Research Article

Journal of Molecular Recognition

Received: 7 January 2014, Revised: 16 May 2014, Accepted: 16 May 2014, Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2396

Characterization of a new maleimido functionalization of gold for surface plasmon resonance spectroscopy

Marc Hovestädt[a,b†], Henry Memczak[a,b†], Dennis Pleiner[a], Xin Zhang[c], Jörg Rappich[c], Frank F. Bier[a,b] and Walter F. M. Stöcklein[a]*

Para-maleimidophenyl (*p*-MP) modified gold surfaces have been prepared by one-step electrochemical deposition and used in surface plasmon resonance (SPR) studies. Therefore, a FITC mimotope peptide (MP1, 12 aa), a human mucin 1 epitope peptide (MUC, 9 aa) and a protein with their specific antibodies were used as model systems. The peptides were modified with an N-terminal cysteine for covalent and directed coupling to the maleimido functionalized surface by means of Michael addition. The coupling yield of the peptide, the binding characteristics of antibody and the unspecific adsorption of the analytes were investigated. The results expand the spectrum of biosensors usable with *p*-MP by widely used SPR and support its potential to be versatile for several electrochemical and optical biosensors. This allows the combination of an electrochemical and optical read-out for a broad variety of biomolecular interactions on the same chip. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: biosensor; surface plasmon resonance; diazonium coupling; maleimidophenyl; cys-peptide; aryl diazonium salts

INTRODUCTION

The development of solid-phase modifications is very important for many fields in life science like biocompatibility, cell cultivation and especially biosensors: for drug development, medical diagnostics and food safety (Doshi and Mitragotri, 2009; Engel *et al*., 2008; Gooding and Darwish, 2012; Jiang *et al*., 2012; Pérez-López and Merkoçi, 2011; Qi *et al*., 2009; Variola *et al*., 2011). It is a challenge to create precisely fitting properties of a defined surface for each application. Additionally, in the last few years in bioanalytical research, much effort was put into combining different methods to obtain faster and even more information about the investigated molecules or biochemical interactions (Hsu *et al*., 2009; Lê *et al*., 2010; Visser and Heck, 2008). Because of this fact, the requirements concerning the used functional surfaces increased. There is a need to establish a uniform surface modification for different intermolecular interaction studies, based on solid-phase applications, to avoid surface dependent mismatches.

Today, surface plasmon resonance (SPR) is one of the most popular optical methods to detect binding events in real time, label free and on short timescales. Hence, it is possible to determine the amount of a coupled ligand or to investigate the kinetics of binding reactions. Generally, gold surfaces are used for this purpose, because of their superior optoelectronic and chemical properties. Different methods are available to immobilize ligands on gold surfaces by self-assembling thiol containing molecules. Many commercial chips consist of self-assembled monolayers (SAMs) of alkanethiols covered with dextran derivatives providing functional coupling groups to bind biomolecules (Löfås and Johnsson, 1990). Although showing excellent biocompatibility, these surfaces cannot be used for electrochemistry, because they are instable under reductive potential. Therefore, several efforts were undertaken to overcome this drawback, for example, the use of combined SAMs or molecules with higher denticity (Almeida *et al*., 2010; Phong *et al*., 2008; Siemeling *et al*., 2012; Wang *et al*., 2009). Furthermore, it is necessary to identify optimum conditions and especially to find the best platform for the investigated interaction. For example, dextran chips reveal unspecific binding using positively charged molecules or lectins as analytes.

The majority of the coupling strategies for proteins are based on undirected attachment to carboxyl or amino groups. These methods often lead to a reduced binding capacity of the ligand caused by its unfavourable orientation (Johnsson *et al*., 1995; Kausaite-Minkstimiene *et al*., 2010). In particular, the correct orientation is crucial for the solid-phase presentation of small molecules like peptides (Wiedemann *et al*., 2004). Therefore,

* *Correspondence to: W. F. M. Stöcklein, Fraunhofer Institute for Biomedical Engineering IBMT, Branch Potsdam, Department of Molecular Bioanalytics and Bioelectronics, Am Mühlenberg 13, 14476 Potsdam, Germany. E-mail: walter.stoecklein@ibmt.fraunhofer.de*

† *Both authors equally contributed to this work*

a *M. Hovestädt, H. Memczak, D. Pleiner, F. F. Bier, W. F. M. Stöcklein Fraunhofer Institute for Biomedical Engineering IBMT Branch Potsdam, Department of Molecular Bioanalytics and Bioelectronics, Am Mühlenberg 13, 14476 Potsdam, Germany*

b *M. Hovestädt, H. Memczak, F. F. Bier University of Potsdam, Institute of Biochemistry and Biology, Karl-Liebknecht-Straße 24/25, 14476 Potsdam, Germany*

c *X. Zhang, J. Rappich Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Institut für, Silizium-Photovoltaik, Kekulestr. 5, 12489 Berlin, Germany*

other, site-directed immobilization techniques exist, for example, biotin-streptavidin, hydrazine and thiol coupling (Panicker *et al.*, 2004). A new promising class for the biocompatible functionalization of various surfaces is aryl diazonium salts (Chehimi, 2012; Mahouche-Chergui *et al.*, 2011), which can be electrochemically reduced in aqueous and non-aqueous solutions to form thin surface aryl layers with the functionality needed (Harper *et al.*, 2008; Zhang *et al.*, 2013).

In the present study, the *p*-MP functionalized surface was investigated for its utilization as substrate in SPR applications, because primary surface properties have already been examined by X-ray photoelectron spectroscopy, cyclic voltammetry and infrared spectroscopic ellipsometry (Zhang *et al.*, 2013).

The *p*-MP surface characteristics are (i) preparation by simple and cheap one-step functionalization, (ii) covalent linking to the surface and (iii) chemo-selective, site-directed immobilization of the ligands by Michael addition of thiols. Thereby, it is considered to be universally applicable for electrochemical and optical biosensing applications.

The *p*-MP modified surfaces used in this study were produced as described by Zhang *et al.* (2013). A mimotope peptide recognized by the anti-FITC antibody and a human mucin 1 epitope peptide were used as model systems (Böttger *et al.*, 1999; Schenk *et al.*, 2007; Zvirbliene *et al.*, 2006). The peptides were synthesized with N-terminal cysteine for the covalent coupling to the maleimido-group (Figure 1). Different essential parameters for SPR chips were investigated, for example, coupling yield of the peptide, unspecific binding of the antibody and maximum amount of bound analyte. Furthermore, the *p*-MP modified surface was tested for an exemplary protein–protein interaction for kinetic analysis via SPR. In summary, we were able to transfer the *p*-MP surface optimized for electrochemical read-out systems to SPR as an optical biosensing method.

MATERIALS AND METHODS

Chemicals

N-Hydroxysuccinimide (NHS), N′-(3-dimethylaminopropyl)-N′-ethylcarbo diimide hydrochloride (EDC), glycine, guanidine hydrochloride and molecular biology grade buffer reagents were purchased from Sigma-Aldrich (Munich, Germany) and used without further purification. All buffers were filtrated using 0.2 μm pore filters. Peptides MP1 (C-SGSGGPPGMVGDWWDW-amide; Biosyntan, Berlin, Germany) and MUC (C-GSSGSTAPPVHNV; GeneCust, Dudelange, Luxembourg) had a purity of higher than 90%. Anti-FITC monoclonal antibody (clone B13-DE1) from mouse was provided by the group of K. Hanack (University of Potsdam, Potsdam, Germany); anti-MUC monoclonal antibody from mouse (clone 14G2) was provided by the group of A. Zvirbliene (Institute of Biotechnology, Vilnius, Lithuania); anti-GFP monoclonal antibody (clone GST-2) from mouse (clone 7.1) and recombinant GFP were purchased from Roche (Mannheim, Germany). Fluorescein sodium salt was purchased from AppliChem (Darmstadt, Germany). The *p*-maleimidophenyl diazonium tetrafluoroborate (*p*-MPDT) was synthesized by S. Janietz (Fraunhofer IAP-Golm) via N-(4-Aminophenyl) maleimide (TCI Europa) and identified by NMR spectroscopy in CD_3-CN (8.55 dblt, 2H; 8.12 dblt, 2H; 7.07 singlet, 2H).

Methods

Electrochemical grafting

A standard $10 \times 12 \times 0.3$ mm glass chip coated with gold and a titanium intermediate layer from Ssens (Enschede, Netherlands) were used for the electrochemical grafting. The *p*-MP layers were electrochemically grafted on Au surface by the use of an

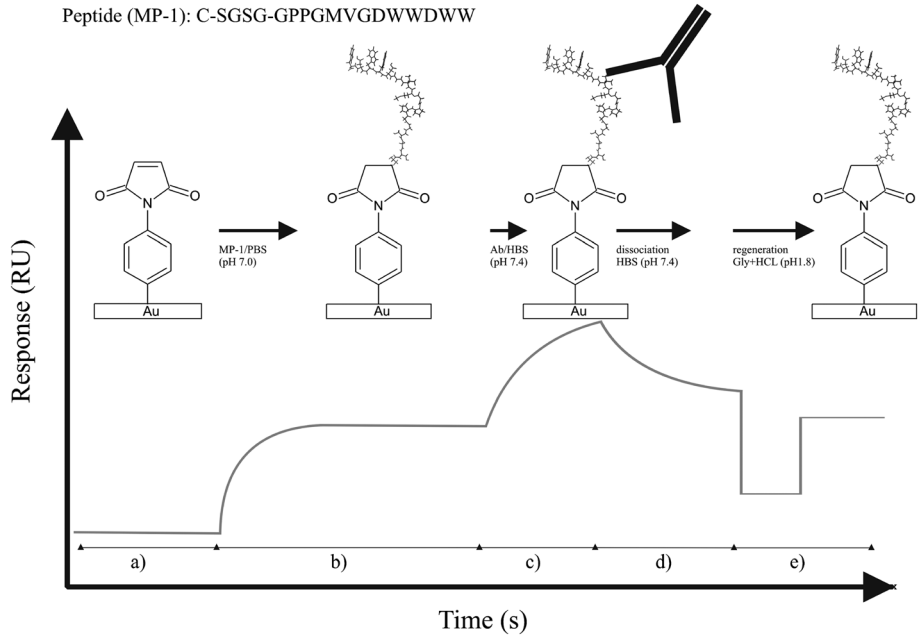

Figure 1. Schematic sensorgram for the immobilization of ligand and characterization of their functionality using surface plasmon resonance. (a) Baseline corresponding to the degree of *para*-Maleimidophenyl (*p*-MP) functionalization, (b) immobilization of cysteine-modified peptide via Michael reaction with maleimide backbone, (c) association phase: binding of peptide-specific antibody, (d) dissociation phase: washing out of antibody in a flow of running buffer, (e) complete regeneration of the sensor surface by solving the noncovalent antibody–peptide interaction.

Journal of Molecular Recognition

electrochemical cell accommodating three electrodes, that is, the gold-coated SPR chip as working electrode, gold wire counter electrode and Ag/AgCl (3 M KCl) reference electrode. All electrodes were connected to a potentiostat/galvanostat (Ivium Technologies, CompactStat™), and the deposition of the *p*-MP layers was carried out from acetonitrile solution containing 2 mM *p*-MPDT and 0.1 M Bu_4NBF_4 by cyclic voltammetry ramping the potential between +0.75 and −0.6 V at a rate of 50 mV/s for several times. After the deposition, the *p*-MP-functionalized Au surface was ultrasonically cleaned in acetonitrile for 2 min and dried in N_2 stream.

Surface plasmon resonance

The SPR measurements were performed using a Biacore™ T100 device (T200 Sensitivity Enhanced, GE-Healthcare Bio-SciencesAB, Uppsala, Sweden) at 25 °C with HBSP (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20, pH 7.4) running buffer. Immobilization of cysteine containing peptide at 100 μg/ml was carried out in phosphate buffer (20 mM Na_2HPO_4, 1 M NaCl, pH 7) at a flow rate of 10 μl/min. Blocking was performed in two steps. Firstly, 1 M NaCl (pH 4.0) with 50 mM cysteine was injected for 20 min (5 μl/min), followed by an overnight exposure of 1 mg/ml BSA in HBSP. Binding experiments were carried out by injection of several concentrations of antibody at flow rates of 10–30 μl/min. Inhibition experiments were performed by mixing antibody with several concentrations of fluorescein, allowing at least 30 min for incubation before injection. Concentration of free antibody was calculated using previously obtained standard curves and normalized to injections of antibody without inhibitor. Binding of anti-FITC to immobilized MP1 was regenerated using 4 M guanidine hydrochloride in 50 mM Tris/HCl, pH 7.5 for 30 s, whereas 50 mM HCl was used for regeneration of the MUC surface. Protein–protein interactions were regenerated with 100 mM glycine-HCl, pH 1.8. Sigmoidal fits of inhibition curves were carried out using a standard four-parameter logistic fit model. All curves were double referenced against an empty flow cell and an injection of buffer to eliminate unspecific binding to the sensor surface and bulk refractive index effects.

Anti-GFP was immobilized using an additional modification step with mercaptopropionic acid, because direct immobilization led to a low amount of active ligand on the surface and significant unspecific binding. Mercaptopropionic acid (1 mg/ml in H_2O) was injected for 30 min at a flow rate of 10 μl/min, followed by a 20 min blocking step with 1 M NaCl and 50 mM cysteine (pH 4.0). Activation of established carboxylic groups was performed by injecting 0.4/0.1 M EDC/NHS for 5 min, followed by 20 min injection of 40 μg/ml anti-GFP in 10 mM acetate buffer pH 5.0. Deactivation of reactive esters was carried out by using 1.0 M ethanolamine pH 8.5 for 7 min. Kinetic data were obtained using five successive injections of recombinant GFP (single-cycle kinetics).

Loading of the chip was calculated considering one resonance unit (RU) corresponding to one pg/mm^2 of bound protein (Karlsson *et al*., 1993). Binding capacity of the ligand was calculated as quotient of the total molar amount of bound antibody per immobilized peptide.

Further analysis of obtained sensorgrams was performed using Biacore™ T200 Evaluation Software v1.0.

RESULTS

The *p*-MP-modified gold surface is able to serve as a substrate for the covalent attachment of cysteine containing peptides and proteins (Zhang *et al*., 2013). In our study, we used the established systems consisting of an anti-FITC antibody, and the mimotope peptide MP1 and the anti-MUC antibody with the corresponding MUC epitope peptide, respectively. The peptides were N-terminally modified with a serine- and glycine-containing spacer and a cysteine for covalent immobilization (Figure 1). MP1 additionally competes with the antigen fluorescein for the same paratope (Böttger *et al*., 1999).

Homogeneity of the unmodified and modified sensor chips

First of all, we tested the homogeneity of the pure gold substrates and the *p*-MP modification. We evaluated the baseline signals of two chips on all four flow cells before and after modification with *p*-MP (Figure 2). The mean response for pure gold was about 35.000 RU; the modification with *p*-MP resulted in an increase by averaged 15.000 RU. The overall standard deviation was 3.2% for the unmodified gold surface and 1.7% after *p*-MP modification. This indicates negligible impacts of inhomogeneneity of the surface modification on the choice of the flow cell for the following interaction analysis. The electrochemical modification can be tracked in real time by cyclic voltammetry and adjusted by variation of the reaction time. Furthermore, the maximum amount of immobilized peptide was determined by calculating the difference of the SPR response before and after immobilization took place. Around 2500 RU of cysteine-modified peptide MP1 corresponding to 1.4 pmol/mm^2 could be bound to the surface (Figure 2). The calculated average surface area per peptide was 1.1 nm^2, representing a dense packaging of the peptides. The standard deviation for this immobilization was about 11%. A systematic correlation between the amount of immobilized peptide and the aforementioned deviations in the individual degree of *p*-MP modification could not be detected. Obviously, other influences play a role as well, for example, storing issues, ageing processes and individual handling of each chip and flow cell.

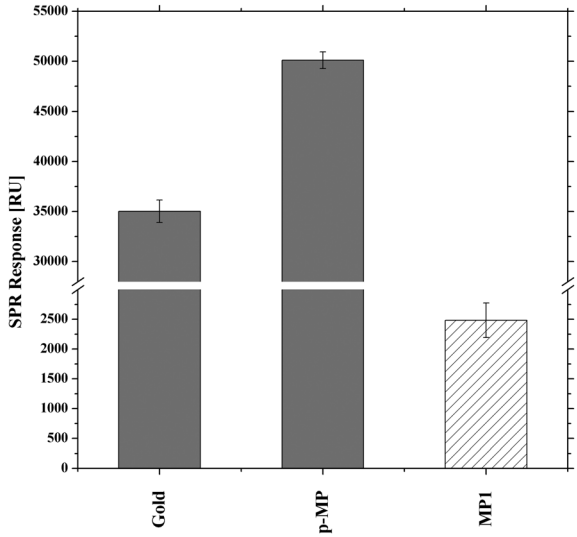

Figure 2. Surface plasmon resonance (SPR) responses for successive surface modifications on gold. The first column represents the SPR signal in HBSP running buffer of eight flow cells on two bare gold chips. The SPR signal in the second column was taken after electrochemical grafting of the gold surface with *p*-MP. The amount of MP1 immobilized represents the individual difference to the *p*-MP functionalized surface. Error bars represent the standard deviation ($n = 8$).

Antibody binding to immobilized peptides

The functionality of the peptide-modified sensor surfaces was verified by the injection of peptide-specific antibody at various concentrations (Figure 3). For both peptide-antibody binding systems, the highest injected concentration approached saturation as indicated by the exponential fit of the binding data (Figure 3, insets). All curves of one showed uniform dissociation behaviour. While the anti-FITC antibody dissociates apparently fast, the anti-MUC antibody showed no significant dissociation within the observed time-scale. A maximum of 1650 RU (11 fmol/mm^2) anti-FITC could be bound to 1600 RU of immobilized MP1 (892 fmol/mm^2). For the anti-MUC/MUC interaction, 1900 RU (13 fmol/mm^2) of antibody is bound to 370 RU (282 fmol/mm^2) MUC peptide.

For further verification of the reproducibility of the SPR response, 10 consecutive injections of the antibody were performed (Figure 4). Repeated injections of anti-FITC antibody did not result in loss of binding capacity as indicated by low variation of the maximum binding response. The maximum binding signal increased slightly during the first cycles, staying stable during the following injections. Overall, the absolute maximum binding response varied by less than 5%. The good reproducibility is provided and reflected by complete regeneration of the sensor surface. A stable baseline could be observed just after injection of the regeneration solution. Obviously, the underlying *p*-MP modification and the immobilized cysteine-functionalized peptide are not affected by the chosen regeneration conditions (4 M guanidine hydrochloride in 50 mM Tris/HCl, pH 7.5). Other conditions were also tested for regeneration. HCl at concentrations of 30–100 mM, or 100 mM glycine-HCl pH 1.8, is sufficient for regeneration of MP1 immobilized onto dextran-based sensor chips, but here, regeneration remained incomplete. NaOH at 20 mM or higher and 0.5% SDS led to strong decreases of the baseline signal, while the binding signal was not affected, indicating desorption of material, and to increased unspecific binding in following injections. Except basic conditions (20 mM NaOH or higher) and SDS, no substantial effects on the immobilized peptide and on the *p*-MP modification have been observed; the surface remained stable. Additionally, unspecific binding to the unmodified reference flow cell was very low (40 RU at 2 μM of anti-FITC antibody). In the case of anti-MUC/MUC binding, regeneration with 50 mM HCl was satisfactory.

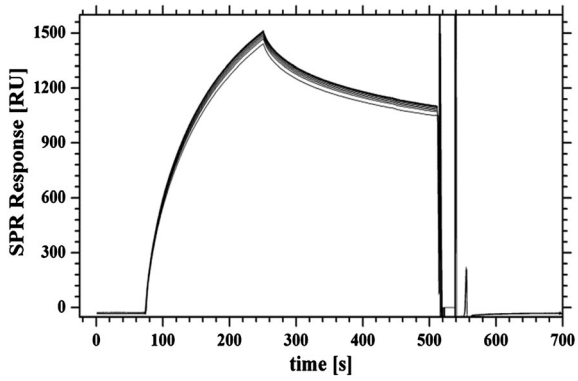

Figure 4. Overlapping surface plasmon resonance (SPR) sensorgrams of 10 consecutive injections of 74 nM anti-FITC antibody and regeneration solution over MP1-modified (1600 RU immobilized) *p*-MP surface.

For the determination of the specificity of the observed antibody–peptide interactions, inhibition assays were performed. MP1 is recognized by the same antibody paratope as fluorescein, but with much lower affinity (27 μM vs 0.3 μM) (Messerschmidt, 2007; Messerschmidt *et al*., 2011). Therefore, the pre-incubation of antibody with fluorescein should lead to an inhibition of the antibody-MP1 interaction. Results of the inhibition experiment with 200 nM anti-FITC antibody and different concentrations of fluorescein as an inhibitor are shown in Figure 5. Fluorescein inhibits the antibody binding to the surface in a dose-dependent manner. From the sigmoidal fit, an IC_{50} value of 226 nM was calculated. The complete inhibition proves the specificity of the observed binding event. The binding of anti-MUC antibody at 10 nM was inhibited by the use of the MUC peptide in the same manner as fluorescein before (Figure 5). Full inhibition of antibody binding was obtained above 100 nM peptide concentration in the solution, an IC_{50} value of 6 nM was calculated from the sigmoidal fit. Both binding examples demonstrate the specific binding of the antibody to the immobilized peptide, as shown by the saturation behaviour of the binding curves and the complete binding inhibition by free antigen in solution.

Additionally, the cross-reactivity of the MP1-modified chip surface with other proteins was investigated, using a protein library covering various molecular weights, glycosylation patterns and isoelectric points (Figure 6). Most of the proteins showed no cross-reactivity with peptide MP1 on *p*-MP. Remarkably, the

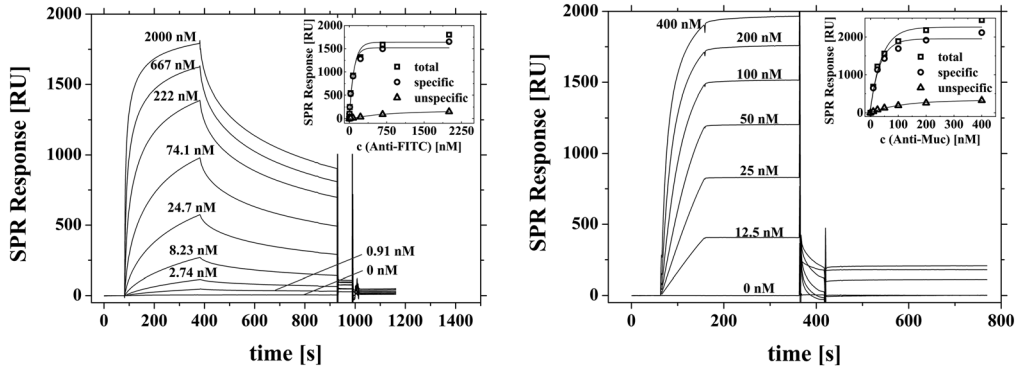

Figure 3. Injections of different concentrations of anti-FITC (left, 0–2000 nM) and anti-MUC (right, 0–400 nM) antibody over a MP1/MUC-modified *p*-MP surface and subsequent regeneration. Insets: correlation between antibody concentration and maximum binding signal obtained 5 s after injection stopped. The concentration-dependent curves were split up into total binding to measuring flow cell, binding to reference flow cell without immobilized peptide (unspecific) and referenced specific signal.

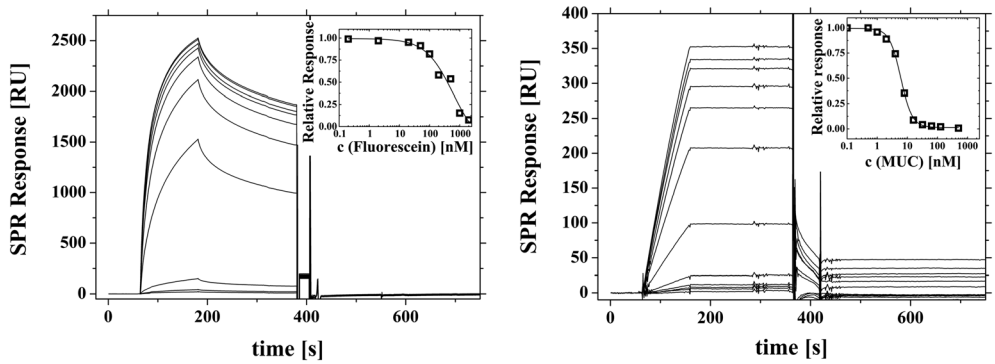

Figure 5. Inhibition of anti-FITC binding to MP1-modified surface by fluorescein (left) and inhibition of anti-MUC binding to MUC-modified surface by MUC peptide (right). Insets: correlation between inhibitor concentration and normalized SPR response with sigmoidal fit of the data.

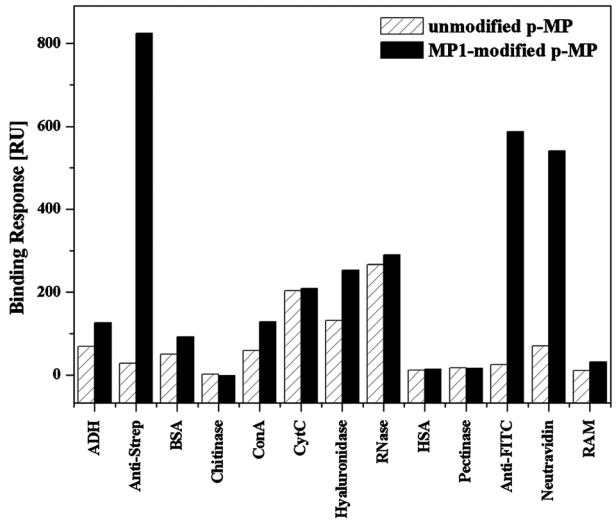

Figure 6. Determination of *p*-MP-dependent cross-reactivity by binding of different proteins to non-modified *p*-MP as well as after MP1 immobilization. All proteins were injected at same concentration of 10 μg/ml, diluted in running buffer.

lectin concanavalin A causing high binding response on standard dextran-based SPR surfaces did not show significant unspecific binding. The positively charged proteins cytochrome C (pI 10.0) and RNase (pI 9.6) showed the highest binding signals to the unmodified surface. This indicates that the overall surface potential of the *p*-MP chip is negative, causing unspecific interactions due to electrostatic attraction of positively charged molecules. Nevertheless, for the investigated MP1/anti-FITC test system, this issue can be corrected by referencing the binding with a non-modified flow cell, while unspecific binding was generally low (Figure 3). Major cross-reactivity for MP1-modified *p*-MP was observed for anti-Strep antibody and neutravidin. Anti-Strep binding to MP1 is also observed for this peptide immobilized to other surfaces and relies not on the specific immobilization method to *p*-MP. As on other surfaces, this anti-Strep/MP1 interaction cannot be inhibited with fluorescein (data not shown). Only neutravidin showed a cross-reactivity, which relies on the specific immobilization of MP1 to *p*-MP, because there was no cross-reaction with *p*-MP itself and with MP1 immobilized on dextran-based standard SPR surfaces. Overall, MP1 immobilized to *p*-MP showed no cross-reaction except to neutravidin. As observed by the very low unspecific binding of ConA, the *p*-MP modification appears to be especially suitable for the use of lectins, which cause unspecific binding to standard dextran-based SPR sensor surfaces.

Kinetics of a protein–protein interaction

In addition to interactions with peptidic ligands, an exemplary protein–protein interaction was kinetically characterized (Figure 7). A 1:1 binding system consisting of immobilized anti-GFP antibody from mouse and recombinant GFP as binding analyte was used. The binding constant for the chosen antibody-antigen interaction was in the low nanomolar range (3.54 ± 0.06 nM) as determined by single-cycle kinetics (Table 1). No substantial unspecific binding to the reference flow cell was observed. A maximum of 26 RU of analyte could be bound, meaning that there was approximately 10% functional ligand on the surface, which is not unusual for proteins immobilized in a random orientation.

DISCUSSION

The arguably most common method for the immobilization of proteins and peptides onto SPR chips is the amine coupling procedure. Usually, carboxylic groups on the surface (planar or gel) have to be activated first. Proteins are then bound by their free amino groups to the succinimide-ester activated surface. Although

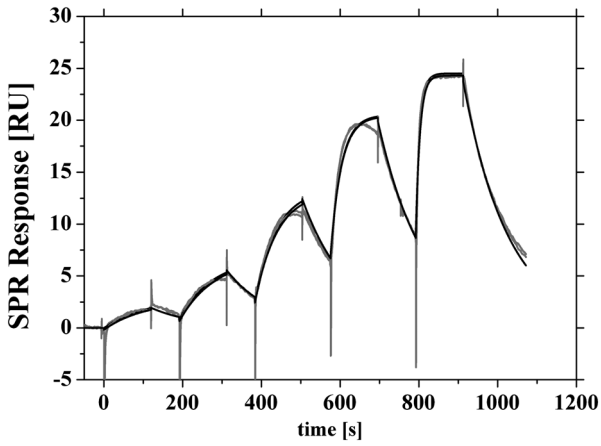

Figure 7. Kinetic analysis of a protein–protein interaction. Anti-GFP antibody was immobilized (1600 RU). GFP was injected at concentrations of 0.46, 1.37, 4.11, 12.4 and 37 nM (grey curves) and fitted (black curves) using the 1:1 binding model.

Table 1. Interaction kinetic data (1:1 binding fit) obtained for the binding of recombinant GFP to immobilized anti-GFP antibody (mean values from triplicate experiments and standard deviation)

K_D (M)	k_a (1/M·s)	k_d (1/s)	R_{max} (RU)	Chi^2
$(3.54 \pm 0.06)\ 10^{-9}$	$(2.44 \pm 0.02)\ 10^{6}$	$(8.64 \pm 0.08)\ 10^{-3}$	26.0 ± 1.3	0.68 ± 0.18

this procedure is less laborious than other methods involving surface activation, it is of limited value for the immobilization of peptides. As preconcentration is necessary for effective coupling, neutral or acidic peptides can hardly be immobilized onto such chips, as the chip surface still is negatively charged, and preconcentration fails. On the other hand, positively charged peptides are readily immobilized. However, any free amine within the sequence can react with the surface, leading to a heterogeneous surface, containing only a fraction of properly oriented peptides. Therefore, there is need for sensors that contain reactive groups for oriented one-step immobilization of peptides independent on their charge.

We tested a maleimido functionalized surface, which is highly reactive for thiol coupling. The only requirement for peptides is that they must contain a cysteine at the C- or N-terminus. The mimotope peptide Cys-MP1, which is negatively charged (pI 3.9) and resists to amine coupling, could be coupled at high ligand density in a defined orientation. This was also possible for neutral cysteine-modified MUC peptide (pI 7.7). The functionality of the immobilized peptides was shown by the binding of their specific antibodies. This antibody showed highly specific binding to the flowcell containing the peptide, compared with the control flowcell without peptide and by the use of other proteins as analytes. A further proof of specificity was the complete inhibition of the binding in the presence of fluorescein (anti-FITC) and MUC peptide (anti-MUC), the peptide itself. The inhibition curve for anti-FITC/MP1 is in accordance with the published affinities, considering bivalent binding and active antibody concentration lower than the concentration measured by absorbance at 280 nm. The K_D value for the antibody binding to immobilized FITC-BSA is 0.7 nM (Schenk *et al*., 2007); the affinity of the single chain variable fragment derived from this antibody for fluorescein in solution is 1.1 nM (Stech *et al*., 2012).

Ligand density is a big issue for sensing low concentrations of weakly binding analytes, and even more, if the analyte binds bivalent or multivalent. High ligand density causes increased mass transfer limited binding and rebinding effects, resulting generally in higher observed binding affinities, especially if the analyte binds bivalent like IgG-type antibodies or multivalent, for example viruses. However, the results shown here for the anti-FITC/MP1 system reflect proper binding. As saturation was observed at an antibody concentration of less than 1 μM, the binding avidity is in the sub-micromolar range, whereas the 1:1 affinity was 27 μM (Messerschmidt *et al*., 2011). As for anti-FITC/MP1, a comparable R_{max} of around 2000 RU for antibody binding in the anti-MUC/MUC system was obtained, corresponding to one antibody per 125 nm^2 or roughly a monolayer. This indicates that this is the maximum binding signal achievable with antibodies on *p*-MP surfaces. In both cases, evaluation of true binding kinetics was not pursued, because of the high ligand density effects described above. Kinetics were demonstrated in the third example for protein–protein interactions.

For the observation of protein–protein interactions, a different immobilization procedure including an additional modification step with mercaptopropionic acid was used. This was necessary because direct immobilization of proteins via their thiol-/amino groups resulted in a low amount of active ligand on the surface (<1%) and significant unspecific binding (~50%). This was observed for two test systems and made quantitative kinetic evaluation difficult. To obtain a higher amount of active ligand using direct coupling of proteins to the maleimido-surface, a more directed immobilization approach, that is, by the introduction of artificial thiols in the ligand, could also be used. Additionally, further chemical modifications with hydrophilic functionalities of the hydrophobic *p*-MP backbone could reduce sticky interactions between hydrophobic parts of the ligand and the surface. For further reduction of unspecific binding, the simple phenyl-backbone of *p*-MP could also be modified with hydrophilic groups, which is mandatory for antifouling, biocompatible surfaces. Nevertheless, the kinetic experiment shows that the *p*-MP chip is principally also suitable for the characterization of protein–protein interactions after additional modification with mercaptopropionic acid and subsequent amine coupling.

CONCLUSIONS

Our results show the capability of *p*-MP-modified gold surfaces to be used as SPR sensor chips. In summary, the new maleimide-functionalization method has considerable advantages over other methods especially for immobilization of small molecules: it is a one-step procedure, leading to a covalent carbon–Au linkage. Furthermore, Cys-containing peptides can be directly immobilized via Michael addition to the functionalized surface. Modification of peptides and other biomolecules with cysteine is straightforward and allows an oriented immobilization, in contrast to methods based, for example, on amine coupling, where a random coupling can reduce the number of molecules in the proper orientation for further binding reactions. High ligand density close to a monomolecular layer of the peptide could be obtained. This could also enable sensitive interaction analysis of multivalent binding partners, for example, nanoparticles, viruses or cells.

The *p*-MP modification on gold and related aryl diazonium salts are suitable for various kinds of electrochemical and optical approaches. This fact is very prominent for the combined use of different measurement systems by avoiding surface dependent deviations. The suitability for SPR, one of the most popular quantitative biomolecular interaction analysis methods, is therefore an important aspect for further development of functional biomaterials based on these electrochemically grafted aryl diazonium salts. Cysteine-modified functional peptides could be immobilized directly, without the need of chip activation, and in a defined orientation. High ligand densities of covalently immobilized peptide could be achieved, allowing sensitive analyte detection. The *p*-MP chip could also be used to immobilize proteins via an additional modification step with mercaptopropionic acid. Protein–protein interactions could be examined kinetically. For the first time, our results extend the spectrum of application

of electrochemically grafted aryl diazonium salts to SPR-based biosensors and show the suitability of these surfaces for the quantitative evaluation of binding properties for peptide–protein and protein–protein interactions.

Acknowledgements

This project was funded by the Federal Ministry of Science and Education (BMBF), Germany (FKZ 03 IS2201 A).

REFERENCES

Almeida I, Cascalheira AC, Viana AS. 2010. One step gold (bio)functionalisation based on CS2-amine reaction. *Electrochim. Acta* **55**: 8686–8695. doi: 10.1016/j.electacta.2010.07.084

Böttger V, Peters L, Micheel B. 1999. Identification of peptide mimotopes for the fluorescein hapten binding of monoclonal antibody B13-DE1. *J. Mol. Recognit.* **12**: 191–197. doi: 10.1002/(SICI)1099-1352(199905/06)12:3<191::AID-JMR455>3.0.CO;2-2

Chehimi MM. 2012. Aryl diazonium salts new coupling agents in polymer and surface science. Wiley-VCH: Weinheim.

Doshi N, Mitragotri S. 2009. Designer biomaterials for nanomedicine. *Adv. Funct. Mater.* **19**: 3843–3854. doi: 10.1002/adfm.200901538

Engel E, Michiardi A, Navarro M, Lacroix D, Planell JA. 2008. Nanotechnology in regenerative medicine: the materials side. *Trends Biotechnol.* **26**: 39–47. doi: 10.1016/j.tibtech.2007.10.005

Gooding JJ, Darwish N. 2012. The rise of self-assembled monolayers for fabricating electrochemical biosensors--an interfacial perspective. *Chem. Rec. N. Y. N* **12**: 92–105. doi: 10.1002/tcr.201100013

Harper JC, Polsky R, Wheeler DR, Brozik SM. 2008. Maleimide-activated aryl diazonium salts for electrode surface functionalization with biological and redox-active molecules. *Langmuir* **24**: 2206–2211. doi: 10.1021/la702613e

Hsu W-L, Lee S-S, Lee C-K. 2009. Ellipsometric surface plasmon resonance. *J. Biomed. Opt.* **14**: 024036. doi: 10.1117/1.3116713

Jiang X-M, Wang L-M, Wang J, Chen C-Y. 2012. Gold nanomaterials: preparation, chemical modification, biomedical applications and potential risk assessment. *Appl. Biochem. Biotechnol.* **166**: 1533–1551. doi: 10.1007/s12010-012-9548-4

Johnsson B, Löfås S, Lindquist G, Edström A, Müller Hillgren RM, Hansson A. 1995. Comparison of methods for immobilization to carboxymethyl dextran sensor surfaces by analysis of the specific activity of monoclonal antibodies. *J. Mol. Recognit.* **8**: 125–131. doi: 10.1002/jmr.300080122

Karlsson R, Fägerstam L, Nilshans H, Persson B. 1993. Analysis of active antibody concentration. Separation of affinity and concentration parameters. *J. Immunol. Meth.* **166**: 75–84. doi: 10.1016/0022-1759(93)90330-A

Kausaite-Minkstimiene A, Ramanaviciene A, Kirlyte J, Ramanavicius A. 2010. Comparative Study of Random and Oriented Antibody Immobilization Techniques on the Binding Capacity of Immunosensor. *Anal. Chem.* **82**: 6401–6408. doi: 10.1021/ac100468k

Lê HQA, Sauriat-Dorizon H, Korri-Youssoufi H. 2010. Investigation of SPR and electrochemical detection of antigen with polypyrrole functionalized by biotinylated single-chain antibody: a review. *Anal. Chim. Acta* **674**: 1–8. doi: 10.1016/j.aca.2010.06.008

Löfås S, Johnsson B. 1990. A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands. *J. Chem. Soc. Chem. Comm.* **21**: 1526–1528. doi: 10.1039/c39900001526

Mahouche-Chergui S, Gam-Derouich S, Mangeney C, Chehimi MM. 2011. Aryl diazonium salts: a new class of coupling agents for bonding polymers, biomacromolecules and nanoparticles to surfaces. *Chem. Soc. Rev.* **40**: 4143–4166. doi: 10.1039/c0cs00179a

Messerschmidt K. 2007. Strukturelle und kinetische Charakterisierung des dual-spezifischen Antikörpers B13-DE1.Thesis, University of Potsdam

Messerschmidt K, Degen J, Micheel B. 2011. Oxidoreductase activity of multifunctional monoclonal antibody B13-DE1. *J. Mol. Recognit.* **24**: 930–934; doi: 10.1002/jmr.1136

Panicker RC, Huang X, Yao SQ. 2004. Recent advances in peptide-based microarray technologies. Comb. Chem. *High Throughput Screen* **7**: 547–556.

Pérez-López B, Merkoçi A. 2011. Nanoparticles for the development of improved (bio)sensing systems. *Anal. Bioanal. Chem.* **399**: 1577–1590. doi: 10.1007/s00216-010-4566-y

Phong PH, Tomono H, Nishi N, Yamamoto M, Kakiuchi T. 2008. Artificially phase-separated binary self-assembled monolayers composed of 11-amino-1-undecanethiolate and 10-carboxy-1-decanethiolate on Au (111): A comparative study of two preparing methods. *Electrochim. Acta* **53**: 4900–4906. doi: 10.1016/j.electacta.2008.01.038

Qi H, Peng Y, Gao Q, Zhang C. 2009. Applications of nanomaterials in electrogenerated chemiluminescence biosensors. *Sensors* **9**: 674–695. doi: 10.3390/s90100674

Schenk JA, Sellrie F, Böttger V, Menning A, Stöcklein WFM, Micheel B. 2007. Generation and application of a fluorescein-specific single chain antibody. *Biochimie* **89**: 1304–1311. doi: 10.1016/j.biochi.2007.06.008

Siemeling U, Memczak H, Bruhn C, Vogel F, Träger F, Baio JE, Weidner T. 2012. Zwitterionic dithiocarboxylates derived from N-heterocyclic carbenes: coordination to gold surfaces. *Dalton Trans.* **41**: 2986–2994. doi: 10.1039/c2dt11976e

Stech M, Merk H, Schenk JA, Stöcklein WFM, Wüstenhagen DA, Micheel B, Duschl C, Bier FF, Kubick S. 2012. Production of functional antibody fragments in a vesicle-based eukaryotic cell-free translation system. *J. Biotechnol.* **164**: 220–231. doi: 10.1016/j.jbiotec.2012.08.020

Variola F, Brunski JB, Orsini G, Tambasco de Oliveira P, Wazen R, Nanci A. 2011. Nanoscale surface modifications of medically relevant metals: state-of-the art and perspectives. *Nanoscale* **3**: 335–353. doi: 10.1039/c0nr00485e

Visser NFC, Heck AJR. 2008. Surface plasmon resonance mass spectrometry in proteomics. *Expert Rev. Proteomics* **5**: 425–433. doi: 10.1586/14789450.5.3.425

Wang W, Zhang S, Chinwangso P, Advincula RC, Lee TR. 2009. Electric potential stability and ionic permeability of SAMs on gold derived from bidentate and tridentate chelating alkanethiols. *J. Phys. Chem. C* **113**: 3717–3725. doi: 10.1021/jp808957r

Wiedemann U, Boisguerin P, Leben R, Leitner D, Krause G, Moelling K, Volkmer-Engert R, Oschkinat H. 2004. Quantification of PDZ domain specificity, prediction of ligand affinity and rational design of super-binding peptides. *J. Mol. Biol.* **343**: 703–718. doi: 10.1016/j.jmb.2004.08.064

Zhang X, Tretjakov A, Hovestaedt M, Sun G, Syritski V, Reut J, Volkmer R, Hinrichs K, Rappich J. 2013. Electrochemical functionalization of gold and silicon surfaces by a maleimide group as a biosensor for immunological application. *Acta Biomater.* **9**: 5838–5844. doi: 10.1016/j.actbio.2012.10.022

Zvirbliene A, Samonskyte L, Gedvilaite A, Voronkova T, Ulrich R, Sasnauskas K. 2006. Generation of monoclonal antibodies of desired specificity using chimeric polyomavirus-derived virus-like particles. *J. Immunol. Methods* **311**: 57–70. doi: 10.1016/j.jim.2006.01.007

