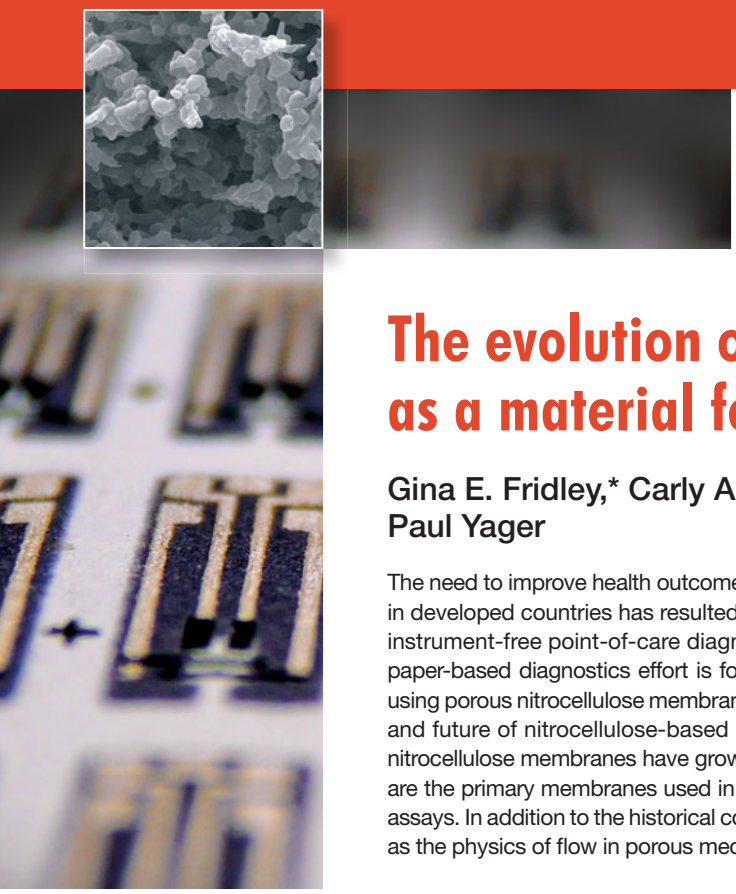

The evolution of nitrocellulose as a material for bioassays

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The need to improve health outcomes in the developing world and to moderate healthcare costs in developed countries has resulted in an increased interest in sophisticated, inexpensive, and instrument-free point-of-care diagnostics using porous materials. One major segment of the paper-based diagnostics effort is focused on developing high-performance point-of-care tests using porous nitrocellulose membranes. This review provides a perspective on the nature, history, and future of nitrocellulose-based assays. Beginning as a protein blotting substrate, porous nitrocellulose membranes have grown to be the most commonly used lateral flow substrate and are the primary membranes used in two-dimensional paper networks for user-friendly multistep assays. In addition to the historical context, we examine assay development considerations, such as the physics of flow in porous media, reagent deposition and storage, and detection methods.

Introduction

In recent years, the urgent need for rapid and low-cost medical diagnostics—both in developed and developing countries—has become clear. One platform that addresses this need is paper-based microfluidics, which eliminates the need for external equipment to move fluids. Porous materials such as paper are ideal substrates for low-cost assays because they are inexpensive and disposable. One of these materials—porous nitrocellulose—is now one of the most commonly used materials in point-of-care (POC) devices, such as the OraQuick Advance HIV-1/2 test, the BinaxNow tests for a variety of infectious pathogens, and, most commonly, the home pregnancy test. This review focuses on the history and evolution of nitrocellulose-based assays, as well as critical aspects of assay performance: flow in porous media, protein adsorption, dry reagent storage, and analyte detection.

Background

History of nitrocellulose

Nitrocellulose is a versatile polymer that has been broadly utilized since the 1800s.[1–3] Also known as cellulose nitrate, nitrocellulose is created commercially by the reaction of cellulose (purified from plants, commonly wood pulp and cotton) with nitric acid, replacing the cellulose hydroxyl groups with nitrate groups.[4] Today, nitrocellulose membranes are created by phase inversion,[5–7] in which nitrocellulose is dissolved in an organic solvent that is evaporated in the presence of a nonsolvent, leaving a precipitated nitrocellulose membrane with high porosity[4,8] (**Figure 1**a). The porosity and pore size of the membrane can be controlled by the solvents used, evaporation speed, temperature, and humidity.[8] The result is a material with the unique combination of tunable pore size, high surface-to-volume ratio, and very low cost.

Porous nitrocellulose membranes were first used to immobilize biomolecules in the 1960s.[4] Nygaard and Hall demonstrated in 1963 that RNA-DNA complexes adsorb onto nitrocellulose membranes, while free nucleic acid strands pass through.[9] Others then began immobilizing nucleic acids on nitrocellulose membranes to probe for interactions between a nucleic acid of interest and other biomolecules.[10,11] In 1975, Southern demonstrated the transfer of DNA from polyacrylamide gels to nitrocellulose.[12] This groundbreaking technique, known as the "Southern blot," allowed specific nucleic acid fragments to be captured for subsequent analysis. The Southern blot inspired the "Northern blot" for RNA transfer[13] and the "Western blot" for protein transfer to nitrocellulose.[14,15] These blotting techniques have been widely employed in biological research and highlight the unique ability of nitrocellulose to interact with three of the most important classes of biomolecules (proteins, DNA, and RNA).[4]

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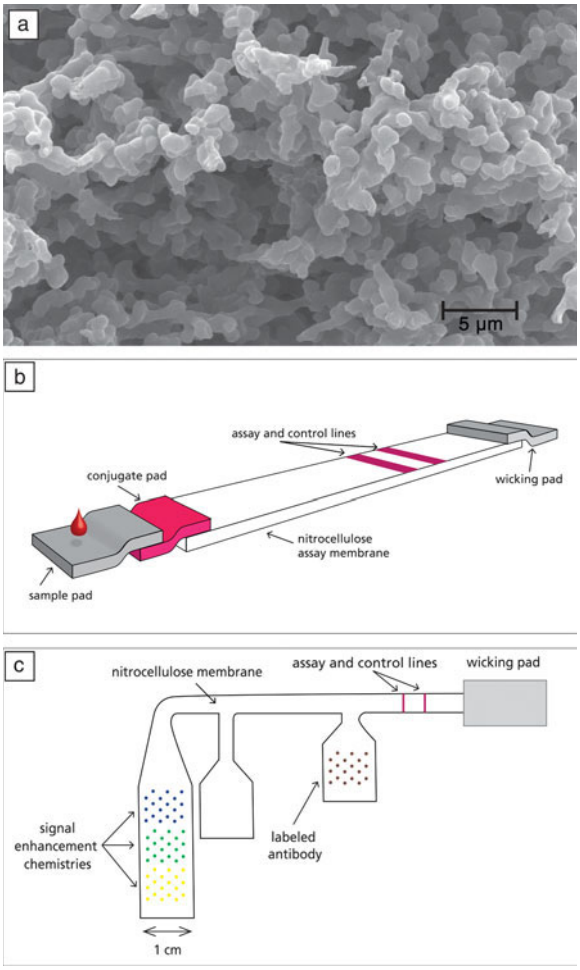

Figure 1. Nitrocellulose and nitrocellulose-based assays. (a) SEM image of a blade-cut edge of a porous nitrocellulose assay membrane. (b) Schematic of a traditional lateral flow assay. (c) Schematic of a multistep two-dimensional paper network assay.

Stemming from the blotting assays, other assays were developed utilizing porous nitrocellulose as a solid support for diagnostic assays incorporating flow. Both flow-through assays and lateral flow (LF) tests feature capture molecules (e.g., target-specific antibodies) immobilized on nitrocellulose through which a sequence of sample and reagents is passed to detect the presence of a target molecule. The flow-through assay uses flow perpendicular to the membrane, driven by a large absorbent pad[16] or vacuum manifold.[17] While the flow-through assay has the advantage of being multiplexable, the highly manual nature of the test typically relegates it to the laboratory. However, two examples of POC flow-through assays are the Multispot HIV test[18] and the DxBox.[19] For most POC applications, the LF test has become the assay of choice.

Lateral flow technology

Over the last 25 years, nitrocellulose has become a prominent material in POC diagnostics through its role in LF technology. Since the first LF test came to market in 1988,[20] this popular technology has developed into a $2.1 billion market involving over 200 companies.[8] Because LF tests are inexpensive, rapid, portable, equipment-free, and easy to use, they are now used in many fields, including clinical, environmental, agricultural, veterinary, drug testing, and bio-defense.[8]

The most common LF devices consist of a "strip" with four contacting porous components arrayed linearly: (1) sample pad, (2) conjugate pad containing dried, gold-conjugated detection antibodies specific to the target analyte, (3) nitrocellulose membrane with capture agents immobilized at control and test lines, and (4) wicking pad to draw the sample through the device (Figure 1b).[8] The nitrocellulose membrane is critical to the functioning of LF devices because analyte binding and detection occur within it. To begin the test, the user adds a microliter-volume of sample (e.g., blood, urine, saliva) to the sample pad. Results are typically available in less than 15 minutes and can be visually read based on the development of color at the test line, due to the accumulation of the gold-conjugated detection antibodies. The device's simplicity permits easy manufacturing, and the dried reagents eliminate the need for refrigeration and extend shelf life.[21]

While LF devices have revolutionized low-cost testing, they have several drawbacks: they are usually qualitative, have lower sensitivity than gold standards (e.g., nucleic acid amplification tests, enzyme-linked immunoassays (ELISA), and bacterial/viral culture), require additional sample preparation, are rarely multiplexable, and cannot perform multiple automated steps.[21] Currently, numerous innovations, such as two-dimensional paper networks (2DPNs),[22–27] are being developed to overcome these disadvantages. Such innovations require an understanding of the scientific and engineering principles involved in assay design.

Considerations for assay development

Physics of flow in porous media

Understanding flow in porous materials is essential when designing nitrocellulose-based assays. The principle of capillary flow describes the passive wicking of fluids that occurs in porous media. Capillary flow is affected by several characteristics of the porous medium: pore size, pore distribution, and porosity; by characteristics of the fluid (e.g., viscosity, surface tension); and by the interaction between the two: the contact angle of the fluid with the porous material.[28–30]

Though most porous media have some tortuosity (i.e., the "twistiness" that is defined by the ratio of the actual path length of a particle flowing through the material to the straight-line distance), they are most easily approximated as a bundle of straight pores. This approximation allows us to use conventional fluidic principles, such as the Reynolds number, to understand the behavior of fluid in these pores. In nitrocellulose membranes (where the most applicable Reynolds number is ~10^{-3}), flow is laminar. Thus, flow streamlines are parallel to the channel walls, and the primary mode of mixing between adjacent streams is diffusion.[30,31]

The "wet-out" process of a fluid wicking into a dry porous membrane is governed by the Washburn equation:[28]

$$L^2 = \frac{\gamma d_p \cos(\theta) t}{4\mu}, \quad (1)$$

where L, the length of the fluid column in the porous material, depends on pore diameter (d_p), fluid surface tension (γ), fluid viscosity (μ), and the cosine of the contact angle (θ). Taking the derivative of L with respect to t, we see that the wet-out linear velocity is inversely proportional to the fluid column length behind the wetting edge:

$$\frac{dL}{dt} = \frac{\gamma d_p \cos(\theta)}{8\mu L}. \quad (2)$$

This slowing is due to the linear increase in viscous resistance as the fluid column lengthens.

From Equation 2, we see that the wet-out rate is inversely proportional to fluid viscosity (μ) and proportional to the membrane pore diameter (d_p). Though the viscosity of a liquid is generally known, the pore diameter within a particular membrane is not. In nitrocellulose membranes, pore size distribution varies widely depending on the material and casting process used. The membrane wet-out rate is also proportional to the cosine of the contact angle (θ) and the surface tension (γ), which account for the liquid-air and liquid-solid interfacial energies. Surfactants are often added to nitrocellulose during manufacturing to decrease the contact angle, thereby improving wettability.[32] The surface tensions of common liquids are well known, though changes in temperature affect wet-out rates, and fluids used in real assays are never pure solvents.

Once a porous membrane has fully wetted out, flow is governed by Darcy's equation for flow in porous media:[29]

$$Q = -\frac{\Delta P}{R} \text{ where } R = \frac{\mu l}{\kappa w h}, \quad (3)$$

where the volumetric flow rate (Q) depends on the paper's permeability (κ), cross-sectional paper area (wh), fluid viscosity (μ), and the pressure drop (ΔP) that occurs over the length (l) of the paper network. This equation assumes that flow is fed by an infinite source, and, as the wicking pad becomes wetted, the capillary pressure it generates does not decrease. These assumptions are approximately correct as long as the wicking pad does not approach saturation.

The utility of both Washburn's equation and Darcy's law for modeling flow in paper devices has been repeatedly demonstrated.[33] Furthermore, methods such as pH-based electrochemical marking[34] have been developed to quantify flow rates in porous devices to enable a systematic validation process for mathematical modeling.

Protein adsorption

The adsorption of biomolecules—typically proteins in commercial lateral flow tests—to the porous nitrocellulose membrane is another key assay consideration. While protein adsorption to nitrocellulose has been observed for years,[35,36] the process is not well understood.[4] However, known principles should be considered when trying to achieve protein immobilization at a test line—or to prevent nonspecific adsorption elsewhere.

First, proteins are thermodynamically driven to adsorb to nitrocellulose.[37] This adsorption is governed by noncovalent interactions between the two.[36,37] Some argue that this attraction is a hydrophobic interaction between the carbon-containing nitrocellulose and the hydrophobic portion of the protein,[8] while others describe it as a primarily electrostatic interaction between the dipoles of the nitrocellulose nitrate groups and dipoles within the protein.[32] Either way, it is well-established that proteins will adsorb to porous nitrocellulose membranes unless something is done to prevent this process.[8] In particular, nitrocellulose-based LF assays are often pre-treated with a protein-containing blocking solution to fill unused binding sites.[8] This blocking step prevents the sample analyte from nonspecifically adsorbing to the membrane before it can reach the capture line.

Second, the kinetics of protein adsorption and desorption to the membrane must be considered, especially for capture molecules immobilized at a test line. Contrary to classic adsorption studies in which protein adsorption—tested in simplified systems at equilibrium—is considered irreversible,[36] the complex systems of LF assays render protein adsorption a kinetic, two-way process. Specifically, the fluid flow velocity,[28] presence of potential stripping agents such as salts and detergents,[38] and reagent drying time[36,37] are important considerations for assays that depend on protein immobilization.

The most commonly used tools for the deposition of proteins include piezoelectric spotters that can deposit small volumes into precise locations on a strip,[26,39] high-volume stripers that "paint" a line of reagent across a large batch of strips,[8] and inkjet printers.[40,41] Each method has advantages and disadvantages; the choice depends on the required throughput, the need for patterning flexibility, and budget. These deposition tools are also important for patterning assay reagents so they may be stored directly on the device.

Dry reagent storage, rehydration, and patterning

When designing POC diagnostics, storing reagents in dry form directly on the device is most desirable. Using dry reagents reduces user steps, removes the need for continuous refrigeration (i.e., cold chain), provides a longer assay shelf life, and facilitates device automation.

Generally, proteins lose function at high temperatures or when dried in an uncontrolled manner.[42] However, a wide body of research has demonstrated that the presence of the non-reducing disaccharides trehalose[42–46] and (less frequently) sucrose[42,47] can stabilize dried proteins for extended periods at elevated temperatures. It is believed that the high glass transition temperature of trehalose (106°C[48]) ensures that molecular mobility of embedded proteins is kept low at elevated temperatures, thus protecting the protein against degradation.[42,43,46] Furthermore, the sugar hydroxyl groups substitute for the waters of hydration of the protein, which are lost upon drying, helping to preserve the protein's native conformation.[42]

Because dried reagents are important for POC diagnostics, several techniques have been developed for dry reagent storage and subsequent rehydration. Reagents are often stored dry within a conjugate pad placed in line with the fluidic pathways for rehydration at the time of use. Conjugate pads have been used in both LF immunoassays[8] and, more recently, 2DPNs.[26] Though these pads are effective for reagent storage, they offer minimal control over the spatial and temporal release of rehydrated reagents and require additional materials and manufacturing steps, which add cost. Recent developments have sought to address the latter of these concerns. Abe et al. showed the ability to rehydrate gold-anti-IgG antibody conjugate dried on blocked cellulose membranes and to use it downstream to detect IgG in buffer.[49] In our group, Fridley et al. developed methods for controlled release of reagents printed directly onto nitrocellulose membranes. By patterning reagents and sugars into specific layouts, we controlled both the spatial and temporal distribution of reagents as they rehydrated on the membrane.[50]

Detection

In an ideal assay, the user would be able to detect the lowest clinically relevant concentration of an analyte in a patient sample. In reality, however, the limit of detection (LOD) of rapid tests often does not reach that ideal. Most commonly, LF tests use labels that can be interpreted by eye to eliminate the need for additional machinery. Three promising detection labels are colloidal gold nanoparticles, colored latex beads, and carbon black.[8] Signal amplification chemistries, such as metallic enhancement of colloidal gold and enzyme-based systems, can also greatly improve the assay LOD and dynamic range.

Numerous labels and chemistries have been developed and are being used in commercial assays.[8] See Chapter 5 of the *Lateral Flow Immunoassay* book for a thorough review of detection labels and amplification chemistries.[8]

The Future

As discussed previously, existing LF tests have inadequate sensitivity and specificity for many medical applications.[21,51,52] Sophisticated paper-based technologies, which are still driven by capillary flow, have evolved from simple LF tests.[22–25,33,53–61] These new paper-based assays demonstrate a breadth of functionalities lacking in LF tests.

Since 2008, our group has pursued the development of next-generation nitrocellulose-based assays. In particular, our work has focused on the development of 2DPNs (Figure 1c), which feature automated multistep processing. We began with the creation of a fluidic toolbox to understand and control fluid flow and reagent delivery in an automated fashion. These tools include methods for fluidic mixing,[33] volume metering,[22,27] fluid transport control,[23,25] and staged reagent delivery.[22,23] We then utilized many of these tools in a 2DPN device to demonstrate a six-fold signal enhancement using staged delivery of gold nanoparticles and gold enhancement solution.[22] Next, we adapted an existing LF pregnancy test into a 2DPN with gold enhancement, demonstrating a four-fold LOD improvement compared to the conventional strip alone.[24]

Finally, we used this body of knowledge to develop two self-contained assays. First, we developed a 2DPN assay for malaria using reagents dried on conjugate pads. This POC assay has the same sensitivity as a laboratory-based malaria ELISA.[26] Based on that successful assay, we developed a gold-enhanced malaria assay with comparable sensitivity that uses only reagents patterned and dried directly on the nitrocellulose membrane. By patterning reagents, both the volume of reagents and the total number of components were decreased.[62] This work exemplifies the promise of 2DPNs to deliver simplified manufacturing and reduced costs for automated multistep assays in the future.

Conclusions

In this article, we have reviewed the past and present of nitrocellulose in assays and provided insights into the technological considerations for development of sophisticated novel diagnostics. Overall, the current state of the art is rich with tools and information that can be exploited to develop improved point-of-care assays. These new capabilities permit features such as multiplexing, on-device sample processing, and signal enhancement, which have the promise of enabling higher-sensitivity assays with rich diagnostic information, while still retaining the low cost and ease of use of traditional lateral flow tests. These assay improvements, especially when combined with recent developments in automated, cell phone-based readers,[58,63–65] are poised to spawn the next generation of tools for medical diagnosis at the point of care, whether that be a doctor's office in Lima, a home in the suburbs of Seattle, or a rural clinic in the mountains of Nepal. Given the strides made in this field already, it is exciting to look forward to future advancements in next-generation nitrocellulose-based devices.

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