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5                    COVALENT ATTACHMENT OF MACROMOLECULES  
ON SUBSTRATE SURFACES

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The subject field concerns functionalizing polystyrene surfaces, particularly of labware, for covalent conjugation of molecules.

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With the increasing expansion of biological research and commercial biological applications, there has been a concomitant increasing need for laboratory equipment capable of specifically binding to complementary ligands and receptors. In order to provide solid substrates, particularly labware, for specific complex formation, it is necessary to bind a wide variety of ligands and receptors, frequently proteins, to the surface. The binding should provide a surface which minimizes non-specific binding, minimizes loss of the surface bound molecules during the processing, and provides for a high density of the compound bound to the surface in a manner which allows for efficient complex formation.

At the present time, there is no simple and reliable method for covalently attaching a macromolecule to the surface of polystyrene labware, particularly when clarity retention is desired, as well as a high density of the attaching macromolecule. Almost all common organic solvents, used to chemically activate or derivatize crosslinked polystyrene for

solid phase peptide synthesis, particularly solvents such as methylene chloride and DMF, readily dissolve or badly haze uncrosslinked polystyrene. Many of the known solid substrate bound macromolecules exhibit a low surface concentration of the macromolecule, or exhibit only partial biological activity of the bound macromolecule. In some cases, the overall charge of the macromolecule is changed, as is the case in protein conjugation techniques employing active esters (which transform an amino group on the protein to an amide). In other cases, the yield of macromolecules bound to the surface of the substrate is low. In still other cases, surface activation of the substrate adds color to the substrate, thus eliminating or reducing the effectiveness of the substrate in assays such as enzyme linked immunosorbent assays (ELISA).

A continuing interest exists, therefore, in the development of improved activated solid substrates, in improved activated solid substrates bound with covalently attached macromolecules, and in the improved processes for producing these compositions.

#### Relevant Literature

Activated substrates, including polystyrene, and various biologically active substances bound to the substrate, are described in U.S. Patent Nos. 4,736,019; 4,657,873; 4,654,299; 4,419,444; and 4,081,329. Functionalization of polystyrene is also described in U.S. Patent Nos. 3,956,219; 3,886,486; 3,974,110; 3,995,094; and 4,226,958. See also, copending application Serial Nos. 055,528 and 051,917, filed May 29, 1987 and May 19, 1987, respectively, and EPO Application Serial No. 88/304516.3. The use of N-hydroxymethyl 2-chloroacetamide in nitrobenzene/H<sub>2</sub>SO<sub>4</sub> to derivatize polymers containing polystyrene is described in EPO Application Serial No. 83/109905.6.

### SUMMARY OF THE INVENTION

Formed substantially uncrosslinked polystyrene products are functionalized employing hydroxymethyl-amides for electrophilic substitution on the phenyl groups. The substituted-methylamides include a substitutable functionality permitting nucleophilic substitution, while being stable under the conditions of aryl substitution. The resulting functionalized polystyrene may be used for reacting with a wide variety of functionalities, particularly associated with macromolecules, to provide for a high density of covalently bonded macromolecules. The method employs a polystyrene insoluble solvent as the solvent for the aromatic electrophilic substitution. The resulting functionalized polystyrene may be reacted with macromolecules to provide for surfaces useful for complex formation between complementary binding pairs.

### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, polystyrene surfaces are functionalized by reaction with hydroxymethylamides or derivatives thereof in a polystyrene insoluble solvent which is tetramethylenesulfone (TMS), under aromatic electrophilic substitution conditions. The amides are characterized by having a functionality capable of nucleophilic substitution under mild conditions, while stable under the conditions of aromatic electrophilic substitution. The resulting functionalized polystyrene may be reacted with various functionalities capable of nucleophilic substitution, particularly macromolecules, to provide for a surface which may be used for complex formation between specific binding pairs. Of particular interest is the functionalization of labware which can be used in the isolation of a wide variety of substances which binds specifically to a complementary

pair member, in assays and as affinity columns.

The polystyrene objects which are activated, will be for the most part non-crosslinked, usually  
5 having fewer than about 0.5% crosslinks, more usually fewer than about 0.1% crosslinks. The polystyrene products are moulded, so as to provide for a smooth surface with few indentations or canyons, so that molecules may be bound to the surface and be  
10 readily available for binding to other molecules.

The solid substrate may exist in any form, including reaction vessels, reagent tubes, beakers, cuvettes, columns, microtiter plates, Petri dishes, fabricated articles, beads, rods,  
15 fibers, strands, membranes, discs, or plates. The articles are formed by moulding polystyrene at least substantially free of crosslink, usually resulting in a clear, smooth surface.

Because of the clarity of the substrate, one  
20 can see through the walls of the labware and can have efficient transmission of light, as needed. This clarity is particularly important in assays, where colorimetry or fluorimetry is involved, and light is observed as transmitted through a wall. The other  
25 factor is the smoothness of the surface, which avoids having large canyons or crevices in relation to molecular dimensions. Where the product has an uneven surface, molecules which are bond to the surface may bind within the crevice or along the walls of the  
30 crevice, making the molecule unavailable for binding to a complementary specific binding member. Therefore, even where one can obtain a high degree of activation of the surface, so that a large number of molecules may be covalently bonded to the surface, only a portion of  
35 the molecules will be available for binding to their complementary member. By contrast, with polystyrene molded surfaces, the surface is fairly uniform and flat

so that molecules that are covalently bonded to the surface extend upwards from the surface, usually allowing easy access to sites on the molecule for binding to complementary pair members. Thus, receptors  
5 such as surface membrane proteins and antibodies may be bound to the surface, where their binding sites are readily available for binding to complementary ligands.

Retention of the desirable properties of the  
10 molded polystyrene is achieved using functionalized Y-methyl acetamides, where the Y-methyl group reacts with the polystyrene in a TMS solvent in the presence of an acid catalyst under mild conditions, and Y is a moiety capable of undergoing nucleophilic  
15 substitution by an aryl group, e.g. benzene, in the presence of an acid catalyst. The  $\alpha$ -acyl functional group of the Y-methyl amide is a group which is stable under the aromatic electrophilic substitution conditions, but can be readily displaced under  
20 nucleophilic substitution conditions, for example, by a Lewis base.

The compounds used for functionalizing the polystyrene preferably have the following formula



wherein:

R is an alkyl group of from 1 to 3, more preferably from 1 to 2 carbon atoms or hydrogen, preferably  
30 hydrogen;

X is a halogen (including pseudohalogen), where the halogen is of atomic number of at least 17, wherein X may be chlorine, bromine, or iodine, and the pseudohalogens, for example arylsulfonate esters, sulfonium salts. Usually, when other than halogen, the substituent on the  $\alpha$ -carbon atom of the acyl group will be of at least about 2 carbon atoms and not more

than 10 carbon atoms, usually not more than about 8 carbon atoms, and may have from 1 to 6, more usually from 1 to 5, and preferably from 1 to 4 heteroatoms, which include halogen and chalcogen (oxygen and sulfur),- where any counterion is not included in the limitations. While many other functional groups may be present, such as cyano and nitro, for the most part they do not play a useful role and the compounds are not generally available.

10 Y may be the same or different from X, usually different, and is a group capable of nucleophilic substitution, particularly oxy-derivatives, halides and pseudohalides, where the oxy-derivatives may be esters, either organic or inorganic, or ethers. Y will usually  
15 be of not more than 20 carbon atoms, usually not more than 12 carbon atoms and up to 8, usually not more than 6 heteroatoms, Y groups include hydroxy halogen of atomic number of at least 17, pseudohalides such as arylsulfonate esters, esters such as  
20 phosphates, carboxylates, imidates, ethers, such as alkyl, aryl, oxyalkyl. Y is preferably OH.

N-hydroxymethyl acetamides which find use are the 2-substitued N-hydroxymethyl acetamides, including N-(hydroxymethyl)-2-chloroacetamide,  
25 N-(hydroxymethyl)-2-bromoacetamide, N-(hydroxymethyl)-2-iodoacetamide, N-(hydroxymethyl)-O-(p-bromobenzene-sulfonyl)glycolamide, N-(hydroxymethyl)-O-(p-toluenesulfonyl)glycolamide, N-(hydroxymethyl)-2-acetamidopentamethylenesulfonium iodide, and  
30 N-(hydroxymethyl)(2-acetamido)pentamethylenesulfonium trifluoroacetate. The synthetic reaction for each of these individual compounds is shown in the Examples.

The N-hydroxy compounds may be derivatized or prepared directly with the particular acid or hydroxyl  
35 replacing group in the reaction mixture during the N-substitution of the  $\alpha$ -substituted acetamide or using

a formaldehyde derivative such as  $\alpha$ -chloromethyl methoxyethyl ether.

The activation of the polystyrene will occur under mild conditions in TMS which neither  
5 dissolves nor swells the polystyrene.

The reaction will be carried out in the presence of an acidic catalyst, preferably a strong acid, particularly a protonic acid, preferably an organic acid, such as trifluoroacetic acid, trifluoromethanesulfonic acid,  
10 methanesulfonic acid, sulfuric acid, phosphoric acid, hydrogen fluoride. The concentration of the acid will generally range from 0.1 to 2M. The reaction will normally be carried out in the substantial absence of atmospheric moisture, which can be achieved by  
15 covering the reaction mixture, providing for an inert atmosphere. The acidic catalyst is also preferably a Lewis acid.

The polystyrene surface is contacted with the N-(Y-methyl) acetamide compound within a temperature and time range suitable for activating the surface of  
20 the substrate. Generally, the temperature will range from  $-5^{\circ}\text{C}$  to  $60^{\circ}\text{C}$ , preferably  $0^{\circ}$  to  $30^{\circ}\text{C}$ , and the activation time is from 5 min to 48 h, usually from 2 to 8 h. The concentration of the N-(Y-methyl) acetamide compound is  
25 typically between 0.01 to 2 M, usually between 0.05 to 0.5 M. For most applications, the use of at least 0.05, usually 0.1 ml of reaction solution per square centimeter of solid substrate surface area is sufficient to provide for the desired  
30 level of activation by reaction of electrophilic groups onto the surface of the substrate.

Before the conjugation of the macromolecule, the surface of the substrate may be freed of any unreacted N-(Y-methyl) acetamide compound by washing

with a buffer and/or water. The particular material employed for the washing step is not critical to the invention, and is chosen for convenience, so long as the conditions do not affect the substitutable group of the amide. Substitution of the substitutable group may be conducted immediately after activation, or at some interval of time, since the surface is stable when stored in an inert environment.

The activated polystyrene surface may now be reacted with compounds capable of substituting the substitutable groups. These compounds for the most part will have atoms having unshared pairs of electrons, such as nitrogen, oxygen and sulfur. For the most part, these compounds will be peptides, particularly proteins. Where peptides are reacted, the reaction will be under mild conditions by contacting solutions of the molecules at concentrations ranging from  $10^{-6}$  to 2 g/ml, more usually from  $10^{-3}$  to 1 g/ml, at a pH in the range of 6 to 10, more usually 7 to 9.5.

The medium will normally be buffered with a convenient buffer, e.g. phosphate, borate, or other inorganic or organic buffer, which cannot compete for reaction with the substitutable group on the surface. The concentration of the buffer will generally be from 0.01 to 0.5 M. Also included in the medium may be various salts, particularly alkali metal halide salts, where the concentration of the salt will generally range 0.01 to 0.5 M.

The temperature will generally range from 0 to 40°C, more usually from 10 to 30°C. The time for the reaction will vary depending upon the substitutable group, the nature of the substituting group, the other components in the reaction medium. Generally, the incubation time will be at least 5 min, usually at least 30 min, and

not more than 24 h, usually not more than 12 h.

The resulting activated polystyrene surface may be analyzed by electron spectroscopy for chemical analysis (ESCA) to determine the percent amount of  
5 elements on the surface of the substrate. Table 1 in Example 4 illustrates the data obtained for a polystyrene tissue culture flask (25 cm<sup>2</sup>).

Groups which may be substituted onto the  
10 polystyrene surface include proteins, particularly biologically active proteins;  
ligands, such as haptens, and antigens; receptors, such as antibodies, monoclonal antibodies and antibody  
fragments, enzymes, and naturally occurring receptors,  
15 such as surface membrane proteins, lectins, and blood proteins (e.g., thyroxine binding globulin); complexing agents, such as cryptands, crown ethers, porphyrins, and phthalocyanines; nucleic acids, such as RNA, DNA, oligonucleotides unnatural nucleotides, e.g. methyl-  
20 phosphonates; peptides; indicator substances, such as dyes, fluorescers, and chemiluminescers; antibody binding proteins (such as Staphylococcal protein A or complement component enzymes); ligands such as cerebrosides, sphingomyelin and gangliosides;  
25 glycosides; enzymes, where the enzymes are used as catalysts rather than receptors, enzyme substrates, inhibitors, or co-factors; or other molecules which may be of interest for the detection of the same or  
different molecules; or used for the manipulation,  
30 isolation, reaction, interaction, or other phenomena, where a result of interest is produced.

After completion of the reaction, the reaction mixture may be discarded and the surface washed with a solvent, buffer, or water to remove any residual non-  
35 covalently bound materials. The particular wash solution employed for this washing step is not critical to the invention, and is chosen for convenience.

The uses of the product of the present invention-- the polyaryl addition polymer either in its activated state, or containing a covalently attached macromolecule or other molecule-- are many and varied,  
5 as follows.

The modified devices find a wide variety of uses in diagnosis and in therapy, particularly ex vivo, in the isolation of cells from blood, other physiological fluids, or dispersed tissue. Because of the  
10 high density of various compositions which may be bound to the surface, where complementary binding members may be found on cells, the cells may be bound to the surface, thus separating subsets of cells from a mixture of cells. In addition, this method of  
15 separation allows for high levels of viability of the cells and ease of cell recovery. Because the clarity of the surface is maintained, the binding of cells to the surface may be monitored, as well as the processing of the cells, such as expansion with mitotic agents and  
20 lymphokines or activation with immune complexes.

The subject surfaces may also be used in diagnostic assays to provide for efficient binding of specific binding pair members to the surface. Because  
25 of the clarity of the surface, light can be transmitted through the sample and the bottom of the container, e.g., a microtiter well plate. Furthermore, because of the high efficiency of binding, particularly where the possibility of binding at multiple sites exists,  
30 washing can be relatively thorough and vigorous, to ensure the substantial absence of any non-specifically bound components of the assay.

EXPERIMENTALExample 1

Several N-hydroxymethyl acetamide compounds  
5 were prepared by suspending a 2-haloacetamide compound  
(25g, 267 mmol in water (75 ml) and 37% formaldehyde  
(8.01 g, 20 ml, 267 mmol), and treating the suspension  
with  $K_2CO_3$  (25 g, 18 mmol). A solution forms after  
stirring for several min. The reaction mixture is then  
10 placed at 4°C overnight, filtered, and dried in vacuo  
(0.01 mm, 18 h, at room temperature).

Using the above process with 2-chloroacetamide  
yielded 12.5 g (38%) of N-(hydroxymethyl)-2-  
chloroacetamide (Compound 1), as colorless crystals:  
15 melting point, 98-99°C.

Using the above process with 2-bromoacetamide  
yielded 47% of N-(hydroxymethyl)-2-bromoacetamide (2),  
ad colorless crystals: melting point, 88-89°C.

Using the above process with 2-iodoacetamide  
20 yielded 43% of N-(hydroxymethyl)-2-iodoacetamide (3),  
as colorless crystals: melting point, 117-118°C.

Example 2

N-(hydroxymethyl)-O-(p-bromobenzenesulfonyl)  
25 glycolamide (4) was prepared by treating a solution of  
O-(p-bromobenzenesulfonyl)glycolamide (7.1 g, 24 mmol)  
in DMF (21 ml) with 37% formaldehyde (2.2 g, 5.4 ml, 72  
mmol) and  $K_2CO_3$  (1 ml of a 50 mg/ml water solution,  
0.36 mmol). After 30 min, the reaction was diluted  
30 with 10 times the volume water, cooled on ice, and  
filtered. The solid was dried in vacuo and  
recrystallized from EtOAc to give 5.9 g (76%) 4 as  
colorless crystals: melting point, 99-100°C.

N-(hydroxymethyl)-O-(p-toluenesulfonyl)  
35 glycolamide (5) was prepared using the same procedure  
as for 4, resulting in a 70% yield of 5 as colorless  
crystals: melting point, 91-93°C.

N-(hydroxymethyl)-2-acetamidopentamethylene-sulfonium iodide (6a)

(6a) was prepared by chilling a solution of 3 (1.0 g, 4.6 mmol) and pentamethylene sulfide 2.4 g, 2.5 ml, 24 mmol) in acetone at 4°C for 48 h. The solid that crystallizes is collected on a glass frit, washed with ether, and dried in vacuo to yield 560 mg (38%) of the iodide salt of 6a, as colorless crystals: melting point 93-95°C.

10 N-(hydroxymethyl)-2-acetamidopentamethylene-sulfonium trifluoroacetate (6)

(6) was prepared by eluting the iodide salt prepared above through a 10-fold excess AG1-X4 (-O<sub>2</sub>CCF<sub>3</sub>) column with water. The UV active (254 nm) fractions were lyophilized, dried in a rotovap three times from anhydrous acetonitrile, and dried in vacuo (0.05 mm, 18 h, room temperature) to give a quantitative yield of 6 (based on the starting iodide), as light yellow crystals: melting point 65.5-67°C.

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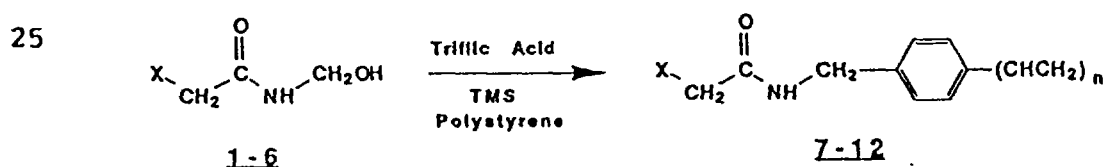
Example 3 Preparation of a substrate surface

A 0.2 M solution of N-hydroxymethyl acetamide derivatives 1-6 in tetramethylenesulfone (TMS), was combined in a 1:1 ratio with a 2 M trifluoromethyl-sulfonic acid (TFMSA) solution in TMS, and the reaction solution was immediately added to the polystyrene surface to be derivatized. Tissue culture flasks (commercially available from Corning) were capped tightly with the screw-top caps provided by the manufacturer. Microtiter plates (96-, 24-, or 6-well, non-irradiated polystyrene) or Removeawells (Immulon 1; commercially available from Dynatech) were covered with adhesive-backed Mylar tape. The reaction solution was left in contact with the polystyrene surface for 5 h at room temperature, washed with water until odorless, and air-dried at room temperature overnight. The surfaces

were stable for at least two weeks in a dry, light-free environment.

#### Example 4

Solutions of each of the N-hydroxymethyl acetamide compounds 1-6 were made at a concentration of 0.1 M with 1 M TFMSA in TMS, and the resulting solutions were used to activate both tissue culture flasks (commercially available from Corning) and microtiter plates (Immulon I, available from Dynatech), by contacting the solution with the flasks or plates for 5 h at room temperature. Only non-irradiated polystyrene was used for the activation step. For both tissue culture flasks and microtiter plates, at least 0.1 ml of reaction solution per square centimeter of polystyrene surface area was used. The reaction mixture was protected from moisture in the air by tightly capping the tissue culture flask, or covering the microtiter plate with Mylar adhesive tape, immediately after the introduction of the reaction solution. The surfaces prepared, surfaces 7-12, are outlined below:



| X—                                                                                                                       | COMPOUND | SURFACE |
|--------------------------------------------------------------------------------------------------------------------------|----------|---------|
| Cl—                                                                                                                      | 1        | 7       |
| Br—                                                                                                                      | 2        | 8       |
| I—                                                                                                                       | 3        | 9       |
| Br—  —SO <sub>3</sub> —               | 4        | 10      |
| CH <sub>3</sub> —  —SO <sub>3</sub> — | 5        | 11      |
|  S <sup>+</sup> —                     | 6        | 12      |

ESCA data for T-25 (25 cm<sup>2</sup>) tissue culture flasks having surfaces 7-12 is shown in the following Table.

| SURFACE   | ELEMENT % |     |      |     |     |     |     |     |
|-----------|-----------|-----|------|-----|-----|-----|-----|-----|
|           | C         | N   | O    | F   | Cl  | Br  | I   | S   |
| <u>7</u>  | 81.8      | 5.9 | 6.9  | ∴   | 5.4 | *   | *   | *   |
| <u>8</u>  | 83.0      | 5.4 | 5.9  | *   | *   | 5.2 | *   | *   |
| <u>9</u>  | 78.1      | 7.8 | 7.0  | *   | *   | *   | 7.1 | *   |
| <u>10</u> | 73.0      | 4.5 | 14.0 | 1.1 | *   | 3.3 | *   | 4.5 |
| <u>11</u> | 78.0      | 4.0 | 14.0 | *   | *   | *   | *   | 4.0 |
| <u>12</u> | 82.0      | 3.6 | 8.4  | 2.3 | 0.2 | *   | *   | 3.1 |

The starting polystyrene was found to have 3-5% oxygen on its surface.

#### Example 5

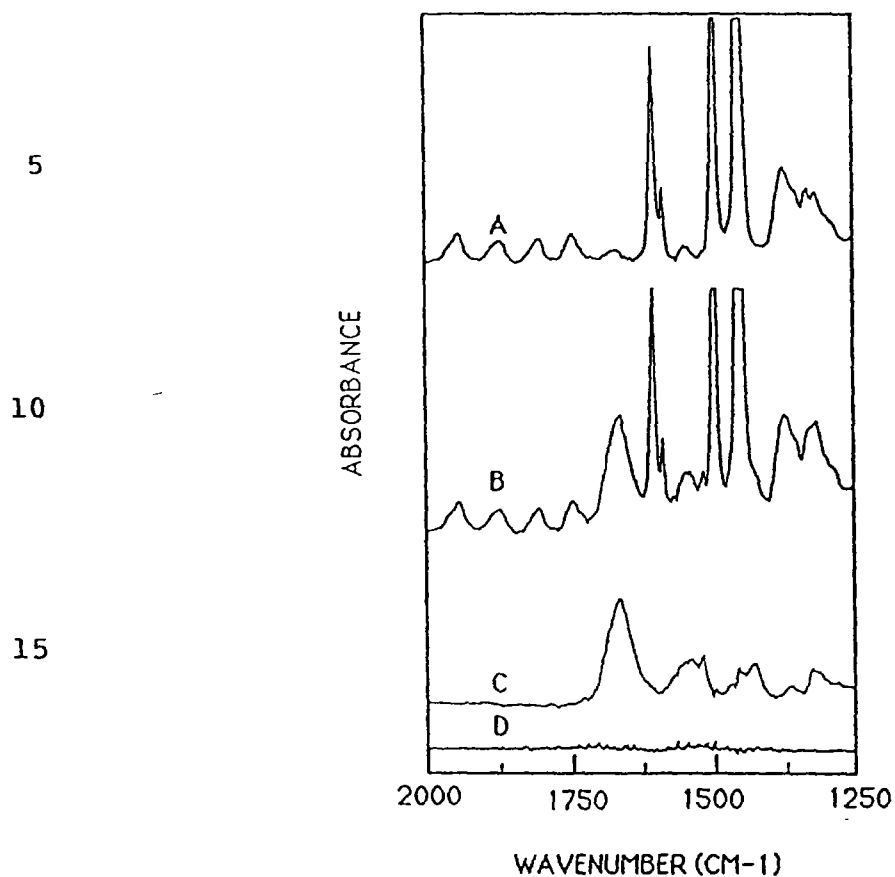
Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) was also applied to analyze the derivatized polystyrene surfaces 7-12. In all cases, a strong amide I and amide II absorbance were detected. A series of strong symmetric and asymmetric O-S-O absorbances was also detected for the sulfonate esters 10 and 11. The data are summarized in the table next page.

SELECTED FTIR ABSORBANCES OF ACTIVATED SURFACES

|    | SURFACE   | FTIR ABSORBANCE ( $\text{cm}^{-1}$ ) |
|----|-----------|--------------------------------------|
| 5  | <u>7</u>  | 1664, 1535                           |
|    | <u>8</u>  | 1660, 1541                           |
|    | <u>9</u>  | 1648, 1539                           |
| 10 | <u>10</u> | 1680, 1541, 1393, 1373, 1189         |
|    | <u>11</u> | 1678, 1541, 1373, 1191, 1178         |
| 15 | <u>12</u> | 1636, 1559                           |

Example 6

A detailed study of the ATR-FTIR spectra of polystyrene and 8 in the  $1250\text{--}2000\text{ cm}^{-1}$  region demonstrates the power of FTIR in performing surface analyses. Spectrum A is underivatized polystyrene from a T-25 tissue culture flask, the starting material for surface 8 (spectrum B). The digitized form of both spectra can be stored, and the contribution from polystyrene in spectrum B can be removed by subtracting spectrum A, generating the difference spectrum C. In spectrum C the amide I, II, and III bands are readily apparent. The doublet nature of amide II may indicate that both s-cis and s-trans forms of the amide are present on the polystyrene surface of 8. When two separate scans of polystyrene are subtracted, the difference spectrum D is found to be devoid of any artifactual features.

ATR-FTIR OF 8

Example 7 Protein binding protocol with  $^{35}\text{S}$ -labeled human IgG.

Remove a well strips having surfaces 7-11, as produced in the previous example, and Immulon 1 were coated with  $^{35}\text{S}$  labeled human IgG (spec. act.  $>3$  dpm/ng) in PBS at  $20\text{ }\mu\text{g/ml}$ ,  $100\text{ }\mu\text{l/well}$  for 16 h at room temperature. Surface 12 was treated in the same fashion, except the coating buffer was  $0.1\text{ M}$  borate, pH 9.2. After incubation, the radioactive solution was carefully removed, and the wells were rinsed 10 times with PBS. The wells were broken apart, placed in  $1\%$  SDS ( $3\text{ wells}/10\text{ ml}$ ) and incubated at  $55^\circ\text{C}$  for 14 h. The wells were removed from the SDS, given one more PBS rinse, and counted in Fisher Scintiverse II. The wells were recounted 24 h later. Agreement between the two counts was always within 5%.

The results are tabulated below, and show that the derivatized polystyrene surfaces bound 2.2 to 2.8 times as much protein as the underivatized Immulon I.

| 5  | SURFACE    | IgG SURFACE<br>DENSITY (ng/cm <sup>2</sup> ) |
|----|------------|----------------------------------------------|
|    | Immulon 1™ | 100                                          |
|    | <u>7</u>   | 225                                          |
| 10 | <u>8</u>   | 260                                          |
|    | <u>9</u>   | 250                                          |
| 15 | <u>10</u>  | 281                                          |
|    | <u>11</u>  | 248                                          |
| 20 | <u>12</u>  | 240                                          |

Example 8 A macromolecule-bonded, activated substrate, produced according to the present invention, was used in an ELISA protocol.

Serial dilutions of a 1 mg/ml solution of rabbit x human IgG (commercially available from Jackson ImmunoResearch) were used to provide concentrations of 100, 70, 30, 10, 3, and  $1 \times 10^{-4}$  mg/ml solutions in 0.1 M borate, pH 9.2. Four polystyrene wells (activated with Compound 7 according to the present invention) with 100  $\mu$ l of each concentration were coated with the antibody for 16 h in a covered microtiter plate at room temperature (no rocking). Four more wells were coated with buffer alone. The antibody solution was shaken out and tapped dry on a piece of toweling. The non-specific binding sites in all wells were blocked with

200  $\mu$ l of 5% BSA in PBS, incubated in a covered plate for 16 h at room temperature. The plate was washed five times with PBS/0.02% Tween 20 (using a Wheaton gun), and all wells were incubated 3 h at room  
5 temperature with 100  $\mu$ l of a  $30 \times 10^{-4}$  mg/ml human IgG solution (in PBS, and available from Sigma). The plate washed 5 times with PBS/0.02% Tween 20, and 100  $\mu$ l of a 1:3000 dilution goat x human IgG(H+L)-AP (alkaline phosphatase) conjugate (commercially available from  
10 Jackson ImmunoResearch), in PBS/0.02% Tween 20, was added to all wells for 3 h at room temperature in a covered plate. The plate was washed 3 times with PBS/0.02% Tween 20, and 3 times with PBS. To all wells was added 100  $\mu$ l of alkaline phosphatase substrate (p-  
15 nitrophenyl phosphate) at a concentration of 1 mg/ml in 1 M triethanolamine, pH 9.6. The color was allowed to develop for 7 min, and was stopped by addition of 3 M NaOH, 25  $\mu$ l/well. The plate was then read on a  
Molecular Devices Vmax Kinetic Microplate Reader at 405  
20 nm.

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|    |                                                          |                                        |
|----|----------------------------------------------------------|----------------------------------------|
|    | Coating concentration<br>rabbit x human IgG<br>(mg/well) | Absorbance 405 nm<br>(Mean of 4 wells) |
| 5  | 0                                                        | 0.000                                  |
|    | $1 \times 10^{-5}$                                       | 0.122                                  |
| 10 | $3 \times 10^{-5}$                                       | 0.386                                  |
|    | $10 \times 10^{-5}$                                      | 1.097                                  |
|    | $30 \times 10^{-5}$                                      | 1.681                                  |
| 15 | $70 \times 10^{-5}$                                      | 1.882                                  |
|    | $100 \times 10^{-5}$                                     | 1.946                                  |

20           It is evident from the above results, that the  
subject methodology is highly efficient, simple, and  
produces functionalized surfaces which find a variety  
of uses. The clarity of the surface allows observation  
of the surface, where cells are being processed,  
25   transmission of light through the container, and the  
ability to monitor events in the container. In  
addition, the subject method provides for a very high  
density of molecules bound to the surface, where it is  
found that with antibodies, there is a high level of  
30   orientation of available binding sites. The smooth  
polystyrene surfaces are relatively free of cracks and  
crevices so that on a molecular scale, the proteins  
bound to the surface are available to binding of other  
molecules and cells.

35           All publications and patent applications cited  
in this specification are herein incorporated by  
reference as if each individual publication or patent

application were specifically and individually  
indicated to be incorporated by reference.

Although the foregoing invention has been  
described in some detail by way of illustration and  
5 example for purposes of clarity of understanding, it  
will be readily apparent to those of ordinary skill in  
the art in light of the teachings of this invention  
that certain changes and modifications may be made  
thereto.

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CLAIMS

1. A method for functionalizing a polystyrene surface, wherein said polystyrene is clear and at least  
5 substantially uncrosslinked to provide a high density of functional groups for covalently linking a moiety of interest to said surface, said method comprising:  
contacting said polystyrene surface with an  $\alpha$ -substituted N-(Y-methyl) acetamide in tetramethylenesulfone in  
10 the presence of an acid catalyst for sufficient time for displacement of Y by the benzene rings of said polystyrene to produce an acetamido substituted surface with retention of clarity, wherein said  $\alpha$ -substituted group is displaceable by nucleophilic substitution and Y is a group capable of  
15 electrophilic displacement by an aryl group in the presence of said acid catalyst.
2. A method according to Claim 1, wherein said acidic catalyst is a protonic catalyst.
3. A method according to Claim 2, wherein said  
20 acid catalyst is trifluoromethylsulfonic acid.
4. A method according to Claim 1, 2 or 3 wherein Y is OH.
5. A method according to Claim 1 wherein said acid catalyst is a Lewis acid catalyst.
- 25 6. A method according to any one of the preceding claims, wherein said  $\alpha$ -substituent is a halogen or pseudohalogen.
7. A method according to any one of the preceding claims wherein said contacting is at a temperature in

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the range of 0 to 40°C.

8. A method according to any one of the preceding claims which comprises the further step of removing said tetramethylenesulfone solution.

5 9. A method according to Claim 8 which further comprises contacting said acetamido substituted surface with said moiety of interest containing a nucleophilic group in a polar solvent, whereby said moiety of interest becomes covalently bound to said surface at a high density.

10 10. A method according to Claim 9, wherein said moiety is a protein.

11. A method according to Claim 10, wherein said protein is an antibody.

12. A functionalised polystyrene moulded surface obtainable by the method of any one of the preceding claims.

13. A polystyrene surface according to Claim 12 which is the surface of an item of laboratory equipment.