J. Clin. Chem. Clin. Biochem.
Vol. 21, 1983, pp. 789–797

Immobilisation of Antibodies and Antigens on Macro Solid Phases – A Comparison Between Adsorptive and Covalent Binding

A critical study of macro solid phases for use in immunoassay systems, Part I

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(Received March 29/August 8, 1983)

Summary: This article describes a comparison between adsorption and covalent binding of protein-conjugates (bovine serum albumin-diazoluminol), antibodies (sheep anti-human thyrotropin) and haptens ([^{125}]thyroxine) to polystyrene, nylon and glass balls.

Different combinations of adsorption and covalent binding were used; these varying with the solid phase in question. Polystyrene balls were preactivated by adsorption of a copolymer of phenylalanine and lysine (poly phe-lys) from aqueous solution. The treated balls were then in most cases chemically activated with pentane-1,5-dial before coupling the substance under test. Polystyrene balls were directly chemically activated by nitration followed by reduction and diazotisation.

Nylon balls were either used as received or partially hydrolysed with either hydrochloric acid or sodium hydroxide before further use. Chemical activation was carried out using either carbodiimides or pentane-1,5-dial, the latter proving to be unsuitable because of cross-linking between amino groups on the ball surface. Glass balls were coated with organofunctional aminosilanes followed by chemical activation with pentane-1,5-dial. The best results were obtained using nylon balls activated with carbodiimides and with polystyrene balls coated with poly phe-lys and chemically activated with pentane-1,5-dial. Glass balls proved to be unsuitable as the coating precision was poor under laboratory conditions.

Immobilisierung von Antikörpern und Antigenen an Makro-Festphasen – Ein Vergleich zwischen adsorptiver und kovalenter Bindung

Eine kritische Untersuchung von Makro-Festphasen zum Gebrauch in Immunoassay-Systemen, Teil I

Zusammenfassung: Es wird die adsorptive bzw. kovalente Kopplung von Proteinkonjugaten (Rinderserumalbumin-Diazoluminol), Antikörpern (Schaf anti-Humanthyrotropin) sowie Haptenen ([^{125}I]Thyroxin) an Polystyrol-, Nylon- und Glaskugeln beschrieben. Je nach Festphase wurden unterschiedliche Kombinationen adsorptiver und/oder chemischer Reaktionen angewendet. An Polystyrolkugeln wurde ein Co-polymer aus Phenylalanin/Lysin (Poly phe-lys) adsorbiert. Diese so „vorbehandelten" Kugeln wurden entweder zur weiteren Adsorption der Testsubstanz verwendet, oder mit Pentan-1,5-dial chemisch aktiviert und die Testsubstanz kovalent gebunden. Eine direkte chemische Aktivierung von Polystyrol wurde mittels Nitrierung, Reduktion und anschließender Diazotierung durchgeführt.

Nylonkugeln wurden sowohl unbehandelt als auch nach saurer bzw. alkalischer Oberflächenhydrolyse verwendet, wobei sich die letzteren als unbrauchbar erwiesen. Zur Aktivierung der Nylonkugeln wurde ein Carbodiimid eingesetzt, über das anschließend die Testsubstanz gekoppelt wurde. Eine Pentan-1,5-dial-Aktivierung scheiterte aufgrund von Quervernetzungen mit den aktiven Aminogruppen an der Oberfläche.

Glaskugeln wurden mit Aminoalkylsilanen beschichtet und anschließend mit Pentan-1,5-dial aktiviert.

Die besten Kopplungsergebnisse wurden mit Polystyrolkugeln erreicht, die mit Poly phe-lys und anschließend mit Pentan-1,5-dial aktiviert wurden. Als brauchbar erwiesen sich auch mit Carbodiimid aktivierte Nylonkugeln.

Glaskugeln konnten wegen der schlechten Präzision der Beschichtung unter Laborbedingungen nicht eingesetzt werden.

Introduction

Since *Catt & Tregear* first described the adsorption of antibodies onto plastic tubes (1, 2) many commercial kit producers have adopted this method or variations thereof, in order to simplify methodology.

There are, however, several disadvantages of this adsorptive technique, some of which are described here. The adsorption of protein molecules, in this case antibody molecules, is based upon hydrophobic interactions and *Van der Waal*'s forces, which may not be strong enough to resist the incubation and wash steps included in assay procedures, especially when the latter involve the use of non-ionic detergents such as Nonidet, Tween and Brij. Up to 70% of the adsorbed antibody can be removed from the surface in such steps (3). At the same time, the precision and sensitivity of the assay is negatively influenced (3).

In other cases, the antibody in question cannot be adsorbed to the surface and retain its immunoreactivity (4). Adsorption of antibodies and antigens onto ion-exchangers is no alternative, as such electrostatic binding is highly susceptible to pH and salt-concentration changes, resulting in a high unspecific binding in addition to the loss in sensitivity and precision mentioned above (5) Covalent coupling of molecules to microparticles such as cellulose, glass or nylon leads to an increase in stability, but does not alleviate the need for centrifugation. In order to improve the ease of handling in assays already published from this Department (6—8), a preliminary study of solid-phases was carried out to test their suitability for immobilisation of reaction partners (4, 9). The results here presented enlarge upon this study, and present in detail the methods of immobilising molecules on macro-solid-phases, here balls of 6—7 mm diameter made of glass, polystyrene or nylon. Balls were chosen to allow for a "tube-transfer" step before measurement, to reduce the unspecific effects contributed by the vessel wall. The use of coated balls allows for easy automation or semi-automation of the method, as already seen in certain commercial kits (Abbott, Hepatitis and Tumour diagnostic radio- and enzymeimmunoassays). The aim of this study, presented in three parts, is to demonstrate that it is possible to produce reactants coupled to polystyrene balls which give rise to assays with excellent precision, a point often not seen using purely adsorptive techniques.

Materials and Methods

Materials

Soda-glass balls (7 mm diameter) were purchased from Sternkopf, Lübeck, Germany. Polystyrene and Nylon balls (6.4 mm diameter) were obtained either from Precision Plastic Ball Co., Chicago, Illinois, U.S.A. or from Spherotech Kugeln, Fulda, Germany.

Aminoalkylsilanes were donated by Wacker-Chemie, Munich, Germany.

Ultrogel A6 was purchased from LKB, Munich.

Luminol was obtained either from EGA-Chemie, Steinheim, Germany or Sigma, Munich.

Copolymers of phenylalanine and lysine (1:1) were purchased from Miles-Yeda, Rehovot, Israel and from Sigma. These had a relative molecular mass (M_r) between 30 and 40 kD.

Radioactive thyroxine, thyrotropin and thyroxine binding globulin were donations of Henning Berlin.

Pentane-1,5-dial was purchased from Sigma (Catalog No 6-5882) as a 25% solution.

Other substances used were purchased from Merck, Darmstadt, Germany, EGA-Chemie or Sigma.

Methods

Adsorptive coating of solid phases

Adsorptive procedures were carried out in salt-free non-buffered aqueous solutions as already described (4). [^{125}I]thyroxine was chosen as hapten, bovine serum albumin-diazoluminol as protein and a sheep anti-human thyrotropin IgG-fraction as an antibody to study adsorption to the different solid phases. These substances were quantified using established physico-chemical methods. They were also used in the covalent binding studies.

Silanisation of glass balls using organofunctional aminosilanes, followed by activation with pentane-1,5-dial

Glass balls were activated according to the methods described by *Weetall* (10) using aminoalkyl silanes. A solution of pure silane (10 ml) was made up in distilled water (190 ml) and added to a two-necked round-bottomed flask (500 ml), cooled in ice and fitted with a water-cooled reflux condenser. The pH was adjusted with 7 mol/l HCl to between 3 and 4. The silanes used were coded GF-90 and GF-91 which had the following structural formulae:

$NH_2(CH_2)_2-O-(CH_2)_3-Si-(O(CH_2)_2NH_2)_2$ (GF-90)

and

$NH_2(CH_2)_2-NH-(CH_2)_3-Si-(OCH_3)_3$ (GF-91).

100 g chromic acid-washed glass balls were added to the flask, which was then heated at 75 °C for 3 h in a fume cupboard with occasional shaking. After washing the balls with distilled water, the silane coating was stabilised by heating at 115 °C in a drying oven for 6 h. The resulting alkylamine coated glass balls were stored in a dessicator at room temperature until use.

The subsequent activation with pentane-1,5-dial was carried out by immersing the balls in a 20 ml/l solution of pentane-1,5-dial in distilled water and allowing them to stand for 30 min at ambient temperature with occasional shaking. The balls were washed twice with 1 l distilled water and once with 500 ml 0.15 mol/l NaCl. The presence of aldehyde groups on the surface of the balls was tested for with *Schiff*'s reagent (*p*-rosaniline reduced with SO_2), as follows: A treated ball was placed in a test-tube and 500 µl *Schiff*'s reagent added. The tube was stoppered and allowed to stand for 10–15 min. The presence of free aldehyde groups was seen as a purple shimmer on the surface of the ball. As a control, a chromic acid treated ball was taken through the same procedure.

After the presence of free aldehyde groups had been demonstrated the coupling of hapten, protein or antibody was carried out. The concentrations and volumes here given are for 100 balls. The coupling solution was prepared by dissolving 500 µg of the molecule to be coupled in 20 ml distilled water and the balls added over a period of 2–3 s. The resulting mixture was lightly shaken and adjusted to an end concentration of 0.05 mol/l phosphate buffer, pH 7.5 using a 0.5 mol/l stock solution. The coupling reaction was allowed to proceed overnight at 4 °C. The resulting *Schiff*'s bases were reduced with $NaBH_4$ or with $NaBH_3CN$ at a concentration of 0.075 mol/l, with a reaction time of 2 h at ambient temperature. If $NaBH_3CN$ was used, the reaction was carried out in a fume cupboard.

Activation of polystyrene balls

a. Adsorption of poly phe-lys with subsequent pentane-1,5-dial activation

A stock solution of poly phe-lys (1 g/l) was made by dissolving 100 mg of the co-polymer in 100 ml distilled water. Solution occurred at 60 °C under stirring and took 30–40 min for the Miles-Yeda compound. The Sigma poly phe-lys dissolved rapidly, solution being attained within 2–3 minutes. The stock solution was stored at 4 °C until use. No evidence of microbial growth was observed for periods up to 4 weeks storage in the refrigerator.

5 ml of poly phe-lys stock solution was made up to 200 ml with distilled water and poured rapidly over 1000 polystyrene balls in a 500 ml conical flask, the resulting mixture being agitated regularly over the first 60 minute period. The flask was allowed to stand at room temperature overnight to complete the coating procedure.

The coated balls were washed once with 500 ml 0.15 mol/l NaCl followed by two washes with 2 × 500 ml distilled water. The activation with pentane-1,5-dial and subsequent coupling of hapten, protein or antibody was identical to that in previous section, except that the amount of sheep anti-human thyrotropin IgG was 2.5 µg/ball instead of 5 µg/ball (see (c) below).

b. Activation via nitration followed by reduction and diazotisation

Amination of polystyrene has already been published (11) and a similar method is described here. The concentrations and volumes are for 1000 balls. 250 ml 10 mol/l HNO_3 were poured over 1000 polystyrene balls in a 500 ml conical flask and heated at 50 °C for 90–120 min, the time depending upon the degree of nitration required. This was seen when a yellow surface remained after washing a ball with copious amounts of distilled water. The nitrated balls were washed with distilled water until the wash fluid had a pH above 2. Sodium dithionite (50 g/l) solution was added until the balls were covered and the flask then allowed to stand for 18–24 h at room temperature. During this time a precipitate of amorphous sulphur formed, at the same time the colour of the balls had changed from golden-yellow to greenish-yellow, confirming that reduction had taken place. The balls were then washed with 4 × 500 ml distilled water and covered with 250 ml 1 mol/l HCl and then stored in an ice-bath. When the solution had reached 3 °C, solid KNO_2 was added to give an and concentration of 2.5 mol/l. The addition of KNO_2 took 20–30 minutes with regular agitation of the flask. The diazotisation was allowed to proceed for 2 h at 0 °C, after which the balls were washed with distilled water until the wash solution remained negative to starch-iodide paper. Coupling of peptides and proteins was carried out using 5 µg substance to be coupled per ball. Diazo-coupling was carried out as follows: 100 diazotised polystyrene balls were added to 25 ml 0.05 mol/l $Na_2B_4O_7$ at pH 9.35 and cooled to 0 °C in an ice-bath. 1 ml of the peptide (500 µg) was added dropwise under continuous agitation over a period of 5–10 min. The coupling was allowed to proceed overnight at 4 °C after which the balls were washed with 1 × 200 ml 0.15 mol/l NaCl followed by 2 × 200 ml distilled water.

c. Preparation of the sheep anti-human thyrotropin IgG and subsequent reaction with [^{125}I]thyrotropin

1 ml of a sheep anti-human thyrotropin serum was precipitated with 2 ml polyethylene glycol (M_r 6000) solution (200 g/l), the precipitate being washed with 1 ml 0.15 mol/l NaCl and then dissolved in 0.5 ml 0.01 mol/l phosphate buffer, pH 8. This was then transferred to a DEAE-cellulose column in a 5 ml syringe and eluted with 0.01 mol/l phosphate buffer, pH 8. The fractions with the maximal binding capacity for [^{125}I]thyrotropin were pooled and the protein concentration determined with the *Lowry* method. The balls were coated as described above, using 2.5 µg protein/ball. The binding of radiolabelled thyrotropin was performed as follows: 50 µl [^{125}I]thyrotropin was added to 200 µl assay buffer (0.05 mol/l Tris-HCl containing 2.5 g/l bovine serum albumin, pH 7.4) and an anti thyrotropin coated ball added. After a 4 h incubation at ambient temperature, the assay mixture was aspirated off, the ball washed with 2 × 1 ml 0.15 mol/l NaCl, transferred to a clean tube and the radioactivity measured. Non-specific binding was tested for in which the [^{125}I]thyrotropin tracer was replaced with [^{125}I]thyroxine binding globulin, the same number of counts being added to these tubes.

Activation of nylon balls

Methods for activation and modification of nylon have been published extensively (12). The nylon balls used in this study were first washed with distilled water followed by 2 mol/l NaCl to remove small particles adhering to the surface.

a. Partial hydrolysis

1000 balls were added to 250 ml 3 mol/l HCl in a 500 ml conical flask, which was then heated to 60 °C for 30 min. After cooling, the balls were washed with 4 × 500 ml distilled water, after which they allowed to stand in 500 ml 0.15 mol/l NaCl for 30 min. The NaCl was changed every 30 minutes until the pH remained constant.

Hydrolysis with NaOH gave rise to difficulties as it was not possible to remove all the NaOH remaining in the pores after reaction. Leakage of alkali was detectable several days after partial hydrolysis with NaOH.

b. Chemical activation of nylon balls

Activation with pentane-1,5-dial was carried out as described above, the subsequent coupling of the substance in question taking place directly after activation.

Activation with N-cyclohexyl-N'-(2-(N-methylmorpholino)-ethyl)-carbodiimide *p*-toluene sulphonate was carried out at pH 4 in a low-ionic strength solution (distilled water adjusted with 0.01 mol/l HCl). The molar ratios of the reactants were calculated so that the relationship between reactants was approximately ball-carboxyl-groups: carbodiimide 1:10. The ligand to be coupled was always in excess. The coupling procedure was as follows: The first reaction between N-cyclohexyl-N'-(2-(N-methylmorpholino)-ethyl)-carbodiimide *p*-toluene sulphonate and nylon balls was carried out by adding the N-cyclohexyl-N'-(2-(N-methylmorpholino)-ethyl)-carbodiimide *p*-toluene sulphonate solution to the balls, keeping the pH between 3.8 and 4.2 with 0.01 mol/l HCl or NaOH over the activation time of 120 min at ambient temperature. The balls were then washed with 2 × 500 ml 0.01 mol/l phosphate buffer, pH 6. A solution of the ligand (5 µg/ball) was added to the balls, the ligand being dissolved in 0.01 mol/l phosphate buffer, pH 6. The second stage of the reaction was allowed to proceed at 4 °C for 24–72 h, after which the balls were washed with 0.15 mol/l NaCl followed by distilled water. The volumes of solutions and reaction vessels were identical to those in (a) above for 1000 balls.

Saturation of unspecific binding sites on the ball surface

After the specific coupling of the substance in question to the ball surface, possible "active" binding sites were blocked by saturation with a protein solution in Tris-HCl buffer. The saturation procedure was the same in all cases, irrespective of the ball material. The balls activated with carbodiimide or pentane-1,5-dial were allowed to stand in a 25 g/l solution of either bovine serum albumin or human transferrin in 0.01 mol/l Tris-HCl pH 7.2 (250 ml/1000 balls), for 24–96 h at 4 °C. In many cases optimal saturation was not achieved until after 96 h.

The balls coated using adsorptive techniques or activated by diazotisation were blocked with bovine serum albumin (25 g/l) dissolved in 0.15 mol/l NaCl, adjusted to pH 7.2 with 0.1 mol/l phosphate buffer.

After saturation, the balls were either stored in 0.05 mol/l Tris – HCl containing 10 g/l bovine serum albumin and 0.1 g/l NaN_3, pH 7.2, or dried under a stream of compressed air and stored in tightly stoppered plastic bottles at 4 °C or room temperature.

Preparation of a bovine serum albumin-diazoluminol conjugate

50 mg luminol (5-amino-2,3-dihydro-1,4-phthalazine dione) (0.35 mmol) were suspended in 10 ml ice-cold 1 mol/l HCl. 60 mg (0.7 mmol) KNO_2 was added under stirring over a 10 minute period, keeping the reaction vessel in an ice-bath. As the reaction proceeded, the green-yellow suspension changed into a clear orange-red solution, which could be stored for up to 72 h at −20 °C before being used, although the best results were obtained with the freshly diazotised luminol.

20 mg bovine serum albumin were dissolved in 10 ml 0.1 mol/l carbonate hydrogencarbonate buffer, pH 9.5 and cooled to 0 °C in ice. 200 µl of the diazotised luminol was added dropwise under continual stirring over 15–20 minutes, keeping the pH between 9 and 9.5 with 1 mol/l K_2CO_3. The reaction was allowed to proceed overnight at 4 °C. Unreacted diazoluminol was removed by passing the mixture over an Ultrogel A6 column (80 × 3 cm) collecting fractions of approximately 2 ml. The elution buffer was 0.01 mol/l phosphate in 0.15 mol/l NaCl, pH 7.4. Around 90% of the bovine serum albumin was eluted in fractions 35–45. The molar substitution rate was detected photometrically (see Instrumentation and Practical Details).

Instrumentation and Practical Details

Radioactivity measurement

Radioactivity measurements were made with a NE 1600 multihead gamma spectrometer (Thorn-EMI/Nuclear Enterprises, Sighthill, GB).

Spectrophotometric measurements

Spectrophotometric determinations were carried out using a Beckman 24 spectrophotometer (Beckman, Munich).

The determination of the molar substitution ratio diazoluminol: bovine serum albumin was carried out using the value $\varepsilon_{347} = 7.1 \times 10^7$ m²/mol for luminol (13). Using this approximation a molar substitution rate of ca. 8:1 was obtained. The lowest detectable amount of conjugate was ca. 3×10^{-10} mol/l or 3×10^{-14} mol/tube using a 100 µl sample.

Chemiluminescence measurement

All measurements were made manually using an LKB 1250 luminometer coupled to a microcomputer (LKB Data Box 1223) with chemiluminescence programme, connected to a Teletype printer (model 43). All equipment was supplied by LKB-Wallac, Turku, SF.

Hydrogen peroxide, used as "start" reagent was dispensed from a programmable constant-velocity dispenser-dilutor (Hamilton-Micro-Lab P, Bonaduz, CH). All metal fittings were replaced by Teflon to eliminate M^{++}-ion catalysis of the chemiluminescent reaction.

The oxidation system used was an alkaline pseudoperoxidase solution. Microperoxidase – MP 11 (Sigma) stock solution was made by dissolving 2 mg in 10 ml 0.05 mol/l Tris-HCl, pH 7.4. This solution was stable at 4 °C in excess of 12 months. Working solutions of microperoxidases were prepared by diluting the stock solution 1:50 with 0.05 mol/l phosphate buffer containing 4 g/l bovine serum albumin pH 7.4.

The reaction system was as follows:

Microperoxidase (working solution)	100 µl
NaOH (2 mol/l)	200 µl

Ball or solution to be measured

Transfer to luminometer and initiate by injecting 120 µl H_2O_2 (100 µl 300 g/kg solution in 10 ml distilled water).

The light signal was integrated over 20 or 30 s, during which time 250 single measurements were made, the integral being given in mV. A pH between 13 and 14 was chosen to slow down the kinetics of the light-generating reaction. A reagent blank was run in which the substance to be measured was replaced by a transferrin-coated ball or by phosphate buffer (0.05 mol/l, pH 7.4).

Results

Binding of [^{125}I]thyroxine to macro solid phases – an example of hapten-binding

Table 1 shows the binding of [^{125}I]thyroxine to the different solid phases. The values given are the bound counts per minute (total activity 1500 counts/min). Values shown are the mean and standard deviations from 10 balls.

Tab. 1. This table shows the binding of radiolabelled thyroxine to the three types of balls together with the effects of different wash solutions. GF 90 and GF 91 are the two aminosilanes. The concentrations of the wash solutions are as follows and are identical for tables 2, 3 and 4.
0.15 mol/l NaCl, 1 ml/l Tween 20 and 1 mol/l KCl. Washing was carried out as explained in the text.
The numbers represent the counts bound (total counts per minute in each case 1500) with the standard deviation. The results are from 10 balls.

	Column 1 16 h incubation at 4 °C				Column 2 16 h incubation at 4 °C + $NaBH_4$				Column 3 32 h incubation at 4 °C
	H_2O wash	NaCl wash	Tween wash	KCl wash	H_2O wash	NaCl wash	Tween wash	KCl wash	NaCl wash
Glass balls									
Adsorption	295 ± 56	287 ± 40	226 ± 74	210 ± 30	–	–	–	–	371 ± 21
Pentanedial	412 ± 20	403 ± 57	275 ± 6	197 ± 22	45 ± 5	51 ± 6	47 ± 12	49 ± 16	459 ± 141
GF 90 + Pentanedial	746 ± 63	742 ± 58	730 ± 20	750 ± 40	685 ± 46	701 ± 87	704 ± 62	671 ± 54	818 ± 56
GF 91 + Pentanedial	885 ± 150	760 ± 150	740 ± 120	646 ± 107	761 ± 91	748 ± 40	773 ± 54	721 ± 98	630 ± 156
Nylon balls									
Adsorption	265 ± 13	264 ± 4	197 ± 2	180 ± 10	–	–	–	–	476 ± 30
Pentanedial	586 ± 60	555 ± 51	460 ± 40	405 ± 40	191 ± 56	234 ± 47	214 ± 20	221 ± 10	683 ± 23
Hydrolysed + Adsorption	1073 ± 132	1051 ± 129	1030 ± 200	1003 ± 101	–	–	–	–	1035 ± 41
Hydrolysed + Pentanedial	1049 ± 72	1064 ± 59	1010 ± 50	945 ± 70	768 ± 17	767 ± 20	763 ± 34	749 ± 28	1042 ± 41
Carbodiimide	574 ± 30	572 ± 28	460 ± 20	396 ± 11	–	–	–	–	755 ± 36
Hydrolysed + Carbodiimide	852 ± 52	860 ± 40	750 ± 114	726 ± 55	–	–	–	–	840 ± 43
Polystyrene balls									
Adsorption	434 ± 22	425 ± 40	204 ± 30	129 ± 52	–	–	–	–	459 ± 40
Pentanedial	528 ± 30	492 ± 16	270 ± 20	198 ± 20	101 ± 59	100 ± 64	120 ± 32	117 ± 16	593 ± 42
Poly phe-lys + Pentanedial	817 ± 26	798 ± 14	640 ± 10	622 ± 2	657 ± 13	653 ± 12	622 ± 14	605 ± 14	874 ± 3
Diazotised	266 ± 40	206 ± 150	190 ± 40	142 ± 31	–	–	–	–	264 ± 20

Column 1 shows the binding after an overnight incubation at 4 °C together with resistance to different wash steps, the latter being standardised as follows: 1 ml of wash solution was dispensed into each tube, after which the tubes were vortexed batchwise for 10 s at a constant speed (Vortex mixer, SMI Serono, Freiburg i. Br., Germany). The wash solution was aspirated off on a vacuum pump with a Pasteur pipette, the ball being transferred to a clean tube before being counted.

Column 2 shows the binding of [^{125}I]thyroxine to pentane-1,5-dial activated balls, the reaction time bing 16 h followed by reduction with $NaBH_4$ (see methods). Column 3 shows the same as column 2, but with a reaction time of 32 h followed by a single NaCl wash and no $NaBH_4$ reduction. The washing and measuring procedure was as above.

Although the ligand is bound covalently and adsorptively to the balls, the difference is seen in the resistance to wash steps and wash solutions. The binding to the chemically activated balls was, as to be expected, higher and more resistant to removal by washing, even when high salt and/or detergent solutions were used. As can be seen from Table 1, the best results were obtained with carbodiimide activated nylon balls. The worst results were from hydrolysed nylon balls followed by the silanised glass balls. Stabilisation of *Schiff*'s bases with $NaBH_4$ made no difference to the results. Balls pre-treated with pentane-1,5-dial alone were unstable to borohydride reduction, all radioactivity being removed from the surface. Prolongation of the second phase of the carbodiimide coupling to 32 h increased the amount of radioactive thyroxine covalently bound to the ball surface.

Binding of [^{125}I]thyrotropin to immobilised sheep anti-human thyrotropin IgG

Table 2 shows the binding of [^{125}I]thyrotropin to the antibody-coated solid phases. The binding is given in terms of fraction radioactivity bound. Again results from 10 balls are shown in each cases. The reaction scheme is shown in Materials and Methods.

Column 1 shows the resistance to NaCl washing, columns 2 and 3 to Tween 20 and 1 mol/l KCl. The results are similar to those in table 1. The high unspecific binding to hydrolysed nylon balls is probably the most interesting result here, demonstrating that pores on the surface are not readily accessible to the wash solutions, at least in the short wash phases used here.

Binding of bovine serum albumin-diazoluminol to the balls

Table 3 shows the binding of bovine serum albumin-diazoluminol in an analogous manner to tables 1 and 2 above. Column 1 shows binding after 16 h reaction, column 2 after 38 h reaction.

The units used are mV · s, i.e. the integral expressed as change in potential at the photomultiplier.

Again the results are similar to those in tables 1 and 2. The stability of the polystyrene ball adsorbed conjugates was studied at greater length, the results being shown in table 4. After treatment with poly phe-lys, the adsorptive capacity was increased, not however, the resistance to detergent and high salt concentration. When poly phe-lys is activated with pentane-1,5-dial, the increase in binding capacity was also present, here however, also the stability to high salt concentrations and detergents in the wash solutions. In fact, the latter combination was adopted for routine use, as can be seen in parts 2 and 3 of this article.

Tab. 2. This shows the fraction of tracer specifically (B_0/T) and non-specifically (UB/T) bound to sheep anti-human thyrotropin coated balls. The specific binding was with ^{125}I-labelled thyrotropin (25000 counts per minute total) and the unspecific binding with ^{125}I-labelled thyroxine binding globulin (25000 counts per minute total). As the latter can only reflect the unspecific binding, only the mean percent binding has been given. Again, as above, the results represent the mean and standard deviation from 10 balls. Carboxynylon was prepared according to *Sundaram* (12).

	Column 1 NaCl wash		Column 2 Tween wash		Column 3 KCl wash	
	B_0/T	UB/T	B_0/T	UB/T	B_0/T	UB/T
Glass balls						
Adsorption	0.13 ± 0.01	0.03	0.12 ± 0.02	0.13	0.12 ± 0.004	0.04
Pentanedial	0.06 ± 0.01	0.03	0.06 ± 0.006	0.03	0.06 ± 0.003	0.03
GF 90 + Pentanedial	0.23 ± 0.04	0.03	0.20 ± 0.02	0.03	0.13 ± 0.05	0.03
GF 91 + Pentanedial	0.08 ± 0.002	0.02	0.05 ± 0.002	0.04	0.07 ± 0.003	0.03
Nylon balls						
Adsorption	0.36 ± 0.006	0.05	0.29 ± 0.0002	0.04	0.23 ± 0.008	0.06
Pentanedial	0.08 ± 0.008	0.04	0.05 ± 0.002	0.04	0.06 ± 0.007	0.04
Hydrolysed + Adsorption	0.56 ± 0.007	0.26	0.49 ± 0.024	0.29	0.48 ± 0.03	0.22
Hydrolysed + Pentanedial	0.64 ± 0.001	0.29	0.58 ± 0.02	0.27	0.57 ± 0.02	0.23
Carbodiimide	0.14 ± 0.006	0.05	0.10 ± 0.01	0.05	0.12 ± 0.002	0.04
Hydrolysed + Carbodiimide	0.56 ± 0.04	0.26	0.52 ± 0.02	0.21	0.50 ± 0.02	0.19
Carboxynylon + Carbodiimide	0.25 ± 0.03	0.04	0.25 ± 0.02	0.04	0.20 ± 0.02	0.05
Polystyrene balls						
Adsorption	0.57 ± 0.02	0.02	0.19 ± 0.007	0.02	0.35 ± 0.006	0.02
Pentanedial	0.11 ± 0.01	0.04	0.06 ± 0.005	0.03	0.12 ± 0.002	0.04
Poly phe-lys + Pentanedial	0.44 ± 0.06	0.04	0.39 ± 0.004	0.03	0.39 ± 0.002	0.05
Diazotised	0.32 ± 0.03	0.04	0.26 ± 0.001	0.03	0.30 ± 0.001	0.03

Tab. 3. This shows the coupling of bovine serum albumin-diazoluminol to the three solid phases. Here the numbers represent the 20 second integral of the light-output curve, the mean and standard deviation from 10 balls being used. Concentrations of wash solutions are as in table 1.

	Column 1			Column 2			Column 3		
	16 h incubation at 4 °C			38 h incubation at 4 °C			16 h incubation at 4 °C + $NaBH_4$		
	NaCl wash	Tween wash	KCl wash	NaCl wash	Tween wash	KCl wash	NaCl wash	Tween wash	KCl wash
Glass balls									
Adsorption	3617 ± 431	321 ± 30	382 ± 109	3260 ± 40	390 ± 20	555 ± 42	–	–	–
Pentanedial	2936 ± 249	233 ± 30	339 ± 96	4082 ± 1400	234 ± 12	393 ± 192	735 ± 200	344 ± 52	648 ± 52
GF 90 + Pentanedial	7805 ± 167	5278 ± 371	4329 ± 214	9899 ± 1122	8599 ± 264	7853 ± 1040	2113 ± 418	3251 ± 1386	2647 ± 546
GF 91 + Pentanedial	16844 ± 11741	7966 ± 748	3259 ± 398	16629 ± 7481	8015 ± 3002	8767 ± 1238	2409 ± 481	3967 ± 1100	1974 ± 1125
Nylon balls									
Adsorption	7903 ± 675	3901 ± 69	4708 ± 689	10733 ± 1088	7352 ± 257	8767 ± 1238	–	–	
Pentanedial	5770 ± 1276	2572 ± 211	2538 ± 449	8840 ± 123	3282 ± 116	5042 ± 399	2455 ± 220	1232 ± 68	1724 ± 91
Hydrolysed + Adsorption	74081 ± 6580	61171 ± 3241	45263 ± 2036	94449 ± 2828	75272 ± 1894	71537 ± 1840	–	–	–
Hydrolysed + Pentanedial	41229 ± 3189	32748 ± 3442	38482 ± 8494	46905 ± 5481	36691 ± 836	36013 ± 694	14172 ± 1790	10016 ± 338	10001 ± 2575
Carbodiimide	15952 ± 378	6265 ± 205	12309 ± 676	20423 ± 884	12932 ± 673	17015 ± 1004	–	–	–
Hydrolysed + Carbodiimide	79364 ± 2690	68255 ± 13744	51093 ± 4858	78855 ± 5966	79136 ± 7075	77045 ± 6385	–	–	–
Polystyrene balls									
Adsorption	2900 ± 578	310 ± 21	593 ± 192	3688 ± 669	513 ± 54	973 ± 109	–	–	–
Pentanedial	3206 ± 608	292 ± 47	674 ± 249	3097 ± 366	344 ± 110	1196 ± 112	1657 ± 196	918 ± 28	1832 ± 98
Poly phe-lys + Pentanedial	14214 ± 490	10293 ± 98	9019 ± 196	15615 ± 254	10223 ± 333	10585 ± 695	8308 ± 786	7709 ± 318	8234 ± 1080
Diazotised	2845 ± 401	2061 ± 66	1556 ± 54	3166 ± 204	2470 ± 36	2170 ± 98	–	–	–

Tab. 4. Here the effects of detergent washing during the activation of polystyrene balls with poly phe-lys and pentanedial are demonstrated. The conjugate used is bovine serum albumin-diazoluminol as in table 3 above. The expression of results is also as in table 3. In all cases, the results are obtained using the poly phenylalanine-lysine from Miles-Yeda.

	Column 1 NaCl wash	Column 2 Tween wash	Column 3 KCl wash
Polystyrene balls			
Adsorption	2900 ± 578	310 ± 21	593 ± 192
Pentanedial	3206 ± 608	292 ± 47	674 ± 249
Poly phe-lys + Adsorption	17331 ± 725	4352 ± 19	4411 ± 392
Poly phe-lys + Pentanedial	14214 ± 490	10293 ± 98	9019 ± 196
Poly phe-lys + Tween wash before Pentanedial activation	7058 ± 392	4019 ± 294	3921 ± 392
Poly phe-lys + Tween wash after Pentanedial activation	10577 ± 392	7058 ± 196	5881 ± 392
Poly phe-lys + Tween wash before and after Pentanedial activation	5931 ± 40	3744 ± 177	3960 ± 332

Discussion

In the experiments described here, no satisfactory results were obtained using glass balls. The actual coupling of materials to glass presented in itself no problem, however, under laboratory conditions the necessary precision was lacking. On the one hand, the silanisation process described by *Weetall* (10) whereby covalent linkage between silane and the silica molecules in the glass are described, must be questioned as the work of *Schmidt* et al. (14) show that the silanisation is mainly due to polymerisation of the silane followed by burning on the coated material at temperatures up to 300 °C. The results of *Schmidt* correspond with observations made during modification of polymers by coating with silanes (15). The use of a coating machine as described by *Schmidt* (14) may very well standardise the procedure so that precisely coated glass balls could be produced, which would give adequate capacity of coupled groups with low unspecific binding. Nylon balls can be used when adsorptive procedures are adopted and where washing with detergent or solutions of high ionic strength can be avoided. Covalent coupling of ligands to nylon can be best effected with carbodiimides, the stability to detergent and high salt concentrations then being given, allowing assays to be performed where such steps are necessary to reduce unspecific binding (16). The activation of nylon with pentane-1,5-dial reduced the binding capacity, possibly due to cross-linkage between adjacent free amino groups.

The most practical and reproducible coating method, and the one which is now in routine use in this laboratory is that using adsorption of poly phe-lys to polystyrene balls followed by activation with pentane-1,5-dial and coupling of the required ligand. The poly phe-lys fulfills two important requisites, firstly the strong hydrophobic interaction between the phenylalanine residues and the polystyrene surface and secondly the introduction of "reactive" amino groups with spacer-arm in the form of the terminal amino group of lysine. The further activation with pentane-1,5-dial gave rise to an eleven membered side-chain spacer.

The assays which have been built up using polystyrene balls treated in this way are described in the third part of this article and involve haptens such as gentamicin and thyroxine, serum proteins such as transferrin and ferritin, as well as hormones such as thyrotropin.

The somewhat extensive amination of polystyrene is possible but cannot be recommended in terms of simplicity, where the above "activation" with poly phe-lys is possible. The poly phe-lys-pentanedial combination alleviates the need for "expert chemical knowledge", and can be carried out in almost any laboratory.

Attempts at a direct acylation of polystyrene using a *Friedel-Craft* reaction had to be abandoned due to the lack of an organic solvent which did not dissolve the polystyrene. Even cyclohexane which is reported not to affect polystyrene (17) dissolved the balls slowly. To conclude, the use of macro solid phases is an important step in automation and simplification of immunoassays. The problem of unacceptable precision due to non- or hardly reproducible coating procedures has deterred many workers from considering such a system. The experimental results described here show that it is possible to optimise solid phase coating so that precision presents no problems. The results presented in the following two parts of this article confirm this optimism.

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