

**HYDROPHILIC, HIGH PROTEIN BINDING, LOW FLUORESCENCE,
WESTERN BLOTTING MEMBRANE**

This application is a continuation of Ser. No. 12/462,635 filed August 6, 2009, which claims priority of Provisional Application Serial No. 61/189,302 filed August 18, 2008, the disclosures of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

"Blotting" or "electro-blotting" refers to the process used to transfer biological samples from a gel to a membrane under the influence of an electric field. The process requires a membrane that can immobilize biomolecular samples for subsequent detection. This places specific requirements on the membranes related to surface area, porosity, and protein binding capacity.

Western blotting is one modification of this technique that involves the immobilization of proteins on membranes before detection using monoclonal or polyclonal antibodies. Prior to protein immobilization on the membrane, sample proteins are separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) to separate native or denatured proteins. The proteins are then transferred or electro-blotted onto a membrane, where they are probed and ultimately detected using antibodies specific to a target protein. Western blotting membranes are typically made of nitrocellulose (NC) or polyvinylidene fluoride (PVDF). The specificity of

the antibody-antigen interaction can enable a single protein to be identified among a complex protein mixture.

To summarize, Western blotting involves application of a protein sample (lysate) onto a polyacrylamide gel, subsequent separation of said complex mixture by electrophoresis, and transferal or "electro-blotting" of separated proteins onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Following the transfer, the membrane is "blocked" to prevent nonspecific binding of antibodies to the membrane surface. Many antibody labeling or tagging strategies are known to those skilled in the art. In the simplest protocols, the transferred proteins are incubated or complexed with a primary enzyme-labeled antibody that serves as a probe. After blocking non-specific binding sites a suitable substrate is added to complex with the enzyme, and together they react to form chromogenic, chemiluminescent, or fluorogenic detectable products that allow for visual, chemiluminescence, or fluorescence detection, respectively. The most sensitive detection schemes make use of chemiluminescent or fluorescent phenomena. In chemiluminescent detection, an enzyme-substrate complex produces detectable optical emissions

(chemiluminescence). These emissions are recorded and measured using suitable detectors such as film or photonic devices. Absence or presence of signal indicates whether a specific protein is present in the lysate, and signal intensity is related to the level of the protein of interest, which in some cases may be quantifiable.

The use of nitrocellulose membranes is ubiquitous in immunodetection assay work, particularly in Western blotting. This is partially due to historical considerations, and partially due to ease of use. Nitrocellulose blotting membranes do not require an organic liquid pre-wet step, a requirement for working with hydrophobic membranes. Hydrophobic membranes require an alcohol pre-wet step followed by a water exchange step (for alcohol removal), before assembly within the blot-transfer assembly. Intrinsically hydrophobic membranes afford a limited time-frame for this assembly; the potential for the membrane to dry out is significant. Once dry, the membrane cannot be re-wet unless the pre-wet sequence is repeated. Once the membrane is contacted to the gel, removal prior to transfer can effectively ruin the gel and the separated protein samples contained. The pre-wet step is time consuming and can considerably impede workflow. A

hydrophilic membrane will remain wet for a longer time interval, and can be re-wet with water if it does dry out before assembly.

Nitrocellulose blotting membranes are water wet-able and show satisfactory performance for most blotting applications. But nitrocellulose is not as mechanically or chemically stable as PVDF. PVDF will maintain its mechanical integrity over a long timeframe, whereas NC will become brittle and discolored. PVDF membrane blots can be stripped of antibodies and be re-probed. NC blots cannot. NC is prone to air oxidation, wherein it can become hazardous. It requires a separate waste stream, and when disposed of must be damped with a wetting agent, usually water.

Hydrophobic PVDF blotting membranes possess equivalent protein binding ability to NC blotting membranes, but exhibit superior blotting performance. Much lower sample concentrations can be detected under the same conditions on these PVDF membranes compared to NC. Low-background fluorescence hydrophobic PVDF blotting membranes exhibit the same enhanced sample detection while enabling the use of fluorescent detection schemes.

It therefore would be desirable to provide a hydrophilic PVDF membrane for immunodetection assays such as Western blotting, with performance characteristics that approach the lower sample detection limits and low background fluorescence that are characteristic of hydrophobic PVDF membranes. This invention addresses these requirements.

SUMMARY OF THE INVENTION

Those skilled in the art of surface modification for the purpose of altering substrate surface energies, and in particular with regard to surfaces intended for contact with biological systems, will concur that hydrophilic surface modifications traditionally exhibit low protein binding behavior. The embodiments disclosed herein build on the serendipitous and unexpected discovery that space polymers derived from certain monomeric acrylamide mixtures, and formed using free-radical polymerization reactions, can give rise to surface modifications that are not only hydrophilic, but also demonstrate a high level of protein binding.

Much of the prior art describes the use of hydroxyl containing monomers, usually carbonyl ester containing acrylate polymers, to produce membrane surface

modifications having hydrophilic character and high resistance to protein binding. However, it is known that polymers from such monomers are not resistant to strong alkaline solutions. For example, a solution of 1.0 normal sodium hydroxide will hydrolyze the carbonyl containing acrylate polymers to acrylic acid containing polymers. Such acrylic acid containing polymers are ionically charged under certain pH conditions, and will attract and bind oppositely charged proteins or biomolecules, thus increasing sorption and membrane fouling. In addition, acrylic acid containing polymers swell in water to an extent that they constrict pore passages, thus reducing membrane permeability and productivity. Moreover, polymers from hydroxyl containing monomers, such as hydroxy acrylates, further react in strong alkaline solutions and degrade into soluble low molecular weight fragments, which dissolve away and expose the underlying substrate porous media or membrane.

Practitioners attempting to develop optimized membranes either for filtration or non-filtration applications in the pharmaceutical and biotechnology industries must overcome significant problems. Facing stringent cost, performance and safety requirements, a practitioner must use materials

and develop manufacturing methods that produce membranes with not only optimized flow and retention characteristics, but be economical to produce, meet cleanliness criteria, be stable to the various chemical environments which are commonly encountered, and be very either very resistant to biomolecule adsorption, or very strongly adsorbing, depending upon the intended end-use. Thus, in this instance, it is very desirable to have a membrane modification that results in a hydrophilic, biomolecule adsorptive surface that is heat stable, which is resistant to degradation by any potential reagent solutions, and which has very low levels of material capable of being extracted there-from.

Protein binding results from early investigations into mixed-acrylamide polymeric surface modifications indicated that certain mixtures of hydrophilic bis-acrylamide crosslinking monomers and monofunctional neutral or charged acrylamides can, when copolymerized using UV-initiated or electron beam-initiated free-radical techniques, produce high protein-binding hydrophilic surface modifications. However, initial starting levels of each monomer had to be severely decreased before observing satisfactory dot blot morphology and blotting transfer performance.

The problems of the prior art have been overcome by the present embodiments, which provide a hydrophilic membrane particularly suited for blotting applications, preferably Western blotting. More specifically, a pre-wet hydrophobic membrane substrate, preferably made of PVDF, is contacted with a monomer solution and subjected to polymerizing conditions to render the substrate permanently hydrophilic.

The resulting membrane exhibits low background fluorescence, high protein binding, excellent retention of protein sample spot morphology, and extended dynamic range (high signal-to-noise ratio, enhanced sample detectability). Where chemiluminescence is used for detection, the level of background fluorescence inherent in the unmodified parent membrane is not as critical. The membrane demonstrates comparable or higher performance in Western blotting applications than conventional nitrocellulose blotting membranes, particularly for detection at low sample concentrations, and is directly water-wettable, eliminating the need for an alcohol pre-wet step prior to use. The membrane exhibits complete, instant, and uniform wetting upon contact with water, and exhibits delayed wetting when contacted with a saturated aqueous aluminum chloride solution. That is, when said

membrane is placed on the surface of this saturated aqueous aluminum chloride solution, it wets through in a minimum time interval of not less than 1 second).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-3 are diagrams of Western blot results using treated membranes in accordance with certain embodiments.

DETAILED DESCRIPTION

The membranes hydrophilically modified in accordance with embodiments of the present disclosure provide immunodetection assay platforms that are comparable to, or exhibit superior blotting performance to nitrocellulose membranes, particularly with respect to expansion of the low end of the dynamic range of sample detectability. For example, in FIG. 1, Western blotting results from a typical development run demonstrate the performance differences between the hydrophilic PVDF blotting membrane of this invention, and the controls (FL - hydrophobic PVDF membrane, and NC - Whatman/S&S BA-85 membrane). Each horizontal strip in the figure contains 5 separate Western transfer blots; three blots on hydrophilic PVDF development samples, and one blot on each of the control membranes. Each horizontal strip of 5 Western blots is the result from

one electrophoresis and transfer experiment (5 gels followed by 5 blots were run in each experiment). By design, each experiment embodies identical conditions on each gel/blot with identical quantities of protein sample (applied in 4 lanes across each gel) before electrophoresis and transfer. The results shown are the recorded (chemiluminescent) transfer blots for the detection of 2 proteins (HSP70 and GAPDH) from a complex sample mixture (lysate), applied at decreasing sample concentrations, from left-to-right on each gel, and corresponding to: 5 µg, 2.5 µg, 1.25 µg, 0.67 µg.

Suitable porous membranes include those formed from aromatic sulfone polymers, polytetrafluoroethylene, perfluorinated thermoplastic polymers, polyolefin polymers, ultrahigh molecular weight polyethylene, polyamides including Nylon 6 and Nylon 66, and polyvinylidene fluoride, with polyvinylidene fluoride being particularly preferred. Porous membranes include both microporous membranes and ultrafiltration membranes, and are preferably in the form of sheets. Generally the average pore sizes include those between 0.001 and 10 microns. Blotting membranes are nominally 0.45µm pore size materials. Preferred starting membranes have a porosity (void volume)

range specification of 68-73%. Blotting membranes are traditionally symmetric. However, the coating could be applied to an asymmetric membrane.

The polymeric coating can be a copolymer or terpolymer formed from at least one polyfunctional monomer modified with at least one hydrophilic functional group, said hydrophilic polyfunctional monomer(s) selected from the group consisting of polyfunctional acrylamides, polyfunctional methacrylamides and diacroylpiperazines, and formed from at least one monofunctional monomer modified with at least one hydrophilic functional group, said hydrophilic monofunctional monomer(s) selected from the group consisting of monofunctional acrylamides, monofunctional methacrylamides, and acryloyl piperazines.

It was found that a porous hydrophobic membrane, preferably one made of polyvinylidene fluoride coated with a crosslinked acrylamide - methylene-bis-acrylamide copolymer was rendered highly hydrophilic. Furthermore, at the copolymer level that was applied to the porous PVDF membrane samples in early iterations of this invention, IgG binding assays revealed protein binding levels to be in the neighborhood of 400 $\mu\text{g}/\text{cm}^2$. This level is typical of the parent hydrophobic PVDF membrane and of conventional

nitrocellulose membranes. The first surprising result was that membranes so prepared were both hydrophilic, and high protein-binding. However, the Western blotting performance of these initial samples (those that exhibited this high level of protein binding) was not satisfactory in terms of maintaining small sample blot size (blot morphology), and in terms of sample capture in blot transfers. By modifying the copolymer coating level, satisfactory blotting performance was realized. At these modified levels, protein binding levels were reduced to between 250 and 325(00) $\mu\text{g}/\text{cm}^2$, but Western blotting performance rose to levels intermediate between nitrocellulose and the preferred parent hydrophobic PVDF membrane. Surprisingly, the present inventors found that the protein binding level is not the best or only predictor of Western blotting membrane performance. Sacrificing some protein binding ability by modifying the coating level on the substrate can result in improved blotting performance.

Thus, component levels and relative concentrations of the modifying formulation are critical to obtain acceptable immunodetection assay performance. A low overall solids concentration in a highly specific component ratio balances water-wetting performance against blotting performance.

The very low background fluorescence level of the substrate membrane is preserved. If, however, the level of surface modification chemistry is too low, the result is a membrane that is not water-wettable to an acceptable extent. If the level of surface modification is too high the resulting membranes exhibit extremely high surface energies. As stated earlier, at higher levels of surface modifying chemistry, the measured protein binding capacity is roughly equivalent to nitrocellulose and hydrophobic PVDF membranes, but poor electro-blotting performance results.

In accordance with certain embodiments, the total solids level in the modifying/reactant solution is to be adjusted to between 0.90% and 1.10% by weight. A total solids concentration in this range with the specified component ratio results in optimal blotting performance. This formulation includes a UV-photoinitiator component.

Suitable amounts of the acrylamide monofunctional monomer and the bis-acrylamide crosslinking monomer in the reactant solution are to be between 0.20% and 2.00% by weight (each), preferably with amounts between 0.30% and 0.60% by weight (each), and most preferably between 0.40% and 0.50% by weight (inclusive, each). The preferred ratio of acrylamide to bis-acrylamide of the monomer reactant

solution is about 1:1 (mass/mass). The preferred overall monomer concentration of acrylamide:methylene-bis-acrylamide monomer reactant solution is between 0.5% and 1.5% by mass. A suitable UV-photoinitiator component is present in 0.01% to 0.20% by weight preferably between 0.05 and 0.15% by weight, and most preferably between 0.09% and 0.11%, by weight. Suitable UV-photoinitiators include Irgacure 500, 754, 2959, and 819DW. Methods for preparation of the modified porous membrane substrate in accordance with certain embodiments include the steps of providing a porous membrane substrate, contacting the surface of the porous membrane substrate with a reactant solution comprising acrylamide and methylene-bis-acrylamide, and a suitable photoinitiator, removing the membrane from the solution, and polymerizing the coating *in situ* on the membrane substrate by exposing the same to radiation of a suitable wavelength and intensity for a suitable time interval. Preferably the porous membrane contacted with the reaction solution is irradiated with an ultraviolet light source. Filters may be used to reduce or eliminate undesirable wavelengths which may cause damage to the porous membrane. The amount of exposure time to the UV light and the intensity thereof should be familiar to those skilled in the art.

In the preferred embodiment of the invention, a laboratory-scale preparation of the reactant monomer solution is made by dissolving 1.00g acrylamide, $[H_2C=CH-C(O)-NH_2]$ monofunctional monomer; 0.80g methylene-bis-acrylamide, $[H_2C(-NH-C(O)CH=CH_2)_2]$ cross-linking monomer; and, 0.20g Irgacure 2959 photo-initiator into 198.00g of Milli-Q® water. An extended mixing interval of about 2 hours is required to fully dissolve the cross-linker and the photo-initiator.

More specifically, the porous hydrophobic starting membrane is pre-wet by immersion in an organic liquid or in an aqueous solution thereof that does not swell or dissolve the porous membrane, and which pre-wets the entire porous surface of the membrane.

The liquid may be a low molecular weight alcohol, or a mixture of water and a miscible organic liquid. Suitable liquids or compositions include methanol, ethanol, isopropanol, water mixtures thereof, acetone/water mixtures, and tetrahydrofuran/water mixtures of sufficiently low surface tension to affect wetting the entire membrane surface.

The purpose of this pre-wetting step is to assure that the entire membrane surface is rendered wettable by water, and subsequently by the aqueous reactant monomer solution. The pre-wetting step must be followed by a rigorous exchange step with water to eliminate the presence of the organic solvent. These pre-wetting solvents or water mixtures thereof can exert a negative influence upon the intended polymerization of the reactant monomers.

Subsequent immersion and gentle agitation of the water-wet porous membrane in the reactant solution allows the entire surface of the porous membrane to be wet with reactant solution. As long as excess water is removed from the membrane prior to immersion in the reactant solution, no significant dilution of the reactant solution will occur.

The sample is withdrawn after a short (two minute) time interval and excess reactant solution is removed from the membrane sample. The reactant solution-wetted membrane is anaerobically exposed to UV radiation to effect the polymerization directly onto the entire porous membrane surface. The resulting coated membrane exhibits: Immediate, complete, and thoroughly uniform wetting when contacted onto a water surface; A high level of Western blotting performance; A high level of protein binding (≥ 250

$\mu\text{g}/\text{cm}^2$ IgG) by radio-labeled assay; and, Low background fluorescence (about 2000 rfu @ 485nm/535nm excitation/emission wavelengths using a TECAN GENios FL fluorescence reader with detector gain set at 86, and running Magellan 5.0 software package), which is about twice the background fluorescence of the untreated (unmodified parent hydrophobic) membrane under the same measurement conditions. When placed on the surface of a saturated aqueous aluminum chloride solution, the membrane will wet through in a minimum time interval of not less than 1 second, and in a maximum time interval that may exceed 60 seconds.

Example 1

Commercially available hydrophobic PVDF membrane from Millipore Corporation (IPFL000000) was immersed in methyl alcohol. The membrane was withdrawn and immersed in water with agitation to extract methanol for 1 minute. The membrane was withdrawn and immersed in fresh water for an additional 2-minute interval and then placed in fresh water before immersion in reactant monomer solution. Excess water was drained from the membrane and the membrane was then immersed in monomer reactant solution with gentle agitation for 2 minutes. Membrane was then exposed to UV

radiation from both sides in a UV curing process at a line speed of 15 to 25 fpm. Membrane was recovered and placed into a water bath to remove unreacted monomer and non-adhering oligomers and polymer. Samples were dried either in air at room temperature overnight, or in a static forced-air oven between 60°C and 80°C for 10 minutes, or on an impingement dryer at 90-110°C at a line speed of 15 to 25 fpm. Average membrane extractables as determined by an in-house TOC (Total Organic Carbon) method were measured to be about 1.44 µg/cm², as shown in Table 1.

Table 1 - Total Organic Carbon (TOC) - Five (5) 47mm disks.

Requestor ID: MIL6UJP37			PVDF Hydrophillic IPFL Membrane Received		
Requestor: Antoni Peters			For Blotting and Immuno Assays Applications		
			Ext. for TOC Analysis		
			Extraction By: A. Pervez		
			TOC By: M. Santos-Rosa & A. Pervez		
			Date: 10/16-19/2006		
Ext.	Ext. Temp.	Ext. Time	Ext. Vol.	Ext. Area	
Solvent	[°C]	[Hours]	[g]	[cm²]	
MilliQ Water	Ambient	24	40	86.75	
					Summary
					Results
	TOC Acid/Oxid	TOC	Corrected TOC	TOC	TOC
Sample ID	[µL/min]	[ppm C]	[ppm C]	[µg C]	[µg C/cm²]
Water Blank	0.20/0.20	0.0639			
T102 072806 A:-MBAM/AC/I-2959	0.75/1.00	3.29	3.23	129	1.49
T102 072806 B:-MBAM/AC/I-2959	0.75/1.00	2.91	2.85	114	1.31
T102 072806 C:-MBAM/AC/I-2959	0.75/1.00	3.33	3.27	131	1.51

Average extractable residual monomer levels were determined by HPLC. Values determined from 3 samples are provided in Table 2.

Table 2. Extractable Monomer Levels by HPLC – Same samples as shown in Table 1.

Sample	Acrylamide ($\mu\text{g}/\text{cm}^2$)	MBAM ($\mu\text{g}/\text{cm}^2$)	Irgacure 2959 ($\mu\text{g}/\text{cm}^2$)
T102 072806A	0.002	0.105	N.D.
T102 072806B	0.005	0.091	N.D.
T102 072806C	0.011	0.094	N.D.

Example 2

The procedure of Example 1 was used to treat PVDF membranes having the specifications, treatment conditions and reactant solutions shown in Tables 3A-D, 4A-D, and 5A-D.

Table 3A

MODIFICATION DATA for Hydrophilic PVDF Western Blotting Membrane

	Monomer	Starting Membrane		Membrane					
				Casting					
Roll No.	Mix	Lot Data		Dryer	Thick (um)	Porosity (%)	Bbl Pt (psi)	Flow Time (seconds)	
	Lot#			Temperature (F)					

RUN R - No Mix Adjustment - Footage VS Monomer Mix Concentration

NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
R01	R Mix 1	IPFL 071007 T214		300	112	72.8	9.4	56.0	
R02	R Mix 1	IPFL 071007 T205		300	113	73.0	9.4	63.0	
R03	R Mix 1	IPFL 071007 T205		300	113	73.0	9.4	63.0	
R04	R Mix 1	IPFL 071007 T205		300	113	73.0	9.4	63.0	
R05	R Mix 1	IPFL 071007 T205		300	113	73.0	9.4	63.0	
R06	R Mix 1	IPFL 071007 T206		300	111	72.4	9.5	65.0	
R07	R Mix 1	IPFL 071007 T206		300	111	72.4	9.5	65.0	
R08	R Mix 1	IPFL 071007 T206		300	111	72.4	9.5	65.0	
R09	R Mix 1	IPFL 071007 T206		300	111	72.4	9.5	65.0	
R10	R Mix 1	IPFL 071007 T214		300	112	72.8	9.4	56.0	
R11	R Mix 1	IPFL 071007 T214		300	112	72.8	9.4	56.0	
R12	R Mix 1	IPFL 071007 T214		300	112	72.8	9.4	56.0	
R12 End	NA	NA		300	NA	NA	NA	NA	NA

Table 3B

MODIFICATION DATA for Hydrophilic PVDF Western Blotting Membrane

Roll No.	Monomer Mix Sample ID	Monomer Mix Component Concentrations				Totalized Footage By Roll
		Actual	Actual	Actual	Actual	
		AC Wt% (HPLC)	MBAM Wt% (HPLC)	I-2959 Wt% (HPLC)	Total Solids Wt% (HPLC)	

RUN R - No Mix Adjustment - Footage VS Monomer Mix Concentration						
NA	R Mix 1 DRUM	0.5156	0.4214	0.1130	1.0500	0
R01	MM1 Start	0.5091	0.4042	0.1102	1.0235	0
R02	MM2	0.5105	0.3957	0.1094	1.0156	500
R03	MM3	0.5038	0.3837	0.1055	0.9931	800
R04	MM4	0.4996	0.3773	0.1001	0.9771	1100
R05	MM5	0.4948	0.3708	0.0999	0.9655	1250
R06	MM6	0.4935	0.3685	0.1020	0.9640	1550
R07	MM7	0.4868	0.3618	0.0995	0.9481	1850
R08	MM8	0.4868	0.3605	0.0974	0.9448	2150
R09	MM9	0.4777	0.3553	0.0956	0.9286	2300
R10	MM10	0.4721	0.3528	0.0956	0.9204	2600
R11	MM11	0.4673	0.3521	0.0932	0.9125	2900
R12	MM12	0.4682	0.3561	0.0918	0.9161	3200
R12 End	MM13 End	0.4574	0.3546	0.0879	0.8999	3300

MODIFICATION DATA for Hydrophilic PVDF Western Blotting Membrane

[illegible]

RUN R - No Mix Adjustment - Footage VS Monomer Mix Concentration											
		NA	NA	NA	NA	NA	NA				
NA		NA	NA	NA	NA	NA	NA	1.0	1.6	1.0	90.0
R01		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	70.0
R02		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	56.7
R03		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	39.3
R04		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	33.0
R05		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	28.8
R06		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	27.8
R07		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	26.9
R08		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	26.4
R09		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	27.4
R10		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	25.2
R11		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	27.1
R12		20	20	H/4	Staggered	10/9.5	10/9.5	1.1	1.7	1.1	27.8
R12 End		NA	NA	NA	NA	NA	NA	1.1	1.7	1.1	25.2

MODIFICATION DATA for Hydrophilic PVDF Western Blotting Membrane

RUN R - No Mix Adjustment - Footage VS Monomer Mix Concentration

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Table 4A

MEMBRANE MODIFICATION DATA - Hydrophilic PVDF Western Blotting Membrane

		Monomer	Starting		Heat Treat T		Membrane
					or Mod Line		Casting
Run	Roll No.	Mix	Membrane		Dryer		Dryer
Segment		Lot#	Lot Data		Temperature	Temperature	Temperature

RUN S - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED							
NA	NA	NA	NA				
Test	R01	S Mix 1	IPFL 071007 T203	205			300
S1	R02	S Mix 1	IPX 070907 R103	205			300
S1	R03	S Mix 1	IPX 091407 T103	205			310
S1	R04	S Mix 1	IPX 120307 T107	205			300
S1	R05	S Mix 1	IPX 091407 T102	205			310
S1	R06	S Mix 1	IPX 070907 R105	204			300
S2	R07	S Mix 1	IPX 120307 T107	200			300
S2	R08	S Mix 1	IPX 120307 T107	Transition			300
S2	R09	S Mix 1	IPX 120307 T107	205			300
S2	R10	S Mix 1	IPX 120307 T107	Transition			300
S2	R11	S Mix 1	IPX 120307 T107	210			300
S2	R12	S Mix 1	IPX 120307 T107	210			300
S2	R13	S Mix 1	IPX 120307 T107	210			300

Segment 1 Variation of starting membrane properties
Segment 2 Variation of line speed and dryer temperature

Table 4B

MEMBRANE MODIFICATION DATA - Hydrophilic PVDF Western Blotting Membrane

Run Segment	Roll No.	Starting Membrane Properties			Monomer Mix Samples and Mix Component Concentrations								Totalized	
		Thick (um)	Porosity (%)	Bbl Pl (psi)	Flow Time (seconds)	Monomer Mix Sample ID	Actual Wt% (HPLC)	Actual AC	Actual MBAM	Actual I-2959	Actual Total Solids	Actual Wt% (HPLC)	Footage By Roll	

RUN S - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED

NA Test	NA	NA	NA	NA	NA	MM1B	0.4979	0.3894	0.1005	0.9878	0.9878	0.1005	150	
S1	R01	112	72.8	9.6	61.0	MM1C start	0.4926	0.3877	0.1005	0.9808	0.9808	0.1005	300	
S1	R02	115	74.8	9.3	55.0	MM2B	0.4893	0.3849	0.0996	0.9738	0.9738	0.0996	450	
S1	R03	115	66.4	9.6	59.0	MM3B	0.4812	0.3785	0.0971	0.9568	0.9568	0.0971	600	
S1	R04	126	72.9	9.4	65.0	MM4B	0.4780	0.3760	0.0963	0.9503	0.9503	0.0963	750	
S1	R05	122	66.2	11.1	78.3	MM5B	0.4761	0.3733	0.0954	0.9448	0.9448	0.0954	950	
S1	R06	130	74.7	10.7	74.0	MM6B	0.4785	0.3757	0.0953	0.9495	0.9495	0.0953	1100	
S2	R07	126	72.9	9.4	65.0	MM7B	0.4699	0.3678	0.0934	0.9311	0.9311	0.0934	1200	
S2	R08	126	72.9	9.4	65.0	MM8B	0.4673	0.3656	0.0926	0.9255	0.9255	0.0926	1305	
S2	R09	126	72.9	9.4	65.0	MM9B	0.4660	0.3644	0.0925	0.9229	0.9229	0.0925	1405	
S2	R10	126	72.9	9.4	65.0	MM10B	0.4655	0.3637	0.0917	0.9209	0.9209	0.0917	1505	
S2	R11	126	72.9	9.4	65.0	MM11B	0.4642	0.3619	0.0910	0.9171	0.9171	0.0910	1605	
S2	R12	126	72.9	9.4	65.0	MM12B	0.4618	0.3598	0.0903	0.9119	0.9119	0.0903	1705	
S2	R13	126	72.9	9.4	65.0	MM13B	Not Available	Not Available	Not Available	Not Available	Not Available	Not Available	1855	

Segment 1 Variation of starting membrane properties
Segment 2 Variation of line speed and dryer temperature

Table 4C

MEMBRANE MODIFICATION DATA - Hydrophilic PVDF Western Blotting Membrane

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RUN S - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED

	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Test	R01	20	20	20	10/9.5	1.0	1.5	1.1	1.1	15.0
S1	R02	20	20	20	10/9.5	1.0	1.5	1.1	1.1	42.0
S1	R03	20	20	20	10/9.5	1.0	1.5	1.1	1.1	40.0
S1	R04	20	20	20	10/9.5	1.0	1.5	1.1	1.1	40.0
S1	R05	20	20	20	10/9.5	1.0	1.5	1.1	1.1	40.0
S1	R06	20	20	20	10/9.5	1.0	1.5	1.1	1.1	40.0
S2	R07	20	20	22	10/9.5	1.0	1.4	1.1	1.1	53.7
S2	R08	20	20	20	10/9.5	1.0	1.4	1.1	1.1	39.1
S2	R09	20	20	20	10/9.5	1.0	1.4	1.1	1.1	37.6
S2	R10	20	20	18	10/9.5	1.0	1.4	1.1	1.1	33.0
S2	R11	20	20	18	10/9.5	1.0	1.4	1.1	1.1	30.5
S2	R12	20	20	20	10/9.5	1.0	1.4	1.1	1.1	24.9
S2	R13	20	20	25	10/9.5	1.0	1.4	1.1	1.1	25.2

Segment 1 Variation of starting membrane properties

Segment 2 Variation of line speed and dryer temperature

MEMBRANE MODIFICATION DATA - Hydrophilic PVDF Western Blotting Membrane

[illegible]

RUN S - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED

[illegible]

Segment 1 Variation of starting membrane properties

Table 5A

MODIFICATION DATA FOR Hydrophilic PVDF Western Blotting Membrane

Run Segment	Roll No.	Monomer Mix Lot#	Starting Membrane Lot Data	Good Footage	Heat Treat Temp or Mod Line Dryer Temperature	Membrane Casting Dryer Temperature
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RUN T - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED

1	NA	010908M1DRUM		0	NA	NA
1	NA	010908M1-MM1 Tank		0	NA	NA
1	NA	010908M1-MM2 Tank Diluted		0	NA	NA
1	R01	010908M1 PVH 050107 T101B		155	200	
1	R02	010908M1 PFL 071007 T203		90	200	300
2	R03	010908M1 IPX 120307 T109		90	200	300
2	R04	010908M1 IPX 120307 T109		90	200	300
2	R05	010908M1 IPX 120307 T109		90	Transition	300
2	R06	010908M1 IPX 120307 T109		90	220	300
2	R07	010908M1 IPX 120307 T109		90	215	300
2	R08	010908M1 IPX 120307 T109		90	215	300
2	R09	010908M1 IPX 120307 T109		90	Transition	300
2	R10	010908M1 IPX 120307 T109		90	220	300
3	R11	010908M1 IPX 091407 T101		10	205.4	300
3	R12	010908M1 IPX 071007 T213		90	205.5	300
3	R13	010908M1 IPX 120307 T104		90	205.8	300
3	R14	010908M1 IPX 070907 T105		90	206.1	300
3	R15	010908M1 IPX 070907 R102		90	205	300
3	R16	010908M1 IPX 070907 R106		90	205	300
3	R17	010908M1 IPX 120307 R110		90	205	300
3	NA	010908M1 NA		0	205	

Segment 1
Segment 2
Segment 3

Diagnostic

Dryer Temperature & Linespeed Variation

Porosity and Thickness Variation - Starting Membrane

Table 5B

MODIFICATION DATA FOR Hydrophilic PVDF Western Blotting Membrane

Run Segment	Starting Membrane Properties				Monomer Mix Samples and Component Concentrations				Totalized	
	Roll No.	Thick (um)	Porosity (%)	Flow Time (seconds)	Monomer Mix Sample ID	Actual Wt% (HPLC)	Actual MBAM Wt% (HPLC)	Actual I-2959 Wt% (HPLC)	Actual Total Solids Wt% (HPLC)	Footage By Roll

RUN T - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED

1	NA	NA	NA	NA	DRUM	0.76	0.60	0.15	1.51	0
1	NA	NA	NA	NA	MM1	0.75	0.58	0.15	1.48	0
1	NA	NA	NA	NA	MM2 Start	0.51	0.40	0.10	1.00	0
1	R01	109-125	Not Available	Not Available	Not Taken	NA	NA	NA	NA	375
1	R02	112	72.8	9.6	61.0	NA	NA	NA	NA	675
2	R03	120	71.0	9.5	63.0	0.50	0.39	0.10	0.99	775
2	R04	120	71.0	9.5	63.0	NA	NA	NA	NA	875
2	R05	120	71.0	9.5	63.0	NA	NA	NA	NA	975
2	R06	120	71.0	9.5	63.0	NA	NA	NA	NA	1075
2	R07	120	71.0	9.5	63.0	NA	NA	NA	NA	1175
2	R08	120	71.0	9.5	63.0	NA	NA	NA	NA	1275
2	R09	120	71.0	9.5	63.0	NA	NA	NA	NA	1375
2	R10	120	71.0	9.5	63.0	NA	NA	NA	NA	1675
3	R11	118	66.7	8.3	46.7	0.48	0.38	0.09	0.96	1775
3	R12	116	72.3	9.0	56.0	NA	NA	NA	NA	1875
3	R13	119	72.4	9.7	66.0	NA	NA	NA	NA	1975
3	R14	130	74.7	10.7	74.0	NA	NA	NA	NA	2075
3	R15	134	73.4	9.9	65.0	NA	NA	NA	NA	2175
3	R16	114	72.4	9.7	67.0	NA	NA	NA	NA	2275
3	R17	113	70.2	9.4	61.0	NA	NA	NA	NA	2375
3	NA	NA	NA	NA	MM5	0.47	0.37	0.09	0.94	2675

Segment 1

Diagnostic

Segment 2
Dryer Temperature & Linespeed VariationSegment 3
Porosity and Thickness Variation - Starting Membrane

Table 5C

MODIFICATION DATA FOR Hydrophilic PVDF Western Blotting Membrane

Run Segment	Roll No.	Nip		Nip		Line Speed ft/min	UV Chamber Conditions				UV P1 Inch HWC	UV P3 Inch HWC	UV P2 Inch HWC	Starting O ₂ Level (ppm)
		Aisle Press psi	Wall Press psi	Nip	Nip		N ₂ Flow	Top/Bottom (SCFM)						

RUN T - POROSITY/THICKNESS, VMF4 DRYER TEMP, LINESPEED

Run Segment	Roll No.	Aisle Press psi	Wall Press psi	Nip	Nip	Line Speed ft/min	N ₂ Flow	Top/Bottom (SCFM)	UV P1 Inch HWC	UV P3 Inch HWC	UV P2 Inch HWC	Starting O ₂ Level (ppm)
1	NA	20	20	20	20	NA	NA	NA	NA	NA	NA	NA
1	NA	20	20	20	20	NA	NA	NA	NA	NA	NA	NA
1	NA	20	20	20	20	NA	NA	NA	NA	NA	NA	NA
1	R01	20	20	20	20	18	10/9.5	0.9	0.9	1.4	1.0	52.0
1	R02	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	77.0
2	R03	20	20	20	20	22-20	10/9.5	0.9	0.9	1.4	1.0	78.0
2	R04	20	20	20	20	18	10/9.5	0.9	0.9	1.4	1.0	44.0
2	R05	20	20	20	20	18	10/9.5	0.9	0.9	1.4	1.0	32.0
2	R06	20	20	20	20	18	10/9.5	0.9	0.9	1.4	1.0	32.0
2	R07	20	20	20	20	22-20	10/9.5	0.9	0.9	1.4	1.0	30.0
2	R08	20	20	20	20	18	10/9.5	0.9	0.9	1.4	1.0	26.0
2	R09	20	20	20	20	18-20	10/9.5	0.9	0.9	1.4	1.0	20.0
2	R10	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	22.0
3	R11	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	38.0
3	R12	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	34.0
3	R13	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	41.0
3	R14	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	21.0
3	R15	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	21.0
3	R16	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	21.0
3	R17	20	20	20	20	20	10/9.5	0.9	0.9	1.4	1.0	21.0
3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Segment 1

Diagnostic

Segment 2

Dryer Temperature & Linespeed Variation

Segment 3

Porosity and Thickness Variation - Starting Membrane

Table 5D

MODIFICATION DATA FOR Hydrophilic PVDF Western Blotting Membrane

Run Segment	Roll No.	Water Wet Speed OK?	Water Wet Uniform X-web?	Water Wet Through OK?	Speckle Level OK?	Opacity/ Translucence OK?	Salt Wet Time Seconds	Salt Wet Time Seconds	Salt Wet Time Seconds	Fluores BKG RFU	QC-Blotting Pass/Fail Control	Western Blotting Live Lysate

RUN T - POROSITY/THICKNESS, VMF4-DRYER TEMP, LINESPEED

1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	R01	Y	Y	Y	Y	Y	2.4/5.6	3.4/6.2	3.1/6.2	3.0/6.0	N/Avail.	>NC
1	R02	Y	Y	Y	Y	Y	2.9/12.6	3.2/11.4	2.9/2.9	3.0/9.0	N/Avail.	>NC
2	R03	Y	Y	Y	Y	Y	1.1/24.1	1.4/10.8	1.1/4.1	1.2/13.0	N/Avail.	>NC
2	R04	Y	Y	Y	Y	Y	1.2/14.5	1.5/19.9	1.3/5.6	1.3/13.3	N/Avail.	>NC
2	R05	Y	Y	Y	Y	Y	1.3/10.5	1.5/15.4	1.3/4.2	1.4/10.0	N/Avail.	>NC
2	R06	Y	Y	Y	Y	Y	1.3/24.7	1.4/9.5	1.3/4.0	1.3/12.7	N/Avail.	>NC
2	R07	Y	Y	Y	Y	Y	2.5/60.0	2.3/25.4	2.5/5.4	2.4/30.0	N/Avail.	>NC
2	R08	Y	Y	Y	Y	Y	2.1/9.7	2.6/14.0	2.6/5.5	2.8/9.7	N/Avail.	>NC
2	R09	Y	Y	Y	Y	Y	2.8/13.1	3.5/22.6	2.5/5.9	2.9/13.9	N/Avail.	>NC
2	R10	Y	Y	Y	Y	Y	3.1/28.7	3.5/15.7	2.5/7.1	3.0/17.2	N/Avail.	>NC
3	R11	Y	Y	Y	Y	Y	24.1	6.5	4.2	11.6	N/Avail.	>NC
3	R12	Y	Y	Y	Y	Y	47.1	35.7	8.6	30.5	N/Avail.	>NC
3	R13	Y	Y	Y	Y	Y	67.9	70.7	34.5	57.7	N/Avail.	>NC
3	R14	Y	Y	Y	N (HIGH)	N (White)	>180	>180	>180	>180	N/Avail.	>NC
3	R15	Y	Y	Y	N (HIGH)	N (White)	>180	>180	>180	>180	N/Avail.	>NC
3	R16	Y	Y	Y	N (HIGH)	Y	>180	57.3	16.4	>180	N/Avail.	>NC
3	R17	Y	Y	Y	Y	Y	19.3	40.8	5.1	21.7	N/Avail.	>NC
3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Segment 1

Diagnostic

Segment 2

Dryer Temperature & Linespeed Variation

Segment 3

Porosity and Thickness Variation - Starting Membrane

Example 3

The protocol used for Western blotting and chemiluminescent detection is as follows:

- Protein samples are electrophoretically separated using Bis:Tris (4~12%) midi gradient gel (Invitrogen, WG1402BOX).
- Samples are electro-blotted at 45V for 1hr 15min using BioRad tank transfer apparatus (Criterion Blotter # 165-6024) onto a hydrophilic membrane prepared as in Example 1.
- Blots are washed 2x (3 min each) in TBS-T (0.1% Tween)
- Blots are blocked for 1hr at RT in TBS-T with 3% NFM (non-fat milk, Carnation).
- Blots are washed 2x (3 min each) in TBS-T (0.1% Tween)
- Blots are incubated with primary antibody in TBS-T for 1hr.
- Blots are washed 3x (5 min each) in TBS-T (0.1% Tween)
- Blots are incubated with secondary antibody in TBS-T for 1hr
- Blots are washed 4x (5 min each) in TBS-T (0.1% Tween)
- Protein bands are visualized using ECL (Millipore)

Immobilon-HRP) and x-ray film.

This protocol was used on the samples prepared in Example 2, and the blotting results are shown in Figures 1, 2, and 3.

Western blotting results from a typical development run demonstrate the performance differences between the hydrophilic PVDF blotting membrane of this invention, and the controls (FL - hydrophobic PVDF membrane, and NC - Whatman/S&S BA-85 membrane). Each horizontal strip in the figure contains 5 separate Western transfer blots; three blots on hydrophilic PVDF development samples, and one blot on each of the control membranes. Each strip of 5 Western blots is the result from one electrophoresis and transfer experiment (5 gels were run in each experiment). By design, each experiment embodies identical conditions with identical quantities of protein sample (applied in 4 lanes across each gel) before electrophoresis and transfer. The results shown are the transfer blots for the detection of 2 proteins (HSP70 and GAPDH) from a complex sample mixture (lysate), applied at decreasing sample solids (concentrations), from left-to-right and corresponding to: 5 µg, 2.5 µg, 1.25 µg, 0.67 µg.

Note that in each row (the result of a single

electrophoresis and electro-blotting experiment, three hydrophilic blotting membranes of the invention are compared to two control blotting membranes. One control consists of nitrocellulose blotting membrane (NC) and the other control is a hydrophobic PVDF blotting membrane (FL). In the case of each single experiment, the FL membrane demonstrates the highest signal intensity for the 4 titers of analyte protein solution, and the NC membrane demonstrates the lowest signal intensity. It can be seen, when comparing the signal strengths between NC control blotting membranes and hydrophilic PVDF blotting membranes of the invention, that at the highest analyte sample titers (left hand side of each blot), that the NC and the hydrophilic PVDF membranes exhibit similar signal strengths. However, as one progresses to lower and lower analyte sample titers (progressing from left-to-right), the signal strength falls off more rapidly for the NC membrane. This demonstrates that the hydrophilic PVDF membrane allows sample detection at lower protein concentrations than NC membrane does.

What is claimed is:

1. A Western blot immunodetection assay for detecting target proteins in a protein sample, comprising:

electrophoretically separating said target proteins from said protein sample;

transferring the separated target proteins onto a porous polymeric substrate having its surface modified with a crosslinked polymeric coating comprising acrylamide and methylene-bis-acrylamide, said coating rendering said surface hydrophilic;

blocking said substrate to prevent nonspecific binding of antibodies to said substrate surface;

incubating said substrate with a primary antibody capable of binding to said target proteins;

incubating said substrate with a labeled secondary antibody capable of complexing with an enzyme of said primary antibody to provide a measurable signal; and

detecting said signal.

2. The Western blot immunodetection assay of claim 1, wherein the measurable signal is detected by visual, chemiluminescent or fluorescent detection.

3. The Western blot immunodetection assay of claim 1, wherein said porous polymeric substrate having its surface modified has a protein binding capacity as measured by an IgG binding test of 250-325 $\mu\text{g}/\text{cm}^2$.

4. The Western blot immunodetection assay of claim 1, wherein the average total organic carbon level of said porous polymeric substrate is below 1.5 $\mu\text{g}/\text{cm}^2$, and wherein

extractable monomer levels, as determined by HPLC, are below 0.02 µg/cm² for acrylamide, and below 0.15 µg/cm² for methylene-bis-acrylamide.

5. The Western blot immunodetection assay of claim 1, wherein said porous polymeric substrate has a background fluorescence value, and wherein said substrate modified with said polymeric coating exhibits a background fluorescence of twice that of said substrate background fluorescence value under identical measurement conditions.

6. The Western blot immunodetection assay of claim 1, wherein said substrate comprises polyvinylidene fluoride.

7. The Western blot immunodetection assay of claim 1, wherein the ratio of acrylamide to methylene-bis-acrylamide is 1:1 (mass/mass).

8. The Western blot immunodetection assay of claim 1, wherein the overall monomer concentration of the acrylamide:methylene-bis-acrylamide monomer reactant solution used to coat said surface is between 0.5% and 1.5% by mass.