Chapter 4

Development of rapid analytical and preparative methods for S-layer proteins - Part II
A lateral flow assay is a rapid, inexpensive, disposable, membrane-based assay that provides visual evidence of the presence of an analyte (e.g., antigen) in a liquid sample within a short period of time. In a lateral flow assay, the reaction of a molecule with ultra-specific receptors during its transport along a membrane can be used to rapidly identify the molecule visually within 5-10 min. The most common example of a rapid lateral-flow assay is the pregnancy test, which identifies the presence of the Human Chorionic Gonadotrophic (HCG) peptide hormone associated with pregnancy.

Lateral-flow immunoassays represent a well-established technology appropriate for use in a wide variety of point-of-care or field-use applications. It offers many advantages over existing immunochemical detection techniques such as:

- Reaction takes place in very short time (maximum 10 min).
- No instrumentation required for detection of positive or negative reaction.
- Useful for on-field detection purpose.
- Cost effective in terms of chemicals or reagents required
- One step process for conjugation and detection.

Biagini et al. (2006) exploited the lateral flow assay system for on-field detection of anti-anthrax IgG in body fluids with a minimal detectable concentration of ~ 3 μg/mL. Lateral flow assays have been developed for the sensitive detection of human pathogen Mycobacterium tuberculosis (Simonson and Lloyd, 2005). In addition, lateral flow assays have been used for rapid detection and quantification of plant pathogens, e.g., Rhizoctonia solani, Thanatephorus orchidicola, T. praticola, R. fragariae (teleomorph:
Ceratorhiza fragariae), and Ceratorhiza goodyerae-repentis (Thornton et al., 2003). Opina and Miller (2002) reported highly accurate detection of Ralstonia solanacearum using immunostrip assay in comparison to ELISA.

To exploit all the above mentioned advantages of lateral-flow assays, the choice of the detection label is a crucial factor. The main criteria involved in selection of a label are mentioned in Table 4.1.

Table 4.1. Features of the label to be used for Lateral Flow Assay

<table>
<thead>
<tr>
<th>Visibility</th>
<th>Sensitivity</th>
<th>Stability</th>
<th>Multi-analyte detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>One step</td>
<td>Colors</td>
<td>Clean result</td>
<td>Ease of preparation</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>Ease of use</td>
<td>Scale-up</td>
<td>Adaptability</td>
</tr>
</tbody>
</table>

A variety of nanoparticles have been explored as detection labels. Polystyrene latex beads coated with antibodies have been widely used in immuno-assays for detection of antigens e.g. anthrax and botulinum toxins (Simo et al., 1994; Griyan et al., 1986; Gambiano and Limet, 1989). However, they are not reliable since they give false positive results (Hanmond et al., 1986; Kumar et al., 1980; Gasparyan, 2001). Paramagnetic nanoparticles such as iron oxide (LaBorde and O'Farrell, 2002) have also been tested for lateral flow assay systems but stringent synthesis and functionalization protocols have restricted their use.

4.1. Gold nanoparticles as labels

Gold nanoparticles are suitable detection labels for a lateral flow assay system because:

1. They are easy to synthesize and can be functionalized as per requirement (Hayat, 1989).
2. Due to their small size, more number of particles can be accommodated at a time, forming a sharp visible band on membrane.

3. They display beautiful colors depending upon their size and shape. Thus, gold nanoparticles of different sizes can be used for different applications (Daniel and Astruc, 2004).

4. Their superior stability, sensitivity, and precision and reproducibility of manufacture make them suitable for use in rapid tests.

5. Gold is essentially inert and forms perfectly spherical nanoparticles when properly manufactured.

6. Proteins bind strongly to the surfaces of gold nanoparticles, thus providing a high degree of long-term stability in both liquid and dried forms (Hermanson, 1996).

7. Under accurate stabilization conditions, the nonspecific interaction of gold conjugates can be reduced to zero (Hayat, 1989; Hermanson, 1996).

Immuno-gold (gold nanoparticle-labeled antibodies) has been routinely used for staining and detection of molecules in electron microscopy (Hayat, 1989). But, in the last few years, gold nanoparticle-labeled antibodies have been used in immunoassays (Hermanson, 1996). Kalvatchev (1995) used IgY antibody labeled with gold nanoparticles for detection of Rickettsia conorii, as an alternative to other known immuno-enzymatic or immunofluorescent methods. IgY-gold nanoparticle conjugates have also been used for quantitative estimation of antigens such as human chorionic gonadotropin hormone, ferritin, and serum protein (Englebienne et al., 2000; Leuvering and Thal, 2000; Leuvering et al., 1981).

Thus, a lateral flow assay system using gold nanoparticle-labeled antibodies offers high sensitivity, reproducibility and reliability. However, synthesis of gold nanoparticles of uniform size is a prerequisite for development of a successful assay. Therefore, the first goal was to synthesize uniform-sized gold nanoparticles.
4.1.1. Synthesis and characterization of gold nanoparticles

Gold nanoparticles of size 20-40 nm are recommended for detection purpose because they form more compact and intense band after reaction with the target. A decrease in the detection signal intensity was observed when nanoparticles of size > 40 nm were used for conjugation (Hayat, 1989; Hermanson, 1996). Taking into consideration these facts, synthesis of gold nanoparticles was carried out by the citrate reduction method (Frens, 1973).

For synthesis of gold nanoparticles, 2 mL of 1% Na$_3$C$_6$H$_5$O$_7$·2H$_2$O (tri-sodium citrate, 99% purity, LOBA Chemicals, India) solution was added to a boiling solution of (2 mL) 0.01% HAuCl$_4$·3H$_2$O (49%, SRL Chemicals, India) under refluxing condition. Initially, the solution turned dark grayish in color, which finally became ruby red coloured with continuous boiling for next 10 min. After 10 min, the solution was allowed to cool to room temperature. The presence of gold nanoparticles was confirmed by UV-Vis spectroscopy. An absorption peak was observed at 521 nm (Fig. 4.1), which is the characteristic surface plasmon resonance peak of gold nanoparticles.

![Fig. 4.1. UV-Vis spectrum of gold nanoparticles synthesized by citrate method](image-url)
Gold nanoparticles to be used for lateral flow assay should be of high quality i.e. uniform in size. Heterogeneous population of nanoparticles may lead to poor performance of the test. Transmission electron microscopy was performed to assess quality of gold nanoparticles synthesized by above mentioned method.

Fig. 4.2. Transmission Electron micrograph of citrate stabilized gold nanoparticles. Scale bar 50 nm.

Fig. 4.2 shows the TEM image of the gold nanoparticles. Uniform-sized spherical gold nanoparticles of diameter 20 ± 2 nm were observed.

4.1.2. Conjugation of antibodies with gold nanoparticles

To achieve a good conjugation, the ratio of reactant concentrations must be optimized. Therefore, several parameters such as the isoelectric point (pI) of the antibody, the pH of the solution, and the quantity of antibody required for efficient labeling, need to be considered.

Antibodies were dialyzed against the borate buffer solution (2 mM, pH 9.0) for 24 h at 4 °C with minimal three changes of buffer during the process. Purified antibodies were conjugated with gold nanoparticles using the protocol described by Hermanson (1996). Initially, the pH of the gold nanoparticles solution (O.D. at 521 nm = 1) was adjusted to 9 using 0.1 M K$_2$CO$_3$ solution.
Antibodies were suspended in 0.2 M borate buffer solution to achieve the final concentration of 1 mg/mL and the minimum quantity of antibodies required for stabilization of gold nanoparticles was determined as per the protocol described by Hayat (1989). Electrostatically-stabilized gold nanoparticles aggregate in the presence of salt. This aggregation property of gold nanoparticles was utilized to detect the minimum amount of antibody required for stabilization of the gold nanoparticle-antibody conjugate. Antibodies were mixed with solution of gold nanoparticles as per the ratio described in Table 4.2 and incubated at ambient temperature for 10 min.

Table 4.2. Determination of minimum concentration of antibodies required for stabilization of gold nanoparticles.

<table>
<thead>
<tr>
<th>Antibody (µg)</th>
<th>Buffer (µL)</th>
<th>Gold nanoparticles (µL)</th>
<th>NaCl (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>35</td>
<td>15</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>500</td>
<td>100</td>
</tr>
</tbody>
</table>

For each sample, the absorbance was recorded at 521 nm and the absorbance was plotted against the antibody concentration (Fig. 4.2).
The sigmoidal nature of the plot was used to calculate the concentration of antibodies where no further change in the absorbance was observed, which is the minimum concentration of antibodies required for conjugate stabilization. Thus, the minimum antibody concentration sufficient enough to stabilize gold nanoparticles in solution was found to 30 μg. After optimizing antibody concentration, the synthesis process was then scaled up to 25 mL volume. The reaction was carried out at 4 °C and the antibody solution was added dropwise with gentle stirring over the period of 5 min. Bovine serum albumin solution (750 μL of 10%) was also added to the above solution for further stabilization of the gold nanoparticles-antibody conjugate. The stirring was continued for 20 min and these gold nanoparticles-labeled antibodies were purified by centrifugation at 50,000 g for 45 min at 4 °C. The soft pellet obtained after centrifugation was suspended in 200 μL sodium phosphate buffer solution (0.01 M, pH 7.4) and stored at 4 °C until further use. The UV-Vis spectra of gold nanoparticles and gold nanoparticles-antibody conjugate after concentration are shown in Fig. 4.3.

Fig. 4.2. Stabilization of gold nanoparticles using antibodies
An increase in the peak intensity of the gold nanoparticles was observed in case of the gold nanoparticles-antibody conjugate, which indicated the formation of a stable conjugate.

It has been reported that the stabilization of gold nanoparticles by the antibody occurs due to the conjugation of gold to the different amino acids present in the antibody, viz., lysine, tryptophan, and cysteine. Each amino acid residue interacts with the nanoparticles by a different mechanism (Hermanson, 1996). Gold nanoparticles synthesized using the citrate-reduction method contain a net negative surface charge due to the presence of citrate ions electrostatically attached to the positively charged gold core (Fig. 4.4). These negatively-charged nanoparticles are attracted to the positively charged lysine residue. Tryptophan can bind to gold through hydrophobic interactions, whereas, cysteine, which contains a thiol (-SH) group, can form conjugates with gold by forming Au-S covalent bonds (Fig. 4.4).
Among all three interactions controlling the attachment of antibodies to gold particles, the gold-cysteine interaction is the strongest due to the presence of covalent bonds, and which is primarily responsible for the stabilization of the gold nanoparticles-antibody conjugate. However, the success of the conjugation process strongly depends on the location of these amino acid residues on the antibody (Chandler et al., 2000).

4.1.3. Lateral Flow Assay System

For construction of the lateral flow assay system, gold-conjugated antibodies (0.5 mg/mL) were coated as a 1 mm narrow line on thin strip of filter paper (Whatmann no. 3) called as the conjugate-pad. Unlabeled antibodies (1 mg/mL) were coated on a 1 mm narrow line on nitro-cellulose membrane (Hi-flow membrane, Whatmann) and allowed to dry at room temperature for 30 min. The membrane was then cut into pieces of 0.5 cm x 2.5 cm dimension. The filter paper strip coated with gold-labeled antibodies was placed at one end of membrane and an absorbent pad was placed at the other end. After wrapping this assembly in a plastic wrapper, strips of ~ 1 cm thickness were cut and fitted in an immunochromatographic cartridge. The schematic of the lateral flow assay cartridge is shown in Fig. 4.5.
The crude antigen solution (1 mg/ mL, cell lysate of B. subtilis) 20 μL was spotted just behind the gold-labeled antibody, i.e. on the sample pad. This was followed by the addition of 100 μL running buffer (phosphate buffered saline). Both the antigen and gold nanoparticles-labeled antibodies flow on the membrane along with the running buffer due to capillary action. As the antigen and gold-labeled antibodies flowed across the membrane by capillary action, a complex was formed between the two species. The migration of this complex was stopped at the point where unlabeled antibodies were precoated on the membrane. This resulted in formation of pink colored band, which occurred within 10 minutes and was clearly visible to the naked eye. Formation of a pink colored band (Fig. 4.6) indicated a positive reaction, i.e., detection of antigen by the antibody-antigen reaction under the lateral flow conditions.

Fig. 4.5. Diagram of Lateral Flow immunochromatographic cartridge. (Biagini et al., 2006).
The total time required for detection of the S-layer was 10 minutes. The sensitivity of the test works out to 2 μg/mL or approximately 100 μL of a culture. Hence, the test would be useful to detect S-layer proteins rapidly from a large number of bacterial cultures.

Thus, a lateral flow assay based on gold nanoparticle-labeled IgY antibodies was developed for an easy, rapid, sensitive and visual detection of S-layer protein. Development of such an assay system for rapid detection of S-layer protein is a major spin off in the present study and is reported for the first time.

4.2. Rapid purification of S-layer protein

To exploit the unique features of S-layers in diverse technological applications (as summarized in Chapter 1), the detection, isolation and purification of the S-layer protein is a crucial factor. Existing method of isolation and purification involve expensive chemicals, complex laborious protocols and are time consuming. Therefore, the development of rapid and efficient processes for large-scale purification of S-layer is the need of the hour.
Immunoaffinity chromatography is a powerful tool for the purification of proteins (Cuatrecasas et al., 1968). It is based on the reversible formation of a tight binding complex between a ligand (antibody) immobilized on an insoluble matrix and a ligate (protein) to be isolated from the solution. Typically, the ligate is adsorbed by a column with the immobilized ligand, whereas non-interacting substances are washed off. By changing the elution conditions, the ligate can be released in a highly purified form. Traditional affinity chromatography exploits high affinity or avidity (binding constant \( K_a > 10^5/M \)) between the interacting molecules, which will result in an effective adsorption of the ligate.

The strong affinity between the antigen and antibody is exploited for chromatographic separation (and hence purification) of respective antigens (Wilchek et al., 1984; Jack and Beer, 1996; Yang et al., 2005). Immunoaffinity separation is widely preferred due to its natural process, high specificity, and scalable production (Pieper et al., 2003; Burgess-Cassler et al., 1989; Kojima, 2001; Fang et al., 2003). Immunoaffinity purification has become a method of choice in recent years for separation of proteins in serum and plasma as well as enzymes using IgY antibodies (Wilchek et al., 1984; Liu et al., 2006; Huang et al., 2005; Yang et al., 2005).

Since, immobilization of proteins and protein-binding bio-ligands is not simple; the choice of the bio-ligand influences all experimental parameters of immunoaffinity chromatography. The choice of the matrix and its activation for bio-ligand coupling is dictated by the activation method and the nature of the bio-ligand. In order to obtain an optimum binding of the bio-ligand, several factors need to be considered while immobilizing the ligand on the matrix:

1. Orientation of the bio-ligand should not affect its activity or binding site.
2. During coupling, the bio-ligand must retain its active state.
3. In case of covalent attachment, the reactivity of the bio-ligand should not decrease after conjugation.
In the present study, anti-S-layer IgY antibodies coupled to a suitable matrix has been used as immunoaffinity beads for the purification of the S-layer of *B. subtilis*.

### 4.2.1. Purification of S-layer protein by immunoaffinity chromatography

#### 4.2.1.1. Activation of matrix by CNBr (cyanogen bromide)

For immunoaffinity chromatography, selection of the matrix is the most important parameter. The matrix should have sufficient porosity to maintain a continuous flow, possess mechanical and chemical stability, high capacity, and very low nonspecific absorption. Sepharose CL-4B was selected as the matrix for the preparation of immunoaffinity beads, due to its large pore size and ease of activation. To ensure efficient, covalent and specific attachment of antibodies to the matrix, Sepharose CL-4B needs to be activated to increase the number of sites available for conjugation. Activation of Sepharose CL-4B by cyanogen bromide was performed according to the protocol developed by Axen et al. (1967). Sepharose CL-4B was washed with 10 volumes of autoclaved Quartz distilled water on Whatmann filter paper. 10 mL of washed Sepharose CL-4B was mixed with an equal volume of ice-cold 2 M Na₂CO₃ solution. Sepharose CL-4B was activated in an ice bath by the dropwise addition of cyanogen bromide (CNBr) dissolved in acetonitrile (0.32 mL per 10 mL slurry) for 1 min with continuous stirring. Stirring was continued for 5 min and the activated Sepharose CL-4B was rapidly filtered and aspirated to semi-dryness (until Sepharose lost its sheen and cracks appeared in cake). The Sepharose CL-4B cake was washed with 10 volumes of ice cold 1 mM HCl solution, followed by 2 volumes of ice cold 0.1 mM HCl solution. At this moment, the Sepharose CL-4B cake regained its sheen.

During the activation process, cyanogen bromide reacts with the hydroxyl (-OH) groups on Sepharose to form cyanate esters or imidocarbonates. These groups react readily with primary amines under very mild conditions; the net result is a covalent coupling of a ligand to the Sepharose matrix (Fig. 4.7). The cross-links in the CL series of Sepharose yield mechanically more robust
beads and do not appreciably change the activation efficiency of cyanogen bromide. Since activated Sepharose is very unstable washing, addition of the ligand and mixing needs to be done very rapidly (preferably in < 90 sec) (Cuatrecasas, 1970).

4.2.1.2. Preparation of immunoaffinity beads

Prior to conjugation, anti-S-layer antibodies were dialyzed against 0.1 M NaHCO₃/0.5 M NaCl solution for 24 h at 4 °C. Dialyzed antibodies were centrifuged at 100,000 g for 1 h at 4 °C to remove insoluble aggregates (if any). Concentration of antibodies in the supernatant was measured by recording absorbance at 280 nm and 10 mg of antibody in 5 mL of 0.1 M NaHCO₃/0.5 M NaCl solution were mixed with 5 mL of activated Sepharose CL-4B. The reaction mixture was incubated at 4 °C for 24 h. The conjugation reaction was terminated by addition of 0.05 M ethanolamine solution (pH 8). The antibody-conjugated Sepharose was allowed to settle and the supernatant containing excess, unreacted antibody was collected. The concentration of antibodies in the supernatant was estimated by measuring the absorbance at 280 nm on a UV-Visible spectrophotometer. The process of Sepharose activation with cyanogen bromide and subsequent conjugation with antibody has been depicted in Fig. 4.7.
Fig. 4.7. Schematic representation of activation of surface groups on Sepharose using cyanogen bromide and cross-linking of antibody (Hermanson, 1996).

Using the concentration of antibody detected in the supernatant, the efficiency of antibody-Sepharose conjugation was found to be 60%. Although, a conjugation efficiency of 80-90% is considered as an ideal value for immunoaffinity, a binding capacity of 40% has been reported for an antibody concentration of 2-3 mg antibody/mL of Sepharose (Springer, 1995). In the present study, the antibody concentration was 2 mg/mL of Sepharose and hence, the calculated efficiency (~ 60%) is an acceptable value for the immunoaffinity purification of S-layer.
4.2.1.3. Immunoaffinity purification of S-layer protein

Antibody-conjugated Sepharose CL-4B beads (5 mL bed volume) were filled in 5 mL pipette tips (acting as a column) and washed with 10 volumes of sterile phosphate buffered saline to remove any unattached antibodies. The cell lysate of *B. subtilis* (protein concentration = 3 mg/mL) was loaded on the column and was circulated for 90 min. The column was then washed with 5 volumes of phosphate buffered saline to remove the unattached protein. The protein bound to antibodies was eluted from the column using 3 mL of glycine buffer solution (50 mM glycine-HCl, pH 2.5, 0.15 M NaCl). Since, protein antigens eluted using an acid or alkalis undergo denaturing, the eluted fraction (3 mL) was immediately neutralized using 1 M Tris-Cl buffer solution (pH 9). The protein concentration in the eluted fraction was estimated by measuring the absorbance at 280 nm using a UV-Visible spectrophotometer. The total protein recovered in the eluted sample was 1.2 mg/mL and hence the protein recovery efficiency of the column was estimated as 40%.

The lower protein recovery can be explained in terms of the size of the protein molecule and its retention time inside the column. S-layer is present as a protein sheet on the cell surface in the natural environment. In the present study, a cell lysate containing a non-denatured S-layer was used as the source of antigen. Thus, the whole S-layer array would be present in the cell lysate, which would sterically hinder its access to the antibody present inside the pores of the Sepharose CL-4B matrix. Thus, only a fraction of S-layer molecules would come in contact with the antibodies, which would decrease the retention of the protein inside the matrix. This argument is supported by the observations of Springer (1995), who reported that an increase in the antigen size hindered its access to the antibody in the pores of the matrix (Springer, 1995). Alternatively, the fraction of S-layer protein attached to the antibody through the binding site would sterically block the incoming protein from accessing the available binding sites (antibodies) in its vicinity. Both processes would lead to low binding efficiencies of the S-layer protein inside the column. Hence, a low concentration of protein would be recovered in the
eluted fraction, leading to low value of the protein recovery efficiency (~ 40 %).

To increase the protein recovery efficiency, a denatured S-layer protein could be used as the source of antigen. In this case, the breakdown of the S-layer array into individual subunits would decrease the steric hindrance inside the column. This would subsequently increase the retention of the protein on the matrix. However, presence of a denaturing agent in the loaded sample would react with the antibody and adversely affect its binding property with the antigen.

Although presence of protein in the eluted fraction was detected by spectrophotometer, it was followed by SDS-PAGE analysis to check the presence of protein bands corresponding to those of S-layer of B. subtilis.

4.2.1.4. SDS-PAGE analysis

SDS-PAGE (10%) was performed as per the protocol (Section 2.5, Chapter 2). After electrophoresis, the gel was processed by the silver staining procedure (Appendix I) to visualize the protein bands.

![SDS-PAGE analysis of protein eluted from immunoaffinity column using glycine buffer. Lane 1- Protein molecular weight marker. Lane 2- Immunoaffinity purified S-layer.](image)

SDS-PAGE analysis of eluted fraction (Lane 2, Fig. 4.8) showed the presence of protein bands having molecular weight 50 and 38 kDa. No other protein
bands were observed, which indicated that S-layer protein was completely separated from the cell lysate. Thus, purification of the S-layer of *B. subtilis* was achieved using an immunoaffinity column containing anti-S-layer IgY antibodies. Additionally, the multi-step protocol required for isolation and purification of S-layer from bacterial cell (Fig. 2.7, Chapter 2) was avoided and the process time was reduced from 72 h to 24 h. Thus, a process for rapid purification of S-layer protein using immunoaffinity chromatography has been developed for the first time.

Thus, protocols were established for the rapid detection and purification of S-layer protein of *B. subtilis*. The next step in the present investigation was to use the purified S-layer protein and exploit its potential as a template for nanopatterning.