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Surface modification for direct immunoprobes

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Abstract: The modification of glass-type surfaces by several hydrophilic polymers of different molecular masses and functional properties [chitosan, dextran, poly(oxyethylene), poly(ethyleneimine) and poly(acrylamide)] with respect to the application for direct immunoprobes was investigated. Activation of the surface was carried out by silanisation and the polymers were coupled to the surface via amide bonds. The carboxyl derivative of a hapten was attached to the functional groups of the polymers by carbodiimide-activated coupling. As a reference system, the ligand was directly coupled to the silanised surface. Non-specific protein adsorption, specific binding of antibodies and regeneration were monitored by evaluation of reflectance spectra obtained by white light interference at a thin silica layer (*RifS*).

All polymer modified layers showed improved properties compared to those with direct attachment of the hapten. The non-specific adsorption was reduced to 5–50 %. Binding of a specific antibody was significantly increased by the polymer modification: Mass transport limited binding of the specific antibody in low concentrations (30 nM) up to a surface coverage value of 2 ng/mm² and a maximum surface coverage in the range of a monolayer of IgG (5–6 ng/mm²) was observed for most of the polymers. The surface coverage found for IgG bound specifically to the dextran-modified surface exceeded a protein monolayer.

Keywords: immunoprobe, surface chemistry, biospecific interaction analysis

INTRODUCTION

Affinity-based methods are well established and are of considerable importance in modern analytical chemistry. The principle of these methods is the strong and highly selective binding of ligands to a corresponding biological receptor. Methods based on antibody affinity are already well established for medical and environmental trace analytics (Ekins, 1994). Typically, the antibody-analyte interaction is carried out in a heterogeneous test format using labelled compounds. Quantification is achieved by detection of the labels which are enzymes, fluorophors, or radioactive isotopes (Price & Newman, 1991). In recent years, several techniques have been described that allow direct, label-free monitoring of affinity interactions at solid–liquid interfaces (Liedberg *et al.*, 1983; Nellen *et al.*, 1990; Cush *et al.*, 1993; Gauglitz *et al.*, 1993; Ebato *et al.*, 1994). These techniques are based on physical phenomena occurring during the binding of proteins to a transducer surface. For probing these effects we have used reflectometric interference spectroscopy (*RIfS*), but the results apply to optical methods based on glass type substrates in general. Direct optical detection is based on the difference in refractive index between water and protein. Protein binding at a surface therefore increases

TABLE 1. Surface properties required for different applications of affinity interaction analysis

Feature	*Quantitative immunoassay (labelled)*	*Quantitative immunoassay (label free)*	*Kinetic investigations (label free)*
immobilisation	low density moderate accessibility	high density moderate accessibility	low density high accessibility
non-specific interaction	minor problem	significant problem	significant problem
regeneration/stability	not required	very important	important

the refractive index locally which can be detected by optical set-ups based on evanescent field or reflectometric interrogation. By these detection schemes any compound binding at the surface is detected. Non-specific adsorption becomes a major problem for these detection schemes because it cannot be discriminated from specific effects.

Using detection at solid phases the surface properties are of fundamental importance for the performance of the system, in particular for direct monitoring of the interaction. Adequate properties have to be achieved by modification of the surface. Our aim was to establish protocols for surface modification that meet the requirement for different applications:

1. The detection of analytes of low molecular mass by an indirect sequential immunoassay.
2. The investigation of thermodynamical and kinetical properties of receptor-ligand interactions.

The main task of surface modification for direct immunoprobes is the immobilisation of one of the interacting compounds in good accessibility and appropriate concentration. Furthermore non-specific interaction with proteins has to be minimised and stability of the layer with respect to the possibility for repeated regeneration has to be provided. A comparison of the properties required for specific applications is given in Table 1.

In common immunoassay protocols surface modification and ligand immobilisation is carried out by adsorption of proteins and protein conjugates. Apart from surface modification by protein adsorption several other modification techniques applied in the field of affinity interaction analysis have been reported. Some established methods are briefly discussed in Table 2.

Frequently surface modification is achieved by attachment of appropriate polymers to shield the surface against non-specific binding and to generate active sites for the attachment of ligands. The polymers should have a low (non-specific) interaction tendency with proteins. Hydrophilic polymers have proven to be most suitable. Several water-soluble and hydrogel-forming polymers exhibiting a low affinity to proteins are known from biocompatibility investigations, e.g. poly(ethylene glycol) (Mori *et al.*, 1982), polysaccharides (Elam & Nygren, 1984), poly(vinyl alcohol) (Miller & Pappas, 1988), chitosan (Hirano *et al.*, 1987), poly(hydroxyethyl

TABLE 2. Comparison of several immobilisation techniques

Method	*Advantages*	*Drawbacks*	*References*
adsorption	very simple protocols universal application	low stability badly defined layers dependence from surface properties	Ahluwalia *et al.* (1991)
Self-assembling monolayers (thiols; poly-ions)	fast, simple protocols well defined layer systems high stability	limited to certain substrates (thiols: restricted to metal surfaces) (poly-ions: strongly charged layers)	Porter *et al.* (1987), Lvov *et al.* (1993)
film balance techniques	highly defined layers moderate flexibility	optimisation of conditions prerequisite, dependence from surface properties limited stability	Ahluwalia *et al.* (1991)
covalent attachment	universal application high stability highly defined layer systems	sophisticated protocols different methods for various surface types	Ahluwalia *et al.* (1991)

methacrylate) (Ratner & Hoffman, 1976), polyacrylamide (Bruck, 1973) and its derivatives. Many of these polymers have to be functionalised to introduce active sites for coupling.

Application of dextran, a polysaccharide, for surface modification is prominent in many fields of affinity interaction analysis and some techniques have been reported (Löfas & Johnsson, 1990; Böcher *et al.*, 1992). But only a few other biocompatible polymers have been investigated with respect to their potential for surface modification in the field of affinity sensing.

Completely different approaches using plasma deposition for improving surface properties with respect to biocompatibility have been reported recently (Ertel *et al.*, 1992; Lopez *et al.*, 1992).

In our case, highly stable, flexible and universal modification of glass type surfaces for the application with various direct optical transducers was required. For these reasons we have investigated the covalent modification of such surfaces in detail. In this study we present efficient protocols for surface activation, attachment of functionalised polymers and coupling of ligands. A selection of the polymers described above was investigated with respect to their suitability for these purposes. Non-specific protein adsorption, specific antibody binding and stability of the modified surfaces were determined by reflectometric interference spectroscopy (*RIfS*), a transducer for direct optical detection of binding events.

EXPERIMENTAL

Materials

4-Aminobutyldimethylmonomethoxysilan (ABDMS) was purchased from ABCR/Karlsruhe. Dextran (*M*=300,000 g/mole), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-hydrochloride (EDC), *N*-hydroxy-succinimide (NHS), poly(ethylenimine) (PEI) branched, (*M*=50,000 g/mole) 50% aqueous solution, Ovalbumin and Pepsin were from Sigma/Deisenhofen. Sodium cyanoborohydride, propionic acid, succinic anhydride, hexamethylenediamine (HMDA), dimethyl formamide (DMF), diisopropylethylamine (DIPEA) and diisopropyl carbodiimide (DIC) were purchased from Fluka/Neu-Ulm (all of analytical grade). α,ω-diamino poly-(oxyethylene) (POE) (*M*=2000 g/mole) was from W. Rapp/Tübingen. Chitosan (CHI) (partially deacylated chitin, *M*=70,000 g/mole) was from ICN pharmaceuticals/Costa Mesa, USA. 4-Chloro-6-(isopropylamino)-1,3,5-triazine-2-(6′-amino)caproic acid (atrazine caproic acid) and polyclonal sheep anti-atrazine antibody were kindly supplied by Dr Ram Abuknesha/GEC London. Interference layers for *RIfS* (10 nm Ta_2O_5 and 500 nm SiO_2 on 1 mm BK7 glass deposited by plasma-induced CVD) were supplied by Schott/Mainz.

Aminodextran (AMD) was prepared by oxidation of dextran followed by reductive amination with ammonia. The oxidation was carried out with a 5 %w/v (310 mM) aqueous solution of dextran by 0.7 % (33 mM) w/v sodium periodate (corresponding to 10% of the anhydroglucose units) for 12 h at 4°C according to Floor *et al.* (1989). The solution was purified by dialysis against water for 3 days. To the dialysed solution a 10-fold molar excess of ammonium chloride was added and reductive amination was carried out by adding sodium cyanoborohydride several times (two fold molar excess altogether) at pH 6 and 100°C during 5 days according to Yalpani and Brooks (1995). The dextran was purified by dialysis against distilled water for 3 days, precipitated by a 10-fold excess of methanol at pH 4, filtered off and dried at 60°C overnight.

Elementary analysis was used to determine the contents of nitrogen. A concentration of amino groups of approx. 2.5% with respect to the anhydroglucose groups was estimated from the C/N-ratio. Direct assessment of the number of amino groups was possible by the ninhydrin-based Kaiser test as described by Sarin *et al.* (1981). Kaiser solutions (50 µl of each) were added to approx. 1 mg amino-dextran and heated to 100°C for 30 min. Glycine solutions treated in the same way were used for calibration. The contents of amino groups in the AMD was determined photometrically to be 2.2% with respect to the anhydroglucose groups.

Poly(acrylamide-co-acrylic acid) (PAM) (*M*= 200,000 g/mole) was prepared by basic hydrolysis of poly(acrylamide) (NaOH, 90°C) and had a functional capacity of 1.39 mmol/g polymer.

Surface chemistry

Activation of the surface was achieved by silanisation with an amino-functionalised silane. To avoid uncontrolled oligomerisation, a monoalkoxy silane was used. Carboxyl groups on the surface were obtained by conversion with succinic anhydride. The polymers were attached to the surface via amide bonding as demonstrated in Figure 1, for an amino-functionalised polymer. Hapten molecules modified by carboxyl groups were coupled to the amino groups of polymer on the surface to investigate specific binding of an antibody. This flexible modification strategy allows the construction of combined layer systems by simple reactions. For comparison the hapten was directly coupled to the aminosilane.

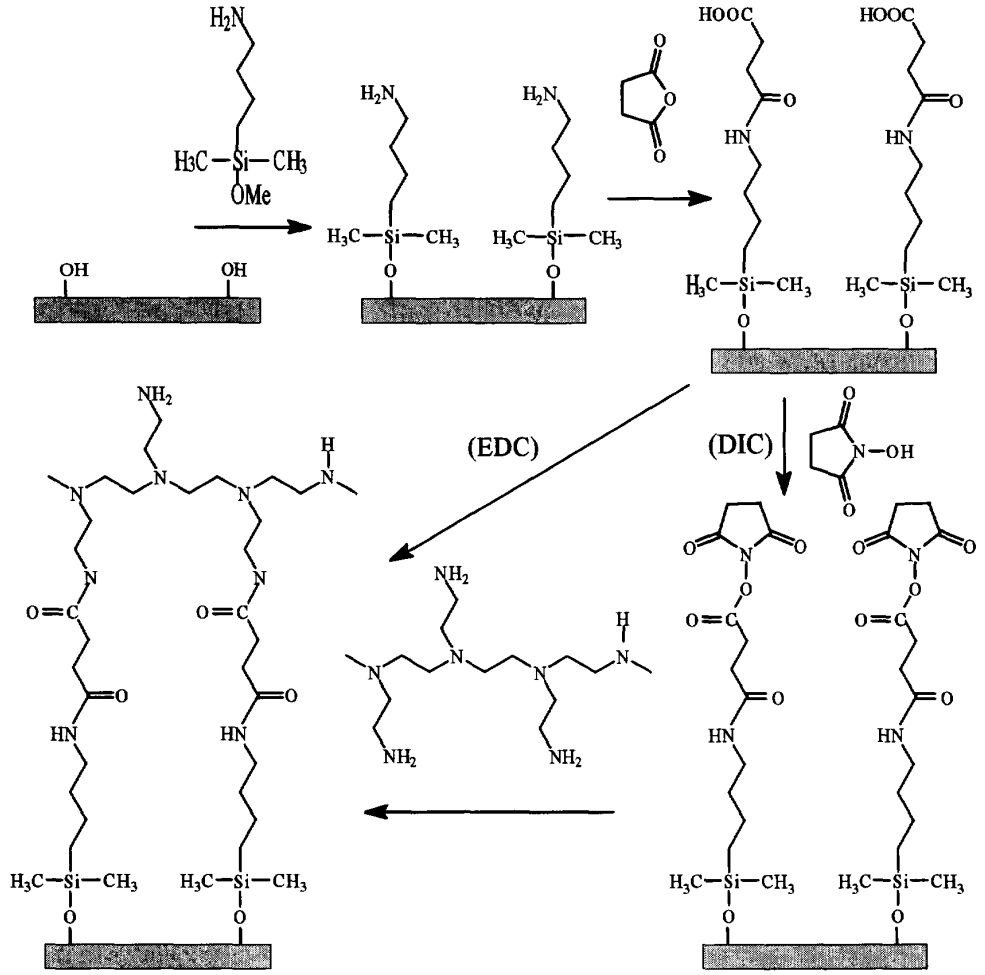

Fig. 1. Activation of the surface and coupling of an amino-functional polymer (e.g. poly(ethyleneimine)) to the surface (directly and via NHS-active ester).

The silica surfaces were cleaned in freshly prepared, hot piranha solution (H_2SO_4/H_2O_2 2:1) for one hour. The slides were thoroughly rinsed in ultrapure water and dried at room temperature. Silanisation was carried out in a solution of 1% v/v 4-aminobutyldimethyl-ethoxysilane (ABDMS) and 0.25% v/v water in toluene for 24 h. After thorough rinsing with toluene, water, 1 mM HCl, and water again, the slides were dried at room temperature.

For the attachment of the amino-group-containing polymers the amino moieties on the silica surface were converted to carboxyl groups according to a protocol described by Janolino and Swaisgood (1982). The slides were covered with 200 mg succinic anhydride per cm^2 surface and 1 ml/cm^2 phosphate buffer (10 mM KH_2PO_4, pH 7,4) was added. The succinic anhydride was dissolved by shaking, while the pH was maintained at 6–7 by adding 3 N sodium hydroxide solution. After 1 h, the slides were rinsed and dried at room temperature.

Polymer coupling

A comparative overview over the polymers used for surface modification is given in Table 3. The corresponding structures are given in Figure 2.

The amino derivatised polymers POE, PEI and AMD were attached to the carboxyl groups on the surface in

TABLE 3. Characteristics of the polymers including the molecular mass per functional group and the concentration used for coupling

Polymer	*Molar mass (g/mole)*	*Functional group*	*Mass per reactive group (g/mole)*	*Concentration (w/w)*
Chitosan (CHI)	70,000	NH_2	162	15%
Poly(ethyleneimine) (PEI)	50,000	NH_2	129	10%
Poly(oxyethylene) (POE)	2000	NH_2	1000	50%
Poly(acrylamide-coacrylic acid) (PAM)	200,000	COOH	712	15%
Aminodextran (AMD)	300,000	NH_2	6500	30%

aqueous solution using the water-soluble carbodiimide EDC. The concentrations for coupling are given in Table 3. The pH of the polymer solutions was adjusted to 3–4 by 2 M HCl and 30 µl of these solutions per cm^2 were incubated on the surface in a humid chamber to avoid drying up. An excess of EDC was added twice during a reaction period of 12 h. The slides were intensively rinsed with water and dried at room temperature.

For coupling of chitosan, the carboxyl groups on the surface were preactivated by 2 M NHS and DIC in DMF for 4 h. After rinsing with DMF and water the polymer solution was incubated on the surface for 18 h. Then the slides were rinsed with water and dried at room temperature.

The attachment of the carboxyl-derivatised PAM required a slightly different modification protocol. The polymer was directly coupled to the silanised surface. The reaction was carried out as described for the other amino polymers. The conversion of the carboxyl groups to amino groups was achieved by reaction with 50 µl of 30% HMDA hydrochloride adding EDC in small portions over a period of 12 h.

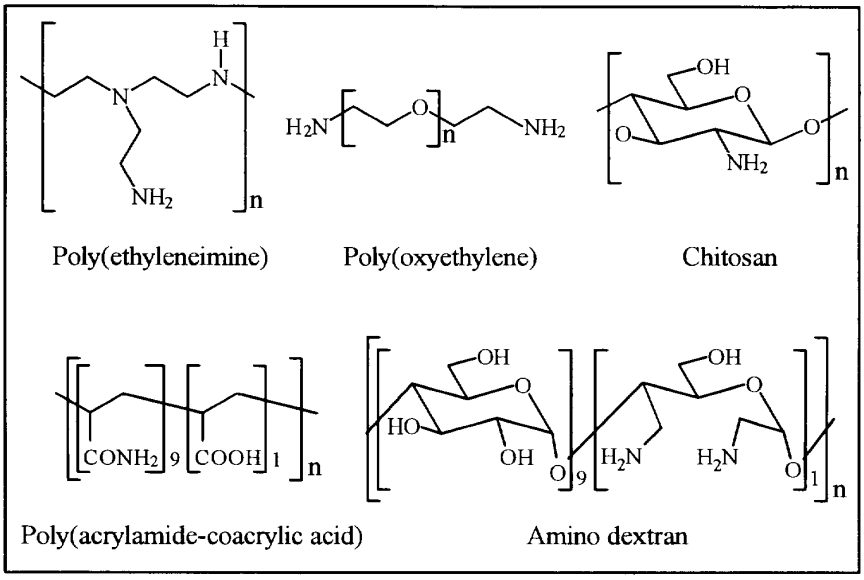

Fig. 2. Structures of the polymers used for surface modification.

Attachment of ligands

Before coupling of the hapten to the amino groups on the surface the slides were dipped in a solution of 1% v/v DIPEA in DMF, rinsed with pure DMF and dried at room temperature. Atrazine caproic acid in DMF (100 mg/ml, 0.33 M) was coupled to the surface using 200 µl/ml DIC (1.3 M). This solution was incubated for at least 6 h on the surface (approx. 10 µl/cm²). Afterwards the slides were rinsed in DMF and water and dried at room temperature.

Decreased surface concentrations of the hapten on the surface were achieved by adding propionic acid to the atrazine caproic acid in different molar ratios. The corresponding amount of atrazine caproic acid was dissolved in a solution of 25 µl/ml propionic acid in DMF (0.33 M). Reaction of these mixtures with the surface was carried out as described above.

Contact angle measurements

Dynamic contact angles were determined by a Wilhelmy type tensiometer (K12, Krüss/Hamburg). Glass slides of 25×15×1 mm³ were modified on both sides and their weight for different depths of immersion was determined. The contact angles for advancing Θ_{adv} and receding Θ_{rec} were determined by linear regression of the dipping curve from the intercept. The receding angle Θ_{rec} was used for comparison of the different surfaces because it was precisely accessible for both hydrophilic and hydrophobic surfaces. The standard deviation of Θ_{rec} obtained from four different measurements (including mounting of the slide) of the same sample was 0.5°.

Characterisation by *RifS*

Protein binding was directly monitored by reflectometric interference spectroscopy (*RIfS*). The detection principle is described in detail by Gauglitz *et al.* (1993). This method is based on interference of white light reflected at thin layers. Binding of proteins at the surfaces increases the optical thickness of the film which is detected as a change in the reflectance spectra (Brecht *et al.* (1995). The experimental set-up is shown in Figure 3.

The sensor mounted in a flow cell of 200 nL volume is connected with a tungsten light source (20 W) and a diode array spectrometer (MCS 210 and 410 series, 512 diodes, 350–780 nm, 12 respectively 16 bit nominal resolution, Carl Zeiss/Jena) by bifurcated fibre optics (PMMA, 1 mm in diameter from Microparts/Dortmund). Perpendicular incidence of the light allows collection of the reflected light with the same fibre. The optical thickness is determined from the interference spectra by a parabolic fit to a selected extremum. Recent tests with radioactive tracers (Brecht & Abuknesha, 1996) have shown that an increase of 1 nm optical thickness corresponds to a protein coverage of approximately 1 ng/mm². Sample handling is carried out by a FIA system (ASIA from Ismatec/Zurich).

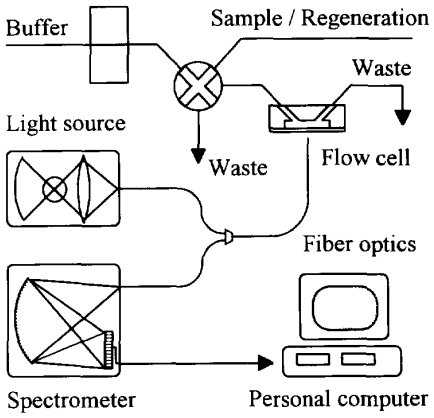

Fig. 3. Experimental set-up for direct monitoring of affinity interactions by RIfS including the light source, the flow cell, the sample handling system, and the diode array spectrometer.

Test scheme

The polymer modified surfaces were investigated with respect to non-specific adsorption, specific binding of antibodies, regeneration and binding capacity after regeneration. All measurements were carried out at least twice. Non specific adsorption was determined by incubation of Ovalbumin in high concentration (1 mg/ml). Specific binding was investigated by incubation of a polyclonal sheep-anti-atrazine IgG in low and high concentrations (30 nM, respectively, 300 nM). All protein solutions were prepared in phosphate buffered saline pH 7.4 (PBS). These three steps were carried out after another for an incubation period of 500 s. Interference spectra were acquired during this period with a time resolution of 2–5 s. Regeneration of the surface after protein binding by change of pH, chaotropic reagents and protease was investigated monitoring the effects with a time resolution of 10 s.

RESULTS AND DISCUSSION

Surface chemistry

The application for direct optical affinity sensing requires transparent and very homogeneous layers to avoid loss of light and interferences by scattering. In this respect, one of the most critical step of the surface modification is the silanisation by highly reactive silanes because undefined oligomerisation of multifunctional silanes is possible. In the case of ω-amino silanes, reactions with the terminal amino groups forming internal zwitterions can also occur. This effect, leading to high surface loading and crosslinking, is useful for other purposes, e.g. chromatographic applications, but is not desired if highly defined surfaces are needed. Therefore, mono-functional amino silanes in low concentrations were used to avoid oligomerisation. Still, after intensive rinsing of the freshly silanised surfaces with toluene and water, very small spots remained which were removed by 1 mM HCl. After this procedure, the surfaces looked very clean and no inhomogeneity could be observed. For all subsequent steps, intensive rinsing with water was sufficient to yield clean and macroscopically homogeneous surfaces.

The surfaces were highly hydrophilic after cleaning in piranha solution and drying under atmospheric conditions (Θ_{rec}=6.0±0.1°). After the silanisation increased hydrophobicity was observed (Θ_{rec}=36±4°, compared to 78±1° for a surface silanised with dichlorodimethylsilane). This wettability was maintained during the reaction with succinic anhydride (Θ_{rec}=36±3°). The coupling of polymers lead to differences in hydrophilicity. Low contact angles were observed for AMD (Θ_{rec}=7±2°) and PEI (Θ_{rec}=0). Coupling of POE increased the wettability of the surface only weakly (Θ_{rec}=25±0.1°). CHI and PAM were not investigated because the homogeneous modification of large surfaces was not possible due to the polymer consistency.

The attachment of the (hydrophobic) hapten decreased the hydrophilicity of the surface. This effect was only weak for AMD and PEG ($\Delta\Theta_{rec}$=5°) but very strong for PEI ($\Delta\Theta_{rec}$=25°). Direct coupling of the hapten to the silanised surface did not significantly change the wettability.

Functional properties

A typical characterisation cycle carried out on a surface modified by chitosan is shown in Figure 4.

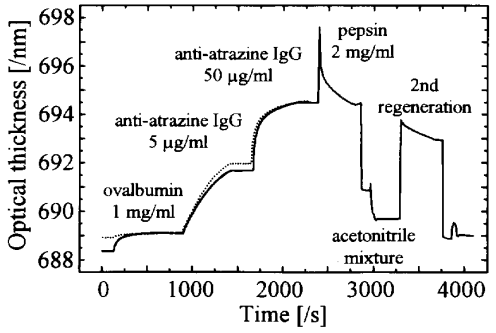

Fig. 4. Non-specific and specific binding of proteins at a chitosan-modified surface followed by two regeneration cycles (——) and second cycle of protein binding (·····).

Non-specific adsorption

The amount of non-specific protein adsorption at the differently modified silica surfaces is shown in Figure 5. Compared to the silanised surface all polymer modified surfaces show significantly lower non-specific adsorption.

Lowest non-specific adsorption is obtained by modification with POE and particularly with AMD. A reduction to 10% respectively 5% with respect to the adsorption on the silanised surface is achieved. For CHI, PAM and PEI no significant differences of non-specific effects were observed, all ranging between 0.8 nm and 1.2 nm (reduction to 40–50 % corresponding to silanised surface). Binding curves for 1 mg/ml Ovalbumin at CHI modified surfaces before and after hapten coupling are shown in Figure 6. At the hapten-modified surface, a seven-fold increase in non-specific protein adsorption is observed. At PEI-modified surfaces

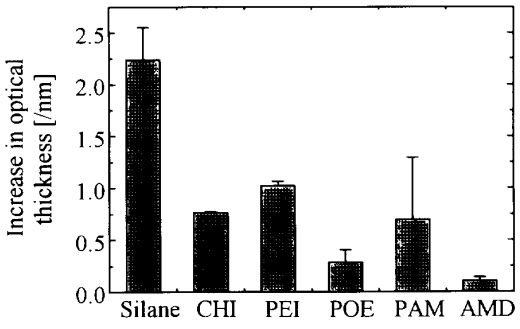

Fig. 5. Non-specific adsorption of 1 mg/ml Ovalbumin at polymer-modified surfaces compared to a silanised surface.

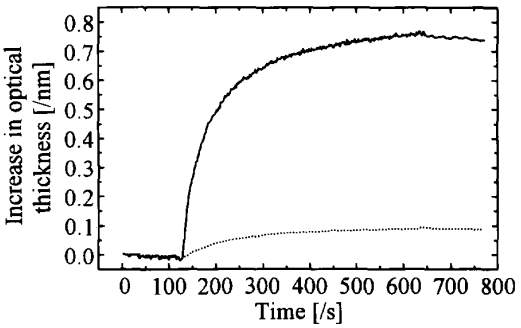

Fig. 6. Non-specific adsorption of Ovalbumin (1 mg/ml) on chitosan-modified surfaces: pure (····) and ligand-modified (—) polymer.

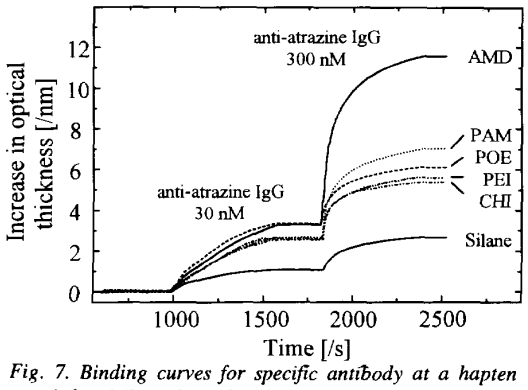

Fig. 7. Binding curves for specific antibody at a hapten coupled to differently modified surfaces.

non-specific adsorption observed at the pure polymer exceeded values found after modification with the hapten (data not shown).

The main reason for the decrease in protein affinity is shielding of the surface by the hydrophilic polymer chains. It can be assumed that mainly polymer–protein interactions are responsible for residual non-specific adsorption. The polymers with a higher density of functional groups showed a stronger (non-specific) affinity to proteins. Protein–surface interactions are mediated by Coulomb forces, hydrogen bridges, van der Waals interaction and hydrophobic interaction. Non-specific adsorption is due to interactions of the protein with the polymer backbone or by interaction with the immobilised ligand. Both effects are observed: the significant increase of non-specific binding due to hapten coupling at CHI modified surfaces indicates that the hapten is involved in non-specific effects. Non-specific effects at PEI modified surfaces were reduced after hapten coupling. In this case Coulomb interactions between the charged polymer and proteins may be involved, which are reduced by the coupling procedure.

Specific interaction

The binding curves for 30 and 300 nM antibody concentration are presented in Figure 7.

The binding curves for antibody concentrations of 30 nM are linear up to surface concentrations of 2 ng/mm^2 for all polymer-modified surfaces. This indicates mass transport limited binding due to a high surface concentration of the hapten and a high affinity of the antibody. In this case, the binding kinetics depend only on diffusion to the surface, which is a function of concentration and flow geometry. The differences in the slope of the binding curves at low surface coverage for different polymer modifications can be ascribed to the slight variations in flow conditions introduced by exchange of transducers and are not significant.

Sufficient surface concentration and accessibility of the hapten is obviously obtained for all polymer-modified surfaces. The binding at the directly attached hapten is significantly slower, perhaps because the accessibility of the hapten is limited due to the short spacing from the surface and 'burying' by non-specifically adsorbed Ovalbumin.

Increased maximum specific binding of antibody is observed at the polymer modified surfaces (comparison in Figure 8). A surface loading of approximately 3 ng/mm^2 is obtained by the directly attached hapten. But also the non-specifically adsorbed Ovalbumin has to be taken into account, giving more than 5 ng/mm^2 of protein altogether. That corresponds well to a monolayer of protein. The surface concentrations of IgG reached on all polymer modified surfaces are in the range of a monolayer (between 5 and 7 ng/mm^2). An exception is the AMD-modified layer, which is capable of binding significantly more than a monolayer of IgG. This proves that dynamic re-arrangement of the dextran layer is

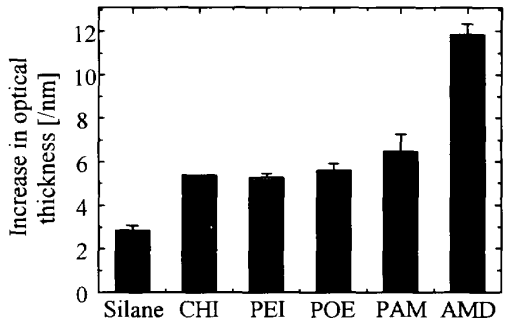

Fig. 8. Maximum loading of modified surfaces by specific antibody binding (300 nM).

possible, allowing the antibody to bind within the polymer matrix. It is well understandable that this effect is not possible at the POE-modified surface because of the short chains of about 40–50 oxyethylene units. The molecular masses and the chain lengths of the other polymers are not much different from AMD (Table 2). However, significant lower protein loadings indicate that no re-arrangement of the polymers occurs. We ascribe this to a loss of flexibility due to the high degree of functionalisation: the high density of functional groups probably leads to an increased number of chemical bonds and physical interactions between polymer chains and the surface and, consequently, the flexibility is reduced.

The maximum antibody binding is reached more slowly at the AMD-modified surfaces than at the other polymers. This effect is well explained by the rearrangement of the layer system during the antibody binding that is necessary for the formation of a 'multilayer structure'.

Regeneration and stability

Regeneration was attempted by procedures typically used for this task (100 mM citric or hydrochloric acid, 100 mM glycine/HCl pH 1.5, 8 M urea, and 6 M guanidinium chloride, pH 1.5). All these methods did not work well with the antigen–antibody system used. The same problems occurred with monoclonal antibodies. Similar experience with pesticide antibodies is reported by Bier *et al.* (1994) who suggests antibody degradation by proteases for regeneration. Using 100 mM NaOH and acetonitrile as described by Minnuni and Mascini (1993) was successful in removing protein but also strongly degraded the surface. Therefore a regeneration protocol was developed which included an incubation of 2 mg/ml pepsin at pH 1.9 (10 mM phosphate buffer) for 480 s and a short pulse of a mixture of acetonitrile, water and propionic acid in the ratio 50:50:1 for 50 s. Regeneration of the highly loaded surfaces is possible by repeated incubation of pepsin and the solvent/water mixture. Remaining material after several regeneration cycles is considered to be strongly non-specifically adsorbed and, therefore, not removable. In fact, after protein loading and regeneration, non-specific adsorption of Ovalbumin is drastically reduced, as it can be observed in the second cycle in Figure 4. Remaining highly active binding sites seem to be blocked by the protein. This effect was observed for all polymers but PEI. Here, the non-specifically adsorbed protein was perfectly regenerable. This indicates a different principle of interaction for PEI than for other polymers. Responsible for the different behaviour of PEI are probably the polycationic properties, as discussed above.

After regeneration, all polymer-modified layers show still strong specific binding, more than 90% of the first response. Best results were obtained by modification with CHI, PEI and AMD, where the maximum loading is not decreased at all. The application of dextran-modified surfaces in immunoassay for pesticide detection have proven to be stable during more than 200 cycles (Brecht *et al.*, 1995). More than 80% of the initial maximum loading was achieved after this period (data not shown).

At the POE-modified surfaces, the specifically bound antibody is removed quantitatively by regeneration and the original baseline is reached, but the second response is reduced by approximately 5%. This effect can be attributed to the telechelic properties of POE. POE, in contrast to all other polymers, is attached to the surface only by a single bonding and, therefore, is more prone to desorption due to hydrolysis.

Different properties are observed for the PAM-layers, where about 10% of antibody remains on the surface in spite of repeated regeneration, but the same maximum loading was reached. This indicates that considerable non-specific, irreversible adsorption of antibody took place.

Similar effects are observed when the hapten is directly bound to the silanised surface. About 50% of the antibody remains on the surface. Thus, different non-specific adsorption sites for Ovalbumin and IgG must be present at these surfaces.

Low ligand density

A defined surface concentration of the ligand is an important aim of the systematical optimisation of surface chemistry. Low hapten concentrations are of particular interest for kinetical and thermodynamical investigations (Table 1). But, even when increased binding rates are required, the hapten loading should be carefully controlled because of non-specific interactions at highly hapten-loaded surfaces as discussed above.

Our approach was to decrease the surface concentration of the hapten on dextran-modified surfaces by adding a competing blocker of the amino groups during the coupling step. Propionic acid was mixed in several ratios between 10 an 1000 to the ligand to titrate the surface concentration of the hapten. No significant differences in non-specific protein adsorption were observed at these layers (data not shown). Specific binding of 30 nM and 300 nM anti-atrazine IgG is shown in Figure 9.

At a hapten-to-blocker ratio of 1:10 the binding kinetics of the 30 nM antibody is not affected but the

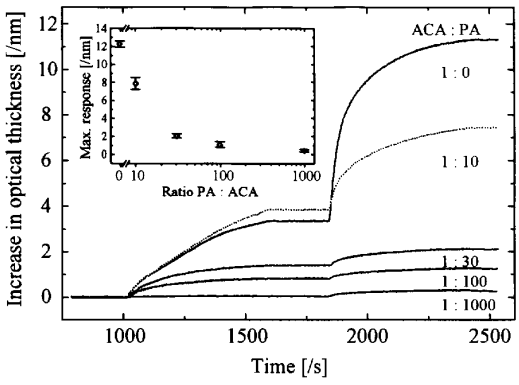

Fig. 9. Binding curves for specific interaction of 30 nM and 300 nM anti atrazine IgG on dextran surfaces with different ratios of hapten (ACA) to propionic acid (PA) during coupling. The inset shows the maximum response as a function of the coupling ratio.

maximum loading is decreased by one third. By further diluting the hapten (hapten-to-blocker ratio of 1:30) the binding of 30 nM antibody is no longer diffusion controlled and saturation is soon observed. The maximum binding is reduced to less than 20% of the original loading. This much stronger effect for 30-fold dilution than for 10-fold dilution proves that the surface concentration of the hapten is significantly higher than the maximum surface concentration of antibodies bound. Even much higher hapten concentration can be expected from the other polymers because of the higher degree of functionalisation.

By further dilution a decrease of the maximum antibody loading down to 300 pg/mm^2 was observed. Using this low hapten surface concentration, strong antibody desorption can be observed, which is much weaker for high concentrations. This is ascribed to the increasing distance between individual hapten molecule at the surface. At low concentrations, the distance between haptens begins to exceed the distance that the two binding sites of the divalent antibodies can bridge ($\approx$10 nm). This restricts binding of antibodies to only one binding site so that the dissociation kinetics of single binding sites become observable without avidity effects due to multi-site binding. Very low hapten concentrations therefore allow the determination of kinetical association and dissociation constants of single binding sites and by this, more detailed affinity characterisation.

SUMMARY AND CONCLUSION

We have reported simple, rugged, and well reproducible protocols for the covalent attachment of functionalised polymers onto glass-type surfaces and the coupling of ligands to these polymers. Versatile coupling chemistry based on amide bonds allowed the application of large experience acquired in solid and liquid phase peptide synthesis (Geckeler, 1995).

The most critical step of the chemistry described here is the defined silanisation by an ω-aminoalkyl silane. The highly hydrophilic glass surfaces required for high coupling yields were achieved by intensive pre-treatment. Polymerisation of the silane was avoided by using monoalkoxy derivatives, but still an intensive rinsing procedure was required. The surfaces obtained by this protocol did not show any macroscopic inhomogeneities.

Polymers were coupled using solutions of high concentration (10–50% w/w). This was, in particular, necessary when polymers with a low degree of functionalisation (AMD, POE) were used to reach a sufficient loading of the surface. Coupling under aqueous conditions was possible with all polymers. This was preferred to have comparable coupling conditions.

Using *RIfS* for direct monitoring of binding events allowed a detailed characterisation of the interaction properties of the surfaces. All polymer-modified layers showed improved interaction properties compared to the directly attached ligand. The non-specific adsorption was strongly decreased by shielding the surface, particularly by modification with AMD. The highly functionalised polymers PEI, CHI and PAM showed significantly stronger non-specific protein adsorption which can be explained by hydrophobic hapten–protein interactions and Coulomb interactions with the polyelectrolytic polymers. The concept of a competing blocker presented above will probably improve the performance: attachment of the ligand in low surface concentration and reducing the charge of the polymer by using an indifferent carboxylic acid (e.g. glycolic acid) as a competing blocker molecule during the coupling can be used to tune the properties of the polymers.

Binding of a specific antibody was significantly enhanced by polymer modification: mass transport limited binding of the specific antibody in low concentrations (30 nM) up to 2 ng/mm^2 was observed indicating high loading of the surface by the ligand. Maximum binding in the range of a monolayer of IgG (5–6 ng/mm^2) was observed for most of the polymers studied. On AMD-modified surfaces a higher maximum loading significantly exceeding a monolayer of IgG was obtained. Rearrangements of the AMD-layer during antibody binding seem possible because of increased

flexibility of the polymer chain. A decrease in binding velocity for a high loading with antibodies supports this assumption.

Reduced hapten density at the surface could be achieved by dilution of the hapten with an indifferent blocking compound during the coupling step. A wide range of surface densities could be adjusted.

Detailed characterisation of the polymer layers is crucial to learn more about the layer properties. Investigations by ellipsometry give information for understanding the different behaviour during protein binding. FT-IR spectroscopy allows characterisation of the chemical reactions during the modification steps. By AFM the arrangement of the polymers on the surface could be investigated. Further information will be provided by more detailed investigation of the binding kinetics for the different polymers. The different properties of the polymers allow applications in different fields according to the discussion in Table 1: AMD, CHI, and PEI are suitable for immunoassay applications because of their tremendous stability. Because of minimum non-specific interaction dextran-modified surfaces are recommended when using direct immunoprobes. CHI and PEI are more suitable for labelled immunoassay because non-specific adsorption can be discriminated and plays a less important role. Using the PAM polymer for attaching carboxyl-derivatised ligands is not very feasible because conversion of the carboxyl groups on the surface to amino groups is necessary. But when coupling amino-functionalised compounds or when pre-activation of the surface is required, e.g. coupling of proteins, simple coupling protocols using PAM are possible.

The potential of modification by POE lies in the field of characterisation of affinity interactions. Well defined layers and high accessibility of the hapten are required for these applications. POE-modified surfaces exhibit such properties. The concentration of amino groups can be easily titrated by adding an ω-terminally blocked, monofunctional POE-derivative during surface modification.

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