Protein-Reactive, Molded Polystyrene Surfaces Having Applications to Immunoassay Formats

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Synopsis

Injection-molded polystyrene surfaces were chemically modified rendering them protein reactive. The process involves chlorosulfonation of the aromatic ring, sulfonamide formation with excess di- and triamines, and reaction of the residual pendant amines with various bifunctional molecules. Surfaces possessing pendant bromoacetyl, iodoacetyl, fluorodinitrophenyl, and trimellitic anhydride were prepared and can exhibit up to ten times more protein immobilization capability compared to unfunctionalized polystyrene, where the coupling takes place presumably via hydrophobic interaction.

INTRODUCTION

Polystyrene is a widely used material for solid supports in the design of heterogeneous immunoassays. The applicable physical forms are numerous, where latexes, microspheres, centimeter-sized spheres, and multiwell microtiter trays are the most common. In a typical configuration, the support is coated with antibody or antigen which serves as the capture phase for the analyte of interest. After exposure of the coated support to the liquid sample to be analyzed, the surface is washed free of nonbinding components. The presence of analyte, which should now be immobilized, is detected by the addition and subsequent binding to it of a second antibody which has been labeled with an appropriate tag, such as ^{125}I in radioimmunoassay (RIA), or a specific enzyme in enzyme immunoassay (EIA).

Polystyrene surfaces are usually coated with the biological capture phase entity, and the binding takes place predominantly via hydrophobic interactions. When this mode of attachment is used, care must be taken that surface-active agents originating from the synthesis and/or processing of the material are removed, since they compete for the hydrophobic binding sites on the plastic surface. During its use in an actual immunoassay, care must be taken so that the coated molecules are not stripped off by a combination of displacement by other proteins or surface active agents and the shear forces of washing.

Certain advantages relative to surface concentration and stability toward displacement can be gained by covalently attaching the proteinaceous capture phase to the support surface. This can be achieved by surface functionalizing the support with carboxylic, amino, aldehyde, epoxy, or other reactive groups through which a protein coupling reaction can be accomplished. In the case of latexes, the functional group can be incorporated as a functionalized comono-

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mer, since the surface of the particle can obtain a relatively high surface concentration of polar groups.[1] Microspheres are usually functionalized after synthesis; however, the material is usually crosslinked during the suspension polymerization so that it can withstand surface functionalization reactions in organic media. Several patents describe such processes related to the chlorosulfonation of polystyrene.[2-4]

In the case of injection-molded polystyrene parts, such as centimeter-sized spheres and microtiter plates, the use of crosslinked resin is not practical in view of its high viscosity in the melt. Crosslinking of the finished part by ultraviolet radiation is difficult in view of the high energy per crosslink needed relative to other common plastics.[5]

In this paper we describe the surface functionalization of linear polystyrene molded surfaces without concomitant dissolution of the derivatized surface. A chlorosulfonation method is used as the first step, followed by a series of reactions which produce several different protein-reactive surfaces.

EXPERIMENTAL

All solvents and reagents were reagent grade and used as received except that the nitromethane was distilled before use. Injection-molded 0.63-cm diameter polystyrene spheres were used in this study. The spheres were uniformly surface roughened by an abrasive wheel to provide more surface area. Surface optical microscopy revealed contours with an average depth and separation of approximately 30 μm. The surface area of the roughened sphere is approximated to be three times that of a smooth sphere, and this factor was used to obtain all surface concentrations in this study. The weight average molecular weight of the polystyrene was 245,000 with a polydispersity index of 4.5.

Synthesis of Hydrazide Surface-Functionalized Polystyrene Beads

Method A. Neat chlorosulfonic acid (250 mL, 3.76 mol) was stirred by a mechanical stirrer in a 1 L three necked round-bottomed flask which had been fitted with a thermometer, dry-ice acetone bath, and nitrogen inlet and outlet. Polystyrene beads (0.63 cm diameter, 900 beads) were added in one portion at 0–5°C. The surface functionalization was allowed to proceed for 20 minutes. The liquid was then removed by suction. A nitromethane wash (250 mL) was added into the reaction flask, stirred with the beads for one minute, and then removed by suction. Hydrazine monohydrate (250 mL, 5.15 mol) was added in one portion. The cooling bath was subsequently removed and the reaction flask was allowed to warm up to ambient temperature over 30–40 min. The liquid was then removed and the beads were washed successively with 400 mL each of H_2O, 1 N HCl, 1 N HCl, H_2O, 1 N NaOH, 1 N NaOH, H_2O, and methanol. The beads were then dried under vacuum at ambient temperature.

Method B. Nitromethane (250 mL, predistilled) was cooled to 0–5°C in a 1 L reactor. A mechanical stirrer was employed at approximately 250 rpm. Chlorosulfonic acid (25 mL, 0.376 mol) was transferred dropwise into the reactor via a syringe. After 5 minutes, the polystyrene beads (450 beads) were added in one portion. After 4 hours, the liquid was removed and the beads

washed with 80 mL of nitromethane. Hydrazine monohydrate (80 mL, 1.65 mol) was then added in one portion and the reactor was allowed to warm up to room temperature with stirring for 30 min. The liquid was then removed, and the following washes were carried out: 80 mL water, 2 × 150 mL 1 *N* HCl, 150 mL water, 2 × 150 mL 1 *N* NaOH, 2 × 150 mL water, 150 mL methanol. The beads were then dried overnight in a vacuum oven at 60°C.

Synthesis of Amino Surface-Functionalized Polystyrene Beads

General procedures for the synthesis of different amino beads, with varied hydrocarbon chain length, are identical to the procedure for the synthesis of the hydrazide bead. The only modification is the substitution of various amines for the hydrazine monohydrate. When ethylene diamine or diethylene triamine was used, a volume equal to that of hydrazine monohydrate was used. Since hexamethylenediamine is a solid, it was first dissolved in either water or diethylene triamine as a 15% solution.

Synthesis of Bromoacetyl Surface-Functionalized Beads

Diethylene triamine functionalized polystyrene beads, (68 beads) were added to 20 mL of dry acetonitrile in a [illegible]00 mL rough-bottomed flask. Bromoacetyl bromide (0.087 mL, 15 μmol/bead) and triethylamine (0.142 mL, 15 μmol/bead) were then added to the reaction flask. A shaker was utilized to provide good movement of the beads in the liquid phase. After 2.5 hours, the beads were separated on a suction funnel. Three washings of the beads were carried out using dry acetonitrile. The resultant beads were then dried under vacuum at 25°C.

Synthesis of Fluorodinitrophenyl, Iodoacetyl, and Anhydride Surface-Functionalized Beads

The procedure for the synthesis of these beads was identical to that used for the bromoacetyl surface-functionalized beads, except dinitrodifluorobenzene, iodoacetic anhydride, or trimellitic anhydride acid chloride was substituted for bromoacetyl bromide at 15 μmol per bead.

Analysis of Surface Functional Groups

Surface functional groups were quantitated indirectly by measuring the disappearance of a reactive species from a solution of known concentration. Nucleophilic surfaces were reacted with a 0.02 *M* phenylisocyanate solution, $\epsilon = 1.05 \times 10^4$ l mol^{-1} cm^{-1} at 226 nm in spectroscopic-grade acetonitrile. Surface electrophilic groups were quantitated similarly using a standard 0.02 *M* acetonitrile solution of aniline, $\epsilon = 1.02 \times 10^4$ l mol^{-1} cm^{-1} at 239 nm. Beads which had been prereacted with analytical reagent, washed, and dried were used as blanks in the wet method to correct for absorption of the analytical reagent.

Surface Immobilization of Antibody

The protein uptake capacities of functionalized and unfunctionalized beads were measured by immobilization of rabbit immunoglobulin G (IgG) or

affinity purified anti-alpha-fetoprotein (α-AFP) which had been ^{125}I labeled. A typical coating solution contained approximately 200 μg protein/mL in 0.05 *M* phosphate buffer, 0.1 *M* in NaCl at pH 7.4, and had counting activities of approximately 5.00 μCi/mL. Approximately 50 beads were totally immersed in the coating solution at 20–80 μg protein per bead and agitated on a rotary shaker at 180 rpm for 24 hours at 25°C. The coating solution was then removed, and the beads were washed once with an equal volume of phosphate buffered saline with respect to the coating solution. The beads were then counted individually and the uptake was expressed as micrograms of protein per cm^2. This was followed by four additional washes and another radioactivity measurement. The beads were then incubated in an equal volume with respect to coating solution of 1% sodium dodecyl sulfate to detach adsorbed protein and recounted giving a measure of the covalently bound material.

RESULTS AND DISCUSSION

The Chlorosulfonation Reaction

The mechanism of the chlorosulfonation reaction is shown in Figure 1. The result is a surface that obtains a significant negative charge due to the incomplete conversion of sulfonate to sulfonyl chloride and some limited hydrolysis which is difficult to prevent. This was demonstrated by the high affinity that an amine-containing yellow dye, 2,3-diamino phenazine, had for the surface after all the sulfonyl groups had been converted to sulfonamide by a nonchromophoric amine.

The surface material also becomes crosslinked as a result of the reaction most likely via sulfone formation as shown in Figure 2. The crosslinked surface material can be separated from the bulk interior by Soxhlet extraction with chloroform. The solvent dissolves the linear polymer leaving the shell material behind (see Fig. 3). The elemental analyses of the shell material from hydrazide and bromoacetyl functionalized beads are given in Table I and confirm a high degree of chemical modification to the native polystyrene.

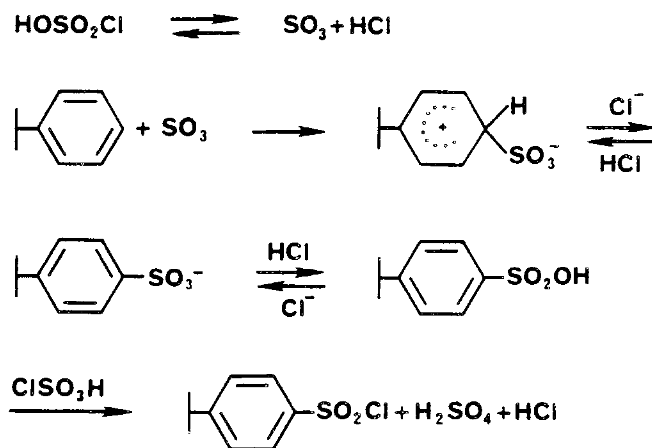

Fig. 1. Chlorosulfonation reaction mechanism.

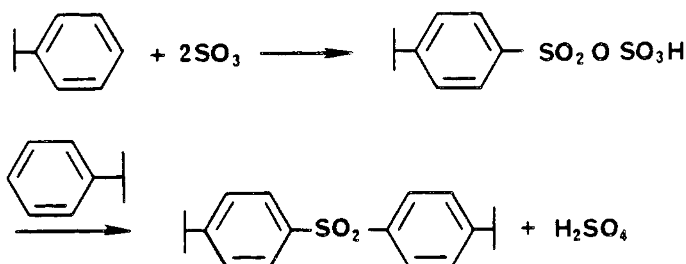

Fig. 2. Crosslinking due to sulfone formation.

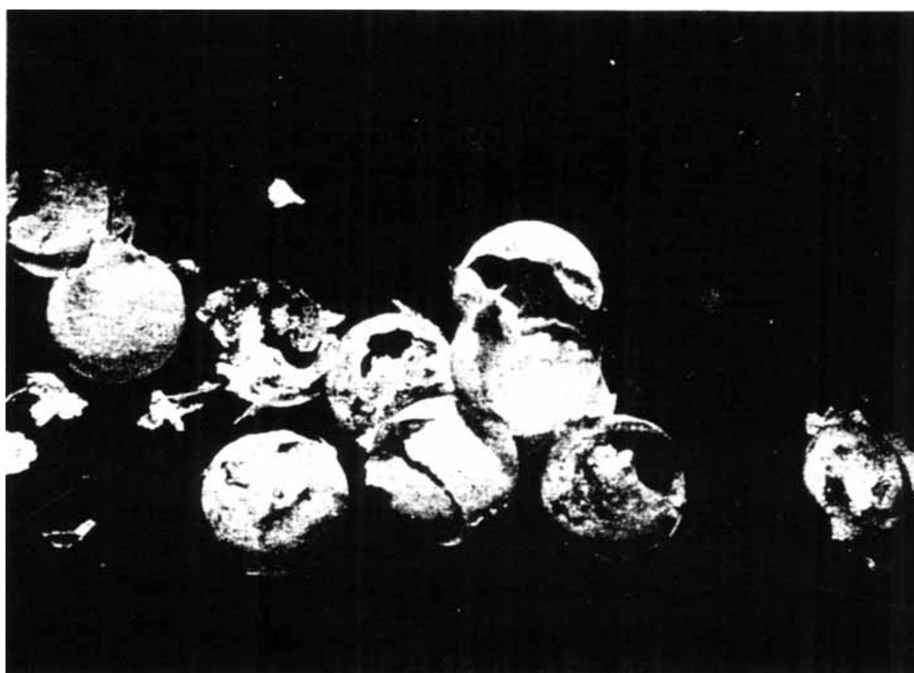

Fig. 3. Crosslinked polymer shell isolated from the bulk linear polystyrene core.

TABLE I
Elemental Analyses (%)

Polystyrene modification	C	H	N	S	Br
Hydrazide sulfonamide	48.48	5.08	14.14	16.16	—
Bromoacetyl diethylene Triamine sulfonamide	49.66	4.79	4.59	11.57	20.61

Concentration of Surface Amine

Quantitative analysis for surface amine (vide supra) showed that 0.94 μmol of primary amine per cm^2 can be attained by these methods; however, surfaces with concentrations higher than 0.70 μmol/cm^2 usually exhibited surface deterioration in the form of flaking.

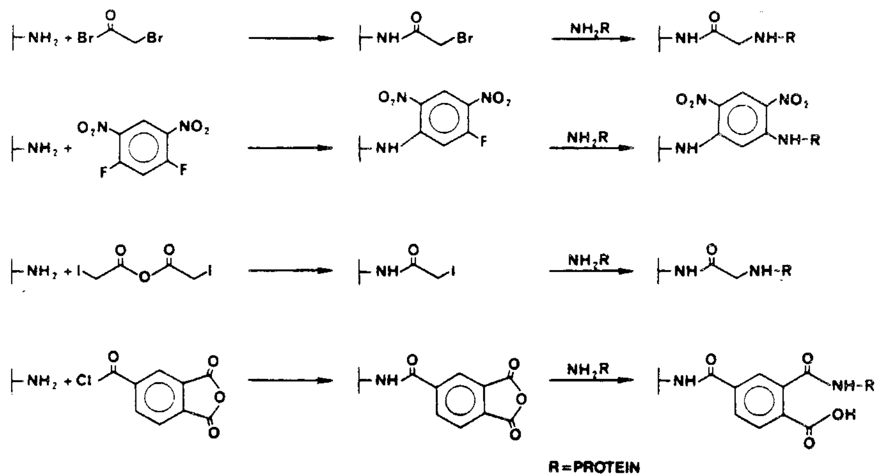

Fig. 4. Surface immobilization schemes.

Protein-Reactive Surfaces

A general method for covalent immobilization of protein on solid surfaces utilized the inherent nucleophilic groups of the protein for points of attachment. The approach taken in this study was to activate the surface amino groups with bifunctional electrophilic molecules, as shown in Figure 4. Bromoacetyl bromide, difluorodinitrobenzene, iodoacetic anhydride, and trimellitic anhydride acid chloride were reacted in excess with the surface amines leaving the second electrophilic group for reaction with the protein. The protein immobilization step was carried out at neutral pH in an aqueous solvent system. Groups of lower reactivity on the solid phase will generally show a better selectivity between the nucleophiles on the protein and the solvent, water, resulting in a high uptake. Conversely, very reactive groups on the solid phase show less selectivity, resulting in low uptake.

In the evaluation of the immobilization capacity of the various surface activated beads, ^{125}I-labeled anti-alpha-fetoprotein antibody was coated by a standardized procedure (vide supra) to give the results shown in Table II. It should be noted that all covalent methods evaluated showed substantial protein uptake capacity compared to the adsorption method. Among the four covalent protein uptake methods the bromoacetyl and the trimellitic anhydride methods showed the highest uptake. It was observed that even at very high surface activation concentrations, not all of the immobilized material was covalently bound. Upon thorough washing with 1% sodium dodecyl sulfate (SDS), the adsorbed antibody was detached, leaving behind only covalently bound protein. It was found that the bromoacetyl activation method was most efficient in covalent immobilization.

Figure 5 compares the results of protein immobilization via bromoacetyl functionalized surfaces with unfunctionalized polystyrene. Exhaustive washing with phosphate buffered saline resulted in a reduction of only 0.13 $\mu g/cm^2$. Washing with 1% SDS lowered the surface protein by an additional 0.13 $\mu g/cm^2$. This indicates that at least 80% of the bound antibody is covalently linked, where the rest was bound via hydrophobic interactions.

TABLE II
Anti-Alpha-Fetoprotein Immobilization

Precursor bead	Activation method	Total bound[a] α-AFP ($\mu g/cm^2$)	Covalently bound[b] α-AFP ($\mu g/cm^2$)
Polystyrene	None	0.032	0.010
Amino bead	Bromoacetyl bromide	0.53	0.42
Amino bead	Difluorodinitro benzene	0.34	0.26
Amino bead	Iodoacetic anhydride	0.36	0.29
Amino bead	Trimellitic anhydride acid chloride	0.53	0.29

[a] Results after binding at pH 7.4, 25°C, 21 hours and four phosphate buffered saline (PBS) washes.
[b] Amount determined after overnight incubation in 1% SDS, then one PBS wash.

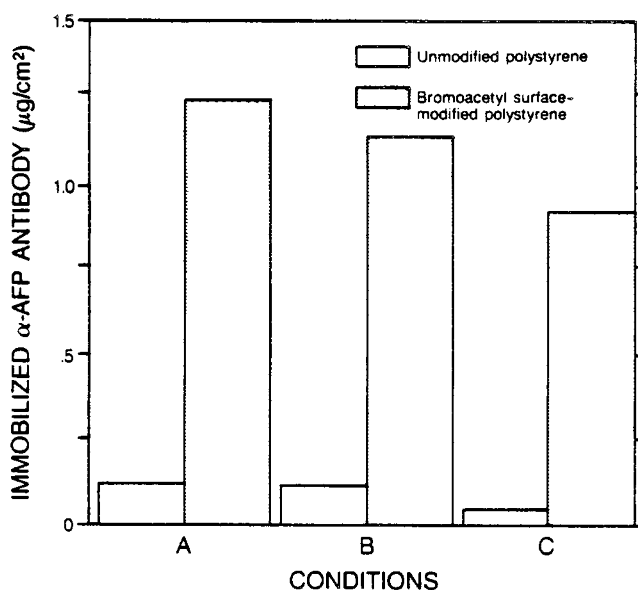

Fig. 5. Immobilization and retention of α-AFP antibody under specified sequence of conditions A–C. (A) 24-hour coating with 50 μg antibody at pH 7.4, 25°C and a phosphate buffered saline wash at pH 7.4, 25°C. (B) Condition A plus four additional washes. (C) Condition B plus 16-hour 1% SDS incubation and a phosphate buffered saline wash at 25°C.

CONCLUSION

Molded polystyrene surfaces were chemically modified to be protein reactive, resulting in a relatively high protein immobilization capability. The surfaces of 0.63-cm diameter molded spheres were chlorosulfonated and coupled with diamines to yield hydrazide and amino-derivatized beads. These surface nucleophilic groups were further activated with bifunctional reagents for protein immobilization. This antibody showed stability against shear stress and displacement by surface active molecules.

These methods hold promise toward the development of immunoassay formats that have increased stability toward protein detachment and shorter

times for performing an immunoassay via a higher surface concentration of immobilized antibody.

References

1. B. W. Greene, *J. Colloid Interface Sci.*, **43** (2), 449 (1973).
2. Chung-Ji Tschang and Heinrich Klenfenz, U.S. Patent 4,235,973 (November 25, 1980).
3. Chung-Ji Tschang and Heinrich Klenfenz, U.S. Patent 4,266,030 (May 5, 1981).
4. J. E. Herweh and A. C. Pochkas, U.S. Patent 3,709,844 (January 9, 1973).
5. J. E. Wilson, *Radiation Chemistry of Monomers, Polymers, and Plastics*, Marcel Dekker, Inc., New York, 1974, p. 413.
6. H. H. Roth, *Ind. Eng. Chem.*, **49**, 1820 (1957).
7. W. H. C. Rueggeberg, T. W. Sauls, and S. L. Norwood, *J. Org. Chem.*, **20**, 455 (1955).

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