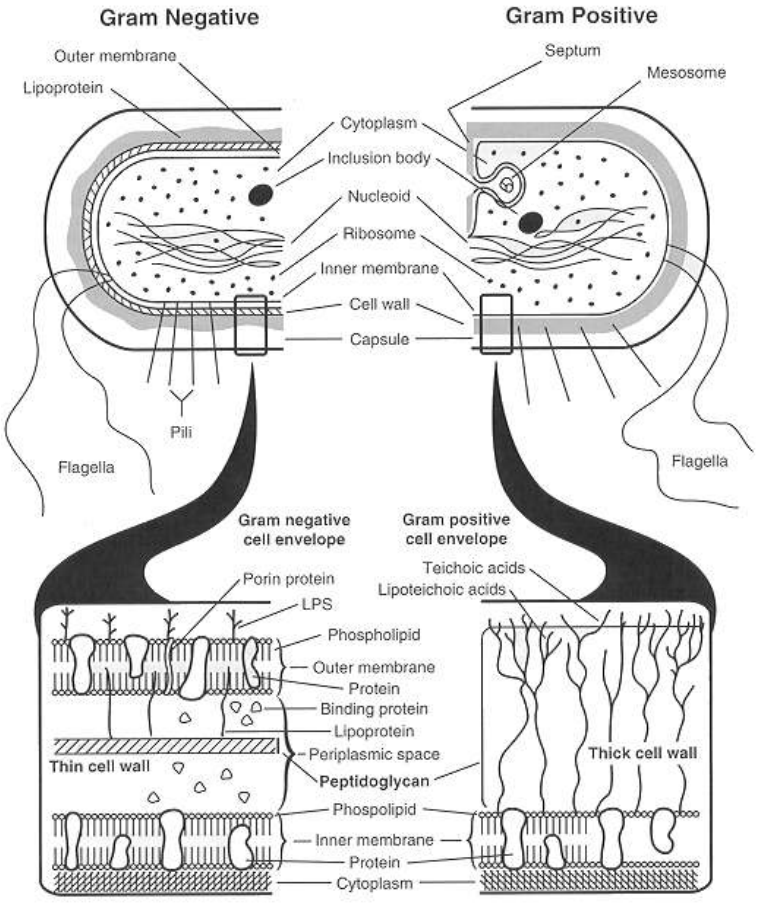

Fig. 1.9: Comparison of the thick cell wall of Gram-positive bacteria with the comparatively thin cell wall of Gram-negative bacteria. Note the complexity of the Gram-negative cell envelope (outer membrane, its hydrophobic lipoprotein anchor; periplasmic space). [adapted from: Medical microbiology, 4th edition. The University of Texas Medical Branch at Galveston, Edited by Samuel Baron].

Moreover, mechanical disruption of the cell wall of Gram-positive organisms or its enzymatic removal with lysozyme results in complete extraction of the crystal violet-iodine complex and conversion to a Gram-negative reaction. Therefore, autolytic wall-degrading enzymes that cause cell wall breakage may account for Gram-negative or variable reactions in cultures of Gram-positive organisms (such as *Staphylococcus aureus, Clostridium perfringens, Corynebacterium diphtheriae,* and some *Bacillus* spp).

1.2.2.2 Structure of LPS

Lipopolysaccharides (LPS, endotoxins) are amphiphilic molecules, which are present in the outer membrane of most Gram-negative bacteria (Fig. 1.10). They are made up of a carbohydrate moiety of varying size, consisting in most cases of a polysaccharide composed of repeating units (O-chain, O-specific polysaccharide, O-antigen) that is linked to an oligosaccharide (the core region), which in turn substitutes a lipid moiety (Lipid A). The LPS exhibit antigenic properties and can act as potent virulence factors and are well known as bacterial endotoxins, which play an important role in the pathophysiology of Gram-negative sepsis.

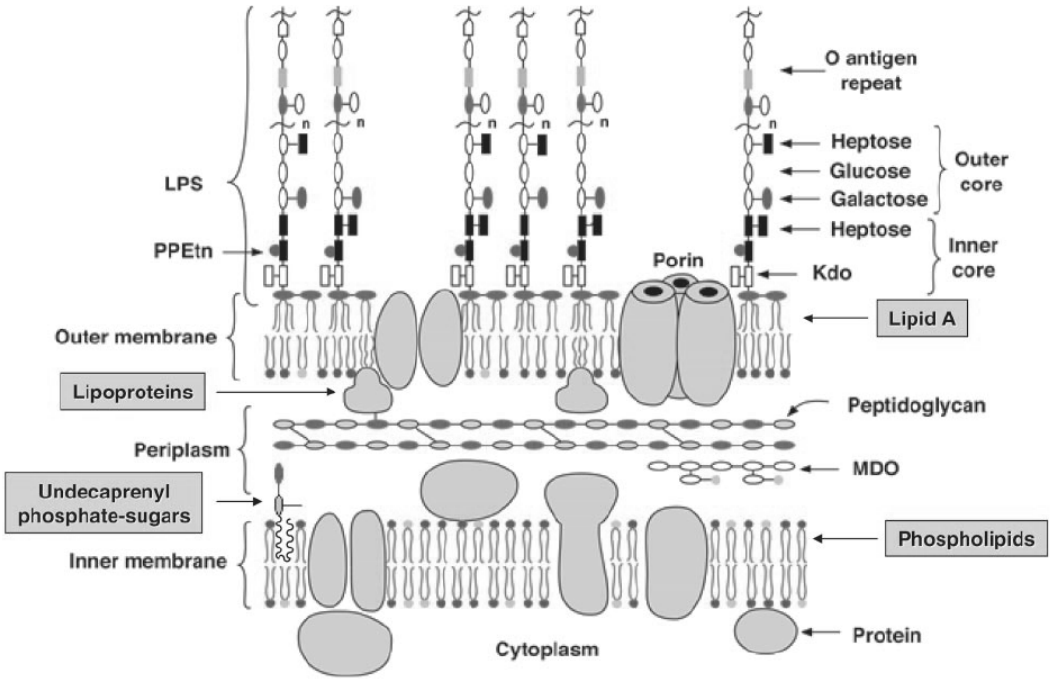

Fig 1.10: Schematic representation showing the different regions of the cell wall of Gram-negative bacteria. The outer membrane shows the LPS, which are embedded into the phospholipid layer through their hydrophobic Lipid A portion. The hydrophilic regions of the LPS consiting of the inner and the outer cores, and the antigenic O-repeat units are also shown (adapted from: Raetz, 1990).

The basic structure of the LPS is explained in Fig. 1.10. The structure and biochemistry of LPS has been extensively studied (Ratez, 1990; Ratez and Whitefield, 2002). Here, we briefly review the basics of the structure and chemistry of LPS, which are important in understanding of the principle behind the working of the SPR-based immnoassays, developed for the detection of the Gram-negative bacteria *Salmonella*.

Lipid A consists of a β-1,6 linked disaccharide of glucosamine, acylated with R-3-hydroxymyristate at positions 2, 3, 2′, and 3′, and phosphorylated at positions 1 and 4′ (Fig. 1.11). The two R-3-hydroxy-acyl groups of the non-reducing glucosamine (Rietschel et al., 1983; Takayama et al., 1983) are further esterified with laurate and myristate (Fig. 1.11). Thus, Lipid A is polar in nature with an overall negative charge due to the presence of the phosphate groups. This hydrophobic property of lipid A has been used in this work for oriented immobilisation of LPS on hydrophobic SPR chips. Lipid A is the membrane anchor of the lipopolysaccharide (LPS) (Fig. 1.11) (Nikaido et al., 1987; Osborn, 1979). In LPS, the 6′ position of lipid (Fig. 1.11) is glycosylated with a non repeating oligosaccharide, designated the core (Strain et al., 1983). There are certain general trends, which have been reported for the lipid A structures found in Gram-negative bacteria. The lipid A of enteric Gram-negative bacteria have an “asymmetrical” pattern with respect to their acyl side chain placement (four fatty acids are associated with the non-reducing end, while only two are linked to the reducing end of glucosamine) as compared to the lipid A of the few non-enteric pathogens that have been characterized as “symmetrical” with respect to acyl chain placement (Helander et al., 1988). Those non-enteric bacteria that tend to grow at lower temperatures (Kropinski et al., 1982; Goldman et al., 1988) possess lipid A bearing somewhat shorter fatty acyl chains. This alteration might function to optimize the physical properties of lipid A for growth at lower temperatures. Lipid A subspecies may exist within the same preparation that contain additional polar substituents at positions 1 and 4′, including 4-amino-4-deoxy-L-arabinose and phosphoethanolamine. The functions of these moieties are unknown, but they generally reduce the net negative charge on lipid A. Polymyxin-resistant strains of *S. typhimurium* have been described with increased amounts of the 4-amino-4-deoxy-L-arabinose on lipid A (Vaara et al., 1981)

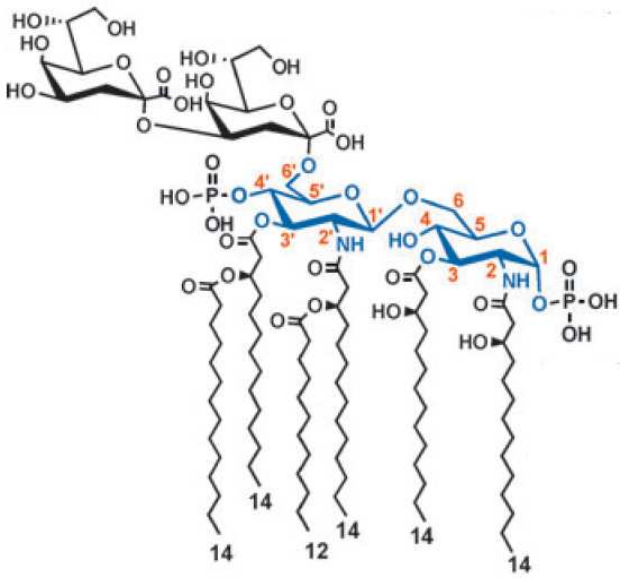

Fig 1.11: Chemical structure of the Lipid A –KDO portion of the LPS. The glucosamine ring structure is shown in blue. The KDO ring structure is shown in black, which linked to 6´ position of glucosamine ring. Numbers in red show the glucosamine ring positions and the numbers in black show the predominant fatty acid chain lengths (adapted from: Raetz, 1990).

The eight-carbon sugar, 3-deoxy-D-manno-octulosonic acid (KDO), is directly linked to lipid A (Fig. 1.11) (Rick et al., 1987). The other core sugars include L-glycero-D-manno-heptose (hep), glucose, galactose, and N-acetylglucosamine (Anderson et al., 1983). The core oligosaccharide of LPS can be divided into two regions an inner subdomain and an outer subdomain. The most common constituent of the inner core in Gram-negative bacteria is the KDO and hep. The outer core consists of hexsoses, primarily glucose, galactose and N-acetylglucosamine. Other than providing an attachment site for O-antigen, the function of the outer core is unknown. It is important to note that the within a genus or family Gram-negative bacteria, the structure of the core region is known to be well conserved.

The next most important domain of the LPS is the O-antigen domain. The O-antigen domain consists of repeating oligosaccharide, attached to a distal glucose residue of the core. The O-antigen repeat (often a tetrasaccharide) may be forty units long. (Imoto et al., 1987). However some LPS molecules may lack the O-antigen, in spite of having the genes responsible for synthesis of the O-antigens. These are referred to as rough strains. In *E. coli* K 12, the O-antigen is missing entirely (Galanos et al., 1977). O-antigen structures are highly variable, compared to those of the core lipid A, possibly helping bacteria evade the immune system. The O-antigen by virtue of its attachment to a terminal sugar of the outer core is exposed and is thus the main

immunogenic factor that distinguishes different Gram-negative bacteria from one another (Anderson et al., 1983). In *Salmonella* there are more than thousand immunochemical variants of the O-antigen (Rietschel et al., 1983). The serogrouping of *Salmonella* is based on grouping together of *Salmonella* isolates, which contain certain common O-antigens, and this forms the basis of serotyping *Salmonella*. The differentiation of *Salmonella* based on the detection of specific O-antigens, present on their cell surface, is also the underlying principle of the detection technique using SPR presented in this work.

LPS can be extracted using aqueous phenol (Westphal and Lüderitz., 1954). This is the commonly used method for obtaining purified LPS fractions from different Gram-negative bacteria. When LPS is extracted from cells with phenol-containing solvents, it is frequently accompanied by organic counterions, such as polyamines (Galanos and Lüderitz., 1975). These can be removed by electrodialysis, or preferably in the case of rough LPS, by partitioning in two-phase mixtures of chloroform, methanol, and water under acidic conditions (Brozek et al., 1989). The ability to extract purified form of the LPS is another important contribution to the field of immunology. Purified LPS fractions from different pathogenic Gram-negative bacteria are frequently used as capture antigens in immunological assays for detection of infections caused by these bacteria. Our work presented here on detection of *Salmonella* infections by SPR also is based on the same principle. LPS also has a high affinity for Ca^{2+} and Mg^{2+} (Nikaido et al., 1987). The incubation of *E. coli* and other Gram-negative bacteria in high concentrations of Ca^{2+} render them competent for uptake of DNA, permitting genetic transformation without loss of viability (MacLachlan and Sanderson., 1985). The role of Ca^{2+} in generating the competent state probably involves interaction of the ion with the LPS.

1.2.3 Salmonellosis

The disease caused by *Salmonella* is called salmonellosis. The establishment of a human *Salmonella* infection depends on the ability of the bacteria to survive the environment outside the digestive system, the ability to survive the gastric acid of the human stomach and the ability of the pathogen to attach (colonise) and enter (invade) intestinal cells. For the latter, *Salmonella* must compete with indigenous gut microorganisms for suitable attachment sites (Barman et al., 2008). Diarrhoea

associated with salmonellosis is thought to appear in response to bacterial invasion of intestinal cells rather than the action of enterotoxins. A main difference with other bacterial intestinal pathogens like *Shigella* and *E. coli*, who are replicating within the cytoplasma of host cells, is that *Salmonella* is confined to vacuoles in which bacterial replication takes place. These vacuoles are called *Salmonella* containing vacuoles (SCVs). The infected vacuoles move and release *Salmonella* cells into the tissue. Prior to invasion of intestinal cells, *Salmonella* has to encounter and attach to these cells (Steele-Mortimer, 2008). This involves several types of fimbriae or pili. Other virulence factors of *Salmonella* include siderophores (to retrieve essential iron from the host) and enterotoxins.

1.2.3.1 Salmonellosis in humans

Human salmonellosis continues to be a major international problem both in terms of morbidity and economic losses (Barnass et al., 1989). Salmonellosis is primarily associated with consumption of food products contaminated with *Salmonella.* The Robert Koch Institute in Germany reported 21,596 cases of salmonellosis during the first 8 months of 2008 [Anonymous, 2008]. In Germany (May, 2007), a batch of contaminated dessert resulted in a salmonellosis outbreak causing at least 239 sick and one death [Anonymous, 2007]. *Salmonella*-contaminated vegetables and fruits were also identified as a widespread source of *Salmonella* infections in humans (Brandl, 2006).

The incubation period is usually within the range of 12-72 h, but occasionally may extend up to a week. In some outbreaks, where large numbers of organisms are believed to have been consumed, incubation periods as short as 2.5 h have been reported (Stevens et al., 1989). *Salmonella* infection most commonly results in a gastroenteritic illness with diarrhoea and, from time to time, a number of other symptoms can occur. In the majority of cases the acute gastrointestinal symptoms will clear with supportive therapy only within 4-5 days. Malaise, lassitude, and weakness may continue for substantially longer time. A possible complication of acute salmonellosis is a reactive arthritis.

1.2.3.2 Salmonellosis in pigs

Clinical porcine salmonellosis can be separated into two syndromes: one involves *S.* Typhimurium, which is associated with enterocolitis, while the other involves

S. Choleraesuis and is usually associated with septicaemia. Septicaemic pigs are inappetent, lethargic and febrile, with temperatures of up to 41.7 °C. Respiratory signs may consist of a shallow, moist cough and diaphragmatic breathing. Clinical signs first appear after 24-36 h of infection (Reed et al., 1986). Diarrhoea is normally not a feature of *S.* Choleraesuis infection until at least the fourth or fifth day of infection. It may last from 5 to 7 days after onset if chronic reinfection is not occurring. Enterocolitis in pigs is typically associated with *S.* Typhimurium infection and occasionally with *S.* Coleraesuis infection. In contrast to the septicaemic disease, the initial sign of infection is often watery, yellow diarrhoea. Infected pigs show inappetence, fever and lethargy. Mortality is usually low. However, morbidity can be high within a few days of infection (Wilcock and Schwartz, 1992). The prevalence of *Salmonella* in the intestine of individual pigs from different sources is extremely variable (Gray et al., 1995, 1996a, 1996b). Individual animals may remain as carriers for up to 36 weeks (Wood and Rose, 1992). *Salmonella* are a major cause of economic losses in the pig production chain, resulting in millions of dollars in lost income to the pork industry. Transmission of *Salmonella* between hosts is thought to occur via the faecal-oral route of exposure. A number of studies have reproduced experimental infection by the oral route and, during acute disease; pigs will shed up to 10^6 S. Choleraesuis g^{-1} faeces (Smith and Jones, 1967) or 10^7 *S.* Typhimurium g^{-1} faeces (Gutzmann et al., 1976). Further studies in pigs indicated that *Salmonella* could be isolated from tonsils, mesenteric lymphnodes and intestine contents just 2 h after oral infection (Blaha, 2001). The occurrence of salmonellosis in suckling pigs is rare, presumably because of lactogenic immunity, but infection is not uncommon (Fedorka-Cray et al., 2000; Wilcock et al., 1976; Gooch and Haddock, 1969).

1.2.3.3 Pathogenesis of *Salmonella* infection

The pathology of *Salmonella* infections is extremely variable. Severity is influenced by serotype, virulence, natural and acquired host resistance, route and quantity of the infective dose (Reed et al., 1986). *Salmonella* usually gain entrance to the host by the oral route and are deposited in the intestine, where they invade the intestinal absorption cells also called enterocytes (Wray and Sojka, 1977). Interperitonial and intravenous infection are also possible. The basic virulence strategy common to *Salmonella* is to invade the intestinal mucosa. Here the gut-associated lymphoid tissue (Peyer's patches) tries to limit multiplication of the bacteria. If this defence

mechanism is not sufficient enough, *Salmonella* are drained from the infected intestinal tissues to the regional lymph nodes, where the bacteria come in contact with macrophages. If the the host defence mechanisms like macrophages successfully capture the bacteria, bacterial multiplication is limited, the infection remains localised in the intestine and the bacteria are unable to spread (Bäumler et al., 2000). After a localised infection, the pathogens spread into the body from the gut-associated lymphoid tissue via the efferent lymphatics and the thoracic duct into the vena cava. Release of endotoxin into the circulation can account for the systemic disease, including fever, pneumonia, meningitis, and septic arthritis (Clarke and Gyles, 1993). Patients with diarrhea usually recover completely, although it may be several months before their bowel habits are entirely normal. A small number of persons with *Salmonella* develop pain in their joints, irritation of the eyes, and painful urination. This is called Reiter's syndrome. It can last for months or years, and can lead to chronic arthritis, which is difficult to treat. Antibiotic treatment does not make a difference in whether or not the person develops arthritis.

Owning to its importance, the mechanism of *Salmonella* invasion of human cells is under intense study. Two *Salmonella* pathogenicity islands (SPI-1 and SPI-2) encoding structural elements of type III secretion systems (T3SS) and effectors injected into the host cells, are necessary for entry and proliferation within mammalian cells (Holden, 2002). Infection occurs in several well-organized and adjusted steps. The first one includes docking of *Salmonella* to the epithelial cell and injection of SPI-1 encoded effectors, which suppress the host immune system and modify the actin and tubulin cytoskeleton (Guignot et al., 2004). Endocytosis is the second step and requires formation of *Salmonella* containing vacuoles (SCVs). Retainment of SCVs in the host cytoplasm is assured by SPI-2 encoded effectors (Waterman and Holden, 2003) and strains not capable to sustain intact SCVs are avirulent (Fields et al., 1986).

1.2.3.4 Immunity to *Salmonella* in animals

The immune system consists of two broad categories, innate and specific immunity. Innate, or non-specific immunity includes serum components, such as complement, and non-specific defence cells, such as polymorphonuclear neutrophils, macrophages and natural killer cells. The antibody, produced by B lymphocytes, provides the active effector function for humoral immunity. Antibodies protect the host against

infection by binding to the surface of infecting organisms and thus preventing them from attaching to and invading host cells (McGhee et al., 1992), and consequently enhance their engulfment and killing by phagocytic cells (Johnson et al., 1985). Cellular immunity is mediated by T lymphocytes and these cells can have either a direct effector function (cytolytic T lymphocytes) or a regulatory function (helper and suppressor T cells) by modifying the activation of B cells or other T cells. This component of the immune response is important in protecting against intracellular pathogens, such as viruses, parasites and bacterial species and acts through direct killing of the infected host cell or the activation of the phagocytic cell defences (Schat, 1994; Lillehoj, 1993). Both humoral and cellular immunity appear to play a role in protection against a *Salmonella* infection. About one week post-infection an antibody response can be detected in the sera of pigs and this response can persist for 10 weeks or more (Gray et al., 1996b). Serum immunoglobulin, IgM anti-*Salmonella* responses appear first, followed by IgG and IgA (Hassan et al., 1991). The IgM and IgA levels gradually decline, while IgG levels can persist for extended periods (Chart et al., 1992). Reinfection results in a rapid, enhanced antibody response (Hassan et al., 1991). Serum immunoglobulins can be passed vertically into the colostrum (Jones et al., 1988). Serum antibodies are detectable even after individuals are no longer culture positive for the organism (Gast et al., 1997). Experimentally and naturally infected pigs have been shown to have a titre to LPS for at least 12 weeks after exposure to *Salmonella*, even after clearing the bacteria (Gray et al., 1996b).

1.2.4 Detection of *Salmonella* and *Salmonella* infections

1.2.4.1 Microbiological detection

The cultural isolation of *Salmonella* is most common in animal-production and food processing industries. Most of the methods were developed for the diagnosis of clinical salmonellosis in humans and animals (Fedorka-Cray et al., 2000). The microbiological isolation and detection of *Salmonella* involves the use following culture media.

Pre-enrichment media

Several pre-enrichment media have been proposed (D'Aoust, 1981). Lactose broth was perhaps the first to receive widespread use. But there has been concern that lactose broth, because of the fermentation of lactose and resulting acidity, would allow the pH to fall to a level that is inhibitory or lethal to *Salmonella* (Hiker, 1975). Buffered peptone water (BPW) has been used without a fermentable sugar and with greater buffering capacities. BPW was found to be more appropriate than lactose broth for isolating *Salmonella* (Fricker, 1987). The pH of the lactose broth cultures after incubation ranged from 4.8-5.5, whereas the range for BPW was 5.8-6.4, respectively. BPW has been the pre-enrichment broth of choice for use in conjunction with Rappaport-Vassiliadis (RV) enrichment media (Waltman, 2000).

Selective-enrichment broth

Selective-enrichment broths are formulated to selectively inhibit other bacteria while allowing *Salmonella* to multiply to levels that may be detected after plating. There are currently three major types of selective-enrichment media: tetrathionate, selenite and Rappaport-Vassiliadis (RV). There are also different formulations within each type of enrichment (Waltman, 2000).

(a) Selenite enrichment media:

Leifson (1936) formulated the first selenite enrichment media, commonly known as selenite F (SF). Bacteria reduce selenite, which increases the pH and reduces the toxicity of selenite. North and Bartram (1953) modified SF by adding cystine [selenite-cystine (SC)]. Stokes and Osborne (1955) modified SF by changing the carbohydrate source from lactose to mannitol and adding sodium taurocholate and brilliant green [selenite brilliant green (SBG)]. In a comparative study, Waltman et al. (1995) compared SF, SC, and SBG with tetrathionate and RV enrichment broths. The tetrathionate and RV enrichment results were better than the results from the use of selenite enrichment. In addition selenite is reported to be toxic and a mutagen (Andrews, 1996).

(b) Tetrathionate enrichment media:

Muller (1923) described a selective enrichment broth that contained iodine and sodium thiosulphate, which he combined to form tetrathionate. Kauffmann (1935)

modified the tetrathionate brilliant green (TBG) enrichment broth of Muller by adding ox bile and brilliant green (MKTT). Several studies have shown that tetrathionate enrichment is better than selenite enrichment (Waltman et al., 1995; D'Aoust et al., 1992).

(c) Rappaport-Vassiliadis (RV) enrichment media:

Rappaport et al. (1956) described an enrichment medium based on the ability of *Salmonella* to: survive relatively high osmotic pressures (using magnesium chloride), multiply at relatively low pH (pH 5.2), selectively grow in presence of malachite green (106 mg L^{-1}), and grow with minimal nutritional requirements (5 g peptone L^{-1}). Vassiliadis et al. (1970, 1976) modified the medium by reducing the concentration of malachite green, which made the medium suitable for incubation at 43°C. Several studies have shown that RV enrichment was better than either tetrathionate or selenite enrichment broths (Waltman et al., 1995; Oboegbulem, 1993). RV enrichment medium has been approved for isolating *Salmonella* from raw, highly contaminated food and animal feeds, replacing the use of selenite enrichment media (June et al., 1996). Goossens et al. (1984) developed a semi-solid medium based on RV enrichment broth [modified semi-solid Rappaport-Vassiliadis (MSRV)]. The MSRV plate is incubated at 41.5°C and the motility of *Salmonella* further selects and differentiates *Salmonella* from other microorganisms. Several studies have shown the advantages of the MSRV culture method (Davies and Wray, 1994; Read et al., 1994; Aspinall et al., 1992).

Plating media

Plating media should be judged not only on their ability to selectively grow *Salmonella,* but also on their ability to differentiate colonies of *Salmonella* from those of other bacteria. Many selective plating media are available for the isolation of *Salmonella.* The plates are incubated at 35-37°C for 20-24 h and colonies suspected to represent *Salmonella* are taken for further investigations (Waltman, 2000). Brilliant green (BG) agar was developed by Kristensen et al. (1925) and later modified by Kauffmann (1935). The selectivity of the agar derives from the presence of the brilliant green dye and the presence of lactose and sucrose (BPLS), which is the basis for the differential capabilities of the media. Almost all *Salmonella* fail to ferment either lactose or sucrose and their colonies appear pink to red, with a

reddening of the media. Several investigators have proposed the use of BPLS (Waltman et al., 1995).

Rambach agar (RAM) was developed based on the finding that *Salmonella* produce acid from propylene gycol (Rambach, 1990). The selective ability results from the presence of bile salts. The differential characteristics involve the fermentation of propylene gycol by *Salmonella* and resulting in a change in colour of the colonies to pink red. This is caused due to the precipitation of the neutral red dye present in the media, due to change in pH to acidic conditions. A chromogenic indicator system for β-galactosidase acitiviy is used to differentiate *Salmonella* from other lactose fermenting members of the family Enterobacteriaceae. In presence of lactose fermentors the chromogenic substance, X-Gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside), results in the formation of blue-green colonies. But some serovars of *Salmonella* do not produce the characteristic red colonies, e.g. *S.* Pullorum, *S.* Gallinarum, *S.* Abortus, and *S.* Dublin (Pignato et al., 1995; Kühn et al., 1994).

Other plating media are also used for isolation of *Salmonella* (Table 1.7). Thus, *Salmonella* give colourless colonies with black centres on *Salmonella-Shigella* (SS) agar; blue-green with black-centred colonies are seen on Hektoen agar; Typically *Salmonella* species will appear as black or red to pink colonies with a black center on Xylose lysine tergitol 4 (XLT4) media due to the ability to reduce thiosulfate to hydrogen sulfide. *Salmonella* grow red with black centres on Xylose lysine desoxycholate (XLD) agar; giving yellow colonies on Gassner agar and red colonies on Brilliant green agar (BGA).

Table 1.7: Appearance of *Salmonella* on most commonly used selective agars (adapted from Waltman, 2000)

Medium	Appearance of *Salmonella* colonies
Bismuth sulphite agar	Black metallic sheen
Brilliant green agar	Red
Brilliant green sulphapyridine	Red
Brilliant green novobiocin	Red
Deoxycholate citrate agar	Colourless BC*
Gassner agar	Yellow
Hektoen enteric agar	Blue-green BC
MacConkey agar	Colourless
Rambach agar	Crimson with pale borders
Salmonella-Shigella agar	Colourless BC*
Xylose lysine desoxycholate agar	Red BC*
Xylose lysine tergitol 4	Red BC*

*BC, black centre due to H_2S production

1.2.4.2 Biochemical identification

Typical *Salmonella* colonies isolated on any of the selective plating media (section 1.2.4.1) should be confirmed by biochemical tests. The principal biochemical tests by which *Salmonella* can be identified are given in Table 1.8. The (+) signs indicate that this factor or reaction must be seen in *Salmonella*, (-) indicates the reaction should not appear. Testing all these reactions requires considerable resources. Therefore it more convinient to use composite media such as triple sugar iron agar (TSI), which is prepared in tubes. It contains glucose, lactose and sucrose, a H_2S detection system and an indicator (Jones et al., 2000).

Table 1.8: Biochemical reactions of typical *Salmonella* spp. (adapted from Waltman, 2000)

Test	Reaction of typical strains	Test	Reaction of typical strains
		Fermentation of:	
Nitrate reduction	+	Glucose	+
Oxidase	-	Mannitol	+
Urea hydrolysis	-	Maltose	+
Indole*	-	Lactose	-
H_2S production	+	Sucrose	-
Citrate utilisation*	-	Salicin	-
Sodium malonate	-	Adonitol	-
Growth on KCN	-	Dulcitol	+
Methyl red*	+	Lysine decarboxylase	+
Gelatin liquefaction	-	Arginine dihydrolase	+
Voges Proskauer *	-	Ornithine decarboxylase	+
		Deamination of phenyalalanine	-

*** These four tests together form a series of tests referred to as the IMViC test (Indole, Methyl red, Voges Proskauer and Citrate). The Voges Proskauer test detects organisms that utilize the butylene glycol pathway and produce acetoin.**

TSI is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It is used to differentiate enteric bacteria based on the ability to reduce sulfur and ferment carbohydrates. As with the phenol red fermentation broths, if an organism can ferment any of the three sugars present in the medium, the medium will turn yellow. The medium is inoculated by stabbing the loop into the centre of the agar down to the base of the reaction tube. The loop is pulled back and the rest of the inocular is distributed on the slope of the agar surface. The tube is incubated at 37°C for 24 h. When glucose (not lactose or sucrose) is fermented by the microorganisms a change of colour from red to yellow can be observed. The intensity of the colour depends on the amount of glucose, which is converted. However, the reaction under the aerobic conditions on

the slant reverts and becomes alkaline (red) because of protein breakdown in the medium. Under anaerobic conditions inside the agar at the bottom of the tube, the medium remains acid (yellow) and H_2S production is characterized by blackening of the medium (Jones et al., 2000). This happens with *Salmonella* and *Shigella*. Some *Proteus* spp. and other species may give a similar reaction, but can be distinguished by their ability to hydrolyse urea. Urea agar in another reaction tube should always be used in parallel. With recent, developments in the area of biochemical detection of *Salmonella* it is now possible to rapidly carry out these biochemical tests and obtain results within hours using test cards, like the VITEK GNI+ card used for the biochemical identification of Gram-negative bacteria (de la Torre et al., 2005).

1.2.4.3 Serological detection

The most commonly used serological assay for detection of *Salmonella* and *Salmonella* infections is the sandwich ELISA. In case of ELISA an antibody (for the detection of *Salmonella*) or antigen (for the detection of *Salmonella* infections) is immobilised onto the surface of a polystyrene microtitre plate (MTP), which captures the test analyte (bacteria or antibodies) from the test sample. This is usually followed by a secondary detection step, using a different antibody, which is specific for another part (epitope) of the analyte molecule. This secondary detection antibody is usually labelled with an enzyme. The labelled component of an immunoassay is usually called the “tracer”. As a sandwich is formed between the capture antibody or the capture antigen, the analyte and the secondary detection antibody this form of the assay is called a “sandwich assay ELISA”. When an enzyme is chemically conjugated to the labelled antibody and a substrate added to the reaction wells of the MTP, the substrate is converted by the enzyme to generate a colour or create fluorescent or luminescent end-products. These end products are then read by optical (UV-visible spectrophotometer, fluorescence spectrophotometer) or electronic equipment. The intensity of the detection signal recorded is directly proportional to the concentration of the bound detection antibody, which in turn correlates with the concentration of the antigen.

As mentioned, ELISAs can be used to detect either the microorganism or a humoral immune response (antibody) to the microorganism. Whereas culture methods may take 3-7 days to identify *Salmonella*, ELISA can detect it in a much shorter period of time, usually in one day or less. Several antigen-capture immunoassays have been

used to detect *Salmonella* in pig faeces, food matrices and also human serum (Lambiri et al., 1990; van Poucke, 1990; Araj and Chug, 1987).

The detection of antibodies to the O-antigen of *Salmonella* has been used successfully in pigs for detection of *Salmonella* antibodies. The antibody ELISA using mixed purified lipopolysaccharide (LPS) from both *S* Typhimurium and *S.* Choleraesuis has been used screening of breeding, multiplying and slaughter pig herds in Denmark since 1993. The screening of breeding and multiplying herds is performed on serum samples, whereas meat juice is used for slaughter pigs (Nielsen et al., 1995). The ELISA used show good sensitivity and specificity to *Salmonella* infections. Meat juice is obtained by freezing a sample of muscle tissue overnight and after allowing it to thaw, thereby releasing antibody-containing tissue fluid. Meat juice has been used routinely in Denmark to trace *Salmonella* infected pig herds because it is inexpensive and rapid compared to the bacteriological methods (Proux et al., 2000). In the ELISA tests the control standards used are *Salmonella* positive sera of hyperimmunized pigs as well as one *Salmonella* negative serum. The details of the ELISA used in this work for screening of serum samples from pigs infected with *Salmonella* are discussed in section 3.2.2.

1.2.4.4 Polymerase chain reaction (PCR)

This assay is based on the ability of *Salmonella* specific primers, through complementary DNA base pairing, to anneal only to the target sequence. Thermostable DNA polymerase (*Taq* polymerase) recognizes the template primer complex as a substrate, which results in the simultaneous copying of both strands of the segment of DNA between the two annealed primers. The denaturation, annealing and elongation steps take place in a cyclical fashion, relying on the thermostability of the *Taq* polymerase, until the target sequence is amplified to detectable amounts (van der Zee and Huis, 2000). Before starting the first cycle in the thermocycler the DNA, primers, the polymerase, deoxynucleotide triphosphates (dNTPs), and a buffer are mixed in a reaction tube. The targeted region of the *Salmonella* genome is amplified by repetition of a three-step process:(a) Denaturation of the double-stranded DNA into single strands by heating (b) annealing specific, complementary, oligonucleotide primers to the single stranded DNA by cooling and (c) enzymatically extending the primers to produce an exact copy of the original double-stranded target sequence.

This process from (a) to (c) is usually repeated in 30 to 40 cycles. In the final step the detection of the amplified target DNA is done using agarose gel electrophoresis. Before PCR it is often necessary to first grow the bacteria on an enrichment medium and then extract and purify the DNA (Hernandez et al. 1995). Zhang and Weiner (2000) reported a method of bacterial DNA extraction from liquid media by using the cetyltrimethylammonium bromide (CTAB). PCR-based assays are the latest technique for the detection of pathogens including *Salmonella* in food samples (Uyttendaele et al., 2003; Nakano et al., 2003). The advantages of using PCR in serotyping of rough strains of *Salmonella* are discussed in section 5.3. As seen from the brief description here on the steps involved in carrying out PCR, it is obvious that it is a time consuming process.

2 Aims and objectives

The literature review on different SPR based assays for the detection of different bacteria including *Salmonella* using SPR, showed the lack of application of the SPR-based technology in detection of bacteria in real food systems (section 1.1.2.7). As seen from the review presented in section 1.2.4, different methods have been developed and are used for the detection of *Salmonella* spp. Conventional culture methods are labour-intensive; take 2–3 days for results and up to 7–10 days for confirmation (Rose, 1998). Various validated immunoassays and biochemical analyses (Gene-Trak®, *Salmonella*-TEK®, Assurance 1–2 Test®, TECRA *Salmonella* VIA®, and VIDAS®), as well as other "rapid" analytical methods, also require pre-enrichment of samples and a minimum time between 24 h and 48 h for the assay (Andrews et al., 2001). These techniques, which are mostly, based either on enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) (Patel, 2000), involve the use of various enzymes and reagents for colorimetric or fluorescent end-point detection, and require additional equipment. Enzyme-linked immunosorbent assays (ELISA), though faster than the conventional culturing methods, still take up to 3 h and also require labelling reagents (Schneid et al., 2006). PCR based assays besides involving steps of DNA extraction and amplification, and hybridisation to colorimetric or fluorescent detection probes, also need to have the pre-enrichment step (Eyigor et al., 2002). In case of multiplex PCR assays, capable of simultaneous detection of different pathogens, the final detection stage usually involves running and interpretation of agarose gels in addition to the normal steps of PCR (Kumar et al., 2006; Wang and Slavik, 2005), thus, adding to time and complexity of the assays. Given the widespread prevalence of *Salmonella* and the consequent threat of salmonellosis, rapid detection of the presence of *Salmonella* in water and foods is of great concern to the food industry (Thornton et al., 1993). Thus, it was proposed to explore the possibility to develop an SPR-based assay for rapid detection of *Salmonella* present in food matrices.

According to the World Health Organisation (WHO), more than 2,559 serotypes of *Salmonella* have been identified till date (Table 1.6, section 1.2.1.3). Studies on trends of the serotypes and host-related factors are necessary for the development of effective prevention plans for salmonellosis (Arshad et al., 2007; Mead et al., 1999).

The control of these outbreaks involves the rapid detection of the *Salmonella* serotype responsible for the outbreak. The conventional method of serotyping (as discussed in section 1.2.1.3) is the SAT, which is a manual process and often false results are reported (Cai et al., 2005). Thus there was an opportunity to use SPR as an alternative to SAT. There was also no literature available for attempts to completely serotype a given *Salmonella* serovar using SPR.

Another area where the literature review brought forth the possibility of using SPR was in the development of rapid serological assays for the detection of *Salmonella* infections. The existing conventional, ELISA and PCR-based serological assays for the detection of *Salmonella* infection are time consuming (section 1.2.4.4). SPR assays for detection of *S.* Enteritidis antibodies in egg yolk (Thomas et al., 2006), *Mycoplasma hyopneumoniae* antibodies in pig serum (Kim et al., 2006) and antibodies in pigs against classical swine fever virus (Cho and Park, 2006) were found in literature. As screening of pig heards for the status of *Salmonella* infections is a manadatory requirement in the EU, the development of a rapid SPR technique for carrying out such screenings was identified as another area of investigation, within the scope of this work.

Thus based on the above observations the scope and objective of this work can be summarised as follows:

1. The initial focus of this work was to evaluate SPR as a possible method for the development of **an immunoassay for detection of *Salmonella* as well as *Salmonella* infections.**

2. The objective was to determine and **optimise the immobilisation technique** for immobilising the capture antibody as well as antigens required for detection of *Salmonella* and *Salmonella* infections, respectively.

3. After establishing the immobilisation techniques, the assay for **detection of *Salmonella* was to be extended for application in food samples e.g milk.** Simultaneously, the detection of ***Salmonella* infections based on the detection of antibodies in serum of infected farm animals using a serological SPR-based assay** was to be established.

4. The final goal was to provide a **SPR-based detection and serotyping** system for *Salmonella* and also a **multiplex zoonoses chip** which can be used for rapid and multiple detection of *Salmonella* infections in farm animals and possibly in humans.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

The chemicals and reagents used in this work along with the name of the suppliers are presented in Table 3.1.

Table 3.1: List of chemicals and reagents with name of suppliers.

Chemicals/Reagents	Supplier
Toluene SupraSolv®	Merck, Darmstadt, Germany
Ethanol Lichrosolv®	Merck, Darmstadt, Germany
Acetone SupraSolv®	Merck, Darmstadt, Germany
Acetic acid	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	Merck, Darmstadt, Germany
Dimethylformamide (DMF)	Merck, Darmstadt, Germany
Dioxane	Merck, Darmstadt, Germany
Disodium hydrogenphosphate dihydrate	Merck, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, Germany
Hydrogen peroxide (30% w/v)	Merck, Darmstadt, Germany
Methanol SupraSolv®	Merck, Darmstadt, Germany
Molecular sieve 3Å	Merck, Darmstadt, Germany
Dipotassium hydrogen phosphate	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Sodium hydrogen carbonate	Merck, Darmstadt, Germany
Sodium carbonate	Merck, Darmstadt, Germany
Sodium Chloride	Merck, Darmstadt, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium dihydrogenphosphate dihydrate	Merck, Darmstadt, Germany
(3-aminopropyl)triethoxysilane (3-APTES)	Sigma-Aldrich Chemie, Steinheim
Sodium acetate anhydrous	Sigma-Aldrich Chemie, Steinheim
Bronidox L® (5-Bromo-5-Nitro-1,3-Dioxane)	Sigma-Aldrich Chemie, Steinheim
Human serum (from human male AB plasma)	Sigma-Aldrich Chemie, Steinheim
LPS from *Salmonella* Enteritidis (L6011)	Sigma-Aldrich Chemie, Steinheim
LPS from *Salmonella* Typhimurium (L6511)	Sigma-Aldrich Chemie, Steinheim
Protein A from *Staphylococcus aureus*	Sigma-Aldrich Chemie, Steinheim
Sodium bromide	Sigma-Aldrich Chemie, Steinheim
N-α, N-α Bis(carboxymethyl)-L-lysine trifluoroacetate salt	Sigma-Aldrich Chemie, Steinheim

Table 3.1(continued): List of chemicals and reagents with name of suppliers.

Chemicals/Reagents	Supplier
(+) Biotin N-succinimidyl ester (Biotin-NHS ester)	Fluka (Sigma-Aldrich Chemie), Steinheim
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)	Fluka (Sigma-Aldrich Chemie), Steinheim
Cobaltous chloride hexahydrate	Fluka (Sigma-Aldrich Chemie), Steinheim
Ethanolamine hydrochloride	Fluka (Sigma-Aldrich Chemie), Steinheim
N, N′-Dicyclohexylcarbodiimide (DCC)	Fluka (Sigma-Aldrich Chemie), Steinheim
N-Hydroxysuccinimide	Fluka (Sigma-Aldrich Chemie), Steinheim
N-Hydroxysulfosuccinimide (Sulfo-NHS)	Fluka (Sigma-Aldrich Chemie), Steinheim
Sodium borohydride	Fluka (Sigma-Aldrich Chemie), Steinheim
Sodium meta periodate	Fluka (Sigma-Aldrich Chemie), Steinheim
Succinnic anhydride	Fluka (Sigma-Aldrich Chemie), Steinheim
Thimerosal®	Fluka (Sigma-Aldrich Chemie), Steinheim
Octadecyltrimethoxysilane	Fluka (Sigma-Aldrich Chemie), Steinheim
Tryptone [peptone from casein (pancreatic digest)]	AppliChem, Darmstadt, Germany
Yeast extract (molecular biology grade)	AppliChem, Darmstadt, Germany
Potassium hydroxide	Carl Roth GmBH & Co., Karlsruhe, Germany.
Streptavidin (from *S. avidinii*)	Prozyme® USA

3.1.2 Buffers

The buffers used in the experiments carried out as part of this work are presented in Table 3.2.

Table 3.2: List of buffers and their respective ingredients and preparation protocol.

Name	Reagents	Quantity	Method of preparation
Phosphate buffered saline PBS (pH 7.3, 30 mM phosphate, 120 mM NaCl)	Sodium Chloride Disodium hydrogenphosphate dihydrate Sodium dihydrogen phosphate dihydrate *Bronidox L®	7.01 g 4.27 g 0.94 g 1.2 g	The weighed ingredients are dissolved in 800 mL Millipore® water. pH is adjusted to 7.3.using a solution of NaOH. The volume is then made up to 1.0 L with water. The buffer is finally filtered through 0.2 µm sterile filter.
Carbonate buffer 0.1 M, pH 8.6	Sodium hydrogen carbonate	8.4 g	The weighed ingredients are dissolved in 800 mL Millipore® water. pH is adjusted to 8.6 using 0.1 M sodium carbonate solution. The volume is then made up to 1.0 L with water. The buffer is finally filtered through 0.2 µm sterile filter.
Carbonate buffer 0.1 M, pH 8.3	Sodium hydrogen carbonate	8.4 g	The weighed ingredients are dissolved in 800 mL Millipore® water. pH is adjusted to 8.3 using 0.1 M sodium carbonate solution. The volume is then made up to 1.0 L with water. The buffer is finally filtered through 0.2 µm sterile filter.
Phosphate buffer 0.05 M, pH 6.0	Potassium dihydrogen phosphate *Bronidox L®	6.81 g 1.2 g	The weighed ingredients are dissolved in 800 mL Millipore® water. pH is adjusted to 6.0 using 0.1 M dipotassium hydrogen phosphate solution. The volume is then made up to 1.0 L with water. The buffer is finally filtered through 0.2 µm sterile filter.
Acetate buffer 0.1 M pH 5.5	Sodium acetate anhydrous *Bronidox L®	0.82 g 0.03 g	The weighed ingredient is dissolved in approximately 30 mL of Millipore® water. pH is adjusted to 5.5 using a 0.1 M solution of acetic acid and the volume made up to 100 mL

***Bronidox L® (5-Bromo-5-Nitro-1, 3-Dioxane) is added as a preservative to the buffers which need long-term storage.**

3.1.3 Samples

In this work, milk was used as a model system to establish an SPR-based assay for the detection of *Salmonella* in food systems. Serum samples from pigs infected with *Salmonella* spp. was used to validate the SPR-based assay for the detection of *Salmonella* infections. In addition, the LPSs used in these assays were also obtained from sources other than Sigma. The details of the samples obtained from external sources are listed in Table 3.3.

Table 3.3: List of samples and materials

Sample	Additional information	Source
Milk	• Cow Milk, Ultra-high temperature (UHT) treated, 1.5 % fat • Cow Milk, Pasteurised 3.5% fat	Local supermarket From local farms in Germany
Pig serum samples: • Serum from pigs infected with *S.* Typhimurium • Serum from pigs infected with .*S.* Choleraesuis	Different levels of infection as classified by ELISA, frozen or in freeze dried form	Labor Diagnostik GmbH, Leipzig, Germany
LPS samples: • *S.* Choleraesuis LPS • *S.* Typhimurium LPS • *S.* Anatum LPS	1 mg mL^{-1} in 0.9 % NaCl 1 mg mL^{-1} in 0.9 % NaCl 1 mg mL^{-1} in 0.9 % NaCl	Labor Diagnostik GmbH, Leipzig, Germany
Salmonella serovars	Details of serovars provided separately in Table 3.4	Institut für Angewandte Mikrobiologie, Justus-Liebig-Universit¨at Gießen, Germany

3.1.4 *Salmonella* serovars

The details of the *Salmonella* serovars used in this work are presented in Table 3.4.

Table 3.4: *Salmonella* serovars with details of their antigenic formula and strain type used in this work.

Serovar	Somatic(O) antigens	Flagellar (H) antigen		Serogroup	Strain type
		Phase 1	Phase 2		
S. Enteritidis	1,9,12	g,m	-	D1	*CCUG 32352
S. Heidelberg	4,5,12	r	1,2	B	*CCUG 21289
S. Infantis	6,7	r	1,5	C1	*CCUG 35612 (rough)
S. Thompson	6,7	k	1,5	C1	*CCUG 12652
S. Typhimurium	4,5,12	i	1,2	B	*CCUG 31969 and *CCUG 11732

***CCUG: Culture Collection, University of Göteborg, Sweden**

3.1.5 Culture media

The culture media used in culturing of the *Salmonella* and *E. coli* used in this work are presented in Table 3.5.

Table 3.5: List of culture media with the ingredients and method of preparation.

Name	Reagents	Quantity	Method of preparation
Luria-Bertani (LB) broth	Tryptone [Peptone from casein (pancreatic digest)] Yeast extract NaCl	10 g 5 g 10 g	The weighed ingredients are dissolved in water to a final volume of 1.0 L and autoclaved
Luria-Bertani (LB) agar	Tryptone [Peptone from casein (pancreatic digest)] Yeast extract NaCl Agar	10 g 5 g 10 g 16 g	The weighed ingredients are dissolved in water to a final volume of 1.0 L and autoclaved

3.1.6 Antibodies

The details of different antibodies used in the development of the SPR-based assays for the detection of *Salmonella* and *Salmonella* infections, and also in the development of the SPR-based serotyping method for serotyping of *Salmonella* are presented in Table 3.6.

Table 3.6: List of antibodies used, their properties and suppliers.

Antibody	Details of antigen/specificity	Origin/Quality	Supplier
anti-H:g,m (Catalogue No. 23851)	H:g,m flagellar antigens of *Salmonella*	Rabbit antiserum, Ready to use	Statens Serum Institut (SSI), DK-2300 Copenhagen, Denmark
anti-H:q,s,t,p,u (Catalogue No. 22667)	H:q,s,t,p,u flagellar antigens of *Salmonella*	Rabbit antiserum, H-multiphase, Ready to use	SSI, Denmark
anti-Poly A-E (Catalogue No. 40190)	O-somatic antigens from serogroups A to E of *Salmonella*	Rabbit antiserum, O-multigroup, Ready to use	SSI, Denmark
anti-*Salmonella* O4 (Catalogue No. TR1302)	Purified O:4-somatic antigen from *Salmonella*	Mouse, monoclonal, IgM Ready to use	SIFIN Institut für Immunpräparate und Nährmedien GmbH Berlin, Germany
anti-*Salmonella* O5 (Catalogue No. TR1303)	Purified O:5-somatic antigen from *Salmonella*	Mouse, monoclonal, IgM Ready to use	SIFIN, Berlin, Germany
anti-*Salmonella* O9 (Catalogue No. TR1307)	Purified O:9-somatic antigen from *Salmonella*	Mouse, monoclonal, IgM Ready to use	SIFIN, Berlin, Germany
anti-*Salmonella* O46 (Catalogue No. TR1315)	Purified O:46-somatic antigen from *Salmonella*	Mouse, monoclonal, Ready to use	SIFIN, Berlin, Germany)
anti-*Salmonella* Group B (Catalogue No. TR1201)	Purified O-somatic antigens from *Salmonella* group B	Mouse, monoclonal, Ready to use	SIFIN, Berlin, Germany
anti-*Salmonella* Group C (Catalogue No. TR1202)	Purified O-somatic antigens from *Salmonella* group C	Mouse, monoclonal, Ready to use	SIFIN, Berlin, Germany
anti-*Salmonella* Group D (Catalogue No. TR1202)	Purified O-somatic antigens from *Salmonella* group D	Mouse, monoclonal, Ready to use	SIFIN, Berlin, Germany
anti-*Salmonella* Group E (Catalogue No. TR1204)	Purified O-somatic antigens from *Salmonella* group E	Mouse, monoclonal, Ready to use	SIFIN, Berlin, Germany
anti-*E.coli* (Catalogue No.20-ER13)	Reactive to all O and K antigenic serotypes of *E. coli*	Rabbit, polyclonal, (5 mg mL^{-1})	Fitzgerald Concord, MA
anti-*Salmonella* sp., IgG fraction capture antibody used for SPR assays (Catalogue No. SAL-007-82026)	Polyvalent for *Salmonella* O and H antigens	Rabbit, polyclonal, (5 mg mL^{-1}), IgG fraction	Capricorn products LLC, Portland ME, USA 04103

3.1.7 Equipments

The different equipments used in this work are listed in Table 3.7.

Table 3.7: List of equipments, version and manufacturer.

Equipment	Version	Manufacturer
pH meter	Lab 850	Schott instruments, Germany
Centrifuge	Heraeus Biofuge Pico	Kendro Laboratories, Langenselbold, Germany
Tip sonicator	Labsonic® 1510,	B. Brauer, Melsungen, Germany
ELISA washer.	Nunc Immuno™ Wash 8	VWR International GmbH, Darmstadt, Germany
UV/VIS spectrophotometer	Nicolet evolution 100	Thermo Life Sciences, Thermo electron corporation, Dreieich, Germany.
ELISA Plate reader	Multiscan EX	Thermo Life Sciences, Thermo electron corporation, Dreieich, Germany.
Contact angle measuring device	Krüss Contact Angle Measurement System G 10, with SCA20 software	Krüss GmBH, Hamburg (available at the working group of Prof. Dr. Wendorff (Fachbereich Chemie, Philipps-Universität Marburg)
SPR device	Plasmonic® SPR device	Plasmonic Biosensoren AG, Wallenfels, Germany
Shaker	Titramax 101	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany
Vortexer	Model 35213	IKA, Staufen Germany
Buffer filtration device	Bottle top filter 1000 mL capacity	Nalgene, VWR International GmbH
Vacuum pump	KNF Laboport®	KNF Neuberger GmbH, Germany
Autoclave	FVA/2 with IBS Integra Biosciences controller	Fedegari Autoclavi SPA
Analytical balance	Sartorius CP225D-OCE	Sartorius AG, Göttingen, Germany

3.1.8 Kits and consumables

The different kits and consumables used in this work are listed in Table 3.8.

Table 3.8: List of kits and consumables and their suppliers.

Kits and consumables	Supplier
Tube-O-DIALYSER™ (1000 MWCO)	G-Biosciences USA
SALMOTYPE® Pig Screen ELISA kit	Labor Diagnostik GmbH, Leipzig, Germany
Cellulose acetate sterile filters (0.2 µm)	Sartorius AG, Göttingen, Germany
Microtitre plates for SPR (U-bottom), Catalogue No. 650101	Greiner Bio-One GmbH Frickenhausen; Germany
Disposable cuvettes for Plasmonic®	Plasmonic Biosensoren AG, Wallenfels, Germany
SPR chip	Plasmonic Biosensoren AG, Wallenfels, Germany

3.2 Methods

3.2.1 Culture and preparation of *Salmonella* antigens

All the *Salmonella* serovars belonging to *Salmonella enterica* subsp. *enterica* (mentioned in section 3.1.4, Table 3.4) were grown in sterile liquid LB medium by incubation for 24 h at 37 °C. The bacteria used for the assay were grown to a concentration of 1×10^{10} cells mL^{-1}. In order to kill the bacteria, thimerosal (1%, w/w) was added to the medium and incubated at ambient temperature for 1.5 days. The contents were vortexed at regular intervals during this time. After this period, to check the effectiveness of the thimerosal treatment, 100 µL of the LB medium, containing bacterial cells, was added onto sterile LB agar medium. In case of a successful thimerosal treatment, there should be no visible growth of bacteria on the LB agar after 48 h of incubation at 28 °C. The liquid medium, containing the killed bacteria, was then centrifuged at 4,000 rpm for 10 min at ambient temperature using table top centrifuge (Heraeus Biofuge pico, Germany). The cells were obtained as pellets at the bottom of the tube. After pouring off the supernatant, the pellets were washed three times with PBS. In each case this was done by suspending the pellets in PBS followed by renewed centrifugation. After the third and final wash, the pellets were suspended in PBS to the initial volume and stored at 4 °C until further use.

Before use the bacterial cells were sonicated, over ice, three times using a tip sonicator (Labsonic® 1510, B. Brauer, Melsungen, Germany) at 50 Watt.

3.2.2 ELISA for detection of *Salmonella* infection

The SALMOTYPE® Pig Screen ELISA kit was used for the detection of serum antibodies in pigs infected with *Salmonella*. The ELISA kit is provided with ELISA plates precoated with LPS mixture of *Salmonella* spp. The LPS mixture is composed of six purified LPS fractions (O-antigen 1, 4, 5, 6, 7 and 12) enabling the detection of 90 % of all common *Salmonella* serotypes. Using this ELISA kit it is possible to classify the sera, which correlate to low, medium and high levels of *Salmonella* infection in pigs. The ELISA kit is supplied with positive and negative controls. The detection signals from these controls are used to obtain normalised OD % values for the tested serum samples. The OD % of the samples is expressed with respect to the concentration of the antibody present in the control sera. Thus, the OD % correlates with the antibody concentrations present in the serum (Korsak et al., 2006).

The steps involved in carrying out the ELISA, for detection of anti-*Salmonella* antibodies in pig serum, as per the manufacturer's instruction is as follows:

1. All the reagents were brought to room temperature (18-23 ^{0}C) before use. The infected and uninfected sera were received from LDL, either frozen or in freeze-dried form. Frozen samples were thawed prior to use. Freeze-dried samples were reconstituted in water to their original volume prior to use. In case of ELISA, sera were diluted 1:100 using the dilution buffer supplied.

1. 100 µL of each of the ready-to-use negative and positive controls, and 1:100 diluted pig serum samples were pipetted into the LPS coated wells of the microtitre plate (MTP).

2. The MTP was covered and allowed to incubate on a shaker (250 rpm) for 30 min at room temperature, following which the wells were emptied using the ELISA washer.

3. The wells were rinsed 3 times using wash buffer with the aid of an ELISA washer.

4. Then 100 µL of ready to use anti-IgG HRP conjugate was added to each well.

5. The MTP was covered and again incubated for 30 min at room temperature on a shaker (250 rpm), following which the wells were emptied using the ELISA washer.

6. The wells were rinsed 3 times using wash buffer with the aid of an ELISA washer.

7. 100 µL of TMB substrate solution was then added to each well.

8. The MTP was covered with a silver foil, and the contents incubated in the dark for 10 min

9. At the end of 10 min the reaction was stopped by adding 10 µL of the stop solution (8 % H_2SO_4)

10. The optical density was then read using a plate reader (Multiscan EX) at 450 nm. Measurements were also taken at the reference wavelength of 620 nm. Before taking the readings the spectrophotometer was calibrated with air as blank.

The optical density was calculated using the formula supplied by the manufacturer.

The principle involved in the ELISA based detection is explained as follows:

The LPS coated on the surface of the ELISA plate captures the anti-*Salmonella* antibodies (IgG) from the serum. The captured antibodies are then labelled with HRP conjugated anti-IgG antibodies, which are specific for the Fc region of the captured IgG. HRP (EC 1.11.1.7) is an oxidoreductase and belongs to the subclass peroxidase which catalyses the reaction involving hydrogen peroxide and a donor, in this case TMB. The ready to use TMB substrate (which contains H_2O_2) is thus converted to a soluble blue coloured product in presence of the HRP. The reaction is stopped by the addition of the stop solution (8% H_2SO_4), and results in the formation of a stable yellow reaction product. The colour intensity of this end product is read at 450 nm. The intensity of this end product is directly proportional to the concentration of the anti-IgG HRP bound to the surface. This in turn correlates to the concentration of the antibody (IgG) present in the infected serum samples. The reaction scheme involving the formation of the coloured complexes due to the reaction of TMB and H_2O_2 has been discussed in detail in literature (Josephy et al., 1982). Briefly, the first blue product is a charge transfer complex of the parent diamine and the diamine oxidation product. This species exists in rapid equilibrium with the radical cation. Under acidic conditions the stable yellow diiamine product is obtained. The reaction scheme as presented by the authors is shown in Fig. 3.1.

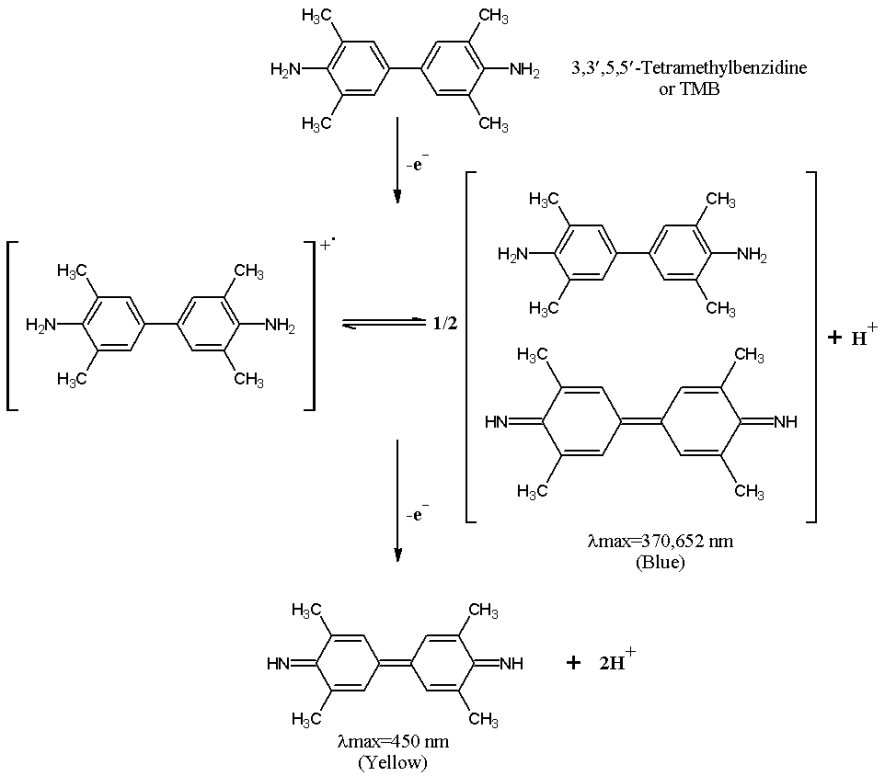

Fig. 3.1: Reaction steps explaining the formation of the stable yellow diiamine product due to the oxidation of TMB (3,3′,5,5′-tetramethylbenzidine) by H_20_2 mediated HRP (adapted from Josephy et al., 1982).

3.2.3 Modification of antibodies

The different methods used for modification of the antibodies required as part of this work are described below.

3.2.3.1 EDC/sulfo-NHS activation of carboxyl functional groups

The carboxyl functional groups present on the Fc-region of antibodies are activated using EDC/sulfo-NHS, resulting in the formation of activated sulfo-NHS esters. This activated antibody is then immobilised on amine-functionalised gold surface of SPR prisms. The activation of the carboxyl groups of the polyclonal antibody was carried out by incubating the antibody (1 mg mL^{-1} prepared in PBS) in an aqueous solution of EDC/sulfo-NHS (0.4 M EDC and 0.1 M sulfo-NHS) for 30 min at 4 ^{o}C. At the end of the incubation period, dialysis of the activated antibody was carried out using a Tube-O-DIALYSER^{TM} (1000 MWCO) against PBS. The dialysis was initially carried out for 6 h at 4 ^{o}C. The dialysis was further allowed to continue overnight at 4 ^{o}C after a change to fresh buffer at the end of the 6 h period. The reaction involves

the formation of an active O-acylisourea intermediate by reaction between the carboxyl functional group of the antibody and EDC.

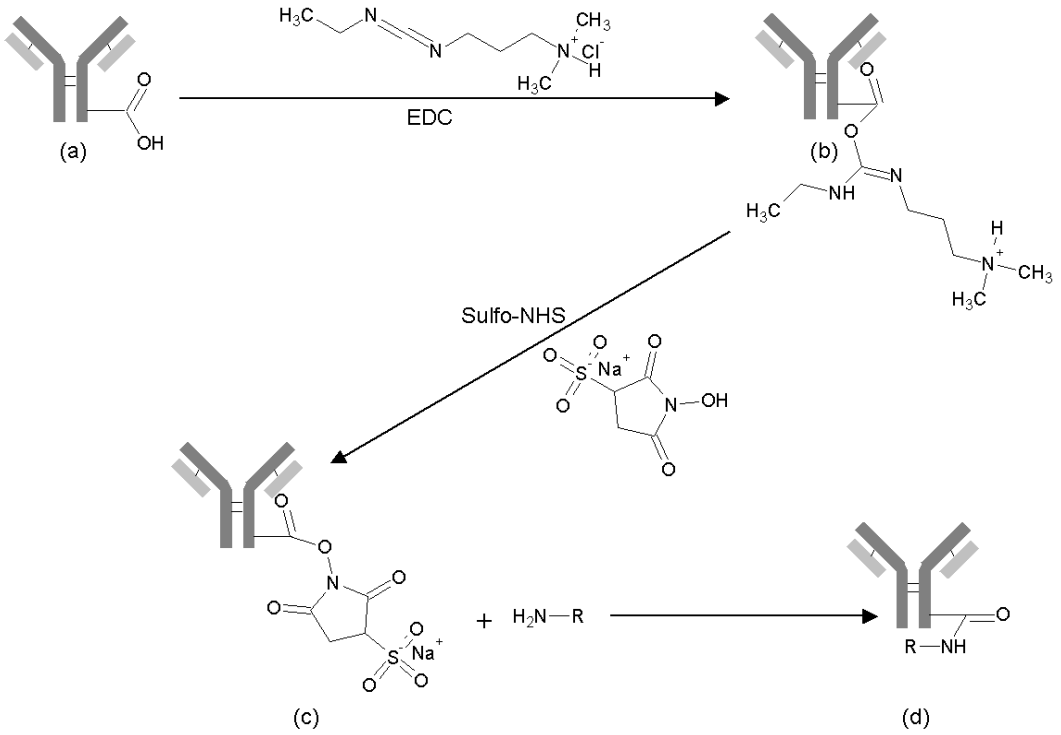

Fig. 3.2: Steps involved in the activation of the carboxyl side chain of antibodies (a) antibody presenting carboxyl side chain in Fc region (b) reaction with EDC first results in the formation of an unstable, active intermediate O-acylisourea (c) the presence of sulfo-NHS results in the formation of a semistable sulfo-NHS ester (d) the sulfo-NHS ester reacts with amine groups on the SPR-chip, resulting in the immobilisation of the antibody due to formation of stable amide bonds.

This active intermediate is converted into sulfo-NHS ester in presence of sulfo-NHS. The sulfo-NHS esters are responsible for binding the antibody by the formation of stable amide bonds on the surface of amine-functionalised SPR chips. The details and results of the immobilisation steps are discussed later in section 4.1.1.2. The steps involved in this reaction are explained in Fig. 3.2.

It is important to note here that sulfo-NHS is used instead of NHS because of better solubility of sulfo-NHS over NHS in water. The sulfo-NHS ester survives longer in aqueous solution than the active ester formed by reaction of EDC alone with a carboxyl group (Hermanson, 1996).

3.2.3.2 Oxidation of the carbohydrate side chain of antibody

The next important method used for modification of antibodies involves the periodate oxidation of the carbohydrate molecules. The carbohydrate molecules are present in the Fc-portion of the antibodies (section 1.1.2.4). It is assumed, as the carbohydrate molecules are sufficiently removed from the antigen binding sites, conjugation of the antibody using the polysaccharide side chains are less likely to compromise the antigen binding activity of the antibody. The polyclonal antibody was diluted (1 mg mL^{-1}) in PBS buffer from a stock solution. The polyclonal antibody was mixed with an aqueous solution of 0.1 M $NaIO_4$ to obtain a final concentration of 500µg mL^{-1} of the antibody. This mixture was then incubated in the dark, for about 30 min at room temperature. This resulted in the formation of reactive aldehyde residues. Unreacted $NaIO_4$ was then separated from the reaction mixture by dialysis (Tube-O-DIALYSER^{TM}, 1000 MWCO). The samples were dialysed at 4 ^{o}C for 2 h against a 2.0 L portion of PBS. This was followed by three additional dialysis cycles, each performed for a minimum of 2 h.

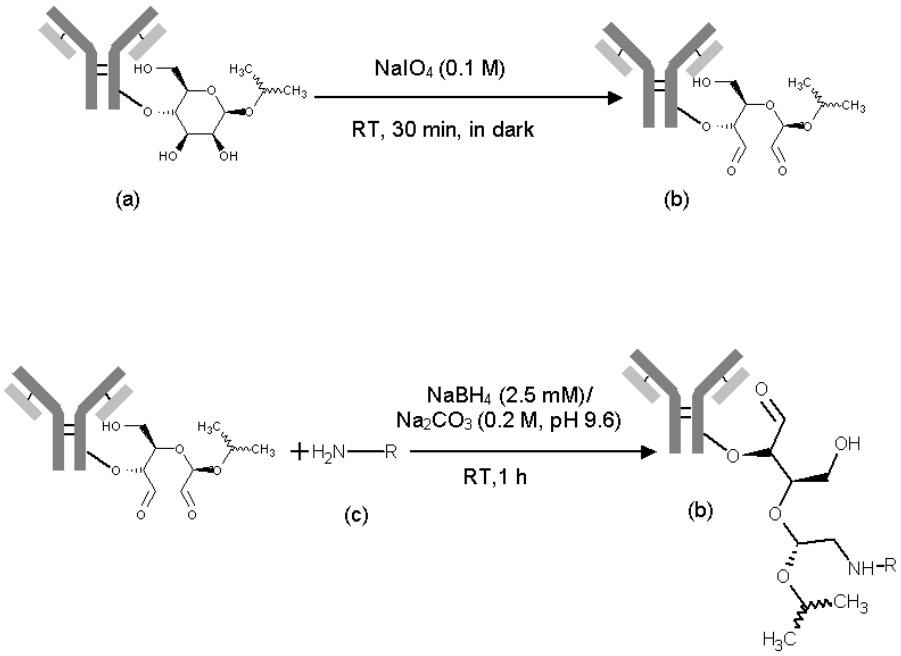

Fig. 3.3: Steps involved in the modification of the carbohydrate side chain of an antibody (a) antibody presenting carbohydrate side chain in Fc region (b) periodate oxidation of the carbohydrate side chain resulting in the formation of reactive aldehyde residues (c) the antibody is then immobilised on an amine functionalised SPR-chip (d) by reductive amination of the reactive aldehyde groups, using $NaBH_4$.

The antibody was then coupled to an amine functionalised gold surface using reductive amination. Reductive amination was carried out using 0.1 M $NaBH_4$ in

sodium carbonate solution (0.2 M, pH 9.6). The steps involved in this reaction are explained in Fig. 3.3.

Details and results of the use of this activated antibody for immobilisation on amine-functionalised gold surface of SPR prism is presented in section 4.1.1.2. The use of this method of activation of antibodies has been reported in literature (Qian et al., 1999).

3.2.3.3 Antibody biotinylation

Biotinylation of antibodies is carried out according to the protocol discussed below. The antibody was first dialysed (1.25 µg mL^{-1}) against carbonate buffer pH 8.6 using a Tube-O-DIALYSER^{TM} (1000 MWCO). The dialysis was initially carried out for 6 h at 4 ^{o}C. The dialysis was further allowed to continue overnight at 4 ^{o}C after a change to fresh buffer at the end of the 6 h period. For each 1 mL of the antibody solution, 50 µL of 1.25 µg mL^{-1} of Biotin-NHS prepared in DMSO (DMSO is dried over molecular sieve before use) is then added. The mixture is allowed to react at room temperature, in the dark for 3 h.

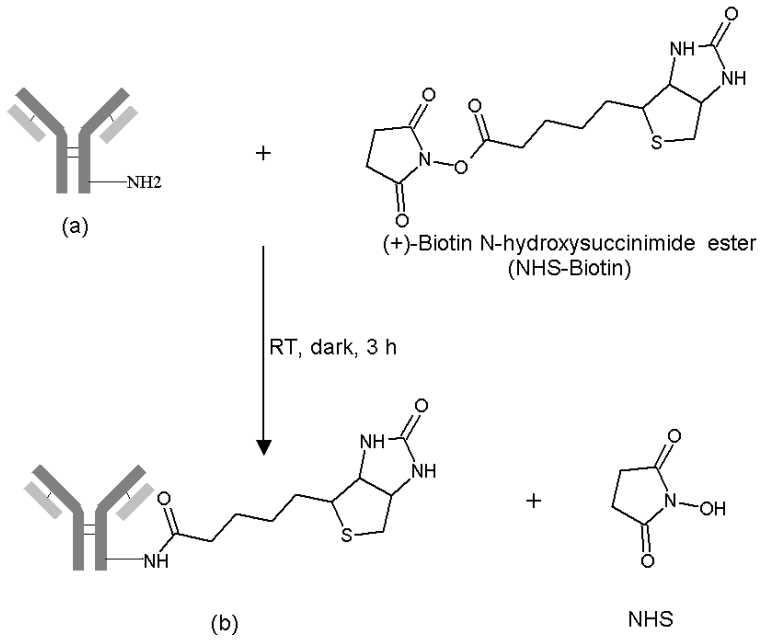

Fig. 3.4: Steps involved in the biotinylation of antibodies (a) antibody presenting amino functional group in the Fc region (b) formation of the stable amide bond after reaction of the amino function on the antibody with the NHS-Biotin, resulting in the formation of the biotinylated antibody. NHS is released during the reaction.

The biotinylated antibody is further dialysed overnight, against PBS at 4^{o}C. The reaction involves the formation of the biotinylated antibody by reaction of the amino

functional group present in the Fc-region of antibodies with Biotin-NHS. The reaction steps involved in preparation of biotinylated antibody are shown in Fig. 3.4. The biotinylated antibodies are then immobilised on a biotinylated SPR chip (section 3.2.4.3). Before addition of the biotinylated antibody the chip is first coated with streptavidin. The details of immobilisation of the biotinylated antibody and the results obtained are presented in section 4.1.1.4.

3.2.4 Surface modification (functionalisation) of SPR chips

3.2.4.1 Cleaning and activation of the gold surface

The cleaning and activating the gold surface of the SPR prisms forms the first and the most important step for successful surface functionalisation of SPR chips. Each SPR prism is cleaned to remove all adhering hydrophobic and hydrophilic substances from the gold surface. The cleaning protocol does not normally destroy the gold coating on the surface of the glass prism. However, weakely adhering gold coatings are removed during this cleaning process. Hence, the cleaning process also serves as a quick quality check for the stability of the gold coating on the prisms. The prisms, which pass this cleaning step, are suitable for use in subsequent coating steps.

The prisms were first cleaned in acetone (5–10 min) followed by incubation in a mixture of 0.1 M potassium hydroxide (KOH) and 30 % hydrogen peroxide (H_2O_2) (1:1, v/v) for 20 min. The prisms were then rinsed with water. This cleaning method also activates the gold surface by introducing hydroxyl groups (Fig. 3.5). These hydroxyl groups act as the reacting groups for the subsequent steps used in creating various functionalised surfaces. The details of steps involved in the creation of SPR-chips with different functionalised gold surfaces are discussed below.

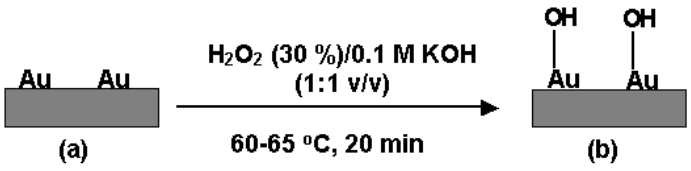

Fig. 3.5: Cleaning and activation of the gold surface of the SPR-chip (a) bare gold surface (b) activated gold surface with hydroxyl groups after the cleaning step.

3.2.4.2 Amine coating of SPR prisms

The cleaned SPR prisms are incubated in a solution 1% (w/w) solution of (3-aminopropyl)triethoxysilane (3-APTES) in toluene. The incubation time is 6 h at room temperature. The incubation is carried out on a shaker bath (250 rpm). The volume of the solution used for each prism is around 6 mL. After the incubation period, the prisms are rinsed 3 times with toluene (20 mL/rinse, 30 to 60 sec/rinse). Depending on further coating strategies the prisms are either rinsed with other solvents or with water and dried and stored under vacuum. The steps involved in creation of the amine functionalised surface are presented in Fig. 3.6.

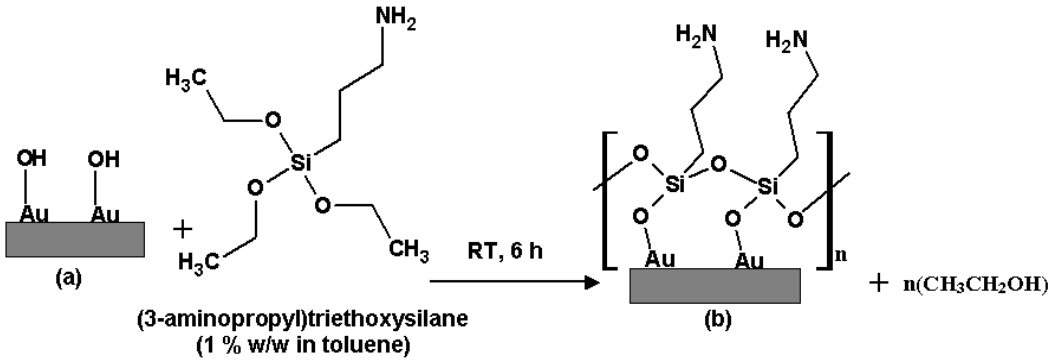

Fig. 3.6: Steps involved in the creation of a SPR-chip with amine functionalised gold surface (a) activated gold surface with hydroxyl groups after the cleaning and activation step (b) amino functionalised gold surface obtained by incubation of the activated prisms in (3-aminopropyl)triethoxysilane (1% w/w in toluene).

3.2.4.3 Biotin coating of SPR prisms

The first step of the biotin coating involves the production of an amine-coated gold surface as described in section 3.2.4.2. After the final washing step in toluene, of the amine coating step, the prisms are immediately rinsed with DMF. This is followed by incubation of the amine-coated prisms in a solution of D-(+)-Biotin-NHS in DMF (1 mg mL^{-1}, 6 mL per prism) for 12 h at room temperature on a shaker (250 rpm). The prisms are finally rinsed in DMF and water, respectively (3 rinses each in DMF and water).

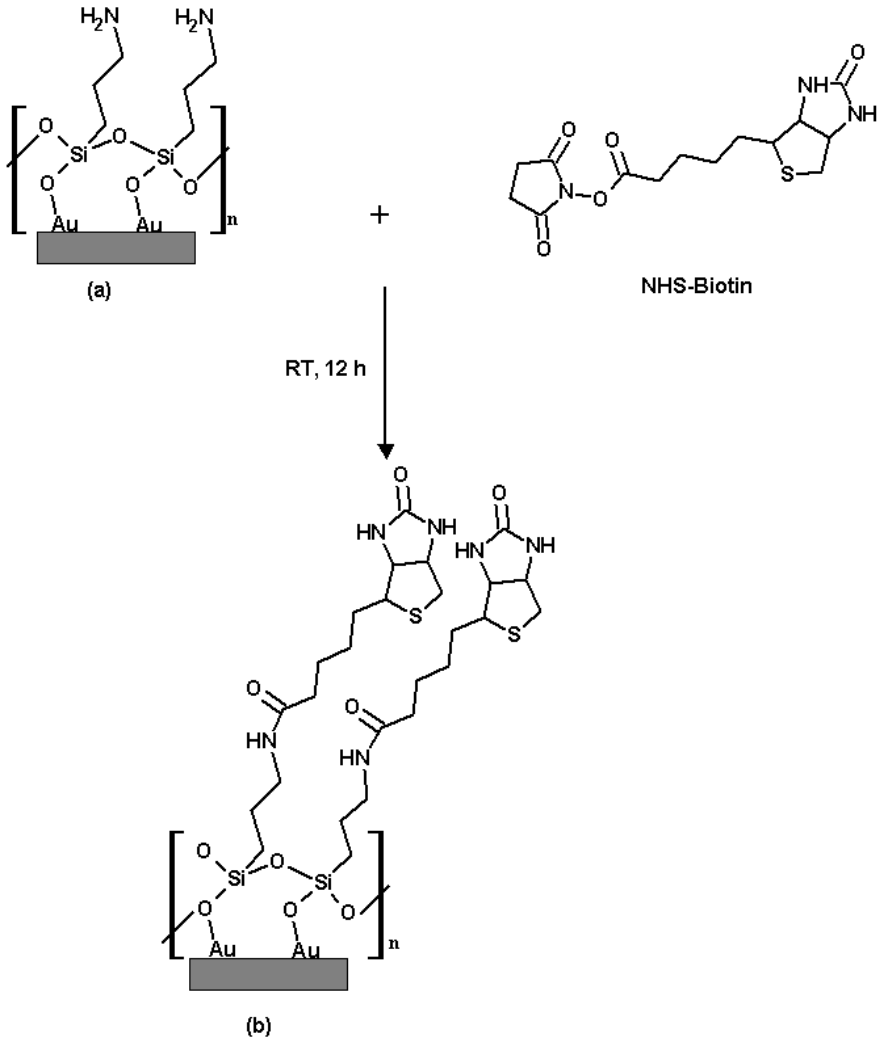

Fig. 3.7: Steps involved in the creation of a SPR-chip with biotinylated gold surface (a) amine functionalised gold surface is reacted with NHS-Biotin resulting in (b) biotinylated gold surface

The biotinylated prisms are then dried and stored under vacuum until further use. The reaction steps involved in the creation of the biotinylated gold surface are explained in Fig. 3.7.

3.2.4.4 Alkylation of SPR prisms (Hydrophobic or C_{18} coating)

The activated SPR prisms were incubated in a solution of octadecyltrimethoxysilane (1 % w/w in toluene) for 6 h at room temperature on a shaker (250 rpm). Approximate volume of the solution used for each prism was 6 mL. After the incubation period the prisms were rinsed three times each in toluene, methanol and water, respectively. The prisms were then dried and stored under vacuum. The hydrophobic SPR-prisms are referred to as C_{18} prisms in this work. The reaction steps involved in the creation of the C_{18} surface are explained in Fig. 3.8. The

hydrophobicity of the chips was determined by carrying out water contact angle measurements (section 4.1.1.7.1).

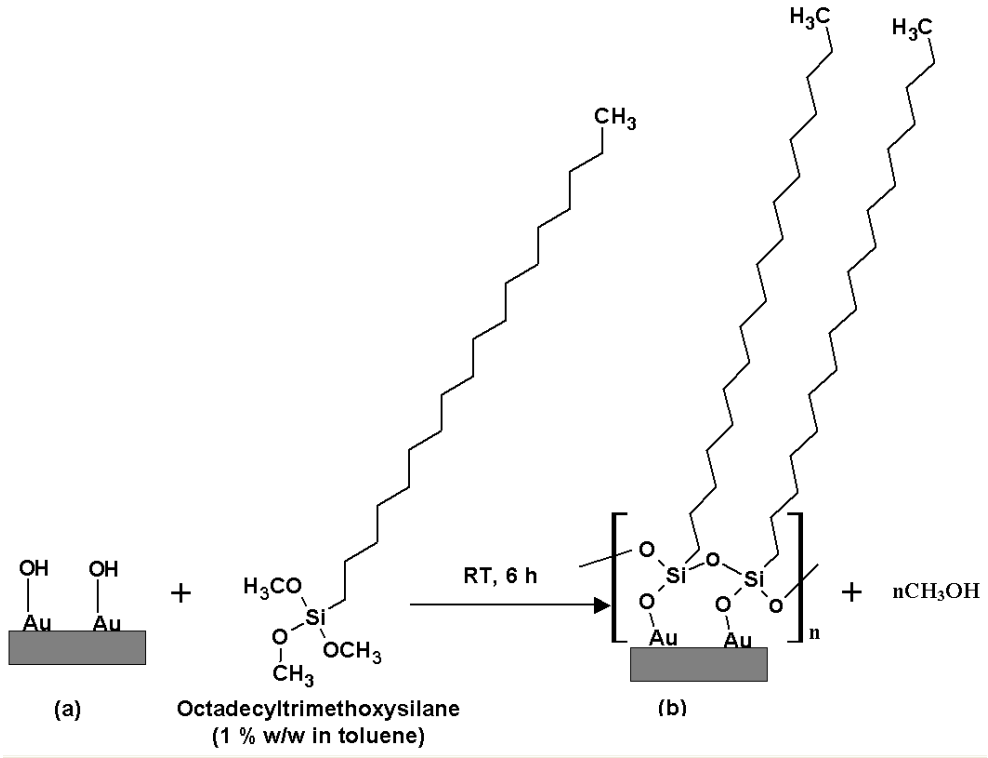

Fig. 3.8: Steps involved in the creation of a SPR-chip with hydrophobic (C_{18}) gold surface (a) activated gold surface with hydroxyl groups after the cleaning and activation step (b) hydrophobic gold surface obtained by incubation of the activated prisms in octadecyltrimethoxysilane (1 % w/w in toluene).

3.2.4.5 Carboxyl coating of SPR prisms

After amine functionalisation of the gold surface of the SPR chip, carried out according to the method mentioned in section 3.2.4.2, carboxyl functionalisation was carried out using the steps described below. The amine functionalised chips, after the final washing step with water, were incubated (15 h, shaker 250 rpm) in a solution of succinnic anhydride (400 µg mL^{-1}, 6 mL per prism) at room temperature. The succinic anhydride solution is prepared in 0.05 M phosphate buffer (pH 6). Approximately, 10 mL of the succinnic anhydride solution is required for each chip. At the end of 15 h, the prisms were washed three times each in water, methanol and dioxane, respectively. Approximately 20 mL of each solution was used for each washing step. The succinnic anhydride treatment resulted in the introduction of a spacer with a carboxyl group (Fig. 3.9).

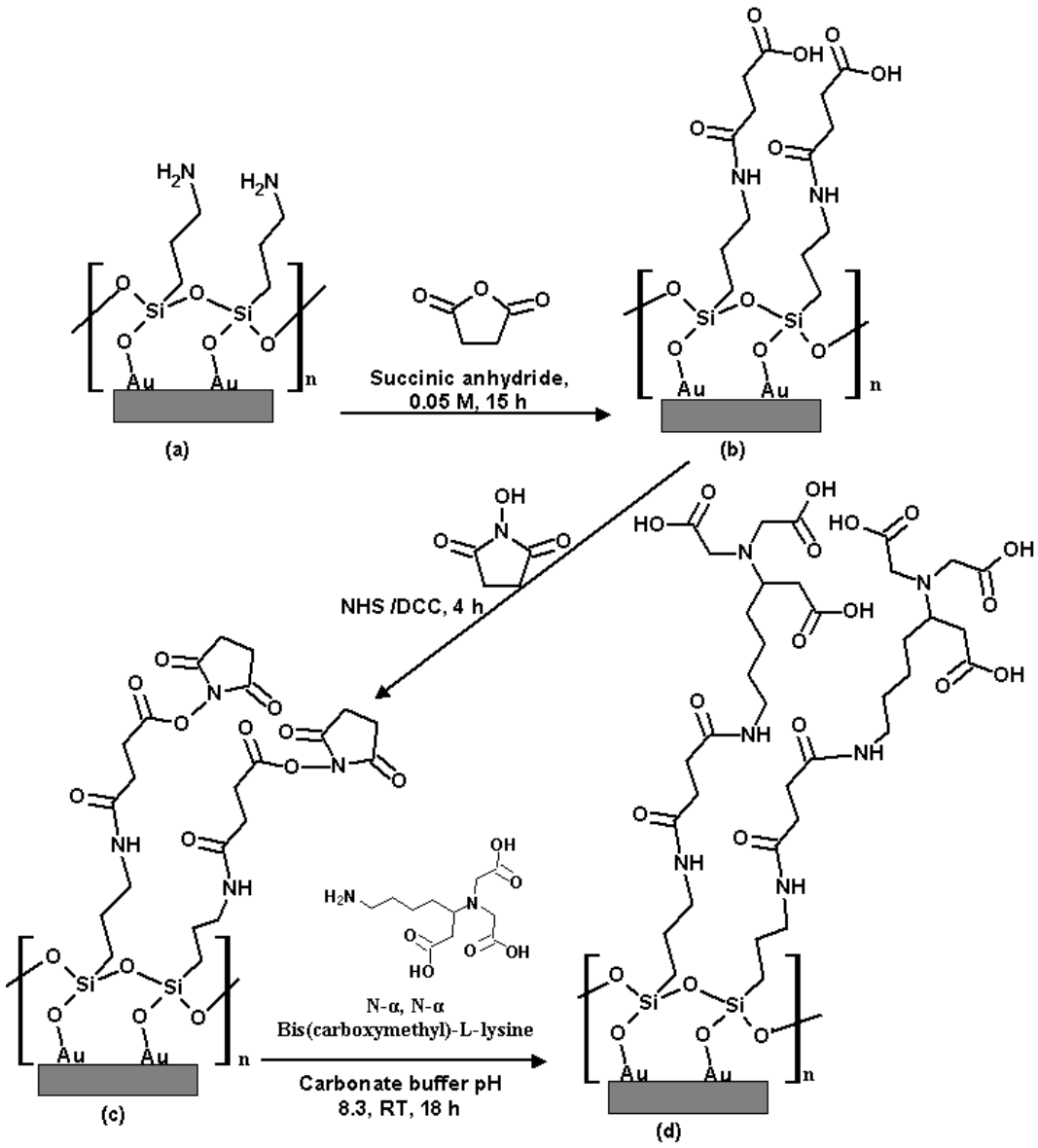

Fig. 3.9: Steps involved in the creation of a SPR-chip with carboxyl functionalised gold surface (a) amine functionalised gold surface (b) introduction of the carboxy spacer by treatment with succinic anhydride (c) activation of the carboxyl function with NHS/DCC resulting in the formation of NHS ester (d) formation of tri-carboxy functionalised gold surface by reaction with N-α,N-α Bis(carboxymethyl)-L-Lysine.

After the final wash in dioxane, the surface was further modified to obtain a tricarboxyl functionalised surface. The carboxyl groups on the surface were first activated with NHS and DCC. The activation was carried out by incubating the chips for 4 h in a solution containing 1.6 g of DCC and 0.8 g of NHS in 40 mL dioxane. (10 mL / prism, shaker 250 rpm). At the end of the incubation period the prisms were washed (three times each, 20 mL for each wash) in dioxane, methanol and water, respectively. At the end of the incubation period the prisms were washed (three times

each, 20 ml for each wash) in dioxane, methanol and water, respectively. This was followed by incubation in a solution of N_{α},N_{α}-Bis(carboxymethyl)-L-lysine trifluoroacetate for 18 h at room temperature. The Bis(carboxymethyl)-L-lysine trifluoroacetate (0.15 mg mL^{-1}) is prepared in 0.1 M sodium hydrogen carbonate buffer (pH 8.3). At the end of the incubation period the tricarboxyl functionalised prisms were rinsed with water and then dried and stored under vacuum.

3.2.4.6 Cobalt-coated SPR prisms

Cobalt (Co^{2+}) coated gold surface was obtained using a tri-carboxyl functionalised chip.

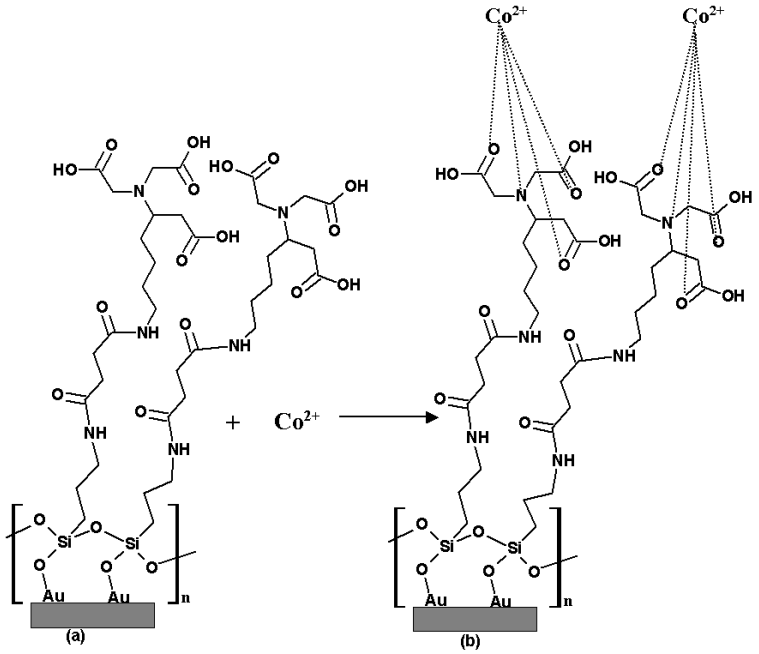

Fig. 3.10: Steps involved in the creation of an SPR-chip with cobalt (Co^{2+}) functionalised gold surface (a) tri-carboxyl functionalised gold surface (b) formation of (Co^{2+}) functionalised surface.

The tri-carboxyl-functionalised chip is prepared according to the protocol described in section 3.2.4.5. The chip was mounted onto the SPR device.10 µL aqueous solution (10 mg mL^{-1}) of cobaltous chloride hexahydrate ($CoCl_2$. $6H_2O$) was added, online onto the tri-carboxyl coated chip, resulting in a concentration of 5 µg mL^{-1} in the cuvette. The steps involved in the creation of the cobalt-functionalised surface are presented in Fig. 3.10.

3.2.4.7 LPS-coated SPR prisms

The C_{18} coated prism forms the basis of preparing the LPS coated prism. The LPS from Gram-negative bacteria is self-assembled on the C_{18} chip by hydrophobic interactions. The LPS coating is carried out online in the SPR device and the incubation time is 15 min. Purified LPS from *Salmonella* spp. dissolved in PBS (10 µg mL^{-1} final concentration inside the cuvette) was used. 10 µL of the LPS solution is used for each cuvette of the SPR device. Details of the LPS coating and optimisation of the LPS concentration are discussed in sections 4.2.1 and 4.2.2.

3.2.4.8 Contact angle (hydrophobicity) measurement of SPR chips

Contact angle analysis characterises the wettability of a surface by measuring the surface tension of a solvent droplet at its interface with a homogenous surface. In more technical terms, contact angle measures the attraction of molecules within the droplet to each other versus the attraction or repulsion those droplet molecules experience towards the surface molecules (Neumann, 1979).

The most commonly used technique is the static or sessile drop method. The experiment normally calls for the addition of a drop of liquid (water) onto the solid surface whose wettability is to be measured. When the liquid has settled (has become sessile) the drop will retain its surface tension and become ovate against the solid surface. The angle between the baseline of the drop and the tangent at the drop boundary is measured. This is the contact angle. The contact angle at which the oval of the drop contacts the surface determines the affinity between the surface and the liquid. A more rounded drop on top of the surface indicates lower affinity because the angle at which the drop is attached to the solid surface is more acute. In this case the liquid is said not to wet the surface and the surface characterised as hydrophobic (Fig. 3.11a). A flat drop indicates a high affinity, in which case the liquid is said to wet the surface and the sur□haracterizedcterised as hydrophilic (Fig. 3.11b). Water contact angle was measured according to the sessile drop method, at room temperature using a “Krüss Contact Angle Measurement System G 10” and the data was analysed using the SCA20 software. The volume of water used for each drop was 50 µL.

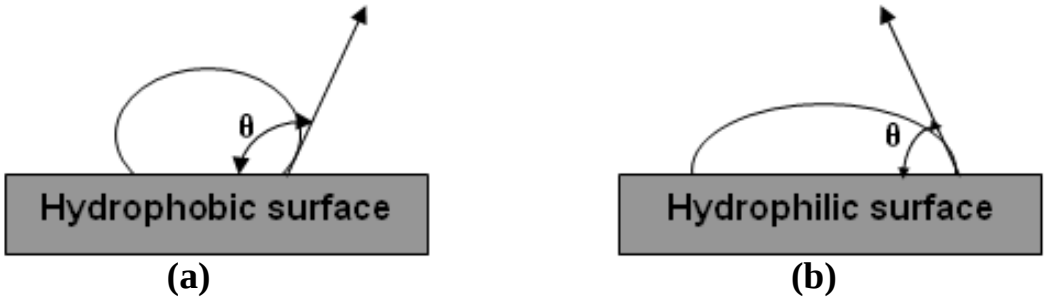

Fig. 3.11: Schematic representation of the appearance of a water drop on (a) a hydrophobic surface and (b) a hydrophilic surface. The angle θ in both cases represnts the respective contact angles.

3.2.5 Plasmonic® SPR device

3.2.5.1 Setup

The SPR-based assays described in this work was developed on the Plasmonic® SPR device (Plasmonic Biosensoren AG, Wallenfels, Germany). The device works on the well-known Kretschmann attenuated total reflection (ATR) configuration (section 1.1.2.1). Each SPR chip is made of a glass prism coated uniformly with gold to a thickness of 50 nm, which acts as the reflecting and sensing surface (Fig. 3.12). The approximate dimensions of the prisms are length = 25 mm, breadth = 10 mm, thickness = 3 mm. The device is characterised by a cuvette-based system, providing 8 parallel channels.

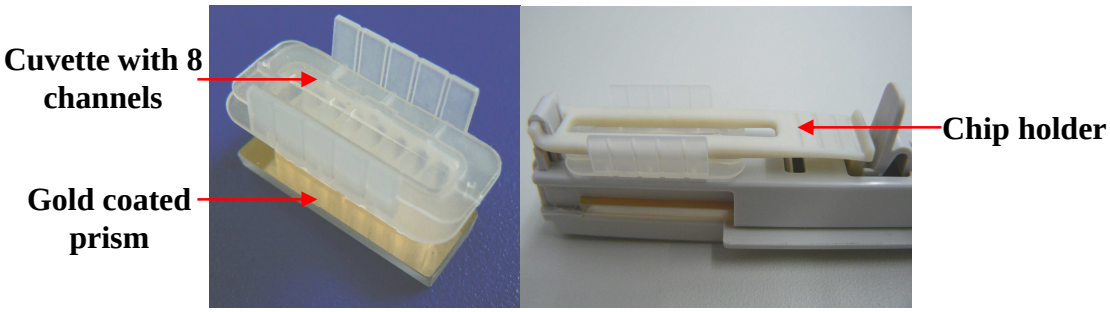

Fig. 3.12: Photographs showing (left) the cuvette with 8 channels placed on top of the gold-coated glass prism (right) the prism with the cuvette mounted on the chip holder

The samples and reagents are loaded into a microtitre plate, from where the autosampler loads them automatically into the channels of the cuvette (Fig. 3.13).

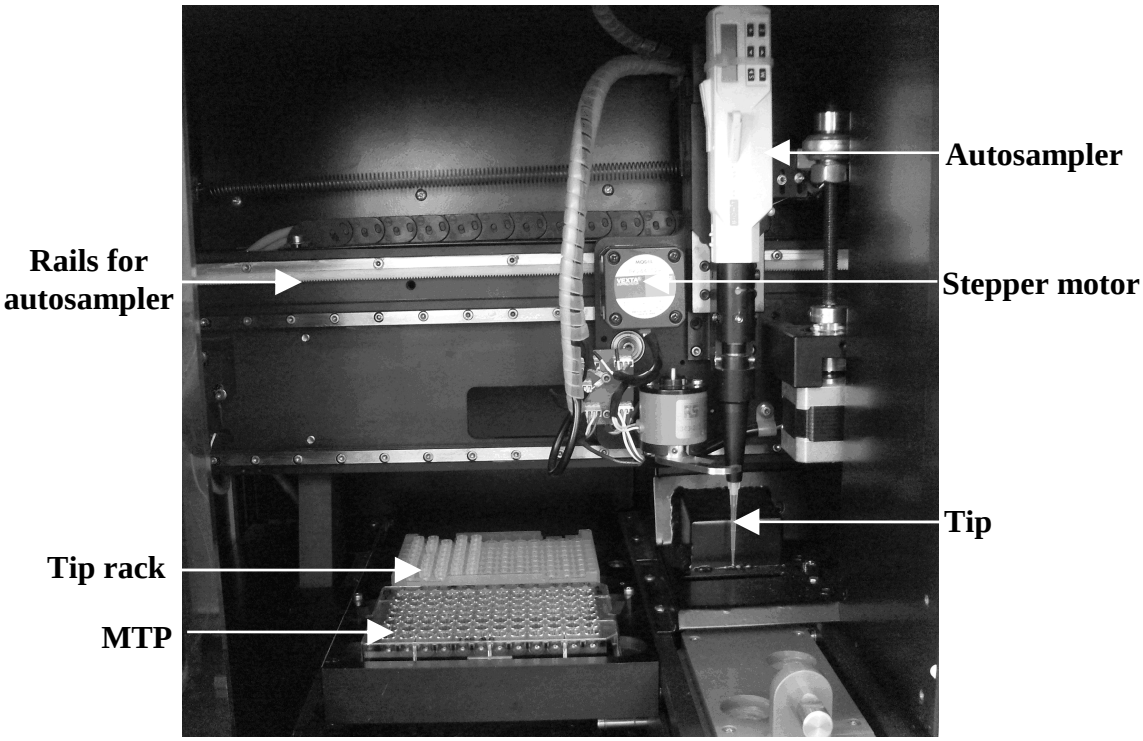

Fig. 3.13: Photograph showing the autosampler of the Plasmonic® SPR device. Movement of the autosampler is by means of a stepper motor. The autosampler moves on rails. The tips and samples are loaded on the tip rack and MTP, respectively. All operations of the autosampler are computer controlled.

The computer controls the operations of the autosampler; this control is based on a coordinate system of operation. The coordinate system of operation is described in Fig. 3.14. Another advantage of this cuvette-based system is that the sample materials can be examined without any danger of blockage, as encountered in case of fluidic-based systems. A sample volume of only 10 μL is required for analysis. The temperature is controlled by means of Peltier elements.

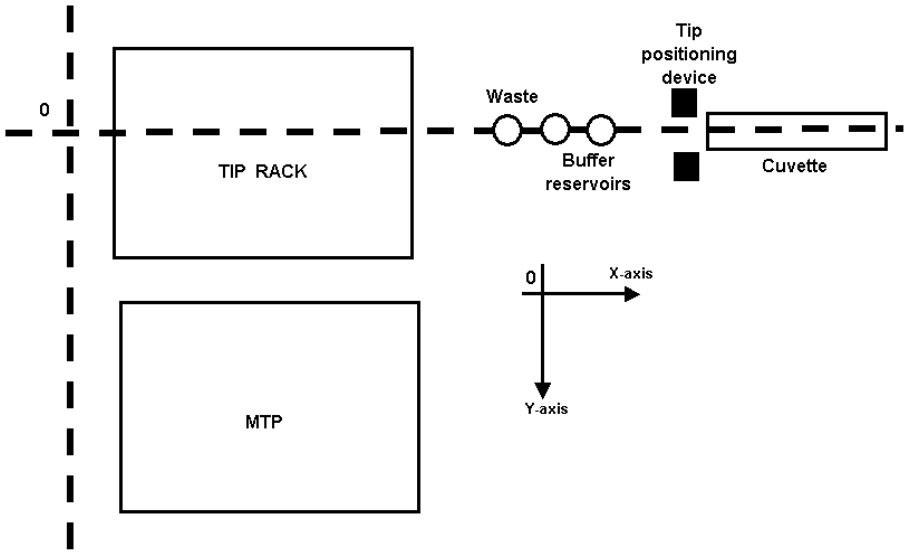

Fig. 3.14: The coordinate system of operation of the autosampler. The origin "O" of the coordinate system corresponds to the intialisation point of the autosampler, which is attained during the instrument start up (defined by reference switches). Operation in the Y-direction is realised by moving the MTP and TIP tray (controlled by a stepper motor). The pipette of the autosampler moves along the X-Z plane (controlled by two stepper motors). Note that the Z-axis is perpendicular to the X-Y plane. A photoelectric barrier is used to determine accurately the X- and Z- position of the pipette tip (tip positioning device)

3.2.5.2 Optics

The unique feature of the Plasmonic® SPR device is its defocusing optics. The source of incident light is a laser diode (786 nm). It emits an elliptical beam of light, which is then converted, using the cylindrical lens system of the device, into a divergent beam. Using the defocusing optics, it is possible to cover all possible angles of incidence required for the real-time determination of the SPR angle on the gold surface. The optical setup of the device is explained in Fig. 3.15a. The reflected light is detected with the help of a charge-coupled device (CCD) camera.

The CCD camera receives all the light reflected from the surface of the SPR chip. The breadth of the CCD camera receiving the reflected light consists of 752 pixels for each channel.

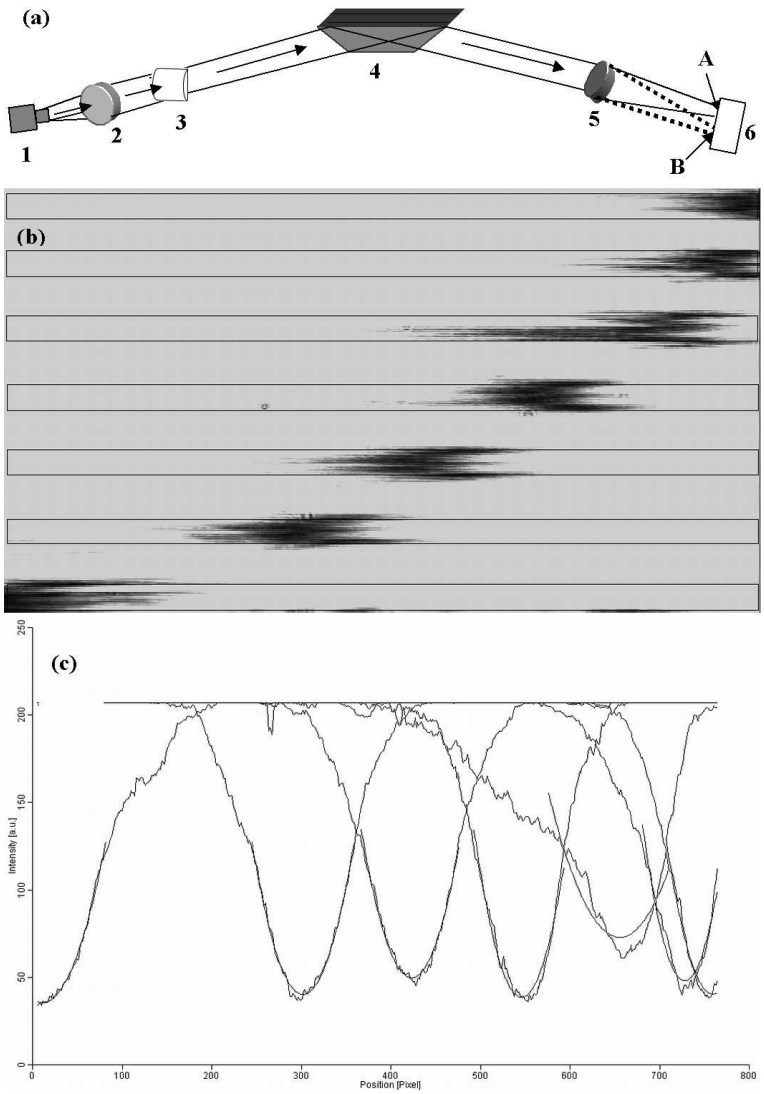

Fig. 3.15: The Plasmonic® SPR device. (a) The optical setup of the Plasmonic® SPR device: 1. Laser diode (786 nm), 2. collimating lens, 3. cylindrical lens resulting in a divergent beam, 4. gold-coated SPR chip, position adjusted to obtain maximum coverage of the gold surface by the divergent beam, 5. Lens to concentrate the reflected beam of light on, 6. CCD camera. Pixel position "A" of minima at the beginning of the run. Change in pixel position to "B" at the end of the run due to a binding event on the surface of the SPR chip. (b) A view of the channels of the device. Plasmons are seen as dark spots, for different concentrations of NaBr: highest refractive index (30% NaBr) on the extreme left and lowest refractive (1.25% NaBr) at the extreme right. (c) Corresponding plot of intensity (Y-axis) of reflected light vs. Position [Pixel] (X-axis) of reflected minima, as seen on the computer screen. Each curve shows different channels corresponding to different concentrations of NaBr solutions.

The change in pixel position of the reflected minima, occurring with each binding interaction on the gold surface, is monitored by the camera. The software allows for the real-time visualisation of the minima for each channel, which are shown as dark

spots on the computer screen (Fig. 3.15b). The software also generates a plot of light intensity (Y-axis) vs. pixel position (X-axis) for each channel.

The pixel position of the reflected minima for the lowest refractive index of the sample solution, starts at 752 pixels and shifts towards 0 pixel with increasing refractive index (Fig. 3.15c). The software continuously monitors this change in pixel position of the minima occurring on the CCD camera for each channel with respect to their position at the beginning of the run. This change correlates with the change in SPR angle, occurring with each binding event. The plot of change in SPR angle with time is called the "sensorgram".

3.2.5.3 Calibration of the SPR device

The calibration of the SPR device is carried out to determine the detection range in terms of refractive index as monitored by pixel position of the reflected minima on the CCD camera. Sodium bromide (NaBr) solution at different known concentrations is used to calibrate the instrument. The instrument was calibrated using NaBr solution starting at a high of 30 % (w/v) and going down to a low of 1.25 % (w/v). A linear detection range of the instrument lies in this concentration range. A good correlation between the change in concentration and change in pixel position was observed in this concentration range (Fig. 3.16). It is important, to clarify at this point that, from the observations of this study, this refractive index linear region of the instrument is well within the range of refractive index change which occurs when carrying out SPR-based immunoassays using bacteria (after removal of the LB broth by centrifugation and resuspending the bacteria in PBS, section 3.2.1) and also when using commercially available antibodies containing preservatives eg, sodium azide (NaN_3).

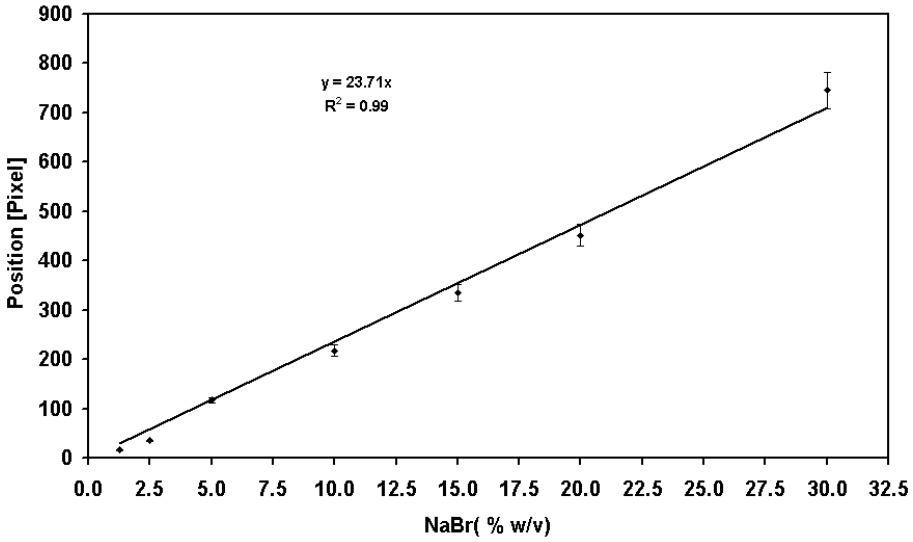

Fig. 3.16: Calibration of the SPR device using different concentrations of NaBr.

3.2.6 SPR-based assays on Plasmonic®

3.2.6.1 Detection modes on Plasmonic® SPR device

Using the Plasmonic® SPR device it was possible to explore two different modes of detection. The two modes are the multi-channel and the sequential detection modes. The device is provided with 8 channels (section 3.2.5). In the multi-channel detection mode, irrespective of the number of analytes (*Salmonella* serovars) captured in any given channel, only one analyte (*Salmonella* serovar) is detected per channel using the secondary detection antibody (Fig. 3.17a). Thus, for detection of each analyte the assay has to be run, in each channel, from the initial biorecognition element immobilisation stage. In the sequential detection mode the secondary detection antibodies, against the captured analytes in each channel, are added sequentially. This enables screening of multiple analytes using one single channel (Fig. 3.17b). Thus, the sequential detction mode has the advantage of saving time as well as reagents. However, there are certain important points, which need to be considered when designing experiments using the sequential detection mode. The investigations and findings on detection of *Salmonella* using the sequential detection mode are discussed in detail in section 4.1.4.2.

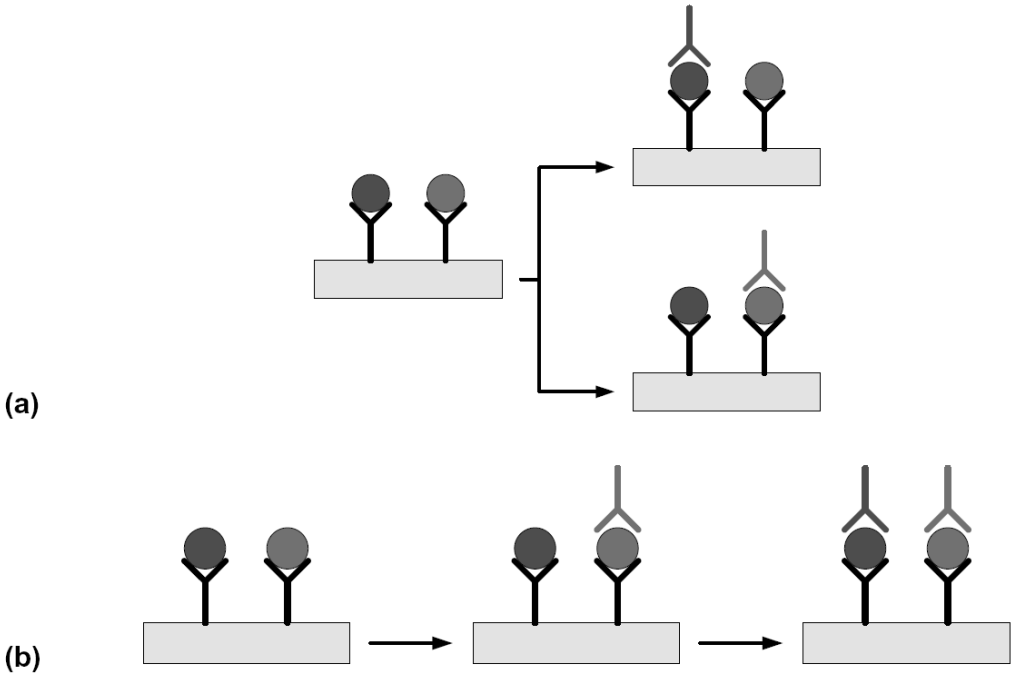

Fig. 3.17: Modes of detection using the Plasmonic® SPR device (a) schematic (multi-channel detection) showing the capture of two different *Salmonella* serovars (dark and light circles) by the capture antibody followed by detection of each analyte, separately in two different channels (b) schematic (single-channel sequential detection) showing the capture of *Salmonella* serovars by the capture antibody and detection of each analyte, sequentially (light coloured detection antibody followed by dark) in the same channel.

3.2.6.2 Assay setup of the SPR-based assay for detection of *Salmonella*

The assays developed for the detection of *Salmonella* were designed as a sandwich assay immunoassays. All experiments were carried out at a constant temperature of 22.00 °C. In the first step of the assay, polyclonal antibody (250 µg mL^{-1}) against *Salmonella* spp. was immobilised on the SPR chip. The immobilised polyclonal antibodies were then used to capture all *Salmonella* serovars that may be present in a given sample. After the step involving immobilisation of the polyclonal antibody and before capture of bacteria, any unbound polyclonal antibodies were washed away with PBS. In the next step, BSA (100 µg mL^{-1}) was added as a blocking agent. The bacteria were then captured. The captured bacteria were then further probed with polyclonal (in case of detection of *Salmonella* spp. in general) or monoclonal (in case of specific detection of *Salmonella* serovars) secondary detection antibodies. The binding of the bacterial cells to the immobilised capture antibody was recorded in

real-time on a SPR sensorgrams, in terms of arbitrary units (AU). Three different readings were taken for each data point. A control experiment was run in each case.

3.2.6.3 SPR-based serogrouping protocol

For the serogrouping of *Salmonella* spp. the same protocol as described above in section 3.2.6.2 was used. After capture of the respective serovars by the polyclonal capture antibody serogroup identification was carried out using the respective group specific antibodies. The details of the different group specific antibodies have been presented in Table 3.6.

3.2.6.4 Serotyping protocol for SPR-based serotyping of *S.* Enteritidis

For serotyping of *S.* Enteritidis the scheme used in the conventional slide agglutination test SAT (Fig. 3.18) was adapted for the SPR serotyping. The steps involved in the serotyping of *S.* Enteritidis are explained as follows. The first step involves screening of the bacteria with polyclonal O-antibody mixture specific for serogroups A to E of *Salmonella*. This mixture is referred to here as Poly A-E. A positive reaction to Poly A-E confirms the presence of genus *Salmonella* belonging to any of the serogroups A to E.

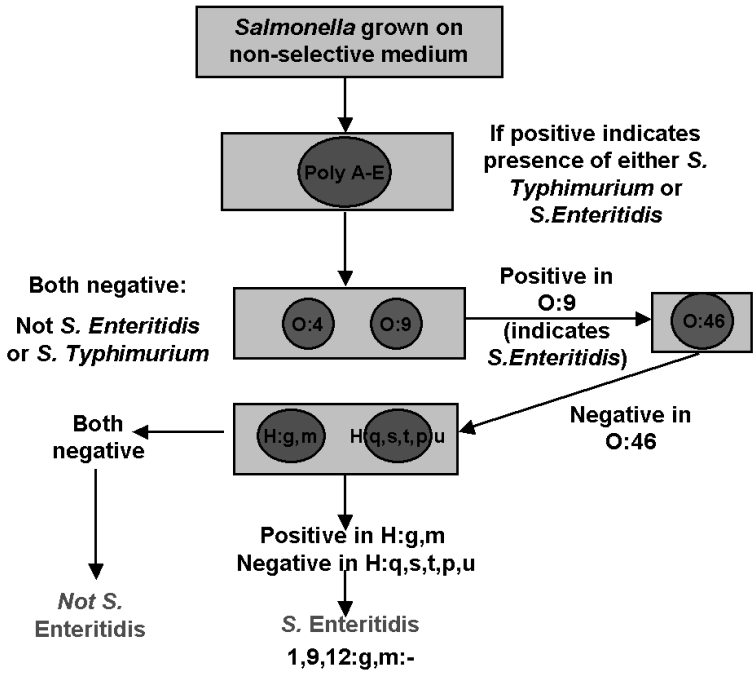

Fig. 3.18: Flow diagram of the steps involved in serotyping of *S.* Enteritidis using the conventional slide agglutination test.

The next step involves determination of the group-specific O9-antigen of *S.* Enteritidis using monospecific anti-O9 antibody. Once the presence of the O9-antigen is confirmed, further test is carried out to determine the presence or absence of the O-antigen O46. To determine the serotype, presence of specific flagellar antigens (H-antigens) needs to be established. In case of *S.* Enteritidis the test involves the response of the bacteria to antibody against H-antigens g,m. The H-antigens g,m are also known to occur in other serovars of *Salmonella* along with the H-antigens q,s,t,p,u. Thus, a final confirmation of the presence of the serotype *S.* Enteritidis is obtained with a positive response to antibody against Hg,m and a negative response to antibody against Hq,s,t,p,u. The SPR-based serotyping is schematically represented in Fig. 3.19.

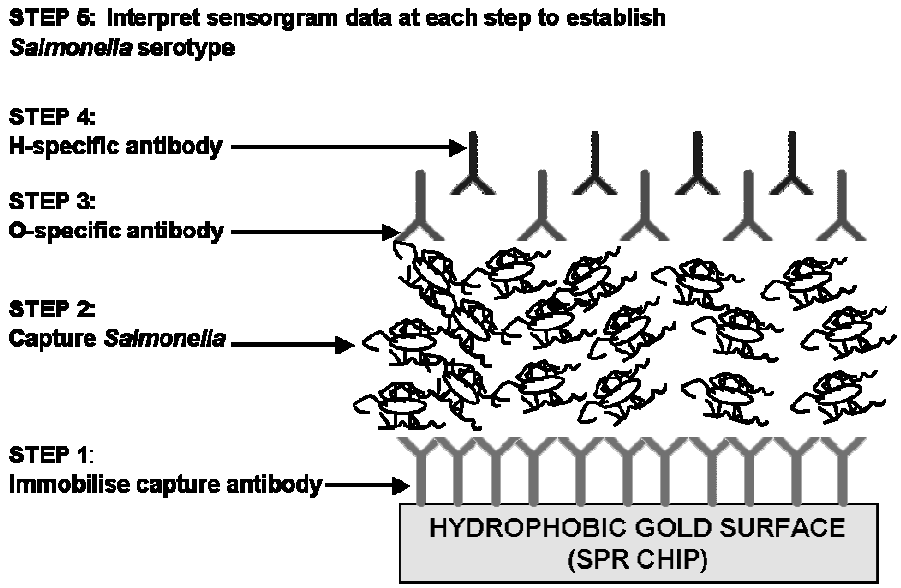

Fig. 3.19: Schematic representation of the steps involved in the SPR-based seroytping of *Salmonella* spp.

3.2.6.5 Assay setup of the SPR based assays for detection of *Salmonella* infection

The serological SPR assay is based on the direct detection of antibodies present in infected serum. The antibodies in the serum samples are detected by capturing them with lipopolysaccharide (LPS). Purified LPS from *Salmonella spp.* whose infection was to be detected, were used as the capture antigen. In the first step the LPS was immobilised on the C_{18} SPR chip surface. After the LPS immobilisation step, the surface was repeatedly washed with PBS to remove all unbound LPS. BSA

(100 μg mL^{-1}) was then added to block any available free C_{18} gold surface. Unbound BSA was washed off with PBS. In case of the mixed LPS-based assay, LPS mixtures from different *Salmonella* serovars were used as the capture antigen. All calibrations runs and runs to optimise the LPS concentration were carried out using anti *Salmonella* O-specific monoclonal antibody (anti-O5) or the respective serogroup specific monoclonal antibodies (in case of mixed LPS-based assay).

A volume of 10 μL of the monoclonal antibody or serum from *Salmonella* infected pigs was added onto the surface of the LPS-coated and BSA-passivated SPR chip, after removing an equal volume of PBS from the cuvette. The total liquid volume in each cuvette is 20 μL. The binding of the serum antibodies to the LPS, immobilised on the SPR chip, was recorded in real time as a SPR sensorgrams. Each sensorgrams recorded the change in SPR angle with time caused due to binding of the antibodies to the LPS. This change was recorded in terms of arbitrary units (AU). Both, unbound serum components and unbound antibodies were then washed away using PBS. In each case, a control was run using serum free of *Salmonella* infection. A minimum of three readings was taken at each antibody concentration.

3.2.7 Determination of antibody concentration

Absorbance of UV irradiation by proteins is the quickest of all methods for quantitating protein solutions. Readings are often performed at 280 nm or 205 nm. The absorbance maximum at 280 nm is due primarily to the presence of tyrosine and tryptophan. Absorbance at 205 nm is due primarily to the peptide bond although other amino acids also contribute. In addition to the speed with which absorbance readings can be made, on major advantage of UV quantitation is that none of the sample is destroyed in determining the concentration. In this work wherever applicable the concentration of the IgM antibodies were measured at 280 nm. This concentration of the antibody was the calculated in accordance with a previous published protocol, using the extinction coefficient ($E^{1\%}_{280nm}$) value of 14 for a 1% solution (Kennedy et al., 1983).

3.2.8 Acid hydrolysis of LPS to remove Lipid A

One of the classical strategies in structural analysis of LPS involves the cleavage of the ketosidic linkage of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), by which the core region is bound to the Lipid A (Qureshi et al., 1982). This ketosidic linkage is

known to be acid labile. Thus, exploiting the acid lability of the ketosidically linked KDO residue, the hydrolysis of LPS was carried using 1 % acetic acid (AcOH) according to method described by Manzo et al. (2001). The sample was dissolved in 1% aq AcOH and hydrolyzed at 100°C for 3 h. Then, the sample was centrifuged (12,000*g*) using a table top centrifuge (Heraeus Biofuge pico, Germany) yielding the O-specific polysaccharide (supernatant) and the Lipid A (precipitate).

3.2.9 Kinetic analysis

3.2.9.1 Theory of kinetic analysis

When a binding interaction occurs between an immobilised ligand A and a ligate (analyte) L. it results in the formation of the complex AL. This interaction is represented by the equation (3.1):

$$A + L \underset{K_{diss}}{\overset{K_{ass.}}{\rightleftharpoons}} AL \qquad (3.1)$$

The rate of formation or association of AL is defined by the equation (3.2):

$$\frac{d[AL]}{dt} = K_{ass.}[A]\,[L] \qquad (3.2)$$

[A]= molar concentration of A (M)

[L]= molar concentration of L (M)

[AL]= molar concentration of AL (M)

t= time in seconds (S)

$K_{ass.}$= association rate constant ($M^{-1}S^{-1}$)

The rate of dissociation of AL is defined by the equation:

$$-\frac{d[AL]}{dt} = K_{diss.}[AL] \qquad (3.3)$$

$K_{diss.}$= dissociation rate constant (S^{-1})

At equilibrium the rate of the forward reaction is equal to the rate of the backward reaction and this condition is defined by the equation 4:

$$K_{ass.}\,[A]\,[B] = K_{diss.}[AB] \qquad (3.4)$$

Rearranging the above equation 4, provides the equilibrium constants:

$$K_A = \frac{[AB]}{[A][B]} = \frac{K_{ass.}}{K_{diss.}} \qquad (3.5)$$

$$K_D = \frac{[A][B]}{[AB]} = \frac{K_{diss.}}{K_{ass.}} \qquad (3.6)$$

K_A= equilibrium association constant (M^{-1}).

The value of K_A indicates the tendency of a ligand and ligate to associate. A high value of K_A indicates a high affinity between the ligand and ligate.

K_D= equilibrium dissociation constant (M).

The value of K_D indicates the tendency of the ligand-ligate complex to dissociate. A lower value of K_D indicates a high affinity between the ligand and ligate or higher stability of the complex.

3.2.9.2 Kinetic analysis using "initial rate analysis" model of Edwards and Leatherbarrow

The "initial rate analysis" model put forward by Edwards and Leatherbarrow (Edwards and Leatherbarrow, 1997) makes the following important assumptions:

In case of optical biosensors, the kinetics of all binding events involving interaction between an immobilised ligand and a ligate (analyte) consists of two phases. A fast initial response phase, which is linear, and a slower second phase which is non-linear. In case of SPR both these phases are recorded in real time on a sensorgram.

The initial binding rates of the linear region of the binding curves for each concentration of the analyte are obtained by linear regression. The concentration of the immobilised ligand is kept constant. This initial rate is dependent on the concentration of the analyte and is described by the equation:

$$\frac{dR}{dt} = \frac{R_{\max}[L]}{K_D + [L]}(K_{ass.}.[L] + K_{diss.}.) = R_{\max}[L]K_{ass.}. \qquad (3.7)$$

R_{max} = maximum binding capacity of the immobilised ligand. This corresponds to the SPR signal obtained for the highest concentration of the analyte tested (AU).

[L] = molar concentration of the analyte (M).

Thus, a plot of the the initial rate (dR/dt) vs. concentration of the analyte results in a straight line. The slope of this line, according to equation (3.7), is the product of the association rate constant ($K_{ass.}$) and the maximum binding capacity of the immobilised ligand (R_{max}).

$$\text{Slope of the plot} = R_{\max .} K_{ass.} \qquad (3.8)$$

Thus using the slope data, K_{ass}.is easily obtained using the equation (3.8).
For calculation of the dissociation rate constant (K_{diss}.), the dissociation phase data of the sensorgram obtained at the saturation concentration of the analyte is used. The data are fitted into the following equation.

$$R_t = R_0 \exp -K_{diss.} t \qquad (3.9)$$

R_t= response at any given time t (AU)

R_0= intial response (AU)

From equation (3.9) it is clear that a plot of $\ln(R_0/R_t)$ on the Yaxis vs. time (t) on the X-axis will result in a linear plot, the slope of which corresponds to $K_{diss.}$
The equilibrium association (K_A) and dissociation (K_D) constants are then calculated using the calculated values of $K_{ass.}$ and $K_{diss.}$ according to the equations (3.5) and (3.6), respectively.

4 Results

4.1 Detection of *Salmonella*

4.1.1 Capture antibody immobilisation

The first step in the development of an SPR-based biosensor, for the detection of *Salmonella* was to successfully immobilise the capture antibody on the surface of the chip. A polyclonal antibody against *Salmonella* spp. (polyclonal to the O- and H-antigens) was chosen for this purpose. A polyclonal antibody was used keeping in mind that the developed assay should be able to detect multiple *Salmonella* spp. Thus, the immobilised polyclonal antibody should be able to simultaneously capture multiple *Salmonella* serovars on the surface of the SPR chip. Different immobilisation strategies were explored. The success of each immobilisation strategy was evaluated, in terms of the antigen binding activity of the antibody. This was done by carrying out an immunoassay for the detection of *S*. Enteritidis. *S*. Enteritidis (1×10^{10} cells mL^{-1}) was captured by the immobilised capture antibody, followed by addition of an O-specific detection antibody against the somatic antigen O9 of the bacteria. In order to reduce complexity and time as far as possible, all these steps required for immobilisation of the antibody were carried out online, in the SPR-device. The immobilisation strategies explored and the results obtained in each case are disussed below.

4.1.1.1 Immobilisation on a carboxyl functionalised gold surface

A carboxyl-functionalised SPR-chip, prepared according to the protocol described in section 3.2.4.5, was used. The first step involved the activation of the carboxyl groups on the surface of the chip using and aqueous solution of EDC/sulfo-NHS (0. 4 M EDC and 0.1 M sulfo-NHS). This resulted in the formation of sulfo-NHS esters on the surface of the chip. The water-soluble carbodiimide EDC is used to form active ester functional groups with carboxylate groups using the water-soluble compound sulfo-NHS. Sulfo-NHS esters are hydrophilic active groups that react rapidly with amines on target molecules (Staros, 1982). Sulfo-NHS esters react with the amine groups of the antibody to form stable peptide bonds. The polyclonal antibody was then added onto the surface of the chip and incubated for 15 min. This

was then followed by inactivation of any unreacted sulfo-NHS esters by the addition of 1 M ethanolamine hydrochloride.

After immobilisation of the capture antibody as described above, the bacteria were added. This was then followed by addition of the anti-O9 detection antibody. The detection signal obtained after subtraction of the control channel was 53 ± 7 AU. The sensorgram obtained is presented in Fig. 4.1a.

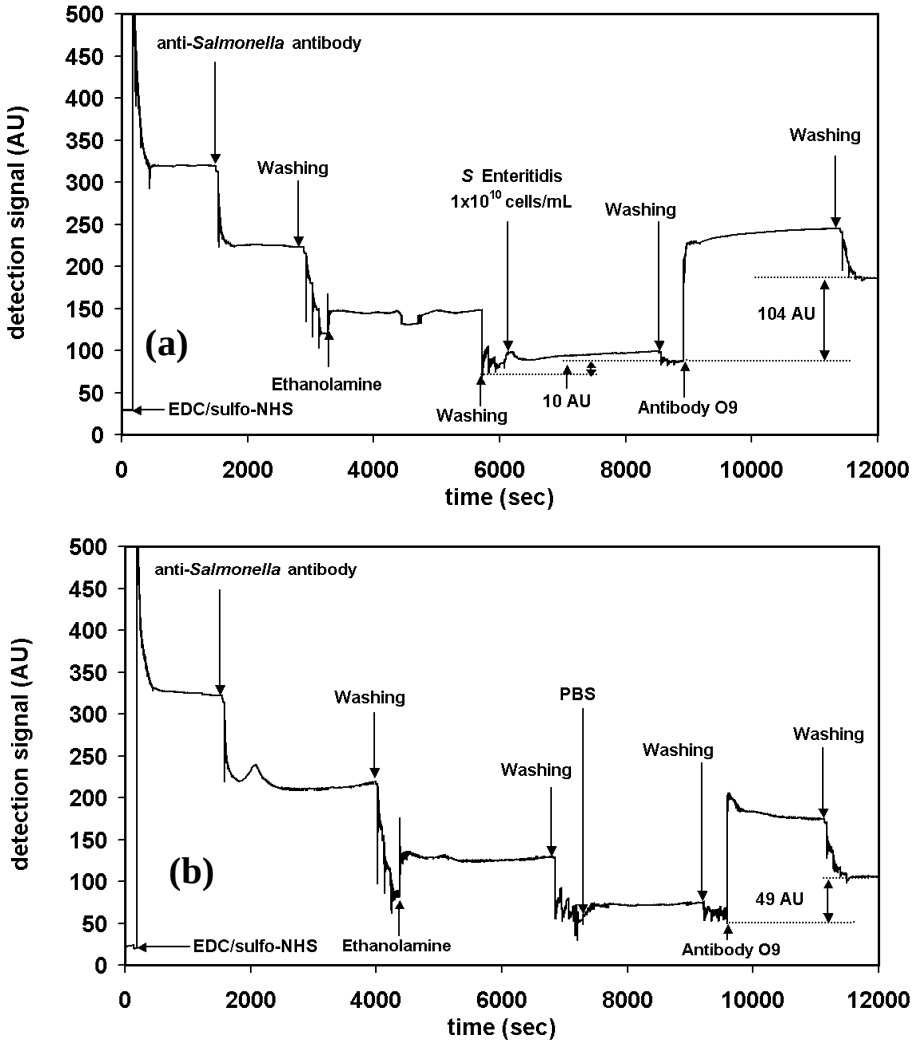

Fig. 4.1: Sensorgrams obtained for the immobilisation of the capture antibody on a carboxyl functionalised chip (a) sensorgram showing the capture antibody immobilisation step followed by capture of the bacteria and then detection with O-specific antibody (b) sensorgram of the control run without bacteria.

It is seen from the data obtained from the control run that this method of immobilisation resulted in high unspecific binding (49 ± 2 AU) of the detection antibody O9 (Fig. 4.1b). Also, it is important to note in the sensorgrams that the

binding of the capture antibody is not visible in terms of rise in the binding curve at the point of adding the antibody. This is due to the high initial bulk refractive index change as a result of addition of the EDC/sulfo-NHS solution in the first step. However, the final detection signal due to the binding of the capture antibody is the difference in height between the point of addition of EDC/ sulfo-NHS and the point just before addition of the ethanolamine.

4.1.1.2 Immobilisation on an amine functionalised gold surface

Immobilisation of the polyclonal capture antibody, on amine-coated surface is carried out by reaction between the amine groups on the surface of the chip and activated carboxyl groups of the antibody. The activation of the carboxyl groups of the antibody was carried out according to the protocol mentioned in section 3.2.3.1. The activated antibody was added, online, on to the surface of an amine-coated chip (section 3.2.4.2) and incubated for 30 min. This was followed by a washing step with PBS. Unreacted sulfo-NHS esters were then inactivated by addition of 1 M ethanolamine hydrochloride. The bacteria were then captured using the immobilised polyclonal antibody. The final step was the addition of the anti-O9 detection antibody. With this strategy for the immobilisation of the capture antibody the final detection signal obtained for the O9 detection antibody was 57 ± 5 AU. In addition, it was observed that the activity of the capture antibody was better retained in comparison to that obtained for the immobilisation strategy on carboxyl surface (section 4.1.1.1). This observation is confirmed by the nature of the binding curve obtained as well as from the detection signal obtained due to binding of the bacteria. The detection signal due to capture of the bacteria was 38 AU. The corresponding signal due to capture of the bacteria in case of the carboxyl surface was only 10 AU. In addition, the control channel in this case showed a comparatively lower unspecific binding of the O9 detection antibody. From these results it can be concluded that immobilisation of the capture antibody on an amine coated surface resulted in improved results as compared to immobilisation on a carboxyl surface. The sensorgrams are presented in Fig. 4.2.

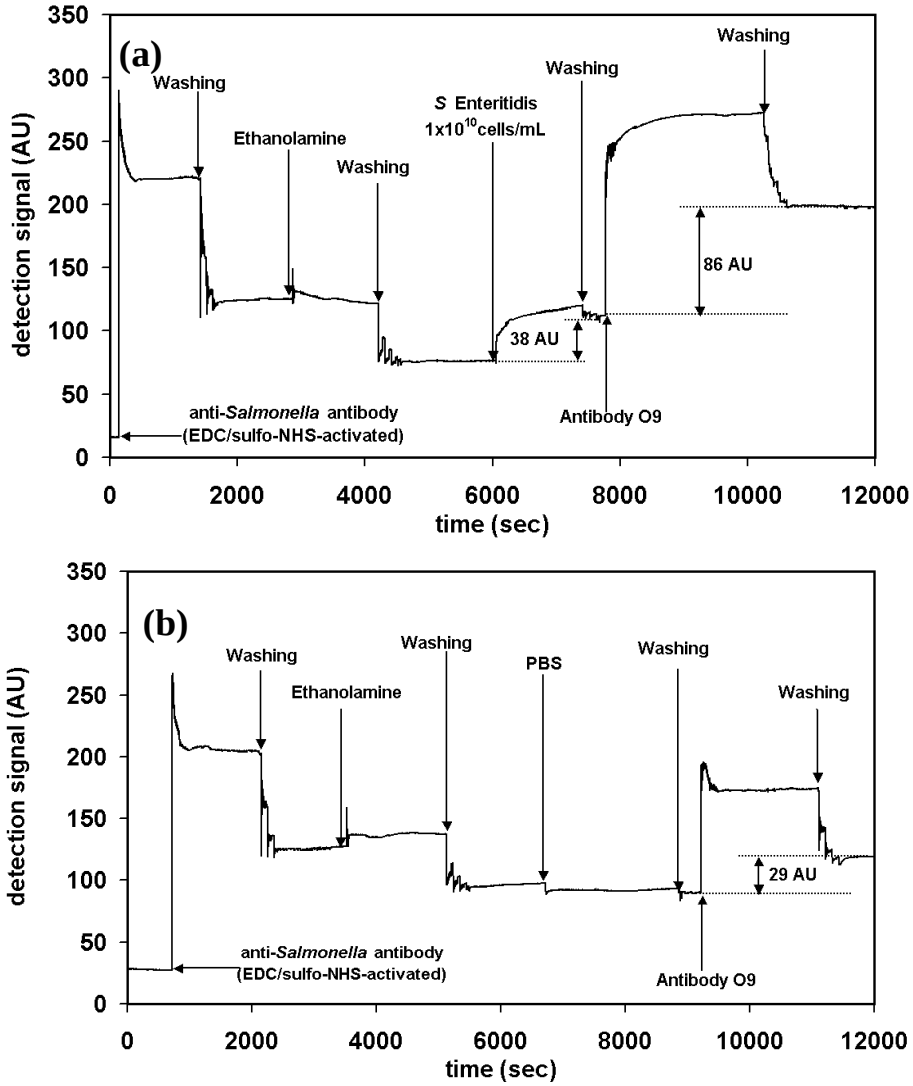

Fig. 4.2: Sensorgrams obtained for the immobilisation of the capture antibody on an amine-functionalised chip (a) sensorgram showing the capture antibody immobilisation step followed by capture of the bacteria and then detection with O-specific antibody (b) sensorgram of the control run without bacteria.

4.1.1.3 Immobilisation by modification of the carbohydrate side chain of the capture antibody

The carbohydrate side chains of immunoglobulins can be modified by oxidation with sodium periodate ($NaIO_4$) to generate reactive aldehyde groups. Aldehyde groups easily react with amines to form Schiff bases. A reducing agent such as $NaBH_4$ is used for reductive amination and stabilisation of the Schiff base (section 3.2.3.2). 10 µL of the polyclonal antibody (500 µg mL^{-1}) with the oxidised carbohydrate side chain was added online to the surface of an amine-functionalised (section 3.2.4.2). SPR chip mounted in the SPR device. The reaction was carried out in presence of

0.2 M Na_2CO_3 solution pH 9.6. The reaction was allowed to happen for 1 h. This was then followed by the addition of 10 µL of a 2.5 mM aqueous solution of $NaBH_4$. The incubation time was 30 min. After this time the unbound antibodies and chemicals were washed away with PBS. 10 µL of BSA (100 µg mL^{-1} in the cuvette) was then added as a blocking agent. The *S.* Enteritidis bacteria were captured by the immobilised polyclonal antibody. Detection of the captured bacteria was then carried out using O-specific antibody against antigen O9. The signal on addition of the detection antibody to the channel containing the bacteria was 64 ± 3 AU.

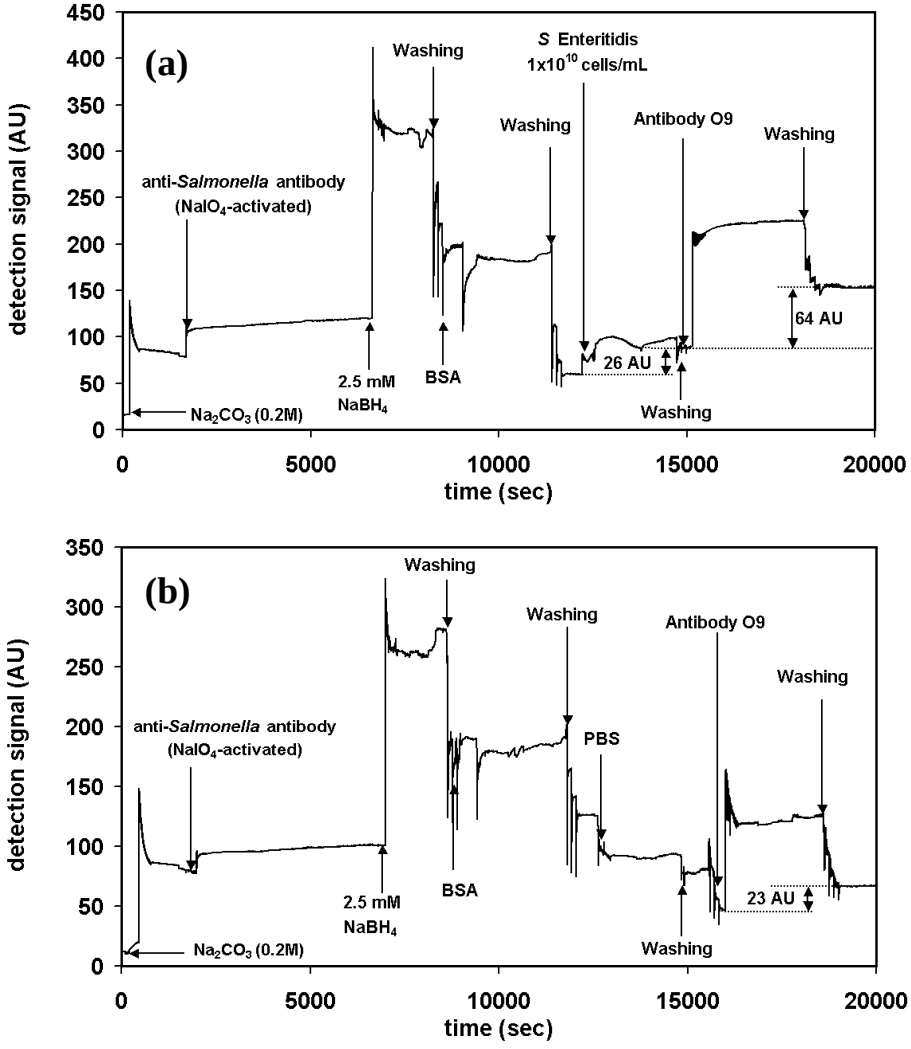

Fig. 4.3: Sensorgrams obtained for the immobilisation of the capture antibody by $NaIO_4$ activation of the carbohydrate side chain (a) sensorgram showing the capture antibody immobilisation step followed by capture of the bacteria and then detection with O-specific antibody (b) sensorgram of the control run without bacteria.

The overall net detection signal obtained was only 41± 4 AU. The sensorgrams are presented in Fig. 4.3.

4.1.1.4 Immobilisation using biotinylated antibody

The anti-*Salmonella* polyclonal antibody was biotinylated according to the protocol mentioned in section 3.2.3.3. The immobilisation was carried out on an SPR chip functionalised with biotin (section 3.2.4.3). The first step involved immobilisation of streptavidin (250 µg mL^{-1}) onto the surface of the biotin chip. The binding of the streptavidin to the biotinylated chip resulted in a detection signal of 77 ± 6 AU. After a washing step with PBS the biotinylated antibody was added to the chip surface coated with streptavidin. However, no significant detection signal was obtained on addition of the biotinylated antibody (5 ± 2 AU).

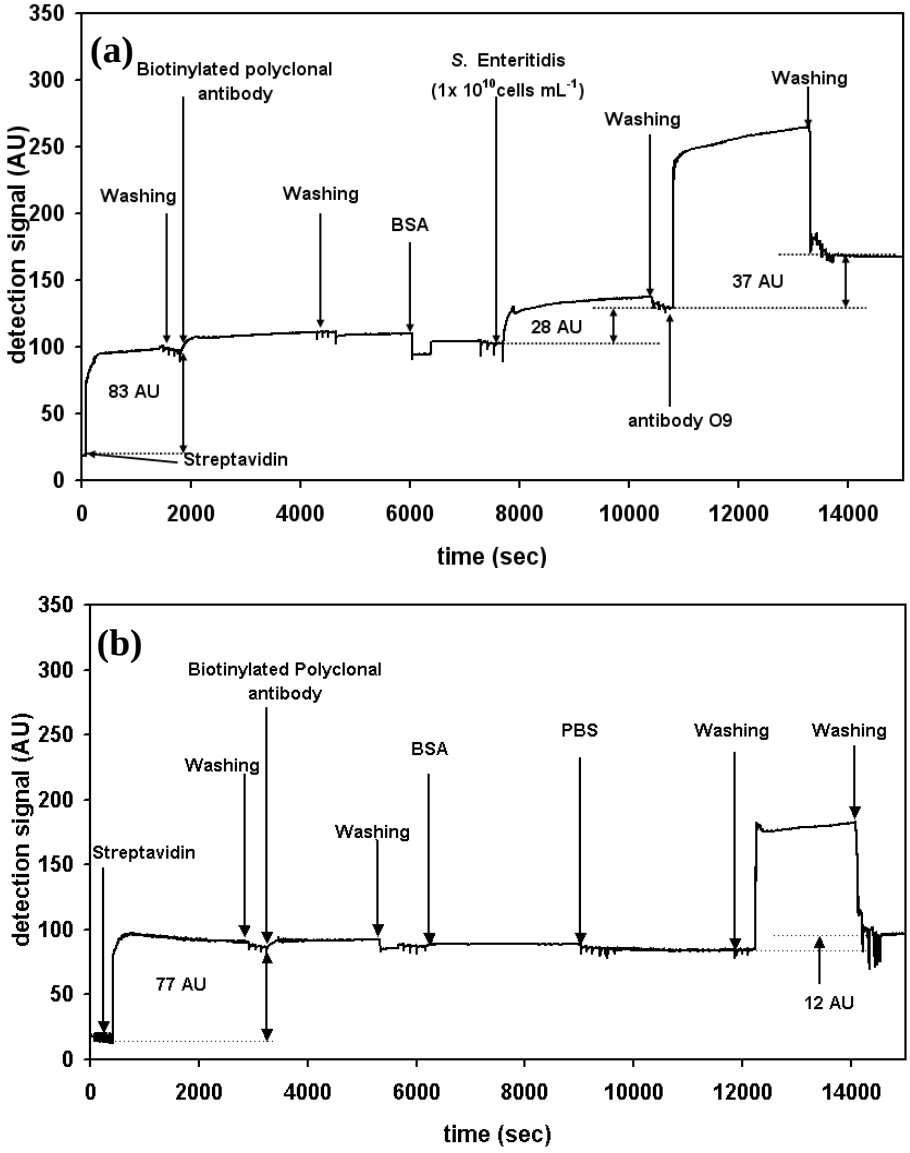

Fig. 4.4: Sensorgrams obtained for the immobilisation of the biotinylated capture antibody on a biotin chip (a) sensorgram showing addition of straptavidin on a botin coated chip followed by the addition of the biotinylated capture antibody, the bacteria and finally detection with O-specific antibody (b) sensorgram of the control run without bacteria.

This could be attributed to specific binding of the biotinylated antibody and lack of any other unspecific binding. This was followed by the blocking step with BSA. Addition of BSA also did not result in any increase in the detection signal indicating complete coverage of the gold surface by the complex formed between streptavidin and the biotinylated antibody. In the next step *S.* Enteritidis was added, resulting in a detection signal of 29 ± 4 AU. This detection signal obtained on addition of the bacteria is a further proof of the presence of the biotinylated antibody on the surface of the chip, inspite of the low binding signal observed on addition of the biotinylated antibody. In the final step of the assay anti-O9 specific antibody was added as the detection antibody. This resulted in a detection signal of 37 ± 3 AU. The detection signal in the control channel was 10 ± 3 AU. The sensorgrams obtained are presented in Fig. 4.4.

4.1.1.5 Immobilisation on a protein A surface

It is clear from the Table 1.1 (section 1.1.2.5) that protein A has a high affinity for antibodies from human, pig, rabbit and guinea pig. The polyclonal anti-*Salmonella* spp. antibody used in our experiments was raised in rabbits and hence a protein A chip is a suitable candidate for the oriented immobilisation of the antibody. Protein A (100 µg mL^{-1}) was immobilised onto a carboxyl functionalised SPR chip by coupling to the amine groups present on the protein. The first step involved the activation of the carboxyl groups on the surface of the chip using an aqueous solution of EDC/sulfo-NHS (0.4 M EDC and 0.1 M sulfo-NHS). This resulted in the formation of sulfo-NHS esters on the surface of the gold chip. Sulfo-NHS esters then react with the amine groups on protein A to form stable peptide bonds. The immobilisation step was carried out online in the SPR device. 10 µL of the protein A solution was added to each cuvette and allowed to incubate for 30 min. This was followed by a washing step with PBS. To this protein A surface the antibody was added. The final concentration of the antibody in the cuvette was 250 µg mL^{-1}. The surface was washed with PBS. 10 µL ethanolamine hydrochloride (1 M) was added as a blocking agent, in order to inactivate any unreacted sulfo-NHS esters. The polyclonal antibody immobilised on the protein A surface was successful in capturing the *S.* Enteritidis bacteria in the next step of the assay. Subsequent addition of the anti-O9 detection

antibody resulted in a positive detection signal (54 ± 3 AU), indicating the presence of the bacteria on the surface of the chip.

The control channel without the bacteria resulted in a detection signal of 22 ± 1 AU. Thus, an overall detection signal of 33 ± 3 AU for the assay proves a partial success of the strategy to immobilise the capture antibody through protein A. The sensorgrams obtained for the capture and detection of *S.* Enteritidis using the protein A mediated immobilisation of the capture antibody on the SPR chip is presented in Fig. 4.5.

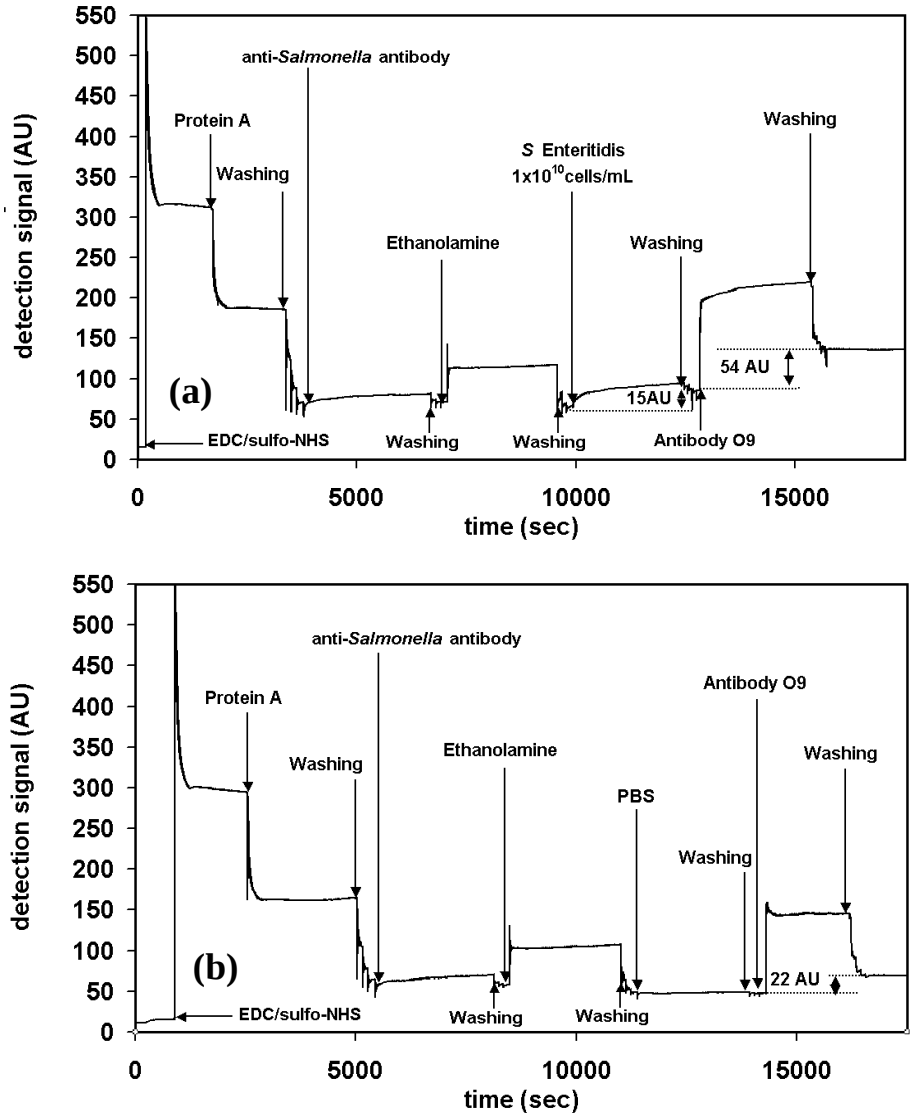

Fig. 4.5: Sensorgrams obtained for the immobilisation of the capture antibody on a protein A surface (a) sensorgram showing the capture antibody immobilisation step followed by capture of the bacteria and then detection with O-specific antibody (b) sensorgram of the control run without bacteria.

4.1.1.6 Immobilisation by chelation on a Co^{2+} surface

A cobalt-coated gold surface was created using a tri-carboxy-functionalised chip. The tri-carboxyl-functionalised chip is prepared according to the protocol described in section 3.2.4.5. The chip is mounted onto the SPR device. 10 µL aqueous solution (10 mg mL^{-1}) of cobaltous chloride hexahydrate ($CoCl_2.6H_2O$) was added online onto the carboxyl-coated chip, resulting in a concentration of 5 µg mL^{-1} in the cuvette. After incubation for 15 min the cuvette was washed with PBS. A good binding signal, which was stable on washing with PBS, was obtained.

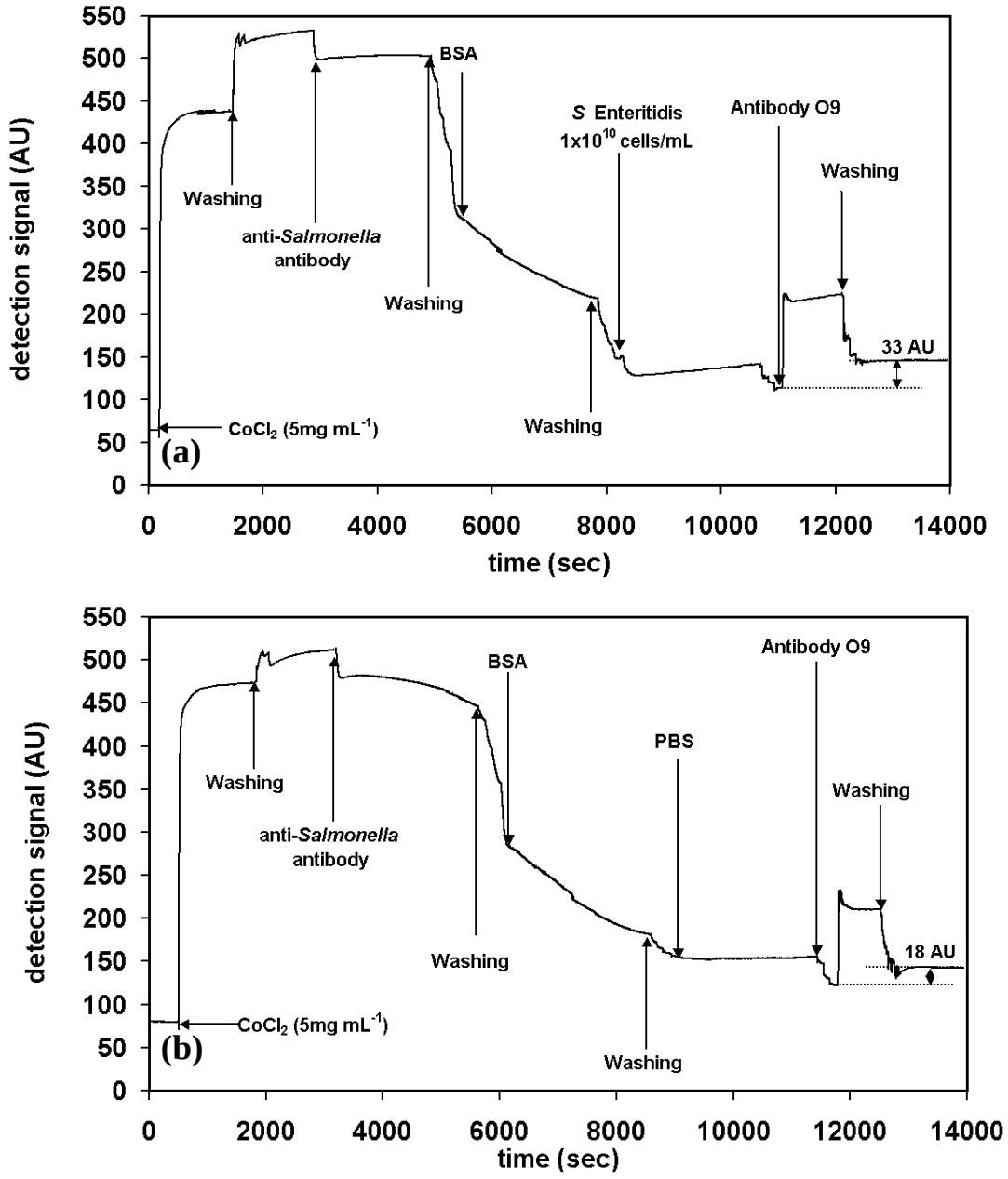

Fig. 4.6: Sensorgrams obtained for the immobilisation of the capture antibody on cobalt (Co^{2+}) surface (a) sensorgram showing the capture antibody immobilisation step followed by capture of the bacteria and then detection with O-specific antibody (b) sensorgram of the control run without bacteria.

The polyclonal anti-*Salmonella* antibody was then added to the surface to obtain a final concentration of 250 µg mL^{-1} and allowed to incubate for 15 min. After addition of the polyclonal antibody and the subsequent washing step it was observed that the cobalt (Co^{2+}) along with the antibody rapidly dissociated from the surface. This is explained as complexation of the polyclonal antibody with cobalt and subsequent dissociation of the complex from the surface of the chip on washing. As most of the polyclonal antibody was washed away as a complex with cobalt, the addition of *S.* Enteritidis did not result in any significant detection signal. The detection signal obtained on addition of the anti-O9 detection antibody was only 33 ± 2 AU (Fig. 4.6 a).

The corresponding detection signal in the control channel was 18 ± 1 AU (Fig. 4.6 b). Hence, this immobilisation strategy was not successful in providing an assay for detection of the bacteria.

4.1.1.7 Immobilisation by hydrophobic interaction on a C_{18} chip.

Hydrophobic interactions occur as a result of interactions between hydrocarbon rich regions of molecules, which pack closely together to minimise their exposure to water (Dill, 1990). This physical phenomenon was used to immobilise the polyclonal anti-*Salmonella* antibody to the surface of the SPR chip. The gold surface of the SPR chip was modified according to the protocol in section 3.2.4.4. After this modification step, the gold surface was rendered hydrophobic due to the presence of C_{18} hydrocarbon chains on its surface. Thus, the proposed hydrophobic interaction is likely to take place between the C_{18} hydrocarbon chain present on the surface of the chip and the hydrophobic regions of the antibody. This method of immobilisation of a biomolecule onto a solid substrate is also referred to as self-assembly. Polyclonal antibody was added onto the surface of a C_{18} chip, mounted on the SPR device, to obtain a final concentration of 250 µg mL^{-1} inside the cuvette. This was followed by the normal protocols of washing, blocking using BSA, capture of the bacteria (*S.* Enteritidis) and finally specific detection of the bacteria using O-specific antibody O9. A high detection signal was obtained on capture of the bacteria by the immobilised antibody (56AU). The success of the immobilisation strategy was further confirmed on addition of the anti-O9 detection antibody. The signal obtained on addition of the detection antibody (75 ±4 AU) was the highest among all the immobilisation strategies explored (Fig. 4.7 a). It was also observed that the control

signal (2 ±1 AU), due to unspecific binding of the anti-O9 antibody (Fig. 4.7 b), was the lowest among all the immobilisation strategies explored.

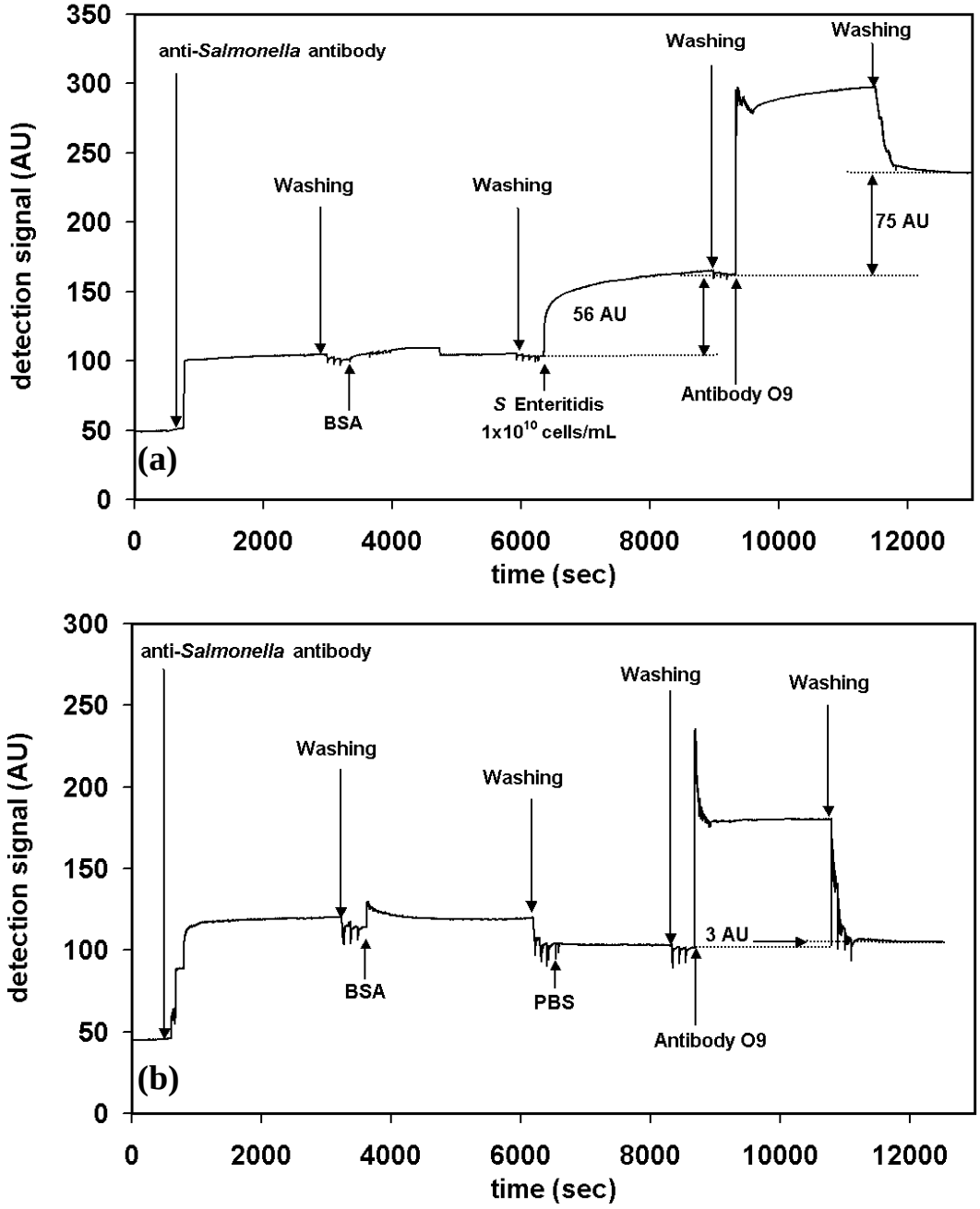

Fig. 4.7: Sensorgrams obtained for the immobilisation of the capture antibody on hydrophobic C_{18} surface (a) sensorgram showing the capture antibody immobilisation step followed by capture of the bacteria and then detection with O-specific antibody (b) sensorgram of the control run without bacteria.

4.1.1.7.1 Hydrophobicity of C_{18} chips and contact angle measurement

Water contact angle was measured at room temperature using a Krüss G10 contact angle measurement system and the data was analysed using the software SCA20 (section 3.2.4.8). The data obtained for water contact angle measured on a cleaned

uncoated gold surface of the SPR prism had and average value of $59^o \pm 4.7^o$. In comparision the value of the water contact angle measured on a C_{18}-functionalised gold surface of an SPR prism was $103^o \pm 2.4^o$. The spread and shape of the drops, as captured by the CCD camera of the device, on both the gold surfaces is shown in Fig. 4.8. The increase in the value of the contact angle confirms the change, in surface property of the gold surface from hydrophilic to hydrophobic on C_{18}-functionalisation (section 3.2.4.4) of the SPR chips. Measurements were taken on atleast three different locations on the surface of each chip. The theory and principle involved in determination of contact angle is presented in section 3.2.4.8.

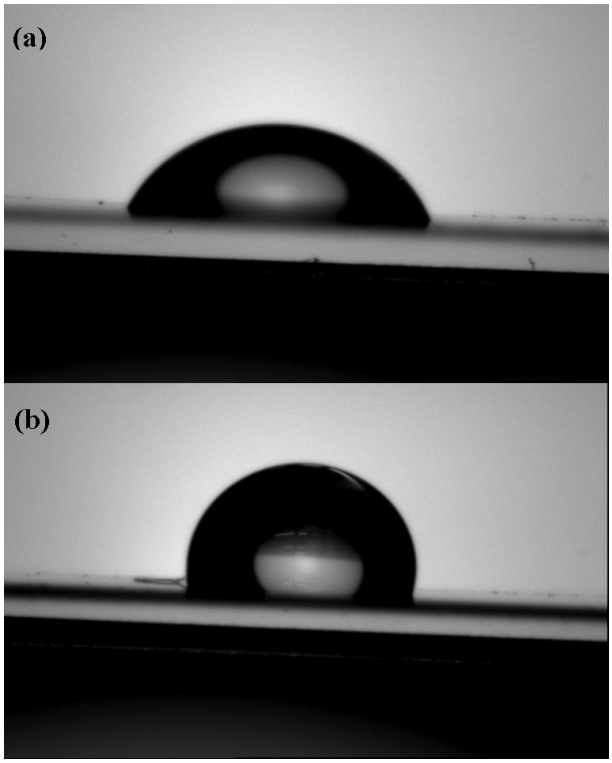

Fig. 4.8: Water contact angle meaurement to check the hydrophobicity of C_{18}-coated SPR chips (a) water drop seen on a cleaned gold surface of an SPR chip (b) water drop seen on the surface of a C_{18}-functionalised gold surface of an SPR chip. It is clearly seen that the water drop on the C_{18} surface has a higher value of the contact angle (hydrophobic) than on the cleaned uncoated gold surface (hydrophilic)

4.1.1.7.2 Effect of pH on the immobilisation of antibody on C_{18} surface

From the above studies it is clear that the most suitable strategy for immobilisation of the polyclonal antibody on the surface of the SPR chip is through hydrophobic interaction. Before carrying out further experiments it was important to determine if the buffer in which the antibody solution is prepared has any effect on the binding of

the antibody to the C_{18} surface. For this purpose the anti-*Salmonella* polyclonal antibody was prepared in PBS (0.15 M, pH 7.3), acetate buffer (0.1 M, pH 5.5), carbonate buffer (0.1 M, pH 8.6) and phosphate buffer (0.05 M, pH 6.0), respectively. On carrying out the immobilisation under each of the above pH conditions, there was no significant difference observed in the detection signals on binding of the antibody to the surface of the C_{18} chip. The average value of the detection signals across the different pH conditions, due to binding of the antibody to the surface of the C_{18} chip, was 71 ± 2 AU (Fig. 4.9). This level of standard deviation is considered insignificant in case of the Plasmonic® SPR device. The experiment was continued further and using the immobilised antibodies. *S.* Enteritidis was immobilised on the surface of the chip. This was followed by addition of the detection antibody specific for the O-surface antigen O9 of *S.* Enteritidis. The average values of the detection signals across the different pH conditions for the capture of the bacteria and for the final detection step with anti-O9 antibody were 40 ± 5 AU and 78 ± 4 AU, respectively. Based on the above results, it is concluded that there is no significant improvement in the binding and activity of the capture antibody on using a buffer other than PBS. Hence, all assays were carried out by preparing the capture antibody dilutions in PBS.

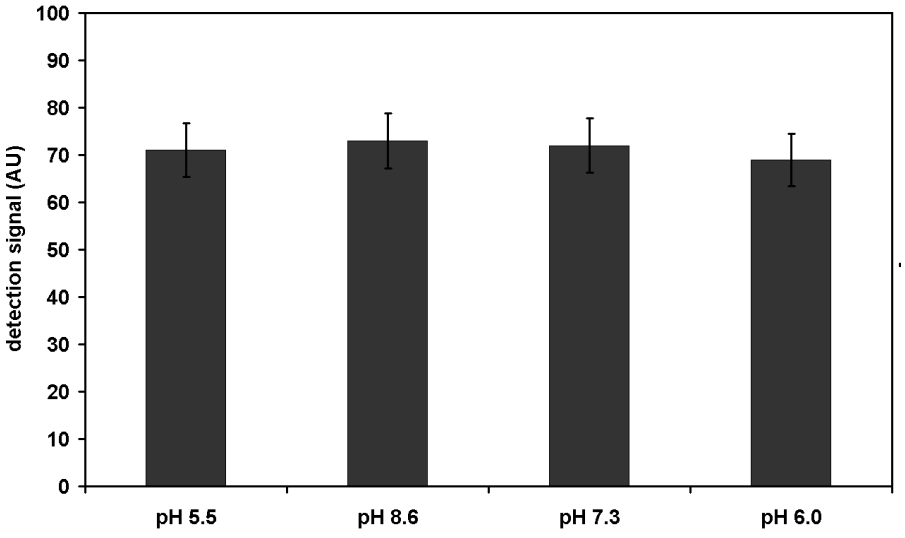

Fig. 4.9: Detection signals obtained for the detection of *S.* Enteritidis on a C_{18} SPR chip with the capture antibody immobilised under different pH conditions.

4.1.1.7.3 Effect of capture antibody concentration

The immobilisation of capture antibody on the surface of the C_{18} chip was evaluated at four different concentrations. The four different concentrations of the antibody were 10 µg mL^{-1}, 50 µg mL^{-1}, 100 µg mL^{-1} and 250 µg mL^{-1}. The corresponding

detection signals, on binding of the antibody to the C_{18} chip were 40 ± 2 AU, 59 ± 3 AU, 70 ± 3 AU and 83 ± 3 AU (Fig. 4.10). Thus, the highest surface coverage of the antibody on the C_{18} surface was obtained at a concentration of 250 µg mL^{-1}. This was the concentration of the polyclonal capture antibody used in all subsequent experiments.

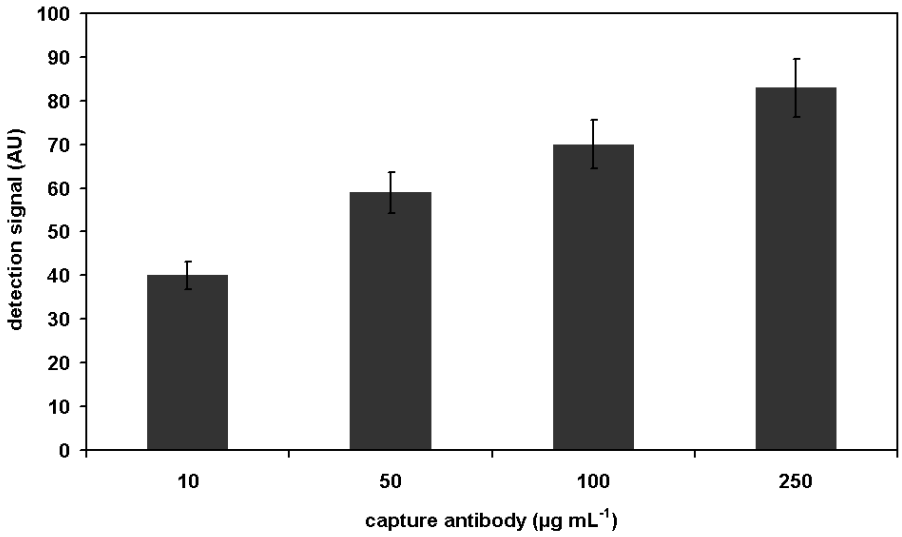

Fig. 4.10: Signals obtained on binding of different concentrations of the polyclonal anti-*Salmonella* antibody (capture antibody) on the surface of a C_{18} SPR chip.

4.1.2 SPR-based sandwich assay model for detection of *Salmonella*

4.1.2.1 Establishment of the assay in buffer

4.1.2.1.1 Direct detection

Initially, the assay was evaluated in PBS. Two different serovars of *Salmonella*, *S.* Typhimurium and *S.* Enteritidis were each evaluated separately. In the first step, addition of the polyclonal antibody onto the hydrophobic C_{18} SPR chip resulted in an average detection signal of 60 ± 1.25 AU. The subsequent washing step with PBS did not result in any change in the detection signal, indicating a stable binding of the polyclonal antibody to the SPR chip surface. Blocking of any free available gold surface with BSA after the immobilisation of the polyclonal antibody did not result

in any detection signal. This was a definite indication of uniform coverage of the C_{18} gold surface with the polyclonal capture antibody.

Bacteria, either *S.* Typhimurium or *S.* Enteritidis were then captured using the immobilised capture antibody. On capture of the bacteria a detectable change in the SPR signal was obtained only at and above a cell concentration of 5×10^5 cells mL^{-1} in case of S. Typhimurium (20 ± 3 AU) and 5×10^8 cells mL^{-1} in case of S. Enteritidis (22 ± 4 AU). The sensorgrams obtained on capture of bacteria by the immobilised polyclonal antibody are presented in Fig. 4.11.

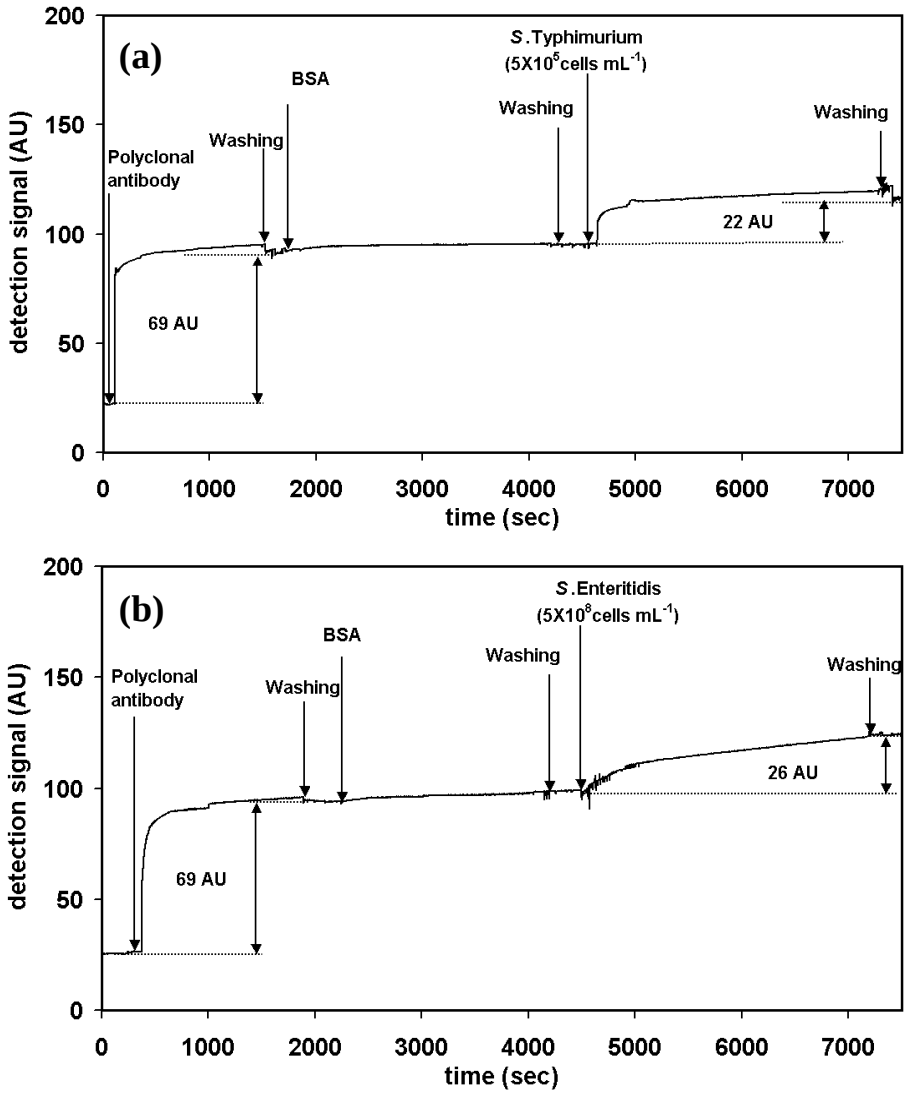

Fig. 4.11: Capture of *Salmonella* by immobilised anti-*Salmonella* polyclonal antibody on C_{18} SPR chip (a) sensorgram for the capture of S. Typhimurium (5×10^5 cells mL^{-1}) in buffer (b) sensorgram for the capture of S. Enteritidis (5×10^8 cells mL^{-1}) in buffer.

4.1.2.1.2 Sandwich Assay

As no significant detection signal was obtained using the direct mode of detection as discussed in section 4.1.2.1.1, the sandwich mode of detection was explored to amplify the signal. The aim was to obtain higher sensitivity for the assay and explore the possibility of specific detection of different *Salmonella* serovars. O-specific antibodies against the specific O-antigens of the respective *Salmonella* serovars were chosen as the detection antibodies. *S.* Typhimurium and *S.* Enteritidis captured, using the immobilised polyclonal antibody, were probed with O-specific antibodies anti-O4 and anti-O9, respectively.

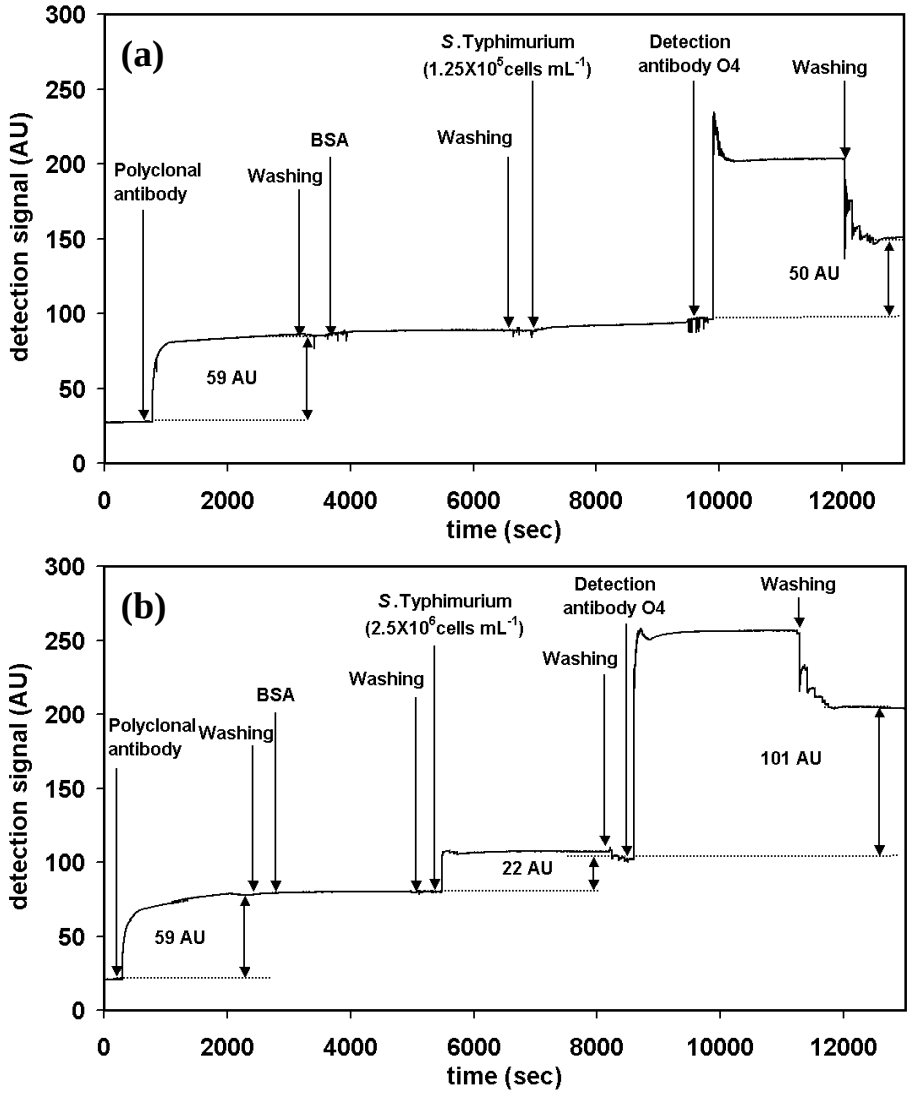

Fig. 4.12: Signal amplification using secondary detection antibody for detection of *Salmonella* by SPR (sandwich assay) in buffer (a) sensorgram for the detection of the lowest concentration of *S.* Typhimurium (1.25×10^5 cells mL^{-1}) using anti-O4 detection antibody (b) sensorgram for the detection of the highest concentration of *S.* Typhimurium (2.5×10^6 cells mL^{-1}) using anti-O4 detection antibody.

Different concentrations of both the *Salmonella* serovars were evaluated separately using the assay in PBS. *S.* Typhimurium was found to have a lower limit of detection (LLD, defined as the concentration of cells resulting in a detection signal, which is the average value of the detection signal obtained due to control plus three times the standard deviation) of 1.25×10^5 cells mL^{-1} (47 ± 3.9 AU) when probed with the anti-O4 detection antibody. The highest concentration of *S.* Typhimurium in PBS evaluated was 2.5×10^6 cells mL^{-1} resulting in a detection signal of 101 ± 8.3 AU. Sensorgrams obtained for the lowest and the highest concentrations of *S.* Typhimurium tested, using the sandwich assay, are presented in Fig. 4.12.

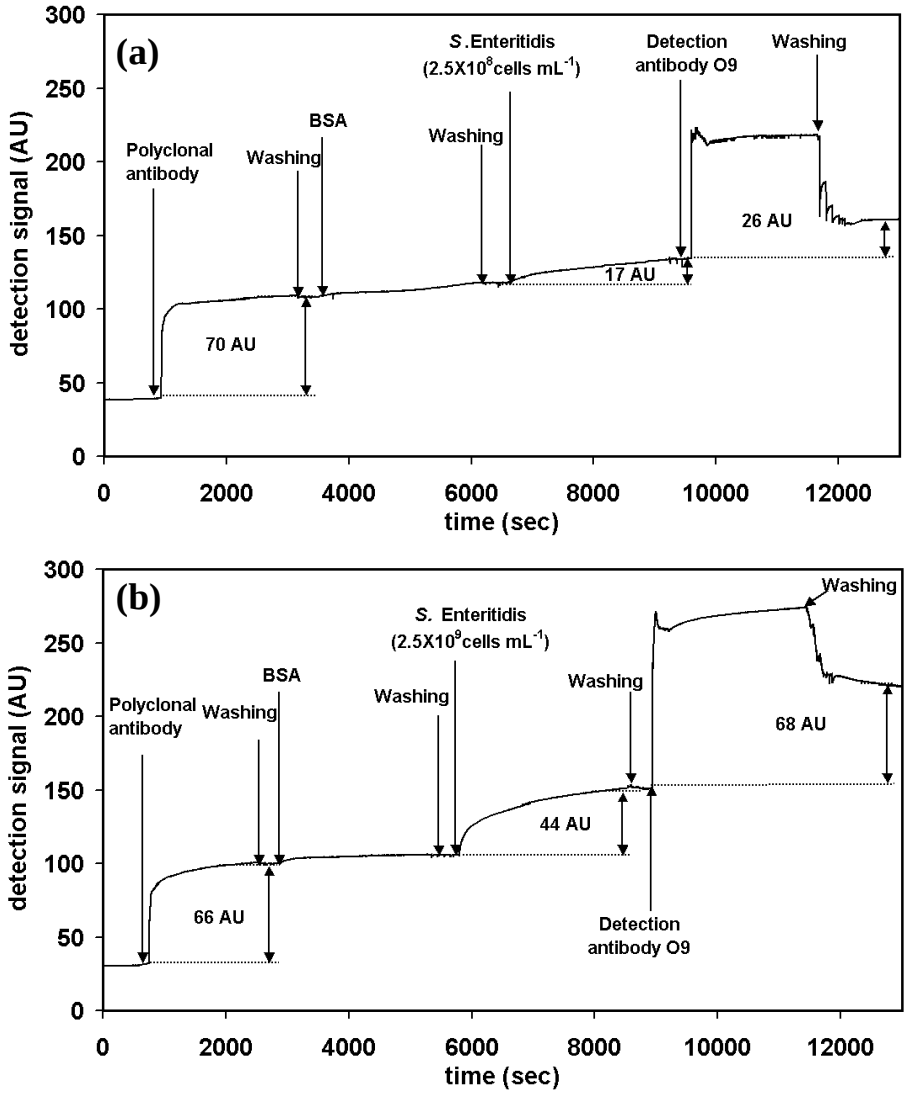

Fig. 4.13: Sandwich assay for the detection of *Salmonella* using SPR in buffer (a) sensorgram for detection of the lowest concentration of *S.* Enteritidis (2.5×10^8 cells mL^{-1}) using anti-O9 detection antibody (b) sensorgram for the detection of the highest concentration of *S.* Enteritidis (2.5×10^9 cells mL^{-1}) using anti-O9 detection antibody.

In case of *S.* Enteritidis the LLD of the assay using the anti-O9 detection antibody was much higher compared to that of the *S.* Typhimurium. In this case the LLD was 2.5×10^8 cells mL ^{1} corresponding to a detection signal of 29 ± 4.3 AU (Fig. 4.13 a). The signal obtained from the highest concentration (2.5×10^9 cells mL^{-1}) of *S.* Enteritidis evaluated was 68 ± 5.4 AU (Fig. 4.13 b).

The value of the control signals for detection of both serovars were 3 ± 2 AU. A control sensorgram is presented in Fig. 4.7 b (section 4.1.1.7). The detection ranges of the SPR-based sandwich assays in buffer for detection of *S.* Typhimurium using the anti-O4 detection antibody, and for detection of *S.* Enteritidis using the anti-O9 detection antibody are presented in Fig. 4.14 a. and Fig. 4.14 b, respectively.

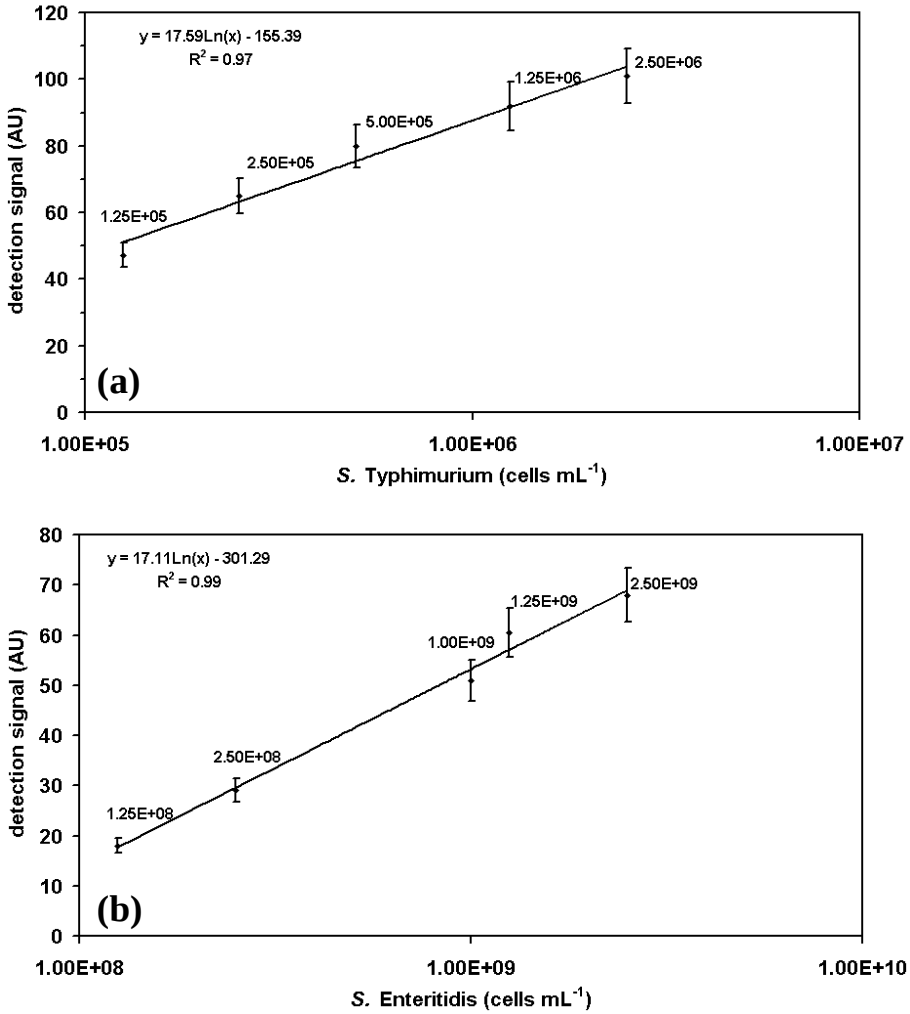

Fig. 4.14: Detection range of the SPR-based sandwich assay for specific detection *Salmonella* (a) detection of *S.* Typhimurium in buffer using O-specific anti-O4 antibody (b) detection of *S.* Typhimurium in buffer using O-specific anti-O9 antibody. Note that the plots are semi-logarithmic with cell concentrations increasing exponentially on the X-axis.

4.1.3 Establishment of the sandwich assay in milk

4.1.3.1 Sandwich assay with polyclonal capture and detection

4.1.3.1.1 Effect of milk matrix on the detection limit

The first step in establishing the assay for detection of *Salmonella* in milk using SPR, was to establish the effect of the milk matrix on the detection limit of the assay with respect to the detection limit in buffer system. For this experiment the sandwich assay, as described in section 4.1.3.1.1, was adopted with the anti-*Salmonella* polyclonal antibody as both the capture and the detection antibody. Different concentrations of *S.* Typhimurium spiked in buffer and milk were used for establishing the detection range and limits of detection. In this case it was essential to first have data for the assay in buffer system for comparison. Therefore, the sandwich assay with polyclonal capture and detection was first carried out in buffer.

Detection range in buffer for polyclonal capture and detection

In the buffer system cell concentrations of 1.25×10^5 cells mL^{-1} and above resulted in significant change in AU and saturation of signal was obtained at 5.0×10^6 cells mL^{-1} (Fig. 4.15 a). The signal due to control had an average value of 3 AU. The lower limit of detection (LLD) was 1.25×10^5 cells mL^{-1} (9 AU). The relative standard deviation of the assay was 3.8%. The linear detection range was between 1.25×10^5 and 2.5×10^6 cells mL^{-1} (Fig. 4.15 b).

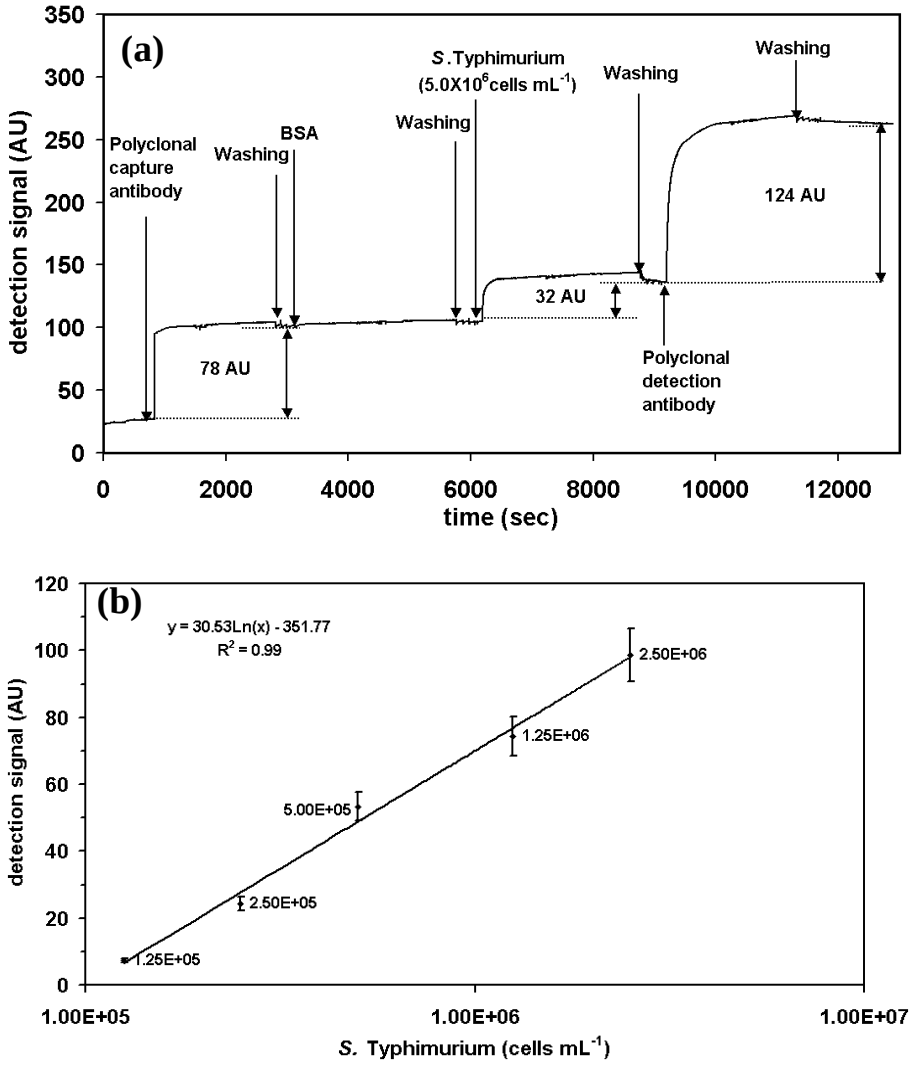

Fig. 4.15: SPR-based sandwich assay with polyclonal capture and detection of *Salmonella* (a) sensorgrams for detection of *S.* Typhimurium (5.0×10^6 cells mL^{-1}) in buffer (b) linear detection range of the assay in buffer. Note that the plot is semi-logarithmic with cell concentrations increasing exponentially on the X-axis.

Detection range in milk for polyclonal capture and detection

Milk spiked with different concentrations of *S.* Typhimurium was used to establish the assay. Uncontaminated milk was used as control. After addition of both milk and milk spiked with *S.* Typhimurium, in different channels, on to the SPR chip, coated with polyclonal capture antibody, significant change in AU was observed. This change is attributed to the overall change in refractive index on the surface of the SPR chip. The refractive index change is caused due to unspecific binding of the milk fat and proteins to the surface, in addition to the binding of *Salmonella* cells to the capture antibody, when present in the sample. This signal, however, was reduced after the subsequent washing step with PBS.

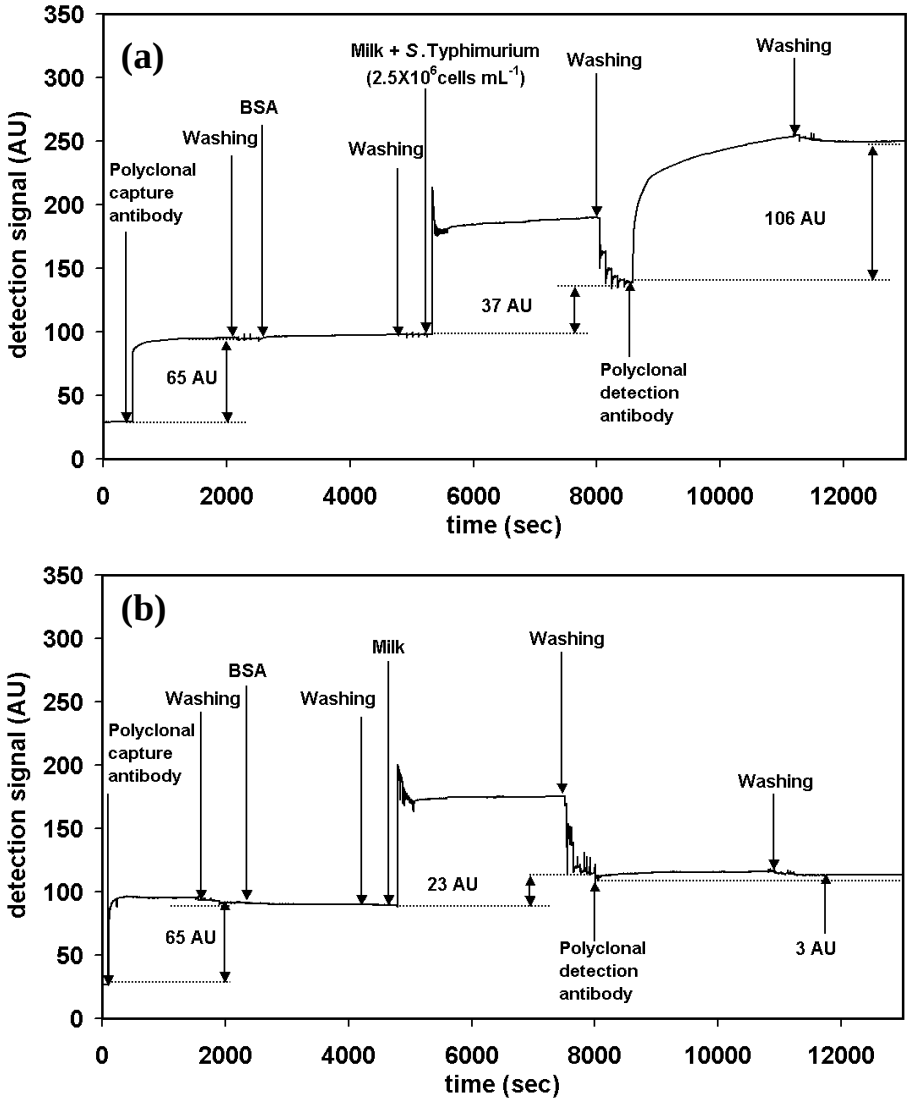

Fig. 4.16: SPR-based sandwich assay with polyclonal capture and detection of *Salmonella* in milk (a) sensorgrams for detection of *S.* Typhimurium (2.5×10^6 cells mL^{-1}) spiked in milk (b) control sensorgram with uncontaminated milk.

On application of the polyclonal detection antibody clear difference in SPR binding signal was observed between contaminated samples (Fig. 4.16 a) and control (Fig. 4.16 b).

The LLD of the assay for the detection *S.* Typhimurium in contaminated milk was also found to be 1.25×10^5 cells mL^{-1} (15 AU). The detection signal in case of uncontaminated milk had an average value of 3 AU. The range of detection extends to 5.0×10^6 cells mL^{-1} (Fig. 4.17). The linear detection range was between 1.25×10^5 and 2.5×10^6 cells mL^{-1}.

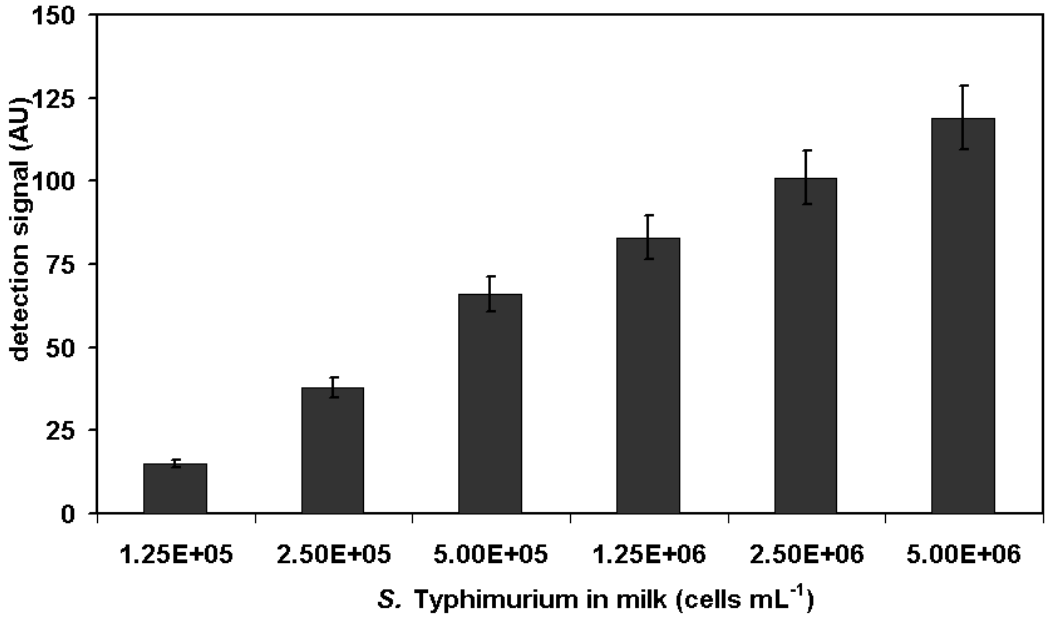

Fig. 4.17: Detection signals with the polyclonal detection antibody of the SPR-based sandwich assay for detection of *S.* Typhimurium in milk samples, spiked with different concentrations of the pathogen.

Comparing the results obtained in buffer system and in milk, it is clear that the presence of milk fat and proteins did not affect the sensitivity of the assay.

4.1.3.2 Specific detection of *Salmonella* serovars in milk

The next step in the development of the SPR-based biosensor for the detection of *Salmonella* was to evaluate the response of O-specific detection antibodies in *Salmonella* contaminated milk samples. The detection signal due to capture of *S.* Typhimurium (5×10^5 cells mL^{-1}) in milk using the polyclonal capture antibody was 43 ± 4.5 AU (Fig. 4.18 a). The corresponding detection signal due to the captured *S.* Enteritidis (3×10^9 cells mL^{-1}) from spiked milk using the polyclonal capture antibody was 75 ± 5.0 AU (Fig. 4.19 a). The captured *S.* Typhimurium or *S.* Enteritidis was then probed with the respective O-specific detection antibodies. The final detection signal obtained for the highest concentration of *S.* Typhimurium (5×10^5 cells mL^{-1}) probed using the O4 detection antibody was 56 ± 3.6 AU (Fig. 4.18 a).

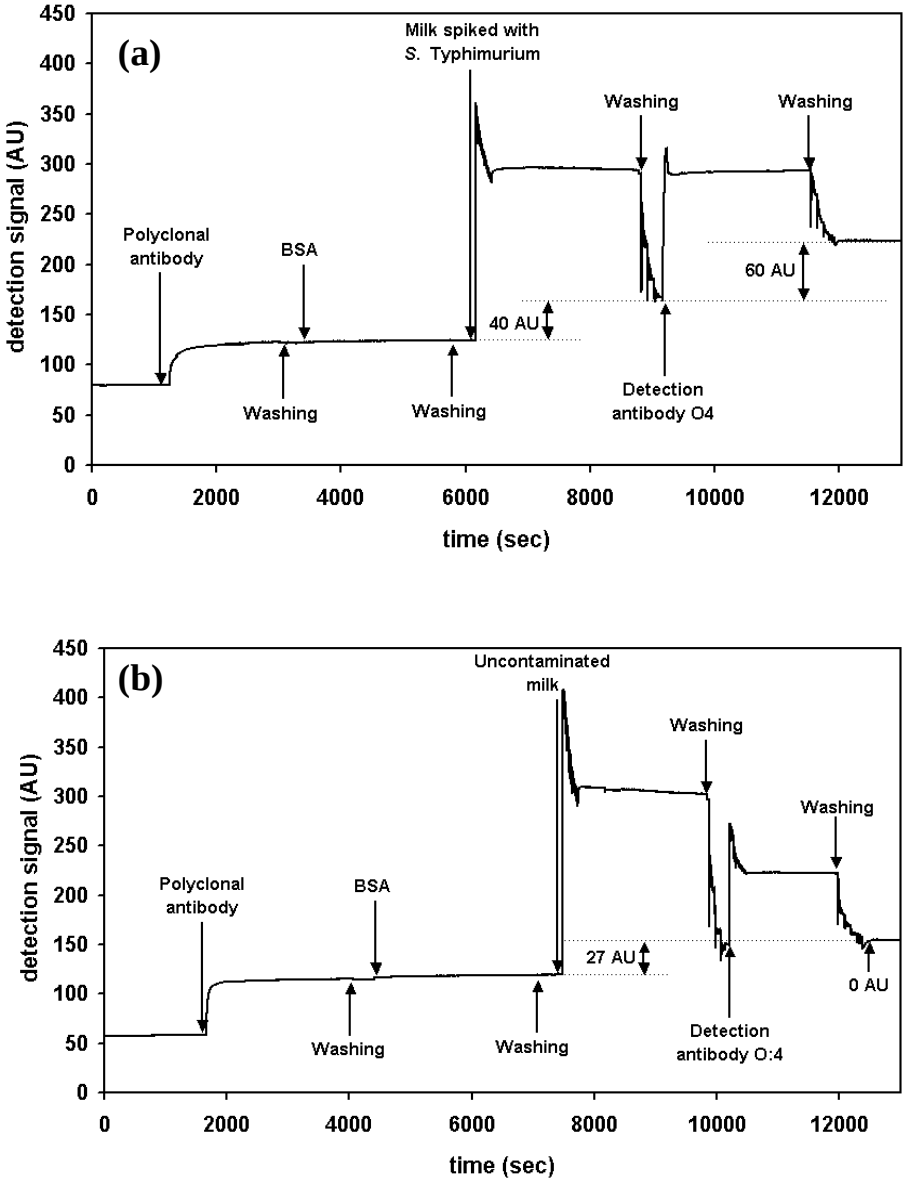

Fig. 4.18: Sensorgrams showing (a) specific detection of *S.* Typhimurium (5×10^5 cells mL^{-1}) in spiked milk using O-specific anti-O4 detection antibody, after capture from milk using the immobilised polyclonal anti-*Salmonella* antibody (b) SPR response to probing of uncontaminated milk (control) with the *S.* Typhimurium specific anti-O4 detection antibody.

The LLD of *S.* Typhimurium in milk using O4 detection antibody was 2.5×10^5 cells mL^{-1}. In both cases the signal due to control (uncontaminated milk) was 0 AU (Figs. 4.18 b and 4.19 b). In case of *S.* Enteritidis the highest concentration probed using the O9 detection antibody was 3×10^9 cells mL^{-1} resulting in a detection signal of 68 ± 5.4 AU (Fig. 4.19 a).

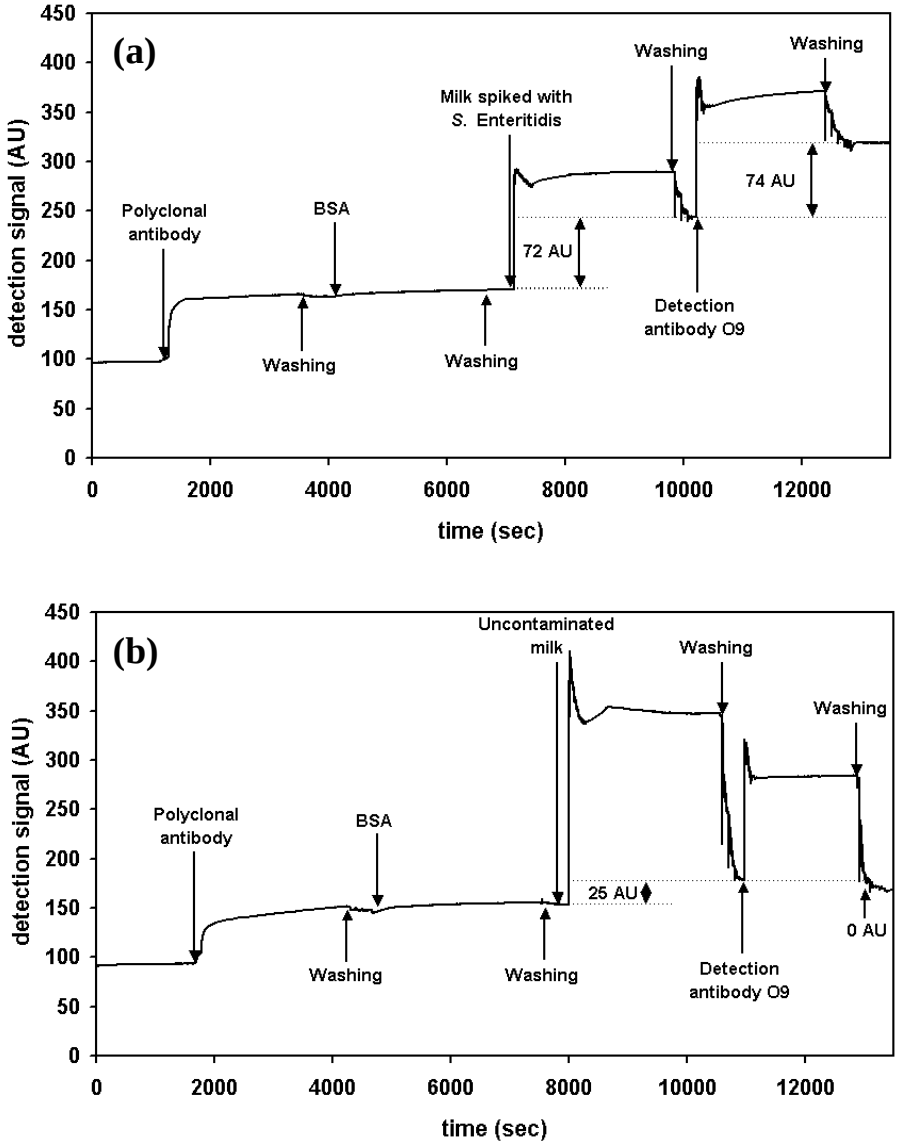

Fig. 4.19: Sensorgrams showing (a) specific detection of *S.* Enteritidis (3×10^9 cells mL^{-1}) in spiked milk using O-specific anti-O9 detection antibody, after capture from milk using the immobilised polyclonal anti-*Salmonella* antibody (b) SPR response to probing of uncontaminated milk (control) with the *S.* Enteritidis specific anti-O9 detection antibody.

The LLD of *S.* Enteritidis in milk, using O9 detection antibody, was 2.5×10^8 cells mL^{-1}. It is important to note here, that even though the LLD in case of *S.* Enteritidis is higher as compared to that of *S.* Typhimurium, the LLD of each of the serovars are similar in buffer (section 4.1.2.1.2) and in milk. Thus, there is no compromise on the overall detection capability of the assay due to the milk matrix, even for specific detection of *Salmonella* serovars.

4.1.3.3 Cross reactivity studies

Cross-reactivity of the O4 detection antibody to *S.* Enteritidis (Fig. 4.20 a) and O9 detection antibody to *S.* Typhimurium was also evaluated (Fig. 4.20 b). As expected, the antibodies were found to be specific for the respective serovars.

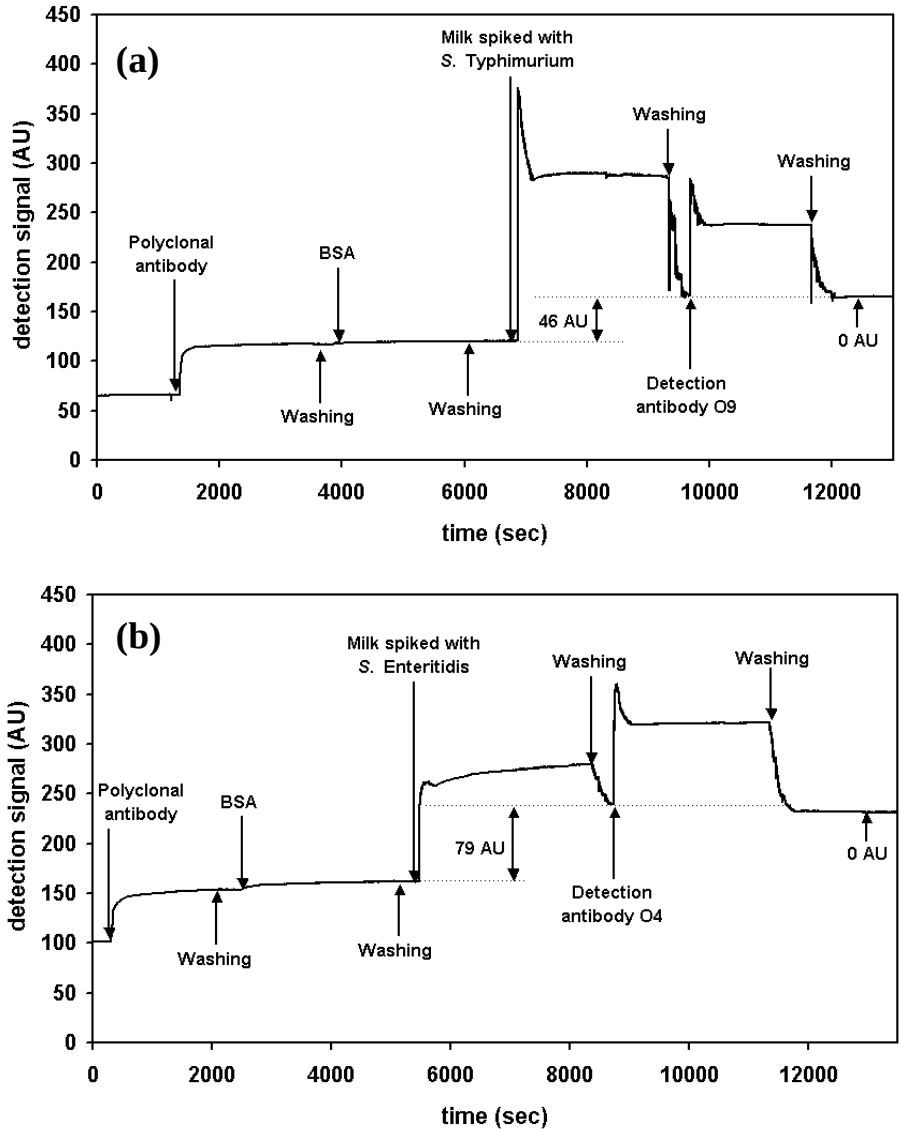

Fig. 4.20: Cross-reactivity check (a) using spiked milk containing *S.* Typhimurium (5×10^5 cells mL^{-1}) to O-specific anti-O9 detection antibody, which is specific for *S.* Enteritidis (b) using spiked milk containing S. Enteritidis (3×10^9 cells mL^{-1}) to O-specific anti-O4 detection antibody, which is specific for *S.* Typhimurium.

Furthermore, no cross-reactivity was observed when milk spiked with *E. coli* K12 (1.0×10^9 cells mL^{-1}) was probed using the O-specific antibodies (Fig. 4.21). In both cases some unspecific binding of the bacteria to the chip surface was observed but there was no response to the secondary O-specific detection antibodies O4 or O9.

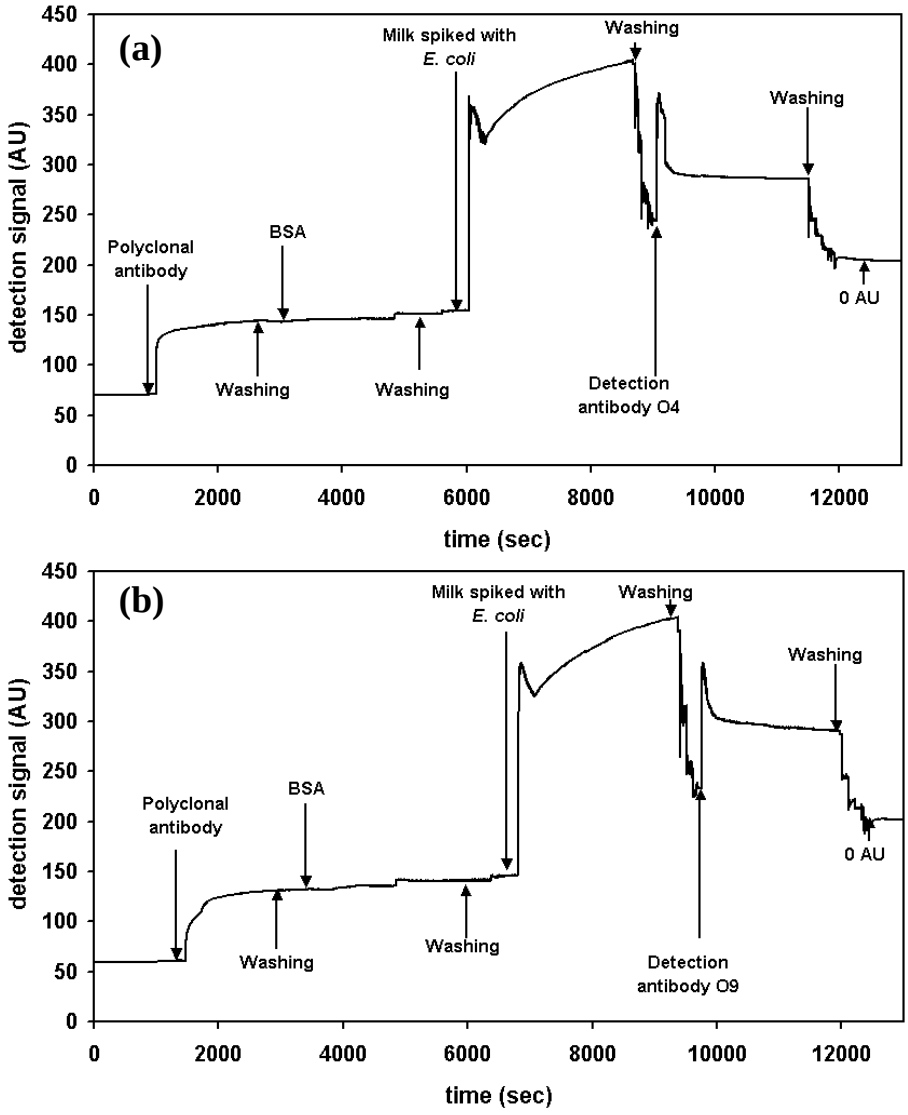

Fig. 4.21: Sensorgrams showing (a) cross-reactivity check using milk spiked with *E. coli* K12 (1.0×10^9 cells mL^{-1}) to O-specific anti-O4 detection antibody, which is specific for *S.* Typhimurium (b) cross-reactivity check using milk spiked with *E. coli* K12 (1.0×10^9 cells mL^{-1}) to O-specific anti-O9 detection antibody, which is specific for *S.* Enteritidis.

The specificity of the sandwich polyclonal capture and detection assay was also checked with respect to *E. coli* (1.0×10^8 cells mL^{-1}), which was captured by the immobilised polyclonal antibody on a C_{18} gold chip. This was followed by detection with the same polyclonal antibody. The SPR sensorgrams showed a binding curve, the slope of which is typical of cell sedimentation (Fig. 4.22). The signal obtained (8 AU) with the polyclonal detection antibody was insignificant in comparison to that obtained for captured *S.* Typhimurium cells, having a concentration of only 5 X 10^6 cells mL^{-1} (Fig. 4.15 a, section 4.1.3.1). Moreover, from the binding kinetics

it is possible to clearly distinguish between the two bacteria. This clearly proves the specificity of the assay for *Salmonella*.

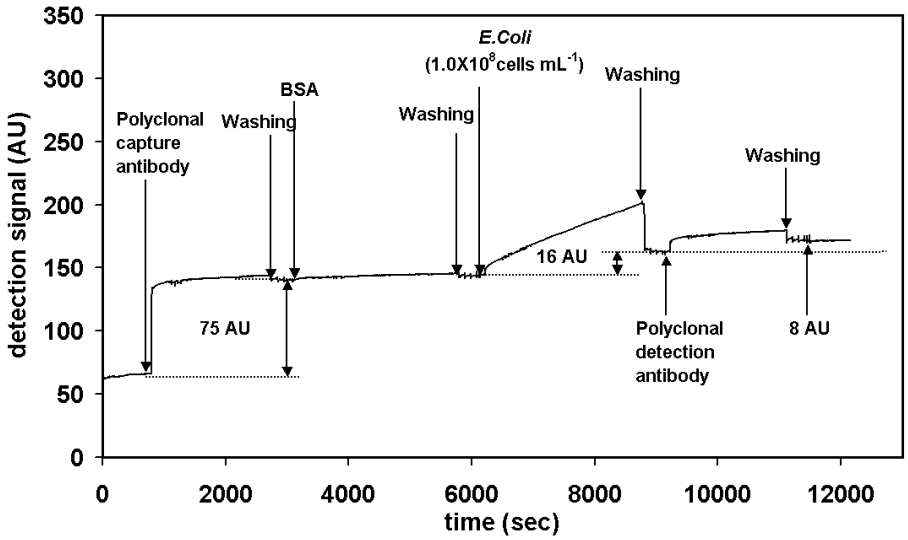

Fig. 4.22: Sensorgram obtained with *E. coli* K12 (1.0×10^8 cells mL^{-1}) in buffer showing the interaction of the cells with the anti-*Salmonella* polyclonal capture antibody, binding kinetics is typical of cell sedimentation, the cells are washed away in the subsequent washing step and no significant detection signal is obtained on addition of the anti-*Salmonella* polyclonal detection antibody.

4.1.4 Detection of different *Salmonella* serovars present in a mixture

4.1.4.1 Individual detection of serovars in a mixture using monoclonal O-specific antibodies

The next step of the assay development was to explore the possibility to detect both the *Salmonella* serovars when present together in a mixture. Initial experiments were designed and carried out to understand the mode of interaction of the *Salmonella* serovars and the antibodies with each other. Milk was spiked with a mixture of *S.* Typhimurium and *S.* Enteritidis having the same final concentration of each bacterium as when tested singly (section 4.1.3.2). In other words, the highest concentration of bacteria in the tested milk samples were 5×10^5 cells mL^{-1} and 3×10^9 cells mL^{-1} of *S.* Typhimurium and *S* Enteritidis, respectively. The control milk samples were prepared by making the necessary volume corrections with respect to the samples spiked with bacteria. The serovar mixture in milk was initially probed with either O4 detection antibody or O9 detection antibody (both diluted 1:2

in PBS) and this resulted in an average detection signal of 45 ± 4 AU. When the same mixture of serovars in milk was probed with a mixture of the undiluted O4 and O9 antibodies (1:1, v/v) the resulting detection signal was 98 ± 7 AU. This detection signal obtained was nearly an addition of the individual detection signals of the respective antibodies (Fig. 4.23). These results clearly indicate that the serovars, when present in a mixture, do not interfere with their interactions with the respective O-specific detection antibodies. The results support the possibility of developing assays for the simultaneous detection of *Salmonella* serovars. Based on the above results, further experiments were carried out to distinguish between the two *Salmonella* serovars when present together in milk. After simultaneous capture of the two serovars from the spiked milk samples by the polyclonal capture antibody, detection was carried out using the undiluted O-specific antibodies. This step required the use of different channels (multi-channel detection) for the O4 and the O9 detection antibodies, respectively. In this case, the results obtained for probing the mixture of serovars with the undiluted detection antibodies were similar to that obtained for the detection of each individual serovar spiked in milk (section 4.1.3.2).

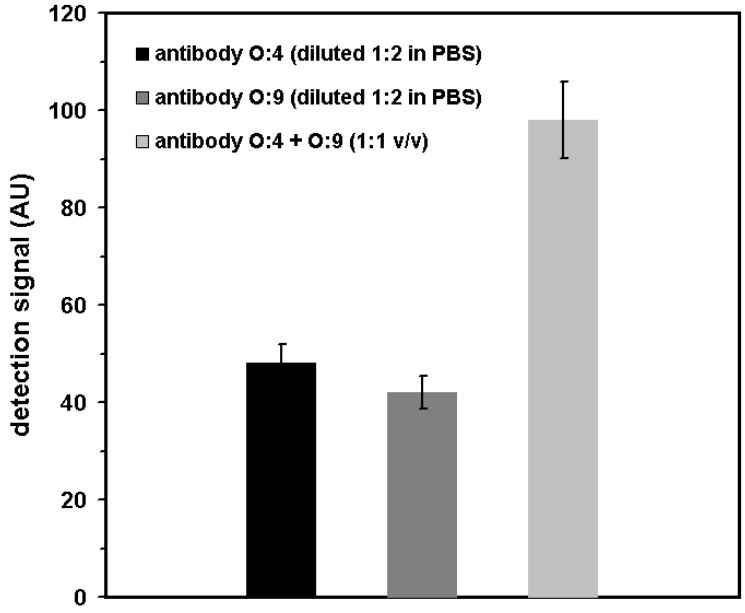

Fig. 4.23: Detection signals of the SPR assay for specific detection of *Salmonella* serovars in milk spiked with a mixture of ***S.*** Typhimurium and ***S.*** Enteritidis, using O-specific detection antibodies. The signal due to control (uncontaminated milk) was 0 AU.

4.1.4.2 Sequential detection of serovars in a mixture

After establishing that neither the serovars nor the detection antibodies interfere with the SPR detection process when present together in a mixture, further experiments were carried out to evaluate the possibility of detecting both the serovars. The possibility of using a single SPR channel in a sequential manner was explored. The sequential mode of detection is explained in section. 3.2.6.1. In the sequential detection mode, mixture of both the *Salmonella* serovars (5×10^5 cells mL^{-1} of *S.* Typhimurium and 3×10^9 cells mL^{-1} of *S.* Enteritidis) present in milk were captured on the C_{18} sensor chip using the immobilised polyclonal capture antibody. The captured bacteria were then probed with either the O4 or O9 detection antibody, followed by O9 or O4 detection antibody. The first detection signals were comparable to that obtained in the multi-channel detection mode (section 4.1.3.2). The average value of the detection signal for *S.* Typhimurium in the mixture, when probed with O4 detection antibody first was 66 ± 3.2 AU (Fig. 4.24 a). The corresponding detection signal for *S.* Enteritidis in the mixture, when O9 detection antibody was the first antibody, was 60 ± 6.7 AU (Fig. 4.24 b). The detection signal for *S.* Typhimurium, when detected in the second place (O4 detection antibody) was 40 ± 7.8 AU (Fig. 4.24 b), and the corresponding detection signal for *S.* Enteritidis detected in the second place (O9 detection antibody) was 28 ± 5.7 AU (Fig. 4.24 a). The data clearly indicate a reduction in detection signal when either of the detection antibodies is added in the second place. In comparison, the detection signal obtained when either of the antibodies is added in the first place is always higher in the sequential detection mode. This reduction in detection signal on addition of the second detection antibody is understood in the context of the exponentially decaying nature of the evanescent wave (section 1.1.2.1). The details on the factors responsible for the reduction in SPR detection signal of the second detection antibody in the sequential detection mode is further discussed in sections 5.2 and 5.3.

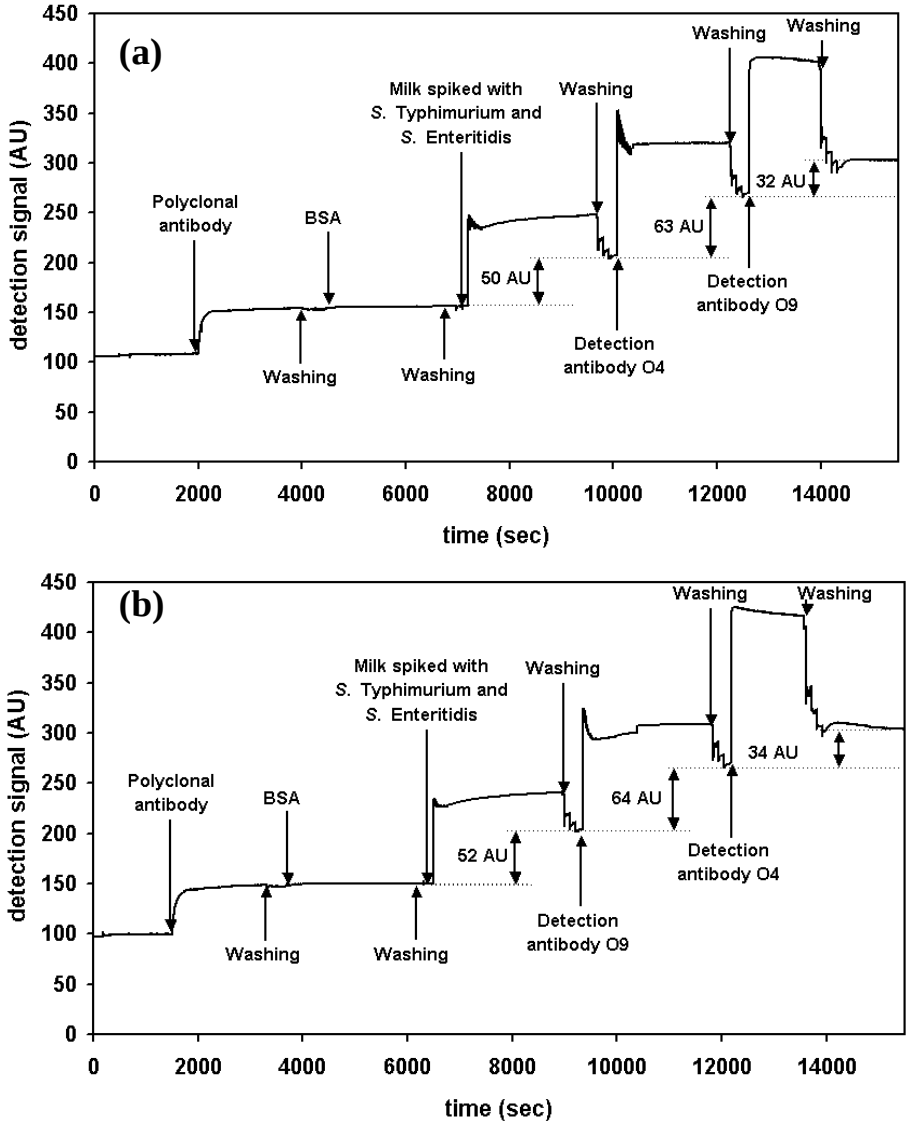

Fig. 4.24: SPR sensorgrams showing the sequential detection of *Salmonella* serovars captured from milk spiked with a mixture of *S.* Typhimurium and *S.* Enteritidis (a) detection of *S.* Typhimurium (anti-O4 detection antibody) followed by the detection of *S.* Enteritidis (anti-O9 detection antibody) (b) detection of *S.* Enteritidis (anti-O9 detection antibody) followed by the detection of *S.* Typhimurium (anti-O4 detection antibody).

4.1.4.3 Sequential detection of mixture of Lipopolysaccharides

Studies were further carried out to elucidate and understand the phenomenon of sequential detection using SPR with respect to the detection of gram-negative bacteria. In order to focus only on the bacterial component responsible for the SPR signal in the assay, purified LPS of both the *Salmonella* serovars were further tested using the SPR assay. Milk was spiked with equal concentrations of both the LPS

(40 μg mL^{-1} each). The spiked milk sample was then probed in the sequential detection mode; anti-O4 antibody followed by anti-O9 antibody and also anti-O9 antibody followed by anti-O4 antibody. In both cases, controls were run using uncontaminated milk with the relevant dilution corrections. The data clearly show that the O9/O4 detection mode was able to detect both the LPSs, which were spiked in the milk sample (Fig. 4.25 b). The SPR detection signals were 47 ± 2.1 AU for O9 as the first and 63 ± 6.1 AU for O4 as the second detection antibody. In case of the O4/O9 mode of detection the signals obtained were 112 ± 8.9 AU and 8 ± 1 AU, respectively (Fig. 4.25 a).

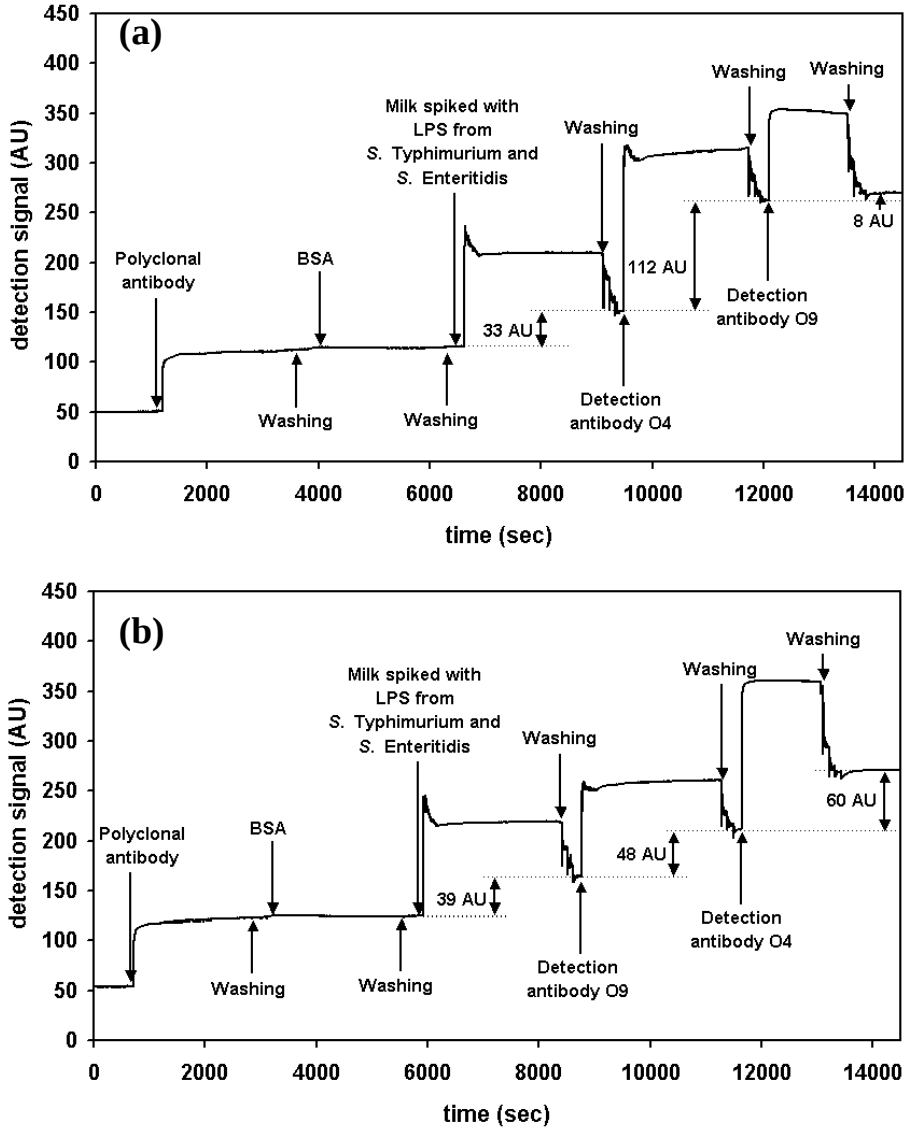

Fig. 4.25: SPR sensorgrams showing the sequential detection of a mixture of *Salmonella* LPS spiked in milk (a) detection of *S.* Typhimurium LPS (anti-O4 detection antibody) followed by the detection of *S.* Enteritidis LPS (anti-O9 detection antibody) (b) detection of *S.* Enteritidis LPS (anti-O9 detection antibody) followed by the detection of *S.* Typhimurium LPS (anti-O4 detection antibody).

Thus, in the O4/O9 detection mode only the *S.* Typhimurium LPS was detected in the milk sample spiked with both the LPSs. It is evident from the data that the binding of the anti-O9 antibody to the LPS from *S.* Enteritidis results in a lower detection signal in comparison to that obtained on binding of the anti-O4 antibody to the *S.* Typhimurium LPS, even though the concentration of both the LPS are the same. This finding agrees well with the observations already made for both these antibodies using whole bacterial cells of both the serovars (section 4.1.4.2). It is thus clear that the signal due to *S.* Enteritidis LPS (anti-O9 antibody) would be lost if detected in the second place of the sequential detection mode.

4.1.4.4 Rule for sequential detection

Based on the studies, using whole cells and purified LPSs of the respective serovars, it can be concluded that sequential detection of serovars using SPR can be easily achieved. However, as in case of all assays, a few optimisation steps in terms of determining the order of detection of the serovars in the sequential detection mode need to be carried out. This would involve initial screening of the individual serovars using SPR in the multi-channel mode to determine their SPR detection signals and detection limits. Using this data, single-channel sequential detection can then be designed. The serovar with the lower detection signal in the individual detection mode has to be detected first, followed by the other serovar of interest. Such an assay would further increase the capability of SPR assays to quickly screen a number of serovars and bacterial strains, reducing time and costs of the assay.

4.1.5 Serotyping of *Salmonella*

As explained (section 1.2.1.3), serotyping of *Salmonella* consists of first assigning the serogroup based on the detection of the cell surface somatic O-antigens. The serotype is then assigned after identification of presence and the type of the flagellar H-antigens.

4.1.5.1 Serogrouping of *Salmonella*

The genus *Salmonella* is divided into different serogroups according to the Kauffmann-White scheme. The assignment of serogroups is based on the presence of shared common O-antigens. Three different strains of *Salmonella* belonging to serogroups B, C and D were selected to evaluate the possibility of serogrouping using SPR. The sequential mode of detection was used for the serogrouping. In this mode, the antibodies, which did not belong to the serogroup of the bacteria to be evaluated, were added as the first detection antibodies. This approach enabled simultaneous evaluation of the serogroup as well as the cross-reactivity of the bacteria to the other serogroup antibodies using one single channel of the SPR device. A control channel without bacteria was run in each case. The sequential detection mode has the advantage of saving time as well as antibodies and reagents compared to multi-channel detection.

4.1.5.1.1 *S.* Heidelberg

S. Heidelberg (CCUG 21289, antigenic formula: 4,5,12:r:1,2), which belongs to serogroup B, was probed using the SPR-based sandwich immunoassay. Sequential detection was carried out with either group D or group C detection antibody as the first detection antibody. This was followed by addition of the group B specific antibody. Analysing the obtained results, it was possible to assign the strain of *S.* Heidelberg to the serogroup B while no cross-reactivity to group B and group C antibodies was obtained. Average net signal due to group B antibody was 40 ± 9 AU (Fig. 4.26).

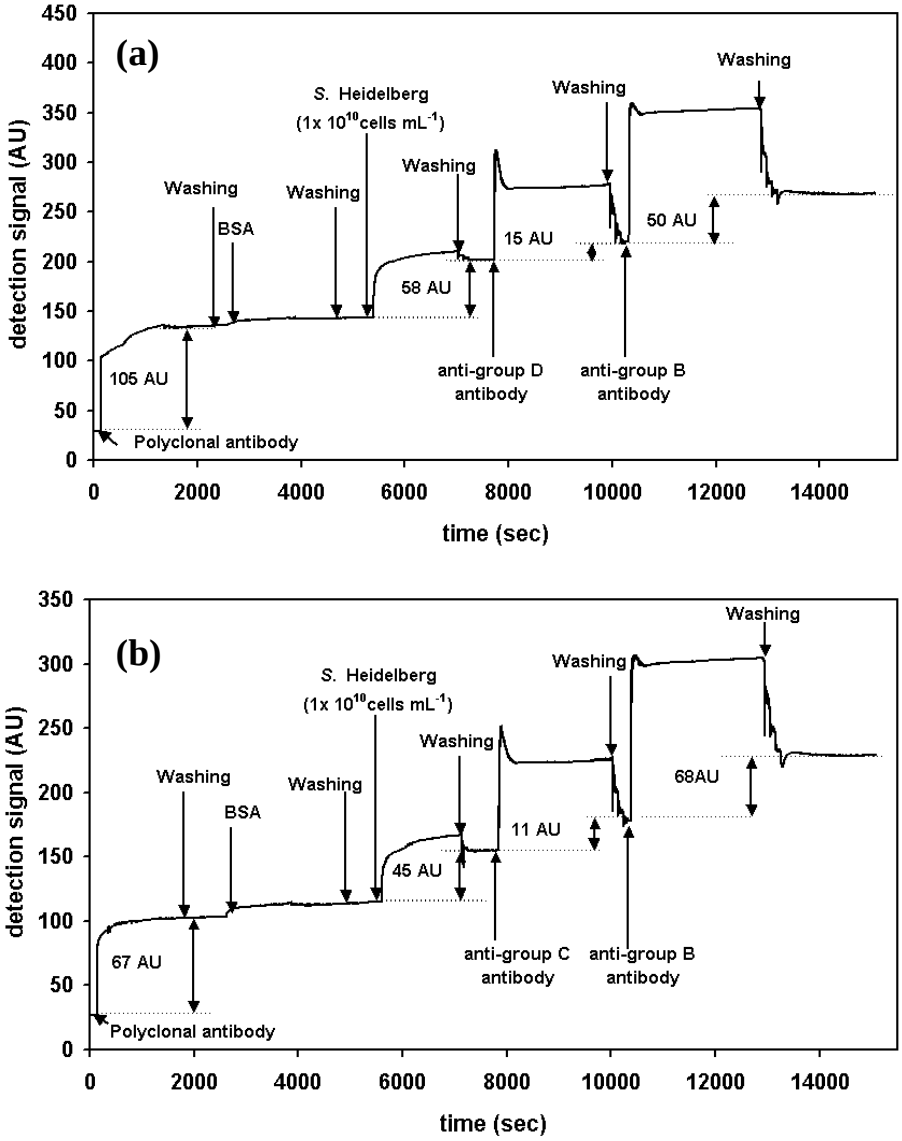

Fig. 4.26: Serogrouping of *S.* Heidelberg (a) sensorgram showing the SPR response of serogroup D-specific, followed by serogroup B-specific antibody to *S.* Heidelberg captured on a C_{18} SPR chip (b) sensorgram showing the SPR response of serogroup C-specific, followed by serogroup D-specific antibody to *S.* Heidelberg captured on a C_{18} SPR chip.

The average net detection signal for the group C and group D antibodies were -8 ± 1 AU and 3 ± 1 AU respectively (Fig. 4.27).

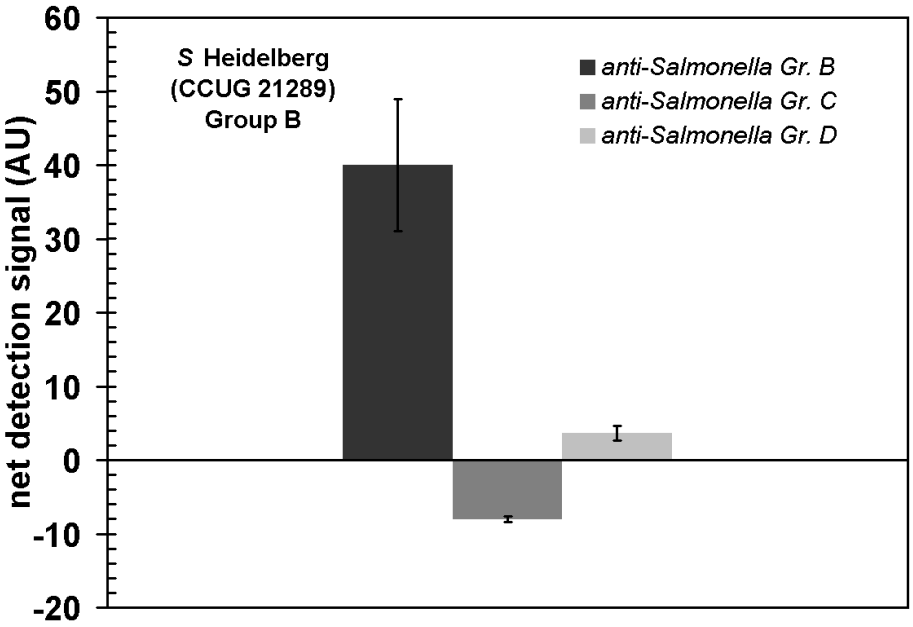

Fig. 4.27: The net SPR detection signals (after subtraction of the control signals) for the serogroups B-, C- and D-specific antibodies on interaction with *S.* Heidelberg captured on a C_{18} SPR chip. Note that negative values of signals indicate higher signal of the detection antibody in the control channel due to unspecific binding as compared to that obtained in the sample channel.

4.1.5.1.2 *S.* Infantis (Group C, Rough strain)

Some strains of genus *Salmonella* are referred to as "rough". These strains of *Salmonella* lack the O-specific antigens. This occurs as a result of mutation in the genes responsible for synthesis of the O-antigens (Raetz, 1996).

In order to observe the behaviour of *Salmonella* rough strains to SPR serogrouping a rough strain of *S.* Infantis (CCUG 35612, antigenic formula: 6,7:r:1,5 or Rough:r:1,5), which belongs to group C, was evaluated. In the first step of the sequential detection, the captured *S.* Infantis bacteria were probed with monoclonal antibody specific for serogroup C. No detection signal was obtained with the group C-specific monoclonal antibodies (-3 AU), thus confirming that the analysed strain was indeed lacking the O-antigens specific for group C (Fig. 4.28 and 4.29).

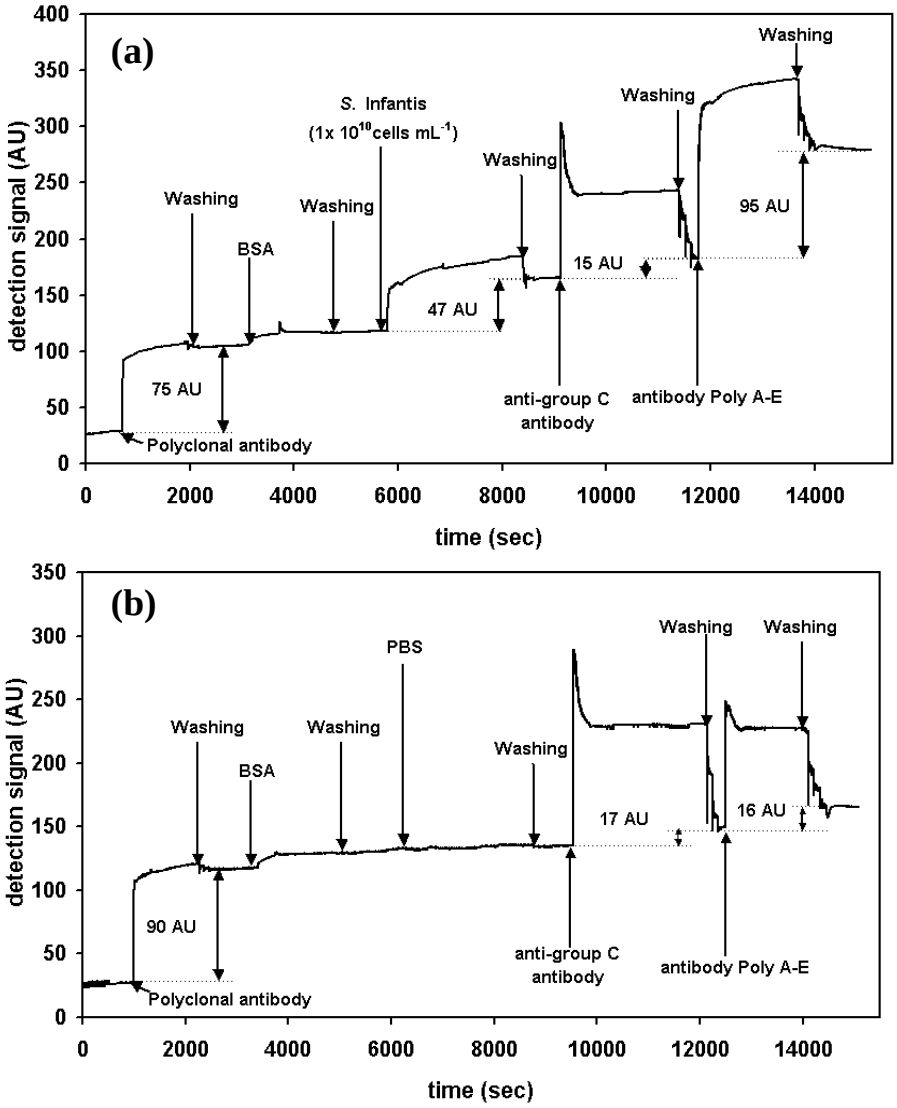

Fig. 4.28: Serogrouping of *S.* Infantis (rough strain) (a) sensorgram showing the SPR response of serogroup C specific antibody, followed by antibody Poly A-E to *S.* Infantis (rough strain) captured on a C_{18} SPR chip (b) sensorgram showing the SPR response of serogroup C specific antibody, followed by antibody Poly A-E for the control channel without the bacteria.

However, in order to determine if it was possible to elicit a response of this rough strain to polyvalent O-antisera without isolation of the smooth form of the strain; Poly A-E (which agglutinates all strains which express O-antigens in the serogroups A-E) was added as the second detection antibody (Fig. 4.28 a). A positive detection signal was obtained with Poly A-E resulting in a net average detection signal of 71 ± 12 AU (Fig. 4.29).

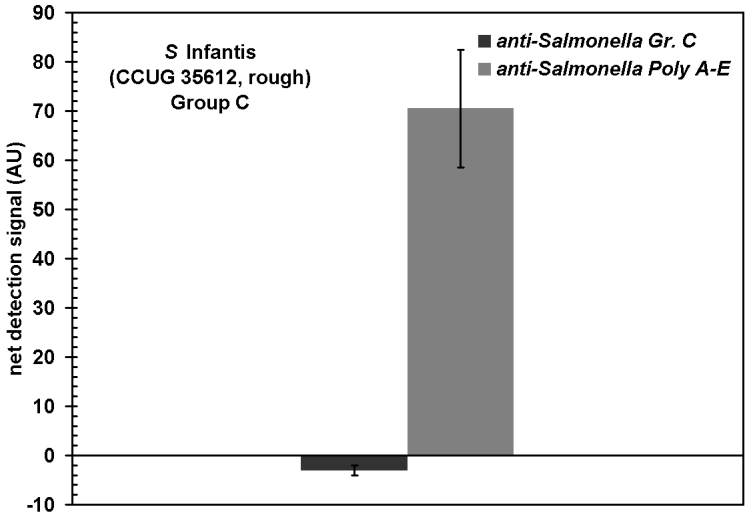

Fig. 4.29: The net SPR detection signals (after subtraction of the control signals) for the serogroup C-specific antibody and antibody Poly A-E on interaction with *S.* Infantis (rough strain) captured on a C_{18} SPR chip. Note that net negative value of signal indicate higher signal of the detection antibody in the control channel due to unspecific binding compared to that obtained in the sample channel.

4.1.5.1.3 *S.* Thompson (Group C*)*

In order to check the performance of the group C-specific monoclonal antibody used for the above experiment, a further set of experiment was carried out with a known smooth strain of *S.* Thompson (CCUG 12652, antigenic formula: 6,7:k:1,5). *S.* Thompson reacted positively to the group C-specific monoclonal antibody (63 ± 2 AU), thus confirming the presence of the O-antigens belonging to group C (Fig. 4.30).

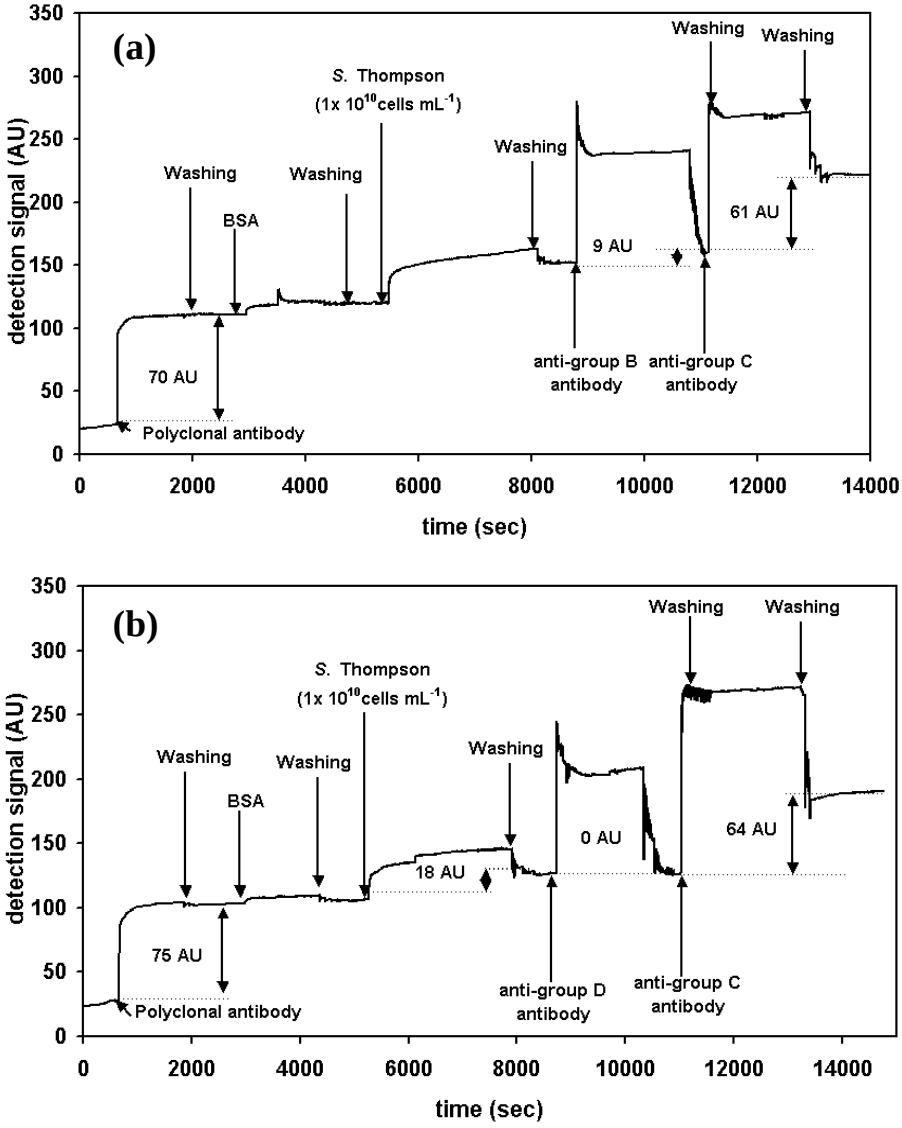

Fig. 4.30: Serogrouping of *S.* Thompson (a) sensorgram showing the SPR response of serogroup B-specific, followed by serogroup C-specific antibody to *S.* Thompson captured on a C_{18} SPR chip (b) sensorgram showing the SPR response of serogroup D-specific, followed by serogroup B-specific antibody to *S.* Heidelberg captured on a C_{18} SPR chip.

The captured bacteria were first probed either with group B (-9 ± 3 AU) or group D (-10 ± 3 AU) specific antibodies to check for cross-reactivity. No cross-reactivity to *S.* Thompson was obtained with neither the group B nor group D antibodies. The data are summarised in Fig. 4.31.

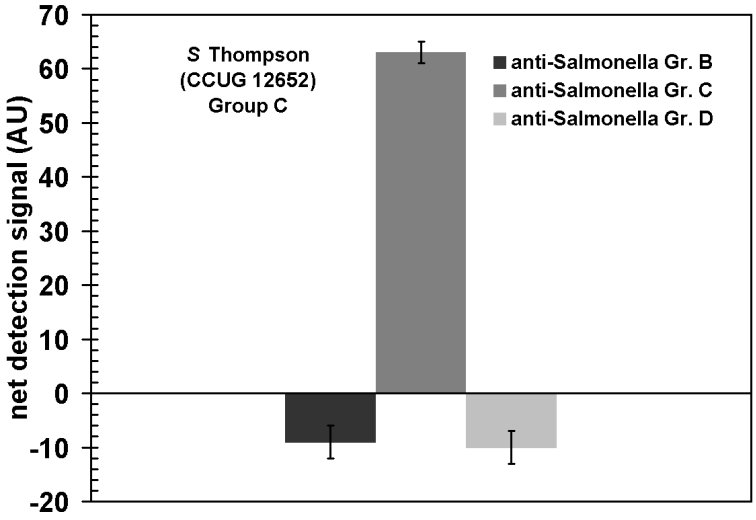

Fig. 4.31: The net SPR detection signals (after subtraction of the control signals) for the serogroups B-, C- and D-specific antibodies on interaction with *S.* Thompson captured on a C_{18} SPR chip. Note that negative values of signals indicate higher signal of the detection antibody in the control channel due to unspecific binding compared to that obtained in the sample channel.

4.1.5.2 Serotyping of *Salmonella* Enteritidis

S. Enteritidis CCUG 32352 was used for validation of the SPR-based serotyping method. According to the Kauffmann-White scheme *S.* Enteritidis has the antigenic formula 1,9,12:g,m:-. As it contains the O9 antigen it belongs to the serogroup D1 (section 1.2.1.1). The response of the bacteria to group D-specific antibody was 77 ± 6 AU (Fig. 4.35). It is monophasic and expresses only one flagellar antigen, which is denoted as g,m in the antigenic formula. The first step in serotyping of *S.* Enteritidis involves detecting its response to the polyvalent O-antisera. This step provides preliminary information if the bacteria under investigation belongs to the genus *Salmonella*. In this case the bacteria were screened for their response to polyvalent O-antisera Poly A-E (polyvalent against O-antigens of *Salmonella* serogroups A-E). The net average detection signal obtained for the Poly A-E antibody was 93 ± 6 AU (Fig. 4.35 and Table 4.1).

Once the bacterial strain was identified to belong to the genus *Salmonella,* the next step was to identify the serogroup. As the strain under investigation was known to belong to the serogroup D1, its response to antibody against the D1 common antigen O9 was investigated. Addition of anti-O9 detection antibody to the bacteria (1×10^{10} cells mL^{-1}), captured on the SPR chip by the polyclonal capture antibody, resulted in a detection signal of 63 ± 7 AU in the multi-channel detection mode. The

SPR sensorgram for the detection of the *S.* Enteritidis strain by the anti-O9 antibody from one of the multi-channel runs has already been presented in Fig. 4.7 (section 4.1.1.7). In comparison the data for the sequential detection using Poly A-E and anti-O9 as the first and second detection antibodies, clearly show a reduction in detection signal of the anti-O9 antibody (Fig. 4.32). The net detection signal obtained using the anti-O9 detection antibody as the second detection antibody was 31 ± 2 AU (Table 4.1).

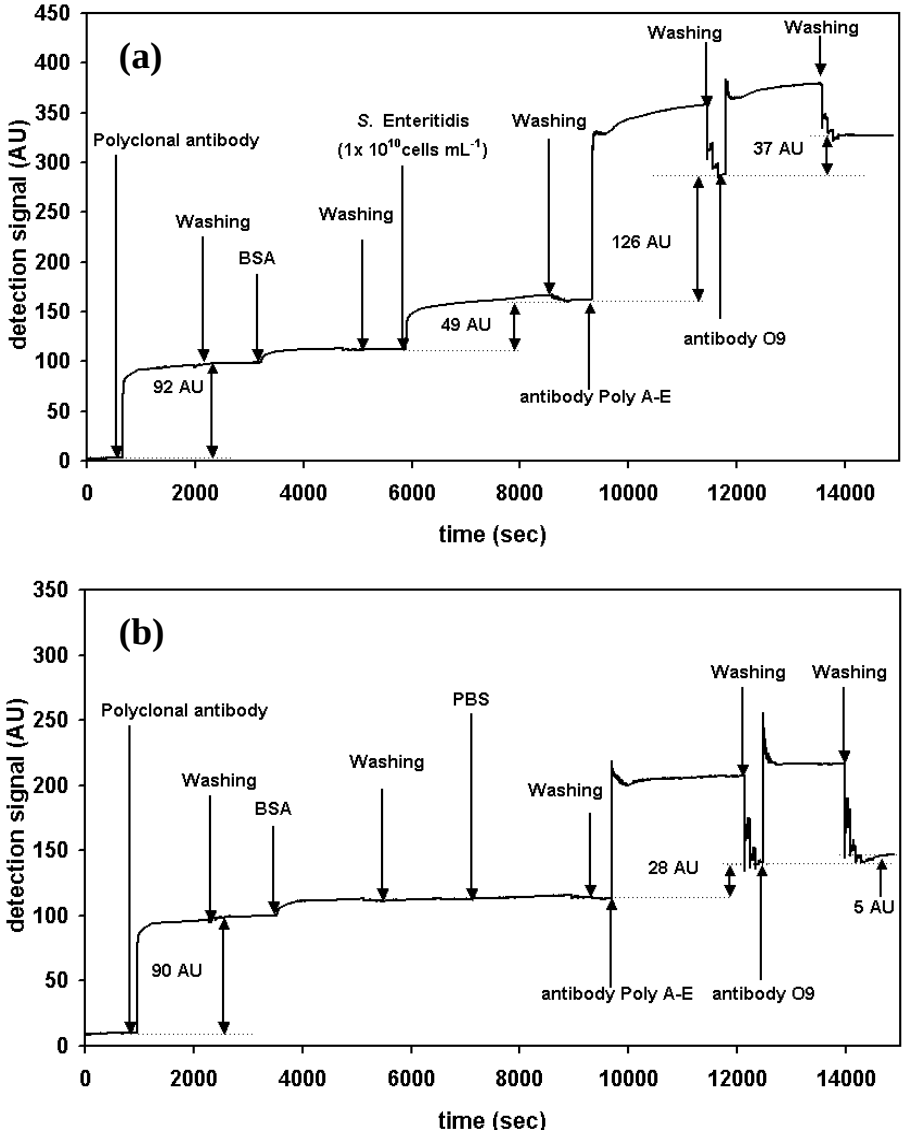

Fig. 4.32: Serotyping of *S.* Enteritidis (a) response of antibody Poly A-E (first detection antibody) and antibody O9 (second detection antibody) in the single-channel sequential detection mode (b) response of the respective antibodies in the control channel.

In the next step of the serotyping scheme, the bacteria were probed with monoclonal anti-*Salmonella* O4 detection antibody in the sequential detection mode along with

the antibody O9 as the second antibody. There was no detection signal obtained for the anti-O4 detection antibody (net detection signal -22 ± 2 AU). Sequential addition of the O9 detection antibody resulted in a significantly high detection signal (net detection signal 62 ± 4 AU, Table 4.1). O4 is the O-antigen present in *S.* Typhimurium (serogroup B). Sensorgrams obtained are presented in Fig. 4.33.

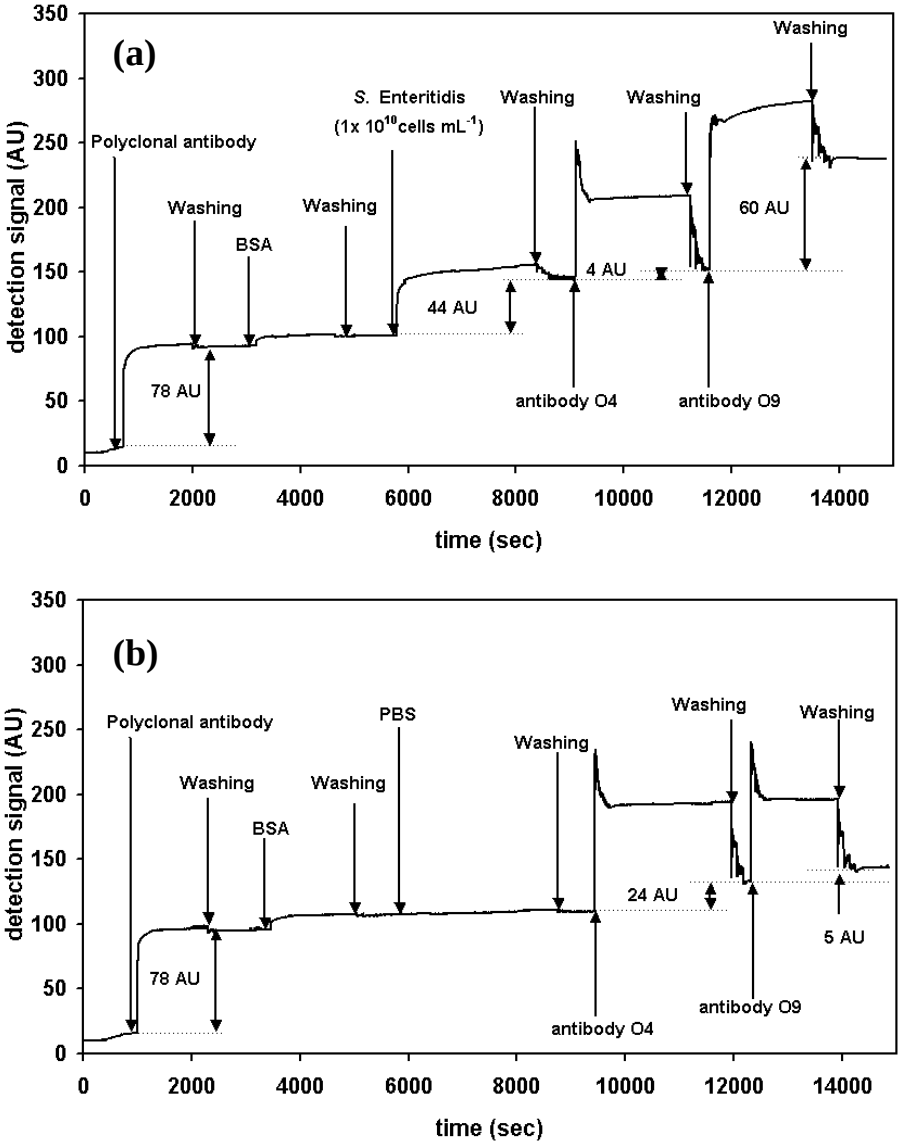

Fig. 4.33: Serotyping of *S.* Enteritidis (a) response of antibody O4 (first detection antibody) and antibody O9 (second detection antibody) in the single-channel sequential detection mode (b) response of the respective antibodies in the control channel.

Serotyping of *S.* Enteritidis also requires the strain under consideration to be evaluated for its reactivity to antibody against the O-surface antigen O46. The reaction of *S.* Enteritidis to the anti-O46 antibody should be negative. O46 is the O-surface antigen that occurs along with surface antigen O9 in *Salmonella* serogroup

D2 (O9, 46) and also in group D3 (O9, 46, 27). This test with anti-O46 antibody rules out the possibility of any false positive detection arising due to the presence of bacteria belonging to the serogroups D2 and D3. Thus, this test is used to confirm that the positive response obtained with the anti-O9 antibody is only due to bacteria belonging to the serogroup D1.

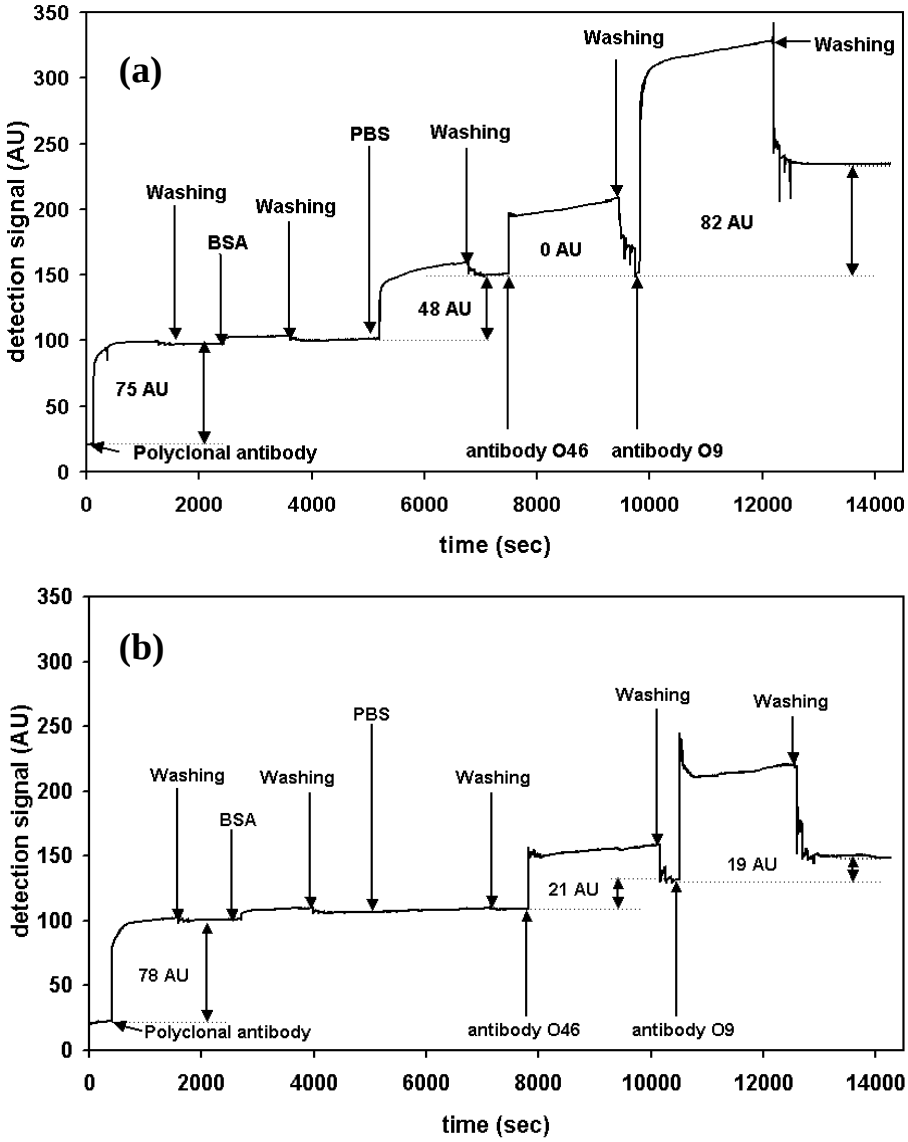

Fig. 4.34: Serotyping of *S.* Enteritidis (a) response of antibody O46 (first detection antibody) and antibody O9 (second detection antibody) in the single-channel sequential detection mode (b) response of the respective antibodies in the control channel. Note the unspecific binding of antibody O46 in the control channel.

Hence, using the sandwich SPR-based assay described above the captured *S.* Enteritidis strain was probed with antibody against the O-antigen O46, in the sequential detection mode; the anti-O9 antibody being the second detection antibody.

The results obtained for strain CCUG 32352 were, as expected, negative for detection with antibody against O46. The resulting net detection signal was -21 ± 1 AU (Table 4.1). The sensorgrams obtained is presented in Fig. 4.34.

The negative value of the detection signal indicates that the O46 detection antibody gave a higher detection signal in the control channel due to unspecific binding. The detection signal obtained for the second detection antibody (O9) was 62 ± 2 AU (Table 4.1). This second detection signal due to the O9 detection antibody is comparable to the signal obtained for the same O9 antibody in the sequential detection with O4 detection antibody as the first detection antibody.

Table 4.1: SPR detection signals obtained for the serotyping of *S.* Enteritidis in the sequential detection mode. (The detection signals are the final net detection signals after subtraction of the control values. The standard deviations shown are obtained for 3 different readings taken for each data point).

1st detection antibody	SPR signal (AU)	2nd detection antibody	SPR signal (AU)
anti-*Salmonella* Gr. D	77 ± 6	anti-*Salmonella* O 9	28 ± 2
anti-*Salmonella* Poly A-E	93 ± 6	anti-*Salmonella* O 9	31 ± 2
anti-*Salmonella* O 4	-22 ± 2	anti-*Salmonella* O 9	62 ± 4
anti-*Salmonella* O 46	-21 ± 1	anti-*Salmonella* O 9	62 ± 2
anti-*Salmonella* H g,m	24 ± 2	anti-*Salmonella* O 9	48 ± 4
anti-*Salmonella* H q,s,t,p,u	-16 ± 1	anti-*Salmonella* O 9	66 ± 5

The data obtained for the serotyping of S. Enteritidis in the sequential detection mode are compiled and presented in Table 4.1. The serotyping results are summarised in Fig. 4.35.

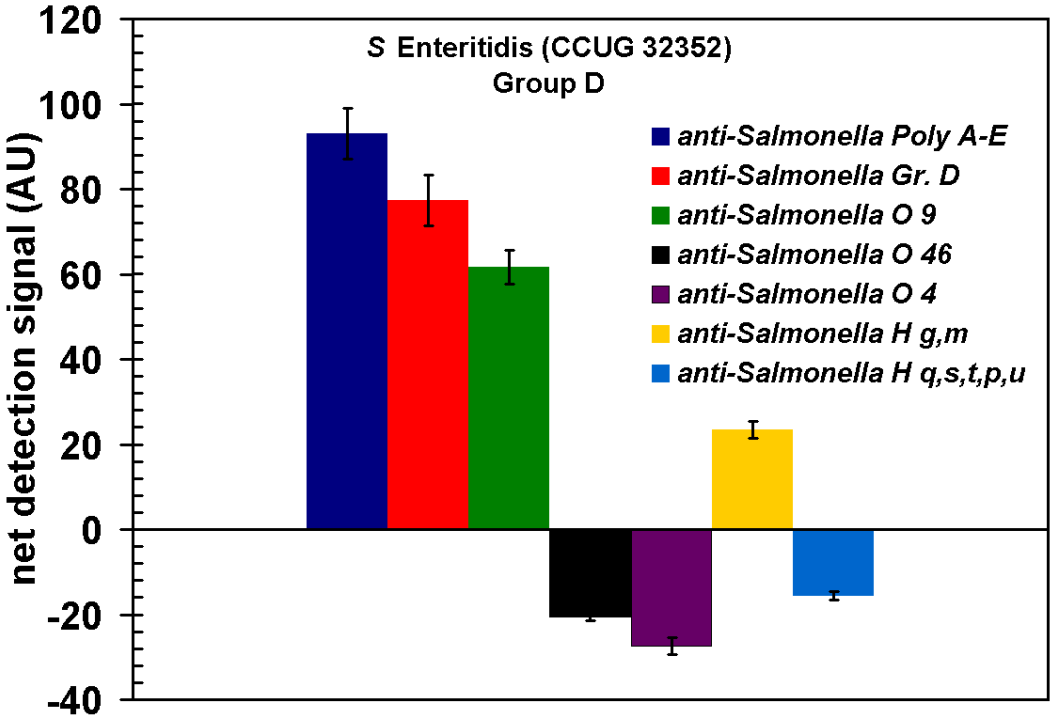

Fig. 4.35: SPR detection signals obtained for the serotyping of *S.* Enteritidis in the sequential detection mode. The detection signals are the final net detection signals after subtraction of the control values. The standard deviations shown are obtained for 3 different readings taken for each data point. Negative values of signals indicate higher signal of the detection antibody in the control channel due to unspecific binding compared to that obtained in the sample channel.

After the successful detection of the group-specific O-antigen O9 for the selected strain of *S.* Enteritidis, the next step was to detect the presence of H-antigen g,m (flagellar antigen). Before performing the sequential detection, the anti-Hg,m antibody was checked in the multichannel detection mode to determine the intensity of the detection signal in comparison to the one obtained with the O9 antibody in the same detection mode. A control channel was also run. This information is necessary to decide if the antibody is to be used as the first or second detection antibody in the sequential detection mode. The net average detection signal obtained was 24 ± 2 AU.

Thus, a lower detection signal was obtained with the Hg,m antibody in comparison to that obtained with the O9 detection antibody. Hence, the Hg,m antibody was used as the first detection antibody in the sequential detection mode. The net detection signal, after subtracting the control signal in the sequential detection mode for the second detection antibody (anti-O9) was 48 ± 4 AU (Table 4.1). The results obtained clearly show the presence of the H-antigen g,m in the strain CCUG 32352.

Sensorgrams for Hg,m-antigen determination of *S.* Enteritidis are presented in Fig. 4.36.

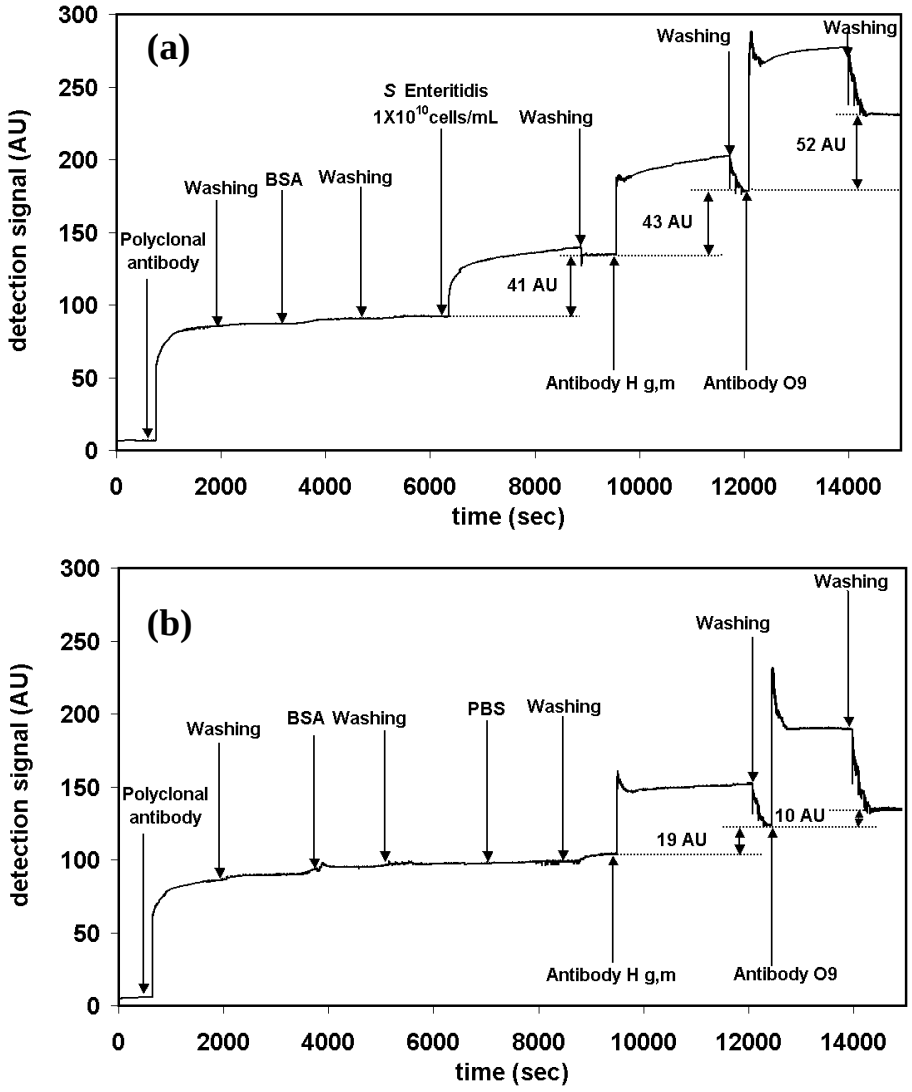

Fig. 4.36: Serotyping of *S.* Enteritidis (a) response of antibody Hg,m (first detection antibody) and antibody O9 (second detection antibody) in the single-channel sequential detection mode (b) response of the respective antibodies in the control channel.

A mandatory requirement for serotyping of *S.* Enteritidis is to confirm a negative reaction of the bacteria to antisera against H-antigens q,s,t,p,u. The strain resulted in a negative response when probed with a mixture of polyclonal antisera against H-antigens q,s,t,p,u. The net detection signal obtained was only -16 ± 1 AU (Fig. 4.37 and Table 4.1). This negative response to Hq,s,t,p,u antisera is essential to rule out the presence of other serogroup D1 serotypes of *Salmonella* which express these antigens along with the Hg,m-antigen. Some examples of such strains from the serogroup D1 are: *S* II [1],9,12:g,m,[s],t:[1,5,7]; *S* II [1],9,12:g,m,s,t:e,n,x;

S Blegdam 9,12:g,m,q:-; *S* Dublin (1,9,12[Vi]:g,p:-); *S* Naestved ([1],9,12:g,p,s:-); and *S* Rostock ([1],9,12:g,p,u:-).

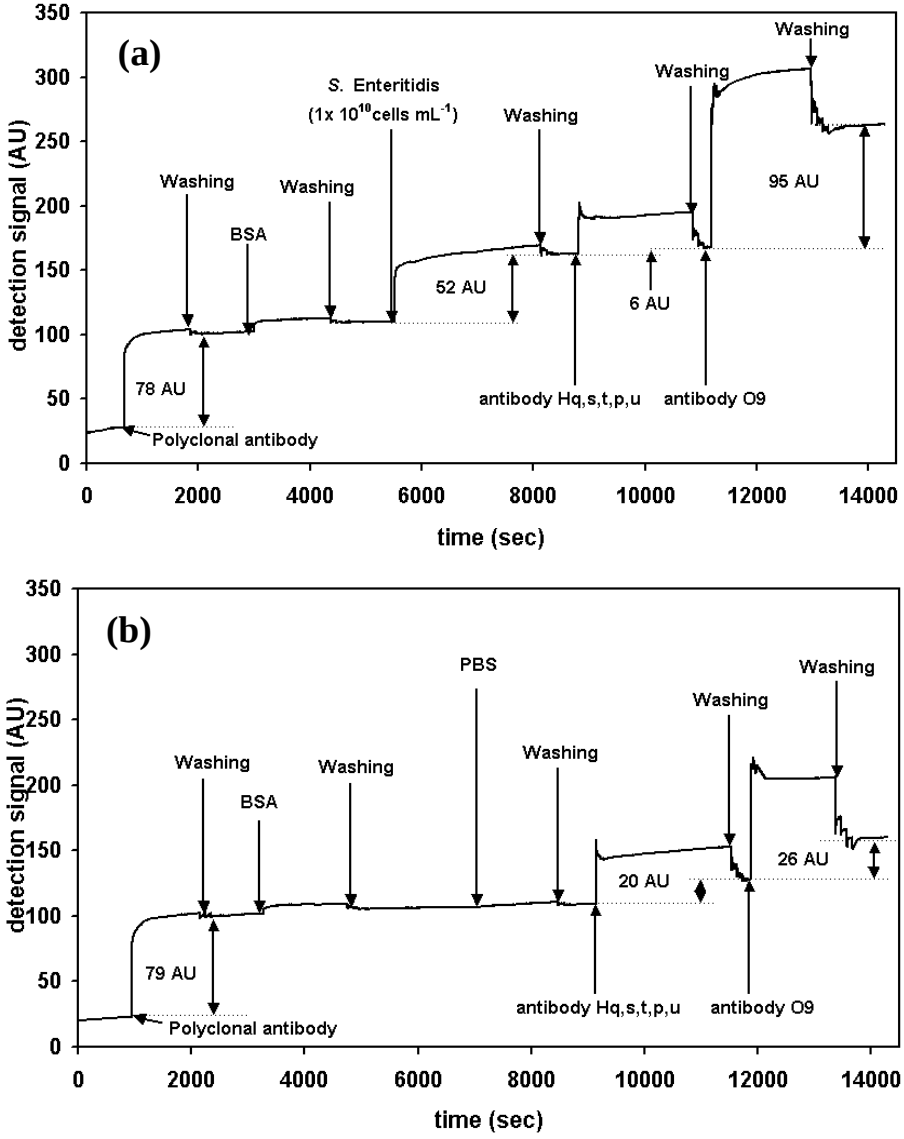

Fig. 4.37: Serotyping of *S.* Enteritidis (a) response of antibody Hq,s,t,p,u (first detection antibody) and antibody O9 (second detection antibody) in the single-channel sequential detection mode (b) response of the respective antibodies in the control channel.

4.2 Zoonoses Chip for SPR-based detection of *Salmonella* infections

According to the WHO a zoonoses is any disease or infection that is naturally transmissible from vertebrate animals to humans. Animals thus play an essential role in maintaining zoonotic infections in nature. Zoonoses may be bacterial, viral, or parasitic, or may involve unconventional agents. *Salmonella* infections are identified

as zoonoses as they can be transmitted from infected farms animals like pigs and chicken. Both pigs and chicken are known carriers of *Salmonella*. Thus, a rapid method for the monitoring of the *Salmonella* status of such animals is being given importance by various governmental and health agencies worldwide. The results obtained using an SPR-based zoonoses chip carried out as part of this study is presented in the following sections.

4.2.1 Immobilisation of LPS on the chip surface

The LPS was immobilised onto the surface of a hydrophobic (C_{18}) chip according to the protocol mentioned in (section 3.2.4.7). To check the effect of immobilisation of the bacterial LPS on the chip surface an experiment was run with one chip with the LPS and one without. Purified LPS from *S.* Typhimurium at 10 µg mL^{-1} was used in the experimental channel. In the control channel, PBS was added instead of the LPS. This was then followed by the addition of O-specific antibody against the O-antigen O5. The O5-antigen is one of the O-specific antigens present on the LPS from *S.* Typhimurium. The difference in results obtained between the experimental channel and the control channel establishes the importance of successful immobilisation of the LPS in developing a zoonoses chip. The detection signal after addition of the anti-O5 antibody in the experimental channel was 93 AU as compared to only 12 AU in the control channel. The sensorgrams obtained for both the channels are presented in Fig. 4.38. This experiment was important to prove the success of the strategy used for the immobilisation of the LPS. The binding of *Salmonella* LPS to the surface of the hydrophobic C_{18} chip can be understood with respect to the structure of the LPS. As discussed in section 1.2.2.2, the LPS of gram-negative bacteria consists of a hydrophobic Lipid A portion and a hydrophilic polysaccharide region, which presents the somatic O-antigens.

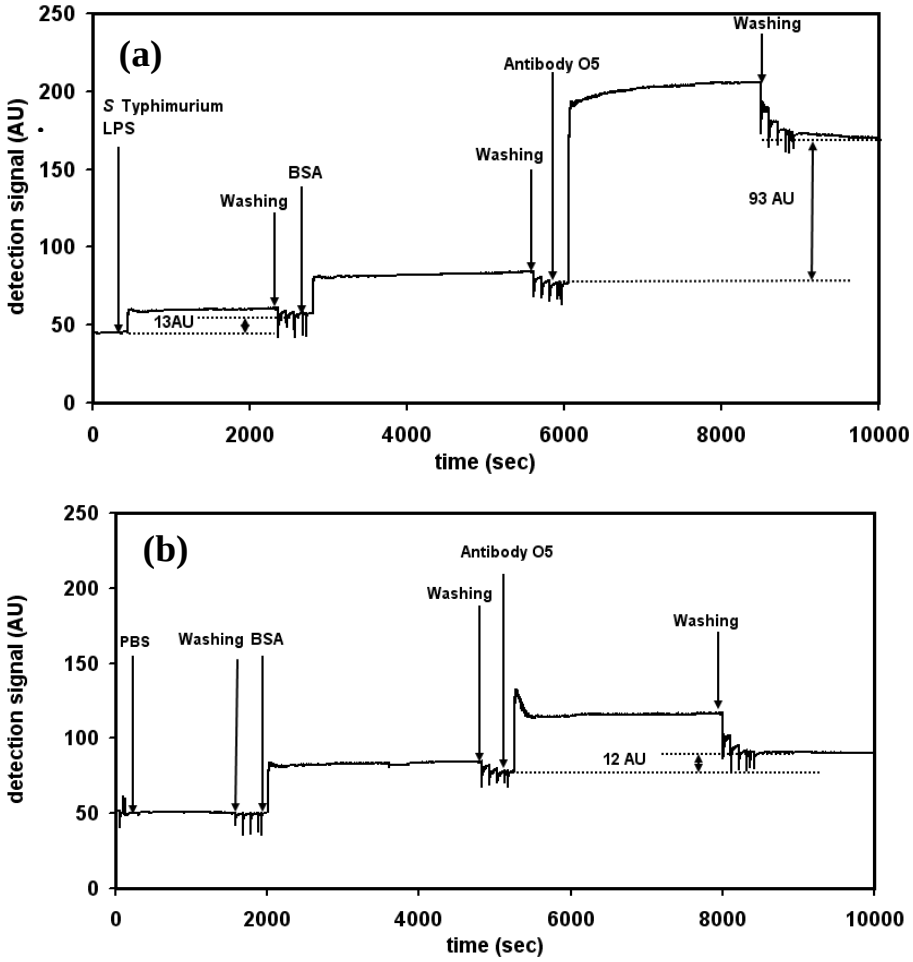

Fig. 4.38: Assay to determine the success of immobilisation of LPS on a hydrophobic C_{18} SPR chip. The assay shows the importance of the LPS in the development of the zoonoses SPR-chip. Sensorgrams obtained for (a) experimental channel with immobilised LPS from *S.* Typhimurim used to detect O-specific O5 antibodies in buffer (b) control channel without LPS on the surface of the chip.

In this assay, successful immobilisation of the LPS to the gold surface is achieved only on proper orientation of the immobilised LPS. The aim of the assay is to detect antibodies against O-specific *Salmonella* antigens in serum of infected pigs. The somatic O-antigens of the LPS should thus be directed away from the chip surface. We were able to achieve proper orientation of the LPS by using a hydrophobic chip surface, which resulted in hydrophobic interaction of the chip surface with Lipid A of the LPS.

4.2.1.1 Effect of heat and acid treatment of LPS on the SPR response of the zoonoses chip

To further understand the phenomenon of LPS immobilisation, an experiment was designed to determine if there was any difference in the detection signal of the anti-O5 antibodies when the Lipid A was removed from the LPS. Lipid A-free LPS was obtained by acid hydrolysis as described in section 3.2.8. The hydrolysis was carried out for 3 h at 100 ^{o}C. Sample taken after hydrolysis and sample of supernatant following the centrifugation step after hydrolysis were both immobilised on a C_{18} SPR chip. The results show that for both the samples there was no significant detection signal obtained on addition of the anti-O5 antibody. In addition LPS without any acid addition was also heated to 100 ^{o}C for 3 h and sample before centrifugation and supernatant after centrifugation were immobilised onto the C_{18} chip. Results obtained were similar as obtained in case of the acid hydrolysed samples. Addition of unhydrolysed LPS on the same chip resulted in significant detection signal with the anti-O5 detection antibody. The results obtained are shown in Fig. 4.39. The results indicate a decrease in antibody binding capacity of the chip surface with use of LPS that is either acid-hydrolysed and heat-treated or only heat-treated. However, from the above experiments it was not possible to draw any conclusion on the role of Lipid A to the binding of LPS to the chip surface.

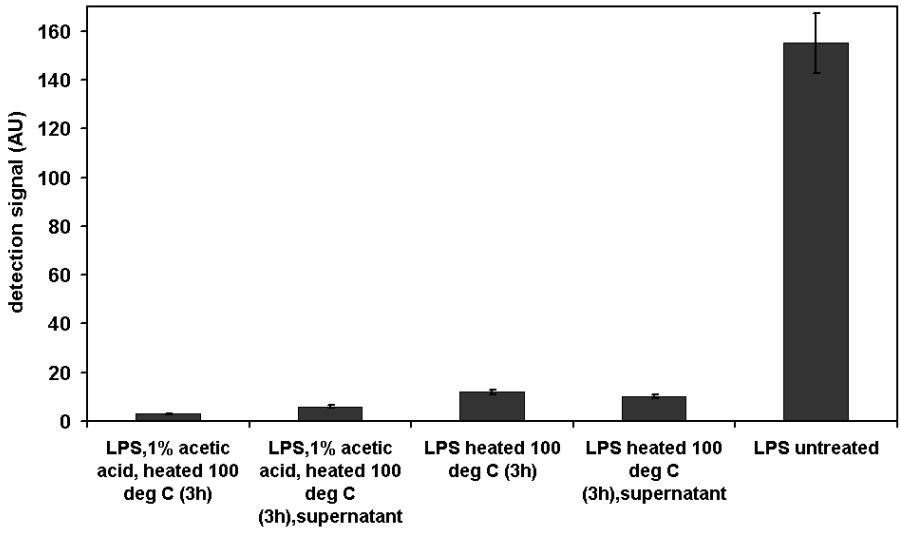

Fig. 4.39: Data showing SPR signals for the detection of O5 antibody (1:10 dilution), obtained using LPS subjected to different treatments prior to immobilisation on the hydrophobic C_{18} SPR chip. Heat treatment of LPS with or without acid results in loss of antibody binding capacity of the LPS chip.

The results conclusively prove that the epitopes (somatic O-5 antigens) on the LPS responsible for binding of the anti-O5 antibody are destroyed by heat. This information is useful in terms of deciding on the storage conditions of the LPS and precoated LPS chips. LPS solutions used for coating and precoated LPS chips should ideally be stored under refrigeration (4 ^{o}C).

4.2.2 Determination of the optimum LPS concentration

An O-specific monoclonal (Mouse, IgM) antibody against the O5 antigen of *S*. Typhimurium was used to establish the optimum LPS concentration required for the SPR assay. Different LPS concentrations (100, 50, 25, 10, and 1 μg mL^{-1}) were evaluated for the detection of the O5 antibody (1:10 dilution in PBS). A concentration of 10 μg mL^{-1} of LPS was found to be optimum (98 AU) for the assay (Fig. 4.40). Increasing the concentration of the antibody beyond 10 μg mL^{-1} did not result in any further significant increase in the detection signal due to the antibody. This was the LPS concentration used in all further experiments.

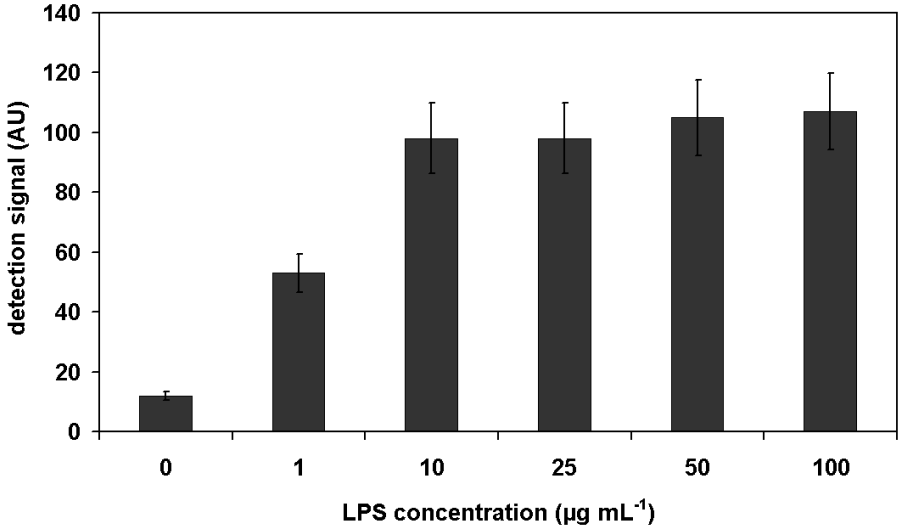

Fig. 4.40: Data showing SPR signals for the detection of O5 antibody (1:10 dilution) obtained with different concentrations of LPS, immobilised on the hydrophobic C_{18} SPR chip. The optimum LPS concentration for the assay is 10 μg mL^{-1}.

As discussed above in section 4.2.1, the control without LPS on the chip surface showed a detection signal of only 12 AU, thus confirming the role of LPS in the assay. The data showing the response of the anti-O5 antibody to the different concentrations of the LPS immobilised on chip surface is presented in Fig 4.40.

In order to establish the commercial viability of the developed SPR assay with respect to the presently used commercial ELISA kits, it was important to optimise the LPS concentration. Using the SALMOTYPE® commercial ELISA kit it is possible to carry out screening of 10,000 serum samples using 1 mg of the LPS (100 µL of 1 µg mL^{-1} of LPS per sample). In case of the SPR-based assay, 10 µL of the LPS at a concentration of 20 µg mL^{-1} is added into the SPR cuvette to obtain a final concentration of 10 µg mL^{-1}, for the analysis of each sample. Thus, for the SPR assay using 1 mg of the LPS, it is possible to carry out analysis of 5000 samples. However, in case of SPR there is the possibility to recover and reuse the LPS solution. The LPS solution can be recovered using the autosampler of the device.

4.2.3 Detection range of the assay in buffer

The assay was then evaluated for detection of different dilutions of the anti-O5 antibody in PBS. The concentration of the anti-O5 antibody was determined using the UV absorption method as described in section 3.2.7. The following dilutions were evaluated: 1:10 (1350 µg mL-1), 1:20 (675 µg mL-1), 1:50 (270 µg mL-1), 1:100 (135 µg mL-1), and 1:200 (67.5 µg mL-1). The average SPR detection signals obtained were 100 AU (1:10), 86 AU (1:20), 69 AU (1:50), 15 AU (1:100), and 10 AU (1:200), respectively. The detection range of the assay was between 67.5 µg mL-1 and 675 µg mL-1.

Higher concentrations of the antibody did not result in any significant increase in the SPR detection signal, thus indicating the saturation limit of the assay. A sensorgram from one of the SPR runs is presented in Fig. 4.41 a, and the response to different antibody dilutions is shown in Fig. 4.41 b.

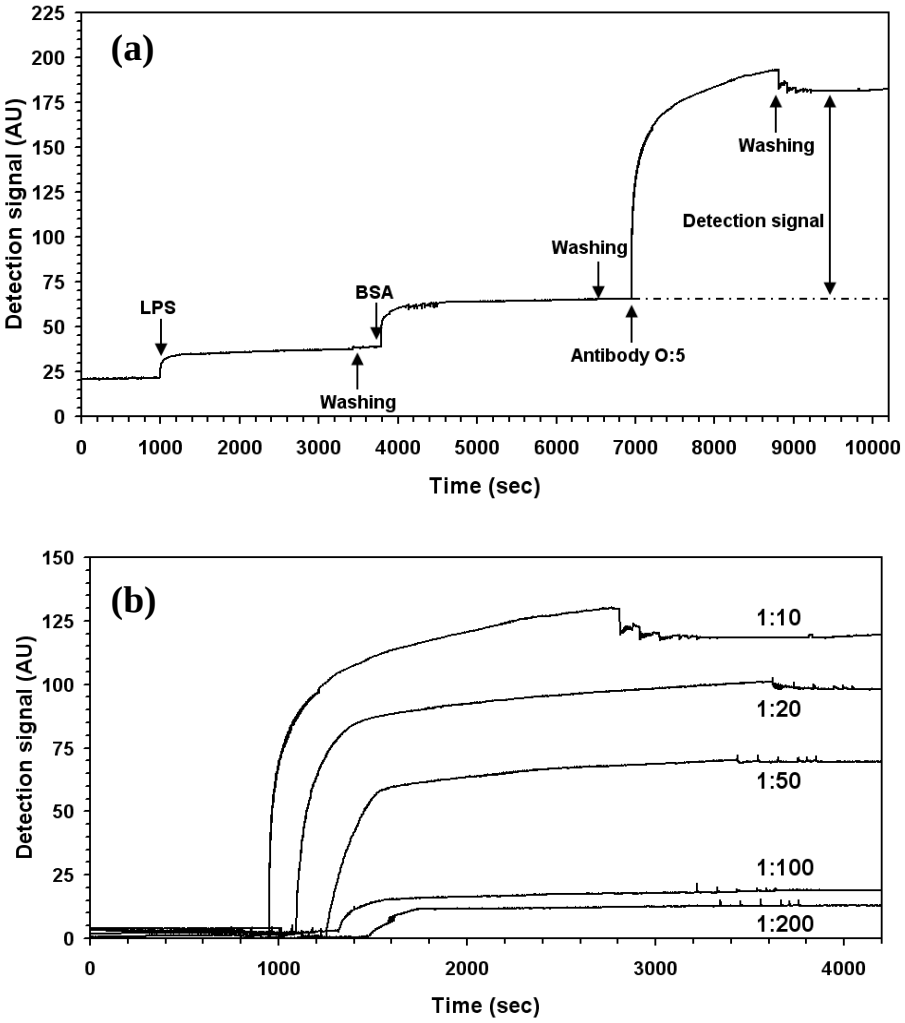

Fig. 4.41: Sensorgram (a) showing binding of LPS to the C_{18} surface of the SPR chip, addition of BSA, and the detection signal due to binding of the O5 antibody (1:10 dilution) to the immobilised LPS. Sensorgram (b) showing detection signals for different dilutions of the O5 antibody captured on a chip coated with 10 µg mL^{-1} of LPS. Only the region of the sensorgram showing the detection signal on addition of the antibodies is shown.

4.2.3.1 Calculation of equilibrium association (K_A) and equilibrium dissociation (K_D) constants

The data obtained from the experiment described in section 4.2.3 was used to calculate the equilibrium association (K_A) and equlibrium dissociation (K_D) constants for the binding of anti-O5 antibody to the *S.* Typhimurium LPS immobilised on the surface of the chip. The "initial rate analysis" model of Edwards and Leatherbarrow described in section 3.2.9.2 was used. The first 10 s of the binding curve (Fig. 4.41 b), obtained at each dilution of the antibody, were used for the calculations. The slope of each line in this region was calculated using linear regression. The slopes obtained are tabulated in Table 4.2.

Table 4.2: Initial binding rates obtained for different concentrations of anti-O5 antibody on binding to a SPR chip coated with LPS from *S.* Typhimurium

Antibody dilution	Antibody concentration (M)	Initial rate or Slope (AU sec^{-1})
1:200	$7.5X10^{-8}$	0.0673
1:100	$1.5X10^{-7}$	0.1693
1:50	$3.0X10^{-7}$	0.5314
1:20	$7.5X10^{-7}$	1.5588
1:10	$1.5X10^{-6}$	3.0490

The slopes (Y-axis) were then plotted against the concentrations of the antibody (X-axis). The linear region of the plot was found to lie between 7.5×10^{-8} M and 1.5×10^{-6} M of the antibody concentration. The linear region of this plot is presented in Fig. 4.42.

The slope of this plot (2×10^{6} AU sec^{-1}) is the product of the maximum response (R_{max}) and the association rate constant ($K_{ass.}$) as described by equation (3.8), in section 3.2.9.2. Using this relation, the $K_{ass.}$ was calculated to be 1.57×10^{4} M^{-1} sec^{-1}. The value of R_{max} corresponds to the SPR response at the highest concentration of the antibody tested. The R_{max} value in this case was 127 AU, which was the detection signal obtained at a 1:10 dilution of the anti-O5 antibody (Fig. 4.41).

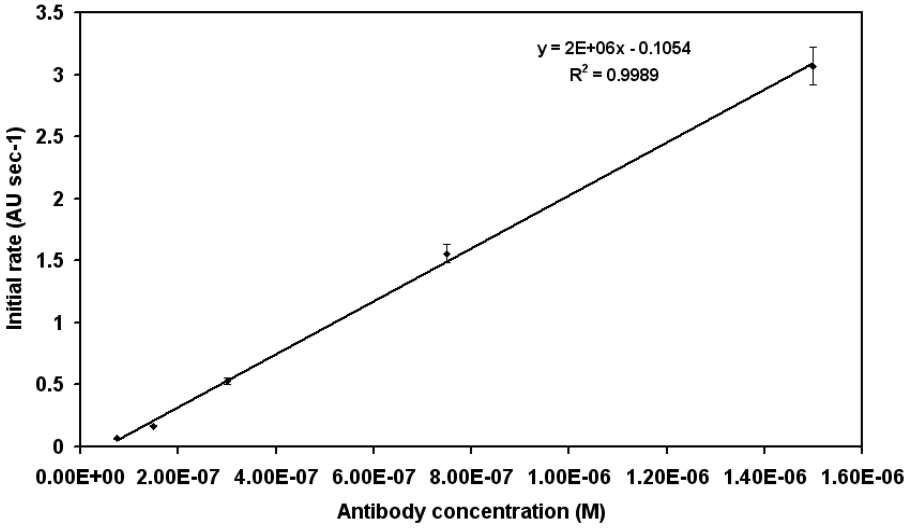

Fig. 4.42: Plot of initial rate vs. time for the determination of the association rate constant ($K_{ass.}$) involved in the binding of the anti-O5 antibody to *S.* Typhimurium LPS, according to the method of Edwards and Leatherbarrow.

The next step was to calculate the dissociation rate constant ($K_{diss.}$). As explained in section 3.2.9.2, the $K_{diss.}$ calculation is based on the data obtained for the dissociation phase of the sensorgram at the highest concentration of the antibody. Applying the equation (3.9) (section 3.2.9.2), to the dissociation phase data for the antibody at 1:10 dilution, a plot was obtained with values of $\ln(R_0/R_t)$ on the Y-axis vs. time on the X-axis. The dissociation rate constant ($K_{diss.}$), which is the slope of this curve, was 5×10^{-4} sec^{-1} (Fig. 4.43).

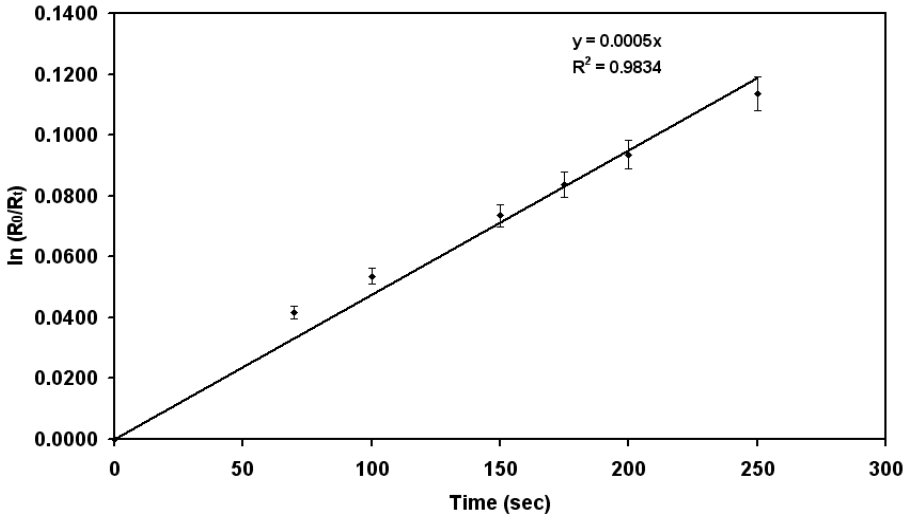

Fig. 4.43: Plot of $\ln(R_0/R_t)$ vs. time for determination of the dissociation rate constant ($K_{diss.}$) involved in the binding of anti-O5 antibody to *S.* Typhimurium LPS according to the method of Edwards and Leatherbarrow. The slope of the plot gives the dissociation rate constant.

Based on the calculated values of $K_{ass.}$ and $K_{diss.}$, the equilibrium association constant (K_A) and the equilibrium dissociation constant (K_D) were calculated using equations (3.5) and (3.6), repectively (section 3.2.9.1). The values obtained for K_A and K_D was 3.14×10^7 M^{-1} and 3.18×10^{-8} M, respectively. The low value of $K_{D,}$ which is in the nanomolar range (31.8 nM) and a corresponding high value K_A indicates a strong binding between the *S.* Typhimurium LPS and the anti-O5 antibody.

4.2.4 Validation of the assay in serum

4.2.4.1 Effect of serum dilution on the detection signal of the assay

To validate the assay in serum, the first step was to see the effect of the serum matrix on the detection signal of the assay. It is well know that in case of ELISA the serum samples need to be diluted to reduce the matrix effect on the final detection signal (Nowak et al., 2007). In case of the SALMOTYPE® ELISA, the serum or plasma samples are diluted to a final concentration of 1:100 (section 3.2.2). Assuming that there could be some kind of matrix effect on the final detection signal in the SPR assay, initial experiments were conducted with serum samples at 1:10 final dilutions in the cuvette. Pig serum samples obtained from the farm (supplied by LDL, Leipzig; Germany) having different levels of *S.* Typhimurium infection, as classified by ELISA were chosen for the experiment. Serum samples classified as negative or low (OD % <10), medium (OD % 20-40) and high (OD % > 40) were used for the assay. At this dilution of the serum, it was possible to distinguish between the samples having high (109 AU) and low level (36 AU) of infection. However, the serum sample classified to have medium level of infection resulted in nearly the same detection signal (36 AU) as that of the low or negative sample.

In SPR assays, the detection signal obtained as a result of a binding event is directly proprortional to the concentration of the analyte binding to the biorecognition element immobilised on the surface of the chip (Keusgen, 2002). In this case the concentration of the *S.* Typhimurium antibodies (analyte) present in the infected serum decreased with the dilution step. Thus, the concentration of the antibody in case of the medium level infected serum, at 1:10 dilution is not sufficient to generate

a signal, which is distinguishable from that of the control. Moreover, it was possible to predict the same from the actual ELISA values of the respective samples. The actual ELISA OD % values for each of the undiluted samples used were 75.8 (high), 37.1 (medium) and 6.0 (low). The medium level of infected serum on 1:10 dilution would thus achieve an ELISA value within the range of the control samples.

The solution to this problem was to maintain a sufficient level of antibody concentration in the serum samples at all levels of infection, which would result in differentiated signals at the negative, medium and high levels of infection. In order to test this hypothesis, it was decided to add the infected sera directly into the cuvette.

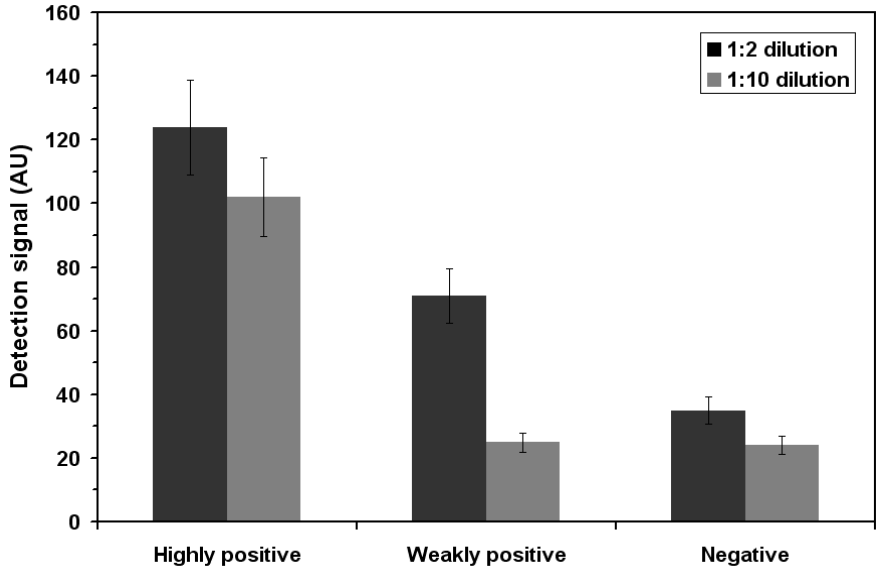

Fig. 4.44: Effect of dilution of pig sera having 3 different levels of *S.* Typhimurium infection – < 10 OD% (negative), 20-40 OD% (weakly positive), > 40 OD% (highly positive) – as obtained using the SPR assay. Direct addition of serum (final dilution 1:2 in the cuvette) provides the best discrimination between the different levels of infected sera.

At this point, it is again important to mention that due to the setup of the Plasmonic® SPR device, addition of sample into the cuvette results in 1:2 dilution. The results obtained for the SPR assay carried out to distinguish between the three levels of infected sera, using undiluted serum samples support our hypothesis presented at the beginning of this paragraph. Using undiluted sera (1:2 dilutions in the cuvette) the assay was able to clearly distinguish between the different levels of infection present in each serum. The results obtained for this study are summarised and presented in Fig. 4.44. Notably, by adding the serum samples directly, without any dilution, the detection signal of the medium level infected sera was now improved by nearly

1.8-fold (63 AU), and it was also clearly distinguishable from that of the negative control. The sensorgrams obtained for the SPR-based detection of the three different levels of infected sera are presented in Fig. 4.45.

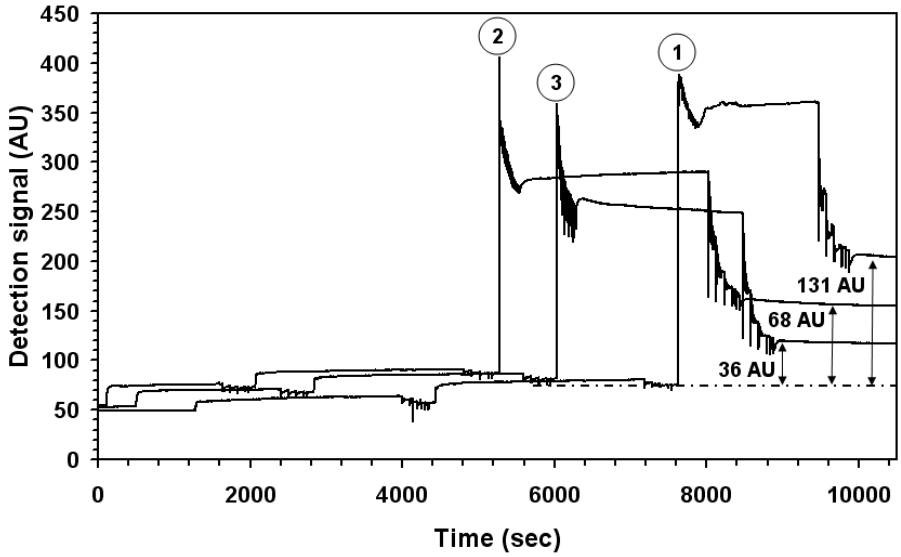

Fig. 4.45: Sensorgram showing curves for the detection of *S.* Typhimurium infection in pigs using ELISA-categorised serum – 1 (75.8 OD%, highly positive), 2 (37.1 OD%) weakly positive), and 3 (6 OD%, negative). The assay does not require any sample preparation and the sera were directly added into the cuvette.

4.2.4.2 Correlation of the assay with commercial ELISA

Based on the above results, all serum samples were directly added into the SPR cuvette. The addition of uninfected serum resulted in an initial change in the sensorgram signal. Change in bulk refractive index of the sample medium caused by the serum components is the most probable explanation for this initial increase in the sensorgram signal. However, after the final washing step, the SPR signal due to control serum was only 32 ± 5 AU. As described above in section 4.2.4.1, sera for 3 different levels of *S.* Typhimurium infection – low (< 10 OD%), medium (20-40 OD%) and high (> 40 OD%) – were tested using the developed assay. The SPR signals obtained with sera falling in the range of low, medium and high level of infection were 43 ± 6 AU, 67 ± 6 AU, and 101 ± 34 AU, respectively. In case of serum samples with > 40 OD%, the lowest level of infected serum tested was 48 OD% and the highest 89 OD%. This explains the high value of the standard deviation in the SPR signals obtained for detection of sera samples in this range. The relative standard deviation (RSD%). of the assay was 12%. The data obtained on

testing all the serum samples using the developed SPR-based assay is presented in Table 4.3.

Table 4.3: SPR and ELISA values of pig sera samples having different levels of *Salmonella* infection. The SPR data was obtained using the developed LPS-based SPR assay on the Plasmonic® SPR device. ELISA was carried using the SALMOTYPE® Pig Screen kit.

Serum Sample	SPR signal (AU)	ELISA value (OD %)	Level of infection
1	34	0	Negative
2	22	0	Negative
3	33	0	Negative
4	33	0	Negative
5	37	0	Negative
6	22	0	Negative
7	36	5.92	Negative
8	34	5.92	Negative
9	35	7	Negative
10	43	7	Negative
11	47	7	Negative
12	37	9	Negative
13	36	10.3	Negative
14	54	17	Medium
15	63	17	Medium
16	57	17	Medium
17	63	34.6	Medium
18	67	37.1	Medium
19	77	37.1	Medium
20	68	37.3	Medium
21	67	37.3	Medium
22	78	37.3	Medium
23	54	48.3	High
24	61	48.3	High
25	67	48.3	High
26	59	63.1	High
27	73	69	High
28	75	69	High
29	131	75.8	High
30	167	75.8	High
31	150	77	High
32	119	77	High
33	120	77.8	High
34	100	77.8	High
35	133	81	High
36	130	81	High
37	132	89.6	High
38	131	89.6	High

The calculated Pearson's correlation coefficient shows a strong correlation ($r = 0.90$, $n = 38$, $p < 0.01$) between the developed SPR assay and the existing ELISA

(Fig. 4.46). Based on the above results, a cut-off value of 50 AU was selected to calculate the sensitivity and specificity of the assay. The cut-off value was defined as the average value of the detection signal obtained for samples within the range 0-10 OD% plus two times the standard deviation. Samples with a detection signal above 50 AU were considered positive. Using this cut-off value, the sensitivity and specificity of the assay were found to be 0.93 and 0.87, respectively, for a total of 38 different tests carried out.

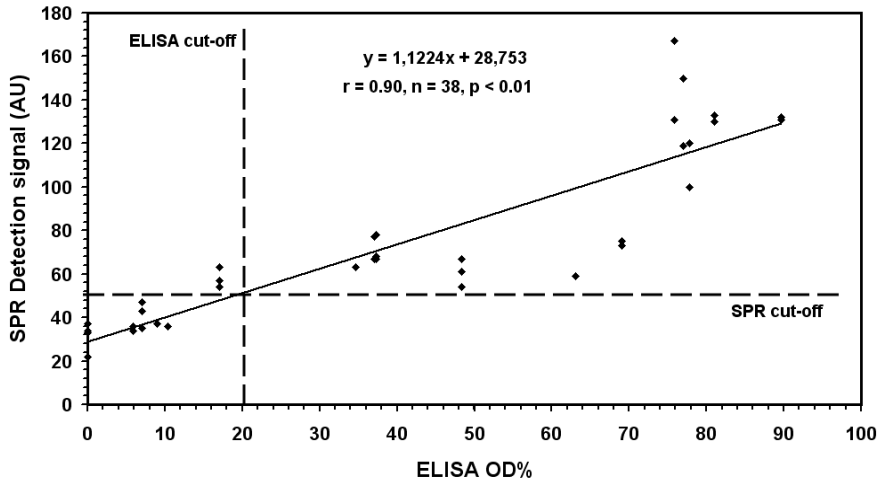

Fig. 4.46: Correlation and regression for detection signals of pig serum samples measured by ELISA (SALMOTYPE® Pig Screen) and SPR (Plasmonic®) for 38 serum samples having different levels of *S.* Typhimurium infection (in some cases a data point may represent more than one sample).

4.2.4.3 Cross-reactivity test

Cross-reactivity of the assay was evaluated against *S.* Choleraesuis and *E. coli* infected serum. Using the SPR assay, serum infected with *S.* Choleraesuis (31.3 OD%) was compared with a *S.* Typhimurium infected serum (37.1 OD%). The detection signal obtained for the *S.* Typhimurium infected serum was 68 AU. The response to *S.* Choleraesuis infected serum (39 AU) was close to that of control serum (36 AU). Similar results were obtained for the SPR assay using pig serum samples spiked with polyclonal (250 μg mL^{-1}) *E. coli* antibodies (35 AU). These results confirm the specificity of the SPR assay for the serological detection of *S.* Typhimurium infection in pigs. The results are summarised and presented in Fig. 4.47.

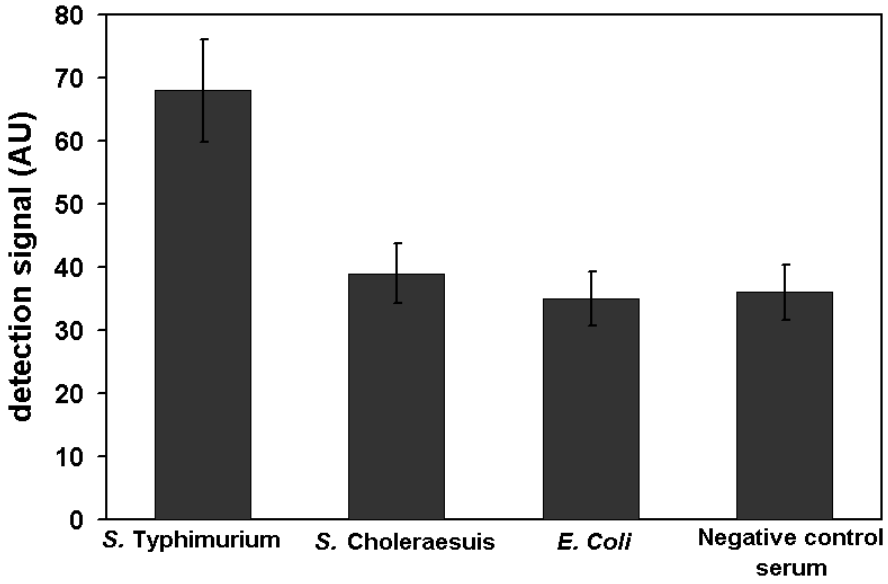

Fig. 4.47: SPR detection signals obtained for cross-reactivity studies with *S.* Choleraesuis and *E. coli* as compared to *S.* Typhimurium infected serum. Detection signal obtained with control serum is also shown.

4.2.5 Mixed LPS assay

The next step in the development of the zoonoses chip was to explore the possibility of creating a chip having multiple *Salmonella* LPS. This aim was to provide a zoonoses chip that would be capable of discriminating between infections caused due to different *Salmonella* serovars.

4.2.5.1 Mixed LPS assay in buffer

Initial assays were carried out in buffer (PBS). The sequential detection mode was explored for the detection of three different serogroup specific antibodies in buffer. For this purpose, a mixture of LPS from *S.* Typhimurim, *S.* Choleraesuis and *S.* Anatum were immobilised on the surface of a hydrophobic (C_{18}) SPR chip, by self-assembly (section 4.2.1). The most commonly used antigen mixture in mixed ELISA is a combination of LPS from *S.* Typhimurium and *S.* Choleraesuis recommended by the Danish Veterinary Institute (Nielsen et al., 1998). In addition to both these LPSs we included LPS from *S.* Anatum in the LPS mix. The concentration of each LPS in the mixture was 10 µg mL^{-1}. The corresponding group antibodies selected for the assay were group B, group C and group E, respectively. These

ready-to-use antibodies, also used for serogrouping of *Salmonella* spp., were used at a dilution of 1:2.5, resulting in a final dilution of 1:5 in the cuvette.

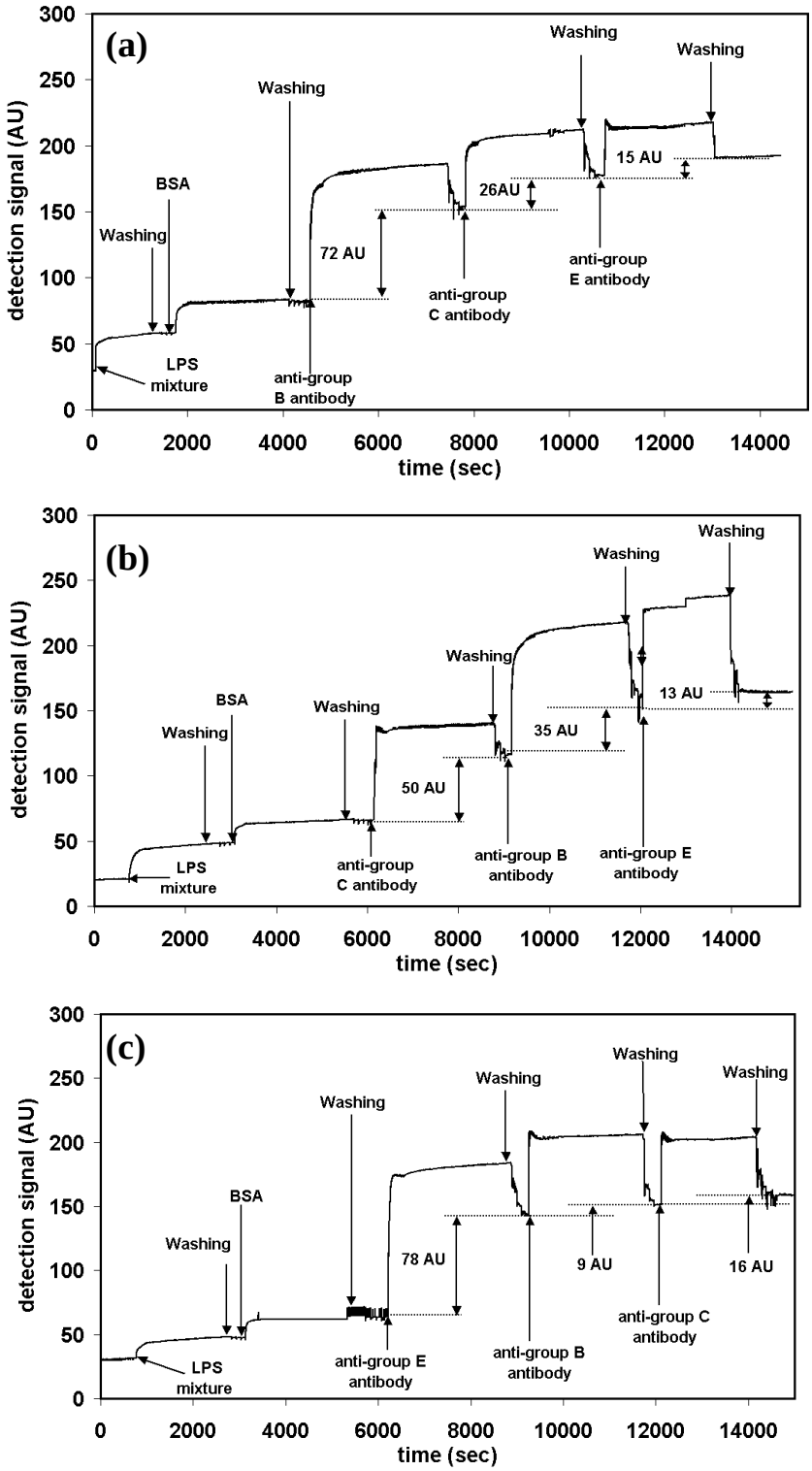

Fig. 4.48: Sensorgram obtained for the sequential detection of group specific antibodies using a mixed LPS-based zoonoses chip. The data shown are for the antibody addition sequence: (a) group B - group C – group E (b) group C – group B – group E and (c) group E – group B – group C.

When each of these antibodies was added sequentially, irrespective of the order of addition, it was found that a strong detection signal was possible only at the first antibody addition step. For the addition sequence: group B – group C – group E the detection signals obtained were 72 AU, 26 AU and 15 AU respectively. For the addition sequence: group C – group B – group E the respective detection signals were 50 AU, 35 AU and 13 AU. Similary, when the antibody sequence: group E - group B - group C was used, the detection signals obtained were 78 AU, 8 AU and 16 AU. The sensorgrams for each of the runs is presented in Fig. 4.48. The results obtained above are clearly explained by the rule established for sequential detection in section 4.1.4.4.

Controls run with serum from uninfected pigs on the mixed LPS chip resulted in a detection signal of 36 ± 2 AU (Fig. 4.49).

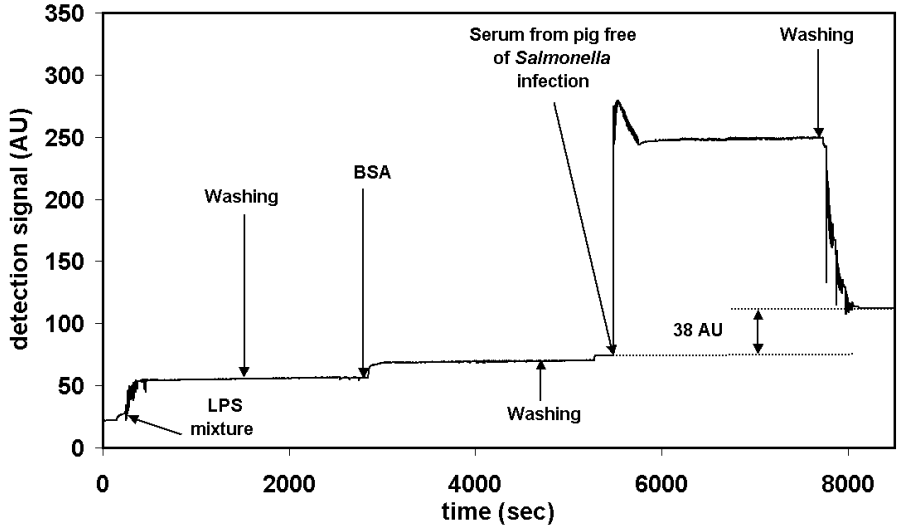

Fig. 4.49: Sensorgram obtained on addition of serum obtained from pigs free of *Salmonella* infection onto a mixed LPS-based zoonoses chip.

4.2.5.2 Mixed LPS assay in serum

After carrying out the initial studies using group specific antibodies, the study was further extended to detect *Salmonella* infections in sera obtained from pigs. The mixed LPS chip was used to evaluate serum samples obtained from pigs infected with either *S.* Typhimurium or *S.* Choleraesuis. The mixed LPS chip had the same final concentration of 10 µg mL^{-1} of each LPS as used above for the assay with buffer. As the assay in buffer showed that sequential detection was not possible, the assay with the serum samples was carried out in the multi-channel detection mode.

S. Typhimurium and *S.* Choleraesuis.sera (hyperimmune sera) having ELISA values > 40 OD % were used for the assay. The detection signals obtained were 85 ± 5 AU for *S.* Typhimurium infected sera and 106 ± 1 AU for the *S.* Choleraesuis infected sera (Fig. 4.50).

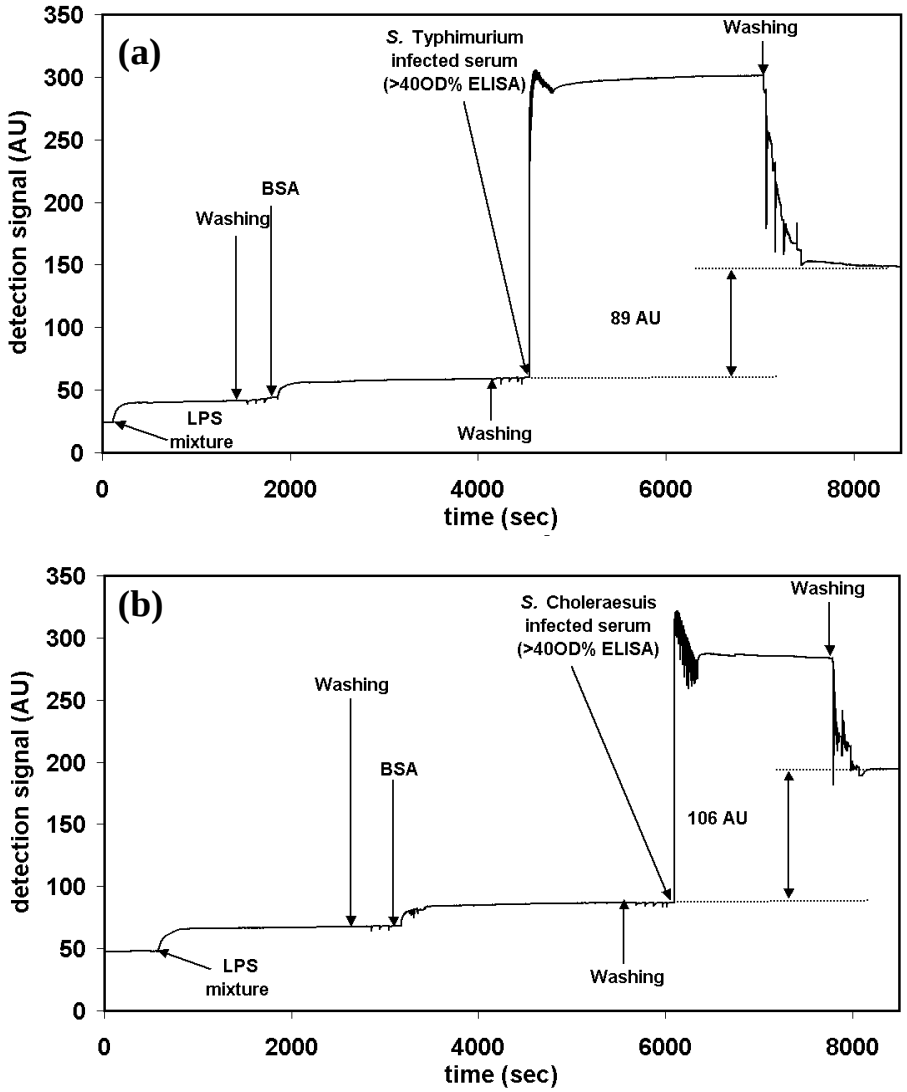

Fig. 4.50: Sensorgrams obtained for the detection of *Salmonella* infections in pigs using a "mixed LPS-based" zoonoses SPR chip (a) sensorgram obtained using serum from *S.* Typhimurium infected pig (b) sensorgram obtained using serum from *S.* Choleraesuis infected pig

Further, *S.* Typhimurium infected sera were run on a chip coated with LPS from *S.* Choleraesuis and alternately *S.* Choleraesuis sera were run on a chip coated with LPS from *S.* Typhimurium. The corresponding detection signals obtained were 46 ± 2 AU and 43 ± 4 AU. These signals were due to the unspecific binding of the serum matrix to the chip surface. The results obtained were well within the cut-off value of 50 AU set for the specific detection of *S.* Typhimurium infection using SPR

(section 4.2.4). Thus, the specificity of the serum antibodies to the respective LPS was confirmed by this experiment. In addition, the data show that the same cut-off value and hence the ELISA correlation, as established for the assay used for specific detection of *S.* Typhimurium infection using SPR, is also valid for the "mixed LPS" SPR assay. The data obtained is summarised in Table 4.4.

Table 4.4: Comparative data of the SPR detection signals obtained for the detection of serum from pigs infected with *S.* Choleraesuis and *S.* Typhimurium, using the mixed LPS assay and the single LPS assay.

Serum samples from pigs infected with	SPR Chip (Mixed LPS, *S.* Choleraesuis + *S.* Typhimurium)	SPR Chip (LPS *S.* Choleraesuis)	SPR Chip (LPS *S.* Typhimurium)
S. Choleraesuis (> 40 OD % ELISA)	106 ± 1 AU	106 ± 1 AU	46 ± 2 AU
S. Typhimurium (> 40 OD % ELISA)	85 ± 5 AU	43 ± 4 AU	85 ± 5 AU

4.2.6 Detection of *Salmonella* infection in humans

In order to determine if the same LPS-based SPR assay could be used for the detection of *Salmonella* infections in humans, experiments were carried out using a zoonoses chip containing immobilised *S.* Typhimurium LPS. As a proof of principle, human serum (from male AB plasma, Sigma) was spiked with the polyclonal anti-*Salmonella* antibody.

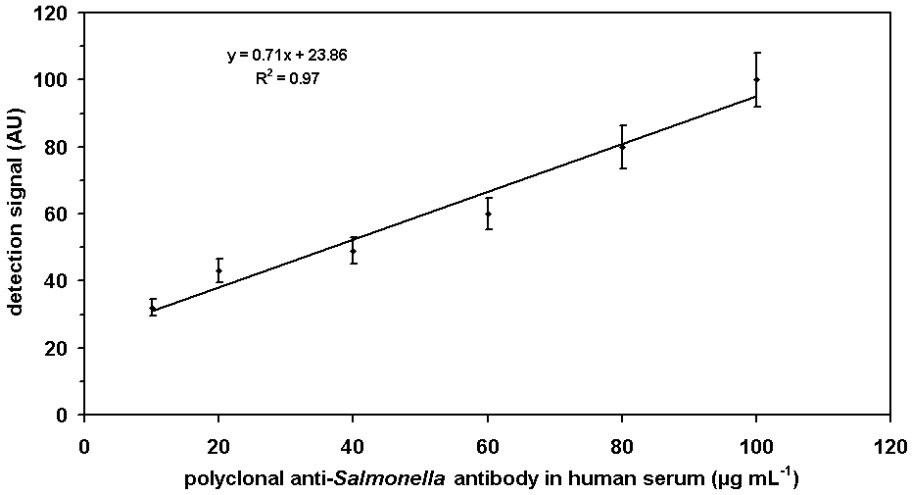

Fig. 4.51: Linear detection range of the SPR-based assay for the detection of *S.* Typhimurium antibodies spiked in human serum.

Different concentrations of the antibody were prepared and detection was carried out using the zoonoses chip. The LPS concentration on the surface of the chip was

10 µg mL^{-1}. The steps of the assay were similar to that used for detection of *Salmonella* infection in pigs (section 4.2.4). The results obtained show that it is also possible to detect the anti-*Salmonella* antibodies when present in human serum. The detection range of the assay extends from 10 µg mL^{-1} to 100 µg mL^{-1} (Fig. 4.51).

The corresponding SPR signals obtained were 32 ± 2 AU and 103 ± 4 AU, respectively. The lower limit of detection obtained for the assay was 20 µg mL^{-1} (49 ± 3 AU). The detection signal due to control was 22 ± 3 AU. Similar to the assay for the detection of *Salmonella* infections in pigs this assay was not affected by the human serum matrix. The sensorgram obtained for one of the runs along with a control (using serum free of the antibody) is presented in Fig. 4.52.

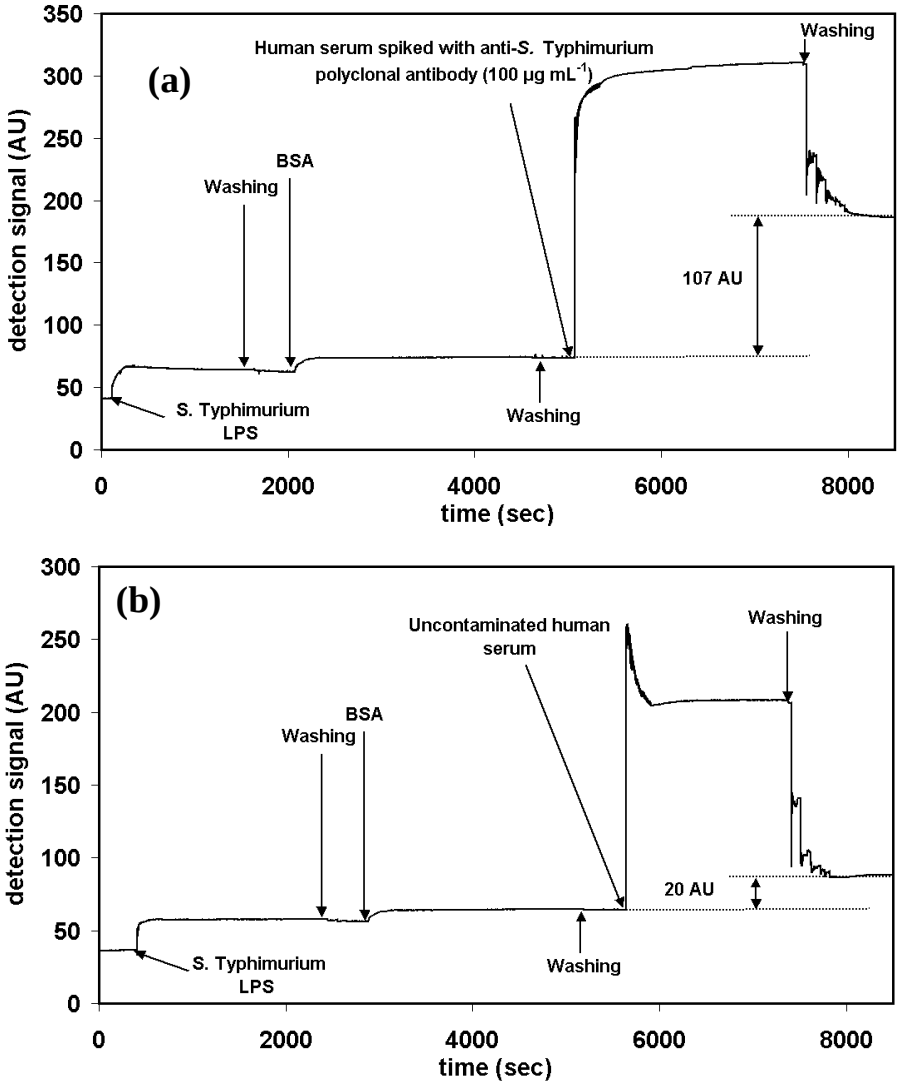

Fig. 4.52: Sensorgrams obtained for the detection of anti-*Salmonella* antibodies in spiked human serum using a *S.* Typhimurium-specific zoonoses SPR chip (a) sensorgram obtained for human serum sample spiked with 100 µg mL^{-1} of polyclonal anti-*Salmonella* antibody (b) sensorgram obtained using uncontaminated human serum.

5 Discussion

5.1 Antibody immobilisation strategy

The first and the most critical step for the development of an SPR-based immunosensor is to successfully immobilise the biorecognition element on the gold surface of the SPR chip (section 1.1.2.3). In this work, the most successful capture antibody immobilisation strategy established for the detection of *Salmonella* using the SPR-based immunoassay was found to be through hydrophobic interaction on a C_{18}-chip (section 4.1.1.7). Alkoxysilanes have been used successfully for the creation of hydrophobic surfaces on glass (Enescu et al., 2004). Owens et al. (2002) demonstrated the possibility of the formation of alkylsilane networks on gold by exposing the gold surface to alkylsilanes in an ultra high vacuum chamber. The advantage of the use of alkylsilanes, over the more conventionally used alkyl thiols, has been discussed by the authors. Alkanethiol and alkylsilane layers exhibit different oxidation behaviours. Oxidation of sulfur atoms causes alkanethiol monolayers to fall apart, while oxidation of alkylsilane layers knit the silicon atoms together by forming a siloxane network. In our case we were able to obtain a stable hydrocarbon layer on the gold surface by using octadecyltrimethoxysilane on a gold surface which had been activated to introduce hydroxyl groups (section 3.2.4.4). A stable covalent bond is assumed to be formed between the oxygen atom of the hydroxyl function on the gold surface and the silicon, by hydrolysis of the methoxy groups. In other words the hydrolysable methoxy groups of octadecyl trimethoxysilane condense with the hydroxyl groups present on the activated gold surface, releasing methanol and leaving the silicon network with the octa-decyl (C_{18}) carbon chain on the gold surface. Our method thus eliminates the requirement of any ultra high vacuum as described by Owens et al. (2002). The integrity of the C_{18} layers on the gold surface is further enhanced by formation of siloxane networks due to hydrolysis of the remaining methoxy groups. Our explanation for the overall success of the assay using this immobilisation strategy is that as the immobilisation of the antibody occurs under physiological conditions, and does not involve contact with any other chemical reagents, the activity of the antibody is not reduced or destroyed. The other immobilisation methods involve activation, of either the antibody (section 3.2.3) or the functional groups on the surface of the chip (section

4.1.1). Water contact angle measurements performed on the C_{18} chip confirmed the highly hydrophobic nature of the chip. The water contact angle obtained for the C_{18} functionalised gold surface was $103^o \pm 2.4^o$ as compared to a value of $59^o \pm 4.7^o$ for a cleaned activated gold surface prior to C_{18}-functionalisation (section 4.1.1.7.1). Consequently, the antibodies are not only tightly bound to the surface by the hydrophobic interactions but the density of the antibodies on the surface is also high. The proof of high surface coverage of the SPR chip by the immobilised antibodies is additionally obtained from the observation, that on addition of BSA (blocking agent) after the antibody immobilisation step no significant signal due to binding of BSA to the surface is observed. This high surface coverage of the C_{18} surface along with addition of BSA as a blocking agent was effective in reducing the unspecific binding of the secondary detection antibody. In comparison higher detection signals were obtained for the unspecific binding of the secondary detection antibody using the other immobilisation strategies (Table 5.1).

Table 5.1: Detection signals obtained for the detection of *S.* Enteritidis using different immobilisation strategies of the capture antibody.

Strategy	Functional group of antibody involved	Sensor surface chemistry	Detection Signal (AU) *S.* Enteritidis (A)	Detection Signal (AU) Control (B)	Net detection Signal (AU) (A-B)
a)	Carboxyl	Amine	86 ± 4	29 ± 2	57 ± 5
b)	Amine	Carboxyl (EDC/NHS)	102 ± 5	49 ± 2	53 ± 7
c)	Carbohydrate	Amine	64 ± 3	23 ± 1	41 ± 4
d)	Fc part	Protein A on Carboxyl (EDC/NHS)	54 ± 3	22 ± 1	33 ± 3
e)	Carboxyl	Cobalt on Carboxyl	33 ± 2	18 ± 1	15 ± 2
f)	Biotinylated (amine)	Biotin	37 ± 3	10 ± 3	25 ± 2
g)	Hydrophobic groups	Alkylsilanes (C_{18})	75 ± 4	2 ± 1	73 ± 4

Among the other immobilisation strategies explored the use of EDC/sulfo-NHS chemistry for coupling (i) the antibody through its carboxyl function to amino functions on the surface of an amine-functionalised chip and (ii) antibody amino function to carboxyl function on the surface of a carboxyl chip, were both partially successful in providing an immunosensor for the detection of *Salmonella* (Table 5.1).

The coupling of proteins (antibodies) using the EDC/NHS activation of the carboxyl groups on carboxymethyl dextran chips has previously been carried out (Johnsson et al., 1991). The concentration of EDC and NHS was optimised and the effect of the presence of NaCl on the activation of the carboxyl functional groups has been investigated. It was shown that in presence of NaCl (0.2 M) activation of the carboxyl groups is hindered. This was due to the disruption in the ion pair formation between the carboxyl group and the EDC, at concentrations below 0.2 M of the EDC. It is assumed that the formation of the ion pair is an important step leading to the formation of the O-isoacylurea. Thus, based on this information, in our experiment which was carried out in PBS (containing 0.13 M NaCl) the concentration of EDC used was 0.4 M. This concentration is high enough to overcome the inhibitory effects of NaCl present in PBS. Johnsson et al. (1991) also studied and optimised the concentration of NHS required for success of this immobilisation strategy. It was found that at NHS concentrations of > 40 mM the rate of conversion of the O-acylisourea to NHS ester is favoured. At NHS concentrations of < 40 mM, water was found to compete with NHS and thus resulting in the hydrolysis of the O-acylisourea, and consequently reducing the coupling yield. The authors also reported that an activation time of 7 min using EDC/NHS converts approximately 30 % to 40 % of the carboxyl groups. Based on the above literature the concentrations of the chemicals and the conditions used in our experiments were justified. To summarise, we used 0.4 M EDC and 0.1 M sulfo-NHS and the activation time of the carboxyl groups on the SPR chip was between 15-20 min. We preferred sulfo-NHS over NHS as it has a better solubility in water and also the resulting sulfo-NHS esters show better solubility in water than their NHS counterparts. However, in case of our assay the secondary detection antibody showed high unspecific binding to the surface of the chip (section 4.1.1.1 and Table 5.1). The probable explanation for this is the incomplete inactivation of the unreacted sulfo-NHS esters by the ethanolamine added as a blocking agent. The use of a higher concentration of ethanolamine or increasing the incubation time with ethanolamine can be explored. Incorporation of an additional blocking step with BSA is another alternative to overcome this problem of unspecific binding. These additional steps will result in increase in the time of the assay and hence has not been included as part of this work.

The results obtained using the immobilisation strategy based on the oxidation of the carbohydrate side chain of the antibody using periodate ($NaIO_4$) followed by coupling to reactive amine groups on an amine-coated chip (section 4.1.1.3) was also only partially successful (Table 5.1). A high unspecific binding of the detection antibody was observed in comparison to that obtained on the C_{18} surface. The use of this method of antibody immobilisation for oriented immobilisation of antibodies has been reported in literature (Qian et al., 1999). The authors address the unspecific binding of the secondary detection antibody by using a blocking step that uses 2% BSA and an incubation time of 2 h. In our case the concentration of BSA used for blocking in all the experiments is 100 µg mL^{-1} and the incubation time is 15-20 min. Given the basic requirement that the SPR-based assays need to be rapid the option of using a higher incubation time for the blocking step is not justified. The authors also suggest an incubation time of 6 h for immobilising the activated antibody on the chip surface, which again is not feasible in our case. The overall net detection signal of only 41± 4 AU (Table 5.1) for the assay also indicates a probable partial loss in binding capacity of the immobilised capture antibody caused due to the modifications carried out on the carbohydrate side chain. Given the results and the constraints in carrying out this immobilisation strategy we conclude that this strategy is not suitable for our specific application. Thus from the comparison of the results obtained using the different immobilisation strategies presented in Table 1.5 it turned out that best results in terms of capturing and detection of *Salmonella* were achieved by immobilisation via hydrophobic interaction.

5.2 Detection of *Salmonella* using SPR

An SPR-based biosensor for the rapid detection of *Salmonella* spp. has been developed in course of this work. The assay can be used both for generic detection of *Salmonella* spp. as well as specific detection of *Salmonella serovars*. Most importantly, for the first time the application of SPR-based detection of *Salmonella* was successfully demonstrated for detection of *Salmonella* serovars in sample matrix. Milk was used as a model system in this study.

A recent review (Ricci et al., 2007) has highlighted the importance of the work presented here on the detection of *Salmonella* in milk. The authors have clearly pointed out that prior to the publication of our work, the final demonstration of the

real applicability of SPR-based systems in food analysis was missing. This review clearly points out that until this time SPR immunosensors developed were proved for the detection of pathogens (in buffer systems) and toxins, and there was hardly any work done to address sample application and method validation in real food matrix. Our own literature search at the beginning of this work also brought forth this important missing aspect in the area of development of SPR biosensors (section 1.1.2.7). During the same time and after the publication of our work (Mazumdar et al., 2007) a number of different research groups have published their work, which addresses the issue of detection of pathogens in food matrix (Homola, 2008).

The assay developed was as a sandwich immunoassay. The use of the sandwich assay mode of detection in case of our assay is clearly justified when a comparison is made of the results obtained in the direct mode and the sandwich mode. Using the direct mode of detection it was possible to detect *S.* Typhimurium only up to a cell concentration of 5×10^5 cells mL^{-1} and *S.* Enteritidis upto a cell concentration of 5×10^8 cells mL^{-1} (section 4.1.2). However, on using the sandwich mode of detection it was possible to detect *S.* Typhimurium and *S.* Enteritidis down to cell concentrations of 1.25×10^5 cells mL^{-1} and 1.25×10^8 cells mL^{-1}, respectively. A number of authors have reported improved detection limits for SPR-based detection of bacteria (Oh et al., 2004a; Waswa et al., 2006; Waswa et al., 2007). The use of protein A or protein G for oriented immobilisation of the capture antibody, by binding to the Fc portion is one such strategy used to improve the detection limit for SPR-based bacterial detection. Waswa et al. (2006) reported results obtained using a protein A-functionalised chip for the detection of *S.* Entritidis and *E. coli* spiked in milk. The detection limit in the direct detection mode is reported to be 25 cfu mL^{-1} for *E. coli* and 23 cfu mL^{-1} for *S.* Entritidis, respectively. However, the authors do not provide any detailed explanation or the reasons behind this improvement in detection limit over other previous attempts using the same strategy. Oh et al. (2004b) used protein G to immobilise and orient the antibodies against *S.* Paratyphi on gold surface, followed by direct detection of the bacteria. In this case the detection range was reported to be between 10^2-10^7 cfu mL^{-1}. In our work the use of protein A to orientation the capture antibody was also explored. The results obtained do not show any added advantage of using protein A in our assay (section 4.1.1.5). The immobilisation of protein A was carried out using EDC/sulfo-NHS chemistry on a carboxyl-functionalised chip similar, to that used by Waswa et al. (2006). The only

difference being that the authors used a chip functionalised with carboxymethyl dextran, which provides a 3-dimensional matrix for immobilisation of the antibody. This is the most probable explanation for the improved detection limit of their assay. Using a 3-dimensional matrix results in higher surface density of immobilised antibodies (Johnsson et al., 1991). However, the requirement for detection of *Salmonella* in foods is to detect 1 cfu mL^{-1} of the bacteria (Baylis et al., 2000), and this is only possible using a pre-enrichment technique prior to biosensoric detection. Given the detection limits of the above strategies on oriented immobilsation of the capture antibody the use of a pre-enrichment step is still required. Thus, the use of protein A and protein G in case of *Salmonella* detection is not justified unless using this approach, the use of the pre-enrichment step can be avoided.

In the past SPR assay systems evaluated for the possible detection of *Salmonella* cells in matrices collected from food-producing animals (Bokken et al., 2003). In this case, the LLD was reported to be 1×10^7 cells mL^{-1}, using a flow-through Biacore® system and carboxymethylated dextran chip. The authors have also expressed their LLD as 1.7×10^3 cells mL^{-1} per test portion of 10 µL, which in our case would translate to 1.25×10^3 cells mL^{-1} for the assay in both buffer system and in milk. A similar assay for detection of *S.* Enteritidis in buffer system had a detection limit of 1.0×10^6 cells mL^{-1} (Koubova et al., 2001). The detection limit of a commercial ELISA-based *Salmonella* detection kit, which includes a sample pre-enrichment step, is reported to be 1.8×10^6 cells mL^{-1} (Blackburn et al., 1994). The LLD of 1.25×10^5 cells mL^{-1} for this assay in a complex matrix like milk is an improvement over all the other assay systems mentioned here. The existing detection limit of our assay would easily enable detection of *Salmonella* in foods, where the initial low number of cells is grown to a detectable level using pre-enrichment techniques (Baylis et al., 2000). The linear detection range of the assay extends up to 2.5×10^6 cells mL^{-1}. The cross-reactivity study, using *E. coli* K12 cells, was also found to prove the specificity of the assay in milk (section 4.1.3.3). No significant detection, in comparison to milk spiked with *S.* Typhimurium, was obtained when the *E. coli* spiked milk sample was checked using the same assay. The positive results of this SPR assay in milk is of significance because there have been problems encountered in developing assays for detection of *Salmonella* in milk. Presence of PCR inhibitors in milk and milk products has been the problem in case of PCR-based assays (Perelle et al., 2004; Baylis et al., 2000). All multiplex PCR assays presently

require the inclusion of internal amplification controls to take care of PCR inhibitors (Hoorfar et al., 2003), thus, adding to the complexity of the assays. The entrapment of the antibody-coated magnetic beads by fat and protein in milk was the cause of failure of an immunomagnetic-electrochemiluminescent assay (Yu and Bruno, 1996). No such matrix effects are encountered in our assay because of the presence of either fat or protein in milk; and there is no compromise on the sensitivity of the assay.

The sandwich SPR immunoassay presented in this work can be used in both the polyclonal detection mode (section 4.1.3.1) as well as the monoclonal detection mode (section 4.1.3.2) based on the specific application requirements. This SPR-based assay can be used for quick screening of various *Salmonella* strains in the polyclonal capture and detection mode. When specific information and confirmation of the serovars is required, it can be obtained by simply replacing the polyclonal detection antibody with a monoclonal detection antibody. The monoclonal antibody can be chosen to detect a specific serovar of interest. The presently used confirmatory tests for *Salmonella* (Rose, 1998) consists of a number of biochemical and serological tests, and are time (7–10 days) consuming and thus impractical for routine use in the food industry. These confirmatory tests, though time consuming, are definitive and extremely sensitive and there is no way that the SPR assays can replace them. However, use of SPR-based assays, in both the polyclonal and monoclonal detection modes, can definitely provide a practical alternative for the monitoring of *Salmonella* and other pathogens in the food industry.

The developed SPR-based immunoassay was also seen to follow some of the phenomenon observed in cases of other immunoassays. The signal saturation of the detection antibody observed in case of the sandwich assay, for determination of *Salmonella,* at higher concentrations of the bacteria (section 4.1.2.1.2) can be explained by the phenomenon of high-dose hook effect. This is a well-known phenomenon observed in case of immunoassays. This is caused by excessively high concentrations of analyte simultaneously saturating both capture and detection antibodies. This prevents the formation of detectable capture antibody/analyte/detection antibody complexes (Davies, 2005). Beyond the saturation point there is usually a decrease in the detection signal with increase in the analyte concentration.

In case of the sandwich assays involving the specific detection of *Salmonella* in milk there is a difference in the detection signals observed for different serovars (section

4.1.4.2). This observation was also valid when using purified LPS in case of *S:* Typhimurium and *S*. Enteritidis. The variability in detection limits between the two serovars can be attributed to the difference in affinity of the two detection antibodies towards the respective bacteria. Reports of differences in affinity of different anti-*Salmonella* antibodies against the O-antigens of *Salmonella* are available in literature (Elkins et al., 1984; Robbins et al., 1965). There is also literature available showing the existence of microheterogeneity in the LPS O-chain of the *Salmonella* serovars Enteritidis and Typhimurium (Parker et al., 2001).

However, these inherent differences in antibody detection signals for different serovars of *Salmonella* is an important factor which needs to be taken into consideration when designing the assays in the sequential detection mode. The results obtained from the studies carried out in the sequential detection mode have brought forth some interesting and important facts related to SPR immunoassays. When sequential detection mode was used to detect a mixture of two different *Salmonella* serovars it was found that a signal reduction occurred for the second detection antibody. The same antibody when added as the first detection antibody resulted in a higher detection signal. This phenomenon is explained as follows:

In case of SPR, an evanescent field is generated at the metal/dielectric interface by the surface plasmon wave (section 1.1.2.1). The unique characteristic of the evanescent field is that the field amplitude is greatest at the interface and exponentially decays as a function of distance from the metal/dielectric interface (Ekgasit et al., 2004). For biomolecular interaction studies using SPR, the evanescent field intensity is effective only up to a depth of 100-200 nm (Lundstrom et al., 1994). *Salmonellae* belong to the family Enterobacteriaceae and are typically 1-5 μm in diameter (Le Minor, 1984). Hence, the size of the bacteria places the bulk of the bound cells outside the SPR evanescent field, much beyond the effective penetration depth of 100-200 nm. This is the main reason for high detection limits obtained for bacterial detections using SPR (Perkins and Squirrell, 2000). Consequently, in the sequential detection mode, further addition steps beyond a particular point would result in a lowering of the detection signal, due to the presence of analytes outside the effective evanescent field. This explains the reduction in signal obtained for the second detection antibody in the sequential detection mode. Hence, when developing an assay in the sequential detection mode, it is important to first screen both the antibodies separately and determine the intensity of the respective detection signals.

As there is early signal saturation and a consequent decrease in the second signal in the sequential detection mode, it is appropriate that the serovar having the lower detection signal is detected in the first place. Thus, based on the understanding of the results obtained for the sequential detection mode we have put forth a rule for sequential detection (section 4.1.4.4). The rule for sequential detection of two *Salmonella* serovars states that the serovar with the lower detection signal has to be detected first, followed by the other serovar of interest (Barlen and Mazumdar, 2007). These observations are in agreement with the only available published data, for the sequential detection of a mixture of anti-BSA antibodies and horseradish peroxide using SPR (Chung et al., 2006).

The time required for the analysis of each sample, including the capture antibody immobilisation step, using the Plasmonic® SPR device is only 1 h. In case of a rapid *Salmonella* detection assay using immunomagnetic separation and ELISA end detection, the time required for only the detection step is reported to be 3 h (Mansfield and Forsythe, 2000). Also the benefit of having the real-time visualisation of binding events as a sensorgram makes the SPR-based assay more robust. It is possible to quickly identify the cause of any deviation in the assay by analysing the sensorgrams. This is not possible in existing immunological methods and usually identifying the cause of failure is quite time consuming and depends on the experience of the operator. The Plasmonic® SPR device has a cuvette system and mixing mechanism for the samples. The autosampler also acts as an intermittent mixing device during the binding events, thus minimising any possible diffusion effects. This arrangement allows sufficient time to all the bacterial cells in the sample, to distribute uniformly on the surface of the SPR chip and then bind to the immobilised capture antibody. The present assay can be used in a fully automated mode with the ability to rapidly analyse a large number of samples. Another important advantage of the SPR-based assay over ELISA is its ability to specifically detect different *Salmonella* serovars even when present as a mixture in a given sample (section 4.1.4). Using ELISA it was not possible to detect individual serovars in samples, which were contaminated with more than one *Salmonella* serovar (Ng et al., 1996). The authors suggest that each of the serovars be first isolated for attaining reliable serogroup differentiation using ELISA.

5.3 Serotyping of *Salmonella* using SPR

The conventional serological technique SAT (section 1.2.1.3) has long been recognised as being labour-intensive (Cai et al., 2005), and various modifications of the method, including attempts of automation, are reported in literature (Gruenewald et al., 1990). Each antigen determination requires a fresh set of reagents and incubation conditions. The use of fluorescent-labelled antisera for serotyping has also been explored (Caldwell et al., 1966). The SPR-based serotyping is label free. PCR-based methods have been developed for serotyping of *Salmonella* (Herrera-León 2007, Fitzgerald et al., 2003, Luk et al., 1993). These molecular methods are highly sensitive, very specific, and reproducible. However, the shortfalls that we see in the PCR-based methods are that they are time consuming and involve multiple steps. Serotyping of each serovar using PCR involves first sequence analysis of both the genes responsible for expressing the O- and H-antigens. This has to be done each time a new isolate is to be serotyped. This is followed by primer design based on the DNA sequence information. The bacterial DNA is then extracted and the amplification carried out using the normal PCR protocol (section 1.2.4.4). Finally, identification of the amplified DNA for data interpretation has to be carried out by running an agarose gel. In comparison, we have proved that using the SPR-based method rapid serotyping of *S.* Enteritidis was possible without any sample preparation. The assay showed exquisite specificity as evidenced by the ability to discriminate between the different antigens while no cross-reactivity was observed. Thus, the SPR-based serotyping is able to deliver results at par with the PCR-based serotyping. The only case where PCR has a definite advantage over SAT and SPR is in the serotyping of the rough strains of *Salmonella*.

Some strains of genus *Salmonella* are referred to as “rough”. These strains of *Salmonella* lack the O-specific antigens. This occurs as a result of mutation in the genes responsible for synthesis of the O-antigens (Raetz, 1996). Genes specific for synthesis of the O-units are located in the rough B (*rfb*) gene cluster, so named because strains lacking the O-antigens produce colonies with a “rough” appearance. The O-antigens are closely related, with only very small differences, *e.g.*, the O-antigens of groups A, B and D differ only in a dideoxyhexose side chain sugar, which is paratose, abequose or tyvelose, respectively, and have similar structures and

biosynthetic pathways (Reeves, 1993). Rough strains are known to self-agglutinate and it is not possible to serotype them using SAT without a subculturing step.

SPR-based serotyping using the rough strain of *S*. Infantis (section 4.1.5.1.2), which belongs to serogroup C, was successful in identification of the bacteria as belonging to the genus *Salmonella*. Positive response obtained for the polyclonal anti-*Salmonella* antibody Poly A-E, to the rough strain of *S*. Infantis is explained as follows:

The outer core region of all *Salmonella* LPS are known to have a common polysaccharide structure (Raetz and Whitefield, 2002; Raetz, 1990). The Poly A-E antibody mixture besides being specific for O-antigens is also polyclonal and has paratopes reacting to the common core polysaccharide region of the *Salmonella* LPS. Thus, a negative response of the bacteria to serogroup C antibody proved that it was a rough strain and lacked the O-specific antigen. The positive reaction to Poly A-E provided the information that it belongs to the genus *Salmonella*. Thus, unlike in SAT, using SPR it is possible to obtain quantitative information about a rough *Salmonella* strain in spite of the absence of the O-antigen. The strain can also be further probed for the identification of the H-antigens, which is not possible for rough strains using SAT. In case of SAT, after the initial self-agglutination test identifies a given *Salmonella* strain to be rough, the next step usually involves isolation of smooth strains by subculturing on blood agar or Mueller-Hinton agar in order to recover the smooth state of the strain, and repeat the agglutination test (Anonymous, 2004). After isolation of the smooth state of the strain, SAT is carried out using Poly O-antisera to determine if the strain under consideration is *Salmonella*. Thus, using the developed SPR-based serotyping, this information about a rough strain is obtained without the need for any subculturing step.

The use of PCR in identification of rough strains of *Salmonella* has been successfully demonstrated (Hoorfar et al., 1999). Primers specific for each serogroup have been designed from the sequence of the respective *rfb* gene cluster (Wang et al., 1992, 1992, Kongmuang et al., 1994). However, PCR for serogrouping can be performed only after carrying out the preliminary *Salmonella* PCR that confirms the species. Alternatively, biochemical confirmatory tests need to be performed which, of course, are time consuming. The *Salmonella* PCR is proved to work successfully only in isolates and amplification may be inhibited when performed in biological or food matrices. We have successfully demonstrated that the SPR-based serotyping is able

to identify *Salmonella* spp. even if the strain is rough and also shown that the method is unaffected by biological or food matrices. This is not possible even with SAT and requires isolation of the smooth form of the strain. Given these advantages of the SPR-based serotyping, it has the potential to be used as a preliminary screening tool to complement the PCR-based serogrouping of rough strains.

The data on serotyping using sequential detection presented in this work for the serotyping of *S.* Enteritidis also brings forth an important aspect of SPR. It is clearly seen (Table 4.1, section 4.1.5.2) that, when the anti-O9 detection antibody was used as the second detection antibody, there was a wide variation in the detection signal, depending on how the first detection antibody interacts with the captured bacteria. Antibodies having similar antigen binding domains (paratopes) as that of the anti-O9 detection antibody resulted in significant reduction in the detection signal of the anti-O9 detection antibody. This is clearly seen from the data obtained for group D-specific antibody and antibody Poly A-E when used as first detection antibodies. This observation was also true for antibody Hg,m. One of the reasons being that the epitopes on the bacteria for the anti-O9 detection antibody are already saturated by these first detection antibodies (in this case group D and Poly A-E). In addition, there is an increase in distance, away from the sensor surface, which cause the second detection antibody to move beyond the limits of the evanescent field. When the first detection antibody did not bind to the bacteria, the resulting detection signal of the second O9 detection antibody was significantly higher. This observation is valid for the first detection antibodies anti-O4, anti-O46 and anti-Hq,s,t,p,u. This differential binding of different antibodies to a given antigen, as observed by SPR forms the basis of quick screening of monoclonal antibodies to identify their epitope specificity pattern (Johne, 1998).

The SPR-based serotyping assay described in this work uses bacteria at a concentration of 1×10^{10} cells mL^{-1}. In case of serotyping by SAT the bacteria to be serotyped are first isolated and then grown to a high concentration of cells, usually overnight, in a non-selective medium (Anonymous, 2003c and 2004). However, as shown this SPR-based serotyping method can be modified and used as a detection technique for the detection of *Salmonella* contamination in different matrices (section 4.1.3). The different antibodies, used in serotyping can easily be used for detection of *Salmonella* contamination and in which case their limits of detection can be established. The SPR-based serotyping can be used to create a quantitative

database of reactivity for different antibodies to different *Salmonella* strains. The data on antibodies can be used in rapid selection of antibodies for developing immunoassays (SPR-based, ELISA etc.) for detection of *Salmonella*.

Serotyping is an integral part of all *Salmonella* surveillance programmes across the world. Serotyping has been recognised as a critical component of public health response to salmonellosis (Herikstad et al., 2002) and is a universal language used for laboratory isolate-based surveillance of *Salmonella*. The use of this common language has enabled quick communication of serotyping data to countries across the globe, thus enabling timely control of major epidemic outbreaks of salmonellosis (Mahon et al., 1997; Killalea et al., 1996; Clark et al., 1973). Serotyping also forms the most important and basic step for more detailed laboratory-based surveillance strategies, which include subtyping and antimicrobial resistance monitoring. The broad range of serotypes of *Salmonella* isolated each year (CDC, 2004) highlights the need for the ever increasing demand on accurate and efficient serotyping methods. Thus, the developed SPR-based serotyping method will serve as an important tool in monitoring and control of salmonellosis worldwide. The importance of serotyping has to be discussed with respect to the cases of *Salmonella* outbreaks and the corresponding number of cases in which the *Salmonella* isolates is serotyped. Statistics show that approximately 1.4 million cases of salmonellosis occur among humans in the United States per year. In approximately 35,000 of these cases the *Salmonella* are serotyped and the results reported electronically to the CDC (Brenner et al., 2000). New tools are being constantly incorporated in the fight against control and prevention of salmonellosis. The *Salmonella* outbreak detection algorithm, which allows users to monitor trends in outbreaks of salmonellosis and detect increases in human infections due to specific *Salmonella* serotypes, has been recently implemented (Hutwagner et al., 2007). The success of such an algorithm is completely based on the accuracy of data of isolates serotyped for outbreaks of salmonellosis. The use of SPR-based serotyping method presented here provides rapid, reliable and quantitative data as compared to the conventional SAT and should thus increase the accuracy of analysis using such an algorithm.

5.4 SPR-based zoonoses chip

The directive 2003/99/EC of the European parliament and the Council of the European Union outlines the strategy for the monitoring of zoonoses and zoonotic agents in Europe

(Anonymous, 2003a). Control of *Salmonella* infection in pigs forms an important part of the Regulation (EC) No 2160/2003 of the European Parliament (Anonymous, 2003b). Article 4 of this regulation states that Community targets shall be established for the reduction of the prevalence of *Salmonella* in herds of slaughter pigs. To set the Community targets, a survey has been initiated to monitor the prevalence of *Salmonella* in slaughter house pigs. As part of this survey, slaughterhouse sampling, by taking ileocaecal lymph nodes to reflect the *Salmonella* status of the pigs sent to slaughter, has been recommended. In addition, serological methods have also been recommended for use within this programme (Anonymous, 2006). Timely detection and control of *Salmonella* spp. in pigs is important for the production of *Salmonella*-free pork (Beloeil et al., 2007; Berends et al., 1997; Pearce et al., 2004; Swanenburg et al., 2001; Vieira-Pinto et al., 2006). Serological monitoring of *Salmonella* infection in pigs forms the basis of all successful *Salmonella* monitoring programmes. The most successful and well known is the Danish *Salmonella* surveillance programme, established in 1995 (Mousing et al., 1997). This programme is primarily based on antibody analysis of meat juice samples using the so-called "mix-ELISA" (Nielsen et al., 1998). The programme relies on these serological results to assign herds to one of three levels of infection – no or few antibody positive-finisher pigs (level 1), moderate antibody positive-finisher pigs (level 2), high proportion of antibody positive-finisher pigs (level 3) –, on a monthly basis (Sorensen et al., 2004). The herd is considered positive if at least one animal is found infected (Steinbach et al., 2002). Furthermore, the success or failure of *Salmonella* control programmes has commercial implications to the pork trade (Bogetoft and Olesen, 2004; Reimer and Carstensen, 2006). Hence, rapid and reliable detection techniques for monitoring of *Salmonella* infection in pigs need to be explored.

In the first phase of the development of the zoonoses chip an SPR assay was successfully established to detect and differentiate between various levels of *S.* Typhimurium infection in pig serum. The SPR results obtained show a high correlation with ELISA data. The Pearson´s correlation coefficient calculated was 0.9 (Fig. 4.46, section 4.2.4.2). The data were obtained by analysing serum samples which were first classified using the commercial SALMOTYPE® Pig Screen ELISA kit. The infection status of slaughter pigs in pig farms has been identified as the first Critical Control Point (CCP) in the implementation of the Hazard Analysis Critical Control Point (HACCP) programme for providing safe pork to the consumer (Borch et al., 1996). Correlation between the serological *Salmonella* status and the actual *Salmonella* burden for a given lot of slaughter pigs has been reported (Steinbach and

Staak, 2001). A major conclusion is that, serology can be used to follow the improvement of an integrated pig production system (Galland and Alt, 2006; Korsak et al., 2006). For the detection of *Salmonella* infection in pigs a good correlation is also proved to exist between the ELISA data, obtained using meat juice, to that obtained using serum (Nielsen et al., 1998). Recently a detailed study has been carried out to make a comparative evaluation of four different approved commercial *Salmonella* test kits used for detection of *S.* Typhimurium infection in pigs (Szabó et al., 2008). The SALMOTYPE® Pig Screen ELISA kit was one of them. The study concluded that there was no significant difference in detection between testing of meat juice and serum samples of infected pigs for *Salmonella* antibodies. In addition, the authors have concluded that during chronic stage of *Salmonella* infection in the life of a fattening pig, the serological tests based on ELISA were more reliable than detection of infection using microbiological culture methods. Thus, the described serological SPR assay, which has been proven to work in a complex matrix such as blood serum, can also be used for serological analyses of meat juice from slaughter pigs. The assay will be a valuable tool for implementation of HACCP programmes in pork production systems, starting from the farm, to the slaughterhouse and if required for evaluation of the infection status in the end product.

The second phase of the zoonoses chip development was to provide an SPR equivalent of the "mix-ELISA" as discussed in the beginning of this section. Though *S.* Typhimurium is a major source of infection in pigs, other serovars of *Salmonella* also infect pigs and are subsequently transferred to humans (Bonardi et al., 2003). As mentioned above various LPS-based ELISAs have been developed and successfully used in the detection of different *Salmonella* infections in pigs. The ELISAs use a mixture of LPSs from different serovars of *Salmonella* and are designated as "complete" or "mixed" ELISAs (Nielsen et al., 1995; Proux et al., 2000). Using such type of ELISAs, it is possible to detect *Salmonella* infection in pigs irrespective of the *Salmonella* serovar. We further extended this concept to enhance the capability of the zoonoses chip based SPR assay to detect infections caused due to other important serovars of *Salmonella* and other pathogenic bacteria infecting pigs and other farm animals. A mixture of LPSs from different serovars of *Salmonella* was successfully immobilised on a single SPR chip (section 4.2.5.2), thus modifying the single LPS-based SPR assay presented in the first phase of the assay development. Using the mixed LPS zoonoses chip it was possible to simultaneously detect antibodies against

Salmonella belonging to Group B, Group C and Group E. This corresponds to response using a zoonoses chip prepared with LPS mix from *S.* Typhimurium, *S.* Choleraesuis and *S.* Anatum. The assay was further validated using the same chip with sera from pigs infected with *S.* Typhimurium and S. Choleraesuis. These two serovars of *Salmonella* are known to be prevalent in pig farms (Vieira-Pinto et al., 2006; de la Torre et al., 2005).

The advantage of using SPR as a tool for kinetic analysis has been long recognised. The main advantage of SPR in kinetic analysis is the possibility of observing kinetic interactions without the use of labels. In most cases, an initial visual inspection of the binding curve gives a preliminary idea about the nature of the binding. This is also seen in this work where we were able to clearly see the difference in the nature of the binding of *Salmonella* and *E. coli* to the immobilised polyclonal anti-*Salmonella* capture antibody (section 4.1.3.3). All serovars of *Salmonella* on binding to the polyclonal antibody resulted in a curve showing typical binding kinetics, with a slope having an initial linear rate and then reaching saturation. In case of *E. coli* there was a steady increase in the slope of the curve and this indicated lack of binding and occurrence of cell sedimentation (Fig. 4.22, section 4.1.3.3). However, the importance of kinetic analysis lies in obtaining quantitative data, which provide critical information on the affinities of the interacting partners. The interpretation and quantitative analysis of kinetic data obtained in SPR experiments can be carried out using different curve fitting models, which in turn are based on a number of assumptions. Karlsson and Fält (1997) have discussed experimental design for kinetic analysis of protein-protein interactions using SPR. According to the authors, the simplest model is the assumption of a one to one interaction. However, in some cases the one to one interaction model cannot be assumed and various factors such as surface effects, mass transfer effects and valency of the interacting partners may result in deviation from this model. In our work we used the "initial rate analysis model" put forward by Edwards and Leatherbarrow (1997). This model uses only the initial linear region of the binding curve and thus excludes the factors, discussed above, which result in non-linearity. Using this model we were able to successfully determine the equilibrium association ($K_{A)}$ and equilibrium dissociation (K_D) constant values for the binding of the anti-O5 antibody to the surface of a zoonoses chip containing LPS obtained from *S.* Typhimurium. The binding constants K_A and K_D calculated were. 3.14×10^7 M^{-1} and 3.18×10^{-8} M, respectively. The low value of

$K_{D,}$ which is in the nanomolar range (31.8 nM) and a corresponding high value K_A indicates a strong binding between the *S.* Typhimurium LPS and the anti-O5antibody (section 4.2.3.1). However, irrespective of the kinetic analysis model used, the most important factor, which needs to be incorporated in the experimental design, is the use of a reference channel, which is run in parallel with the experimental channel. This is critical in case of samples resulting in bulk refractive index changes. Serum and milk samples belong to this category. The initial rate analysis model is not applicable in case of such samples as the actual initial binding kinetics of the interacting partners is masked by the bulk refractive index change caused due to the sample matrix. This problem can be overcome by subtracting the signals obtained in a reference channel, which is run simultaneously. This is presently not possible using the cuvette-based Plasmonic® SPR device. The device allows addition of samples into each channel in a sequential manner and hence the reference data is not obtained simultaneously for each addition to the sample channels. This problem has been addressed in the new hand-held SPR device being developed by our group. The importance of having such a reference channel has also been highlighted for the use of SPR in screening of drug candidates in the pharmaceutical industry (Cooper et al., 2002).

The rapid detection of *Salmonella* infections in humans is of critical importance especially with respect to the diagnosis and treatment of typhoid fever, a widely endemic disease (Olsen et al., 2004). The disease is caused by the bacteria *S.* Typhi. Presently used tests for the detection of typhoid fever are the Widal agglutination test. This is a serological assay, which detects antibodies in infected serum based on agglutination in presence of the antigen. The assay is thus subjective and very often results in false results (Nsutebu et al., 2002). Hence, more reliable and rapid methods, such as ELISA and more recently the TUBEX® test, have been developed. The TUBEX® test is a rapid competitive inhibition immunoassay based on colorimetric detection of anti-*S.* Typhi antibodies in human serum. Using this assay, a detection range of 40-100 $\mu g\ mL^{-1}$ of the anti-*S.* Typhi antibody in human serum has been reported (Tam et al., 2008). Our results on SPR detection using human serum samples spiked with the polyclonal anti-*Salmonella* antibody show a range of 10-100 $\mu g\ mL^{-1}$ of the antibody (Fig. 4.51 and section 4.2.6). However, the proof of principle presented here was carried out using a zoonoses chip functionalised with LPS from *S.* Typhimurium. This assay can be used as a diagonistic tool for detection

of *S.* Typhimurium infections in humans. A specific SPR-based immunoassay for the detection of anti-*S.* Typhi antibodies in humans suffering from typhoid fever can easily be obtained by functionalising the SPR chip with LPS from *S.* Typhi. Future work should thus focus on developing and validating such an assay for rapid detection of typhoid fever. Such an SPR-based assay would be of immense medical importance as a rapid point-of-care application using a hand held SPR device.

Based on the results and discussions we conclude that the work presented here has been able to successfully demonstrate a technology platform based on SPR for the detection, serotyping and clinical diagnosis of *Salmonella* and related infections. The SPR-based biosensor developed as part of this work has the potential to be developed as a rapid point-of–care tool for pharmaceutical and food applications, health and environmental monitoring and basic microbiological screening of bacterial strains. However, to realise the full potential of this technology further work needs to be carried out. Ways of achieving higher detection limits of the assays such as the use of gold nanoparticles need to be explored. The developed assays need to be further validated in the field and hence projects that would focus on this aspect need to be designed. The capability of SPR as a basic microbiological tool needs to be exploited and hence the possibilities and limitations of the using the technology in this area can only be fully understood when a study is undertaken to serotype even larger number of *Salmonella* serovars and other bacteria. Clinical application of SPR for serological detection of bacterial and other infections will find practical application only when the developed assays here are made available in a user-friendly format. This involves work on the development of pre-coated LPS chips. Assays need to be validated for serological detection of infections using larger number of real samples and also such assays need validation on SPR-devices, which are designed to work at the point-of-care. In addition work needs to be extended to use the SPR technology platform to gain insight into the interaction of bacteria and other pathogenic microorganisms with various receptors and components of the immune system. Such studies would provide valuable information that can be used in designing therapies to fight such infections.

6 Summary

Surface plasmon resonance (SPR) biosensors enable rapid and label free sensing of biomolecular interactions. Immobilising specific biorecognition elements on the surface of a gold-coated prism to capture specific analytes in the liquid phase forms the basis of SPR-based biosensors. The liquid phase is in contact with the gold surface. Binding events occurring between the analyte and the biorecognition element results in change of refractive index of the liquid phase. The change in refractive index is proportional to the concentration of the analyte binding to the surface. The SPR response in turn is directly correlated to the change in refractive index. This property of SPR has been successfully exploited to monitor binding interactions of different interacting chemical and biological components. SPR-based biosensors have found valuable applications in the area of health, environment and food safety. The phenomenon of SPR has been used in this work to obtain a biosensor which can: (a) detect the pathogenic bacteria *Salmonella* in complex food matrices such as milk, (b) distinguish between multiple serovars of *Salmonella* when present together in milk, (c) assign serogroups and serotypes to isolated *Salmonella* strains, and (d) also detect *Salmonella* infections in farm animals and humans. The Plasmonic® SPR device was used for this work.

Rapid detection of *Salmonella* contamination is a major challenge for the health care and food industry. *Salmonella* contamination is well known in all foods, and animals and humans are the carriers of the bacteria. As part of this work a rapid, simple and specific immunoassay was developed for detection of *Salmonella* in milk. The SPR assay was developed as a sandwich model using a polyclonal anti-*Salmonella* antibody as the capture and detection antibody. Using the assay *S.* Typhimurium was detected in milk within 1 h, whereas the cultural techniques require 3–4 days for presumptive positive isolates and further time for confirmation. This assay is able to detect *S.* Typhimurium down to a cell concentration of 1.25×10^5 cells mL^{-1} in both milk and buffer system. Since no negative effects on the sensitivity of the assay were encountered due to the milk matrix, no sample preparation or clean-up steps are required. The results obtained are comparable with existing, approved rapid *Salmonella* detection techniques. The sample volume requirement for the assay is only 10 µL.

In the next phase of this work the possibility of simultaneous and specific detection of *Salmonella* serovars by using SPR was explored. This sandwich immunoassay involves the use of a polyclonal anti-*Salmonella* antibody to simultaneous capture multiple *Salmonella* serovars from a sample. This is followed by specific detection of the captured serovars using O-specific anti-*Salmonella* antibodies. The assay was further extended to sequentially differentiate between the two *Salmonella* serovars on a single SPR chip in a single channel. Assay validation using purified LPS from both the serovars of *Salmonella* was also carried out. The lower limits of detection for *S.* Typhimurium and *S.* Enteritidis were 2.5×10^5 cells mL^{-1} and 2.5×10^8 cells mL^{-1}, respectively.

Furthermore, an SPR-based method for serotyping of *Salmonella* was also demonstrated. The method is also based on the principle of sandwich immunoassay. The *Salmonella* to be serotyped are captured on an SPR chip using a polyclonal capture antibody. SPR sensorgrams are then obtained for the immunoreactions of the somatic O- and flagellar H-surface antigens of the captured bacteria, to their respective antibodies. The sensorgram data are then compiled to determine the antigenic formula in accordance with the Kauffmann-White scheme. *S.* Enteritidis was completely serotyped using this SPR-based method. Additionally, *Salmonella* belonging to serogroups B, C and D were successfully assigned to their respective serogroups. This SPR-based serotyping provides quantitative data, and thus, eliminates the possibility of false detections as encountered in the conventional method of serotyping using the slide agglutination test (SAT). Most importantly, the SPR-based assay was also proved to work for the identification of rough *Salmonella* strains. Direct serotyping of rough strains is not possible using SAT.

Rapid and reliable detection of *Salmonella* infections in animals and humans is one of the most important steps in the control and treatment of salmonellosis. Thus, the scope of this work was further extended to develop an SPR-based zoonoses chip for rapid serological testing of *Salmonella* infections in pigs. First a serological assay was developed for specific detection of *S.* Typhimurium infection. Using a lipopolysaccharide (LPS) coated chip, it was possible to bind and detect the anti-*S.* Typhimurium antibodies in serum samples of pigs infected with the bacteria. The developed SPR assay was able to differentiate between sera obtained from pigs having different levels of *Salmonella* infection. A strong positive correlation was observed between the SPR-based assay and a commercial ELISA kit ($n = 38$,

$r = 0.90$, $p < 0.01$). The sensitivity and specificity of the assay were 0.93 and 0.87, respectively. The SPR-based assay is label-free and does not require any sample preparation or dilution steps. The total time required for the analysis of each serum sample is only 45 min. The assay was found to be specific for *S.* Typhimurium and shows no cross reactivity to *S.* Choleraesuis or *Escherichia coli* antibodies. The developed assay has the potential to be used as a reliable and high-throughput tool for *Salmonella* monitoring programmes in pork production. The above assay was further modified with the aim to provide a zoonoses chip capable of detecting multiple *Salmonella* infections. A LPS coated chip, containing mixture of LPS from *S.* Typhimurium, *S.* Choleraesuis and *S.* Anatum was successful in detecting the respective antibodies in buffer and serum samples of infected pigs. In addition the *S.* Typhimurium specific zoonoses chip was also successfully tested using pooled human serum, which had been spiked with the polyclonal anti-*Salmonella* antibody.
Besides, presenting the details of the SPR-based biosensor for the detection of *Salmonella and Salmonella* infections, this work also provides a complete understanding of the science involved in the development of SPR-based biosensors for the detection of pathogens and pathogenic infections. This includes strategies that can be used for successful immobilisation of biorecognition elements on the chip surface.

Zusammenfassung

Biosensoren, die auf dem Prinzip der Oberflächenplasmonresonanz (SPR) beruhen, ermöglichen die schnelle und markierungsfreie Messung von biomolekularen Wechselwirkungen. Die Basis für den spezifischen Nachweis von Analyten in Lösung bildet die Immobilisierung spezifischer, biologischer Erkennungselemente auf der Oberfläche der goldbeschichteten SPR-Prismen. Die zu untersuchende Lösung befindet sich auf der Goldoberfläche der Prismen. Wechselwirkungen zwischen biologischen Erkennungselementen und den Analytmolekülen führen zu einer Änderung des Brechungsindexes der Lösung. Die Änderung des Brechungsindexes ist proportional zu der Menge an Analyt, die über die Erkennungselemente an die Oberfläche gebunden ist. Das erhaltene SPR-Signal korreliert direkt mit der Änderung des Brechungsindexes. Aus diesem Grund wird SPR erfolgreich für die Beobachtung von biomolekularen Wechselwirkungen

eingesetzt. Biosensoren, die auf dem SPR-Prinzip basieren, finden Anwendung in Bereichen wie dem Gesundsheitswesen, der Umweltüberwachung und der Lebensmittelsicherheit. In der vorliegenden Arbeit wurden SPR-Assays entwickelt, die (a) den Nachweis pathogener Bakterien, wie z.B. Salmonellen, in einer komplexen Lebensmittelmatrix, wie z.B. Milch, ermöglichen, (b) eine Unterscheidung zwischen mehreren Salmonellen-Serovaren erlauben, (c) die Serogruppierung und Serotypisierung solcher isolierter Serovare zulassen und schließlich (d) in die Lage versetzen, Salmonelleninfektionen in Tieren und Menschen nachzuweisen.

Die Entwicklung von Schnelltests zum Nachweis von Kontaminationen mit Salmonellen stellt ein Hauptziel im Gesundheitswesen und in der Lebensmittelindustrie dar. Salmonellen-Kontaminationen kommen in allen Lebensmitteln vor. Sowohl Tiere als auch Menschen können Träger dieser Bakterien sein. Als Teil dieser Arbeit wurde ein schneller, einfacher und spezifischer Immunoassay für den Nachweis von Salmonellen in Milch entwickelt. Der SPR-Assay wurde als Sandwich-Modell entwickelt, bei dem ein polyklonaler anti-*Salmonella*-Antikörper sowohl als Fänger- als auch als Detektionsantikörper eingesetzt wurde. Mit diesem Assay konnte *Salmonella* Typhimurium in Milch innerhalb einer Stunde nachgewiesen werden, wohingegen Kulturtechniken drei bis vier Tage allein für eine erste erfolgreiche Isolierung benötigen. Dieser Assay ist in der Lage, *S.* Typhimurium ab einer Konzentration von $1{,}25 \times 10^5$ Zellen mL^{-1} sowohl in Milch als auch in Puffer nachzuweisen. Da durch die Milch keine negativen Matrixeffekte auf die Sensitivität des Assays auftreten, kann auf eine Probenvorbereitung und sonstige Aufreinigungsschritte verzichtet werden. Die erzielten Ergebnisse sind mit denen bereits existierender, etablierter Salmonellen-Schnelltests vergleichbar. Das benötigte Probenvolumen beträgt jedoch nur 10 µL.

In der nächsten Phase dieser Arbeit wurde die Möglichkeit des simultanen und spezifischen Nachweises von diversen Salmonellen-Serotypen mittels SPR untersucht. Der im entwickelten Sandwich-Immunoassay verwendete polyklonale anti-*Salmonella*-Antikörper wurde benutzt, um gleichzeitig mehrere verschiedene Serotypen aus einer Probe zu binden. Darauf folgte ein gezielter Nachweis der gebundenen Serotypen mit O Antigen-spezifischen anti-*Salmonella*-Antikörpern. Der Assay wurde darüber hinaus noch erweitert, um den sequentiellen Nachweis zweier Serotypen auf einem Chip in einem einzigen Messkanal zu ermöglichen. Eine

Validierung des Assays wurde ebenfalls unter der Verwendung von aufgereinigtem Lipopolysaccharid (LPS) der betreffenden Serotypen durchgeführt. Die Nachweisgrenzen von *S.* Typhimurium ($2{,}5 \times 10^5$ Zellen mL^{-1}) und *S.* Enteritidis ($2{,}5 \times 10^8$ Zellen mL^{-1}) wurden ebenfalls bestimmt.

Darüber hinaus wurde eine auf SPR basierende Methode zur Serotypisierung entwickelt. Diese Methode basiert ebenfalls auf dem Prinzip des bereits beschriebenen Sandwich-Immunoassays. Die zu typisierenden Salmonellen wurden wieder mit Hilfe des polyklonalen Fänger-Antikörpers auf dem SPR-Chip immobilisiert. Im nächsten Schritt können SPR-Sensorgramme für die spezifischen Immunreaktionen der somatischen O- und der flagellaren H-Antigene der Bakterien mit den zugehörigen Antikörpern aufgenommen werden. Die in den Sensorgrammen erhaltenen Daten wurden dann verarbeitet, um die Antigen-Formel der Bakterien nach dem Kauffmann-White-Schema zu bestimmen. Es war möglich, *S.* Enteritidis mit dieser Methode vollständig zu serotypisieren. Zusätzlich erfolgte eine korrekte Zuordnung von Salmonellen zu ihren jeweiligen Serogruppen (B, C, D). Da die SPR-basierte Serotypisierung nicht nur qualitative sondern auch quantitative Informationen liefert, wird die Möglichkeit falsch positiver Ergebnisse eliminiert, wie sie bei konventionellen Typisierungsmethoden wie dem Slide Agglutination Test (SAT) auftreten können. Bemerkenswert ist außerdem, dass dieser Assay im Gegensatz zum SAT auch zur Identifizierung sogenannter „rauer“ *Salmonella*-Stämme („rough“ strains) verwendet werden kann.

Der schnelle und zuverlässige Nachweis von Salmonelleninfektionen bei Tieren und Menschen ist einer der wichtigsten Schritte in der Kontrolle und Behandlung von Salmonellosen. Deshalb war ein weiteres Ziel dieser Arbeit die Entwicklung eines SPR-basierten Zoonose-Chips für Schnelltests auf Salmonelleninfektionen in Schweinen. Zunächst wurde ein serologischer Assay für den spezifischen Nachweis von *S.* Typhimurium-Infektionen bei Schweinen entwickelt. Ein mit LPS beschichteter SPR-Chip wurde verwendet, um anti-*Salmonella*-Antikörper in Serumproben von infizierten Schweinen nachzuweisen. Mit dem entwickelten Assay war es sogar möglich, zwischen verschiedenen Infektionslevels zu unterscheiden. Es konnte eine gute Korrelation zwischen dem SPR-Assay und einem kommerziell erhältlichen ELISA-Kit nachgewiesen werden ($n = 38$; $r = 0{,}90$; $p < 0{,}01$). Auch die Sensitivität (0,93) und die Spezifität (0,87) des Assays wurden ermittelt. Der SPR-Assay funktioniert markierungsfrei und erfordert keine Verdünnungsschritte oder

sonstige Probenvorbereitung. Insgesamt benötigt man für die Analyse jeder Probe nur 45 Minuten. Der Assay erwies sich als spezifisch für *S.* Typhimurium und zeigte keine Kreuzreaktivität zu *S.* Choleraesuis- oder *Escherichia coli*-Antikörpern. Der entwickelte Assay hat das Potential als zuverlässiges High-Throughput-Werkzeug für *Salmonella*-Monitoring-Programme in der Schweineproduktion Verwendung zu finden. Der Assay wurde weiterhin mit dem Ziel modifiziert, einen Zoonose-Chip bereitzustellen, der mehrere Salmonelleninfektionen gleichzeitig nachweisen kann. Ein mit einer Mischung aus LPS von *S.* Typhimurium, *S.* Choleraesuis und *S.* Anatum funktionalisierter SPR-Chip konnte erfolgreich zum Nachweis der jeweiligen Infektionen in Form der zugehörigen Antikörper verwendet werden, und zwar sowohl in Puffer als auch in Serumproben infizierter Schweine. Außerdem wurde mit dem für *S.* Typhimurium spezifischen Chip auch gepooltes menschliches Serum positiv getestet, welches zuvor mit polyklonalem anti-*Salmonella*-Antikörper versetzt wurde.

Darüber hinaus bietet diese Arbeit eine komplette Übersicht über das Vorgehen bei der Entwicklung eines SPR-basierten Biosensors für den Nachweis von Pathogenen und der dadurch hervorgerufenen Infektionen. Dies beinhaltet unter anderem auch Strategien für die erfolgreiche Immobilisierung von biologischen Erkennungs-elementen auf Chipoberflächen.

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Erklärung

Ich versichere, dass ich meine Dissertation

“**Surface plasmon resonance (SPR) biosensor for rapid detection of *Salmonella* and *Salmonella* infections** ”

selbständig und ohne unerlaubte Hilfe angefertigt habe.
Ich habe mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch an keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den _____ _____
(Saikat Datta Mazumdar)

Curriculum Vitae

Name:	Saikat Datta Mazumdar
Nationality:	Indian
Date of Birth:	01.02.1971
Place of Birth:	Shillong, Meghalaya, India
Marital status:	Married

Academic Qualifications:

Doctoral Research on "Surface plasmon resonance (SPR) biosensor for rapid detection of *Salmonella* and *Salmonella* infections", since July 2005. AG Prof. Dr. Michael Keusgen, Institut für Pharmazeutische Chemie, Philipps Universität Marburg, Germany.

Master of Science in Food Science and Technology (1992 to 1994):
Central Food Technological Research Institute (CFTRI), Mysore, India.

Bachelor of Sciences (**1989 to 1992**):
North Eastern Hill University, Shillong, India.
Gold medallist.

Pre-university Sciences (**1987 to 1989**):
St. Edmund's College, Shillong, India.

School from Class I to X-ICSE (1977 to 1987):
St. Edmund's School, Shillong, Meghalaya, India.

Professional Experience:

Research Associate (**1998 to 2004**):
Hindustan Unilever Research Centre, Whitefield, Bangalore, India.

Executive Research and Development (**1997 to 1998**):
Modern Food Industries (I) Ltd., Delhi, India.

Executive Production (**1994 to 1996**): Parrys Confectionary Ltd., Chennai, India.

Marburg, den _____ _____

(**Saikat Datta Mazumdar**)

Appendix

List of Publications

(a) Published

Mazumdar, S. D.; Hartmann, M.; Kämpfer, P.; Keusgen, M. Rapid method for detection of *Salmonella* in milk by surface plasmon resonance (SPR). Biosensors & Bioelectronics 2007, 22, 2040-2046.

Barlen, B.*; Mazumdar, S. D.*; Lezrich, O.; Kämpfer, P.; Keusgen, M. Detection of *Salmonella* by surface plasmon resonance. Sensors 2007, 7 (8), 1427-1446.
* Joint first authors

(b) Communicated to Journal

Mazumdar, S. D.; Barlen, B.; Kramer T.; Keusgen, M. A rapid serological assay for prediction of *Salmonella* infection status in slaughter pigs using surface plasmon resonance. Journal of Microbiological Methods (communicated April 2008).

Mazumdar, S. D.; Barlen, B., Kämpfer, P.; Keusgen, M. Surface Plasmon Resonance (SPR) as a Rapid Tool for Serotyping of *Salmonella*. Biosensors & Bioelectronics (communicated August 2008).

Barlen, B.*; Mazumdar, S. D.*; Keusgen, M. Immobilisation of Biomolecules for Biosensors. Physica Status Solidi (a) (communicated August 2008).
* Joint first authors

Posters and Conferences

ENGINEERING OF FUNCTIONAL INTERFACES 2008 (EnFI 2008); June 12 TO 13TH 2008; Fachochschule Aachen, Jülich; Germany

- **POSTER PRESENTATION:**

Immobilisation of Biomolecules for Biosensors. S. Datta Mazumdar, B. Barlen, M. Keusgen

BIOSENSORS 2008 - THE TENTH WORLD CONGRESS ON BIOSENSORS; MAY 14 TO 16TH 2008; INTERNATIONAL CONVENTION CENTRE, SHANGHAI, CHINA

- **ORAL PRESENTATION:**

A new hand-held surface plasmon resonance (SPR) device for rapid point-of-care detection of bacterial infections. B. Barlen, S. Datta Mazumdar, N. Danz, F. Sonntag, M. Keusgen.
[#] Presenting author

- **POSTER PRESENTATION:**

Surface plasmon resonance (SPR) as a rapid tool for serotyping and detection of *Salmonella*. S. Datta Mazumdar, B. Barlen, O. Lezrich, P. Kämpfer, M. Keusgen.

5TH DEUTSCHES BIOSENSOR SYMPOSIUM 18 TO 21ST MARCH 2007, RUHR-UNIVERSITÄT BOCHUM, GERMANY

- **POSTER PRESENTATION:**

Development of SPR assays for the detection of *Salmonella* infection in farm animals and humans. B. Barlen, S. Datta Mazumdar, M. Hartmann, M. Keusgen.

JOINT MEETING OF THE GERMAN PHARMACEUTICAL SOCIETY OCTOBER 4 TO 7TH 2006, MARBURG, GERMANY

- **ORAL PRESENTATION:**

Surface plasmon resonance (SPR) as a rapid tool for serotyping and detection of *Salmonella*. S. Datta Mazumdar, M. Hartmann, P. Kämpfer, M. Keusgen

BIOSENSORS 2006 - THE NINTH WORLD CONGRESS ON BIOSENSORS; MAY 10 TO 12TH 2006; SHERATON CENTRE, TORONTO, CANADA

- **ORAL PRESENTATION:**

Artificial Pathogenic Bacteria on a Surface Plasmon Resonance (SPR) Platform. W. Vornholt, M. Hartmann, S. Datta Mazumdar[#], M. Keusgen
[#] Presenting author

- **POSTER PRESENTATION:**

Rapid Method for Detection of *Salmonella* in Milk by Surface Plasmon Resonance (SPR). S. Datta Mazumdar, M. Hartmann, M. Keusgen