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NITROCELLULOSE: A TRIED AND TRUE POLYMER FINDS UTILITY AS A POST-GENOMIC SUBSTRATE

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1. ABSTRACT

High-throughput techniques for genomics and proteomics differ greatly from traditional bio-molecular research techniques in the amount of data that can be obtained from a given experiment. However, many of these novel techniques rely heavily on the traditional concepts of molecular immobilization followed by hybridization, binding or analysis. These concepts, which predate even traditional blotting techniques, have become so widely used that the complexity of their fundamental precepts is often overlooked. In this review, we explore the history and use of one of the most common immobilization surfaces, nitrocellulose. This unique polymer has been used as a surface in biological research for more than 6 decades, and continues to find utility in the post-genomic era with high-throughput techniques.

2. INTRODUCTION

Analysis of biological systems has been affected by recent technological developments that increase the amount of information an experiment can provide. Advances in sequencing, instrumentation, array technology and high-throughput proteomics approaches are a few examples of technologies that have altered the manner in which basic biological research and drug discovery research are performed. These technologies have already begun to find utility in the genomic and post-genomic era as tools in molecular biology research labs as well as in genetically based medical diagnostics research. For example, expression arrays are now fairly common tools for screening thousands of genes simultaneously to identify a handful for more in-depth analysis by traditional techniques (1-5). In addition, analysis of single nucleotide polymorphisms (SNP) by high-throughput array methods may provide an extremely powerful diagnostic tool (6), and techniques aimed at increasing the throughput of mass spectrometry will have a dramatic effect on the study of proteins (7).

Traditional molecular biology techniques, such as Southern blots, differ greatly from high-throughput technologies in the amount of data that can be generated in a given experiment. However, despite this difference, at the most fundamental level, the old and new are strikingly similar. Virtually all biomolecular screening techniques, traditional and high-throughput, are based on the ability to isolate and immobilize the molecule(s) of interest on a medium and then study them with the appropriate molecular system – often through hybridization or binding to a known molecule.

The choice of solid phase for immobilization can range from truly solid such as glass and plastic to porous such as latex and cellulose. Each of the surfaces offers

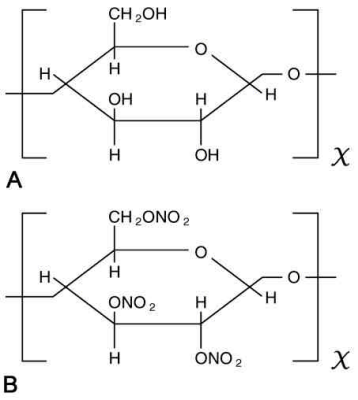

Figure 1. Structure of monomer unit of (A.) Cellulose and (B.) Nitrocellulose. The nitrocellulose unit shows the tri-substituted congener. X = 500 – 2,500.

advantages and disadvantages depending on the intended application. Microporous surfaces and membranes including cellulose, nitrocellulose, polyvinylidine difluoride (PVDF) and nylon are among the most common substrates used for traditional molecular biological techniques. Interestingly, the unique properties of microporous surfaces that make them suitable for traditional assays like Southern blots, such as large volume to surface area ratio, high binding capacity, reproducibility and ease of processing also make them suitable and desirable for the high-throughput methods that the post-genomic era requires.

Microporous surfaces have been used extensively for sample collection, long-term sample storage and analysis of biological samples for decades. Nitrocellulose membranes were used routinely in the 1960's and early 1970's as a platform for the immobilization of nucleic acids (8-11). Most significantly, the demonstration in 1975 by Southern that nitrocellulose could be used as a capture membrane for capillary transfer of DNA (12), and the subsequent demonstrations by Towbin, *et al*. (13), and Burnette (14) that proteins could be transferred from polyacrylamide gels to nitrocellulose membranes, helped define the next 20 years of molecular biology. In addition to the ubiquitous blotting applications that evolved in molecular biology research labs after 1975, microporous surfaces and other cellulosic materials have become synonymous with long term storage of biological samples, neonatal blood screening, and rapid lateral flow immunodiagnostics (15-25). Other traditional applications where microporous surfaces are routinely used include filtration/concentration, ion exchange, and amino acid sequencing.

In order to be useful as a medium for biomolecular analysis, a surface must immobilize the molecule(s) of interest in a near-quantitative fashion; it must allow for short and long term storage of the immobilized molecule(s); it must allow for a solution phase species to interact with the immobilized molecule(s); and it must not interfere with the detection strategy that is used. Microporous surfaces, such as nitrocellulose, meet all of these requirements. Furthermore, the three-dimensional structure of microporous surfaces makes them especially attractive for assays where structural stability and long-term storage of the immobilized species is required. For example, commercially available immuno-diagnostic tests that use nitrocellulose as the assay platform have shelf-lives of several years, and are in many instances semi-quantitative (23-25). This is especially interesting to note given that two of the greatest concerns in the early development of high-throughput protein arrays are stability and quantitation (26).

Microporous surfaces useful for biological reactions include cellulose, nitrocellulose, cellulose acetate (CA), polyether sulfone (PES), PVDF and nylon. Typically, these surfaces are used in membrane or sheet form with an approximate thickness of 100 micrometers, and average pore sizes that range from 0.05 to 10 micrometers in diameter, depending on the application. Interaction of biomolecules with each of these surfaces is unique and not fully understood, but generally occurs in a non-covalent manner. Derivitization is possible under some circumstances in order to add functional groups, but is not required for immobilization (27,28).

Of the surfaces listed above, nitrocellulose is perhaps the most versatile, finding utility in immobilization of nucleic acids, proteins and glycoproteins (12,13,29). It has been used for traditional blotting applications, high-throughput array applications, immunodiagnostic applications and mass-spectrometry coupled proteomic applications. In this review we will discuss the history of nitrocellulose in molecular biology reactions, along with the attractiveness of this polymer for high-throughput applications in the post-genomic era.

3. THE BASICS OF NITROCELLULOSE

3.1. Chemistry

Nitrocellulose is a polymer derived from cellulose (Figure 1). In one of the most common manufacturing processes, nitrate groups substitute the hydroxyl moieties on each sugar unit through treatment with nitric acid. Dry nitrocellulose is readily soluble in organic solvents forming a lacquer; evaporation of the solvents results in the deposition of the polymer as a thin film. Pores can be introduced into the film to create a microporous membrane by including a non-solvent, such as water in the lacquer. Pore formation results from differential evaporation of the solvent and non-solvent. Thus, porosity and pore size can be controlled easily by the amount of non-solvent in the lacquer (Figure 2).

The resulting film is a three-dimensional, hydrophobic structure, although addition of a surfactant during the casting process allows deposition of molecules in aqueous buffers (Figure 3). The most commonly used surfactants are anionic detergents such as sodium dodecyl sulfate (SDS) or Triton X100. Immobilization of deposited nucleic acids or proteins can typically be achieved through drying, either at elevated temperature for nucleic acids or at room temperature for proteins.

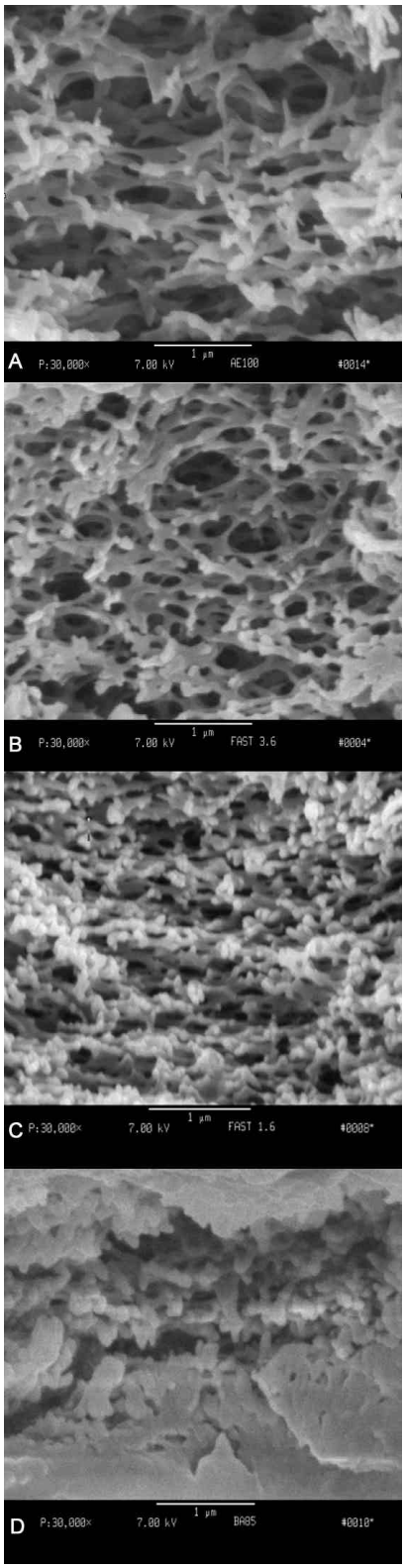

Figure 2. S.E.M. images of various pore size nitrocellulose membranes. (A.) Average pore size = >5micrometers; (B.) Average pore size = 1micrometer; (C.) Average pore size = 0.2micrometers; (D.) Average pore size = <0.1micrometer.

3.2. The history of nitrocellulose in molecular biology

The first use of nitrocellulose for molecular biology techniques came with the recognition that RNA-DNA duplexes formed in solution would bind to a nitrocellulose filter whereas free RNA would pass through (8). Gillespie and Spiegelman improved this technique by immobilizing single stranded DNA on the filter and then "probing" with radio-labeled RNA (9). Immobilization was easily accomplished by drying at moderate temperature. Later, nitrocellulose filters were used to study DNA-DNA, DNA-RNA and protein-DNA interactions (10,11,30,31). These techniques were successful due to the unique properties of nitrocellulose that created strong interactions with some forms of biomolecules but not with others. For example, protein-DNA interactions could be studied because nitrocellulose has a stronger affinity for proteins than for double stranded DNA. Therefore, proteins immobilized on filters could be "probed" with radio-labeled double stranded DNA that, under the right conditions, would not bind to the membrane (31).

Perhaps the most significant advancement of these techniques came from Southern's discovery that nucleic acids could be immobilized on nitrocellulose filters by capillary transfer from an agarose gel (12). This discovery meant that DNA segments of varying lengths could be separated prior to immobilization. A further advance was made when techniques were developed to accomplish the same feat with protein transfer from SDS-PAGE, a technique that has come to be known as the Western blot (13,14).

3.3. Immobilization and binding theory

Since the early reports on immobilization, transfer and differential affinities, a great deal of work has been done to understand the mechanism and theory behind immobilization of nucleic acids and proteins to nitrocellulose membranes (10,11,31-37). Several lines of evidence indicate that biomolecules interact with nitrocellulose via a non-covalent, hydrophobic interaction: drying and/or baking is necessary to immobilize molecules; low concentrations of methanol and high concentrations of salt increase immobilization efficiency; and immobilization efficiency is dependent on molecular length and size, an observation that can be explained theoretically by binding energies involving hydrophobic interactions (32). Despite these observations, the exact mechanism of interaction between nitrocellulose and biomolecules is not known. Compared to other microporous membranes, nitrocellulose is unique in its ability to distinguish, through binding energies and hydrophobic interactions, among single and double stranded nucleic acids, small and large proteins, short and long nucleic acids, and complexed vs. non-complexed molecules (32).

4. BLOTTING ON NITROCELLULOSE

Immobilization of DNA and proteins on nitrocellulose or other microporous membranes through blotting procedures has become one of the most widely used molecular biology techniques (38,39). Typically, complex mixtures, such as cell lysates or genomic DNA,

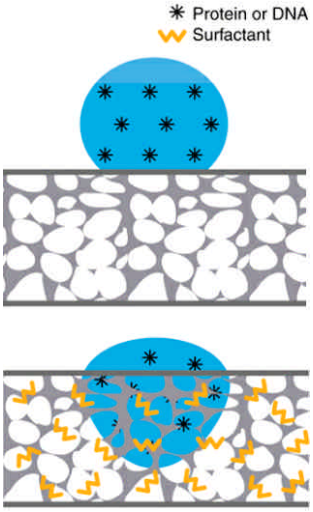

Figure 3. Schematic representation of hydrophobicity of nitrocellulose membranes. The nitrocellulose polymer is inherently hydrophobic. Aqueous solutions of biomolecules cannot penetrate a membrane that is pure nitrocellulose. If a surfactant is added during polymerization, then the hydrophobicity decreases, allowing aqueous solutions to penetrate.

are separated in one or two dimensions by gel electrophoresis. Transfer of molecules from gel to membrane can be accomplished by capillary- or electro-transfer. After immobilization by drying or crosslinking, the blot is probed with a known, labeled solution phase probe, and the binding or hybridization event of interest can be visualized by radioactivity or enzyme linked color or light production.

Blotting techniques have been used so extensively during the past two decades of biological research that they are now commonplace in most research labs. Several improvements and modifications have been made to the original techniques, including alternate transfer buffers, improved blocking solutions, and improvements in chemiluminescent detection (38-41). However, despite these improvements, the basic procedures have remained relatively unchanged. This is due in large part to the versatility of microporous membranes like nitrocellulose. Both Southern and Western blotting can be performed on nitrocellulose with baking or drying as the only immobilization step required; the availability of multiple pore sizes (0.05 – 10 micrometers) allows molecules of all sizes to be retained depending on the need; and the non-covalent, hydrophobic interaction of nitrocellulose with biomolecules appears to be universal. One distinct disadvantage of nitrocellulose membranes, compared to other membranes, is the inability to be stripped and re-probed multiple times due to the fragile nature of the polymer in membrane form. Casting the membrane directly on a solid support such as mylar sheets or glass helps strengthen the structure and allows for reprobing in some applications. Other membranes such as nylon and PVDF are inherently more durable than nitrocellulose, which is one reason they have found increased acceptance in the blotting field.

Despite the robustness of traditional blotting procedures, significant advances have recently been made regarding nitrocellulose and its use in protein blotting. Some of these advances concern detection methodologies and others involve new techniques for working with proteins. These reports help demonstrate the versatility of nitrocellulose as a surface for traditional and high throughput approaches.

4.1. Novel techniques for proteomic applications

Glycoproteins such as mucin are large molecular weight species that contain up to 90% carbohydrate by weight. Traditionally, these proteins are assayed colorimetrically in solution. Recently, Thornton, *et al*., published protocols demonstrating how the assay could be conducted on nitrocellulose membranes, using the periodic acid-Schiff's reagent to detect bound glycoprotein (29). Conducting the assay on nitrocellulose instead of in solution allowed for greater sensitivity and the ability to detect mucin from very dilute solutions. This latter point is a direct result of the microporous nature of the membrane structure having a large volume to surface area ratio providing a tremendous number of binding sites per unit area compared to non-porous surfaces.

Screening of phage display libraries to find antibodies to a specific antigen requires purified antigen for optimal selection. Liu and Marks developed a method whereby antigens electroblotted to nitrocellulose membranes could be used to select monoclonal scFV antibodies from a phage display library (42). Background is a critical issue with this technique since many blocking procedures can also reduce specificity and sensitivity. These authors found that due to the hydrophobic nature of the polymer, nitrocellulose had much lower background than other membranes such as nylon and DEAE-cellulose. By performing 2-D PAGE prior to blotting, antibodies could be rapidly obtained from as little as 1nanogram of antigen, a fact that makes this a powerful tool for high-throughput proteomics.

Sironi, *et al*., adapted an enzyme assay to nitrocellulose and were able to demonstrate high sensitivity due to the structure of the membrane (43). The assay measured tyrosine carboxypeptidase levels by looking at the release of [^{14}C] tyrosine from radiolabeled tubulin that had been adsorbed to the membrane. This system was reported to be 2 to 3 times more sensitive than the more common soluble assay procedure. The authors speculated that the increased sensitivity was the result of tubulin orientation on the membrane, or more likely that the membrane structure trapped the enzyme in close proximity to the substrate increasing the effective concentration of both species.

4.2. Fluorescent detection of blotted proteins on nitrocellulose

Historically, proteins have been detected on Western blots by radioactively labeled antibodies or antibodies coupled to enzymes such as alkaline phosphatase or horse-radish peroxidase that generate colored precipitates or luminescence when incubated with

the appropriate substrate (38,39). Although these methods are easy and can produce very good sensitivity and linearity, they do have limitations. First, the enzyme-linked systems involve multiple steps and are indirect measurements steps that do not directly measure the protein level of interest. Second, for radioactivity and chemiluminescence, the signal is often captured as a black spot on x-ray film, artificially limiting the dynamic range to that of the film and eliminating the possibility of multiplex analysis. In traditional blotting techniques, these factors are not always significant, but they do have the potential to limit the usefulness of the techniques. Several methods have been developed to allow fluorescent detection from nitrocellulose membranes. Thus, the benefits of instrument mediated fluorescent measurements can be combined with the benefits of using nitrocellulose as an immobilization substrate.

Fluorochromes that have been used to detect proteins on nitrocellulose include 5-dimethylaminonaphthaline-1-sulfonyl (dansyl chloride), Cy3, Cy5, PBXL-1, FITC and SYPRO Rose Plus - a europium based metal chelate (44-48). These dyes have excitation maximum wavelengths ranging from the UV region (dansyl) to the red region (Cy5). One significant advantage of fluorescent detection methods compared to enzymatic methods is the ability to directly label an antibody with the reporter molecule. Furthermore, in many cases, the antibody can be derivatized with multiple reporters resulting in direct amplification of the signal as opposed to enhancement of an enzymatic reaction product.

Perhaps the most significant advantage that fluorescent detection from nitrocellulose achieves is the ease with which the data can be quantified. Unlike techniques where the signal is first captured on film and subsequently digitized, fluorescent data are captured digitally when using standard imaging systems. This avoids artificial contraction of the data range on film. Most of the reports on fluorescent detection from membranes demonstrate an increase in sensitivity and linearity relative to chemiluminescent methods – in spite of the fact that the amount of chemiluminescent signal is proportional to the time of exposure (40). Furthermore, none of the authors reported background issues related to taking light based measurements from a microporous surface. Background issues were partially overcome by wetting the membrane prior to analysis making the membrane less opaque.

5. NITROCELLULOSE IN IMMUNOASSAYS AND DIAGNOSTIC TESTS

Immunoassays are routinely used in medical diagnostics to identify and quantify disease associated antigens or antibodies. These types of assays originated with radioimmunoassay (RIA) or saturation assays that used an isotopically labeled tracer to measure the amount of analyte present (49,50). In these tests, the bound analyte had to be physically separated from unbound (free) analyte. Later, adaptation of immunoassays to the solid phase, such as enzyme linked immunosorbant assay (ELISA), improved the ease and sensitivity of the tests (51,52). During the past two decades, several approaches have been taken to adapt immunoassays (enzyme linked as well as direct label) to lateral flow configurations, using nitrocellulose as the solid phase (23-25).

In lateral flow immunoassay a liquid sample interacts with antibodies that are immobilized on a microporous analytical surface as the liquid migrates through the interstitial space of the surface. The lateral flow of liquid by capillary action separates bound antigen from free with no mechanical manipulation required. Furthermore, if the immobilized antibody is present in excess relative to the antigen being tested, then, based on the laws of mass action and fractional occupancy, the amount of antigen bound will be in proportion to the amount present in the sample (50,53). In many assays, the sample first interacts on an absorbent pad, with an antibody linked to an enzyme or colored colloidal metal atom, forming a mobile antigen/antibody complex. The complex then migrates through the analytical surface. When the complex reaches the immobilized antibody, a sandwich is formed as the antigen binds, by a second epitope, to this antibody. This event can be seen immediately if a direct label is used, or after color development if an enzyme is used. The result is a line on the membrane if the antigen is present. The most common example of this type of assay is the test for human chorionic gonadotropin (hCG) for pregnancy (23-25).

Nitrocellulose membranes have several desirable properties that make them the microporous analytical surface of choice for lateral flow immunoassays (23,24). First, antibodies can be immobilized on nitrocellulose in an inert but stable manner. With the proper immobilization buffer, the immobilized antibody is stable for years (Tonkinson and Stillman, unpublished observations). Second, as discussed earlier, the average pore size in nitrocellulose membranes can be controlled and manipulated very easily (Figure 2). Pore diameter can range from 0.05 micrometers to 10 micrometers or greater if a support matrix is used. Pore size is the determining factor for the rate at which liquid migrates through the membrane. Thus, a membrane with pores averaging 8 micrometers will have a flow rate greater than a membrane with average pores of 5 micrometers. Pore size and flow rate also influence sensitivity of a lateral flow assay, by influencing the density of capture antibody molecules, and by controlling the rate at which the antigen passes through the capture antibody region.

Equilibrium binding experiments have demonstrated that nitrocellulose membranes are able to bind an average of 70micrograms protein/cm^2, although this value can vary depending on the formulation, pore size and method of immobilization (23,24). This capacity is substantially greater than for other cellulose-based membranes such as cellulose acetate, as well as PVDF, but is slightly lower than for nylon membranes. However, blocking non-specific protein binding sites on nitrocellulose is much easier than on nylon, leading to better specificity and signal/noise ratios. Most importantly for lateral flow, blocking of the membrane can be

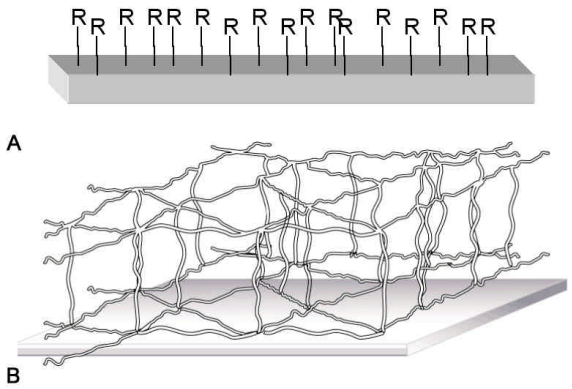

Figure 4. Comparison of 2-dimensional and 3-dimensional surfaces for arraying applications. (A.) A 2-dimensional activated surface, where R could be NH2, COH, COOH, or other reactive moiety. (B.) A 3-dimensional microporous structure on a glass surface.

accomplished "on-the-fly" as the assay runs. To accomplish this, blocking agents such as detergents, albumin or synthetic polymers are impregnated in the sample application pad, and migrate with the liquid front, binding to nitrocellulose along the way.

6. NITROCELLULOSE AND PROTEIN ARRAYS

6.1. Protein arrays and immobilization

Along with the maturation of the field known as genomics has come an increased awareness of the advantages of studying proteins using high-throughput methods. Not surprisingly, efforts to characterize the proteome have come to be known collectively as proteomics (54,55). Proteomic techniques are typically centered on the study of protein-protein interactions and differential expression. Some of the more common techniques associated with high-throughput proteomic analysis include 2-D PAGE, MALDI-TOF mass spec, and protein arrays (55).

Protein arrays have evolved from simple tools that allowed expression libraries to be rapidly screened, into post-genomic tools that allow for the targeted identification of multiple antigens in a complex lysate with the possibility of quantification (56-59). Unlike DNA hybridization, which involves the interaction of a single molecular species through Watson-Crick base pairing, protein binding events are much more complex, involving multiple types of interactions through numerous structural and chemical motifs. In addition, protein structure and stability are important considerations for examining binding events. These distinctions, coupled with the fact that a proteome is much more complex than its corresponding genome, make protein arrays more difficult to reduce to practice than DNA arrays. Despite these complexities, publications describing protein arrays have appeared in the literature for several years and the number has increased dramatically in the last three years (50, 53-73).

Several surfaces have been used for protein arrays including glass (61,65,70), polystyrene (63,65,66), polyacrylamide gel pads (67), PVDF (59,60), cellulose (63,64) and nitrocellulose (68-73). The use of two-dimensional surfaces, such as glass and polystyrene, stems from the DNA array concept that a two-dimensional surface may provide better binding and hybridization properties than a three-dimensional surface (74, 75) (Figure 4). However, immobilization of proteins on two-dimensional surfaces is not always trivial. For example, in order to immobilize them on glass the surface must first be activated. With DNA arrays, a simple poly-amine coating will suffice for non-covalent immobilization of PCR products. Although this strategy has been reportedly used for antibody arrays, proteins typically must be immobilized in a manner that preserves structure and activity, and in some cases, covalent attachment must be used for sufficient immobilization (26,58). These requirements mean that attachment strategies are typically more complex.

Strategies for protein immobilization on glass microarray substrates have included simple non-covalent adsorption to poly-lysine coated glass (58), covalent attachment to slides coated with aldehyde-silane (26), covalent attachment of peptides to slides pre-coated with BSA-NHS (26), and attachment via a bi-functional NHS coating on amino silane activated glass (61). Each of these strategies was used successfully to immobilize a limited number of antibodies or antigens to demonstrate proof-of-principle, although many of the strategies involved lengthy procedures to prepare the surface for arraying. The most simple of these strategies, passive adsorption to poly-lysine coated slides, was reported by Haab, *et al.*, to have limited specificity and sensitivity (58).

6.2. Nitrocellulose membranes as an immobilization medium

Given the historical use of nitrocellulose as a medium for immobilizing and studying proteins and the prevalence of nitrocellulose as a surface for lateral flow immunoassays, it is not surprising that microporous structures, including nitrocellulose, have found acceptance in the protein and tissue array field (Figures 5,6). Nitrocellulose has been successfully used as a surface for macro- and microarrays for screening phage display libraries (68), titrating antibodies (70), and identifying protein-protein and protein-nucleic acid interactions (69). Other applications include quantitative high-throughput immunoassays (70,71), immunophenotyping (72), and screening multiple patient samples simultaneously for specific diagnostic markers (73). These reports demonstrate compatibility with multiple types of detection methods including radioactive, chemiluminescent, fluorescent and colorimetric.

DeWildt, *et al*., demonstrated that a traditional method of screening microbial expression libraries for antibodies could be adapted to an array format that increased through-put by several orders of magnitude (68). Most significant, however, was the increase in accuracy and the ability to screen against impure solutions. These benefits directly resulted from using a microporous structure as the substrate, since it was compatible with robotic arraying, allowed for arraying of each clone in

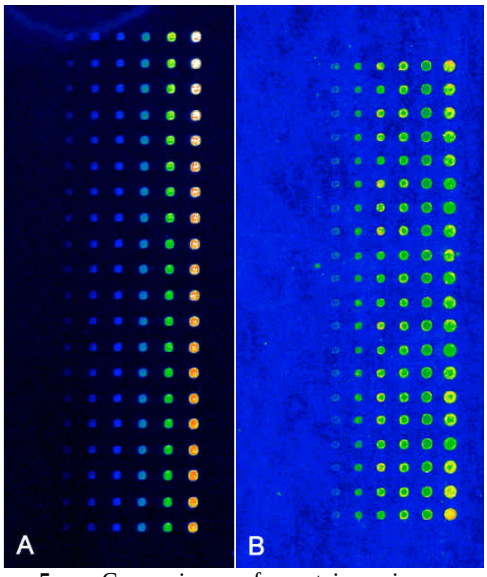

Figure 5. Comparison of protein microarray on nitrocellulose-coated glass (A.) vs. aldehyde-derivatized glass (B.). Cell lysates were made from CEM lymphocytic leukemia cells and then arrayed in serial dilution on the 2 surfaces. After fixation and blocking, arrays were probed using and anti-PCNA antibody followed by a Cy5 labeled secondary antibody. Slides were imaged in a ScanArray 4000 (Packard Instruments, Inc.). The Nitrocellulose slide was imaged at 25% laser power and 60% PMT voltage. The aldehyde slide was imaged at 80% laser power and 80% PMT voltage.

duplicate, and avoided multiple rounds of screening typically performed with ELISA.

One of the most distinct advantages of an array format compared with traditional blotting is the ability to screen numerous binding events in parallel. Ge reported the use of nitrocellulose in the development of a universal system to monitor protein-protein, protein-RNA or protein-DNA interactions among 48 purified transcription factors and co-activators arrayed on nitrocellulose membranes (69). In this system, approximately 7.5 picomoles of each purified protein was arrayed on the membrane using non-denaturing conditions, and the membrane was then probed with either a radioactively labeled protein or oligonucleotide. According to the author, the method is significant because of its universality, the ability to quickly compare affinities of the arrayed proteins for the solution phase species, and its specificity.

Several recent reports have detailed the use of nitrocellulose with protein or tissue arrays that provide diagnostic and prognostic information. Joos, *et al.*, used antigen arrays to demonstrate that accurate titers could be determined for up to 18 autoimmune antibodies simultaneously (70). Antigens routinely used as serological markers for diseases such as autoimmune thyroiditis and lupus were arrayed in dilution on glass or nitrocellulose. The arrays could be probed with as little as 100 microliters serum for 30 minutes followed by secondary antibody incubation and visualization by chemiluminescence. Using this system, the authors were able to accurately determine antibody titers for multiple diseases simultaneously with a small amount of material. It is interesting to note that the sensitivity of the assay was five fold better on nitrocellulose than on aldehyde activated glass (8 vs. 40 femtograms) corresponding to 0.05attomoles per spot that could be specifically detected on the nitrocellulose array.

6.3. Protein arrays on nitrocellulose thin films

Two very recent reports detail the use of nitrocellulose coated glass slides as a surface for cancer arrays. Belov, *et al.*, arrayed 60 antibodies specific to cell surface antigens (72). The array was used to screen leukocytes from peripheral blood, using dark-field microscopy to visualize aggregates of bound cells on each spot – a technique made possible by the translucent nature of the thin film nitrocellulose. The assay demonstrated very good linearity in terms of spot intensity vs. number of bound cells. Furthermore, there was very good correlation of the array results with confirmatory flow cytometric tests. This type of approach would not supplant flow cytometry for diagnosis of leukemias, but rather provide an initial screen of a sample against dozens of different antibodies.

Paweletz, *et al.*, created protein/tissue arrays on nitrocellulose thin films from material garnered with laser capture microdissection (LCMD) (73). This type of micro-immunohistochemistry platform was used to follow progression of invasive prostate cancer by looking at phosphorylation states of the oncogenes Akt and ERK, two markers of invasive carcinoma. The arrays were created by arraying lysates from cells obtained by LCMD and were visualized by a colorimetric approach using 3,3'-diaminobenzidine tetrahydrochloride as a chromagen. The authors note that this system demonstrated remarkable precision, dynamic range and reproducibility. As protein arrays move from research labs into diagnostic medicine, these three factors will become vital to success, underscoring the need for reliability of the immobilization surface.

One drawback of a microporous structure for array technology is the high background that is often obtained when taking fluorescent measurements. This background is attributable to the porous nature of the membrane that creates a substantial amount of light scatter. Scorilas, *et al.*, were able to overcome this problem by using a europium chelate system to visualize protein microarrays on nitrocellulose films (71). This fluorescent system is useful for time-resolved fluorometry since the reporter molecules have fluorescent lifetimes of 600-1000 microseconds. In this study, fluorescent measurements from the nitrocellulose film demonstrated antibody detection limits of 0.25 picograms/spot.

Finally, data from our own labs indicate that nitrocellulose thin films are superior to 2-D modified glass surfaces for some protein array applications. Figure 5 shows arrays of cell lysates on a nitrocellulose film slide and an aldehyde derivatized glass slide that were probed with an anti-proliferating cell nuclear antigen (PCNA) antibody. The signal strength was much greater on the

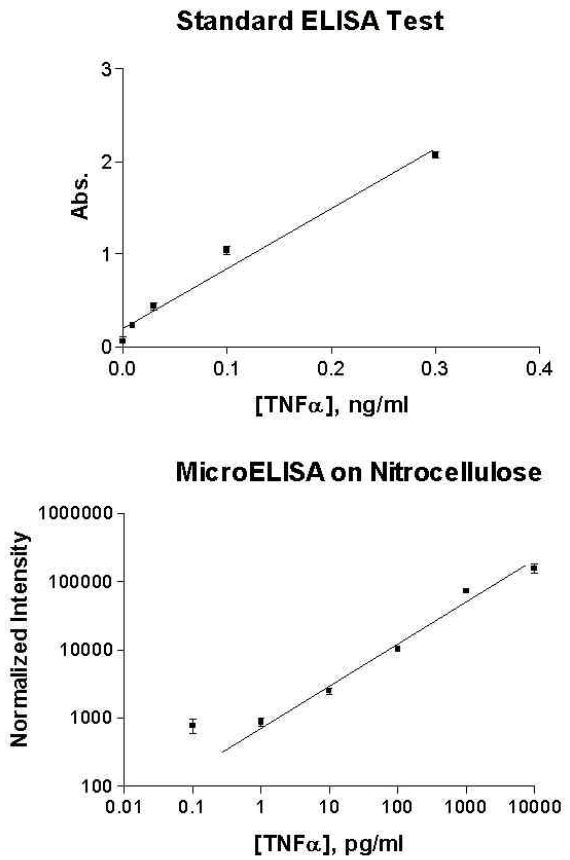

Figure 6. Comparison of linearity and sensitivity for sandwich assays for TNFα. Top: Standard curve from traditional colorimetric ELISA performed in a microtiter plate. Bottom: Standard curve from fluorescent sandwich assay performed on nitrocellulose thin film.

nitrocellulose film than on the 2-D surface. This observation can be attributed to the microporous nature of the film allowing more of the cell lysates to be retained. In addition, nitrocellulose thin films have been demonstrated to be useful for quantitative methods such as micro-array sandwich assays based on traditional micro-titer plate based enzyme linked immunosorbant assays (ELISAs) (Figure 6). A fluorescent based sandwich assay for TNFα performed on a nitrocellulose thin film had greater dynamic range and better sensitivity than the standard curve performed with a traditional colorimetric ELISA. The benefits were derived in part from fluorescent detection system compared to absorbance, but are were also due to the nature of capture antibody immobilization on a three-dimensional surface compared to a two-dimensional surface.

7. NITROCELLULOSE AND MASS SPECTROMETRY

Perhaps the most interesting post-genomic area where the nitrocellulose polymer plays a role is in mass spectrometry. Analysis of proteins, peptides and nucleic acids by mass spectrometry has advanced significantly during the past ten years. This is specifically related to the introduction of plasma desorption (PD) and matrix assisted laser desorption (MALDI) coupled to time-of-flight (TOF) analysis. These techniques are becoming increasingly common since they represent a powerful tool for high-throughput genomic and proteomic analysis.

Nitrocellulose has been used as a substrate for PDMS and MALDI-TOF mass spectrometry for a number of years (76-86). Typically, nitrocellulose polymer is dissolved in organic solvent such as acetone or alcohol and then deposited onto a stainless steel sample stage where ionization will occur. Rapid evaporation of the organic solvent leaves a thin film of nitrocellulose. Methods of depositing the nitrocellulose on the stage include electrospraying, air-brushing, or simple spot deposition with micro-fluidic pumps. The protein, peptide or nucleic acid mix of interest can then be deposited on the nitrocellulose.

For MALDI-TOF, where a matrix such as alpha-cyano-4-hydroxy-trans-cinnamic acid is used to help in spectroscopic analysis, the matrix can either be dissolved with the nitrocellulose, forming part of the thin film onto which the protein is spotted, or it can be applied with the sample after the film has formed (78,80-82,84). Interestingly, Landry, *et al*., found that peptide samples could be mixed with the nitrocellulose and matrix in the soluble organic phase and then deposited on the stage (84). The authors claim that this method resulted in ten-fold enhancement of detection compared to deposition of the peptide on the nitrocellulose film.

The advantages of using nitrocellulose as a mass spectrometry ionization surface include high binding capacity for the biological sample, the ability to store samples without degradation, and enhanced sensitivity and reproducibility (7,76-78). Due to the high binding capacity of nitrocellulose, the surface is very attractive for use with small samples. Several studies have demonstrated that the three-dimensional microporous nature of nitrocellulose membranes and films results in a binding capacity that is much greater than with two-dimensional surfaces (2,77,83). Jespersen, *et al*., reported that enough sample was captured on the film that after ionization for mass spec, enough peptide sample was left that it could be extracted for Edman degradation, HPLC analysis or further solution phase manipulation (77). This allowed for multiple pieces of information to be obtained from a small sample.

The nature of the nitrocellulose/protein interaction helps create an environment in which little or no degradation of immobilized protein occurs over time. In a study by Bodarenko, *et al*., protein samples stored on nitrocellulose coated substrates showed no loss in molecular ion yields (PDMS) over a 2-3 week period, whereas prepared protein samples stored in a vacuum lost 10% of their yield in just 36 hours (76).

Nitrocellulose has been reported to increase sensitivity and reproducibility when used as a substrate for MALDI-TOF mass spec. Several reports indicate that this is due to the ability to wash the surface after the sample is applied in order to remove contaminating alkali metal

contaminants, resulting in an enhanced $[M+H]^+$ ion yield (78). Preston *et al*., demonstrated that nitrocellulose is capable of enhancing the ion yield, thereby improving sensitivity and reproducibility even without washing (78). The authors speculated that this was a result of the nitrocellulose binding the impurities during ionization and/or acting as a crystallization moderator. The latter possibility would stem from the ability of the polymer to act as a nucleation site for the deposited peptide and matrix or from the polymer covering the (microscopically) irregular stainless steel surface.

The advantages of using nitrocellulose as a surface for MALDI-TOF have led to its current use in a wide variety of mass spec applications as techniques become more ubiquitous and high-throughput in nature (82-86). An interesting application developed by Liang, *et al*, coupled solid-phase immunoassay with mass spec to create a highly sensitive micro-immunoassay (82). The procedure took advantage of both the protein immobilization properties of nitrocellulose as well as the benefits stated previously of using nitrocellulose with MALDI-TOF mass spec applications. In this instance, polyclonal IgG antibodies were assembled on the nitrocellulose surface. The corresponding antigen could then be extracted from a complex solution and analyzed unambiguously by mass spec.

Several reports have detailed the high-throughput analysis of protein chips by mass spectrometry (7,83,86). In one system, liquid chromatography is used to separate a protein mixture (83,86). Fractions are deposited on the mass spec surface, either aluminum or silicon, which are then coated with a nitrocellulose/matrix coating. Using this system, hundreds of proteins can be examined in less than 4 hours. In a study aimed at coupling this technology to diagnostics, Borrebaeck, *et al*, created antibody arrays on polystyrene (a traditional immunoassay plastic) silicon, or silicon coated with nitrocellulose (7). The arrays were then incubated with a protein mixture and analyzed by MALDI-TOF. Of the surfaces examined, the nitrocellulose-coated silicon demonstrated the highest sensitivity and capacity by several orders of magnitude. This corresponded to a mass spec detection of antigen in the 600 attomole range.

7. PERSPECTIVES

The genomic revolution has provided biomedical researchers with powerful new tools to use in the post-genomic era. Perhaps the most useful of these is an altered perspective on how information can be collected. Instead of thinking about a linear process, researchers can take advantage of high-throughput techniques to study parallel processes; instead of thinking about the state of a gene or a family of genes, one can now examine the state of multiple gene families, simultaneously. As thinking has changed in this era, it is interesting to note that many of the new techniques being developed are simply variations on old themes. The fundamental technique of examining one molecule by hybridizing or binding it to a second molecule is one such example.

Nitrocellulose is a polymer that has been used for decades to immobilize bio-molecules so that they can be studied. While nitrocellulose membranes will undoubtedly be used for quite some time as surfaces for traditional blotting, this unique polymer is finding utility in other forms with many post-genomic high-throughput techniques. Researchers are discovering that the properties that made nitrocellulose a utilitarian tool for such a long time continue to be required as sensitivity and throughput demands are increased. Undoubtedly, as we continue to move forward in the post-genomic era, many other historical lessons will be utilized.

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