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Hydrophilized and functionalized microtiter plates for the site-specific coupling of antigens and antibodies: application to the diagnosis of viral cardiac and autoimmune diseases

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Abstract

Extensive adsorption of macromolecules (antigens and antibodies) took place when they were covalently bound to non-hydrophilized polystyrene microtiter plates. Precoating polystyrene surfaces with gelatin reduced this non-specific adsorption only partially, whereas precoating with uncharged polymers such as poly(ethylene glycol) (PEG) and polysaccharides eliminated the problem totally.

On such hydrophilized plates functionalized with epoxide groups, antigens and antibodies were randomly bound. On those functionalized with acid hydrazide, antibodies were site-specifically bound by their carbohydrate residues. These site-specifically bound antibodies retained greater reactivity and more of their native antigenic structure than randomly coupled antibodies. They therefore permitted the measurement of a minor analyte (D-dimer) in the presence of an excess of major components such as fibrinogen.

Enzyme immunoassays which were uninterpretable on non-hydrophilized plates because of the adsorption of immunoglobulin gave meaningful results on hydrophilized plates. This held true for immunoglobulin aggregates formed artificially either by successive cycles of freezing and thawing or by the acid treatment of serum to dissociate immune complexes. The latter approach permitted us to obtain unequivocal evidence for the presence of antibodies specific for human T-cell lymphotropic virus type 1 (HTLV1) in immune complexes in sera with no detectable free antibody. For naturally occurring aggregates which are often found in the sera of patients with autoimmune diseases, the use of hydrophilized plates also permitted antibody levels to be measured in instances where background noise was greater than the signal on non-hydrophilized plates.

This combination of hydrophilization and site-specific coupling of monoclonal antibodies of a defined specificity should provide a distinct advantage when developing routine covalent enzyme linked immunoassays (CELIAs) for minor analytes, in a mixture with major constituents, as is often encountered, in both human and veterinary medicine and in the food industry.

Keywords: Antibodies; antigens; hydrophilized and functionalized microtiter plates; site-specific coupling.

Introduction

We have previously shown that glycoproteins such as immunoglobulin G (IgG) and viral antigens can be covalently bound by their carbohydrate or protein residues to insoluble supports such as latex particles [1] or microtiter plates [2]. Evidence for the site-specific coupling of rabbit IgG via its carbohydrate residues was obtained by treating the latex–rabbit-IgG spheres (0.8 μm) with a dena-

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turing buffer at 95°C in the presence of 2-mercaptoethanol to dissociate heavy (H) from light (L) chains and then subjecting the treated spheres to electrophoresis on polyacrylamide gels. Since the spheres do not enter the gel, any H or L chains which were directly bound to the spheres themselves by covalent bonds remain at the top of the gel. On the contrary, those H and L chains which were not attached directly to the spheres but indirectly via S–S bonds to their corresponding covalently bound L and H partners were liberated by the reductive treatment with 2-mercaptoethanol and migrated according to their molecular weight.

After staining the gels with silver nitrate, it was found for IgG covalently bound by amide bonds, randomly distributed over the protein molecule, that the ratio H/L was 0.71. For IgG bound by hydrazone bonds through their sugar residues on the crystallizable fragment (Fc) portion of the H chains, the H/L ratio was 0.35. If we compare these values to the ratio 0.78 obtained for free non-immobilized IgG in solution, it is clear that the decrease in H chains is due to their retention on the spheres which did not penetrate the gel. Thus the vast majority of IgG molecules must have been site-specifically coupled via the sugar residues on their Fc portion.

Site-specific coupling offers both theoretical and practical advantages. In the case of antibodies bound via their Fc portion, the antigen-binding fragments (Fabs) should be oriented away from the surface of the insoluble support and hence be more reactive. Evidence to support this concept has been put forward by other groups [3] using antibodies bound via their sugar residues to hydrazine-functionalized Sepharose beads.

In the case of viral antigens, we have been able to show that enzyme immunoassays, which were performed with cytomegalovirus (CMV) antigens linked by hydrazone bonds on their carbohydrate residues to functionalized microtiter plates, permit the neutralizing antibody (Neut-Ab) titers of human IgG preparations to be obtained. With amide-bound CMV antigens, the titers obtained were not correlated with the Neut-Ab titers [2]. The site-specific coupling here opens up possibilities to develop a routine solid phase method for obtaining specific information which today is only available by laborious analytical methods.

Thus, site-specific immobilization, i.e. coupling via the carbohydrate residues, is of considerable practical value for solid phase immunoassay both for antibody and antigen binding. However, the more advanced the immobilization techniques and the more sophisticated the reagents, such as monoclonal antibodies (MAbs) on the one hand, and synthetic peptides as antigenic determinants on the other, the more important is the nature of the solid support surface. Today, solid phase immunoassays are performed on hydrophobic plastics, mainly polystyrene. The vast majority of analyses are made by simple adsorption of the antibody or antigen to the solid surface. When the procedure involves covalent coupling, polystyrene plates containing various reactive functional groups are being used. The main problem with these plates is the high degree of non-specific adsorption of proteins. Both the normal plates and the reactive ones have a strong tendency to adsorb proteins and it is a well-known fact that such unwanted adsorption gives rise to so-called false positive answers in solid phase immunoassays.

There are two main forces behind protein adsorption to solid surfaces. Hydrophobic interaction, which is an entropy-driven attraction, dominates at hydrophobic surfaces. Electrostatic double-layer forces are important on charged surfaces. In order to prevent proteins adhering to the solid, coating or grafting the surface with hydrophilic, non-charged polymer chains has been found to be effective [4–6]. In this way both types of attractive force will be reduced. If the layer of attached polymer is dense enough, a practically non-adhering surface is obtained.

Such modified polystyrene plates are of interest for solid phase immunoassays for several reasons. Firstly, since non-specific adsorption is reduced, the signal-to-noise ratio is increased, thus reducing the risk of false positive answers. Secondly, since there is little attraction between the attached anti-

body or antigen and the surface, the reagent molecule will orient itself away from the surface and be more available for molecules in the bulk solution. Thirdly, since the attached species will not be in direct contact with the surface, surface-induced conformational changes will not occur. Such distortions may eventually lead to loss of immunoactivity. Indeed, it has been found that MAbs which have been selected on the basis of their interaction with a protein adsorbed to polystyrene or poly(vinyl chloride) (PVC) subsequently showed no reaction with a protein in its native state in solution. This is well documented for complement factor C3 [7] and similar observations have recently been made with fibrin [8].

We have recently described a way of covalently attaching proteins to properly hydrophilized polystyrene plates [9]. Reactive electrophilic groups were introduced on the grafted polymer chains. In order for the protein not to be repelled by the hydrophilic layer, the immobilization reaction was carried out in microemulsion instead of aqueous buffer. A high loading of protein, randomly coupled via amino or thiol groups of the polypeptide chain, was obtained by this method. On subsequent soaking in water the grafted layer regained its protein repellent character.

The present work describes the combination of the techniques of site-specific immobilization via carbohydrate residues of glycoproteins and microemulsion-based immobilization to hydrophilic surfaces. Applications related to diagnostic problems will be reported.

Materials

Polystyrene microtiter plates were purchased from Nunc (Denmark). Polystyrene Primaria® microtiter plates functionalized with amino groups were from Becton Dickinson (France). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), isooctane, Aerosol OT (AOT) and gelatin (300 bloom) were purchased from Sigma (France). Alkaline phosphatase labeled sheep anti-rabbit IgG, alkaline phosphatase labeled goat anti-human IgG or IgM were purchased from Biosys (France). Rabbit IgG anti-human fibrinogen, peroxidase labeled rabbit IgG anti-human fibrinogen and peroxidase labeled swine anti-rabbit IgG were from Dakopats (Denmark). Purified fibrinogen (grade L) was obtained from Kabi Vitrum (Sweden). [1-^{14}C]sodium pyruvate (30 mCi mmol^{-1}) was obtained from Amersham (UK) while [1,2-^{14}C]-ethanolamine (4 mCi mmol^{-1}) was from NEN (USA).

Human T-cell lymphotropic virus type 1 (HTLV1) was a gift of Dr. C. Desgranges, INSERM Unité 271, Lyon, France. Purified influenza virus (B/Hong Kong/8/73) was kindly supplied by Dr. N. Kessler, Laboratoire de Bactériologie et Virologie, Faculté de Médecine, Lyon, France.

Methods

Functionalization of non-hydrophilized polystyrene microtiter plates

The preparation of polyaminostyrene plates according to the method of Chin and Lanks [10] and their subsequent conversion to polycarboxystyrene and polyhydrazidostyrene have been previously described [2,11].

As polyaminostyrene plates are now available commercially (Primaria®), they were treated directly with a solution of 0.06 *M* succinic anhydride in 0.05 *M* sodium tetraborate (pH 9.5) to obtain a polycarboxystyrene support. The next step consisted in activating the carboxyl groups generated on the polystyrene surface using a solution containing 0.01 *M* NaH_2PO_4, 0.05 *M* EDC and 0.05 *M* NHS. The *N*-succinimidyl ester (NSE) groups bound to the polystyrene were used for the covalent coupling of proteins via their amino groups or further derivatized to polyhydrazidostyrene for coupling glycoproteins via their carbohydrate residues. The sequence of functionalization reactions is shown in Fig. 1.

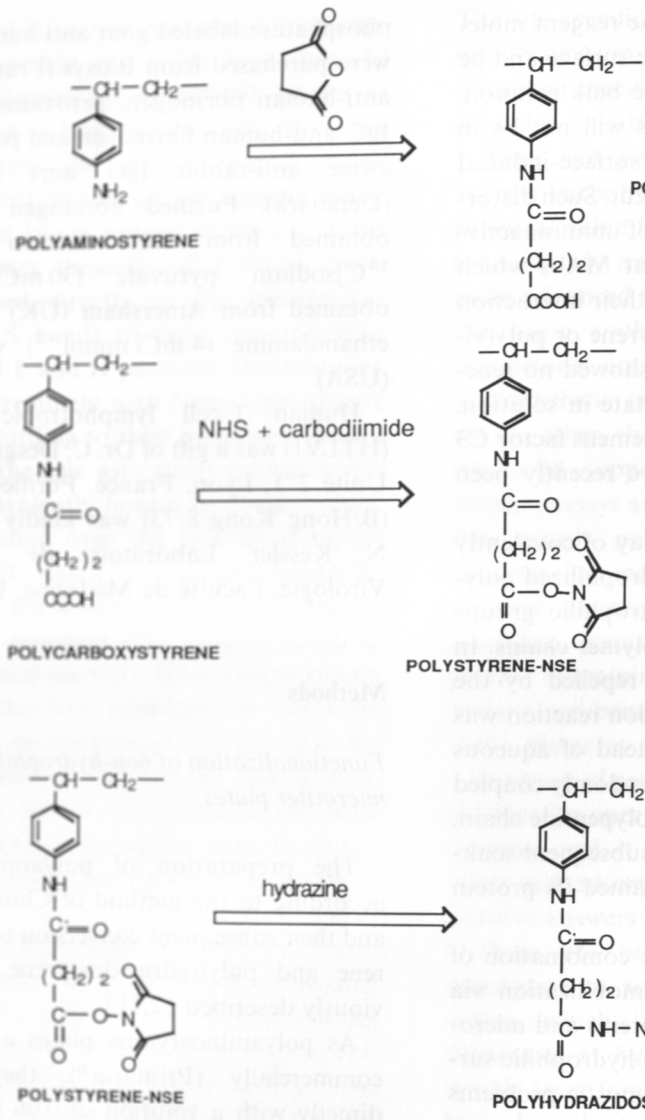

Fig. 1. Scheme of chemical reactions used to obtain polycarboxystyrene, polystyrene–NSE and polyhydrazidostyrene.

Preparation and functionalization of gelatin-coated polystyrene microtiter plates

To wells of the polystyrene–NSE plates were added 100 µl of the solution of gelatin (5 and 10 µg ml^{-1} in phosphate-buffered saline (PBS)). After a period of incubation of 4 h at room temperature, the plates were extensively washed in deionized water. Terminal functional groups such as *N*-succinimidyl ester (NSE) and acid hydrazide were generated on this partially hydrophilized support as described above.

Preparation and functionalization of hydrophilized polystyrene microtiter plates

The poly(ethylene glycol) (PEG) derivative is a tetrafunctional PEG epoxide of molecular weight 14 000–15 000 g mol^{-1} prepared by ethoxylation of di(trimethylol)propane followed by reaction with

epichlorohydrin, as has been described before [12]. The poly(ethylene imine) (PEI) was from BASF, Germany (Polymin SN, a 30% aqueous solution of the polymer). The polysaccharide is a non-ionic cellulose ether obtained from Berol Nobel, Sweden. It has an average molecular weight of 80 000 g mol^{-1} and is prepared by reaction with ethylene oxide and ethyl chloride to a cloud point of around 37°C. The degree of substitution of ethyl groups and the molar substitution of oxyethylene groups, calculated per anhydroglucose unit, are 1.4 and 0.9 respectively. The solid surface is polystyrene microtiter plates.

Reactive PEG plates

The polystyrene plates were washed with ethanol under ultrasonication for 3 min. They were then treated with 2 g l^{-1} potassium permanganate in concentrated sulfuric acid for 30 s at room temperature to induce negative charges on the surface [13]. The plate obtained is referred to as an activated plate. A PEI–PEG adduct was prepared by adding 0.024 g of a 30% solution of PEI to a solution of 1.0 g PEG epoxide in 9 ml of a 0.05 *M* carbonate buffer, pH 9.5. The mixture was kept at 45°C for 3 h under stirring and was then brought into contact with an activated plate. After 2 h at 40°C, the plate was thoroughly rinsed with distilled water and dried to avoid hydrolysis of the remaining PEG epoxide groups. The plate obtained is referred to as a reactive PEG epoxide plate for random coupling. For site-specific coupling, 100 µl of 0.1 *M* adipic dihydrazide dissolved in 0.05 *M* Na_2CO_3 were added to epoxide functionalized wells. After 16 h at 4°C the plates were extensively washed with deionized water.

Reactive polysaccharide plates

The plates were washed with ethanol as above. A solution of 1% polysaccharide in distilled water was brought into contact with the plate and left for 1 h at room temperature. The plate was then thoroughly rinsed with distilled water and dried. Immediately prior to use, aldehyde groups were generated by treating the polysaccharide surface with 1% sodium periodate for 1 h at room temperature. The plate so obtained is referred to as a reactive polysaccharide plate.

Covalent coupling of proteins and glycoproteins on non-hydrophilized gelatin-coated and hydrophilized microtiter plates

The MAb 81D1c2 directed against the isopeptide N^{ε}(γ-glutamyl)lysine had been developed in the Laboratoire d'Immunochimie. Full details of its preparation, reactivity and selectivity have previously been described [14].

For random coupling of IgG and influenza virus on non-hydrophilized and gelatin-coated plates, 100 µl of a PBS solution of IgG or influenza virus (120 ng per well) were introduced into the wells of the polystyrene–NSE plates. Evaluation of adsorption was carried out under the same conditions but in wells in which the carboxyl group had not been activated. After 4 h at 4°C, the plates were washed with a buffer containing 0.14 *M* NaCl, 0.1% Tween, 0.05 *M* Tris adjusted to pH 8.1 with citric acid (TCT).

For random coupling of IgG and HTLV1 on hydrophilized microtiter plates, protein was dispersed in a microemulsion according to the procedure described by Bergström and Holmberg [9]. This was done to increase the amount of protein immobilized on the hydrophilized surface. The microemulsion was composed of 10 wt% aqueous phase, 10 wt% AOT and 80 wt% isooctane. For one plate, 10 ml of microemulsion containing protein at 10 µg ml^{-1} (final concentration) were obtained as follows: 100 µg of protein was diluted in 50 m*M* carbonate buffer of pH 9.2. To this aqueous phase, 7.2 g of a solution containing 800 mg AOT solubilized in 6.4 g isooctane were added. This mixture was vortexed for 15 min to obtain a clear microemulsion. Portions of 100 µl of the microemulsion were added to each well containing the epoxide functionality. After incubation for 2 h at 37°C the plates were rinsed extensively with TCT and finally with deionized water. Evaluation of adsorption was carried out by adding

protein in microemulsion to wells which had been pretreated for 16 h with a solution of 0.1 *M* NaOH in order to hydrolyze epoxide groups. Before use in immunoassay, the plates were rehydrated for 16 h with PBS.

For site-specific coupling of IgG on non-hydrophilized gelatin-coated plates and on hydrophilized plates, IgG was oxidized using sodium periodate as previously described by Quash et al. [1]. Portions of 100 µl of oxidized IgG diluted in 0.05 *M* phosphate buffer of pH 6, 0.14 *M* NaCl were added to wells of non-hydrophilized gelatin-coated plates and to those of hydrophilized plates both of which had been derivatized with hydrazide. Evaluation of adsorption on hydrazide plates was carried out by adding non-oxidized IgG to the wells under the conditions described above. After 1 h at 4°C and washing with TCT, the plates were used for immunoassays.

Adsorption of rabbit IgG anti-fibrinogen on hydrophilized and non-hydrophilized plates after successive freeze and thaw cycles

The polystyrene plates were washed with ethanol and treated with a 1% solution of polysaccharide in distilled water for 1 h at room temperature. After thorough rinsing with water, the hydrophilized and non-hydrophilized plates were incubated with a 2 mg ml^{-1} solution of rabbit anti-fibrinogen IgG that had been subjected to freeze and thaw cycles. Adsorption of antibody was measured by standard enzyme-linked immunosorbent assay (ELISA) using peroxidase conjugated swine anti-rabbit IgG and reading absorbance at 490 nm. The same series of experiments was also performed on non-hydrophilized polystyrene plates.

Preparation of gelatin-coated and hydrophilized plates with a bound hapten (lipoic acid)

The preparation of plates with covalently bound lipoic acid has been described in detail (French patent 2664904, 24 January 1992). The plates were washed with TCT before use.

Immunoassay procedures

To determine the reactivity of rabbit IgG as an antigen, 100 µl of peroxidase labeled swine anti-rabbit IgG diluted 1/1000 in PBS containing 0.1% Tween 20 (PBST) were added to the wells with IgG covalently coupled (assay) or without IgG (control). After 1 h at 37°C, the plates were washed and peroxidase activity was measured using a solution containing 1 mg ml^{-1} *o*-phenylenediamine and 5 m*M* H_2O_2 in 0.1 *M* citrate buffer of pH 4.5. Optical density measurements were carried out at 450 nm on a Titertek Multiskan MCC/340 microreader.

To determine the reactivity of rabbit anti-fibrinogen IgG as an antibody, 100 µl of human fibrinogen (10 µg ml^{-1} for hydrophilized plates and 1 µg ml^{-1} for non-hydrophilized plates in PBST) were added to the wells with randomly and site-specifically bound rabbit anti-fibrinogen IgG. Wells without IgG were used to measure the adsorption of fibrinogen. The plates were incubated for 1 h at 37°C and then washed with TCT. The fibrinogen bound to the wells by adsorption and by reaction with the rabbit anti-fibrinogen IgG was detected using 100 µl of peroxidase labeled rabbit anti-fibrinogen diluted 1/2000 in PBST. After 1 h at 37°C, the plates were washed and the peroxidase activity was measured as described above.

To determine the reactivity of monoclonal Ab 81D1c2 with D-dimer, dilutions of purified D-dimer (1 µg ml^{-1}), normal human plasma (1/100) and normal human plasma (1/100) spiked with D-dimer to a final concentration of 1 µg ml^{-1} were all made in PBST. Portions of 100 µl of each preparation were added to the wells with immobilized MAb (81D1c2). After 1 h at 37°C, the plates were washed with TCT. The D-dimer bound to the wells via the MAb was detected using 100 µl of peroxidase labeled anti-fibrinogen IgG diluted 1/2000 in PBST. After 1 h at 37°C, the plates were washed and peroxidase activity was measured as described above.

To determine lipoic acid Ab titers, 100 µl of the dilutions (1/100) of serum in PBST were incubated

for 1 h at 37°C in wells containing covalently bound hapten (assay wells) and in wells without hapten (control wells). After three washings with TCT, 100 µl of alkaline phosphatase labeled goat anti-human IgG or IgM diluted 1/2000 in PBST were added to each well. After three washings with TCT, the alkaline phosphatase activity was measured by the addition of *p*-nitrophenyl phosphate at 2 mg ml^{-1}, in solution in 1 *M* diethanolamine buffer of pH 9.8 and 0.05 *M* $MgCl_2$. Optical density measurements were carried out at 405 nm on a Titertek Multiskan MCC/340 microreader.

To determine the free Ab and immune complex (IC) bound Ab against HTLV1 in human sera, the technique used for measuring Ab titers previously described by Thomas et al. [15] was modified as follows.

Buffers. Buffers having the following compositions were used: buffer A for IC dissociation consisted of 0.01 *M* glycine and 0.28 *M* NaCl adjusted to pH 2.25 with 1 *N* HCl; buffer B for IC association consisted of 0.05 *M* veronal and 0.2 vol.% Tween 20 adjusted to pH 7.8 with 1 *N* HCl; buffer C (diluting buffer) was obtained by mixing 1 volume of buffer A with 1 volume of buffer B to give a final pH of 7.1.

Incubation of non-dissociated and dissociated sera. For measuring free Ab, serum was serially diluted in buffer C in the wells of the plate to which HTLV1 had been covalently bound. The plate was incubated for 2 h at 37°C.

For measuring free and IC bound Ab, 10 µl of serum were diluted 1/50 in a polypropylene tube containing 490 µl of buffer A. After incubation at 4°C for 15 min, 100 µl of the acid diluted serum were added to the wells of a plate with covalently coupled HTLV1 and to which 100 µl of buffer B had previously been added. After thorough mixing, 100 µl of this sample were serially diluted in buffer C in adjacent wells and the plate was incubated for 2 h at 37°C. At the end of this period, plates were washed with TCT. For measuring free Ab or free and bound Ab linked to the antigen in the wells, 100 µl sheep alkaline phosphatase labeled anti-human IgG diluted 1/2000 in PBST were added to the wells and the plates were incubated for 1 h at 37°C. After three washings with TCT, alkaline phosphatase activity was measured by adding the solution of *p*-nitrophenyl phosphate. Optical density measurements were carried out at 405 nm on a Titertek Multiskan MCC/340 microreader.

For free antibody, sera were considered positive when the optical density (OD) values for a 1/100 dilution were greater than or equal to 0.2. For free antibody and IC bound antibody, sera were considered positive when the OD values for a 1/100 dilution were greater than or equal to 0.5.

Results

The hydrophilization procedure

Two types of hydrophilic coating were used, one PEG based and the other polysaccharide based. PEG chains were grafted to a long, cationically charged polymer, PEI, and the PEG–PEI adduct was adsorbed onto the negatively charged plastic surface. Double-layer forces are likely to be responsible for the very strong attraction between the adduct and the surface. The polysaccharide which is a surface-active cellulose ether is adsorbed directly onto a non-polar plastic surface and hydrophobic interactions are believed to account for most of the driving force for binding. Both procedures give excellent results in terms of hydrophilization efficiency, and leaching experiments have shown that the surface hydrophilicity is constant after soaking for 2 h in water at 37°C. From a practical point of view the coatings may therefore be regarded as irreversibly attached.

We have seen earlier that the amount of adsorbed polysaccharide, as well as its conformation on the surface, is governed by the conditions used, such as temperature during the adsorption step and electrolyte concentration of the bulk solution. In principle the higher the temperature and the electrolyte concentration the greater is the

amount of adsorbed polysaccharide. Studies to examine this have not been made in the present work. However, this issue is dealt with in a very recent paper [16].

Determination of the number of active ester groups and acid hydrazide groups per well on non-hydrophilized plates

This was carried out using [^{14}C]-ethanolamine and [^{14}C]-pyruvate for measuring the number of active ester and acid hydrazide groups respectively.

The results given in Table 1 show that under the experimental conditions used, there were 31 nmol ethanolamine and 18 nmol pyruvate fixed per well. To determine the total number of binding sites per well [^{14}C]-ethanolamine and unlabeled ethanolamine, from 10 to 1000 nmol, were added per well. From the reciprocal plot of nanomoles ethanolamine bound vs nanomoles ethanolamine introduced, it was found that the total number of active ester groups per well was 107 nmol (data not shown).

With the reactivity of functionalized groups assured, we examined their capacity for binding macromolecules.

Determination of the amount of coupled or adsorbed macromolecules on non-hydrophilized plates

Antibody

This was investigated using [^{3}H]-propionate-labeled IgG anti-fibrinogen which was either oxidized for hydrazone coupling to polyhydrazidostyrene or left intact for amide coupling to active ester groups on polystyrene.

It is apparent from Table 2 that adsorption accounts for 60% of the amide-coupled IgG and for 80% of the hydrazone-bound IgG. The absolute amount (44 ng) of oxidized IgG adsorbed onto polycarboxystyrene is 3.5 times greater than that found with non-oxidized IgG (12 nmol). One reason for this difference may be the formation of Schiff bases between residual amino groups on the polycarboxystyrene surface and aldehyde groups generated on the IgG by periodate oxidation. No attempts have been made to monitor Schiff base formation by surface analysis methods, e.g. electron spectroscopy for chemical analysis.

To measure the contribution of adsorption to coupling under conditions used for enzyme immunoassays (EIAs) a similar experiment was performed but this time the amount of bound Ab was revealed with the help of a sheep anti-rabbit IgG. It is apparent from Fig. 2 that both maximum coupling and adsorption are achieved with the introduction of 2 μg IgG per well. It should be noted, though, that the antigenicity of coupled IgG as assessed with the sheep anti-rabbit IgG is four times greater than that of adsorbed IgG. Since adsorbed IgG represents 80% of hydrazone-bound IgG (Table 1) the strong antigenicity obtained with the latter may be indicative of a high degree of retention of antigenic sites on hydrazone-bound IgG.

TABLE 1

Number of reactive groups per well

Reactive group in well	Amount of reagent coupled (nmol)	
	Ethanolamine	Pyruvate
Active ester (NSE)	31	—
Acid hydrazide (AH)	—	18

Each well was treated with 51 nmol of reagent ([^{14}C]-ethanolamine or [^{14}C]-pyruvate). The amount fixed was determined by the difference between the amount introduced into each well and the amount remaining in solution after coupling.

TABLE 2

Amount of [^{3}H]-IgG bound to the wells of microtiter plates

Reactive group in well	Amount of bound IgG (ng)	
	Native IgG	Oxidized IgG
None	12	44
Active ester (NSE)	20	—
Acid hydrazide (AH)	—	54

Each well was treated with 1.5 μg [^{3}H]-IgG. The amount fixed was determined as described in Table 1.

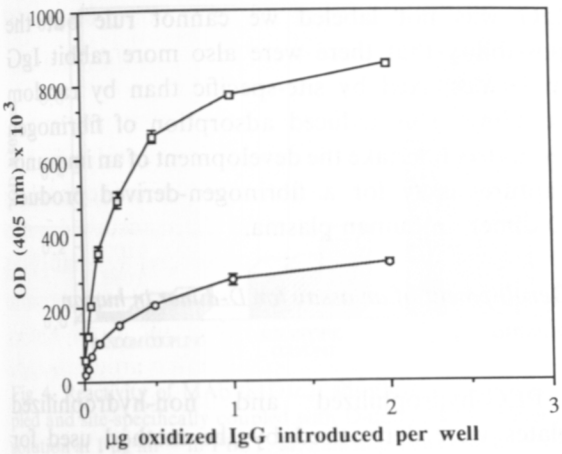

Fig. 2. Fixation to polyhydrazidostyrene of oxidized rabbit IgG by hydrazone bonds (□) and non-oxidized rabbit IgG by adsorption (○). The amount of rabbit IgG fixed was determined with an alkaline phosphatase labeled sheep anti-rabbit IgG. After washing with TCT, alkaline phosphatase activity was measured as described in the legend of Table 5.

Antigen

This was examined using influenza virus as the antigen. It is apparent from Table 3 that also in this case adsorption represents around 80% of bound antigen.

In view of the results described above with both antibodies and antigens, it was clear that the full potential of site-specific coupling of macromolecules could not be correctly appraised on non-hydrophilized polystyrene plates because of concomitant adsorption.

TABLE 3

Amount of influenza virus (B/HK/8/73) bound to wells containing active ester (NSE) groups and to non-reactive wells

Reactive group in well	OD after 10 min
None	0.391
Active ester (NSE)	0.502

The result is expressed as OD (450 nm) after 10 min. Each well was treated with 120 ng virus. The amount of bound virus was determined with a rabbit anti-influenza serum. The amount of bound rabbit IgG was measured with a peroxidase conjugated sheep anti-rabbit IgG. Peroxidase activity was revealed with a solution containing 5 m*M* H_2O_2 and *o*-phenylenediamine (1 mg ml^{-1}) in 0.1 *M* citrate buffer pH 4.5. Values represent the OD at 450 nm after 10 min.

Reactivity of antigens on gelatin-coated plates

In order to diminish adsorption, proteins such as gelatin, bovine serum albumin and fetuin, containing hydrophobic and hydrophilic sites were tested for precoating the wells. It was reasoned that these proteins would adhere by their hydrophobic residues to hydrophobic sites on the well and possibly also form amide bonds between the active esters on the functionalized polystyrene and amino groups on the protein. Once bound, the carboxyl groups on the protein and the residual ones on the wells could then be activated with NHS and carbodiimide for coupling antigens and antibodies.

The results obtained with influenza virus linked by amide bonds to such gelatin-precoated wells are shown in Fig. 3. It is clear that there is a 50% drop in adsorbed influenza virus with no decrease in coupled influenza virus when 0.5 µg gelatin are used for precoating. With 1.0 µg gelatin per well, the OD for the coupled antigen decreases 50% and that for the adsorbed one by 75%.

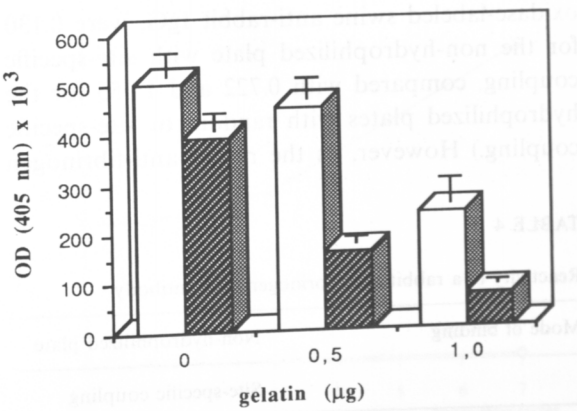

Fig. 3. Effect of precoating polystyrene–NSE wells with different amounts of gelatin on randomly coupled (□) and adsorbed (▨) influenza virus. The amount of bound influenza virus was determined with rabbit anti-influenza serum. The amount of bound rabbit IgG was measured with a peroxidase conjugated sheep anti-rabbit IgG. Peroxidase activity was revealed as described in the legend of Table 3.

Comparison of the adsorption of fibrinogen to non-hydrophilized and hydrophilized plates with covalently bound anti-fibrinogen

Fibrinogen is notorious for its capacity to adsorb onto plastic surfaces. It was therefore an ideal model to assess whether hydrophilization would in fact bring about reduced adsorption. Accordingly, 1 μg rabbit anti-fibrinogen IgG was added to wells of non-hydrophilized plates. In the case of PEG epoxide hydrophilized plates, 1 μg IgG was added directly to the wells when random coupling was carried out. For site-specific coupling the epoxide groups were first derivatized with adipic dihydrazide, as described above.

From the results presented in Table 4, it is apparent that the ratio of adsorbed to coupled protein is reduced fourfold on hydrophilized plates in the case of randomly coupled IgG and 15-fold in the case of site-specifically coupled IgG. The values also indicate that fibrinogen adsorption was less on hydrazone-coupled than on randomly coupled IgG.

In favor of this interpretation is the greater availability of antigen sites on the hydrazone-bound than on randomly bound IgG to the sheep anti-rabbit IgG. (OD values, determined with peroxidase-labeled swine anti-rabbit IgG, were 0.130 for the non-hydrophilized plate with site-specific coupling, compared with 0.722 and 1.055 for the hydrophilized plates with random or site-specific coupling.) However, as the rabbit anti-fibrinogen IgG was not labeled we cannot rule out the possibility that there were also more rabbit IgG molecules fixed by site-specific than by random coupling. This reduced adsorption of fibrinogen led us to undertake the development of an immunocapture assay for a fibrinogen-derived product, D-dimer, in human plasma.

Development of an assay for D-dimer in human plasma

PEG-hydrophilized and non-hydrophilized plates were derivatized by the method used for rabbit IgG in the preceding section, but this time the capture antibody used was an MAb 81D1c2 directed against the $N^{\varepsilon}(\gamma$-glutamyl)lysine isopeptide which is present on reticulated fibrin (D-dimer) but not on monomeric fibrin. The preparation, characterization and specificity of this MAb have previously been described [14]. As a peroxidase labeled rabbit anti-human fibrinogen serum was used to reveal the presence of any bound D-dimer, it also permitted us to assess any non-specific binding to the plates of fibrinogen present in the human plasma samples without added D-dimer.

The results (Fig. 4) show quite clearly that on the plates onto which the MAb was adsorbed the OD value obtained with the plasma alone is greater than that found either with D-dimer in PBS or with the same plasma spiked with D-dimer. This result in itself shows that there is extensive non-specific binding of fibrinogen to non-hydrophilized

TABLE 4

Reactivity of a rabbit anti-fibrinogen IgG antibody

Mode of binding	Non-hydrophilized plate	Hydrophilized PEG treated plate	
	Site-specific coupling	Random coupling	Site-specific coupling
Adsorbed	0.098	0.125	0.057
Covalently coupled	0.157	0.847	1.504
Adsorbed/coupled	0.620	0.140	0.040

Each well was coated with 1 μg rabbit IgG and reactivity determined by the addition of 0.1 μg and 1 μg fibrinogen to non-hydrophilized and hydrophilized plates respectively. The amount of fibrinogen present on the plates was measured with a peroxidase labeled rabbit anti-fibrinogen antiserum. Peroxidase activity was measured as described in Table 3.

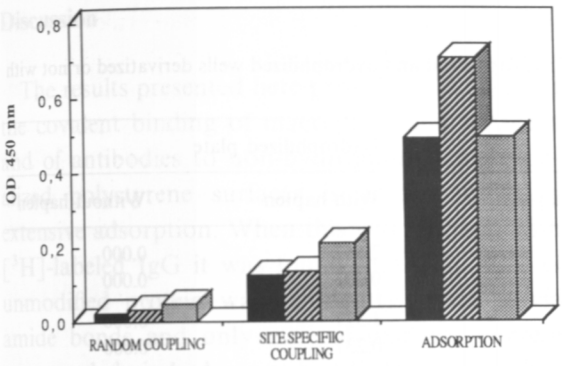

Fig. 4. Reactivity of MAb (81D1c2) adsorbed, randomly coupled and site-specifically coupled with D-dimer: ■, D-dimer in solution at 1 μg ml^{-1} in PBST; ▨, human plasma diluted 1/100 in PBST; ▩, human plasma spiked with D-dimer (1 μg ml^{-1}) diluted 1/100 in PBST. The amount of bound D-dimer was revealed with a peroxidase conjugated rabbit anti-fibrinogen serum. Peroxidase activity was measured as described in the legend of Table 3.

plates. With hydrophilized plates, however, OD values for both site-specifically and randomly coupled antibodies are much lower, with differences becoming apparent between the plasma with and without D-dimer. It should also be noted that the antibodies which were site-specifically coupled via hydrazone bonds show twofold greater reactivity than those which were randomly coupled. These combined results provided good evidence on the one hand for the elimination of non-specific adsorption of fibrinogen on hydrophilized surfaces, and on the other hand for the increased reactivity of Ab bound via the sugar residues on their Fc portion.

These findings therefore provided the impetus to examine whether this type of plate would also allow us to eliminate the problem of non-specific adsorption of immunoglobulins which frequently takes place on non-hydrophilized plates when assays are performed on sera which have been frozen and thawed on several occasions.

Adsorption of antibodies after successive freeze and thaw cycles

For this experiment, the rabbit anti-fibrinogen serum was subjected to up to seven freeze and thaw cycles as described in the Methods section and its adsorption after one, three and seven cycles was compared on non-hydrophilized and on polysaccharide-hydrophilized plates.

Figure 5 shows quite clearly that no adsorption takes place on hydrophilized plates regardless of the number of temperature cycles the protein had undergone. On non-hydrophilized plates, OD values of up to 1.0 are obtained.

This result, with artificially induced Ig aggregates, prompted us to verify whether similar results would be obtained with natural Ig aggregates which are often found in the sera of patients with autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis.

Determination of the titers of antibodies reacting with a hapten: lipoic acid in the sera of patients

This was investigated using lipoic acid which had been covalently bound to polysaccharide-covered plates. It is apparent from Table 5 that for non-hydrophilized plates, there is marked adsorption of IgM onto the plates without lipoic acid. For serum B, this adsorption produces an OD which is even greater than that found for lipoic

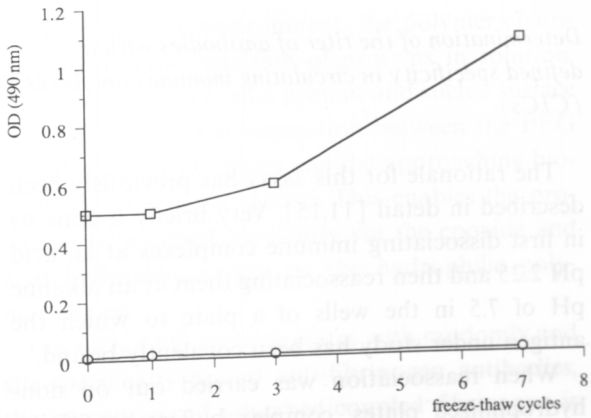

Fig. 5. Adsorption of rabbit anti-fibrinogen IgG after successive freeze and thaw cycles on non-hydrophilized (□) and on polysaccharide-coated microtiter plates (○). Rabbit IgG adhering to the plates was measured with peroxidase conjugated swine anti-rabbit IgG. Peroxidase activity was measured as described in the legend of Table 3.

TABLE 5

Reactivity of immunoglobulins (IgG and IgM) in human sera with non-hydrophilized and hydrophilized wells derivatized or not with lipoic acid

Serum		Non-hydrophilized plate		Hydrophilized plate	
		With hapten	Without hapten	With hapten	Without hapten
A	IgG	0.905	0.183	2.567	0.000
	IgM	2.616	0.314	0.404	0.000
B	IgG	1.331	0.521	1.377	0.000
	IgM	0.236	0.499	0.237	0.000
C	IgG	2.167	0.890	3.233	0.000
	IgM	3.477	1.969	1.183	0.000

The amount of bound Ig was determined with the help of an alkaline phosphatase labeled goat anti-human IgG (γ chain specific) or IgM (μ chain specific). Alkaline phosphatase activity was revealed with a solution containing *p*-nitrophenyl phosphate (2 mg ml^{-1}) in 1.0 *M* diethanolamine buffer pH 9.8, 0.5 m*M* $MgCl_2$. Values represent the OD at 405 nm after 20 min.

acid coated wells. In the case of IgG, non-specific adsorption, though less, is by no means negligible and varies from one serum to the next.

On the hydrophilized plates, the non-specific adsorption of both IgG and IgM is completely eliminated. The results obtained with these three sera are used to illustrate this finding, but no non-specific adsorption of IgG has been observed on over 58 sera examined to date on polysaccharide-hydrophilized plates.

Determination of the titer of antibodies with a defined specificity in circulating immune complexes (CICs)

The rationale for this assay has previously been described in detail [11,15]. Very briefly it consists in first dissociating immune complexes at an acid pH 2.25 and then reassociating them at an alkaline pH of 7.5 in the wells of a plate to which the antigen under study has been covalently bound.

When reassociation was carried out on non-hydrophilized plates, complex buffers containing 10% calf serum and 10% glycerol had to be used to prevent extensive non-specific adsorption of acid-treated Ig to the wells.

In view of the efficacy of PEG- and polysaccharide-hydrophilized plates in eliminating both natural and induced non-specific adsorption of Ig, a series of experiments was undertaken to determine whether the assay for Ab of a defined specificity in CICs could be performed in a simple way on hydrophilized plates.

To investigate this, plates with randomly coupled HTLV1 were used and sera were obtained from patients with clinically confirmed HTLV1 infection. It is apparent (Table 6) that evidence for anti-HTLV1 Ab in CICs was obtained with both seropositive and seronegative sera.

TABLE 6

Determination of the HTLV1 antibody status of 34 human sera by CELIA

Serum Ab status	Number	Percentage positive sera
Free Ab present	17	50
Free Ab absent	17	
Free and IC-bound Ab present	25	74
Free and IC-bound Ab absent	9	

1 μg HTLV1 antigen was introduced into each well of the PEG-hydrophilized plates. For measuring free Ab, diluted sera were put into contact directly with the bound HTLV1. For measuring IC-bound antibody, sera were first dissociated in the buffer A (pH 2.25) and then reassociated in the presence of the alkaline buffer B (pH 7.8) in wells to which HTLV1 had previously been coupled. Bound IgG was measured as described in Table 5.

Discussion

The results presented here provide evidence that the covalent binding of macromolecular antigens and of antibodies to non-hydrophilized functionalized polystyrene surfaces is accompanied by extensive adsorption. When this was assessed with [^{3}H]-labeled IgG it was found that only 40% of unmodified protein was covalently attached via amide bonds and only 20% of oxidized protein was coupled via hydrazone bonds. However, when the amount of immobilized IgG was measured with an alkaline phosphatase sheep anti-rabbit IgG, the ratio of adsorbed to hydrazone-bound IgG was 1:3. It should be noted though, that the reactivity of both the adsorbed and hydrazone-bound IgG towards the anti-rabbit conjugate is really a measure of the availability of antigenic sites. Hence the decrease in availability of these sites on the adsorbed IgG compared to those on the hydrazone-bound IgG could indicate either that the adsorbed IgG has undergone distortion or that the hydrazone-bound IgG has retained more of its native configuration. There is some evidence in the literature in favor of the modification of antigenic sites on adsorption. Indeed, it has been shown that MAbs selected on the basis of their reactivity with adsorbed fibrin were unable to recognize native fibrin in solution [8]. It would therefore seem reasonable to conclude that site-specific coupling, even on non-hydrophilized plates, provides at least one advantage over adsorption in that it may decrease conformational distortions of the bound IgG.

In an attempt to reduce adsorption, non-hydrophilized functionalized plates with activated esters were precoated with readily available proteins such as gelatin and BSA. It was reasoned that in addition to the hydrophobic bonds between the gelatin and the polystyrene, the activated ester groups on the plates would further strengthen the binding of the gelatin to the plate by amide bonds. This approach, using 0.5 μg gelatin per well, brought about some improvement but did not eliminate the problem of non-specific adsorption. As neither the use of fetuin and BSA nor the increase in precoating time brought about any further improvements, polystyrene surfaces grafted, not with charged proteins, but with non-charged hydrophilic polymer chains were used as the solid support. Covalent coupling was carried out in microemulsion via the epoxide groups present on the PEG layer and via the carbonyl groups generated on the polysaccharide layer after mild periodate oxidation.

Carrying out the immobilization reaction in a water-in-oil microemulsion of low water content turned out to be an efficient method to bind proteins to the grafted polymer chains, as we have seen before [9]. Microemulsions having aliphatic hydrocarbons as their main components are non-solvents for the hydrophilic polymers used for grafting, i.e. PEG and polysaccharide. Assuming that the composition of the microemulsion at the surface is similar to that in the bulk (which is not self-evident since microemulsions are microheterogeneous, consisting of domains of hydrocarbon and water), the hydrophilic chains grafted to the surface will not reach out into the liquid phase but will form a compressed surface layer. This behavior has recently been demonstrated by ellipsometry [17]. In such an environment, the polymer chains will not induce repulsion of proteins in solution. Close contact between a protein and such a surface is possible since the interaction between the PEG or polysaccharide chains and the approaching biomolecule may be attractive. This enables the protein to be coupled covalently via the epoxide and acid hydrazide groups on the hydrophilic polymer chains.

On such PEG epoxide plates with randomly and site-specifically bound anti-fibrinogen antibodies, the percentage of adsorbed/coupled fibrinogen is reduced fourfold and 15-fold respectively compared with that on non-hydrophilized plates with randomly and site-specifically coupled antibodies. Here again the availability of antigenic sites on the immobilized antibodies is less on non-

hydrophilized than on hydrophilized plates, and even on the latter, randomly bound antibodies exhibit less reactivity than those which were site-specifically bound. As radiolabeled antibodies were not used in this experiment the amount of antibody bound to the wells is not precisely known. Hence the choice between altered antigenicity on adsorption and decreased quantity of antibody will have to await further experimentation.

The elimination of the problem of non-specific adsorption of fibrinogen on hydrophilized plates permitted us to develop a specific assay for nanogram quantities of D-dimer in the presence of milligram quantities of fibrinogen using this same anti-fibrinogen antiserum. Compared with other assays described in the literature, this one presents two advantages. Firstly, the MAb used is directed against the epitope N^{ε}(γ-glutamyl)lysine which is characteristic of cross-linked and not of monomeric fibrin. Secondly, the necessity for diluting the plasma to 1/1000 in order to reduce non-specific adsorption of plasma proteins, a step which reduces the sensitivity of the test, was obviated on hydrophilized plates. It should be noted that it is only with site-specifically coupled MAbs that a difference was seen between plasma alone and D-dimer-spiked plasma.

Further investigations are being undertaken to see whether the measurement of other minor analytes in a mixture with major components, as is often required in both human and veterinary medicine and in the food industry, can be achieved by combining site-specifically coupled MAbs of defined specificity and hydrophilized plates.

Even at this early stage of development, the combined technology of hydrophilization and site-specific coupling has had a positive impact on the performance of serological assays on retrospective serum samples which tend to form aggregates of Ig after freezing and thawing. This was not only verified with artificially formed aggregates (Fig. 5) but further confirmed on over 50 serum samples with naturally occurring Ig aggregates all of which were obtained from autoimmune patients. It should therefore facilitate epidemiological studies on samples from serum banks.

One serodiagnostic procedure to which this combined technology is particularly applicable concerns the measurement of antibodies of a defined specificity in CICs. There exists a plethora of routine methods for measuring CIC levels regardless of their antigenic specificity. These are usually adaptations of the classical complement fixation test in which immobilized complement factor C1q [18] or radiolabeled complement factor C1q [19] is used, depending on the sensitivity to be attained. The assay described for measuring CIC-bound antibodies of a defined specificity requires so much preliminary work-up of the sample that its adoption on a routine basis is precluded [20]. The acid dissociation and alkaline reassociation method we have previously described [15] can be done routinely and does give reliable results. However, with certain sera the acid dissociation step induces such extensive non-specific adsorption of Ig that no differences are observed when the sera are subsequently reassociated in the presence of antigen covalently bound to the wells of non-hydrophilized plates. The use of hydrophilized plates with covalently bound antigens has considerably reduced this problem. In the survey carried out for CIC-bound anti-HTLV1 antibodies, unambiguous evidence was obtained for their presence even in sera with no free antibodies. Since this method uses the same conjugates and equipment as classical enzyme immunoassays, it could be rapidly and easily implemented when screening individuals suspected of harboring evolutive viral, bacterial and parasitic infections.

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