Chapter 6
Nitrocellulose Membranes for Lateral Flow Immunoassays: A Technical Treatise

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6.1 Introduction

Lateral flow tests on the market today come in a variety of configurations. In simplest form, the test strip comprises several porous materials mounted on an adhesive backing and covered with an adhesive tape. In more complex designs, the strips are placed into plastic housings. The housing is used to expose the sample pad, maintain proper alignment of the materials, and indicate positions of the test and control lines. While an adhesive backing is normally used for mounting of the porous materials during the manufacturing process, the tape used to cover the materials may be reduced or eliminated depending on the internal design of the housing. In the most complex designs, the housing is designed to hold the materials in the desired alignment. The housing, the materials, and the manufacturing process are integrated so that alignment does not require adhesive materials. Also, a desiccant tablet is often placed inside the housing. A recently introduced pregnancy test even includes on-board electronics that produce the word "pregnant" on a visual display instead of requiring the user to look for a positive test line.

Regardless of the complexity of the test strip, nitrocellulose membranes are common to all lateral flow immunoassay tests. For several reasons, the general perception is that the nitrocellulose membrane is the most critical part of a lateral flow test [1–3]. First, it is the surface upon which the critical immunocomplexes form. Second, it is the surface from which the signal is detected, either visually or electronically. Third, historically, it has been the most difficult material to manufacture consistently. While it is true that the membrane is critical for the formation of the immunocomplexes, overall functionality of the test strip depends on all of the materials, chemistries, design elements, and manufacturing processes.

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R.C. Wong, H.Y. Tse (eds.), *Lateral Flow Immunoassay*,
DOI 10.1007/978-1-59745-240-3_6,

This chapter discusses the utilization of nitrocellulose membranes in lateral flow immunoassay test strips. Consideration is given to the manufacturing and testing of the membrane, as well as its physical and chemical properties. Integration of the membrane into finished test strips is discussed relative to the optimization of liquid flow through all of the porous materials to ensure uniform signal development at the test and control lines.

6.2 Historical Perspective

Nitrocellulose membranes have been manufactured for filtration purposes for many decades [4]. In the 1970s, molecular biologists discovered the utility of nitrocellulose membranes as a substrate for molecular detection [5–7]. Building on this experience, lateral flow tests were patented and introduced in the 1980s [8–10]. The primary change with the lateral flow format was the mode of exposure of the probe molecules to the bound biomolecules. Rather than exposing the surface of the membrane to the probe molecule in a comparatively large volume of buffer [5–7], the probe molecule was carried through the pores parallel to the plane of the membrane as liquid moved from one end of the membrane to the other [8–10]. To facilitate liquid flow, membranes had to have a nominal pore rating of >3 μm. Membranes with pores size ≤ 0.45 μm, which were used in molecular biology applications, did not have lateral flow rates sufficiently fast to be of practical use. Fortunately, membrane manufacturers were already producing membranes with pore ratings up to 8 μm. Problems arose with the consistency of membrane performance, however, because these membranes were qualified for normal flow filtration and not for lateral flow tests. Consequently, manufacturers had to develop release criteria related to such use. Requirements for faster-flowing membranes also caused manufacturers to develop membranes with pore sizes estimated to be 15–20 μm. Because the nominal pore size parallel to the plane of the membrane cannot be measured, lateral flow membranes are classified on the basis of the lateral flow times (Table 6.1; also see Section 6.4.2 and Ref. 2).

Table 6.1 Lateral flow membranes manufactured by Millipore Corporation

Designation	Flow time (s/4 cm)	Relative flow rate	Relative sensitivity
Hi-Flow Plus 240	240	Slow	High
Hi-Flow Plus 180	180	↓	↓
Hi-Flow Plus 135	135	↓	↓
Hi-Flow Plus 120	120	↓	↓
Hi-Flow Plus 90	90	↓	↓
Hi-Flow Plus 75	75	Fast	Low

6.3 Membrane Manufacture

6.3.1 Raw Materials

Nitrocellulose membranes are produced by slow and controlled precipitation of polymer from a solvent system [3, 11]. The manufacturing process begins by preparing a lacquer, which is a proprietary combination of nitrocellulose polymers and a defined solvent system. While the components of the solvent system are easily controlled for consistency, the polymer represents a significant challenge. Nitrocellulose is manufactured as an industrial commodity, with only a small amount of total production being converted to membranes. The polymer is typically characterized on the basis of solution viscosity at defined concentrations and related back to mean molecular weight. While this measurement indicates the bulk properties of a given batch of nitrocellulose, blending different grades of polymers to produce a desired viscosity leads to variation in the molecular weight distribution between lots. This variation, affecting both the dissolution properties when the lacquer is prepared and the precipitation characteristics when the membrane is cast, has to be manageable within the process controls of the casting equipment.

Surfactant is another key raw material [1–3]. Nitrocellulose is naturally hydrophobic, and membranes made from nitrocellulose do not wet out in water. To produce membranes that are wettable, manufacturers add a surfactant as a component of the lacquer or apply it to the membrane at the end of the casting process. Manufacturers use different surfactants, and the final concentrations in the membranes vary. The surfactants used are proprietary, but they have been screened for general compatibility with the antibody systems used in lateral flow tests. Still, experience has demonstrated that membranes with similar flow times but from different manufacturers need to be tested empirically for compatibility with specific test chemistries.

6.3.2 Membrane Casting

The primary goal of the casting process is to produce a membrane that is consistent for structure, thickness, and capillary flow time [2]. This is particularly challenging considering that the lacquer is applied to a moving belt in a layer that is a fraction of a millimeter thick. As the belt carries the lacquer through the casting machine, the solvents are evaporated from the membrane under moving air at controlled flow rate, temperature, and humidity. To achieve the large pore sizes typical of lateral flow membranes, evaporation has to be very slow to allow the polymer strands to associate with each other prior to precipitation. Until enough solvent has evaporated to precipitate the polymer, any disturbance to the lacquer can lead to unacceptable thickness variations and structural artifacts. Significant improvements in casting technology specific for these membranes have been made through the years.

Early generations of lateral flow membranes were cast directly onto stainless steel belts and collected at the end of the machine as unbacked membranes. Membranes without a backing are difficult to handle because of their inherent brittleness, and significant yield losses are encountered each time a roll of membrane is unwound and rewound. As membrane manufacturers gained experience in the casting of these membranes, it was discovered that they could be cast directly onto polyester film with sufficient adherence that the nitrocellulose did not easily delaminate [2, 3, 11]. This improved the handling characteristics of the membrane. Fortuitously, the films did not interfere with the performance of the membrane in the finished test strip. Unbacked membranes continue to be produced for test strip by manufacturers whose equipment is optimized for unbacked membranes, but backed membranes are preferred for new tests because of the handling advantages. The thickness of the backing ranges from 2 to 7 mil (50 to 225 μm), depending on the membrane manufacturer.

6.4 Membrane Testing

6.4.1 Sampling Plan

Lateral flow membranes are cast in a continuous, web-based process and collected as master rolls typically 100 m in length. This length has become the preferred length for the test strip manufacturers using reel-to-reel processing equipment. For quality control purposes, a sample is taken between each pair of rolls and tested. This sampling plan produces data sets for the "head end" and "tail end" of each master roll. Both data sets are considered in the assessment of an individual master roll. At each internal sampling point, the data set serves as the "tail end" set for the first roll and the "head end" set for the roll that follows. An example of this sampling plan, as applied by Millipore Corporation, is shown in Fig. 6.1 for capillary flow time. At each location, six coupons are

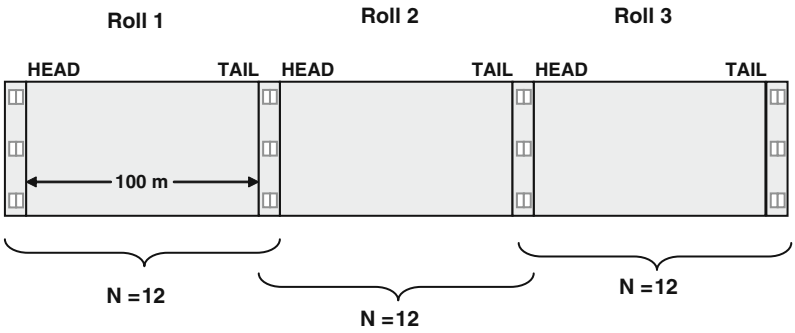

Fig. 6.1 Sampling plan for the evaluation of capillary flow time
The sampling plan used for the evaluation of capillary flow time is presented diagrammatically. Dimensions are not to scale.

tested. The release of the master roll is based on the 12 measurements combined from the head and tail ends. Since one data set applies to two master rolls, a failure within that data set is the cause for rejection of both master rolls. Between sampling points, there is a possibility for membrane to be out of specification. Sampling at 100-m intervals represents a balance between maximizing product yield while minimizing the release of out-of-specification material. Shorter sampling intervals reduce the roll length and increase the cost of testing.

6.4.2 Capillary Flow Time

Lateral flow membranes are evaluated on the basis of capillary flow time (also referred to as wicking time, see Chapter 8), which is the time required for water to travel up and completely fill a 4-cm long strip of membrane [2, 3, 11]. This value is very easy to measure. The selection of 4 cm as the length of the test strip was made for practical reasons. Capillary flow time can be measured on shorter strips. Initially, however, the flow is very rapid. On membranes that flow very fast, it is difficult to call the endpoint of the test consistently, leading to imprecision in the measurement. By extending the length of the strip to 4 cm, the endpoint is more obvious.

Capillary flow time (s/4 cm) is inversely related to capillary flow rate, which is the distance traveled per unit time [2]. Flow rate is difficult to measure reliably, because the flow rate decays exponentially as the water front moves up the membrane. Thus, it is constantly changing. While capillary flow time is measured during membrane testing, it is the flow rate that determines how rapidly a test strip runs and how sensitive the detection system will be. Capillary flow rate is related to the size of the pores parallel to the plane of the membrane. As pore size increases, the flow rate of the membrane increases. For a given membrane type, the membrane manufacturer will specify the capillary flow time. Strip manufacturers should also understand the degree of variability that can be expected within a lot of membrane and the statistical analysis that the membrane manufacturer uses to assess variability. A membrane with highly variable capillary flow time can be very difficult to fabricate into test strips with predictable performance characteristics.

When considering the test strip as a whole, the membrane is the material that normally determines the overall flow rate of the system and the time required to achieve a signal. Development of signals at the test and control lines is a non-equilibrium process, because the analyte and detector particles are being actively carried in the liquid stream and can interact with the capture reagents only for the brief time that they are sufficiently close at the molecular level. As soon as the last detector particles have passed the test line, no further signal development will take place. The effects of membrane flow rate and test line placement on sensitivity have been discussed extensively elsewhere [2, 11].

6.4.3 Membrane Thickness

Membrane thickness is measured using a standard thickness gauge. This parameter is important for several reasons. First, the volume of liquid required to saturate a given area of membrane is determined by the pore volume, which in turn is determined by the thickness of the membrane [2]. For example, when a capture reagent buffer is dispensed onto the membrane, it is likely to spread farther on a thinner membrane. This can affect the width of the capture reagent line, which in turn defines the width of the signal line when the strip is run. Second, dispensing of the capture reagents onto the membrane can be affected by thickness variation [11]. With aerosol application, the cross-sectional area of the stream on the membrane surface can change because the gap between the dispenser tip and the membrane surface varies. With a contact dispenser, variation in the angle between the tip and the membrane surface can affect the consistency of the liquid stream. Third, membrane thickness is important when the strip is placed into a plastic housing [2]. Where the membrane is subject to compression to maintain contact with pad materials, thickness variation can lead to crushing of the membrane and the pads.

It must also be recognized that membrane manufacturers supply membrane within different ranges. Standard nitrocellulose membranes can be as thin as 100 μm and as thick as 150 μm. The range for a specific membrane is determined by the engineering design of the equipment used for its manufacture. When comparing membranes with similar flow times from different manufacturers, consideration must be given to thickness differences for the reasons outlined in the preceding paragraph.

6.4.4 Visual Quality

Visual quality is a subjective assessment of the structural uniformity of the membrane when viewed under various lighting regimes. The membrane should appear uniformly white with no obvious irregularities [2]. Variations in the precipitation of the nitrocellulose are frequently manifested as visual defects in the membrane. When visual defects are extensive, the entire membrane lot may have to be rejected. This typically indicates a fundamental problem with the casting process. If the visual defect is intermittent or infrequent, it may be feasible to cull out the affected area. If an area with a visual defect is encountered during test strip manufacture, it should not be used.

6.5 Membrane Performance

6.5.1 Protein Binding

Protein binding is essential to the function of the membranes in a lateral flow test, but the properties of the nitrocellulose itself are not normally an issue [1–3].

Because nitrocellulose is inherently hydrophobic, it has a high adsorptive capacity for proteins. Lateral flow membranes typically adsorb more than 100 μg of IgG per cm^2. At the concentrations of capture reagents typically applied to the membrane, there is five- to tenfold more binding capacity than necessary. Adsorptive capacity decreases with the molecular weight of the protein. A weak signal is often interpreted as reduced protein binding on the membrane, but this is usually due to solution chemistries that interfere with adsorption to the nitrocellulose or promote desorption when the sample wicks through the test and control lines [11].

To maximize adsorption, antibodies and other proteins should be applied to the membrane in buffers that are preferably free of salt, surfactants, and sugars [2, 11]. The buffer should also be at a low concentration so that crystals dried in the membrane are not of sufficient abundance to occlude the pores. If binding activity of the antibody requires the addition of compounds that might interfere with adsorption, the concentrations used should be no higher than required to maintain antibody functionality.

6.5.2 Blocking

Blocking of the membrane to prevent nonspecific binding of the detector particle and analyte is not absolutely essential to fabrication of a functional lateral flow immunoassay test strip. There are many test strips on the market that do not use a blocking agent; however, blocking agents are required for functionality of some tests because of the nature of the particular sample and antibody system [1, 2, 12]. If a blocking agent is desired, one strategy is to include it as a component of the buffer system dried into the sample pad. The blocking agent dissolves upon addition of the sample and co-migrates with the sample along the strip. From a manufacturing standpoint, this is the simplest approach to adding a blocking agent.

The second option is to apply the blocking agent directly to the membrane by spraying on a defined amount of blocking solution or dipping the membrane into a reservoir of blocking solution. The concentration and type of blocking agent must be determined empirically for compatibility with the sample and antibody system. Care must be taken to avoid excess blocking agent, which can dry down as crystals that occlude the pores. A wash step in buffer alone may be required to remove the excess.

Although not necessarily used for blocking, applying blocking agents can improve the flow characteristics of the membrane. As discussed earlier, surfactants are added to nitrocellulose membranes to make them wettable. While membrane manufacturers test membrane flow with water, test strips are run using a variety of solutions including buffers, urine, saliva, serum, tissue extracts, and environmental samples [13]. These may not have the same flow characteristics as water and often flow at considerably slower rates. Also,

application of the capture reagents to the membranes results in zones with a different chemical environment resulting from drying of the buffer salts, capture reagents, and any other additives into the membrane. Application of the capture reagent can also cause redistribution of the surfactant. The sample may not flow through these areas with the same efficiency as through the rest of the membrane. By applying a blocking solution to the membrane, the nitrocellulose becomes uniformly coated with a single chemical species, the blocking agent. This can improve flow consistency through the membrane.

6.5.3 Membrane Handling

From initial manufacturing until completion of test strip fabrication, membranes are processed through multiple pieces of equipment, brought into contact with other materials, and treated with various chemistries. While the variety of processing schemes is too complex to describe comprehensively in this chapter, these general guidelines apply to both manual and automated assembly of test strips.

1. Minimize contact with the surface of the membrane. When a sharp edge comes into contact with the membrane, it produces a dent. Depth depends on the force applied, but in all cases the discontinuity can affect flow consistency and signal uniformity.
2. If a contact dispenser is used to apply capture reagents to the membrane, the tip must not leave a groove in the membrane [11]. Normally, the tip needs to be composed of a flexible plastic. Tip design and material composition need to be matched to the design of the dispensing equipment.
3. Contact with the edge of the membrane roll should be avoided. If the membrane is unbacked, damage at the edge can serve as the starting point for a break across the width of the membrane when tension is applied. If the membrane is backed, nitrocellulose can flake off the edge and contaminate the manufacturing system.
4. Surfaces that come into contact with the membrane, such as rollers, must be kept clean. If a surface is contaminated with debris, an impression of the debris will be made in the membrane when downward force is applied. In reel-to-reel systems, surface defects sometimes appear at a regular interval, corresponding to the diameter of one of the rollers. The defect also appears identical at each occurrence. This indicates that a piece of debris was stuck to the roller when the membrane was processed through the machine. Cleanliness also applies to manufacturing settings where membrane sections are stacked on top of each other.

6.5.4 Membrane Storage

Membrane storage conditions vary depending on the stage of the test strip manufacturing process [1, 2]. Up until the point that reagents are going to be

applied, the membrane can be stored under ambient conditions (15–30°C, 20–80% relative humidity). A condensing atmosphere should be avoided as liquid in the pores can cause redistribution of mobile components, such as the surfactant. When the membrane is being prepared for application of the capture reagents, it should be allowed to equilibrate to the humidity of the dispensing room, particularly if the membrane is being brought in from a drier environment. Humidity from the air acts to hydrate the surface of the nitrocellulose and improves the absorption of the capture reagent solutions. Once the capture reagents have been applied, the membrane should be dried completely to produce maximum adsorption of the capture reagents. It should then be stored under desiccation or in a dry room at <15% relative humidity. If the membrane is to be blocked, it will have to be brought back to ambient humidity first, processed through the blocking solution, dried, and returned to dry storage. If possible, assembly of the test strips should take place in a dry room.

6.6 Flow Properties

The flow properties of the membrane related to pore size and surfactant have been discussed previously. There are, however, other aspects of the flow that merit discussion because of their impact on the performance of the test strip and the ability to generate results that are predictable and consistent. This involves not only flow through the membrane but also flow through the other porous materials and the test strip as a whole. Depending on the test design, the ability of the strip to allow particle flow has to be considered (see Section 6.6.2). There are two important aspects to liquid flow. First, liquid flows preferentially along the path of least resistance [11]. Second, it is important to recognize that the membrane cannot compensate for flow problems elsewhere in the test strip [11]. Since the functionality of the test depends directly on liquid flow [1, 2, 11], it is important to understand how to optimize flow within the strip.

6.6.1 Porous Pads

6.6.1.1 Manufacture

Porous materials are used as the sample, conjugate, and absorbent pads [2]. Most commonly, nonwoven materials are used: glass fiber for the conjugate pad and cellulose papers for the sample and absorbent pads. Other materials are sometimes used, including various types of woven fabrics. Porous plastic wicks are an integral part of many urine-based tests, where the user is instructed to place the wick in the urine stream to collect the sample. The porous structures of these materials are quite different from membranes because of differences in the ways they are manufactured. Glass fiber pads and cellulose papers are manufactured by suspending the appropriate fibers in a large quantity of water. The

dilute slurry is applied to a rapidly moving, porous screen that permits the removal of the water under vacuum. The fiber mat is processed through various rolling and drying steps to yield the final material. Large master rolls are then processed into narrower widths and shorter lengths as required by test strip manufacturers.

The key attributes of the pad materials are the bed volume and the thickness [2]. The bed volume, defined as the volume of air in the pores per unit surface area (e.g., $\mu L/cm^2$), determines how much volume of sample is required to saturate the structure and what volume of reagents can be dried into the structure during test strip manufacture. The concentration of the sample pad buffer and detector particle solution can be modulated within the constraints of the bed volume specification. The thickness is also important as strips that are placed into plastic housings are under physical constraint (discussed in 6.6.4).

6.6.1.2 Sample Pad

For almost all of the tests currently marketed, the sample enters the test strip through the sample pad. This material is impregnated with buffer salts, surfactants, and other chemical agents that make the sample suitable for interaction with the detection system. If the analyte of interest is present in the sample, it must be capable of binding to the capture reagents on the detector particles and the membrane. The purpose of the sample pad is to modulate any chemical variability in the sample so that the signal produced is proportional to the concentration of analyte.

6.6.1.3 Conjugate Pad

From the sample pad, liquid is transferred onto the conjugate pad. The primary function of the conjugate pad is to hold the detector particles in a dry state so that they are functionally stable until resuspended by the sample [2]. As the liquid moves through the glass fibers, the detector particles need to be released rapidly and quantitatively, as well as consistently between individual test strips. Every effort should be made to ensure that the distribution of detector particles in the conjugate pad is uniform. The consistency of the signals at the test and control lines cannot be any better than the uniformity of distribution on the conjugate pad. Movement of particles in the membrane is dictated almost completely by the direction of liquid flow.

One artifact commonly seen is channeling of the detector particles on the membrane. Although this is often attributed to a problem with the membrane, it is actually a problem with the transfer of liquid from the conjugate pad onto the membrane. If the flow is channeled through different parts of the conjugate pad, either due to non-uniform transfer from the sample pad, highly variable pore diameters in the conjugate pad, or non-uniform transfer to the membrane, the detector particles move into areas where the flow is fastest. This appears as streaks of detector particles on the membrane. In extreme cases it can result in

patchy color development at the test and control lines. Darker zones are coincident with the streaks; lighter zones are coincident with areas between the streaks. Avoiding this artifact requires selection of pad materials with minimal variability in fiber density and distribution.

6.6.1.4 Absorbent Pad

The only function of the absorbent pad is to serve as a sink for the liquid that is processed through the strip [2]. The key attribute of the absorbent pad is the bed volume. There has to be enough pore volume in the absorbent pad to accommodate the full volume of sample that needs to be processed. Once the absorbent pad is full, liquid will stop flowing through the strip.

After a test strip has been run and the results noted, it should be discarded. If a visual record of the result needs to be retained, an electronic image should be made. Alternatively, the absorbent pad and the other pad materials can be removed from the strip and the membrane archived directly. Used test strips should not be stored with the pads retained on the test strip. With prolonged standing, liquid evaporates from the exposed surfaces of the porous materials. The absorbent pad then serves as a reservoir, leading to backflow of liquid onto the membrane. This backflow carries excess detector particles back onto the strip, and can lead to nonspecific and misleading signal development at the test line. This is particularly problematic for samples considered to be negative for the target analyte.

6.6.1.5 Pad Configurations

Another aspect of the liquid flow in the pads is the degree of overlap that they have with each other and the membrane. As the sample flows through the pads, it resuspends the dried chemistries and carries them onto the membrane [1–2]. While each pad fills with liquid, this does not necessarily mean that there is actually flow in any specific region. An obvious example of this is the upstream region of the sample pad in a strip placed into a housing that has a discrete well for sample application. This upstream area wets out when the sample is applied, but does not contribute anything to the development of the signal because the primary flow path is in the downstream direction toward the membrane. Where the pads overlap, it is assumed that the liquid flows through the entire volume of the material. If the overlaps are not configured properly, though, dead spaces can occur where there is little or no flow of the sample. The strip design should allow for complete transfer of the detector particles from the pad. If the flow path has been optimized, there should be no residual color in the conjugate pad after the strip has finished running.

The dimensions of the pads vary between different test strips. Sample pads tend to be much longer than conjugate pads so that they can hold enough dry chemistry to be effective for the entire volume of sample processed through the strip. The conjugate pad tends to be smaller because it holds only a defined amount of detector particle. When these pads are integrated with the

membrane, they are arranged in defined positions. For consistent test strip performance, the alignments should be maintained under as tight a specification as practical [12]. It should be obvious that variation in the positioning of the materials changes the degree of overlap and, consequently, the flow properties of the finished strip.

6.6.2 Membrane Flow

Fabrication of the membrane into a finished test strip presents the opportunity to introduce multiple artifacts that adversely affect flow. Figure 6.2 shows different types of flow patterns on the membrane. When the top of the flow front trails behind the bottom, it is often accompanied by unusual wetting patterns and, thus, is visually obvious. When the top edge is leading, this is often difficult to detect unless the separation is greater than 1 mm. Variations in the flow rate at the top and bottom of the membrane indicate problems with the structural uniformity of the pores at the microscopic level or problems with the chemical uniformity of the nitrocellulose surface through the depth of the membrane (Fig. 6.2A). While structural problems relate to membrane manufacturing, chemical uniformity can be related to contaminants that have been introduced onto the membrane surface. Variations in flow patterns can also be seen across the

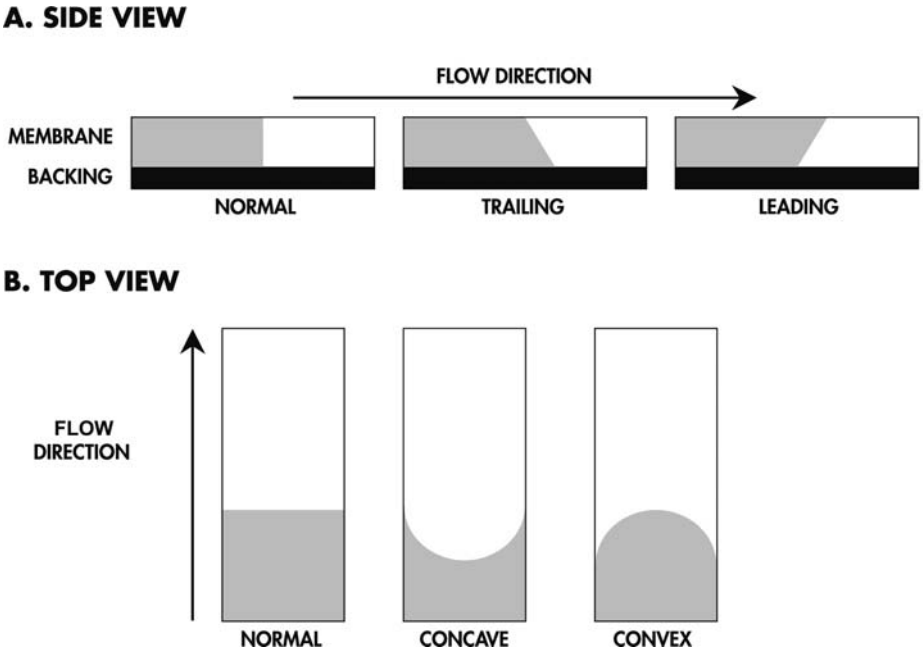

Fig. 6.2 Liquid flow profiles through lateral flow membranes

width of the strip (Fig. 6.2B). If the strip is not placed in a housing, these variations are readily observable. If, however, the strip is inside a housing, they may go unnoticed because the edges of the strip are often obscured by the housing. These types of flow variations are most typically caused by problems with the fabrication process that have resulted in damage to the membrane.

In the finished test strip, every exposed surface of the membrane may have come into contact with another material or been subjected to a mechanical process. Some examples (see Fig. 6.3) are:

a) The leading and trailing edges of the membrane were in contact with metal blades when the membrane roll was originally slit by the manufacturer.
b) The sides of the membrane were in contact with metal blades when the individual strip was cut from the master card.
c) The leading and trailing edges of the membrane are in contact with the conjugate and absorbent pads in the finished strip.
d) Most of the surface of the membrane is in contact with adhesive tape (when included in the strip design).

There are several problems that can arise from this contact. First are problems along the cut edges. The structures of the leading and trailing edges are typically fine, and membrane manufacturers are keenly aware of the need to have cleanly cut edges on their membranes. The most significant problems occur along the sides of the strip. Ideally, the membrane will be in full contact with the adhesive tape on the top and the backing on the bottom, with the nitrocellulose extending fully to the edge (Fig. 6.4). Damage can occur, however, when individual strips are cut because of the range of mechanical properties of the various materials. In some cases, the membrane separates from the adhesive tape. In other cases it separates from the backing. If a channel is opened up (Fig. 6.4), this will provide an unobstructed path for the sample to flow rapidly down the edge, resulting in a concave flow front (Fig. 6.2B). In

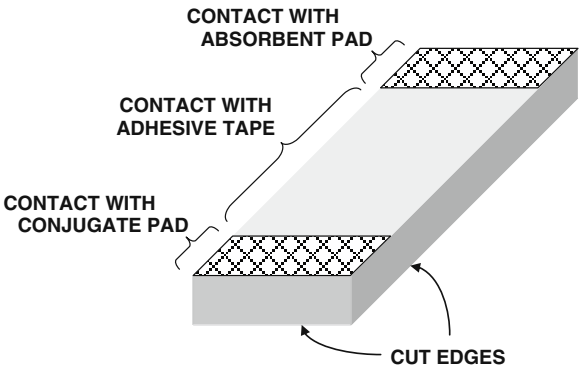

Fig. 6.3 Contact areas on membrane after integration into a finished test strip

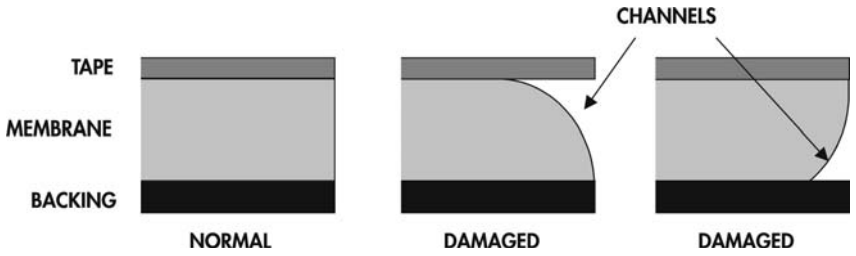

Fig. 6.4 Deformation of membrane edge after cutting
Arrows indicate channels that have opened up as a consequence of the membrane being pulled away from the tape or backing.

other cases, cutting crushes the edges of the strip, reducing the pore size. This causes the sample to flow more slowly along the edges, resulting in a convex flow front (Fig. 6.2B). Even in the absence of adhesive tape, improper cutting can damage the edge of the membrane (Fig. 6.5A).

When the cutting process damages the edges of the membrane, it usually damages the edges of the pad materials, too. Where the conjugate pad and absorbent pad overlap the membrane, their fibrous structure is often pressed down into the surface of the membrane (Fig. 6.5B). This damages the pore structure and can lead to non-uniform transfer of the sample between the pads and the membrane. In extreme cases, the edges of the pads can be so severely crushed that the mechanical force on the pads causes them to bow upward along the central axis and out of contact with the membrane. When this occurs, the primary point of liquid transfer is along the edges. The detector particles take this path, causing the signal lines to be more intense at the edges.

Avoiding these problems requires that the cutting equipment be appropriately designed for the materials involved. Cutting blades should be kept sharp, and the equipment should be kept as clean as possible. The blade(s) also need to be kept free of adhesive. Depending on its flow properties, adhesive can build up on the blades. The adhesive can then stick to the edges of the strip, causing the materials to pull apart as the blade completes the cutting stroke. Excess adhesive can be removed from the blade by wiping the surface with a swab that has been dipped in alcohol. If the blade is removed as part of a maintenance procedure, replacement should be carefully monitored for conformance to mechanical specifications. Slight shifts in the placement of the blade can cause significant problems with the consistency of the cutting due to alterations in the direction and magnitude of the forces applied to the materials.

6.6.3 Strip Width

In the context of the discussion above, it is valid to ask how wide should the strip be. From a cost standpoint, a thinner strip is more economical to

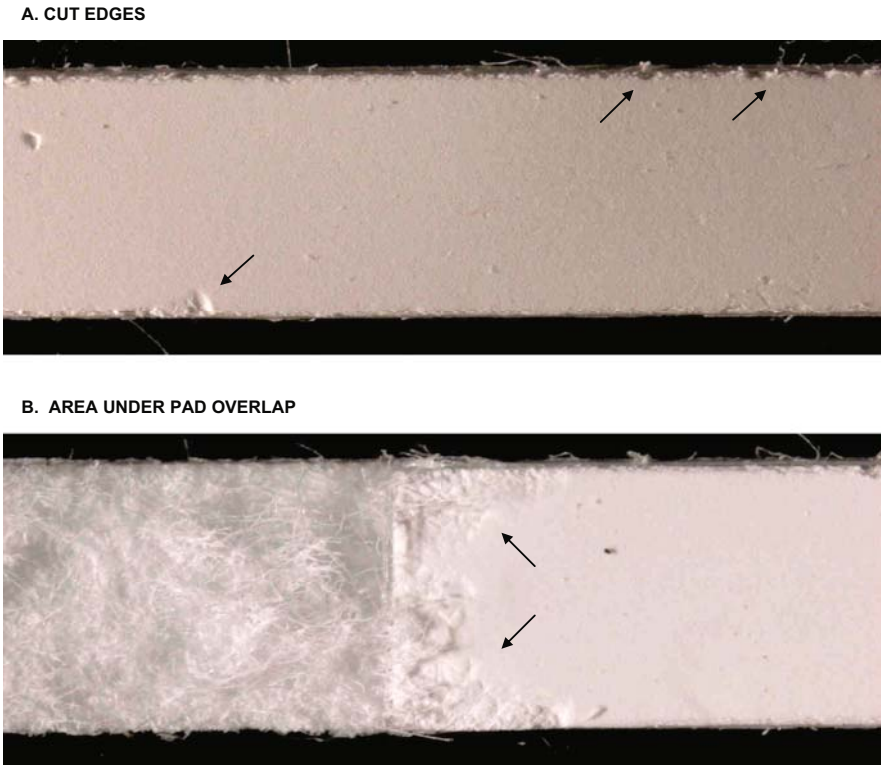

Fig. 6.5 Membrane damage resulting from processing into finished test strips
A. Arrows point to regions with a high degree of damage to the edge resulting from the cutting mechanism.
B. Arrows point to areas of the membrane surface that have been severely damaged by contact with the pad.

produce. However, there are practical limits to how thin a strip should or can be cut. First is the difficulty in reading the signal. Although strong signals are fairly easy to see, the readability of a weak signal depends on the contrast between the signal line and the adjacent membrane area. If there is too little membrane to provide adequate contrast, a weak signal may be missed. Second is a mechanical limitation of the pad materials. Because the pads are typically nonwoven materials, adhesion to the card backing is determined by the small fraction of fibers that are actually in contact with the adhesive. The force of the cutting stroke can easily overwhelm the adhesion, causing the pad material to delaminate and fall apart. Third is the proportion that any edge effects contribute to the overall appearance of the test line. If 0.5 mm on each edge of the strip is subject to aberrant flow, this represents 33% of a strip 3 mm wide. If the strip is 6 mm wide, only 16% of the width is affected. For a quantitative test, it

may be desirable to use a wider strip so that the membrane area scanned by the reader encompasses a zone with uniform flow.

6.6.4 Housing Design

Test strip manufacturers often are unskilled in the design of a housing, particularly the features that define and limit the flow path of liquid through the test strip. When a housing is used, the internal features have to be designed so that the flow path is clearly defined. When the housing includes a well for application of the sample, the bottom of the well needs to be in direct contact with the sample pad around the entire perimeter. If there is any gap between the sample pad and plastic, liquid can run out across the surface of the pad and pool inside the housing. The depth of the well and the internal dimensions of the housing have to be determined to allow for full contact without completely crushing the sample pad. Overcompression of the sample pad can limit the rate of sample absorption.

Internal to the housing, bars or pins are often used to hold the pad materials in contact with each other. Consideration needs to be given to the inherent thickness variation of the materials present at the point of compression. If this is done improperly, materials tending to the low end of their thickness specification can lead to a strip that is not thick enough to contact the top of the housing. Conversely, materials tending to the high end can lead to a strip that is too thick and overcompressed when placed in the housing [12].

The shape of the internal features is also important in defining the flow path. When no adhesive tape is present, a compression bar should span the width of the test strip, especially where the conjugate pad overlaps onto the membrane. If the bar is too narrow, liquid can move across the top of the conjugate pad from the sample pad, travel across the top of the conjugate pad around the edges of the bar, and then cascade down the front face of the conjugate pad, where it pools on the surface of the membrane. This opens a flow path for the liquid, which reduces flow through the conjugate pad. If it occurs before all of the conjugate has transferred onto the membrane, sensitivity will be reduced.

The next area of the housing that needs to be considered is the viewing window. The depth of the viewing window needs to be defined so that the bottom edge is not pressed into the membrane. This will alter the flow path through the depth of the membrane. There is no requirement that the bottom of the viewing window be in direct contact with the surface of the test strip. Many test strips enclosed in housings still include an adhesive tape or a piece of clear plastic that serves to protect the membrane from incidental splash when the test strip is run. This also serves to protect the membrane from mechanical damage.

Finally, there is the positioning of the strip in the housing along both dimensions. Many housings include guide bars that determine the longitudinal positioning of the strip so that the test and control lines are properly registered with

the viewing window and labels on the exterior of the housing. It is important to recognize that shifts of only a millimeter can have a negative impact on the registration of the pad materials with the compression points within the housing. (The same problem can occur if the pad materials and membrane are not kept in constant alignment when the test strip is assembled.) Lateral positioning of the strip in the housing requires matching the position of the guide bars to the width of the cut strip. This is straightforward, and lateral placement of the strip is not usually a problem. The viewing window is often narrower than the width of the strip so that aberrant flow on the edges is hidden from the user.

The choice of housing needs to be considered early in product development. An off-the-shelf cassette will have predetermined design features that will dictate the dimensions of the materials used in the test strip. In some cases, this constraint may not allow development of an economically viable test strip. Custom-designing a housing allows for greater flexibility in the configuration of the test strip, but there will be additional costs associated with specifying the design, testing prototypes, and committing to manufacture of the final design.

6.6.5 Particle Flow

Beyond the effects of test strip design and membrane processing on liquid flow, consideration needs to be given to the interaction between the membrane and the particles that are present in the flow stream. Particles may be an integral part of the detection system (e.g., colloidal gold, latex beads, magnetic beads) or comprise the analyte of interest (e.g., spores, bacterial cells). The ability of a particle to migrate through the membrane is related to two membrane attributes. First is the pore size. The pores in the membrane have to be sufficiently large to accommodate the particle. From a theoretical standpoint, the slowest flowing membranes with pore sizes estimated at $\sim$3 μm should be able to accommodate most test systems. The second attribute is the flow rate of the membrane. As the diameter of the particle increases, physical resistance to forward movement increases. As the flow rate of the membrane decreases, there is less force from the moving liquid to push the particle forward.

When the pore size and flow rate are considered along with the desire to have tests that finish running in a reasonable amount of time, there are practical limitations on which membranes can be used for different types of particles. For the systems using only colloidal particles, which typically average 40 nm in diameter [13], there are no practical limits to particle flow with the lateral flow membranes on the market today. For larger particles, there is a threshold on flow rate below which particle migration will not occur in a timely manner. With latex and magnetic beads, which can be 0.5 μm or greater in diameter, practical limitations on particle flow restrict their usage to medium- to fast-flowing membranes. Spores and cells have to be evaluated on a case-by-case basis.

Another aspect of particle flow relates to their physical state in solution. Ideally, the particles will be monodisperse regardless of diameter [13]. If the particles associate as dimers, trimers, and larger aggregates, their ability to flow through the membrane will be very limited. For detector particles, aggregation is avoided by applying appropriate chemistries to the test strip. Aggregation of colloidal gold can be detected by a color change from cherry red to purple, blue, or, in extreme cases, clear. For latex and magnetic beads, there is no distinct color change that takes place with aggregation. One place to look for aggregates, however, is at the interface between the conjugate pad and the membrane. When the conjugate pad is peeled away after the test is finished, residual color is sometimes seen on the membrane surface. For biological samples, monodispersion can be more difficult to achieve because of the chemical nature of the particles and the type of sample being processed. Large aggregates are likely to be filtered out by the sample pad and may never reach the membrane to be detected.

6.7 Final Comments

Nitrocellulose membranes are a critical material in lateral flow assays. Membrane performance in a finished test strip depends on the consistency of the membrane from both a structural and chemical standpoint. Optimal performance of the membrane, as determined by the sensitivity and specificity of the test, depends on the efficiency and consistency of immunocomplex formation at the test and control lines. The other materials and chemistries are also critical to membrane performance. In combination with the appropriate manufacturing processes, they need to be optimized to allow for the highest degree of consistency.

6.8 Summary

Nitrocellulose membranes are considered to be the most critical material in lateral flow test strips. The membranes supplied today have standard filtration membranes as progenitors and are challenging to manufacture. Lateral flow membranes across a range of flow speeds and relevant quality control tests have been developed to accommodate the requirements of test developers. Proper utilization of nitrocellulose membranes requires understanding the key physical and chemical properties that relate to test strip functionality. Other important aspects include handling the membrane properly to minimize physical damage and integrating the membrane into the test strip to optimize flow consistency.

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