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A Novel *N*-Substituted Pyrrole Based Surface Modification for Biosensing

*Fabian Rüger, Jens Schäfer, Udo Bakowski, Michael Keusgen, and Doru Vornicescu**

Immobilization of proteins, mostly antigens, antibodies or enzymes on a solid surface, is one of the most important steps in the development of every biosensor. Different chemical methods as well as site-directed strategies have been developed in order to obtain highest functionality and stability for the sensoric device. In the present work, a novel method for the immobilization of biomolecules on a surface plasmon resonance (SPR) device is described. New *N*-substituted pyrroles are synthesized and deposited on a gold surface using electrochemical polymerization. To evaluate the established four different surfaces, the binding behavior between a fusion protein consisting of the lectin concanavalin A (ConA) as well as streptavidin (Sav) binding domains and the sugar mannan, which has been chemically coupled to the poly-pyrrole (PPy) surface is investigated. A pyrrole-*N*-C_{16}/pyrrole co-polymer surface as well as a pyrrol-1-yl hexanoic acid/pyrrole (PHCP) surface give best results.

1. Introduction

Immobilization of antigens, antibodies or enzymes on a solid surface is an important step in the development of protein-based biosensor.[1] A rather convenient method is the immobilization of streptavidin (Sav), which can bind to biotinylated proteins by self-organization.[2] This strategy is rather convenient because Sav is stable under various chemical conditions and can be immobilized chemically or by adsorption. Also, proteins can be biotinylated under smooth conditions.

First attempts to immobilize biomolecules on solid surfaces were undertaken with aldehydes to achieve a certain level of cross linking. Epoxides and active esters could be used in the same manner. With these methods high surface loadings could be achieved. As a disadvantage of these strategies, most of the proteins will be inactivated. Therefore, smoother methods are required.

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In the present work, a novel method for surface modification as a platform technology for further immobilization of biomolecules on Surface Plasmon Resonance (SPR) gold layers is described. In order to achieve maximum versatility, simple *N*-substituted pyrrole (NSP) building blocks were designed bearing functional groups like carboxylic acids. The obtained pyrrole/NSP co-polymers should be polymerized via electro-polymerization. After the deposition, established assays like sugar-lectin binding assays should be introduced in order to test the obtained SPR surfaces. The utilization of the lectin-sugar pairs, combined with the Sav-biotin strategy, will take a central role in the here presented experimental layout.[3,4] A recently designed fusion protein consisting of two complementary binding sites will be used for functional testing of the obtained surfaces (**Figure 1**). The first binding domain of the fusion protein is concanavalin A (ConA), a lectin derived from *Canavalia ensiformis*, and the second one is streptavidin (Sav), a protein obtained from *Streptomyces avidinii*. ConA binds specifically to polysaccharides like mannan and dextran. The binding between ConA and polysaccharides can be cleaved by the addition of methyl α- D-mannopyranoside as a 10% solution.[5,6] This allows a smooth regeneration of the sensor surface. As already stated above, the Sav binding domain can be bound to biotinylated proteins, DNA, and further biological recognition elements.[7] In contrast to the ConA-mannan, the Sav-biotin interface is very stable and can be only cleaved under harsh conditions.

2. Experimental Section

All used chemicals were of p.a grade or the highest available purity. They were purchased from Sigma-Aldrich Chemie GmbH (München, Germany), Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and CANDOR Bioscience GmbH (Wangen, Germany).

2.1. Synthesis of Different Pyrrole Monomers

The amine compound (18.0 mmol), together with sodium acetate (18.0 mmol, 1.476 g, 1eq) and tetraheptyl-ammoniumbromide (50 mg, ≈1 mol.%) are added to a mixture of water (20 mL) and acetic acid (99%, 12.5 mL). After addition of toluene (25 mL), the two-phasic mixture was brought to a mild reflux and kept there for 5 min. 2,5-Dimethoxy-tetrahydrofurane (18.9 mmol, 2.498 g, 2.449 mL, 1.05eq) was added within 15 min and the reaction

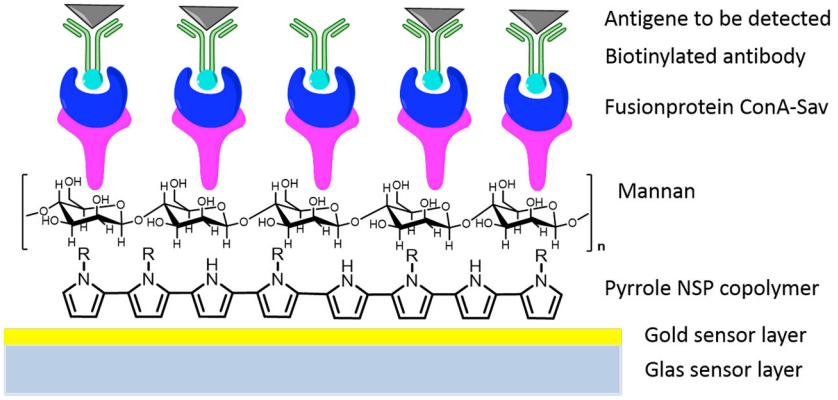


Figure 1. Schematic cross section through the layers which are subject of this investigation.

mixture was heated under reflux for further 6 h. After cooling to room temperature and separation of the organic toluene phase, the aqueous layer was extracted with toluene (two times with 50 mL). The pooled organic phases were washed with a small amount of water (three times with 20 mL) and dried over anhydrous sodium sulphate.

Evaporation of the solvent under reduced pressure, followed by column chromatography gave the desired products in yields between 67 and 95%. Stationary phase: silica gel 60 0.04–0.063 mm, mobile phase: cyclohexane – ethyl acetate mixtures: For pyrrol-N-C_{16} and pyrrol-N-C_{18} 35:1; 4-(1H-pyrrol-1-yl) phenol 10:1 and for 6-(1H-pyrrol-1-yl) hexanoic acid 5:1.

All N-substituted pyrroles used in this work were synthesized using a modified Clauson Kaas reaction (**Figure 2**).[8–10]

2.2. Production of the Poly Pyrrole (PPy) Coated SPR Gold Surfaces by Electro-Polymerization

SPR gold (50 nm) covered prisms, on which the polymer layer should be deposited, were cleaned by the following procedure prior to electro-polymerization: The prisms were rinsed with acetone for 5 min, washed with H_2O followed by treatment with 6 M NaOH for 2 min.

The so pre-washed prisms were incubated in a solution of 0.1 M KOH $+H_2O_2$ (30%) (ratio 1:1) for 30 min at 70–80 °C and washed with H_2O followed by acetonitrile. The freshly cleaned prisms were used as anode during electro-polymerization, the cathode was an identical gold coated prism. The distance between the electrodes was kept at 15 mm.

Several different mechanisms were postulated for the formation of poly pyrroles on the surface of the anode. One of the most widely accepted proposals is given in **Figure 3** and described in a modified form by Gourlay et al.[11] To improve the quality of the layers, co-polymers of plain pyrrole and NSP were generated (**Figure 4**). This procedure led to relatively even and stable polypyrrole films. The following composition was used for the two-electrode cell: 0.1 M tBu_4NBF_4, *N*-substituted pyrrole (0.05 M) and pyrrole (0.05 M) in MeCN (99.5%).

The voltage was kept at 0.9 V using a laboratory voltage source during deposition, the current was monitored until a stable value was achieved. The exposition time depended on the used pyrroles and took between 5 and 20 min. After removal of the chip from the cell, it was rinsed with acetonitrile, EtOH and water to remove traces of monomers and tBu_4NBF_4. Washing steps with ethyl acetate, methanol and H_2O followed by adjacent drying at room temperature until further use.

2.3. SPR Measurements

After preparation of the gold coated glass prisms as described above, SPR measurements were performed in order to test through the binding behavior of the fusion protein ConA-Sav. The main focus was on which PPy surface supports the binding of analyte to the system best. The SPR method allows observation in real time of the

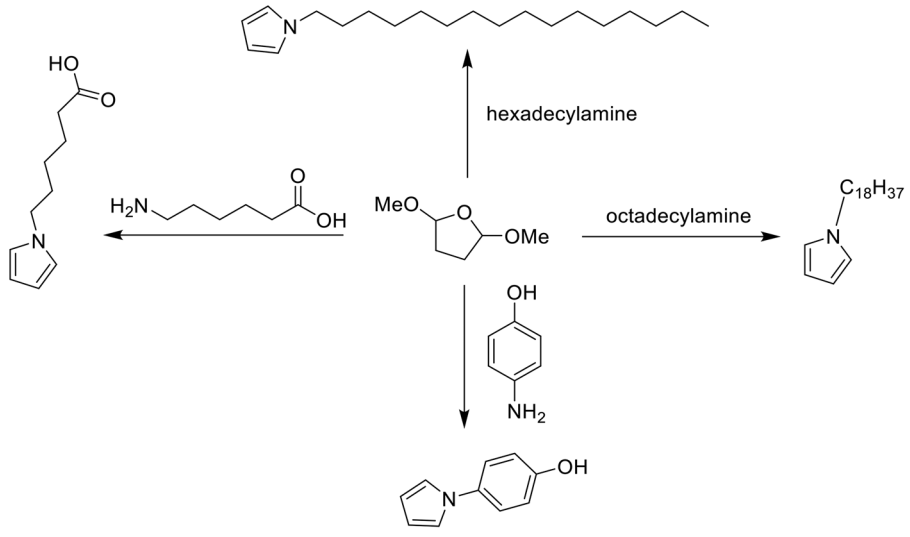


Figure 2. Clauson Kaas reaction yielding different N-substituted pyrroles (NSP's).

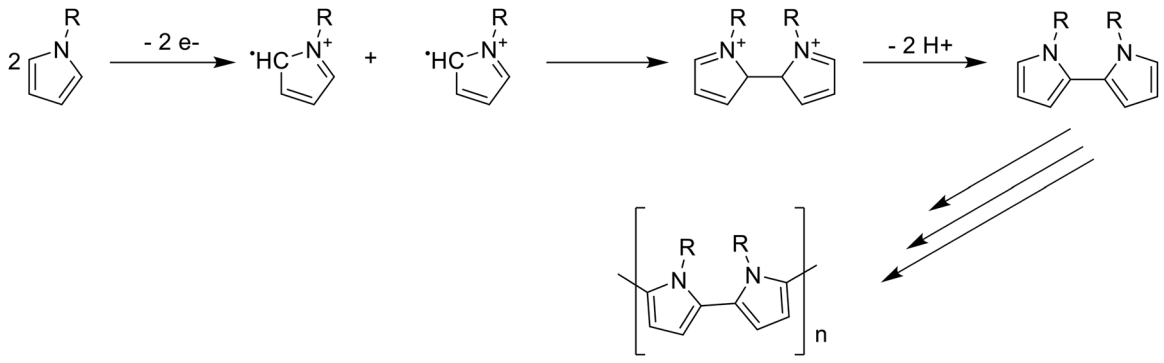


Figure 3. General scheme of electro-polymerization of pyrrole building blocks.

binding process between the ConA-Sav fusion protein and the mannan coated chip free of labelling or other kind modifications of the binding partners.[12] An 8-channel cuvette based device from the company Plasmonic® Biosensor in Wallenfels Germany (Plasmonic SOne®) served as device for the measurements.

The SPR device detects changes in refractive index occurring within the few hundred nanometers above the gold surface (called 'evanescent field') when biomolecules in solution bind to their partners which already had been immobilized onto the sensor surface in a preliminary step. The response signal is measured in pixel corresponding to the reflection angle shift and plotted against time on the display. It describes the occurring interactions in real-time.[8]

After recording the baseline with PBS buffer (30 mmol L^{-1} phosphate, 120 mmol L^{-1} NaCl; pH 7.3), mannan (1.0 mg mL^{-1}) was added on the PPy coated SPR chip by use of mix mode. A blocking step was performed by adding of Smart Blocking Solution® (CANDOR) followed by five washing steps with the same buffer covering the vacancies and removing the unbounded material. According to the measurement setup, the addition of ConA-Sav fusion protein (400 µg mL^{-1} dissolved in PBS buffer) onto the chip surface, followed by washing with PBS buffer was performed. The production and characterization of the ConA-Sav is described by Dassinger et al.[12]

To regenerate the surface and remove the specifically bound fusion protein, methyl α-D-mannopyranoside 10% (w/v) diluted in PBS buffer was added. All adding/washing steps were performed at 22.0 °C by using the 'mix-mode' setting. Display and processing of the data were performed by using of Origin Pro® 8.0 software (Origin Lab, USA).

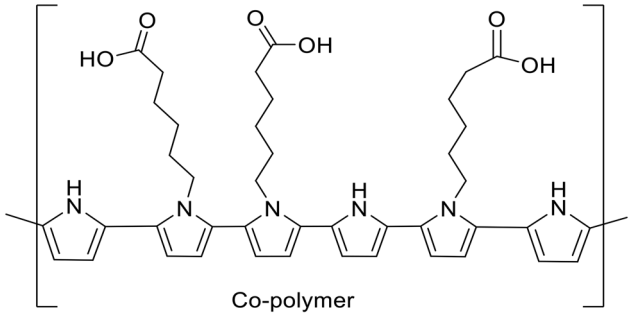


Figure 4. Potential structure of a pyrrole/6-(1H-pyrrol-1-yl) hexanoic acid co-polymer (PHCP).

2.4. AFM Measurements

To evaluate the surface morphology of the pyrrole coated surface, atomic force microscopy (AFM) measurements were performed at different stages of coating. Chips were carefully taken out of the SPR devices, rinsed with water and dried. Measurements were done with a vibration dampened JPK Nano Wizard I ™ (JPK Instruments, Berlin, Germany) equipped with a TopViewOptic™ in an acoustic enclosure, AFM cantilevers HQNSC14/AlBS (Micromasch, Estonia) with a resonance frequency of 160 kHz and a nominal force constant of 5 N/m were chosen for all the measurements. To avoid damaging the surfaces, intermittent contact (air) mode was chosen. The scan speed was proportional to the scan size.

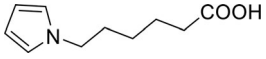
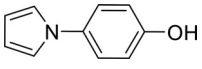
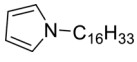
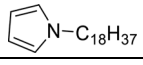
The determination of the RMS (root mean square) roughness Rq of the surface was done with JPK Data Processing Software of the height images (trace). The variable z_i^2 defines the vertical deviation to an averaged ideal surface.

3. Results and Discussion

3.1. Synthesis of Different Pyrrole Monomers

Different N-substituted pyrroles could be successfully synthesized from different rather inexpensive starting materials using 2,5-dimethoxy-tetrahydrofurane and different amino compounds. The obtained yields were between 67 and 95% (**Table 1**). The standard reaction time was set to 6 h, but also shorter times

Table 1. Yields of some NSP's synthesized using Clauson Kaas reaction.

Type	Pyrrole structure	Yield [%]
A[a)]		95
B[b)]		67
C[c)]		73
D[d)]		82

For analytical data, see supporting information: [a)] S1; [b)] S4; [c)] S2; [d)] S3.

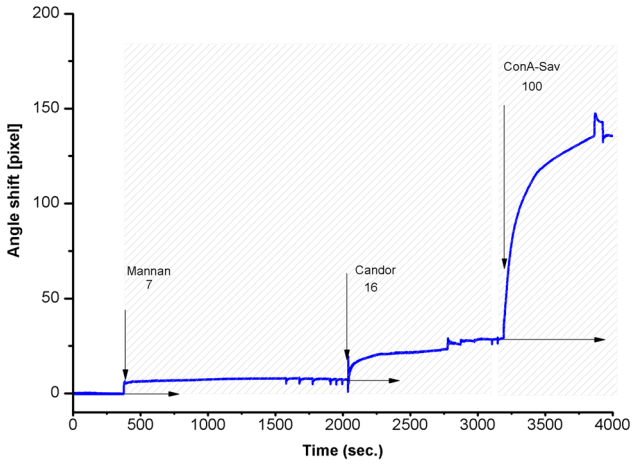


Figure 5. SPR-based mannan-ConA-Sav binding on PHCP co-polymer studies: 1. addition of mannan (1 mg mL^{-1}); 2. blocking step with Candor; 3. addition of ConA-Sav fusion protein (300 µg mL^{-1}).

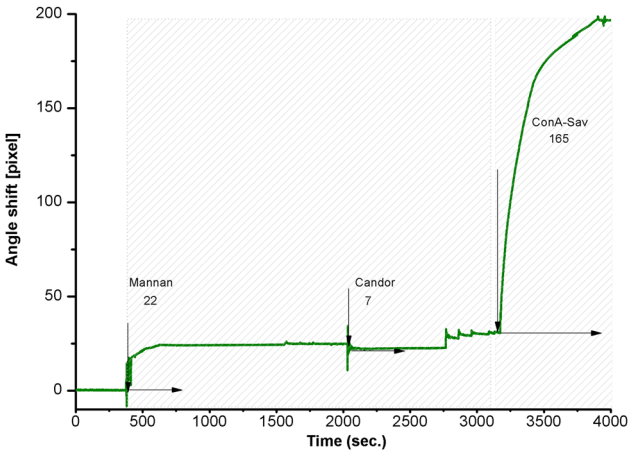


Figure 7. SPR-based pyrrole-N-C16 pyrrole co-polymer studies: 1. addition of mannan (1 mg mL^{-1}); 2. blocking step with Candor; 3. addition of ConA-Sav fusion protein (300 µg mL^{-1}).

seem to be possible in the case of aliphatic amines with low steric hindrance. Disappearance of the amine compound spot (thin layer chromatography) is a good endpoint indication for the reaction. The obtained NSP's showed purities of above 90% prior to column chromatography and could be used directly for electro-polymerization without further purification.

3.2. Production of the Poly pyrrole (PPy) Coated SPR Gold Surfaces by Electro-Polymerization

After cleaning the prisms, the gold surface of the SPR sensor was modified by electro polymerising a 1:1 mixture of NSP's and pyrrole (U: 0, 9V, t: 15 min.). This procedure leads to the formation of a visible, slightly grey, thin layer on the gold surface. Tests using an established mannan/ConA-Sav fusion protein assay were successfully adapted on pyrrole/6-(1H-pyrrol-1-yl) hexanoic acid co-polymer (PHCP) (Table 1 (Type A)), pyrrole- N-C16 pyrrole co-polymer (Table 1 (Type C)) as well as pyrrole/4-(1H-pyrrol-1-yl) phenol pyrrole co-polymer (Table 1 (Type D)) surfaces. Because no significant difference in the structure as also how it behaves during the SPR measurements, the results for pyrrole- N-C18 pyrrole co-polymer (Table 1 (Type B)) were not shown.

3.3. SPR Measurements

The evaluation of the established PPy coated SPR surfaces was done by a functional test according to Figure 1. Firstly, different PPy-films were treated with a mannan solution, followed by the addition of the ConA-Sav fusion protein (Figures 5–8). After coating with mannan, a blocking step with Candor-buffer has been introduced. This step is necessary to guarantee that after

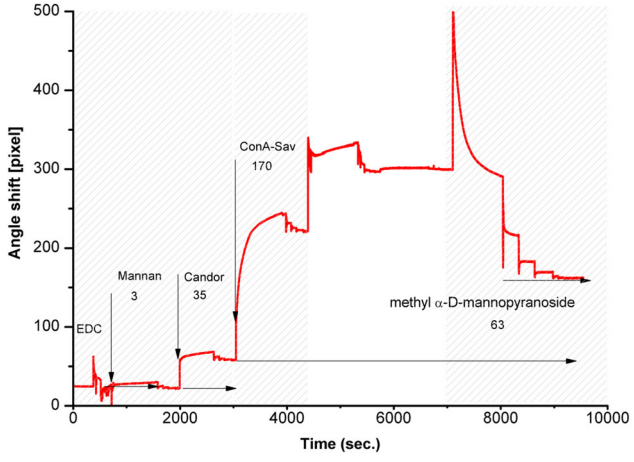


Figure 6. SPR-based covalently coupled mannan-ConA-Sav binding on PHCP co-polymer studies: 1. surface activation with EDC; 2. addition of mannan (1 mg ml^{-1}); 3. blocking step with Candor; 4. addition of ConA-Sav fusion protein. (300 µg ml^{-1}); 5. regeneration with 10% methyl α-D-mannopyranoside.

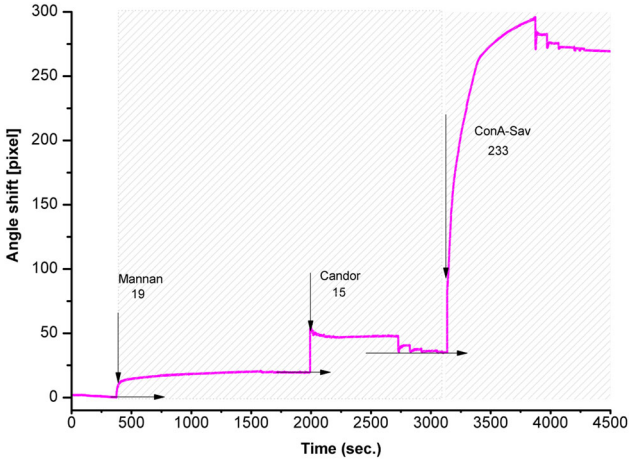


Figure 8. SPR-based 4-(1H-pyrrol-1-yl)phenol pyrrole co-polymer studies: 1. addition of mannan (1 mg mL^{-1}); 2. blocking step with Candor©; 3. addition of ConA-Sav fusion protein (300 µg mL^{-1}).

Table 2. SPR signal increase after addition of the fusion protein to different mannan treated PPy surfaces as given in **Table 1**.

NSP used	Signal increase (pixel)
A	100
A + EDC	170
B	233
C	165
D	153

Table 3. RMS roughness (root-mean-squared), Ra roughness and scanned area of the three surfaces B, C, and D as given in **Figure 9**.

Type of surface	RMS [nm]	Ra [nm]	Scanned area [μm]
B	2.560	1.996	1 × 1
C	4.237	3.014	2.5 × 2.5
D	7.323	6.099	2.5 × 2.5

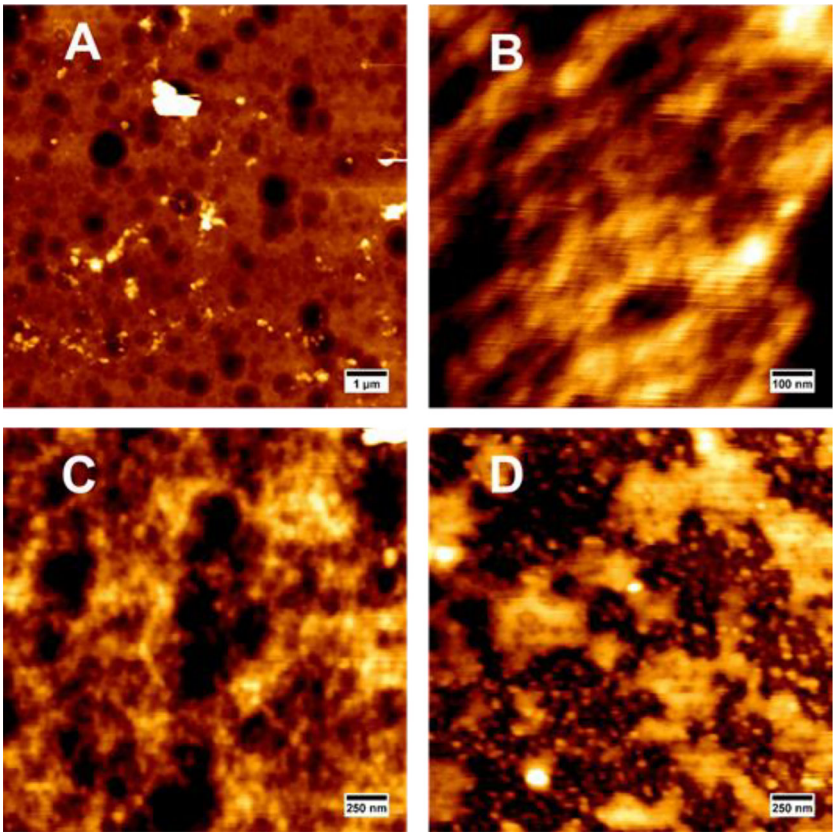


Figure 9. AFM height images (cantilever HQ:NSC14AlBS) of modified chips: a) overview of pure gold surface without modification; b) detailed image of pure gold surface without modification indicating smooth surface (roughness 2.56 nm); c) surface modified with pyrrole and mannan; d) surface modified with pyrrol-N-C16 pyrrole co-polymer and mannan + ConA-Sav (increased roughness of mannan and ConA-Sav clusters visible).

addition of the fusion protein only a SPR-signal for a specific binding is visible.

Figure 5 displays the results for immobilization of the PHCP co-polymer followed by mannan coating. Interestingly, the loading seems to be rather high and a specific binding event could be monitored after addition of ConA-Sav. This led to the assumption that there is a chance of hydrogen bridge or ionic interactions between the PHCP and the mannan probably accompanied by van der Waal's interactions. The formed layer seems to be stable.

In the next step, The carboxylic acid functionality of the PHCP co-polymer was chemically activated by 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and the mannan has been added after activation. In the experiment, the mannan should be covalently bound to the PHCP co-polymer.

The corresponding SPR measurement including on-chip activation is shown in **Figure 6**. The binding of the fusion protein to the covalently immobilized polysaccharide gave reason for the assumption that the chemical reaction has been successful, leading to a thinner more evenly ordered layer of mannan. Moreover, the obtained SPR signals were much higher as in the case of the experiment shown in Figure 5.

As shown in Figure 6, also a regeneration step with methyl α-D-mannopyranoside has been performed. Regeneration is possible by applying a sugar solution whose binding to the fusion protein is thermodynamically favored compared to mannan.[13,14] A relative high regeneration rate (43%) of the mannan surface could be achieved. A complete regeneration could not be achieved because of steric hindrance. The methyl α-D-mannopyranoside must reach the binding site of the ConA to allow cleavage of the mannan-ConA binding. This appears to be difficult in a heterogeneous system deposited on a solid surface.

As a further experiment, ConA was immobilized on pyrrole-N-C_{16} co-polymer surface by unspecific hydrophobic interactions (**Figure 7**). Interestingly, obtained results were nearly comparable to those given in Figure 6. The advantage of this method is, that it is very simple and should work for nearly all types of proteins. Also in this case, liberation of the ConA-Sav fusion protein from the chip surface by washing with a methyl α-D-mannopyranoside solution was performed, but only insufficient regeneration could be achieved (data not shown). As explained above, steric hindrance could be the reason for the low regeneration rate. Modification of the gold chip surface with a 4-(1H-pyrrol-1-yl)phenol pyrrole co-polymer shows high binding performance towards the fusion protein, although not as unpolar and without a long alkyl chain, the surface seems to bind mannan in a similar fashion to pyrrole-N-C_{16} and pyrrole-N-C_{18} co-polymers (**Figure 8**). The substantially higher binding rate of ConA-Sav could be explained with a higher ratio of

"exposed" mannan. The binding rates for the fusion protein on different mannan coated PPy surfaces are shown in **Table 2**.

3.4. AFM Measurements

After the SPR measurements, chips were carefully removed out of the SPR device and analysed by AFM as depicted in Figure 8. An area of 2.5 × 2.5 µm was observed.

In general, the surface showed a somewhat rough, but uniform coating. Results of the roughness study are given in **Table 3**. The surface after mannan coating showed structures of about 50 nm with round or oval shapes. Due to the polymeric structure of mannan, these shapes do fit rather well to its polysaccharide structure.

After loading with the fusion protein (43 kDa), the surface looks quite similar. As it is visible in Figure 8(d), a fine supra-structure can be observed. This could be caused by the ConA-Sav fusion protein. Generally, the loading seems to be rather homogeneous. However, because of the roughness of the mannan-loaded surface (Figure 8(c)), the deposition step needs further improvement. For example, by optimization of the concentration of the mannan solution during the loading step, additionally the mix-mode of the SPR device could be modified.

4. Conclusion

Four different PPy surfaces could be successfully established. Different gold SPR chips, modified with the same PPy, showed very similar binding characteristics. The so produced PPy films adhered strongly to the gold layer and are stable within a broad pH range. Widely used dextran surfaces show a negative charge above pH 3.5, this could lead to unspecific interactions with proteins.[15] By using different NSPs the net charge of the layer can be controlled. Binding inhibitors covalently to the PPy could eliminate the risk of unspecific interactions. The rough surface evaluated by AFM measurements could be optimized towards a smoother surface by carefully improving and modifying the electro-polymerization as well as concentrations of the monomeric pyrroles in solution. Real time monitoring of the electrochemical polymerization process with the SPR technique is something that could be adopted for a complete control of the whole process.[16] The following immobilization steps have some potential for optimization. Out of these surfaces, the pyrrole-N-C_{16} co-polymer surface as well as the PHCP co-polymer surface with and without chemical activation gave sufficient results in a binding assay with the ConA-Sav fusion protein.

A simple regeneration of the PHCP co-polymer chip surface by 10% methyl α-D- mannopyranoside solution was possible in a final washing step. The regeneration rate of the surface was up to 43%, thus providing the possibility to reuse the chip surface and allows for regaining the analyte.

There are further variations of the method thinkable. A very promising variation, which is under investigation, is a co-polymer with N_2, N_2-bis(carboxymethyl)-N_6-pyrrolyl-L-lysine **(S 5)** which, after treatment with Ni^{2+} or Co^{2+} salts, is suitable for the immobilization of His-tagged proteins. This method is applicable to many different proteins, allows immobilization by self-organization, as well as a swift regeneration of the surface.

The final step of the entire assay as shown in Figure 1 would be the immobilization of a biotinylated protein, typically a biotinylated antibody capturing the analyte. This has been also tested in these studies, but until now only with little success because of inactivation of the antibody. The reason for this phenomenon is not clear in the moment. The reason can be insufficient washing steps, inappropriate buffer systems or denaturation of antibodies by contact with uncoated chip areas. The solution for this problem is a main topic of ongoing research.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

atomic force microscopy (AFM), electro polymerization, *N*-substituted pyrroles, poly-pyrroles (PPy), surface plasmon resonance (SPR)

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