Chemistry
A European
Journal

Chemistry Europe
European Chemical Societies Publishing

Accepted Article

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To be cited as: *Chem. Eur. J.* 10.1002/chem.202000989

Link to VoR: https://doi.org/10.1002/chem.202000989

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10.1002/chem.202000989

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RESEARCH ARTICLE

Site-directed antibodies immobilization by resorc[4]arene-based immunosensors

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Abstract: One of the main problems in the development of immunosensors is to overcome the complexity of binding antibody to the sensor surface. Most of immobilizing methods lead to a random orientation of antibodies with a lower binding site density and immunoaffinity. In order to control the orientation of antibody immobilization, several resorc[4]arene derivatives were designed and synthesized. After the spectroscopic characterization of resorc[4]arene self-assembled monolayers (SAMs) onto gold films, the surface coverage and the orientation of insulin antibody (Ab-Ins) were assessed by Surface Plasmon Resonance (SPR) technique and compared with a random immobilization method. Experimental results combined with theoretical studies confirmed the dipole-dipole interaction as an important factor in antibody orientation and demonstrated the importance of the upper rim functionalization of resorcarenes. Accordingly, resorcarene **5** showed a major binding force towards Ab-Ins thanks to the H-bond interactions with the amine protein groups. Based on these findings, the resorcarene-based immunosensor is a powerful system with improved sensitivity providing new insight into sensors development.

Introduction

In recent years, biosensors and immunosensors attracted the attention of researchers as a diagnostic tool in several fields. [1-4] Biosensors are sensing devices characterized by the coupling of a physico-chemical transducer (i.e., electrochemical) with enzyme, antibody, aptamers, etc. [5-8] The research has been focused on the improvement of a technology characterized by selective, sensitive, fast response, and miniaturized biodevices for the monitoring and screening of analytes in several matrices. [9-12] One of the main issues to be solved to reach these features is related to the peculiar nature of biosensor, i.e., the immobilization of the biotransducer on a solid surface. An ideal immobilization technique should ensure high biotransducer loading maintaining its activity. Considering the heterogeneous nature of the biotransducer-analyte interaction, another aspect to be taken into careful account is the correct orientation of the protein to allow the optimal interaction with the analyte in order to enhance biosensor performance. This aspect is of paramount importance in the case of immunosensors, where antibodies are characterized by an asymmetrical structure with their recognition sites taking different positions in space following different immobilization procedures, and thus hindering analyte interaction. Antibodies are characterized by two identical fragment antigen-binding (Fab) regions and a crystallizable fragment (Fc) region responsible for recruiting components of the immune system.
Antibody immobilization on a solid surface can be obtained following two main approaches: random and site-oriented immobilization. A critical drawback of random antibody immobilization is the reduced amount of anti-body molecules available for the interaction with antigen. [13-14] To maximize the amount of immobilized active anti-body, it is necessary to control the antibody orientation promoting the accessibility of the Fab region for antigen interaction. [15-20] Thus, a suitable immobilization approach is always sought to preserve the maximum antibody functionality. In this respect, a promising approach proved to be the photochemical immobilization technique [21], which enable the oriented immobilization of the antibody onto untreated gold surface thus providing a convenient orientation of the Fab region. Supramolecular chemistry has recently attracted great interest as a surface modification tool to achieve the pre-organization of synthetic receptors and to improve the functional properties of SAM. Accordingly, the large pool of macrocycles available, with their synthetic modularity and different complexation properties, have represented an important challenge for the site-directed immobilization in immunosensor development. [22] Among them, calixarenes are one of the most ubiquitous host molecules in supramolecular chemistry. These macrocycles, characterized by a unique three-dimensional surface, can be functionalized at both the upper and lower rims with several functional groups in order to tailor their recognition properties towards a specific class of analytes. Therefore, the use of calixarenes as artificial liker systems to immobilize antibodies properly oriented has attracted increasing interest in the last two decades. [17,22,23] Within the calixarene family, resorcinol-derived cycloligomers, namely resorcarenes, behave as abiotic artificial receptors having enforced cavities of molecular dimension. [24,25] The choice of the groups attached at the upper rim plays a pivotal role determining the shape, the rigidity and the complexation properties, whereas the functionalization of the lower rim is functional to surface anchoring. [26-30] Efficient sensor devices were designed and developed by using several resorcarene-based cavitands. [22] However, to date, limited use has been made of resorc[4]arene derivatives as an artificial linker for the site-directed antibody immobilization, giving rise to advanced immunosensors. Herein, several resorc[4]arenes, variously substituted at the upper rim and featuring long thioether alkyl chains at the lower rim, were designed and synthetized. The surface coverage and the orientation of Ab-Ins on resorc[4]arene SAMs were evaluated by SPR technique and compared with a random immobilization methodology, highlighting a substantial increase in immunosensor sensitivity.

10.1002/chem.202000989

Results and Discussion

Development and synthesis of resorc[4]rene-based supramolecular linkers

Different types of highly sensitive protein microarray were developed by Lee et al. using two bifunctional calixarene-based linkers. [31] In particular, calix[4]arene bearing two thiol groups at the upper rim and a crown bridge between the aromatic rings oriented parallel to each other at the lower rim allowed the formation of a SAM on gold surface. The binding affinity of the crown moiety towards different proteins, antibodies and membrane receptors by supramolecular interactions (e.g., dipole-dipole and hydrogen-bonding) allowed the fabrication of simple and convenient sensor for application in clinical analysis. [23, 31-36]

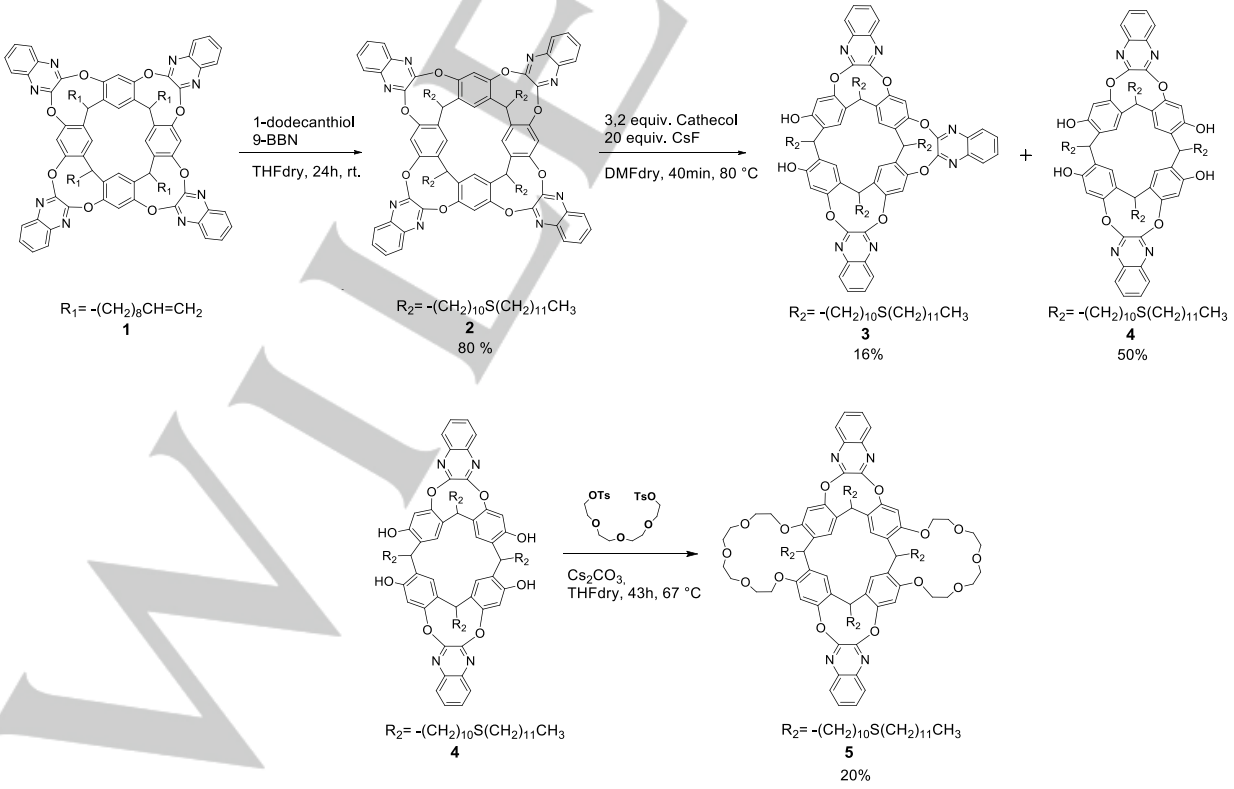
To construct supramolecular artificial linkers for antibodies immobilization, we rationally designed and synthesized resorc[4]arenes **2**-**5** (Scheme 1). The influence of size and polarity of the quinoxaline and crown ether headgroups was evaluated towards site-directed antibody immobilization; in addition, the long tetrasulfide chains were introduced at the lower rim to evaluate their monolayer organization on gold sensor disk surface. The synthetic procedure to afford the resorc[4]arenes **2**-**5** involved the thio-ene transformation of terminal vinylidene groups and the selective removal of the quinoxaline moieties from the upper rim.

The starting substrate **1**, prepared as previously reported [37], reacted with 1-dodecanethiol via anti-Markovnikov addition in the presence of 9-borabicyclo[3.3.1]nonane (9-BBN) as catalyst, in order to obtain resorc[4]arene **2** in 80% yield. The best conditions to drive the excision of quinoxaline units were assessed based on previously reported studies. [38]

By using 3.2 equiv. of chatecol and 20 equiv. of base (CsF), the triquinoxaline derivative **3** and diquinoxaline **4** were obtained in 18% and 48% yields, respectively, along with a small amount of the unreacted resorc[4]arene **2**. Further bis-crown **5** preparation was attempted by Williamson etherification, reacting **4** with 1.1 equiv. of tetra(ethyleneglycol)ditosylate in the presence of Cs_2CO_3 affording the desired compound in 20% yield.

Ab-Ins immobilization on resorc[4]arene-modified sensor disk

To evaluate the influence of the upper rim functionalization on the Ab-Ins interaction, the affinity and the maximum absorption capacity were assessed for biscrownresorc[4]arene **5** and its analogues (**2**-**4**) throughout SPR experiments (Figure 1). Accordingly, the maximum binding capacity (Bmax), the equilibrium dissociation constant (K_D) and the Ab-Ins mass loading were calculated (Table 1). These kinetic parameters combined with the SPR angle variation (Figure 1) indicated how replacing two quinoxaline groups with biscrown ether bridges increases the antibody affinity, whereas the removal of one or two quinoxaline moieties markedly reduced the immobilization process. In particular, the Ab-Ins mass loading calculated from the baseline changes before and after protein injection (considering that an angle shift of 0.120°, is approximatively equivalent to an amount of interacting protein of 1 ng/mm^2 [39]) allowed us to define the different binding affinity of resorc[4]arene derivatives.



Scheme 1. Synthesis of resorc[4]arene-based linkers (**2**-**5**).

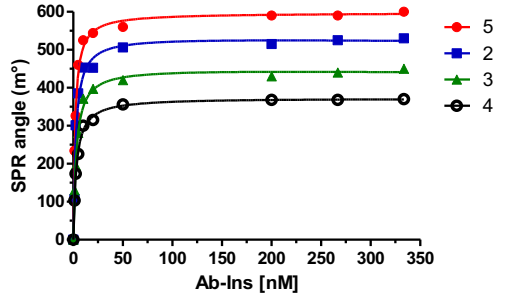


Figure 1. Measurements of Ab-Ins binding to resorc[4]arene derivatives.

One of the right orientation to obtain an optimal Ab-Ag interaction is the "end-on" configuration of antibody with the Fc fragment disposed towards the sensor disk surface. Accordingly, it has been demonstrated that the larger isoelectric point value of the $(Fab)_2$ fragment with respect to the one of the Fc portion induces a dipole moment (DM) of the Ab molecule from Fc to $(Fab)_2$. Several studies have shown how the interaction between Ab and calixarenes is driven by either hydrophobicity and surface charge density, claiming the key role of dipole-dipole attractions on the end-on Ab orientation. [17,31,36] Based on these findings, semiempirical and molecular mechanics (MM) methods were employed to calculate the dipole moment of the resorc[4]arene-based linkers (**2**-**5**) by using AM1 model and MMFF force field (FF), respectively. The DM values were then compared with the obtained K_D (Table 2).

Table 1. Kinetic parameters related to the Ab-Ins–resorc[4]arene (**2**-**5**) interaction. Data obtained are the average of three experiments.

SAMs	2	3	4	5
Bmax(m°)	554.0	456.4	375.2	600.1
K_D (nM)	2.344	2.954	3.023	1.795
R square	0.9768	0.9959	0.9976	0.9955
Ab-Ins loading (ng/mm²)	4.54	3.74	3.08	4.92
Ab-Ins loading (nmol/mm²)	$3.03x10^{-5}$	$2.49x10^{-5}$	$2.05x10^{-5}$	$3.28x10^{-5}$
Ab-Ins loading (n°molecules/mm²)	$1.82x10^{10}$	$1.50x10^{10}$	$1.23x10^{10}$	$1.97x10^{10}$

Notably, good linearity (R^2=0.9953) exists between the DM values of **2**-**4** estimated by semiempirical calculations with the corresponding K_D (Figure 2), while a bit lower correlation (R^2=0.9773) is observed employing DMs estimated through MM calculations. Interestingly, a significant deviation from this behavior is instead observed in the case of **5**. Starting from the experimental value of K_D, the corresponding theoretical DM of 14.3 debye is 3.8 debye higher respect to the value assessed by semiempirical molecular modeling.

Table 2. K_D values of Ab-Ins/Resorc[4]arene complexes compared with the theoretical dipole moment of resorcarene derivatives.

SAMs	K_D	DM (debye) by AM1	DM (debye) by MMFF
2	2.344	12.38	12.27
3	2.954	10.35	10.48
4	3.023	9.93	9.88
5	1.79	10.57	12.11

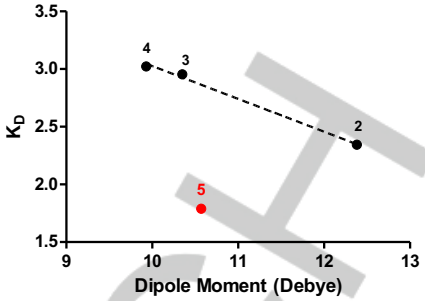


Figure 2. Linear correlation between dipole moments assessed through semiempirical calculations and affinity constants.

Besides the dipole-dipole interaction, the uncorrelated data (K_D*vs* semiempirical MD) of biscrown resorc[4]arene **5** could be attributed to the hydrogen bonding between the oxygens of the crown ether bridges and the secondary amine groups of basic amino acid residues of Ab. [40] To further evaluate the pH-dependence of Ab-Ins supramolecular binding on a **5**-modified gold disk, we monitored the SPR angle variation as a function of increasing pH values. The experimental data revealed a significant correlation between Ab-Ins immobilization and pH, improving in acid condition with respect to the basic one. In line with the hypothesis proposed by Lee and coworkers, the results obtained support the host-guest interaction between amino groups of Ab lysine residues and crown ether moieties of **5**, since a pH-increment over to 6.5 induces a quick reduction in the SPR angle variation (Figure 3).

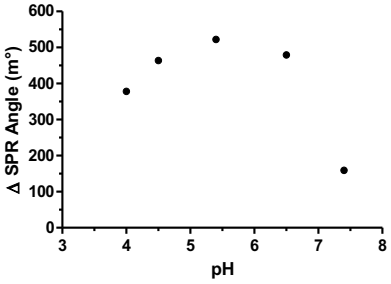


Figure 3. Ab-Ins interaction on **5**-modified SPR sensor disk (15 mg/L).

In addition, π-π interactions between the hydrophobic side chains of antibody and the two quinoxaline groups of **5** may also be involved in the strengthened binding. [31]

Surface coverage and site-oriented antibody immobilization

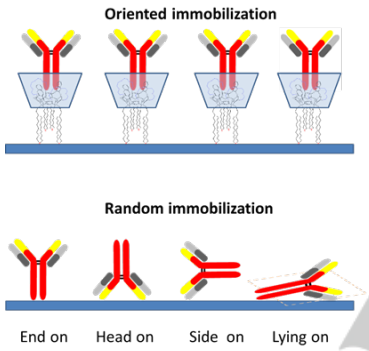
To assess whether the Ab-Ins site-oriented recognition occurs by resorc[4]arenes (**2**-**5**), the surface coverage of the modified gold disk was established at different Ab-Ins concentrations considering the SPR angle variation (Table 3). Notably, the change of SPR signal of sensor disk surfaces is proportional to the amount of the bound protein and decays exponentially with the distance from the gold coat, every 122 m° angle shift of layer thickness below 300 nm corresponds to 1 ng/mm² of immobilized protein. [39] Under saturation conditions, the surface densities of Ab-Ins on SAMs of resorc[4]arenes **2** and **5** were comparable with those obtained with the 11-mercaptoundecanoic acid (MUA)-

SAM. Although the steric hindrance of resorcarene macrocycles with respect to MUA could reduce the number of available sites for the Ab-Ins interaction, the quantity of Ab immobilized on **2**- or **5**-SAM per unit area (ng/cm^2) is only slightly lower, suggesting a site-oriented immobilization of antibody.

Table 3. SPR Shifts and Surface Coverages of Immobilization of [Ab Ins] 10 mg/L and saturation (50 mg/L R[4] and 300 mg/L for MUA) on the resorcarene (15 mg/L) and MUA (a: 30mg/L) SAMs.

SAMs	[Ab Ins] 10 mg/L SurfaceCoverage (ng/cm^2)	[Ab Ins] saturation SurfaceCoverage (ng/cm^2)
2	371	454
3	304	374
4	246	308
5	430	492
MUA	277	500

The supramolecular recognition by properly functionalized resorcarene linkers occurs in line with the “end-on” configuration providing a surface density similar to MUA-SAM that, instead, furnishes the unfavorable random immobilization (e.g. "side on" or "lying on" configurations) (Scheme 2).



Scheme 2. Oriented and random antibody immobilization on solid surface.

To estimate resorcarene concentration onto the gold sensor surface, the measurements of reductive desorption were performed considering the relation between SAM stability and electrochemical potentials. Accordingly, the electrons involved in the desorption reaction of the non-chemisorbed linker and, thus, the number of resorcarene molecules located on the gold surface was calculated by integration of the reductive desorption peak. Considering that 4 electrons are involved for each macrocyclic molecule, the resorcarene concentration calculated is 4.16x10^{-13} mol/mm^2 and the number of resorcarene molecules on the sensor disk surface was 2.5x10^{11} molecules/mm^2. [41] Furthermore, taking into account an average diameter of each molecule with a circular geometry of about 7x10^{-7}mm^2 and an hexagonal dense packing, surface covered per molecule should theoretically be 3.85x10^{-13} mm^2, which corresponds to 2.36x10^{12} molecules/mm^2 of resorcarene. The difference obtained between the experimental and the theoretical values is probably due to an incomplete packed arrangement of the SAM molecules onto the gold surface. Combining this evidence with the value of Ab-Ins molecules/mm^2 of **5** (Table 1), we estimated 12-13 molecules of resorc[4]arene for each Ab-Ins molecule.

Evaluation of immunosensor sensitivity

In order to evaluate the immunosensor sensitivity towards antigen detection, SPR signal variation due to Insulin-(Ab-Ins) binding was detected for both **5**- and MUA-(Ab-Ins) sensor disks (Figure 4). Accordingly, the insulin association experiments on the modified gold disks were performed by varying the Insulin concentration from 0.5 to 200 μg mL^{-1}. A table reporting the first data and last saturation data in Figure 4 has been also added (Table 4).

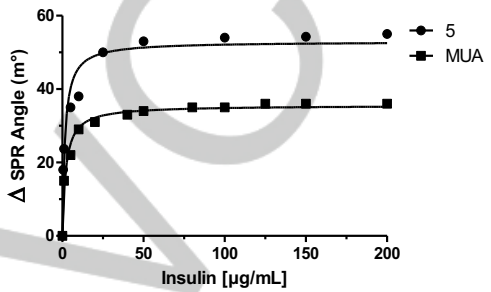


Figure 4. Insulin loading on **5**- and MUA-(Ab-Ins) immunosensors detected by SPR signal variation.

Table 4. SPR Shifts of resorcarene 5 derivative and MUA in the Ab-Ins/insulin interaction.

	ΔSPR Angle (m°)		
Insulin [μg/mL]	**5**	**MUA**	**Increase %**
0.5	18.0	12.0	+34.0
1.0	24.0	15.0	+37.5
5.0	35.0	22.0	+37.2
10.0	38.0	29.0	+23.7
200.0	55.0	36.0	+34.6

The experimental data at low values of [Insulin] highlight an increase in the SPR signal for resorcarene-based immunosensor **5**, as compared to those related on MUA-based immunosensor, which is maintained also in the saturation values. The results obtained confirmed the efficacy of oriented antibody immobilization and the improvement in Insulin-(Ab-Ins) interaction by using preorganized molecular receptors.

Regeneration of biscrown resorcane-based sensor disk

Due to the supramolecular interaction between **5** and Ab-Ins and its strict dependence on pH (Figure 3), regeneration experiments of resorcarene-based sensor disk were carried out by using basic pH-buffers to assess the best condition for the removal of Ab-Ag complex. In particular, NaOH solutions over the concentration range of 10 to 1000 mM were tested (Figure 5). From the data reported in Table 5, solutions with high-pH values proved able to induce an increased level of antibody dissociation resulting, however, more aggressive towards the sensor disk surface (appearance of lighter areas in the disk).

Table 5. Regeneration experiments of **5**-based sensor disk performed by using different concentrations of NaOH.

[NaOH] (mM)	%Ab removed %(m°/m°)	%Ab'/Ab %(m°/m°)
10	41.5%	66.8%
100	58.3%	53.7%
500	69.3%	60%
1000	100%	85.8%

To prevent irreversible damage of the gold disk, a regeneration procedure involving a two-step mechanism and the use of a more diluted NaOH solution was envisaged. [42] Accordingly, an initial phase of Ab-Ins displacement was performed with the use of ethanolamine (1 M, pH 9), that having smaller size and an amino group was able to replace 72% of the immobilized Ab on resorcarene-based sensor disk (regardless of the concentration of Ab injected). Further removal of the adsorbed amine was induced by NaOH solution of 10 mM, overcoming the attraction forces involved in the interaction between the artificial linker and the positively charged ethanolamine. Besides being a very useful regeneration procedure, this methodology reduced the washing times necessary to reach the best pH value for antibody immobilization.

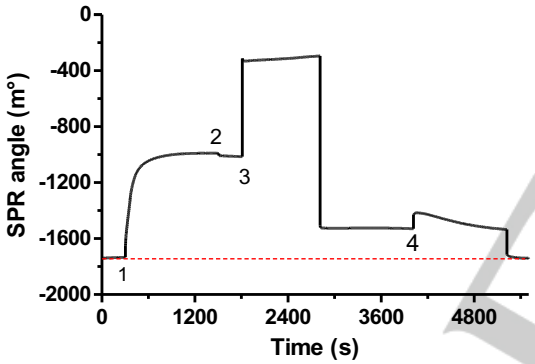


Figure 5. Regeneration of **5**-modified sensor disk 1) immobilization Ab-Ins (10 μg mL^{-1}); 2) 10 mM acetate buffer pH 5.4, 10; 3) 1 M ethanolamine, pH 9; 4) 10 mM NaOH.

Conclusion

In summary, in view of designing an Ab-Ins sensor with superior performance, newly resorc[4]arene-based linkers were synthesized. Resorc[4]arenes **2**-**5**, featuring long thioether alkyl chains at the lower rim to a monolayer on Au surface, were variously substituted (i.e., quinoxalines and crown moieties) at the upper rim to elucidate the influence of functionalization on the Ab-Ins interaction. Accordingly, the affinity and the maximum absorption capacity were evaluated throughout SPR experiments and compared with a random immobilization methodology. The results obtained from SPR measurements, combined with theoretical calculations of the dipole moment of the resorc[4]arene-based linkers (2-5), indicate that the crown groups at the upper rim of 5 play a critical effect on the selective affinity toward the immobilizing antibody in the SAMs, leading to a significant increase in immunosensor sensitivity. The main results of this work showed that the novel well-designed biscrown resorc[4]arene SAM could be considered a useful tool for proteins immobilization contributing to the development of more efficient immunosensing devices.

Experimental Section

Materials

All reagents were commercially available and were used without further purification.N-(-Dimethylaminopropyl)-N′-ethyl carbodiimide (EDC), N-hydroxysuccinimide (NHS), Bovine serum albumin and general reagents were purchased from Sigma-Aldrich and used without further purification. Monoclonal anti-insulin antibody (Ins-Ab) was purchased from Gentaur (Brussels, Belgium). Human insulin expressed in E.coli was purchased from Upstate Biotechnology (NY, USA). All aqueous solutions were prepared using deionized water (specific resistivity ≥ 18.2 MΩ cm) obtained from a Direct-Q 3 UV apparatus (Millipore, France). The gold disks SensorDisc Au bare gold for SPR analysis were purchased from Xantec Bioanalytics (Duesseldorf, Germany), The SPR measurements were performed by AutolabSpringle SPR of EcoChemie (Utrecht, The Netherlands). The electrochemical measurements were performed in a 5 mL thermostated glass cell Metrohm, (Switzerland) with a conventional three-electrode configuration, using a gold working electrode Metrohm (Switzerland) of 3 mm diameter, an Ag/AgCl/KClsat was used as reference electrode Metrohm, (Switzerland) and a glassy carbon rod as counter electrode Metrohm, (Switzerland).The electrochemical experiments were performed using a potentiostat μ-Autolab type III Metrohm (Herisau, Switzerland), interfaced to a personal computer running GPES manager software (version 4.9, Metrohm) for both instrument drive and data collection and elaboration. The linear andnon linearregressions were calculated by using the software GraphPad Prism 5 from GraphPad Software Inc. (USA).

Sensor disk functionalization and antibody immobilization

The SPR experiments were performed by an Eco ChemieAutolab SPR system (Ecochemie, The Netherlands). It works with a laser diode fixed at a wavelength of 670 nm, using a vibrating mirror to modulate the angle of incidence of the p-polarized light beam on the SPR substrate. The instrument is equipped with a cuvette. The planar gold SPR disks were purchased from XantecBioanalytics (Germany). The gold sensor disks (25 mm in diameter) were mounted on the hemicylindrical lens (with index-matching oil) to form the base of the cuvette. An O-ring (3-mm inner diameter) between the cuvette and the disk prevents leakage.An autosampler (Eco Chemie) with controllable aspirating–dispensing–mixing pipette was used to add samples into the cuvette and provide constant mixture by aspiration and dispensing during measurements. This experimental arrangement maintains a homogeneous solution and reproducible hydrodynamic conditions. The temperature of the cuvette was maintained at 25 ± 1 °C. Data were transmitted to a laptop computer and analyzed by an SPR software from Eco Chemie.

Preparation of SPR immunosensors

The planar gold SPR disks were extensively cleaned in a freshly prepared piranha solution (3:1 H_2SO_4 98%:H_2O_2 30%). After 1 h, the disks were thoroughly rinsed with water, dried in a stream of

nitrogen gas and immediately incubated overnight in a 30 μg mL^{-1} of MUA in ethanol or 15 μg mL^{-1} of resorc[4]arene derivatives in chloroform. After self-assembled monolayer (SAM) formation, the disks were washed with ethanol and water and dried with nitrogen gas.

MUA modified sensor disk Ab-Ins immobilization

The carboxyl functions on the MUA SAM layer were activated with a mixture containing 0.5 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and 0.1 mM N-hydroxysuccinimide (NHS) in water. After removing the EDC/NHS mixture and rinsing the disk, acetate buffer containing a solution of Ab-Ins was pumped over the sensors surface for 20 min to achieve a covalent cross-linking by amino reactive groups of antibodies with the aldehyde terminals, followed by a deactivation step of non-reacted activated groups with ethanolamine to reduce the nonspecific adsorption and a treatment with 10 mM glycine pH 2.5 to eliminate nonspecifically adsorbed antibody molecules.[43]

Resorc[4]arene modified sensor disk Ab-Ins immobilization

The resorc[4]arene modified sensor disk were rinsed then acetate buffer 10 mM containing Ab-Ins was pumped over the sensors surface for 20 min to achieve the oriented immobilization between the Fc part of the antibody and the hydrophobic groups on the resorc[4]arene derivatives, followed by a deactivation step of non-reacted groups with bovine serum albumin (BSA) 0.01 mg mL^{-1} to reduce the nonspecific adsorption.

The immobilization of Ab-Ins has been evaluated by monitoring the variation of SPR signal (m°) as a function of standard increasing concentration of antibody in the interval: 10 e 330 nM (Fig. S1).

Insulin detection

The kinetic analysis of the reaction of insulin and Ab-Ins modified sensor disk, can be summarized as follows: (see also Figure 2): 1) establishment of the baseline after coating the antibody, the flow cell was washed with the coupling buffer, 10 mM phosphate-buffered saline (PBS) pH 7.4 with 0.1 M NaCl for the insulin coupling in a range of concentration between 0-200 μg mL^{-1} at flow rate of 100 μL/min. The resonant angle was monitored until the baseline was stabilized. 2) Association the sample containing the antigen (50 μL) was injected in the flow cell and incubated during 20 min at stopped flow, while the SPR signal was monitored. After that, the flow cell was washed with the coupling buffer for 1 min. 3) Dissociationthe nonspecific adsorptions were removed and the resonant angle was set up for about 10 min.

Sensor disk regeneration

The modified sensor disk was obtained by immersing the sensor chip in a 15 mg L^{-1} solution of resorc[4]arene **5**. The immobilizations at pH 5.4 were carried out in acetate 10 mM buffer. An Ab-Ins concentration of 10 μg mL^{-1} was used for the experiments. NaOH concentrations in the range 10-1000 mM were tested for regeneration. A concentration of Ab-ins equal to 10 μg mL^{-1} was used for the second time so as to be able to calculate the % of regeneration.

SAM Reductive desorption

The resorcarene concentration onto the sensor gold surface has been evaluated by means of reductive desorption, in fact it is known that high negative potential cause thiols to desorb from the surface. [44]

The experiment was realized by performing linear sweep voltammetry in 0.5 mol L^{-1} KOH, between -0.2 and -1.5 V vs. Ag/AgCl. The scan rate was 20 mV s^{-1}. The integration of the resulting reductive desorption peak allows us to calculate the electron involved in the reaction and then the number of resorcarene molecules present onto the gold surface.

Associated content

Supporting Information. Synthetic procedures, NMR spectra, computational analyses and SPR measurements of resorc[4]arenes **2**-**5**.

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Funding Sources

This work was supported from PON (Piano Operativo Nazionale) ARS01_00432 PROGEMA – “Processi Green per l’Estrazione di principi attivi e la depurazione di matrici di scarto e non”, 03/2018 – 09/2020 and PRIN 2017 - “Targeting Hedgehog pathway: Virtual screening identification and sustainable synthesis of novel Smo and Gli inhibitors and their pharmacological drug delivery strategies for improved therapeutic effects in tumors” by Italian Ministry of Education, University and Research (MIUR) and Sapienza University of Rome. This work was also supported by MIUR - Dipartimenti di Eccellenza - L. 232/2016 and Sapienza University of Rome (project RM11816436113D8A), and by Regione Lazio project entitled “NanoBioPOCT25-OHD”.

Acknowledgements

The authors acknowledge networking contribution by the COST Action CM1407 “Challenging organic syntheses inspired by nature - from natural products chemistry to drug discovery”. This manuscript is dedicated to the memory of Professor Maurizio Botta (University of Siena, Department of Biotechnology, Chemistry and Pharmacy).

Keywords: Resorc[4]arene• Macrocycles • Immunosensor • site directed immobilization • Surface Plasmon Resonance

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10.1002/chem.202000989

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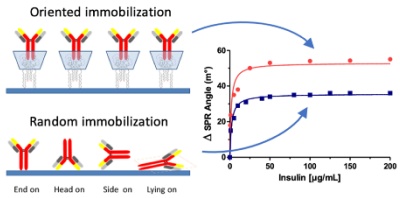

Herein the immobilization of antibody for optimized immunosensors development has been obtained by using resorc[4]arene as site-directing tool; this allows to overcome the complexity of binding antibody to the sensor surface avoiding the random orientation with a lower binding site density and immunoaffinity. Surface Plasmon Resonance (SPR) technique has been employed for the characterization of the modified surface as well as to evaluate the heterogeneous kinetics of the model insulin/insulin-Ab interaction.