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Polyglycerol based coatings to reduce non-specific protein adsorption in sample vials and on SPR sensors

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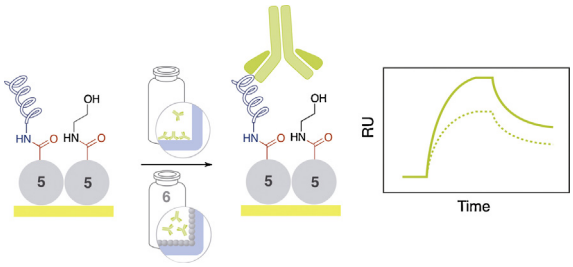
HIGHLIGHTS

- Synthesis and evaluation of novel immobilization matrix.
- Evaluation of sample vial coating for handling low analyte concentrations.
- Combination of both coatings to render relevant components of an immuno assay protein resistant.
- Coatings considerably increase accuracy and sensitivity of assay.

GRAPHICAL ABSTRACT



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ABSTRACT

Coatings based on dendritic polyglycerol (dPG) were investigated for their use to control nonspecific protein adsorption in an assay targeted to analyze concentrations of a specific protein. We demonstrate that coating of the sample vial with dPG can significantly increase the recovery of an antibody after incubation. First, we determine the concentration dependent loss of an antibody due to nonspecific adsorption to glass via quartz crystal microbalance (QCM). Complementary to the QCM measurements, we applied the same antibody as analyte in an surface plasmon resonance (SPR) assay to determine the loss of analyte due to nonspecific adsorption to the sample vial. For this purpose, we used two different coatings based on dPG. For the first coating, which served as a matrix for the SPR sensor, carboxyl groups were incorporated into dPG as well as a dithiolane moiety enabling covalent immobilization to the gold sensor surface. This SPR-matrix exhibited excellent protein resistant properties and allowed the immobilization of amyloid peptides via amide bond formation. The second coating which was intended to prevent nonspecific adsorption to glass vials comprised a silyl moiety that allowed covalent grafting to glass. For demonstrating the impact of the vial coating on the accuracy of an SPR assay, we immobilized amyloid beta (Aβ) 1-40 and used an anti-Aβ 1-40 antibody as analyte. Alternate injection of analyte into the flow cell of the SPR device from uncoated and coated vials, respectively gave us the relative signal loss ($1 - RU_{uncoated}/RU_{coated}$) caused by the nonspecific adsorption. We found that the relative signal loss increases with decreasing analyte concentration. The SPR data correlate well with concentration dependent non-specific adsorption experiments of the analyte to glass surfaces performed with QCM. Our measurements show that rendering both the sample vial and the sensor surface is crucial for accurate results in protein assays.

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1. Introduction

Molecular diagnostics based on immunological assays is widely used in research as well as in clinical laboratories [1,2]. Accuracy as well as sensitivity of such assays are figures of merit [3,4]. Both accuracy and sensitivity of the systems are not only determined by the method itself, but strongly depend on the properties of all the components in the assay system [5]. To this end, the surface properties of the different components in contact with the analyte play an important role as nonspecific, irreversible binding of molecules may deteriorate the overall performance of the system [6]. The importance of nonspecific binding is obvious for the active assay surface, therefore, surface coatings are used to minimize this effect and simultaneously enable a stable and specific immobilization of the molecules to be detected [2]. Nonspecific binding, however, may also occur prior to the interaction with the active assay surface, since the analyte gets in contact with various surfaces in the course of the preparation [7,8]. As the analyte concentrations are typically low (nM), significant loss of analyte may occur because of nonspecific binding to surfaces other than the intended active one, which may lead to false negative results.

Of special concern is the surface of the sample vials as the contact time for the analyte is generally by far the longest there. Dixit et al. [7] addressed this problem by treating polypropylene (PP) and chrome-coated glass tubes by a bovine serum albumin (BSA) solution and characterized the analyte loss with the bicinchoninic acid assay, surface plasmon resonance spectroscopy (SPR), and enzyme-linked immunoassay (ELIA). They demonstrated that the human fetuin A (HFA) loss due to nonspecific adsorption can be significantly reduced by passivation of the surface with BSA. Doran [9] fabricated protein resistant surfaces by coating with dimethyldichlorosilane followed by physisorption of polyethylene glycol (PEG) and various pluronic surfactants and determined the protein loss via enzyme-linked immunosorbent assay (ELISA). Pluronic and PEG coated glass showed a considerable reduction of nonspecific mouse IgG1 adsorption. Goebel-Stengel et al. [8] tested the recovery of radiolabeled endocrine peptides after incubation in untreated glass vials treated with AquaSil Fuid siliconizing agent, various polymeric tubes, and tubes of these materials subsequently modified by addition of BSA. While more peptides adhered to siliconized glass surfaces, the surface modification by BSA significantly increased the recovery compared to untreated and siliconized tubes. Despite the beneficial effects achieved by adsorption of BSA, hydrophilic polymers like PEG or surfactants and the simplicity of the experimental approach, physisorbed layers have drawbacks if a highly pure sample is required. For such situations, a covalently immobilized coating to prevent nonspecific binding would be advantageous.

Dendritic polyglycerol (dPG) is a versatile platform, which combines the ability to include specific functionalities for covalent attachment to surfaces or the immobilization of biomolecules with low intrinsic interaction to proteins [10,11]. It was shown that glass vials covalently coated by silylated dendritic polyglycerol (dPG) have a significantly reduced nonspecific adsorption of therapeutically relevant antibodies applied at high concentrations [12]. In addition, it was previously shown that a dPG based coating could render SPR gold sensors protein resistant [13]. In the present paper, the use of dPG coatings will be expanded in different directions. While covalent coatings have already been successfully used at high analyte concentration, it is important to show that such coatings can be used with solutions of low analyte concentration, too. In addition, we expanded the work on protein resistant dPG-layers on gold by incorporating carboxyl groups into the dPG to allow for further covalent immobilization of biomolecules, thereby, enabling its use as a matrix for biosensors. To demonstrate that the choice of an appropriate sample vial is essential for preventing false results caused by protein loss we chose SPR and quartz crystal microbalance (QCM) as complementary biosensor techniques, as both methods offer high sensitivity and real-time measurement capabilities. Although, SPR exhibited a higher absolute sensitivity and allowed to detect faster processes due to the smaller flow cell volume, QCM offered the opportunity to study a variety of surfaces such as glass, which renders both methods complementary for the purpose of this study [14]. To demonstrate the ability of carboxyl functionalized dPG to serve as a matrix and to show the impact of a protein resistant coating on sample vials we chose amyloid beta 1-40 (Aβ 1-40) and a corresponding monoclonal antibody 75-40 as model system. Amyloid beta peptides which are the main constituents of amyloid plaques associated with Alzheimer's disease and known to engage easily in non-specific interactions due to the amyloidogenic character, we chose as a highly relevant system for our study [15].

2. Experimental

2.1. Materials

All chemicals and solvents were reagent or HPLC grade, used as received, and purchased from Sigma–Aldrich (Steinheim, Germany) unless stated otherwise. Dialysis was performed in regenerated cellulose tubes from Spectrum Laboratories (Spectra/Por® 6 Dialysis membrane, molecular weight cut-off (MWCO) 1000 g mol^{-1} purchased from Roth, Karlsruhe, Germany). The deionized water used was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ cm. NaCl, NaOH and HCl were bought from VWR International (Darmstadt, Germany). *tert*-Butyl bromoacetate was bought from ABCR (Karlsruhe, Germany).

2.2. Methods

^{1}H and ^{13}C NMR spectra were recorded on a Jeol ECX spectrometer operating at 400 MHz and a Bruker Avance 3 operating at 700 MHz respectively at concentrations of 20–100 mg mL^{-1}. The chemical shifts are reported in δ (ppm) values and were referenced as indicated. IR spectra were recorded on a Nicolet Avatar 32 FT-IR with a Smart iTR accessory. Electrospray-ionization time-of-flight high-resolution mass spectrometry (ESI-TOF-HRMS) experiments were conducted on an Agilent 6210 ESI-TOF (Agilent Technologies). Surface-IR spectra were recorded on a Thermo Nicolet 8700 FT-IR. Gel permeation chromatography (GPC) experiments were carried out on an Agilent 1100 Series instrument equipped with refractive index detector. Pullulan standards have been used for calibration. The measurements were run in an aqueous sodium nitrate solution (0.1 M) as the eluent (1 mL min^{-1}, 25 °C), using three columns in-line, Suprema Lux 100, Suprema 1000, Suprema Lux 3000 (dimensions: 8 × 300 nm, particle size: 10 μm, PSS Mainz, Germany). SPR measurements were performed on a Biacore 3000 SPR system (GE Healthcare, Uppsala, Sweden). QCM measurements were performed on a Q-Sense E1 system (Q-Sense AB, Gothenburg, Sweden). Gold chips were cleaned with a UV/ozone ProCleaner™ (Bioforce Nanosciences, Ames, Iowa, US) equipped with a mercury vapor lamp which generates light in the range of 254 and 185 nm.

2.3. Synthesis

dPG (M_n 4.1 kDa, M_w 8.1 kDa) was synthesized by a one-step anionic ring-opening polymerization [16,17]. Triethoxy silyl modified methylated polyglycerol (Sil-dPG, **6**), that contains on average one triethoxy silyl moiety and used for rendering glass

surfaces protein resistant was synthesized via a five-step sequence as reported earlier [12]. First, 5% of the hydroxyl groups of dPG(OH) were converted into the corresponding mesylate with methanesulfonyl chloride, which in turn was converted into the azide by means of sodium azide. Methylation of the residual hydroxyl groups using methyl iodide and reduction of the azide groups with Pd/C yielded amine functionalized dPG(OMe), which was coupled to 3-(triethoxysilyl)propyl isocyanate via urea bond formation to yield triethoxysilyl modified dPG(OMe).

*2.3.1. tert-Butyl acetate functionalized dPG (**2**)*

dPG (4500 g mol^{-1}, 3 g, 0.67 mmol, 1 eq) was dissolved in DMSO (60 mL) and KOH (0.15 g, 2.68 mmol, 50 eq) was added under stirring. The resulting suspension was heated to 70 °C and stirred for 3 h. After addition of *tert*-butyl bromoacetate (2.61 g, 1.98 mL, 13.33 mmol, 20 eq) the mixture was stirred overnight. Subsequently, the reaction mixture was concentrated under reduced pressure, dissolved in MeOH, and centrifuged to separate the product from inorganic salts. In order to separate it the remaining salts and by-products the supernatant was dialyzed against MeOH/water (1:1) and dried under reduced pressure to yield a yellowish honey like product (2.9 g, 83%).

^{1}H NMR (700 MHz; MeOH-d$_4$): δ = 4.24–4.17 (m, 5H, CH_2COO*t*Bu); 4.07–4.01 (m, 5H, CH_2COO*t*Bu); 3.91–3.45 (m, 294H, dPG-backbone); 1.53–1.46 (s, 48H, CH_3 of *tert*-butyl); 1.42–1.37 (m, 2H, CH_2CH_3 of starter); 0.93–0.86 (m, 3H, CH_2CH_3 of starter) ppm. ^{13}C NMR (700 MHz; MeOH-d$_4$): δ = 171.7 ($CH_2COOC(CH_3)_3$); 83.1–82.8 ($CH_2COOC(CH_3)_3$); 81.8–81.3, 80.0–79.5, 74.0–73.8, 72.8–72.1, 70.8–70.6 (dPG backbone); 69.9 ($OCH_2COOC(CH_3)_3$); 64.4–64.3, 62.7–62.3 (dPG backbone); 28.4 ($CH_2COOC(CH_3)_3$); 23.6 (CH_2CH_3 of starter); 8.2 (CH_2CH_3 of starter) ppm.

*2.3.2. Carboxymethyl-dPG (CM-dPG) (**3**)*

2 (2 g) was dissolved in TFA (20 mL) under stirring. After 24 h, the solvent was removed under reduced pressure to yield **3** as solid compound (1.6 g, 90%).

^{1}H NMR (700 MHz; MeOH-d$_4$): δ = 3.99–3.47 (m, 307H, dPG-backbone, CH_2COOH); 1.40 (m, 2H, CH_2CH_3 of starter); 0.89 (m, 2H, CH_2CH_3 of starter) ppm. ^{13}C NMR (700 MHz; MeOH-d$_4$): δ = 177.6–176.8 (CH_2COOH); 81.3–80.9, 79.9–79.3, 74.0–73.6, 72.6–72.0 (dPG backbone); 71.3 (OCH_2COOH); 70.6–70.4, 64.4–64.3, 62.5–62.4 (dPG backbone); 23.7 (CH_2CH_3 of starter); 8.2 (CH_2CH_3 of starter) ppm.

*2.3.3. 1,2-Dithia-3-(1-amino-n-pentyl)cyclopentane (thioctic amine) (**4**) [18]*

(±)-α-Lipoamide (3 g, 14.6 mmol, 1 eq) was dissolved in dry THF (300 mL) in a round-bottom flask and a solution of $LiAlH_4$ (2.2 g, 58.4 mmol, 4 eq) in THF (150 mL) was added under stirring. The solution was heated to 70 °C and stirred for 12 h under argon atmosphere. After addition of water (30 mL), the solution was stirred at 0 °C for 1 h. The solvent was removed under reduced pressure, followed by the addition of methanol (50 mL). The insoluble solid was removed by centrifugation and the supernatant was concentrated under reduced pressure. The resulting residue was dissolved in water (300 mL) and treated with concentrated hydrochloric acid to adjust the pH of the solution to pH 6.5. The solution was stirred for 18 h at rt. Subsequently, the product was repeatedly extracted with 1-butanol and the solvent evaporated to give the crude product, which was dissolved in MeOH/water (1:1) and stirred under oxygen atmosphere for 6 d to reform the internal disulfide that was reduced during the reaction. After the solvent was removed under reduced pressure, the crude product was subjected to column chromatography on silica with chloroform/methanol/acetic acid (150:10:3 – 90:10:3) as an eluent to yield a yellow powdered solid (2.2 g, 79%).

^{1}H NMR (400 MHz; $CDCl_3$): δ = 3.59–3.52 (m, 1H, $CHCH_2CH_2SS$); 3.20–3.07 (m, 2H, $CHCH_2CH_2SS$); 3.01–2.98 (t, J = 7.4, 2H, NH_2-$CH_2CH_2CH_2CH_2CH_2$); 2.49–2.41 (m, 1H, $CHCH_2CH_2SS$); 1.94–1.85 (m, 1H, $CHCH_2CH_2SS$); 1.84–1.74 (m, 2H, $NH_2CH_2CH_2CH_2CH_2CH_2$); 1.73–1.59 (m, 2H, $NH_2CH_2CH_2CH_2CH_2CH_2$); 1.53–1.39 (m, 4H, $NH_2CH_2CH_2CH_2CH_2CH_2$) ppm. ^{13}C NMR (700 MHz; $CDCl_3$): δ = 56.5 ($CHCH_2CH_2SS$); 40.4 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 40.1 ($CHCH_2$-CH_2SS); 38.6 ($CHCH_2CH_2SS$); 34.7 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 28.8 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 27.5 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 26.4 ($NH_2CH_2CH_2CH_2CH_2CH_2$) ppm. MS (ESI-ToF): m/z calculated for $C_8H_{18}S_2N$ [MH$^+$] 192.0881, found 192.0888.

*2.3.4. Thiocticamine functionalized CM-dPG (**5**)*

3 (1.2 g, 0.27 mmol, 1 eq) was dissolved in water (20 mL) under stirring to which 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC × HCl) (0.1 g, 0.53 mmol, 2 eq) and *N*-hydroxysuccinimide (NHS) (0.06 g, 0.53 mmol, 2 eq) was added. After 10 min, a solution of **4** (0.1 g, 53 mmol, 2 eq) in methanol (10 mL) was added and the reaction mixture was stirred for 3 h. Subsequently, the reaction mixture was dialyzed against water/methanol (3:1) and the solvent evaporated to yield **5** as yellowish solid (1.0 g, 83%).

^{1}H NMR (700 MHz; MeOH-d$_4$/D$_2$O (1:1)): δ = 3.97–3.52 47 (m, 310H, dPG-backbone, OCH_2COOH, $CHCH_2CH_2SS$); 3.21–3.20 (t, J = 6.6, 2H, $CH_2CONHCH_2CH_2$); 3.14–3.10 (m, 2H, $CHCH_2CH_2SS$); 2.53–2.45 (m, 1H, $CHCH_2CH_2SS$); 1.98–1.94 (m, 1H, $CHCH_2CH_2SS$); 1.79–1.73 (m, 1H, $NH_2CH_2CH_2CH_2CH_2CH_2$); 1.65–1.60 (m, 1H, $NH_2CH_2CH_2CH_2CH_2CH_2$); 1.58–1.53 (m, 2H, $NH_2CH_2CH_2CH_2CH_2CH_2$); 1.48–1.43 (m, 2H, CH_2CH_3 of starter); 1.42–1.34 (m, 4H, $NH_2CH_2CH_2CH_2CH_2CH_2$); 0.88 (m, 2H, CH_2CH_3 of starter) ppm. ^{13}C NMR (700 MHz; MeOH-d$_4$/D$_2$O (1:1)): δ = 177.2–176.4 (CH_2COOH); 174.1–173.1 ($CH_2CONHCH_2$); 81.1–80.5, 79.5–79.2, 73.5–73.2, 72.1–71.5 (dPG backbone); 71.0 (OCH_2COOH); 70.3–70.0, 63.8–63.6, 62.1–61.9 (dPG backbone); 41.4–41.3 ($CH_2CONHCH_2$); 39.8–39.7 ($CHCH_2CH_2SS$); 39.3–39.2 ($CHCH_2CH_2SS$); 35.5–35.3 ($CH_2CONHCH_2CH_2CH_2CH_2CH_2$); 29.8–29.5 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 27.3–27.1 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 26.3–26.2 ($NH_2CH_2CH_2CH_2CH_2CH_2$); 23.2 ($CH_2CH_3$ of starter); 8.1 (CH_2CH_3 of starter) ppm.

2.4. Coatings

2.4.1. Monolayer formation on glass

Borosilicate vials (d = 9 mm) were coated with triethoxysilyl modified dPG(OMe) according to a procedure described previously [12]. In short, the vials were cleaned by immersion into piranha solution (H_2SO_4/H_2O_2 = 3:1), rinsed with Milli-Q water, and subsequently coated by filling the vials to the neck with an ethanolic solution of triethoxysilyl modified dPG(OMe) and acetic acid as catalyst. The filled vials were kept at 40 °C for 8 h before rinsing with Milli-Q water and drying in a stream of N_2.

2.4.2. Monolayer formation on gold

Prior to monolayer formation, SPR and quartz crystal sensors were cleaned by treating with a UV/ozone cleaner for 10 min. Subsequently, the chips were immersed for 20 min in pure EtOH to reduce the gold oxide which formed in the previous step [19], thoroughly rinsed with EtOH, and dried in a stream of N_2. Monolayers of **5**, mercaptoundecanoic acid (MUA) and mercaptohexadecanoic acid (MHDA) were prepared by immersing gold chips in a 0.5 mM aqueous solution of **5** for 30 min and in a 3 mM ethanolic solution of MUA/MHDA for 48 h, respectively.

2.5. Ellipsometry

Ellipsometry thickness measurements of CM-dPG coated sensors were carried out on a SENpro ellipsometer (SENTECH

Instruments GmbH, Germany) at variable angles between 55 and 70° in the spectral range of 380–930 nm and fitted using the analysis software SpectraRay/3. The reported values are the average of three measurements. The applied model contains two unknowns, the thickness of the coating and its refractive index. Whereas the RI = 1.473 was taken from literature [20], the thickness was determined based on a Cauchy relationship.

2.6. QCM

In order to test the potential loss of an antibody due to nonspecific adsorption to glass surfaces, QCM measurements (Q-Sense E1, Q-Sense AB, Gothenburg, Sweden) were applied using SiO_2 coated quartz crystal sensors as a model system. Before testing nonspecific antibody adsorption injection tubes and flow cell were passivated by nonspecific BSA adsorption. Antibody adsorption was tested under static conditions according to the following protocol: (i) injection of PBS with a flow rate of 0.1 mL min^{-1} until the signal became stable; (ii) injection of antibody 75-40 with a flow rate of 1.0 mL min^{-1} until the volume of the flow cell was fully exchanged; afterwards the flow was stopped; and (iii) injection of PBS after the signal due to antibody adsorption reached a steady state. dPG(OMe) coated glass vials were used as a container for the antibody solutions. Since the energy dissipation was small compared to the frequency shift, a linear relationship between frequency shift and mass of the adsorbate, namely, the Sauerbrey relation was applied for calculating the mass of the respective adsorbates ($\Delta m = C \times \Delta f$, with Δm as the change in mass, C a sensor dependent constant (-17.7 ng cm^{-2} Hz^{-1}), Δf as the frequency change divided by its overtone).

2.7. SPR spectroscopy

2.7.1. Nonspecific protein adsorption

Nonspecific protein adsorption experiments using fibrinogen, BSA, pepsin, and lysozyme were carried out on a Biacore 3000 (GE Healthcare) under dynamic conditions according to the following protocol with a flow rate of 10 μL min^{-1}: (i) PBS (10 μL); (ii) protein (1 mg mL^{-1}, 250 μL); and (iii) PBS (30 μL). Tested proteins are fibrinogen, pepsin, albumin, and lysozyme.

2.7.2. Specific binding assay

In order to evaluate the impact of a protein resistant vial coating on the outcome of an SPR immuno assay both coated and uncoated glass vials were used as a container for the analyte solution. Prior to the actual immunoassay, amyloid beta 1-40 was immobilized according to the following procedure with a flow rate of 5 μL min^{-1}: (i) EDC (0.2 M), NHS (0.05 M) in Milli-Q water (25 μL); (ii) Milli-Q water (10 μL); (iii) amyloid beta 1-40 (50 μg mL^{-1}) in aqueous acetic acid (10 mM, pH 4.3); (iv) Milli-Q water (10 μL); (v) ethanolamine (1 M) in Milli-Q water (25 μL); and (vi) Milli-Q water (25 μL). Specific binding of anti-amyloid beta 1-40 to immobilized amyloid beta 1-40 was tested with a flow rate of 20 μL min^{-1} according to following protocol: (i) PBS (20 μL); (ii) anti-amyloid beta 1-40 (100 μL) (1–50 μg mL^{-1}); (iii) PBS (200 μL); (iv) HCl (20 mM, 20 μL); (v) PBS (20 μL); (vi) HCl (20 mM, 20 μL); and (vii) PBS (20 μL). All measurements are reference compensated by subtracting the signal of a parallel reference channel without ligand. Analyte solution was withdrawn alternately from uncoated and coated vials. The relative signal loss was calculated according to $1 - RU_{uncoated}/RU_{coated}$ ($RU_{uncoated}$ = maximum signal of analyte from uncoated vial, RU_{coated} = maximum signal of analyte from coated vial) for consecutive measurements starting with the uncoated vials.

2.8. Contact angle

Static water contact angles were determined by applying the sessile drop method. In brief, a drop of Milli-Q water (2 μL) was placed on bare and CM-dPG(OMe) coated gold sensors and allowed to equilibrate for 30 s. The given contact angles are the mean of 5 measurements applying a Young–Laplace fitting to the contour of the drop according to $\Delta p = \sigma \times (1/r_1 + 1/r_2)$, where Δp is the Laplace pressure, σ is the surface tension, and r_1 and r_2 are the radii of curvature. Contact angle measurements were carried out on a Dataphysics Contact Angle System OCA (DataPhysics Instruments) and evaluated using the software package SCA202 version 3.12.11.

3. Results and discussion

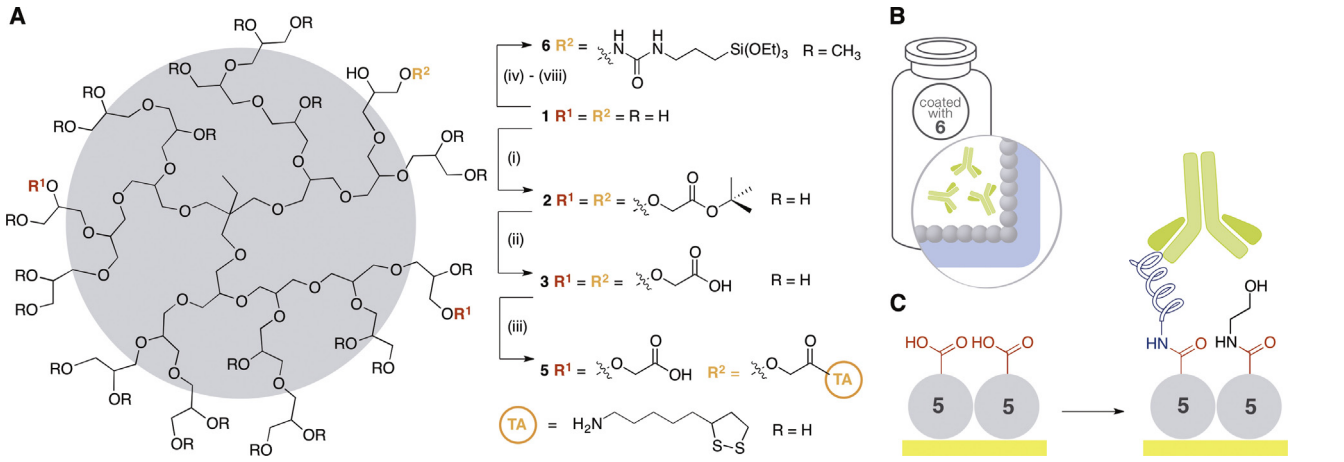
3.1. Synthesis and characterization

To realize our idea of a protein resistant sensor and sample vial surface, two coatings starting from dendritic polyglycerol dPG were synthesized. Synthesis and characterization of dPG has been published elsewhere [16,17] In short, dPG was synthesized via a one-step anionic ring-opening polymerization employing trimethylol propane as starter. For the sensor surface coating, carboxyl groups were chosen as the functional moiety to enable ligand immobilization. Carboxyl groups were incorporated into the dPG by partial etherification of the hydroxyl groups of dPG with *tert*-butyl protected bromoacetic acid followed by acidic deprotection as illustrated in Scheme 1. For grafting carboxymethylated dPG (CM-dPG) to the gold surface of the SPR sensor, thioctic amine was used as linker moiety. Therefore, commercially available thioctic amide was reduced by $LiAlH_4$ and subsequently coupled to CM-dPG via amide bond formation to yield **5** with statistically one thioctic moiety. End-group modifications were confirmed by ^{1}H NMR and ^{13}C NMR, whereas molecular weights were determined by gel permeation chromatography. Mercaptoundecanoic acid (MUA), mercapto-hexadecanoic acid (MHDA) on gold as well as commercially available carboxymethyl dextran coated gold chips were used as control surfaces. The compound used for coating sample vials, Sil-dPG (**6**) was synthesized as reported earlier [12].

3.2. Preparation of monolayers and characterization

Both CM-dPG and Sil-dPG were applied to the respective substrate in a simple one step procedure to form a coating. CM-dPG monolayers were prepared by immersing cleaned gold substrates into an aqueous solution of **5** for 60 min. Coating of **5** was confirmed by ellipsometry and contact angle measurements. We measured the thickness of a dry monolayer of CM-dPG on gold via ellipsometry to be 1.23 ± 0.09 nm using optical constants determined by Yeh et al. [20]. This is in good agreement with a previously determined radius of gyration of 1.49 nm for dPG in acetone, a poor solvent where the system was expected in an almost collapsed state [21]. Water contact angle measurements also indicated an efficient coating. Whereas a bare gold surface exhibited a water contact angle of $71.3 \pm 2.4°$ gold substrates coated with **5** showed a water contact angle of $24.5 \pm 3.3°$, which was in good accordance with contact angles obtained for dPG (20 ± 3) [13]. MUA and MHDA were chosen as reference coatings since they are commercially available, easy to apply and have shown good results with respect to protein resistance in biosensors [5,22]. SAMs of MUA and MHDA showed contact angles of 43.9 ± 2.3 and 59.6 ± 1.8 for MUA and MHDA, respectively, revealing the hydrophobicity of these compounds.

For the glass coating, dPG(OMe) was preferred to dPG(OH) which has a shorter shelf life due to the high amount of hydroxyl



Scheme 1. (A) Synthesis of carboxymethylated dendritic polyglycerol (CM-dPG, **5**) and Sil-dPG (**6**) starting from dendritic polyglycerol (1): (i) KOH, DMSO, *tert*-butyl bromoacetate, 70 °C; (ii) TFA, rt; (iii) NHS, EDC, thiocticamine in MeOH, H_2O, rt; (iv) methanesulfonyl chloride, pyridine, 0 °C; (v) NaN_3, DMF, 60 °C; (vi) KOH, DMSO, MeI, rt; (vii) Pd/C, H_2, MeOH, rt; and (viii) TEA, 3-(triethoxysilyl)propyl isocyanate. Thiocticamine was synthesized by reduction of thiocticamide by means of $LiAlH_4$ followed by oxidation of thiol groups to the corresponding dithiolane under O_2 atmosphere. (B) Borosilicate vial covalently coated with **6** and filled with analyte solution. (C) SPR sensor covalently coated with **5**.

groups causing a hydrolysis of the alkoxysilyl groups. The coating was applied and characterized as reported earlier [12].

3.3. Nonspecific adsorption to SPR sensors

The key feature of the coatings for their intended use in protein assays is their ability to render the surface resistant to nonspecific protein adsorption [23,24]. CM-dPG (**5**), MUA, and MHDA were selected as potential immobilization matrices for the SPR sensor [5,25,26]. Since CM-dextran has been shown to prevent the adsorption of proteins, commercial CM5 sensor chips were used as control [27,28]. Nonspecific protein adsorption was tested with four proteins differing in molecular mass and isoelectric point (pI): fibrinogen (Fib 340 kDa, pI 5.5), albumin (BSA, 65 kDa, pI 4.8), lysozyme (Lys 14 kDa, pI 11.0), and pepsin (Pep 34 kDa, pI 2.0). In label-free assays monitoring the integral change at the surface such as SPR, protein resistant coatings are essential, since nonspecific and specific binding events both contribute to the measured signal. To account for nonspecific binding (as well as signals arising from changes in the properties of the solution) reference-compensated measurements are typically performed, i.e., a second channel of the sensor surface lacking the ligand is run in parallel and subsequently subtracted [29]. This approach can account for nonspecific adsorption to the immobilization matrix, but neglects the nonspecific contributions of the ligand [30]. Therefore, knowledge of nonspecific binding to the ligand is essential. We tested the impact of the chosen ligand amyloid beta 1-40 on nonspecific protein adsorption using the same set of proteins. Amyloid beta 1-40 was immobilized using EDC and NHS chemistry with a slightly reduced pH to allow for a preconcentration of the peptide at the carboxyl groups of the matrix [27].

Fig. 1 shows representative SPR-time traces that were obtained by injecting a solution of fibrinogen. Two classes of surfaces could be discriminated. The bare gold, MUA and MHDA coated sensors exhibited a strong increase in the response signal, indicating a large amount of adsorbed protein. Characteristic differences were observed for these systems, in particular, films containing longer alkyl chains showed an increased adsorption. This is in line with observations by Choi et al. for mercaptopropionic and mercaptoundecanoic acid films of polycrystalline gold, which were attributed to the structural heterogeneity of the gold surfaces causing significant disorder of alkyl chains [22]. On the other hand, adsorption to dPG, CM-dPG, CM-dextran coated sensors resulted in approximately one order of magnitude smaller signals indicating a reduction in the amount of adsorbed fibrinogen. Since the protein resistance of the matrix itself was being tested, no reference-compensation was applied. Therefore, a fast signal drop after injection of PBS was observed, which could be attributed to effects within the bulk of the solvent, i.e., the difference in the refractive index of the fibrinogen solution and the running buffer. A closer look reveals that the signal increase after injection of the fibrinogen solution was not caused by fibrinogen adsorption but was mainly due to bulk effects, i.e., the difference in the refractive index of the fibrinogen solution and the running buffer. The inset further shows that the incorporation of carboxyl groups into dPG caused a twofold increase in the nonspecific fibrinogen adsorption.

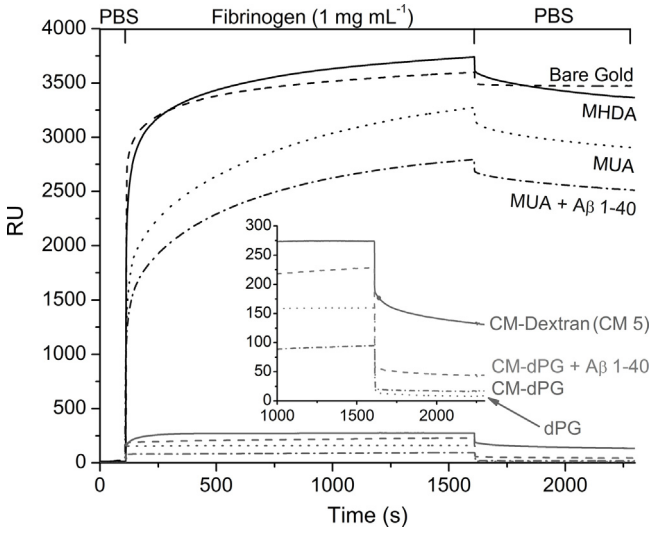


Fig. 1. SPR sensorgrams of nonspecific fibrinogen adsorption on coated and bare sensor chips. Prior and subsequent to injection of fibrinogen, phosphate buffered saline (PBS) was injected into the flow cell. Time points for the respective injections are given as vertical bars at the top of the plot. The adsorption time (1500 s) of fibrinogen was limited by the injection volume of 250 μL for the flow rate of 10 μL min^{-1}. Fabricated CM-dPG (**5**) coating was compared to a commercial CM-dextran sensor chip, a mercaptoundecanoic acid (MUA) coated sensor, and a mercaptohexadecanoic acid (MHDA) coated sensor. The inset shows an enlargement of the sensorgrams at the bottom of the plot.

Table 1
Nonspecific protein adsorption on coated and bare SPR sensor chips given in % relative to the adsorbed amount of the respective protein on a bare gold surface.

Protein	CM-dPG (**5**)		MUA		MHDA	CM-dextran	Gold
	w/o Aβ 1-40	w Aβ 1-40[a]	w/o Aβ 1-40	w Aβ 1-40[a]			
Fibrinogen	0.5 ± 0.1	1.9 ± 0.2	69.4 ± 9.1	62.9 ± 6.5	87.8 ± 10.7	3.3 ± 0.3	100.0 ± 13.0
Pepsin	0.9 ± 0.1	2.0 ± 0.3	7.7 ± 1.1	2.1 ± 0.3	30.0 ± 4.1	1.3 ± 0.2	100.0 ± 17.0
Albumin	0.8 ± 0.1	1.4 ± 0.2	5.9 ± 1.1	2.7 ± 0.4	39.1 ± 5.6	1.9 ± 0.3	100.0 ± 18.7
Lysozyme	0.5 ± 0.1	1.3 ± 0.1	15.1 ± 1.0	4.7 ± 0.5	67.6 ± 6.0	1.4 ± 0.1	100.0 ± 4.4

[a] For CM-dPG and MUA coated surfaces relative amounts of adsorbed proteins after immobilization of Aβ 1-40 are given additionally.

Furthermore, one can observe that CM-dPG even outperformed CM-dextran with respect to fibrinogen adsorption, though part of the adsorbed fibrinogen was reversible bound as can be seen from the slow decrease in the signal after injection of PBS. The fact that a CM-dPG coating rendered the sensor surface resistant against nonspecific adsorption of fibrinogen, a very sticky plasma protein, to an even larger extent than commercial CM-dextran coatings enables its use as coating for an immunoassay. In comparison to the other tested proteins, this difference between CM-dPG and CM-dextran was less pronounced (Table 1). An advantage of CM-dextran over CM-dPG is the larger coating thickness of up to 100 nm which offers an increased loading capacity.

In the next step, we tested the impact of amyloid beta 1-40 immobilization on nonspecific protein adsorption. As the values in Table 1 and Fig. 1 show, immobilization of amyloid beta (Aβ) 1-40 on CM-dPG resulted in an increased nonspecific fibrinogen adsorption by almost a factor of 4. These results indicate the high hydrophobicity of this trans membrane protein fragment, as one of a series of amyloid beta fragments, which are the main constituent of the senile plaques found in Alzheimer's disease [31]. In the case of MUA, the results were quite contrary, because fibrinogen adsorption decreased upon immobilization of Aβ 1-40, and showed the higher hydrophobicity of MUA compared to Aβ 1-40. With immobilized Aβ 1-40, protein adsorption was still too high on MUA, to be suitable for a protein assay. Despite the increase in nonspecific protein binding, CM-dPG still showed excellent protein resistant properties. The same trend could be observed for the other proteins on CM-dPG and MUA coated surfaces. But significant differences were found in terms of the quantitative changes (Table 1). Based on the results discussed above, CM-dPG coatings functionalized with Aβ 1-40 were further used as the active surface of the protein assay. Due to the tendency of Aβ 1-40 to increase nonspecific binding the amount of immobilized ligand was chosen as small as possible in subsequent experiments (approximately 1 RU).

3.4. Nonspecific adsorption to glass

The excellent protein resistant properties of dPG(OMe) coated glass surfaces were shown by Höger et al. [12], who demonstrated that dPG(OMe) coated glass vials could be used to store therapeutic relevant proteins in high dosage forms with only a minor loss due to nonspecific adsorption. Furthermore, we have recently shown via QCM measurements that dPG(OMe) can resist the adsorption of fibrinogen. Experiments, however, have only been done with high concentrations of the protein in the range of 1–2 mg mL^{-1}. Although, the reduction of nonspecific adsorption was highly significant, the percentage loss of antibody due to nonspecific adsorption to the SiO_2 surface was rather small. We defined the loss as the adsorbed mass of the antibody divided by the initially present mass in the flow cell (loss = m_{ads}/m_0). In order to evaluate the concentration-dependent percentage loss in a glass container, we selected QCM and SiO_2 coated quartz crystal sensors as the model system. QCM offered a considerably higher sensitivity and higher accuracy than the size exclusion chromatography we applied in a recent study to investigating a non-planar surface like in glass vials. With QCM we were able to measure nonspecific adsorption of proteins with concentrations of 75-40 down to 2 μg mL^{-1}. This QCM experiment is intended to serve as a control for the protein assay. In order to model a container, the QCM experiment was performed under static conditions. Therefore, the volume above the crystal sensor was fully exchanged by the antibody solution of known concentration and the flow instantly stopped to determine the loss of antibody due to nonspecific adsorption in a closed system.

Fig. 2 shows the nonspecific sorption isotherm for 75-40, a monoclonal antibody against Aβ 1-40, on a bare SiO_2 surface measured via QCM (see also Table 2). The plotted values represent the steady state adsorption for the respective antibody concentration. Based on these results we calculated the percentage of protein loss, which is given in Fig. 3. Since the dissipated energy was small, we calculated the amount of adsorbed antibody by applying the Sauerbrey equation, which directly relates the frequency change to the adsorbed mass [32,33]. Calculated masses, however, refer here to the wet mass, i.e., coupled and entrapped water have to be taken into account [34,35]. Therefore, we subtracted 59% of the mass, which was the literature value for the water content of an adsorbed IgG layer [34]. The obtained mass was divided by the total antibody mass that was initially present in the flow chamber above the quartz crystal sensor to give the loss of antibody upon adsorption. As can be seen from Fig. 3, the percentage loss of 75-40 due to

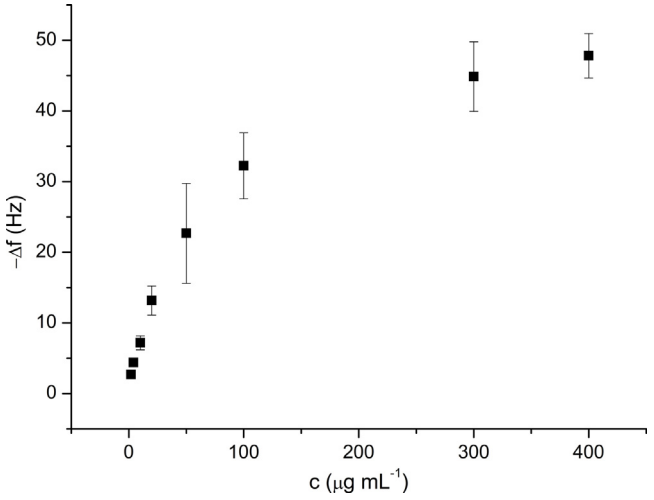


Fig. 2. Nonspecific antibody (anti-Aβ 1-40, 75-40) adsorption as a function of antibody concentration obtained by QCM measurements. Normalized frequency changes of the third overtone divided by three as a function of the injected concentrations. Antibody solution was injected into the flow cell until the volume was fully exchanged. Subsequently the flow was stopped. This means that the nonspecific adsorption was measured under static conditions to simulate a closed system like a sample vial.

Table 2
QCM data obtained from adsorption experiments of antibody 75-40 to an SiO_2 coated quartz crystal sensor.

c_0[a]	m_0 in flow cell (ng)[b]	$-\Delta f$ (Hz)[c]	Adsorbed mass (ng) from the observed frequency change[d]	Mass of adsorbed antibody (ng)[e]	%Loss due to adsorption[f]
2	80	2.7 ± 0.3	37.7 ± 3.7	15.5 ± 1.5	19.3 ± 1.9
4	160	4.4 ± 0.6	61.2 ± 8.2	25.1 ± 3.4	15.7 ± 2.1
10	400	7.2 ± 1.0	99.6 ± 13.7	40.8 ± 5.6	10.2 ± 1.4
20	800	13.2 ± 2.1	182.8 ± 28.5	75.0 ± 11.7	9.4 ± 1.5
50	2000	22.7 ± 7.1	315.2 ± 98.2	129.2 ± 40.3	6.5 ± 2.0
100	4000	32.2 ± 4.7	448.0 ± 65.0	183.7 ± 26.6	4.6 ± 0.7
300	12,000	44.9 ± 4.9	623.3 ± 68.5	255.6 ± 28.1	2.1 ± 0.2
400	16,000	47.8 ± 3.1	664.3 ± 43.6	272.4 ± 17.9	1.7 ± 0.1

[a] Concentration of antibody solution prior to injection.
[b] Antibody mass in flow cell at the beginning of the measurement was calculated with a flow cell volume of 40 μL and the given concentration c_0.
[c] Frequency change of the third overtone divided by three upon adsorption of antibody at equilibrium.
[d] The calculated mass includes coupled and trapped water.
[e] Mass of adsorbed antibody which was calculated by subtracting 59% of the mass for the water in the adsorbate layer.
[f] %Loss was calculated by dividing column 5 by column 2.

nonspecific adsorption increased with decreasing concentration. The lowest concentration we measured in this experiment was 2 μg mL^{-1} as in the case of 1 μg mL^{-1} the observed signal was close to the detection limit. For an initial concentration of 2 μg mL^{-1}, we calculated a percentage loss of almost 20%. At 400 μg mL^{-1}, the upper limit of our measurement range, the percentage loss was below 2%. These results indicate that the protein recovery rate of low concentration protein solutions can be considerably increased by coating glass vials with dPG(OMe). This is particularly important when handling proteins for bioanalytic assays which are in general highly diluted samples in the concentration range of μg mL^{-1} or lower. On the other hand, when dealing with high protein concentrations like during storage, nonspecific adsorption in terms of protein loss might be negligable [12].

3.5. Antibody loss in coated and uncoated vials

In the preliminary experiment, we demonstrated that the percentage loss of the antibody 75-40 due to nonspecific adsorption to glass surfaces was concentration dependent. We therefore postulate that, when applied as a sample container in protein assays, glass vials will cause a significant signal loss and thereby give false results. To show the effect of a protein resistant coating on an immuno assay outcome, uncoated and dPG(OMe) coated vials were used as containers of analyte solution for the SPR experiments. For proving our hypothesis antibody solutions with concentrations ranging from 1 to 50 μg mL^{-1} were prepared in dPG (OMe) coated glass vials and subsequently distributed to an uncoated and a dPG(OMe) coated glass vial, respectively. After filling the final sample vials with the antibody solution they were kept at room temperature for 6 h in the auto sampler before the measurement was started.

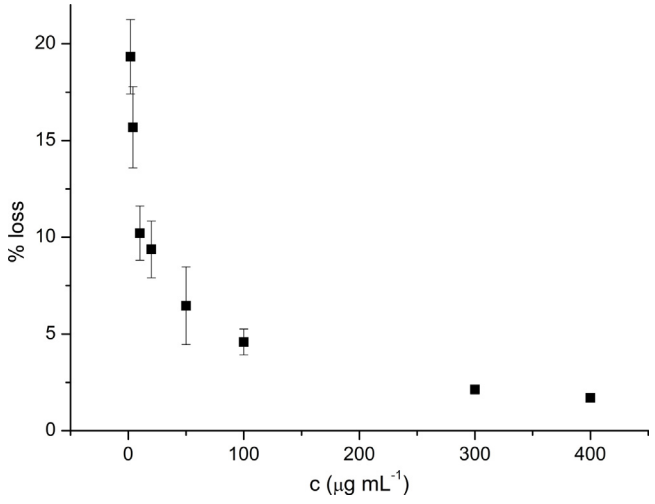
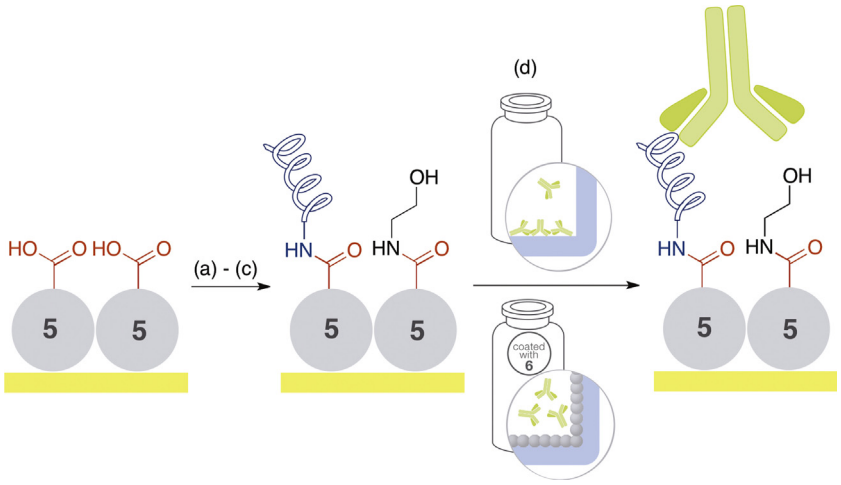


Fig. 3. Percentage loss of antibody (75-40) due to adsorption to a SiO_2 surface calculated from the QCM measurements. The adsorbed amount of antibody was determined by subtracting the mass for the coupled water in the adsorbate. The percentage loss was calculated according to loss = m_{ads}/m_0, where m_{ads} is the mass of adsorbed antibody measured by QCM, and m_0 is the mass of antibody present in the flow cell at the beginning of the measurement, which was determined prior to injection. Values are given in percent.

The SPR sensor surface was coated with **5** and only a minimum amount of Aβ 1-40 was immobilized to keep a similar level of nonspecific adsorption in the measurement and reference channel (Scheme 2). Analyte solution was injected alternately from uncoated and coated vials. Regeneration of the ligand after each measurement cycle was achieved by injecting short pulses of HCl (20 mM). Fig. 4 shows representative SPR-time traces for the binding of 75-40 to immobilized Aβ 1-40 at various concentrations. Dotted lines are the response curves obtained from the analyte solution stored in uncoated vials and solid lines from analyte solutions stored in Sil-dPG coated vials. Systematically, the maximum SPR signal for coated vials was higher than for uncoated vials. Because of the concentration dependence of the nonspecific binding [36], these results demonstrate that the protein concentration of solutions stored in coated vials was considerably higher, which can be explained by a suppression of nonspecific protein adsorption even at low concentrations.

From these SPR curves, we determined the percentage of signal loss in the uncoated vials by calculating the ratio of the signal of two successive measurements at the highest point of the curve, subtracting the result from 1 and multiply it by 100. In Fig. 5, the resulting values are plotted against the concentration of the analyte solution (see also Table 3). These findings support our hypothesis that nonspecific protein adsorption can cause a significant signal loss and thereby yield inaccurate or even false negative results in particular for low protein concentrations. In contrast to QCM measurements, SPR measurements were performed at slightly lower antibody concentrations, because of the lower absolute sensitivity of SPR for a given immobilized amount of Aβ 1-40, which was kept as low as possible in order to reduce nonspecific adsorption. At the lowest concentration, 1 μg mL^{-1}, the percentage signal loss was 23% which is close to the value obtained in QCM measurements for 2 μg mL^{-1} (19%). Please note that the surface-to-volume ratio for the vial and the volume above the QCM-crystal differs by a factor of 3. Whereas the glass vials exhibit a surface-to-volume ratio of 5.9 cm^2 mL^{-1} for the filled height, QCM exhibits a surface-to-volume ratio of 19.6 cm^2 mL^{-1}.



Scheme 2. SPR protein assay: SPR sensor coated with **5**, followed by immobilization of Aβ 1-40 and binding of anti-Aβ 1-40 (75-40) to immobilized Aβ 1-40 according to the following protocol: (a) EDC (0.2 M)/NHS (0.05 M) in Milli-Q water; (b) Aβ 1-40 (50 μg mL^{-1}) in aqueous acetic acid (10 mM, pH 4.3); (c) ethanolamine (50 μg mL^{-1}) in aqueous acetic acid (10 mM, pH 4.3); and (d) alternate injection of analyte solution (75-40) from uncoated and coated sample vials, respectively.

If we combine these consideration with a simple proportionality between coverage and the surface area of the adsorbent, the determined percentage loss between QCM and SPR correlate fairly well with the expectations. Hence, we can conclude that the QCM measurements shown here can be considered as a viable model for a closed container system.

It should be kept in mind that in the present SPR experiment a protein resistant coating was used only for the vials. All other surfaces such as the tubing of the auto sampler were not passivated. As the nonspecific adsorption is found to increase with increasing concentration the effect of surfaces showing nonspecific adsorption led to a stronger loss of the solutions with high protein concentration from the protected vials [36]. Therefore, the effect observed here will be a lower bound of the effects in the vials.

Sil-dPG itself may also be applied as immobilization matrix on metal oxide or silicon surfaces as used in resonant mirror and

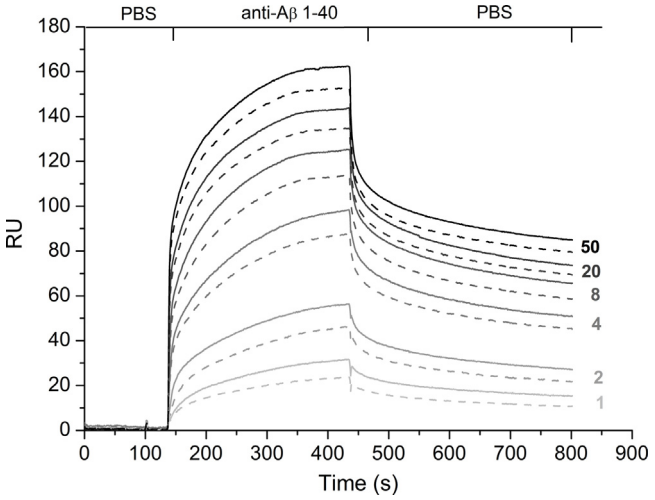


Fig. 4. Representative SPR sensorgrams of 75-40 (anti-Aβ 1-40) binding to immobilized Aβ 1-40. Antibody was alternately injected from uncoated (dashed line) and dPG(OMe)-Sil coated (solid line) glass vials and injected into the flow cell. Time points for the respective injections are given as a vertical bar at the top of the plot. Numbers written beside curves are the respective antibody concentrations in μg mL^{-1}.

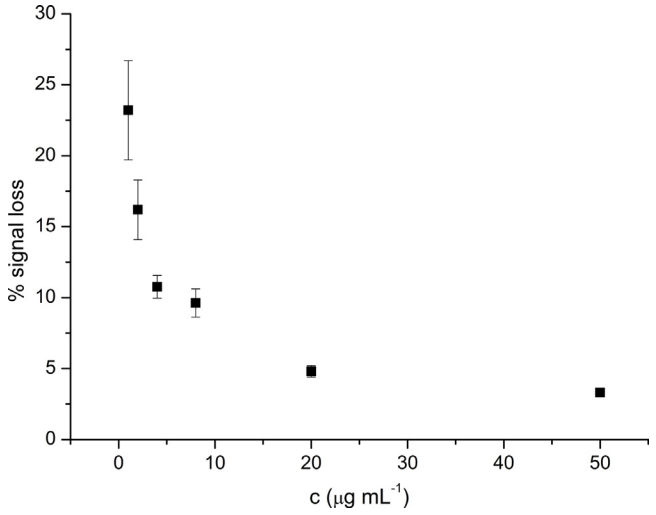


Fig. 5. Percentage signal loss due to adsorption of antibody to the sample vial calculated from the maximum response value of the respective SPR sensorgram. The percentage signal loss was calculated according to signal loss = 1 − $RU_{uncoated}$/RU_{coated}, where $RU_{uncoated}$ and RU_{coated} are the signals obtained for a binding event of antibody taken from an uncoated vial and a coated vial, respectively.

Table 3
SPR data obtained from SPR binding experiment of anti-Aβ 1-40 to immobilized Aβ 1-40.

c antibody (μg mL^{-1})	$RU_{uncoated}$[a]	RU_{coated}[b]	%Loss[c]
1	26.9 ± 3.3	34.9 ± 3.2	23.2 ± 3.5
2	44.9 ± 3.4	53.7 ± 5.7	16.2 ± 2.1
4	84.7 ± 3.6	93.3 ± 5.4	10.8 ± 0.8
8	105.9 ± 9.0	117.1 ± 7.6	9.6 ± 1.0
20	135.3 ± 7.2	142.1 ± 8.2	4.8 ± 0.4
50	150.5 ± 3.2	155.7 ± 7.1	3.3 ± 0.2

[a] SPR response after 5 min of injection of antibody taken from uncoated vial.
[b] SPR response after 5 min of injection of antibody taken from coated vial.
[c] %Loss = 1 − $RU_{uncoated}$/RU_{coated}.

microring resonance biosensors, respectively. Therefore, a further transformation of residual hydroxyl groups in dPG to reactive functional groups such as amine or carboxyl groups would be necessary.

4. Conclusion

Both coatings tested in this study performed very well in their respective application area. CM-dPG, a newly synthesized polyglycerol derivative, which exhibited a superior protein resistance compared to commercially available CM-dextran, was shown to be ideally suited as matrix for protein assays. Sil-dPG showed considerably increased protein recovery rates of low concentration protein solutions. We could demonstrate that especially at low concentrations nonspecific adsorption in sample vials can cause a severe loss of protein. By coating the sample vials with Sil-dPG we could considerably reduce the percentage protein loss due to nonspecific adsorption. Therefore, coating of sample vials is essential to reduce the risk of false negative results in protein assays due to the loss of analyte. For borosilicate glass, which is one of the most commonly used material for the handling of proteins in bioanalytical assays, Sil-dPG offers an easily applicable system, therefore being an excellent alternative to blocking agents like BSA, in particular for assays with tight requirements for purity of the sample. In summary, our results demonstrate that a protein resistant assay surface is not sufficient to obtain accurate results. Instead coating of the handling containers needs to be considered in the assay routine to ensure highly accurate assay outcomes.

Acknowledgements

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