Reconditioning of ELISA Plates

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ABSTRACT

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Cleaning of enzyme-linked immunosorbent assay plates was achieved by sonication in a 0.5% solution of detergent at 60 C for 30 min, rinsing several times in distilled water, and drying at 55 C for 1 hr. Repeated cleaning and reuse of plates resulted in a decline in plate efficacy, likely the effect of reduced coating immunoglobulin adsorption to the polystyrene. Treatment of wells with 1% nitrocellulose after cleaning resulted in a significant improvement in plate performance, which allowed them to be used up to six times with only a marginal reduction in sensitivity compared to new plates. Polystyrene and polyurethane films were less effective regardless of pH or several chemical amendments.

Enzyme-linked immunosorbent assay (ELISA) is a well established method for the detection of plant viruses and is particularly useful for mass-indexing programs. The expense of the disposable polystyrene microtiter plates used in this test is significant. Several attempts have been made to reuse ELISA plates with attention being focussed primarily on cleaning procedures (2,3,4,9). Our experience with Immulon 2 MicroELISA plates (Dynatech Laboratories, Alexandria, VA) indicated that cleaning provided effective protein removal but resulted in serious loss of plate efficacy. This is likely due to removal during washing of the selective protein-adsorptive coating applied during the manufacture of these plates.

The objective of this research was to evaluate the effect of using various plastic coating agents for reconditioning the surface of ELISA plate wells. Several chemical additives to each plastic coating were also evaluated for their ability to enhance adsorption through possible alteration of functional sidegroups of the plastic molecules.

MATERIALS AND METHODS

Virus and antiserum. Healthy and peach rosette mosaic virus (PRMV)-infected tissue was obtained from leaves excised from greenhouse-grown *Chenopodium quinoa* Willd. Antiserum against PRMV was that used previously (11).

ELISA plate treatments. An ethanolic solution of polystyrene was prepared by dissolving an Immulon 2 plate in 100 ml of chloroform, diluting this to 400 ml with ethanol, and removing the precipitated polystyrene by centrifugation for 15 min at 10,000 *g*. The concentration of polystyrene in the supernatant was determined spectrophotometrically to be 2.6 mg/ml. A water-based polyurethane (Varathane 2, Flecto Coatings Limited, Oakland, CA) was diluted to 25, 5, and 1% in distilled water. A 1% stock solution of nitrocellulose was prepared by dissolving 5 g of nitrocellulose trans-blot transfer medium (BioRad Laboratories, Richmond, CA) in 95 ml of dimethylsulfoxide (DMSO) and diluting the solution to 500 ml with ethanol. Each plastic solution was amended with one of the following: 0.5% diethanolamine, 0.01% ethidium bromide, 0.05% formaldehyde, 0.05% glutaraldehyde, 0.01% mercaptoethanol, 2% polyethylene glycol, 2% polyvinyl alcohol, or 0.1% silane and aliquots of each were adjusted to pH 4, 7, or 10 with a 0.2 M aqueous solution of either HCl or NaOH. Plastic solutions and additives were prepared singly at each pH for control treatments.

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ELISA. The double antibody sandwich ELISA followed the protocol of Clark and Adams (6), as modified by Lister (10). Immunoglobulin (Ig) and conjugate were prepared as described by Clark and Adams (6). Wells were coated with 200 μl of anti-PRMV Ig at 2 μg/ml at 38 C for 3 hr. After washing, 200 μl of crude healthy or PRMV-infected plant tissue macerates (tissue:ELISA extraction buffer ratio, 1:19 [v/v]) were each loaded into one-half of the wells of each treatment. Alkaline phosphatase (Type 7, Sigma Chemical Co., St. Louis, MO) was conjugated to purified anti-PRMV Ig (2.5 μg Ig/ml PBS-Tween) and used at a dilution of 1/1,000. Substrate reactions were stopped by adding 50 μl of 3 M NaOH and absorbancies were measured at 405 nm in a Beckman DU-8 spectrophotometer equipped with a microplate reader accessory. Each treatment was expressed as the mean absorbance divided by that of the untreated control wells.

Plastic coating evaluations. Fifty-four ELISA plates from one lot were coated with PRMV Ig and one half of the wells of each plate were loaded with either healthy or PRMV-infected plant tissue macerates. Following completion of ELISA, the plates were sonicated at 60 C for 30 min in a 0.5% solution of Sparkleen detergent (Fisher Scientific, Pittsburgh, PA), rinsed in several changes of distilled water, and dried at 55 C for 1 hr. These plates, subsequently referred to as second-generation plates, were then coated with the various plastic treatments (Table 1). Each treatment was applied to eight wells of each plate such that four wells were previously PRMV-positive and the remaining four wells contained healthy plant sap. Four replicate plates of each set of treatments were used. Treated plates were incubated at 38 C for 1 hr.

Nitrocellulose trials. Nitrocellulose dilutions were made in 20% DMSO in ethanol (DMSO/ethanol) to give final nitrocellulose concentrations of 0.25, 0.50, 0.75, or 1.0% and the pHs were adjusted to 7.0 with 0.01 M NaOH. Sixteen wells in each of eight second-generation plates were treated with each dilution. Control wells received either DMSO/ethanol or distilled water. Following incubation at 38 C for 1 hr, the plates were shaken out, dried, and ELISAs were performed as described previously.

Repetitive tests of 1% nitrocellulose coatings were made by using eight second-generation plates. Twenty-four wells in each plate were loaded with either 1% nitrocellulose, DMSO/ethanol, or distilled water and ELISA was performed with one half of the wells of each treatment being used for virus-positive samples. After absorbancies were read, plates were cleaned by sonication in Sparkleen, recoated as in the previous test, and reused in this fashion for 10 sets of ELISA tests.

Scanning electron microscope observations. Wells from new, used, and nitrocellulose-coated Immulon 2 plates were excised by using a Dremel cutting tool (Dremel Mfg., Racine, WI) and rinsed in Freon TF (Dupont Canada, Toronto) to remove residual styrene fragments. Wells were mounted on the adhesive surface of foil-backed tape affixed to aluminum specimen stubs and were

TABLE 1. Effect of plastic treatments on reuse efficacy of ELISA plates[a]

Plastic treatment	Plastic additives at different pH																										
	No treatment			Diethanolamine (0.5%)			Ethidium bromide (0.01%)			Formaldehyde (0.05%)			Glutaraldehyde (0.05%)			Mercapto-ethanol (0.01%)			Polyethylene glycol (2%)			Polyvinyl alcohol (2%)			Silane (0.1%)		
	4[b]	7[b]	10[b]	4	7	10	4	7	10	4	7	10	4	7	10	4	7	10	4	7	10	4	7	10	4	7	10
No treatment	1.0	1.0	1.0	1.0	1.1	0.90	...	0.98	1.0	1.1	0.89	0.73	1.2	1.0	0.67	0.80	1.0	0.96	0.81	0.86	0.91	0.10	0.14	0.15	0.96	0.30	0.21
Nitrocellulose (1%)	1.8	3.8	3.3	0.67	0.88	0.82	...	1.9	1.7	0.91	1.7	0.73	0.52	1.0	0.99	...	...	...	0.61	0.70	0.73	...	...	...	...	...	...
Polystyrene (0.05%)	1.1	1.0	0.97	0.89	0.94	0.95	...	1.0	1.0	0.97	0.95	0.83	1.1	0.99	0.83	0.91	0.90	0.79	0.69	0.74	0.81	0.20	0.09	0.11	0.78	0.82	0.61
Polyurethane 25%	0.80	0.84	0.45	0.42	0.81	0.72	...	0.77	0.80	0.81	0.99	0.88	0.61	0.70	0.67	0.50	0.49	0.39	0.60	0.77	0.70	0.13	0.06	0.11	0.40	0.28	0.29
5%	0.99	0.86	0.42	0.75	0.80	0.79	...	1.1	0.99	0.92	1.2	0.96	0.82	0.69	0.58	0.68	0.96	0.87	0.68	0.77	0.72	0.16	0.11	0.13	0.62	0.74	0.52
1%	1.3	0.88	0.58	0.85	1.1	0.98	...	1.2	0.97	0.96	1.1	0.89	0.99	1.0	0.87	0.82	0.91	0.79	0.79	0.82	0.86	0.19	0.10	0.11	0.89	0.71	0.53

[a]Values represent the mean absorbance of wells treated with plastic solutions divided by the mean substrate absorbance of wells not receiving any plastic treatment. All wells represented in this table were treated with PRMV-infected plant sap and represent virus-positive test values.

[b]Test solution pH.

subsequently sputter-coated with gold. The samples were examined with a Hitachi S-570 scanning electron microscope operating at 10 kV.

Plates cleaned by sonication in Sparkleen were further treated with a solution of 0.1% protease and 0.1% trypsin in 0.02 M potassium phosphate buffer containing 0.85% NaCl at 38 C for 3 hr. Following rinsing in distilled water and drying, plates were prepared for scanning electron microscopy. Virus-conjugate binding to plates was assessed by planographic measurements taken from micrographs of the plate surface.

RESULTS

Significant improvements in ELISA readings from second-generation plates were obtained by precoating plates with 1% nitrocellulose at pH 7.0 (Table 1). Lower concentrations were less effective and resulted in film separation from the wells (Fig. 1). Baseline values for healthy reactions were comparable to those of new plates, while virus-positive reactions were approximately 85% of new plate values.

Treatments containing other additives did not result in any improvement over pure nitrocellulose coatings. Baseline values for healthy reactions did not differ significantly over any of the plastic treatments. Polystyrene treatment made no appreciable improvement, and 1% polyurethane at pH 4.0 produced only marginal enhancement.

Repetitive recycling of plates followed by nitrocellulose treatment was satisfactory for up to six plate reuses (Fig. 2). Absorbance values of virus-positive reactions in nitrocellulose-treated wells were typically 80–90% of new plate values, while values in untreated plates were generally only 10–25% after the second reuse. Healthy baseline values and interwell variability of nitrocellulose-treated plates compared favorably to new plates (Table 2). After six reuses, opalescence and deterioration increased, further reducing the effectiveness of the plates.

Virus-conjugate complexes were uniformly distributed on ELISA plates as seen under the scanning electron microscope (Fig. 3). This attachment declined with repeated plate reuse while increased amounts of cellular plant debris were found (Table 3). Sonication of plates in Sparkleen detergent removed 95% of the

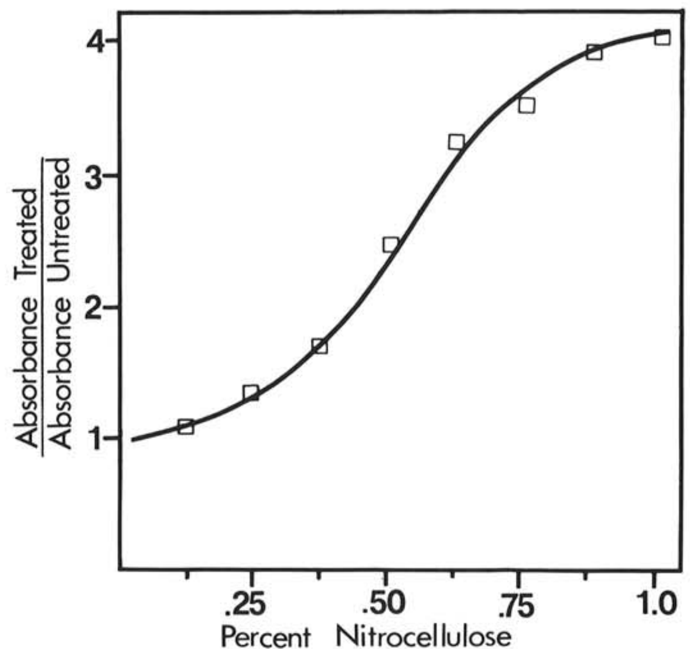


Fig. 1. Efficacy of second-generation enzyme-linked immunosorbent assay plates as influenced by the concentration of coating nitrocellulose. Substrate absorbance is expressed as the mean absorbance of nitrocellulose-treated wells divided by the mean absorbance of untreated wells at a given nitrocellulose concentration.

virus conjugate complexes, although some residual plant material remained (Fig. 4). While virus-conjugate complexes were digested by protease and trypsin, the residual material was unaffected.

Treatment of plates with 1% nitrocellulose formed a thin film in each well which overcoated any debris remaining in the wells (Fig. 5). Virus-conjugate binding to the membrane was greater than on uncoated second-generation plates, although marginally less than that of new plates. No evidence of film tearing or separation from the polystyrene was evident.

DISCUSSION

Attempts to reuse ELISA plates have met with varied success (3–5). While cleaning with alkali is effective, the capability of the polystrene surface to adsorb protein is often changed. This is evident in Immulon 2 plates that have been formulated with a surface coating to enhance immunoglobulin adsorption. Subsequent reduction in plate efficacy after additional reuses possibly results from removal of this coating during the recycling procedure. Treatment of new plates with protein A enhances antibody retention by the polystyrene (1), but it is not cost effective for large scale treatment of used plates.

Treatment of plates with polystyrene is difficult since the solvent interacts with the plate and reduces its transparency. Emulsion formulations were equally unpromising (*unpublished*). Treatment of plates with an ethanolic solution of dilute polystyrene partitioned from chloroform did not result in any significant improvement in plate performance. Marginal improvements with acidified polyurethane coatings were still inferior to new plate performance and were unaffected by various chemical additives.

Antibody-sensitized films have been used extensively in immune electron microscopy as a mechanism for selectively trapping virus particles (7,8,12). Nitrocellulose has generally been preferred to formvar films, possibly because of its reduced hydrophobicity. The high affinity of nitrocellulose for protein adsorption makes it a convenient coating for ELISA wells with reduced immunoglobulin binding capacity. Treatment of plates with 1% nitrocellulose significantly improved plate efficacy by providing a thin, uniform film on which the double antibody sandwich could be formed. Normal plate processing procedures did not cause film tearing or separation from the wells, although gentle washing procedures are recommended. In addition to improving the efficacy of used plates,

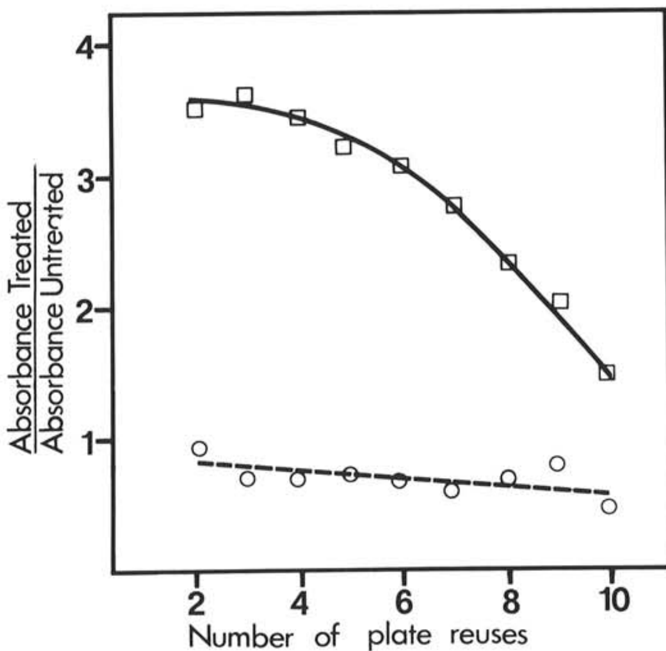
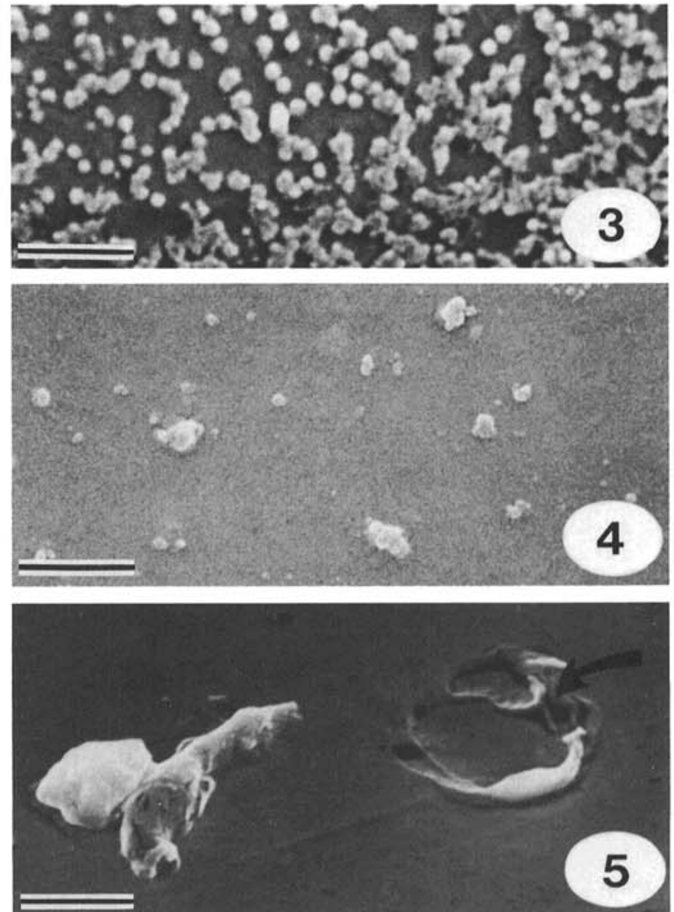


Fig. 2. Efficacy of second-generation enzyme-linked immunosorbent assay plates following repetitive uses. Legend: □——□, plates treated with nitrocellulose after each use; and o------o, plates treated with 20% DMSO in ethanol after each use.



Figs. 3–5. Enzyme-linked immunosorbent assay (ELISA) plate surfaces. **3,** Virus-conjugate complexes on a new ELISA plate. Bar represents 0.5 μm. **4,** Plant cell debris remaining on a used ELISA plate following cleaning by sonication in a detergent solution. Bar represents 5 μm. **5,** ELISA wells overcoated with nitrocellulose. Arrow indicates area where the film has been perforated. Cellular debris and residual protein is overcoated with nitrocellulose. Bar represents 5 μm.

TABLE 2. Absorbance values associated with ELISA plate reuse[a]

Reuses (no.)	(1%) Nitrocellulose treatment, pH 7.0		DMSO/ethanol treatment, pH 7.0		Distilled water		New plate	
	PRMV-infected	Healthy	PRMV-infected	Healthy	PRMV-infected	Healthy	PRMV-infected	Healthy
0	...	...	...	...	...	...	1.79 ± 0.03	0.03 ± 0.02
2	1.60 ± 0.03	0.09 ± 0.02	0.43 ± 0.06	0.18 ± 0.04	0.38 ± 0.08	0.28 ± 0.04	...	...
4	1.56 ± 0.05	0.04 ± 0.04	0.38 ± 0.12	0.22 ± 0.05	0.29 ± 0.09	0.22 ± 0.06	...	...
6	1.33 ± 0.10	0.07 ± 0.03	0.36 ± 0.11	0.23 ± 0.03	0.37 ± 0.16	0.27 ± 0.08	...	...
8	1.04 ± 0.10	0.08 ± 0.07	0.41 ± 0.10	0.20 ± 0.04	0.38 ± 0.07	0.30 ± 0.11	...	...
10	0.99 ± 0.20	0.16 ± 0.13	0.65 ± 0.26	0.30 ± 0.21	0.40 ± 0.09	0.29 ± 0.16	...	...

[a]Values represent the mean absorbance ± standard deviation of twelve wells in each of eight plates.

TABLE 3. Attachment of virus-conjugate to ELISA plates before and after cleaning and reuse[a]

Plate reuses	Coverage of well surface by virus-conjugate deposits (%)
0	92.21
1	48.06
2	28.12
3	36.31
4	28.98

[a] Values represent the mean percentage virus-conjugate coverage on each of three wells sampled in five plates.

nitrocellulose treatments reduced healthy baseline reactions to values equivalent to those obtained with new plates, making visual assessment easier. Scanning electron micrographs indicated that such baseline reductions were likely attributable to overcoating of cellular debris in the wells with nitrocellulose.

Plates could be effectively reused up to six times when recoated with nitrocellulose after each cleaning. After this point, opacity developed in the plastic which was likely associated with degenerative breakdown of the polystryene, resulting in poorer binding of the nitrocellulose. Used in combination with an effective cleaning procedure, nitrocellulose treatment provides a convenient means of reusing plates without appreciable loss in plate efficacy or reproducibility.

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