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ORIGINAL PAPER

Characterisation of morphology of self-assembled PEG monolayers: a comparison of mixed and pure coatings optimised for biosensor applications

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Abstract For detection of low concentrations of analytes in complex biological matrices using optical biosensors, a high surface loading with capture molecules and a low nonspecific binding of nonrelevant matrix molecules are essential. To tailor biosensor surfaces in such a manner, poly(ethylene glycols) (PEG) in varying lengths were immobilised covalently onto glass-type surfaces in different mixing ratios and concentrations, and were subsequently modified with three different kinds of receptors. The nonspecific binding of a model protein (ovalbumin, OVA) and the maximum loading of the respective analytes to these prepared surfaces were monitored using label-free and time-resolved reflectometric interference spectroscopy (RIfS). The three different analytes used varied in size: 150 kDa for the anti-atrazine antibody, 60 kDa for streptavidin and 5 kDa for the 15-bp oligonucleotide. We investigated if the mixing of PEG in different lengths could increase the surface loadings of analyte mimicking a three-dimensional matrix as was found using dextrans as sensor coatings. In addition, the effect on the surface loading was investigated with regard to the size of the analyte molecule using such mixed PEGs on the sensor surface. For further characterisation of the surface coatings, polarisation modulation infrared reflection absorption spectroscopy, atomic force microscopy, and ellipsometry were applied.

Keywords Surface chemistry · Poly(ethylene glycol) · Reflectometric interference spectroscopy (RIfS) · Ellipsometry · Biosensor · Atomic force microscopy (AFM) · Polarisation modulation infrared reflection absorption spectroscopy (PM-IRRAS)

Introduction

Poly(ethylene glycol) (PEG) is a material of growing importance not only in the bioengineering world but also in the fields of drug delivery, pharmaceuticals, biotechnology and biosensing applications [1, 2]. Owing to its high resistance to protein adsorption in aqueous media, PEG is used as coatings for biomedical devices e.g. for implants where protein adsorption onto the surface leads to deleterious responses like thrombosis and embolism [3]. Additionally, its nonfouling, nonimmunogenic and nonantigenic properties make PEG a good matching coating material for such devices and also for different conservation procedures [4]. The origin of these beneficial properties is still intensely investigated and not fully understood yet, as no presented theory could elucidate the protein resistance of PEG under all investigated conditions. Two main explanations for its behaviour are steric stabilisation forces and the mobility of the PEG chains, which apply for most studies [5].

The highly reliable and reproducible suppression of nonspecific adsorption of biomolecules also ensures the specific recognition in biosensors that are coated with PEG.

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Biosensors play an increasing role in the field of environmental analytics, drug development, molecular diagnostics and point of care diagnostics (POCT) [6]. Time-resolved and label-free detection techniques like quartz crystal microbalance (QCM), surface acoustic wave (SAW) sensors, surface plasmon resonance (SPR) and reflectometric interference spectroscopy (RIfS) have received particular attention owing to the possibility of monitoring thermodynamic and kinetic constants without detracting from the biomolecular interaction by introducing radio or fluorescence labels [7]. These techniques necessitate a suitable surface coating that provides a high number of binding sites for immobilisation of receptor molecules while suppressing nonspecific adsorption. Many studies have reported highly potent PEG-modified surfaces in this field of research [8]. The large number of different kinds of functionalised PEGs makes them a universal and powerful tool for immobilisation of all kinds of receptors [9, 10]. When immobilised covalently, the PEG coating remains stable after many hundreds of regeneration cycles even under harsh conditions, giving reproducible results over extended working lifetimes [8]. Other widely used biopolymers for sensor surface coatings are amino dextran [11] or carboxymethylated dextrans [12–14]. Comparing these two polymers when immobilised onto a planar surface, dextrans form a three-dimensional matrix with a huge number of bindings sites (about 20 ng/mm^2 of protein immobilisation). In contrast, PEGs form a two-dimensional, planar structure ("polymer brush"), onto which at most one monolayer of proteins can be immobilised (5–6 ng/mm^2) [11]. Although dextrans allow a higher loading capacity of receptors, the PEGs show much better results in inhibiting the nonspecific binding of proteins onto the modified sensor surface [11].

A combination of the two properties of high loading capacity and minimal nonspecific adsorption of biomolecules in one surface coating would provide a "perfect" sensitive layer. Recently different approaches have been investigated based on either the graft polymerisation of PEG to form a three-dimensional PEG structure mimicking dextran surfaces [30] or by mixing polymer molecules with different properties [31]. The first method shows very good surface loadings as expected from three-dimensional structures. Nevertheless, these layers are not well defined. Therefore, we focused on the mixed PEG layers by immobilising different mixture ratios of PEG with varying lengths onto the surface to form a three-dimensional-like structure with higher binding capacity but keeping the benefit of minimal nonspecific binding. Recently several studies investigated different aspects using a variety of methods. To gain an overall picture of the surface properties we investigated the prepared surfaces with a set of methods providing complementary results. We used AFM to characterise the surface topography, ellipsometry for determination of layer thickness, polarisation modulation infrared reflection absorption spectroscopy (PM-IRRAS) as a chemically specific technique for characterisation of the constitution of the layers and RIfS for time-resolved, label-free analysis of bimolecular interaction in biosensor applications. RIfS is an easy to use and robust detection method based on white light interference at thin solid films. The binding of biomolecules to a modified transparent surface is monitored through the change in apparent optical thickness of the sensitive layer [15, 16]. Because of its simple setup and temperature independence [17], RIfS offers the possibility of hyphenation with important techniques like electrophoresis [18] and mass spectrometry [19].

Regarding glass-type surfaces which are widely used in biosensor applications e.g. for a multiplicity of arrays (DNA, protein, etc.), the dependency of the nonspecific adsorption of biomolecules and the dependency of maximum loading capacity and morphology in regard to the length of the coated PEGs should be characterised. For preparation of improved sensitive layers, the characterisation and comprehension of the polymer surface properties are mandatory.

Methods

Materials

RIfS transducer chips of 1-mm-thick D263 glass with layers of 10-nm Ta_2O_5 and 330-nm SiO_2 were obtained from Schott AG, Mainz, Germany.

Alternatively for the PM-IRRAS experiments a titanium layer was DC-sputtered on <100> silicon wafers followed by a silicon oxide layer. The coating was done under vacuum conditions and the thickness of the titanium and silicon oxide layers were 200 nm and about 5 nm, respectively. Details of the preparation will be published elsewhere [20].

Common organic compounds and biochemicals were purchased either from Fluka, Neu-Ulm, Germany; Sigma-Aldrich, Deisenhofen, Germany; or Merck, Darmstadt, Germany.

3-Glycidyloxypropyl-trimethoxysilane (GOPTS) and diisopropylcarbodiimide (DIC) were purchased from Fluka, Neu-Ulm, Germany.

Diaminopoly(ethylene glycol) (DAPEG) with molecular masses 900 Da, 2,000 Da, 3,000 Da and 6,000 Da were purchased from Rapp Polymere, Tübingen, Germany.

D-Biotin and (2-(1H-benzotriazol-1-yl)-1,1,3,3--tetramethyluronium tetrafluoroborate) (TBTU) were purchased from Sigma-Aldrich, Deisenhofen, Germany.

4-Chloro-6-(ethylamino)-1,3,5-triazine-2-(6′-amino)caproic acid (ACA) and polyclonal sheep anti-s-triazine antibodies were kindly supplied by Dr. Ram Abuknesha from Kings College, London, UK.

The oligonucleotides were purchased from IBA, Göttingen. The sequence of the immobilised DNA oligonucleotide was 5′-CGC TAC AAC CTA CAT-3′. This oligonucleotide was 5′-functionalised with (C_6NH_2). The complementary oligonucleotide consisted of the sequence 5′-ATG TAG GTT GTA GCG-3′ with an LNA base at every third position.

Surface chemistry

Cleaning and activation of the substrate

The substrate surface was cleaned by treatment with 6 M NaOH or KOH for 1 min followed by ultrasonication with freshly prepared piranha solution (60:40 concentrated H_2SO_4/H_2O_2 v/v) for 15 min. Afterwards, the slides were thoroughly rinsed with Milli-Q water and dried in a nitrogen stream.

Cleaning and activation of the Si wafer

The substrate surface was cleaned by treatment with Milli-Q water and ethanol. Afterwards, the slides were dried in a nitrogen stream. The surface was activated with oxygen plasma for 15 min.

Silanisation

GOPTS (15 μL) was pipetted onto an activated dry slide and covered with another slide ("sandwich technique"). After silanisation for 1 h the slides were rinsed with dry acetone and dried in a nitrogen stream.

Immobilisation of polymer

For immobilisation of diaminopoly(ethylene glycol), 10 μL of DAPEG/methylene chloride solution was pipetted onto a slide at a concentration of 4 mg/mL. For the investigation of the influence of different concentrations during the preparation, 9 mg/mL, 6.5 mg/mL, 4 mg/mL, 1.5 mg/mL and 0.5 mg/mL were used. Subsequently, the slides were transferred into an oven where immobilisation took place in an open-topped vessel overnight at 70 °C. Afterwards, the slides were rinsed with Milli-Q water and dried in a nitrogen stream. For pure PEG layers only one length of PEG molecules was used.

Immobilisation of mixed PEG

For preparation of mixed PEG layers, solutions containing an overall PEG concentration of 9 mg/mL of the respective mixture were produced. Therefore, the percent by weight (w/w) ratios (25:75, 50:50, 75:25) were adjusted, dissolved in methylene chloride, vortexed for 30 s and immobilised on the sensor surface as mentioned above.

Immobilisation of ligands

ACA [21], biotin [22] and DNA oligonucleotides [17] were immobilised as reported before.

Characterisation with RIfS

The interaction of analytes with the surface was monitored with RIfS. The principles and the experimental setup of this technique for monitoring binding events at interfaces were discussed in detail before [23, 24]. The change in apparent optical thickness of a thin silica layer (ca. 330 nm) upon analyte binding is detected by interference of white light reflected at the interfaces of a multilayer system using a diode array spectrometer. Binding curves were recorded as apparent optical thickness versus time.

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 (ca. 23 °C). Nonspecific adsorption to the surface was investigated by incubating 1 mg/mL ovalbumin for 650 s after 750 s baseline (buffer). Subsequently after 160 s flushing with buffer, the maximum loading via specific binding to immobilised binding partner was determined by incubating 50 μg/mL antibody or streptavidin, respectively, and 0.5 μg/mL oligonucleotide for 650 s followed by 120 s baseline (buffer). The sensors could be reused several hundred times by flushing the surface with 0.5% SDS pH 1.9 for 140 s followed by 100 s baseline (buffer). Prior to the measurements, the surface was flushed on-line with PBS until equilibration of the biopolymer surface, and then the surface was cleaned by flushing with regeneration solution. Typical measurement cycles are shown in Fig. 1.

Characterisation with PM-IRRAS

IR spectroscopy was performed with a PM-IRRAS setup using a VERTEX 70 spectrometer with a PMA 50 polarisation modulation unit (Bruker, Ettlingen, Germany) equipped with a photoelastic modulator (Hinds Instruments, USA). Details of the experimental setup are described elsewhere [25]. In brief, the resolution for the presented experiments was set to 4 cm^{-1}, the half-wave retardation was calibrated to 1,500 cm^{-1} and 256 spectra were averaged for each measurement. The angle of incidence was 70° and the experiments were performed under dry air conditions. The substrates used for the IR experiments consisted of a layered structure with SiO_2 on Ti to exploit the surface selection rules of IRRAS on metallic surfaces ([20], see also [26]). The spectra were processed as reported

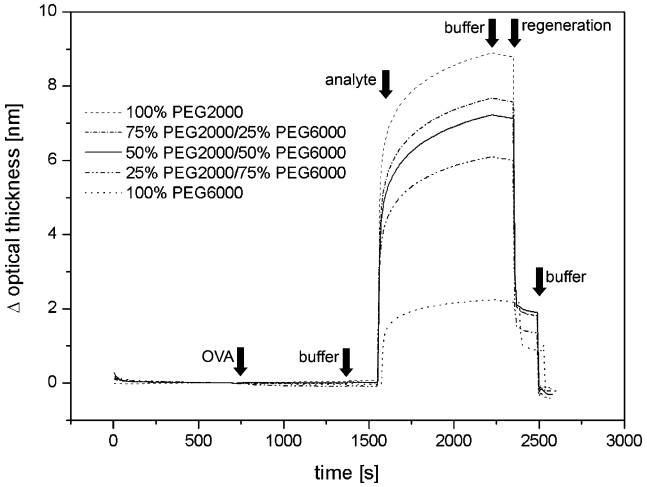


Fig. 1 Typical binding curves obtained with RIfS. After 750 s baseline (buffer), incubation with ovalbumin (650 s) illustrates the nonspecific binding to the surfaces. Subsequently after 160 s baseline, the maximum loading was determined by incubating with the specific binding partner (antibody) for 650 s followed by 120 s baseline. Measurements were carried out for pure PEG2000 and PEG6000 surfaces as well as for the mixed surfaces (PEG2000/PEG6000)

[27]. PM-IRRAS provides information about the composition of sensor surfaces via measuring the IR absorption of immobilised molecules.

Characterisation with AFM

The Multimode™ SPM atomic force microscope was from Digital Instruments, Santa Barbara, USA, using a J-scanner (97) S/N 3286 JV from Veeco Instruments GmbH, Mannheim, Germany with a vertical working range of 5 nm to 5 μm with a noise ≤ 50 pm. Images of coated RIfS transducers were done in tapping mode under air using SuperSharpSilicon AFM tips, Type: SSS-NCH with a spring constant (C) ≈21 N/m and in liquid using a fluid cell (MTFL, Veeco Instruments GmbH, Mannheim, Germany) and NP-S20/Sharpened AFM tips with a spring constant of C≈0.06 N/m. Pictures were taken in air and in liquid (Milli-Q water). AFM can operate in many different modes and media of special interest for biological systems giving information about surface topography, interaction forces and sample composition at the nanometre scale.

Characterisation with spectral ellipsometry

Ellipsometric measurements were done using a spectral ellipsometer ES4G from Sopra, Paris, with a high pressure xenon lamp L2174–01 from Hamamatsu. A double monochromator with a gap length of 400 μm was used. The measuring range was from 230 to 930 nm with a spectral resolution of 0.05 nm. Ellipsometry is a versatile tool for nondestructive determination of physical and optical properties of thin layers like refractive index, physical thickness, absorption coefficients, surface roughness and composition gradients.

Results and discussion

PEG chains with different molecular weight (900 Da, 2,000 Da, 3,000 Da and 6,000 Da) and thus different lengths were immobilised onto the sensor surfaces. The different lengths and flexibility of such chains should show different behaviours regarding nonspecific binding of biomolecules and also regarding loading capacity with immobilised receptors. Nonspecific binding was investigated by monitoring the adsorption of 1 mg/mL ovalbumin (OVA) and BSA on the surface. For analysis of specific binding, the pure coatings containing only one PEG length were functionalised using three different systems representing different types of biosensing problems: triazin derivative/anti-simazine antibody [21], biotin/streptavidin [22] and DNA/LNA hybridisation [17]. The analyte size was 150,000 Da for the antibody, 60,000 Da for streptavidin and 5,000 Da for the 15-mer LNA. In general, a higher change in optical thickness should be observed with larger interaction partners due to the size sensitivity of the detection method.

First, IR measurements were performed to prove the successful immobilisation of the different length PEG molecules on the sensor surface. Figure 2 shows the IR spectra of the PEG layers in the region of the ethylene glycol CH_2 (EG CH_2) wagging and scissor modes [28], demonstrating the presence of the PEG coatings on the surfaces. Note that the C–O–C stretching mode of PEG is shadowed by a strong SiO_2 substrate mode [26]. To demonstrate the specific binding of streptavidin, the PEG layers were then functionalised with biotin and exposed to a streptavidin solution. The IR spectra clearly show the specific binding of streptavidin to immobilised biotin, evidenced by the signal in the amide I and amide II region

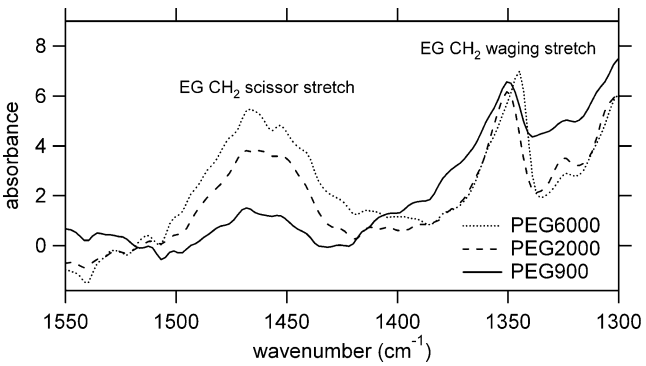


Fig. 2 PM-IRRAS absorption spectra of PEG coatings. EG CH2 wagging and scissor mode region of PEG900, PEG2000 and PEG6000 coatings, measured with PM-IRRAS. The absorption bands indicate the presence of PEG

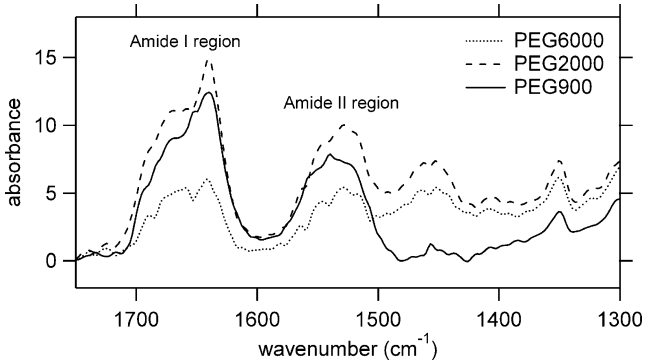


Fig. 3 PM-IRRAS absorption spectra in the region of the amide bands. PM-IRRAS spectra of biotinylated PEG coatings after 1 h exposure to 100 μg/mL streptavidin solution. The amide modes I and II can clearly be observed, indicating a binding of the protein. Additionally, the EG CH_2 wagging and scissor modes of the PEG coating can be detected

between 1,500 and 1,700 cm^{-1} [29] and the presence of PEG on the surface shown by the absorption of the EG CH_2 wagging and scissor modes (Fig. 3). The intensity of the amide I and amide II bands are the lowest for the PEG6000-coated sensor, showing less specific binding of streptavidin. In contrast, the bands of PEG2000-coated surfaces show highest intensity and thus significantly increased specific binding. Exposure to BSA showed no signal in the amide region (data not shown). This indicates that the PEG–biotin coating covers the whole surface, passivates it against nonspecific protein adsorption, like BSA, and binds streptavidin specifically.

For more detailed investigations, the specific binding of three different analytes was monitored using time-resolved reflectometric interference spectroscopy (RIfS). Figure 4 shows the maximum surface loadings of this analytes as well as the nonspecific binding of ovalbumin to the four different PEGylated surfaces. Regarding the sensor surfaces prepared with pure PEG the obtained signals of the specific interaction of the antibody were significantly higher than the signals for the binding of streptavidin to immobilised biotin except for the PEG6000 surfaces. As expected, the oligonucleotide hybridisation showed the lowest signals by far due to the small size of the analyte. For the antibody system, the PEG2000 showed the highest maximum loadings. PEG900 and PEG3000 showed slightly decreased maximum loadings, whereas considerably less antibody binding to PEG6000 surfaces was monitored. For the system streptavidin/biotin, the highest maximum loading was found using the PEG3000 coatings, whereas PEG2000 showed only slightly less signal. Using PEG900, the specific interaction decreased by 23%, whereas PEG6000 also showed the lowest signals as seen in the antibody system. The reproducibility in homogeneity of all PEG6000 surfaces was remarkably low, as demonstrated by the high standard deviation. At least eight different sensors were prepared for each interaction system. No influence of the PEG chain length was found for the oligonucleotide hybridisation.

The nonspecific binding of OVA to most of the prepared sensor surfaces was not detectable and did not exceed 1% of maximum loading except for the antibody system on PEG6000 (2%), the streptavidin system on PEG900 (1.6%) and the oligonucleotide system on PEG900 (8%). As a result, all these sensor surface coatings showed enough shielding of nonspecific binding for biosensor applications.

The best maximum loading properties were found using the medium-sized PEG (PEG2000 and PEG3000), whereas the

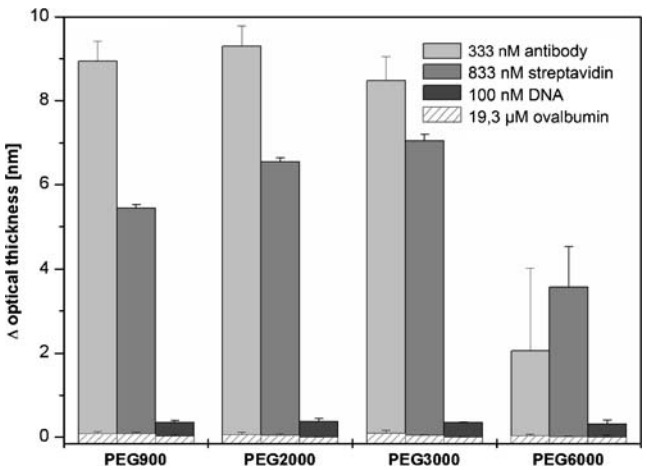
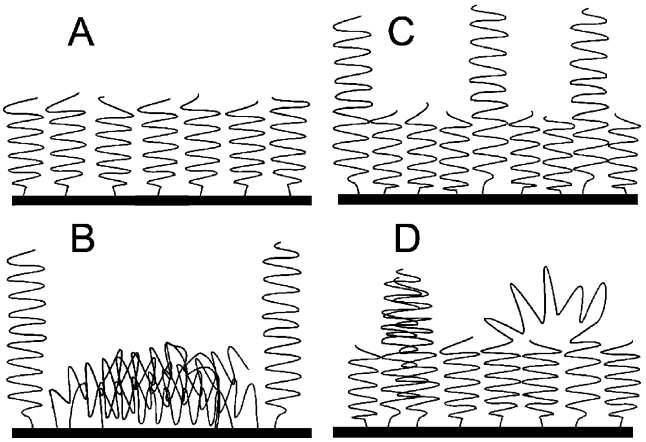


Fig. 4 Comparison of the binding properties to pure PEG layers. The maximum surface loadings for the three analytes and the nonspecific binding of ovalbumin to the surface are shown for all four different PEGylated surfaces using pure PEG. The *error bars* represent the standard deviation of binding signals. A new sensor surface was used for each measurement



Scheme 1 Illustration of the immobilised PEG chains. The short PEG chains form a uniform polymer brush (**a**), whereas the longer chains tend to entangle or collapse on the surface (**b**). This leads to the formation of dents in the polymer layer. For the mixed polymers, a more three-dimensional surface morphology is expected (**c**) where the longer chains protrude the rigid polymer brush of short PEG. However, the realistic arrangement also shows entanglement and PEG chains which are immobilised on both ends (**d**)

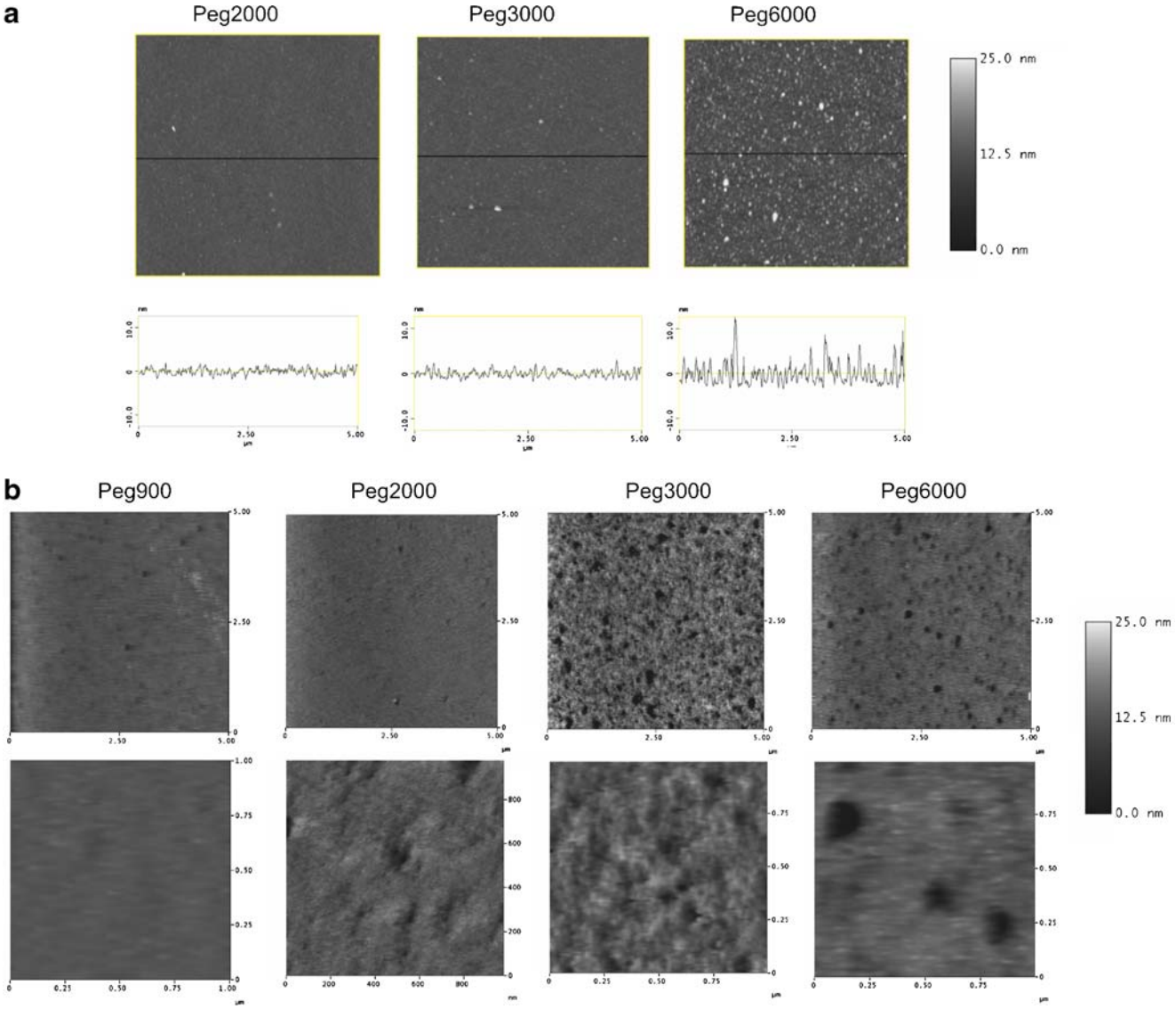


Fig. 5 Surface topography determined by AFM. Height images of pure PEG monolayers, imaged by tapping mode AFM in air (**a**) (5 μm×5 μm) and in distilled water (**b**) (5 μm×5 μm and 1 μm×1 μm). With increasing chain length of the PEG, the homogeneity of the surface decreases and a larger number of dents in the layer appear. The *lateral view* shows a profile between −10 and 10 nm

small PEG900 and the large PEG6000 showed decreased values for maximum loadings, with almost the same shielding effect. Short PEG chains form a rigid polymer brush on the surface when immobilised (Scheme 1a). Therefore, the flexibility of the chains is rather low and the binding capacity is limited. Regarding the antibody system, the difference of the maximum values amount to 4%, whereas for the streptavidin/biotin system the difference rises up to 17%.

The longer PEG chains, however, seem to collapse and/or are immobilised with both ends to the surface (Scheme 1b). Hence, a diminished number of binding sites are available resulting in a lower maximum loading capacity but with remaining shielding effect. Also, the increased standard deviations for PEG6000 surfaces indicate a random change of morphology. Regarding the antibody system, the difference of the maximum values amount to 77%; regarding the streptavidin/biotin system the difference is 46%. The significant loss of signal could originate from loss in binding sites due to partial occurrence of immobilisation on both ends of the chains. Compared with PEG900, the specific binding of streptavidin to the PEG6000 surfaces gives a higher signal than the binding of the antibody. This fact could be due to steric hindrance on the surface caused by the distortion of the PEG chains.

Because of the large steric hindrance of the huge antibody molecules, the surface loading is not significantly influenced by the rigidness of PEG900ylated sensor surface. However regarding PEG6000, the surface loading capacity is highly decreased (77%) caused by the fact that fewer binding sites are present and in addition are less approachable by the

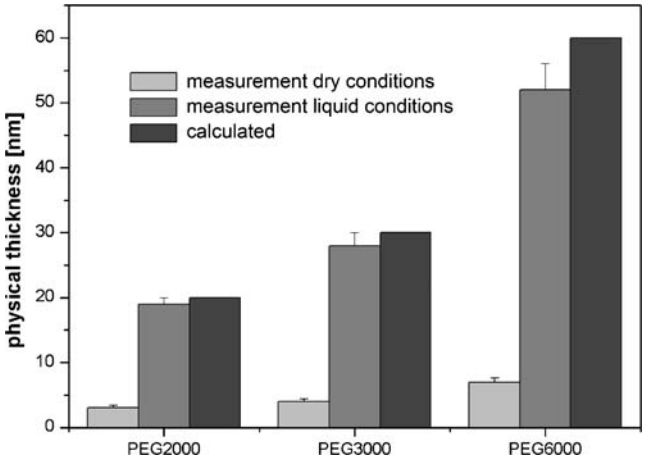


Fig. 6 Physical thickness of the PEG layers determined by spectral ellipsometry: ellipsometric results of pure PEG2000, PEG3000 and PEG6000 were compared with theoretically calculated physical thickness of the PEGylated surfaces. The *error bars* represent the standard deviation of multiple sensors. For the short PEG chains, the results correlate very well, whereas for the longer chains a significant discrepancy was found

analyte molecules. Regarding the medium-sized streptavidin, the steric hindrance between these analyte molecules is lower and thus the effect of the higher flexibility of the PEG chains leading to better accessibility of the immobilised binding partner is observable. Therefore, the surface loading capacity (medium-sized analyte) increases from PEG900 through PEG2000 to PEG3000 corresponding to the flexibility of the immobilised chains. For PEG6000, the surface loading is also smaller because of fewer available binding sites (as mentioned above), but the less accessible ones can be reached more easily by the medium-sized analyte molecules (especially oligonucleotides) resulting in a higher loading capacity than for the larger antibodies on PEG6000. Evidently, there is no steric hindrance for the small oligonucleotide molecules resulting in similar surface loading capacities.

Obviously, the steric hindrance of the analyte molecules is dominating on the PEGylated surfaces with short chains, whereas on surfaces with long PEG chains a summation of this effect with the steric hindrance of the polymer chains occurs.

In addition to the measurements with RIfS and PM-IRRAS, the different PEG surfaces were characterised using atomic force microscopy (AFM). Figure 5a shows images of the PEG2000, PEG3000 and PEG6000 surfaces using tapping mode AFM in air. A 5 μm × 5 μm section of the prepared polymer layers on the glass substrate was scanned in each case. Comparing the surface coatings with each other, the height image of PEG2000 shows an almost homogeneous cover of the transducer. However, the PEG3000 monolayer forms slight irregularities, whereas PEG6000 shows considerable clustering of the PEG chains which results in a significant loss in homogeneity. The analysis of the line height profiles confirms the inhomogeneity of the immobilised PEG6000. The difference in height along the drawn line ranges from 0 to 2 nm for PEG2000 and PEG3000, whereas the height profile for PEG6000 ranges from 2 to 10 nm.

Biomolecular interactions have to be measured in a native environment like buffered solutions. In aqueous conditions the PEG coatings absorb a large amount of water (hydrogel properties) and swell to a multiple of its thickness when dry. After swelling, the morphology of the immobilised PEG chains on the sensor surface changed and correspond to the state during the RIfS measurement. Thus, AFM measurements in liquid provide supplementary information about the actual state. As shown in Fig. 5b, PEG900, PEG2000 and PEG3000 form a relatively homogeneous surface coating, while a slightly increasing number of dents appears. The PEG6000 surfaces show significantly more and enlarged dents supporting the theory of entangled and collapsed PEG chains. These two effects should be reflected in the average physical thickness of the polymer layer compared with the expected theoretical thickness. Therefore, ellipsometric measurements in buffer were carried out to determine the average thickness. Due to the fact that the parameters of physical thickness and refractive index are highly correlated for thin films, a refractive index of 1.37 was assumed to determine the physical thickness of the PEG layers. The refractive index of PEG is 1.5 in the solid phase [8]. After swelling in buffer, PEG layers contain more than 85% buffer leading to a refractive index of 1.37. Actually, the agreement between calculated and measured results falls for increasing PEG chain lengths. For PEG6000, the real physical thickness is 13% smaller than the calculated one, as shown in Fig. 6. In addition, the difference in physical thickness of the PEG

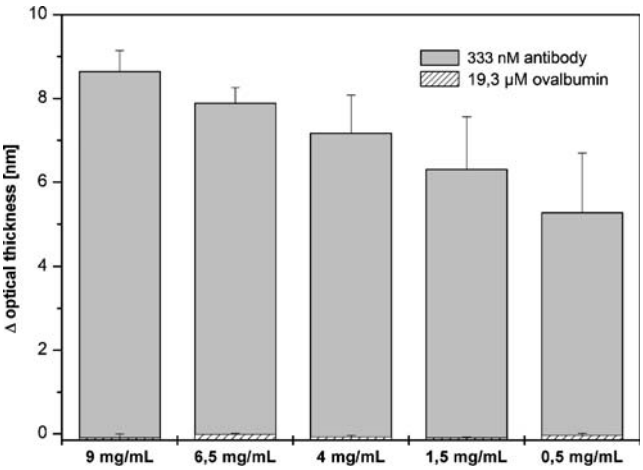


Fig. 7 Binding properties of PEG2000 surfaces using different polymer concentrations during the preparation. Increasing polymer concentration during the preparation step leads to higher surface loadings of antibody, whereas there is no observable influence on the nonspecific binding to the sensor surface

Table 1 Binding properties to mixed PEG layers determined using RIfS (change in optical thickness given in nm)

PEG layer	75%/25%		50%/50%		25%/75%	
	Antibody (specific)	OVA (nonspecific)	Antibody (specific)	OVA (nonspecific)	Antibody (specific)	OVA (nonspecific)
2000/900	7.3±1.0	0.06±0.05	8.0±1.4	–	8.2±0.1	0.04±0.02
2000/3000	9.3±0.5	0.2±0.2	10.0±0.6	0.1±0.1	9.2±0.9	0.2±0.2
2000/6000	7.5±0.2	–	6.4±2.1	–	4.5±2.3	–
3000/900	9.2±0.7	0.01±0.02	8.10.2	0.02±0.02	9.2±0.5	0.08±0.02

layers in dry and liquid conditions caused by swelling can be seen here.

For biosensor applications, the medium-sized PEG (PEG2000 and PEG3000) show the best properties and therefore PEG2000 was used for further investigations of the influence of the polymer concentration during the preparation procedure. As shown in Fig. 7, higher concentrations of polymer solution give higher surface loadings with analogues shielding against nonspecific binding of OVA. Comparing the lowest concentration with the highest one, an increase of 38% in specific binding signal was achieved. Still higher concentrated PEG solutions did not show any further effect.

For mimicking a three-dimensional structure on the surface (Scheme 1c), the four different PEG polymers were mixed in varied ratios as mentioned in the Materials and methods section. As reported in the literature [11], these structures should increase the maximum loading drastically (dextran/PEG = 2:1). As shown in Table 1, the mixed PEG surfaces did not show any significant improvement of maximum loading capacity. The obtained higher signals for 2000/3000 (50:50) and 3000/900 (75:25 and 25:75) were distributed within the standard deviation range.

As shown in Fig. 8, the same behaviour as described above for maximum loading capacity for the antibody system and the streptavidin/biotin system was found. For the larger analyte, the decrease in maximum signal is also more pronounced than for the smaller analyte molecules. Also, the standard deviations are higher for surfaces with larger than 50% of PEG6000. This could be due to formation of island structures on the surface with an increasing fraction of longer PEG chains (Scheme 1d), which seems to be consistent with investigations in the literature [27]. Similar to all previously described PEG surfaces, the nonspecific binding of OVA was negligible.

Conclusions

Covalent immobilisation of pure and mixed self-assembled monolayers of PEG with different lengths was performed using our well-established surface chemistry. These surfaces show negligible nonspecific adsorption of ovalbumin. The specific binding of three different sized analyte molecules to these surfaces was characterised using the label-free and time-resolved detection method reflectometric interference spectroscopy (RIfS). The results showed that the medium-sized PEG offer the best properties for biosensor applications. The size of the analyte molecules influenced the maximum loading capacities due to steric hindrance effects. The long-chained character leads to entanglement and collapse of PEG6000 and thus to an inferior applicability for sensor surface coatings.

This assumption was further investigated and fortified by means of polarisation modulation infrared reflection absorption spectroscopy (PM-IRRAS), atomic force microscopy and spectral ellipsometry. Preparation of mixed PEGylated surfaces did not obtain the expected improvement concerning surface loading capacity. Using this variety of surface characterisation methods, it was possible to highlight every aspect influencing the properties and hence explain the behaviour of the biopolymer in sensor applications.

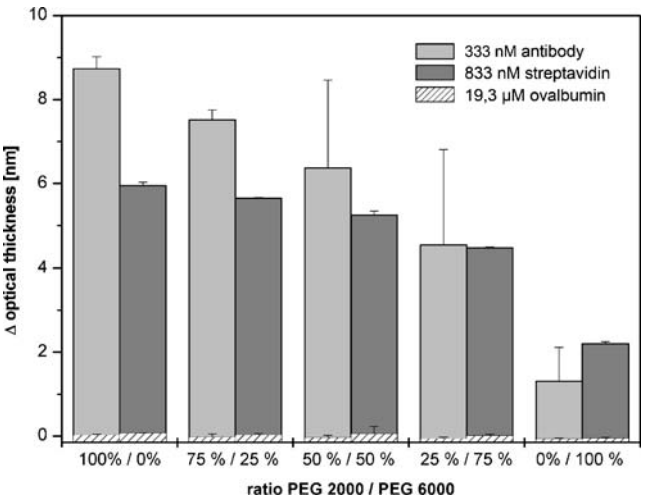


Fig. 8 Binding properties to mixed PEG layers (PEG2000/PEG6000). The maximum surface loadings for the two analytes as well as the nonspecific binding of ovalbumin to the surface are shown for pure PEG2000, PEG6000 and mixed surfaces (PEG2000/PEG6000). The *error bars* represent the standard deviation of binding signals. A new sensor surface was used for each measurement

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