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# A high-density poly(ethylene glycol) polymer brush for immobilization on glass-type surfaces

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**Abstract**

Label-free heterogeneous phase detection critically depends on the properties of the interfacial layer. We have obtained high-density monomolecular poly(ethylene glycol) (PEG) layers by solvent-free coupling of homo-bifunctional PEGs (2000 g/mol) at 75°C to silica surfaces silanized with glycidyloxipropyltrimethoxysilane (GOPTS). Characterization by ellipsometry and contact angles revealed that PEG layers up to 3.4 ng/mm$^2$ with low roughness and flexibility were obtained. Specific and non-specific binding at these PEG surfaces was monitored by reflectometric interference spectroscopy (RIfS). No significant non-specific adsorption upon incubation of 1 mg/ml ovalbumin was detectable ( $< 10$ pg/mm$^2$), and 150 pg/mm$^2$ upon incubation of 10% calf serum, less than 10% of the amount adsorbed to the solely silanized surfaces. The terminal functional groups of the PEG layers were utilized to couple ligands and a protein. Specific protein interaction with these immobilized compounds was detected with saturation loadings in the range of protein monolayers (2–4 ng/mm$^2$). The excellent functional properties, the high stability of the layers, the generic and practical coupling procedure and the versatility for immobilizing compounds of very different functionality make these PEG layers very attractive for application in label-free detection with silica or metal-oxide based transducers. 

*Keywords:* Poly(ethylene glycol); Immobilization; Affinity interaction; Heterogeneous phase detection; Reflectometric interference spectroscopy

## 1. Introduction

During the last decade, label-free monitoring of affinity interactions has become an increasingly attractive method for biomolecular interaction analysis (Fägerstam et al., 1992; Cush et al., 1993), for analytical application (Brecht and Gauglitz, 1997), and for drug screening (Brecht et al., 1996; Gunnarsson, 1997). Most signal transducers for label-free detection are based on optical interrogation principles, such as surface plasmon resonance (SPR), grating couplers, resonant mirror, ellipsometry and interferometry (reviewed by Brecht and Gauglitz, 1995). These methods are based on a heterogeneous phase format with one of the interacting compounds being immobilized at the transducer surface. Label-free transducers detect changes in polarizability or mass, i.e. properties, which are common to all molecules. Owing to the lack in selectivity of detection, suitable properties of the interfacial layer are of fundamental importance for a successful application of such methods for detecting and characterizing biomolecular interactions. The most crucial requirement is to gain selectivity of the transducers by minimizing non-specific binding at the transducer surface, in particular when using complex sample matrices. At the same time, functional sites must be provided, which allow controlled immobilization of chemical or biochemical compounds. The native properties of immobilized compounds should be maintained at the surface and its accessibility must not be affected by the interfacial layer. Furthermore, stability of the interfacial layer

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is required for reliable long-term operation. However, providing these properties by surface modification must not affect the sensitivity of the signal transducer. Thus, optical transducers typically require thin and homogeneous films to allow efficient detection.

Covalent attachment of mono-molecular, densely covering layers has proved to be a suitable method for adjusting surface properties and providing appropriate functional groups for anchoring ligands or proteins. On gold surfaces, oriented self-assembly of long-chain alkane thiols allows very efficient and stable modification (Nuzzo and Allara 1983; Ulman, 1991). Owing to the very dense and ordered coverage, solely the terminal group of the alkane defines the physico-chemical properties of the surface. The terminal group of the alkane thiol layer can be used for further chemical modification on the surface (Löfas and Johnsson, 1990). Furthermore, alkane thiols with coupled ligands can be used for self-assembly (e.g. Duschl et al., 1996; Rickert et al., 1996; Sigal et al., 1996). Optical detection, however, is in most cases performed on glass-type substrates such as silica or transition metal oxides. Glass-type surfaces do not allow such selective covalent attachment of defined and oriented monolayers. However, glass-type surfaces can be chemically modified by silanization with rather reactive silanes containing alkyl chains terminated with functional groups (Ulman, 1991). Densely ordered assembly of alkyl silane monolayers is generally not obtained by silanization, partly for sterical reason (Stevens, 1999), but also because of side reactions leading to oligomerization and formation of multiple layers. Side reactions are particularly critical when using silanes containing functional groups required for immobilization. Thus, silanized surfaces are often hydrophobic and display very strong non-specific adsorption (Piehler et al., 1996), which can only be reduced by attaching layers of hydrophilic polymers of considerable thickness (20–80 nm at polymer loadings of up to 8 ng/mm$^2$) (Piehler et al., 1999). These layers reduce non-specific binding, but proportionally interfere with the binding event (Piehler et al., 1999) and can also affect the sensitivity of the transducer.

In this study, we present a practical and generic approach for effectively shielding glass-type surfaces with ultra-thin layers of (homo-) bifunctional polyethylene glycols (PEG), at the same time incorporating functional groups for immobilization. PEGs are non-branched polymers, which have high exclusion volumes due to high conformational entropy and therefore repel (bio-) polymers including proteins. Thus, surface-attached PEG layers substantially decrease non-specific binding of proteins and other macromolecules to artificial surfaces as initially shown by Mori et al. (1982). Furthermore, its protein repelling properties make PEG chains suitable for protein immobilization (Holmberg et al., 1992). Efficient protection against non-specific protein adsorption was achieved by self-assembling of appropriate PEG terminated alkane thiols on gold surfaces (Prime and Whitesides, 1991; Mrksich et al., 1995). Also, successful assembling of oligoethylene glycol terminated silanes on silica surfaces has been reported (Lee and Laibinis, 1998), but such layers lack functional sites suitable for immobilization. Covalent attachment of functionalized, mono-molecular PEG films onto silanized glass-type surfaces in solution never succeeded to immobilize sufficiently high densities to protect the surface efficiently against non-specific protein adsorption (Gölander et al., 1992; Piehler et al., 1996). In this study, however, we have obtained PEG layers with very high surface densities by solvent-free coupling of homo-bifunctional PEG to silica surfaces activated with glycidyloxipropylsilane (GOPTS). The resulting surfaces were used for direct attachment of small ligands and for the immobilization of proteins. Characterization by ellipsometry and contact angle measurements indicate high-density and homogeneous morphology of these layers. Specific and non-specific binding to these surfaces was assessed by reflectometric interference spectroscopy (RIfS).

## 2. Materials and methods

### 2.1. Materials

Homo-bifunctional PEGs, diamino- (DAPEG) and dicarboxy- (DCPEG) functionalized, with a molecular mass of 2000 g/mol were purchased from RAPP Polymere, Tübingen/Germany. Common organic compounds and biochemicals were purchased either from Fluka, Neu-Ulm/Germany or Sigma, Deisenhofen/Germany. Glycidyloxipropyltrimethoxysilane (GOPTS) was purchased from ABCR, Karlsruhe/Germany. A Thrombstop analogous thrombin inhibitor (TI) with a free, reactive amino group was supplied by Dr Thomas Friedrich, BASF, Ludwigshafen/Germany. 4-Chloro-6-(isopropylamino)-1,3,5-triazine-2-(6′-amino)caproic acid (CTCA) and polyclonal sheep anti s-triazine antibodies and Fab fragments were supplied by Dr Ram Abuknesha (GEC Research, Borehamwood/UK). Wild-type barnase and the mutant 80C as well as wild-type barstar were obtained from Dr. Gideon Schreiber (Weizmann Institute of Science, Rehovot/Israel).

### 2.2. Preparation of the PEG layers

Functionalized PEGs (DAPEG and DCPEG) were covalently coupled to silica surfaces after silanization with GOPTS. Substrates were either RIfS transducers (silica deposited on glass slides) for functional characterization or native oxidized silicon wavers for ellipsometry and measurement of contact angles. Prior to

silanization, the substrate surface was cleaned by treatment with 3 M NaOH for 5 min followed by a freshly prepared solution of 60% concentrated $H_2SO_4$ and 40% $H_2O_2$ (piranha solution, strongly corrosive) for 1 h. After rinsing with water, the surface was dried at room temperature. GOPTS at a surface concentration of $\sim 5$ µl/cm$^2$ was incubated for 1 h, avoiding exposure to the atmosphere by assembling two slides face-to-face. The slides were then washed with dry acetone and dried in a nitrogen stream. Afterwards, the surfaces were immediately treated with the pure PEG derivatives by melting them on the surface at 75°C for 36 h. The surface was then thoroughly rinsed with water and dried at room temperature under atmospheric conditions. Reference surfaces were prepared by using diaminopropane (DAP) and ω-hexadecanethiol (HDT) instead of PEG in this reaction. The chemical reactions utilized for the coupling to the GOPTS-activated surface are schematically shown in Fig. 1. For ellipsometric and contact angle measurements, the surface chemistry was carried out on native oxidized silicon wavers. After silanization, the wafers were sonicated in acetone and after coupling the PEGs the wafers were sonicated and rinsed in ethanol and water.

### 2.3. Coupling of ligands

Coupling of ligands to the terminal functional groups of the immobilized PEGs was carried out at high concentrations in DMF in a face-to-face assembly in DMF vapor saturated chambers. The triazine derivative CTCA (100 mg/ml in DMF) was directly coupled to the terminal amino groups of surface-attached DAPEG using diisopropyl carbodiimide (DIC) as described in detail before (Piehler et al., 1996). An amine-functionalized Thrombstop analogue thrombin inhibitor (TI, BASF, Ludwigshafen) was coupled to the carboxyl groups of DCPEG modified surfaces by incubating 5 µl/cm$^2$ of a solution of 100 mg/ml TI in DMF for 12 h at room temperature. γ-Maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS) in DMF was coupled to the amino groups of DAPEG modified surfaces by incubating it at a concentration of 100 mg/ml in DMF for 12 h at room temperature. Conversion of amino groups into carboxyl groups was carried out by incubating a solution of 5 M glutaric anhydride (GA) in DMF overnight.

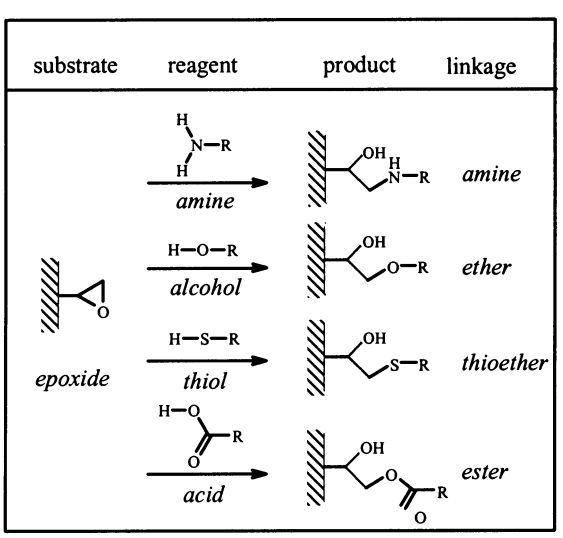


Fig. 1. Reactions of various functional groups with the epoxy-moiety of GOPTS.

### 2.4. Ellipsometry

Surface loadings of PEG layers were determined by ellipsometry using a null-ellipsometer (AutoEL, Rudolph Research) with a He–Ne laser light source, $\lambda = 632.8$ nm, and an angle of incidence at 70°. The refractive index was assumed to be 1.5 for all deposited layers in a three phase model 'substrate/organic layer/air'. An average of measurements at three spots of a sample gave the resulting thickness of the layer, and 4–5 samples were measured for each type of surface. The thickness of the native silica layer on the surface of the silicon was measured after treatment with piranha solution and was found to be between 1.1 and 1.5 nm. The surface loading was estimated from the layer thickness assuming a density of 1.2 g/cm$^3$ corresponding to the refractive index of 1.5 (Piehler et al., 1999).

### 2.5. Contact angles

Advancing and receding contact angles were measured with a goniometer (NRL 100, Ramé-Hart) using fresh ultrapure water (MilliQ, Millipore) in laboratory atmosphere. The samples were taken from the same batches as for the ellipsometric measurements. One measurement of the advancing and receding contact angle was done per sample, results from four to five samples were averaged.

### 2.6. Functional characterization

The interaction of proteins with the surface was monitored by RIfS (Gauglitz et al., 1993). The principles and the experimental set-up of this technique for monitoring binding events at interfaces were discussed in detail before (Brecht et al., 1993; Piehler et al., 1996). The change in apparent optical thickness of a thin silica layer ($\sim 500$ nm) upon protein binding is detected by interference of white light reflected at the interfaces of the layers using a diode array spectrometer. Binding curves $S(t)$ were recorded as apparent optical thickness versus time. A change of the surface loading of 1 ng/mm$^2$ protein leads to a signal $S \approx 1.6$ nm (unpub-

Table 1
Comparison of the surface loading and the contact angles after silanization with GOPTS and further modification with HDT and different PEGs

| Compound | $d$[a] (nm) | $\Delta\Gamma$[b] (ng/mm$^2$) | $\Delta\Gamma$ (pmol/mm$^2$) | $\Theta_a$ (°) | $\Theta_r$ (°) | $H$ (°) |
|---|---|---|---|---|---|---|
| GOPTS | $1.1 \pm 0.1$ | $1.3 \pm 0.1$ | $5.7 \pm 0.4$ | $48 \pm 2$ | $31 \pm 1$ | $17 \pm 2$ |
| HDT | $1.2 \pm 0.1$ | $1.4 \pm 0.1$ | $5.5 \pm 0.3$ | $93 \pm 2$ | $64 \pm 2$ | $29 \pm 3$ |
| DAPEG | $2.8 \pm 0.3$ | $3.4 \pm 0.3$ | $1.6 \pm 0.2$ | $30 \pm 1$ | $27 \pm 1$ | $3 \pm 1$ |
| DCPEG | $1.8 \pm 0.4$ | $2.2 \pm 0.5$ | $1.1 \pm 0.3$ | $30 \pm 2$ | $29 \pm 1$ | $1 \pm 2$ |

[a] Determined with a fixed refractive index of 1.5.
[b] Determined from the thickness assuming a density of 1.2 corresponding to the refractive index of 1.5 for all compounds.

lished calculations). All measurements were carried out in phosphate buffered saline (PBS) containing 50 mM phosphate and 150 mM NaCl with a pH of 7.4 at room temperature ($\sim$25°C). Non-specific adsorption at the surface was investigated by incubating 1 mg/ml ovalbumin or 100 μl/ml calf serum. Maximum loading by specific binding to immobilized ligands was determined by incubating 50 μg/ml anti-triazine antibody (Fab or IgG) or 50 μg/ml bovine thrombin. All protein solutions were incubated for $\sim$500 s at a flow rate of 50 μl/min. The surface was regenerated on-line by incubation of 2 mg/ml pepsin pH 2 for several minutes and a short pulse (30 s) of a mixture of water, acetonitrile and propionic acid (50:50:1), or by a short pulse of 50 mM HCl. For montitoring sulfhydryl-specific coupling of proteins to GMBS-activated surfaces, the transducer was mounted in a simple cell with a cylindrical 150 μl cavity (4 mm diameter). Solutions were directly pipetted into this cavity to allow incubations at high concentrations for prolonged time. Wild-type barnase and the mutant 80C were sequentially incubated at concentrations of 20 μM for 30 min. Subsequently, barstar was incubated at a concentration of 20 μM for 20 min.

## 3. Results and discussion

### 3.1. Surface loading and wetting of the attached layers

The surface loading obtained during each step of surface modification was determined by ellipsometry while the surface coverage and morphology of each layer was evaluated from advancing ($\Theta_a$) and receding ($\Theta_r$) contact angles. Silanization with GOPTS gave a very reproducible surface loading of 1.3 ng/mm$^2$ (cf. Table 1). The corresponding density of 5.7 pmol/mm$^2$ GOPTS at the surface is three times higher as reported for amino functionalized silanes (Lin et al., 1994; Elender et al., 1996; Piehler et al., 1999). Because silanization was carried out in the absence of water in the incubated, pure GOPTS, and the amount of attached silane was very reproducible, it is very unlikely that this higher surface concentration is due to formation of multiple layers. Both thickness and surface loading are in excellent agreement to GOPTS monolayers obtained under highly controlled conditions (Tsukruk et al., 1999) and by other comparable silanes (Jo and Blum, 1999). The contact angles of $\Theta_a = 48°$ and $\Theta_r = 31°$ of the GOPTS surface are in the range of those found for amino-functional silanes under similar conditions (Lin et al., 1994). However, the hysteresis $\Theta_a - \Theta_r$, $H = 17°$ is significantly lower than reported for these layers (24–29°). These results suggest that GOPTS layers of higher density and less heterogeneity were obtained compared to amino-silane layers.

The epoxy-groups on the surface can be reacted with a variety of nucleophiles. Reaction with the highly reactive, sulfhydryl-functional HDT at 75°C gave approximately stoichiometric conversion of these epoxy-residues (96% as determined from the molar surface loadings). The wetting of the resulting surface by water was very low with contact angles of $\Theta_a = 93°$ and $\Theta_r = 64°$. However, the strong hysteresis of this layer (29°) indicate, that despite of the relatively high density no ordered assembling of these layers takes place as happens spontaneously on gold surfaces. A SAM of HDT on gold shows a significantly more densely packed monolayer ($\sim$2.4 ng/mm$^2$, 70% higher) and contact angles of $\Theta_a = 110°$ and $\Theta_r = 102°$, i.e. much lower hysteresis (Lestelius et al., 1997).

The surface loading obtained by coupling bifunctional PEGs to the epoxy-groups of the silane was 3.4 ng/mm$^2$ for DAPEG and 2.2 ng/mm$^2$ for DCPEG. The reproducibility of the surface loading was good for the DAPEG (standard deviation of $\sim$10%), but significantly lower for DCPEG (standard deviation $>$20%). The lower coupling yield and the lower reproducibility are probably caused by the lower reactivity of the carboxyl group and the competing reaction with traces of water. However, these surface loadings are to our knowledge the highest ever observed for mono-molecular layers of PEG of this molecular weight. Methods to increase surface densities by using coupling conditions close to the clouding point reached a maximum of 1.2 ng/mm$^2$ for PEGs of comparable molecular weight (Lin et al., 1994). Only when carrier polymers such as poly(ethylene imine) were used, higher PEG loadings of 2 ng/mm (Gölander et al., 1992) or up to $\sim$4 ng/mm (Brink et al., 1992) were obtained. However, poly(ethylene imine) forms very heterogeneous and flex-

ible layers, thereby increasing the active surface, a fact which is indicated by the strong hysteresis in the contact angles of these surfaces ( ~ 20°, (Kiss and Gölander, 1991)).

The contact angles we found for the PEG-modified surfaces ($\Theta_a = 30°$) were significant lower compared with the GOPTS-surface indicating increased hydrophilicity of the surface. The hysteresis of the contact angles of $H = 1°$ for DCPEG and $H = 3°$ for DAPEG was significantly lower than found for mono-molecular PEG layers obtained by other chemistries under optimum coupling conditions ($H = 10°$; Lin et al., 1994). Such low hysteresis indicates very low flexibility and low roughness of these layers, which can be explained by the high surface density of PEG. Coupling homo-bifunctional PEG-chains does not exclude the attachment with both terminal groups to the surface. However, the relatively short length of the PEG chains ( ~ 45 monomer units) should somewhat limit the probability for such two-sided attachment, which would also be in contrast to the observed high polymer density on the surface.

Table 2
Non-specific binding of ovalbumin (1 mg/ml) and calf serum (100 μl/ml) observed at differently modified surfaces

| Exposed compound | Ovalbumin ([ng/mm$^2$) | Calf serum (ng/mm$^2$) |
|---|---|---|
| ABDMS[a] | 1.5 ± 0.2 | 2.2 ± 0.3 |
| GOPTS | 0.11 ± 0.02 | 1.8 ± 0.3 |
| DAP | 0.15 ± 0.03 | 1.1 ± 0.2 |
| GA | 0.1 ± 0.02 | 2.5 ± 0.3 |
| CTCA | 0.15 ± 0.03 | 1.2 ± 0.2 |
| DAPEG | <0.01 | 0.15 ± 0.03 |
| DCPEG | <0.01 | 0.15 ± 0.04 |
| DAPEG-CTCA | <0.01 | 0.13 ± 0.04 |
| DCPEG-TI | <0.01 | 0.17 ± 0.03 |

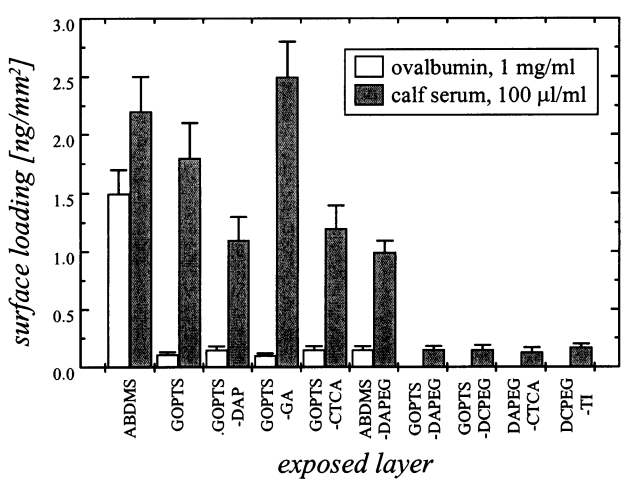


Fig. 2. Non-specific adsorption upon incubation of 1 mg/ml ovalbumin and 10% calf serum at differently modified surfaces in comparison (data from Table 2).

### 3.2. Non-specific binding

After silanization with GOPTS, the surface showed non-specific adsorption of ovalbumin in the range of 100 pg/mm$^2$ (Table 2 and Fig. 2). This is much less than observed at glass-type surfaces silanized with ω-aminobutyldimethylethoxysilane (ABDMS) ( ~ 1.5 ng/mm$^2$, (Piehler et al., 1996)) or ω-amino-propyl-trimethoxy-silane (APTS) ( ~ 1.4 ng/mm$^2$, unpublished data). Because the silica surface shows negligible non-specific binding before silanization, the attached silane provides the binding sites for non-specific binding (Piehler et al., 1996). Apparently, the much more dense layer formed by GOPTS compared to ABDMS protects better against non-specific interactions, which most likely take place with the hydrophobic alkyl backbone of the silane. Upon exposure to 10 % serum — i.e. a complex mixture of proteins in high concentration — significantly stronger non-specific adsorption at the GOPTS surface was observed with a saturation loading of nearly 2 ng/mm$^2$.

Non-specific binding of ovalbumin was not significantly altered after reacting the terminal epoxy-group with DAP to form an amino-functional surface, and after its conversion into carboxylic groups by GA. Also coupling the ligand CTCA to free amino groups of DAP did not significantly affect non-specific binding, indicating that the hydrophobic backbone of the silane is mostly responsible for non-specific interactions. For non-specific binding upon incubation of calf serum, much stronger binding to negatively (GA) charged surfaces was observed than at positively (DAP), indicating that more complex interactions take place. For this reason, shielding of surfaces against such complex protein matrices is such a critical issue for label-free detection.

Strongly decreased non-specific binding was observed after reacting the epoxy group of GOPTS with PEGs. For both DAPEG and DCPEG, no significant adsorption of ovalbumin (i.e. less than 10 pg/mm$^2$ within the incubation period of 500 s) at a concentration of 1 mg/ml was observed. This is significantly less binding than what has been observed at PEG-covered glass-type surfaces before (minimum of 100–150 pg/mm$^2$ (Lin et al., 1994; Piehler et al., 1996), cf. Fig. 2). More importantly, also adsorption during incubation of 10% calf serum was strongly reduced to about 150 pg/mm$^2$, i.e. less than 10% of the amount observed at surfaces treated only with GOPTS (Table 2 and Fig. 2). Such efficient shielding of the surface we even could not achieve by dextran layers up to surface loadings of 8 ng/mm$^2$ (Piehler et al., 1999). The highly improved shielding of these layers compared to DAPEG cova-

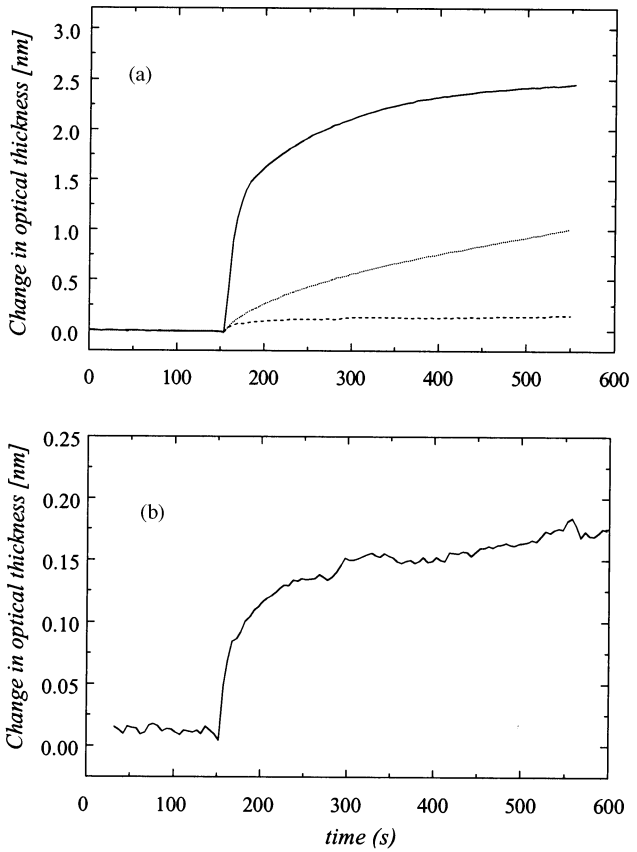


Fig. 3. Comparison of the kinetics of non-specific adsorption upon incubation of 10% calf serum at differently modified surfaces. (A) Binding to treated with GOPTS (—), AMD (---) and DAPEG (– – –) surfaces in comparison. (B) Enlarged binding curve for DAPEG.

lently coupled to ABDMS activated surfaces by amide chemistry (Piehler et al., 1996) is demonstrated in Fig. 2. Furthermore, the very low level of non-specific binding at the PEG-modified surfaces was maintained during coupling of the ligands.

Saturation of non-specific binding at these PEG-modified surfaces was as fast as observed at GOPTS-modified surfaces, while at surfaces modified with dextran (cf. Piehler et al., 1999) much slower binding kinetics was observed (Fig. 3). The fast binding kinetics indicates that the residual non-specific binding to the PEG surfaces is probably due to some not densely enough covered areas on the surface. This observation is in good agreement with the different mesh properties of PEG-layers compared to dextran layers (Needham et al., 1997; Piehler et al., 1999).

### 3.3. Binding to immobilized ligands

Non-specifc binding at the PEG-modified layers did not change upon coupling of ligands (cf. Fig. 4). Specific binding of anti-triazine antibodies to the triazine derivative CTCA covalently attached to the terminal amino groups of a DAPEG layer is shown in Fig. 4. A maximum loading of 4.5 nm (2.8 ng/mm$^2$) Fab and 6 nm (3.8 ng/mm$^2$) IgG were observed. These maximum surface loadings agree to those observed for binding of IgG and Fab to CTCA directly attached to DAP-modified GOPTS surfaces (data not shown), where the surface density of CTCA sites can be expected to be substantially higher. We therefore assume that these numbers correspond to monolayers of these proteins. This corresponds to a surface of $\sim 30$ nm$^2$ for each Fab unit, which agrees well with its dimensions of $\sim 5$ nm in all dimensions. The saturation signals observed for Fab and IgG species of the antibody gave a ratio of 1.36. This value is close to the theoretical value of 1.5 calculated from the molecular mass of IgG (75 kDa per binding sites) and Fab (50 kDa per binding sites), assuming that each binding site of the bivalent IgG binds to a ligand. At dextran layers, significant lower ratios have been observed because cross-linking within the polymer layer affected its flexibility and the accessibility of the attached ligands (Piehler et al., 1999).

Specific binding of thrombin to a thrombin inhibitor coupled to a DCPEG-modified surface is shown in Fig.

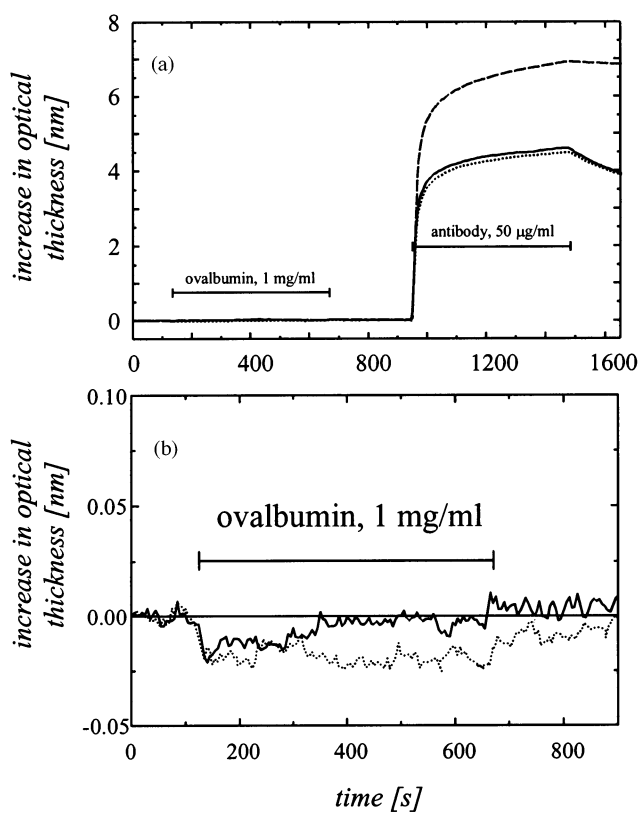


Fig. 4. Non-specific and specific binding to CTCA immobilized on a DAPEG layer. The response during incubation of 1 mg/ml ovalbumin is shown enlarged in the inset. Saturation signal of specific binding anti-triazine antibody IgG (– – –) and Fab (—), and the saturation signal with Fab after 100 regenerations with 50 mM HCl (---).

5. A maximum loading of ~4 nm (2.5 ng/mm$^2$) thrombin was observed, again in the range of a monolayer of this protein (38 kDa). For kinetic analysis of biomolecular interaction at interfaces using dextran layers, rebinding and slower mass transport within the interfacial layer was found to be a considerable problem (e.g. Schuck, 1996). Kinetic analysis of the dissociation phase of the binding curve shown in Fig. 5 gave a dissociation rate constant of 0.02 s$^{-1}$. Investigation of the same interaction using extended dextran hydrogel layers (~8 ng/mm$^2$) gave a substantially lower dissociation rate constant of 0.005 s$^{-1}$ at a similar saturation loading of 3.5 ng/mm$^2$. These results indicate that rebinding is lower at PEG layers, probably due to its flat arrangement compared to the extended hydrogel structure of the dextran layers (Piehler et al., 1999).

### 3.4. Immobilized proteins

The terminal group of the immobilized PEG can be used for covalent attachment of proteins to monitor protein–protein interactions. As an example for such application, immobilization of the ribonuclease barnase (12.5 kDa) and binding of its inhibitor protein barstar (10.3 kDa) is shown in Fig. 6. A barnase mutant with a free cysteine residue (250 μg/ml, 20 μM) was immobilized on a DAPEG surface using GMBS as a sulfhydryl-specific coupling reagent. The specificity and efficiency of this immobilization procedure is demonstrated in Fig. 6A. While the wild-type protein lacking free thiol groups does not at all bind to GMBS-activated surface, the cysteine mutant is irreversibly immobilized with a saturation surface loading of 4.2 nm (~2.6 ng/mm$^2$). The surface loading corresponds to a coverage of 0.2 pmol/mm$^2$ (i.e. 8 nm$^2$ per protein molecule). Because each barnase molecule is attached only to a single maleimido-residue, approximately 1/8

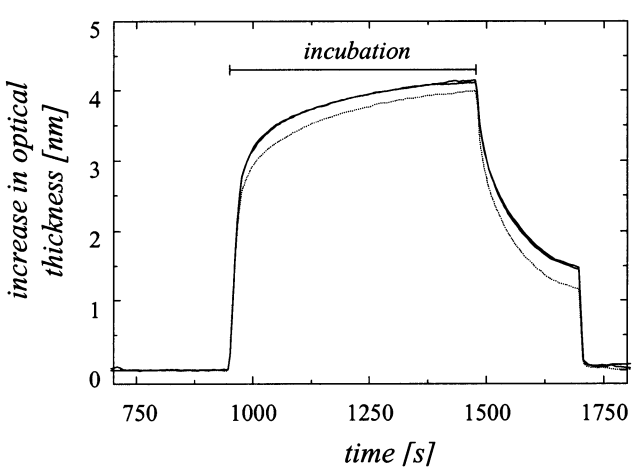


Fig. 5. Binding of thrombin (50 μg/ml) to the thrombin inhibitor immobilized via DCPEG. First binding curve (—) and binding curve after 100 regeneration cycles (---).

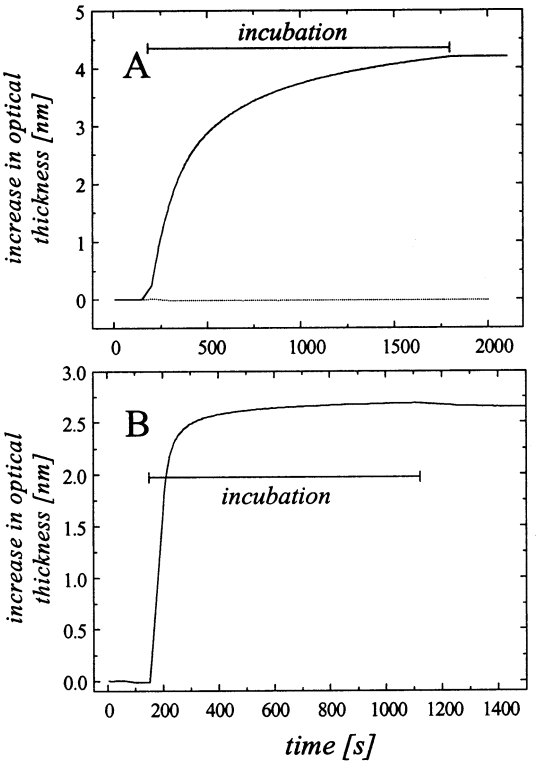


Fig. 6. Protein–protein interaction on a PEG layer. (A) Site-directed immobilization of the barnase mutant 80C (250 μg/ml) on DAPEG2000 activated with GMBS (—) compared with binding of wild-type barnase at the same concentration (---). B: binding of the barnase inhibitor barstar (120 μg/ml) to the immobilized barnase.

of the PEG chains on the surface are occupied by a protein molecule.

After blocking of residual activated amino groups with free cysteine, barstar, the intracellular inhibitor of barnase (120 μg/ml), binds specifically and fast to the immobilized barnase (Fig. 6B). The saturation loading of barstar is 2.7 nm (1.7 ng/mm$^2$). This corresponds to a stoichiometry of 80% taking the difference in molecular weight into account. Thus, only 20% protein on the surface is either not active or the binding sites of are not accessible.

### 3.5. Stability of the layers

The stability of DAPEG and DCPEG layers with ligands coupled to the terminal groups (CTCA and TI) was investigated by a series of 100 regenerations with 60 s pulses of 50 mM HCl within 12 h continuous running (data not shown). Less than 100 pm decrease of the baseline was observed throughout the entire experiment, which is within the drift of the detection system. Even more importantly, non-specific binding of both ovalbumin and calf serum was not significantly increased by this treatment. Specific binding for both thrombin binding to TI and antibody binding to CTCA decreased by less than 5% (cf. Figs. 4 and 5).

## 4. Summary and conclusions

Application of label-free transducers for detecting and characterizing biomolecular interactions requires surfaces with low non-specific affinity and functional sites for controlled immobilization. We have established an efficient method to cover glass-type surfaces with dense, thin layers of functionalized PEG. A solvent-free coupling chemistry on surfaces activated with GOPTS was developed to yield such high-density layers for homo-bifunctional PEGs with different terminal functional groups. The PEG layers obtained by this method showed high surface loading in combination with low contact angles as well as very low hysteresis of the contact angles. From these results we conclude that the coupling method presented here achieves very high density of PEG chains at the surface, higher than achieved by other coupling methods investigated before. The improved efficiency of coupling of PEG compared to other procedures can be explained by two reasons: (i) using GOPTS for the silanization is advantageous compared to amino-functionalized silanes, because a higher density and better ordered layer is obtained, probably because of the absence of nucleophilic groups in GOPTS. Not only is non-specific binding considerably decreased, but also a higher density and better accessibility of functional groups is obtained. (ii) coupling of PEG under solvent-free conditions achieves a higher density of immobilized PEG chains because the exclusion volume is minimized in the absence of solvent molecules.

The functional properties of these PEG layers obtained by the high density, two-dimensionally arranged PEG chains are very suitable for heterogeneous phase detection for several reasons.

(1) Extremely low non-specific protein adsorption was observed both for individual proteins and complex mixtures. The high degree of shielding has not been achieved before by mono-molecular layers on glass-type surfaces, neither with PEG using different coupling techniques, nor with dextrans or other hydrophilic polymers. Such efficient shielding allows the application of complex sample matrices, which is particularly interesting for label-free detection.

(2) The terminal group of the PEGs is available for covalent coupling of ligands and proteins as demonstrated in this study by different applications. The linkages of these ligands via PEG chains were stable throughout hundreds of regeneration cycles. The versatility of the coupling chemistry allows for applications of various homo- and hetero-bifunctional PEGs. However, modification of the terminal group of the PEG does not alter shielding against non-specific binding.

(3) Owing to the planar arrangement of the immobilized ligands, the binding dynamics is significantly less affected by rebinding and mass-transport than in much more extended dextran layers, making these surfaces very suitable for biomolecular interaction analysis. Because of the low absolute amount of polymer deposited on the surface, the thin and dense nature of the layer and their high stability, only minimum effects on the sensitivity of signal transduction can be expected.

The ultra-thin PEG layers obtained by this technique on glass-type surfaces can be used in a similar way as alkanethiolates on gold, but have very different physico-chemical bases: alkanethiolate SAMs are formed spontaneously by a very selective reaction, and are orientedly stabilized by co-operative inter-chain attraction. The dense PEG layers presented in this study, conversely, are forced by a non-selective reaction under extreme reaction conditions (high temperature, high concentration). In aqueous environment, the orientation of the polymer chains in the layer is dominated by inter-chain repulsion due to the high exclusion volume of the PEG chains, leading to its excellent protein repelling properties.

These principal differences have to be considered for designing surface chemistry based on such PEG layers. Using the method described here for direct assembling of pre-synthesized constructs of PEG with terminal functional groups or even ligands is critical due to the possibility of side reactions. Construction of more sophisticated layers is better achieved by sequential coupling on the surface. By adding hetero-bifunctional PEGs terminated with one non-reactive group (e.g. methoxy), mixed PEG-layers could be prepared to obtain lower concentrations of interaction sites. Another possibility is coupling of other polymers (e.g. dextrans) to PEG layer as it was carried out on SAM (Löfas and Johnsson, 1990).

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