J Incl Phenom Macrocycl Chem (2010) 66:185–194
DOI 10.1007/s10847-009-9702-y

ORIGINAL ARTICLE

Development and molecular recognition of Calixcrownchip as an electrochemical ALT immunosensor

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Received: 20 July 2009 / Accepted: 6 November 2009 / Published online: 9 December 2009

Abstract The developments of immunosensors with a variety of formats are increasingly finding applications in clinical diagnostics and biological researches. A strategy for the immunoassay and preparation of Calixcrownchips is proposed. This strategy is based on the immobilization of antigens or antibodies on the surface of Calixcrown and the direct electrochemistry of horseradish peroxidase (HRP) that was labeled to an antibody or antigen, with its activity determined by using tetramethylbenzidine (TMB) as an electrochemical substrate. The present study includes general considerations of the competitive immunoreaction protocols. Alanine aminotransferase (ALT) monoclonal antibody (anti-ALT-mAb) was successfully immobilized on thiol derivative of Calixcrown fixed to a gold surface. ALT antigen was detected by competitive immunoreactions based on microarrays of anti-ALT-mAb or antigen immobilized on the surface of the Calixcrownchip. For the anti-ALT-mAb immobilized microarray the dynamic range is 0.05 ng/mL–10 μg/mL, the detection limit is 0.05 ng/mL and the sensitivity is 10 nA/(ng/mL) respectively. The Calixcrownchip immunosensor microarray provided much better technical performance than a comparable enzyme sensor with immobilized-anti-ALT-mAb. To investigate the complexation site, the structures of the complexes formed between the crown-5-ether moiety of Calix[4]arene and protonated Arginine and Lysine were determined by minimizing the complex formation energies. The complex stability depends on the number of amine groups in the alkyl chain of the amino acid and also the number of methylene groups between the amine groups of the amino acid.

Keywords Immunosensor · Calixarene · Alanine aminotransferase · Protein immobilization · Electrochemistry

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Introduction

Biosensors based on protein–protein interactions, self-assembled monolayers (SAMs) and adsorption of proteins on solid interfaces have been evolving for several decades [1]. Sulfur–gold interactions are highly specific. This allows the introduction of various functional groups to a sulfur containing molecule without interfering with the adsorption to a gold surface [2].

The Calixcrown molecule used in the present study is 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) (Fig. 1). This molecule can have three possible conformations; Cone (u-u-u-u), Partial Cone (u-u-u-d) and 1,3 Alternate (d-u-d-u) conformers (Fig. 1). It is a bifunctional molecular linker. The thiol groups, which are typically linked to the Calixcrown lower rim, act to decrease the conformational rigidity when it is fixed to a gold surface to form a self-assembled monolayer (SAM), and allows tight binding of capture proteins to the crown moiety of the Calixcrown molecules. A Calixcrownchip can be used as a powerful tool with a wide range of applications; including protein–protein interaction, protein–DNA interaction, and enzyme activity assay and also for diagnostic applications with clinical samples from prostate cancer and HIV patients [3–6]. Increasing efforts were made during the last decade to link the specificity of

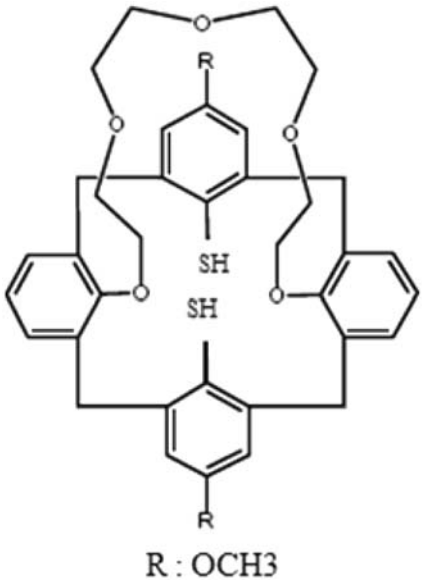


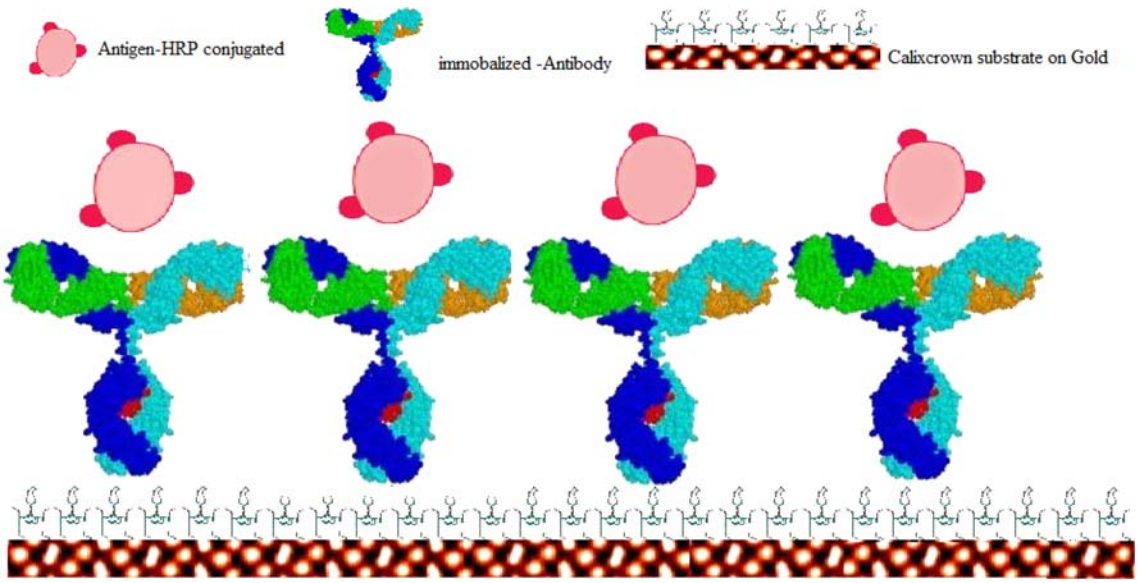
Fig. 1 1,3-Dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) in the Cone conformation for clarifying the hydrogen atoms are eliminated

immunoassays with the sensitivity and the low recognition limits presented by modern electrochemical techniques. Techniques, including amperometric, potentiometric, surface-plasmon resonance, fiber optics and continuous-flow type, have been developed and applied to biological species and clinical diagnostics [7–10].

ALT (Alanine aminotransferase) is principally found in the liver and kidneys, with less significant amounts in the heart and in skeletal muscles [11–13]. Lack of ALT could interfere with nitrogen shuttling and deprive the liver of an energy source, sometimes causing liver failure. In a previous study [14] the amperometric detector of ALT-enzymatic activity contained three parts: an anti-ALT antibody-immobilized outer membrane, then a pyruvate oxidase-absorbed inner membrane, and finally a self assembled monolayer mediator-coated gold working electrode and an Ag/AgCl reference electrode. Chronoamperometric measurement of the immunosensor was performed. The major problem in antibody- and antigen-microarray chips has been difficulty in reproducibility, possibly because of the random orientation of protein on the chip. A vertical orientation of protein molecules could result in a high-density attachment of protein, and lead to a higher specificity and reproducibility with reduced analytical error.

In this current investigation the direct electron communication between ALT Antibody and Antigen, immobilized in Calixcrownchip biosensors, were investigated and evaluated, using horseradish peroxidase (HRP) conjugated complementary proteins (Scheme 1). It has been indicated that the immunosensor microarray provided much better technical performance than our previous reported of enzyme activated technique [14]. For instance the detection limit was evaluated to be 200 times smaller. Although our previous biosensors systems for ALT had shown appropriate speed and precision, they were schematically more complicated than Calixcrownchips.

It has been shown that Calixcrown interacts with antibody and recombinant protein containing extra Arginine residues, immobilizing them with the correct orientation [15]. The vertical orientation of the protein molecules



Scheme 1 Scheme of the protein immobilization strategy, Calixcrown monolayer on the gold surface after adsorption of Antibody and reaction with an Antigen-HRP conjugated

resulted in a high-density attachment of protein, and lead to high specificity and reproducibility with minimal analytical error. It was also shown that the immobilization of yeast 9Arg-GAL4 mutant protein containing extra 9Arg residues at N-terminal was much greater than that of the control GAL4 protein on ProLinker^{TM}-coated slide glass [15].

Materials

Chemicals

1,3-Dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) (purity: 93%, bp: 224 °C, MW: 703.0 g/mol) was purchased from Proteogen Company (ProLinker-Cr5B). Oxidized TMB (3,3′,5,5′-tetramethyl-benzidine), the 16-sensor array (2.5 × 7.5 cm) was microfabricated with a thin, optical-grade layer of gold electrodes deposited on plastic. Each sensor in the array contained three electrodes: working, reference and auxiliary (counter). All other chemicals were analytical grade, and distilled deionized water (DDW) was used in all measurements. A phosphate-buffered saline solution (PBS, pH 7.4) was prepared by dissolving 2 Mm KCl, 136 mM NaCl, 1.4 mM KH_2PO4 and 8 mM Na_2HPO_4 in DDW and adjusting pH to 7.4 with HCl.

Human anti-ALT monoclonal antibody production, expression and purification

The encoding human ALT sequence was amplified from the total RNA of HepG2 cells using Taq DNA polymerase (Takara, Japan). The primers used were: 5′-ATAGAATT CATGGCCTCGAGCACAGGTGACCGG-3′ and 5′-AGT AAGCTTGGAGTACTCGAGGGTGAACTTGGC-3′ [16]. A polymerase chain reaction was performed for 30 cycles using standard conditions. The amplified fragment (1,503 bp) was digested with *Eco*RI/*Hin*dIII and sub cloned into the expression vector pET21a (Novagen, USA). Positive clones were verified by DNA-sequencing and transformed into BL21 cells for expression. The expression and purification of the recombinant protein in the pET system was performed according to the manufacturer's instructions.

The human recombinant ALT was mixed with an equal volume of complete Freund's adjuvant and injected into the peritoneal cavity of female BALB/c mice (6–8 weeks old). Each injection contained 250 mg of protein in a volume of 0.5 mL. The four booster injections were usually conducted at 2–3 week intervals prior to sacrificing mice for cell fusion. Cell fusion, selection, and ELISA for screening of positive hybridoma cells were as described previously [17, 18]. For large-scale production of mAbs, ascitic fluids were produced by injection of 1×10^7 hybridoma cells into the peritoneal cavity of mice that had been pretreated with Pristane a week earlier. After clearing, the ascitic fluids were put onto a protein-G column. The antibodies were eluted with 0.1 M glycine–HCl, pH 2.5, neutralized with 1 M Tris, pH 8.0, and dialyzed against PBS. Then they were stored at −80 °C until ready to be used. The protein concentration was determined according to the Bradford method [19]. Result were as reported in a previous paper [14].

Calixcrownchip substrate

The bonding of the thiolate group to the gold surface is very strong (approximately 150 kcal mol^{-1}). This technique provides a more stable immobilized protein, and it is applied widely. Gold slides were precleaned by soaking in a solution containing (1:1:1 mixture of H_2SO_4, H_2O_2 and D.D.W) for 10 min at 41 °C, and thoroughly washed in distilled water (DW). The slides were then dried under a stream of N_2 gas. The gold slides were immersed in a solution of 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) for optimum time and concentration at room temperature. The slides were then rinsed in sequence of $CHCl_3$, C_2H_5OH, and DW and finally blown dry in nitrogen gas.

Methods

Electrochemical measurement

Horseradish peroxidase (HRP) was used as the immune-label with 3,30,5,50-tetramethylbenzidine (TMB) as the redox substrate and activity determined by using cyclic voltammetry. CV was performed using an initial potential of 0.0 V and a switching potential of +1.0 V with a scan rate of 20 mV s^{-1} using a CH-electrochemical analyzer model CHI with standard accessories. Initial cyclic voltammetry was performed with a PBS buffer, pH 7.2. Figure 2 shows cyclic voltammograms obtained before (a) and after (b) the addition of an acid stop solution. In the presence of the acidic stop solution TMB undergoes a single 2-electron oxidation step at +0.42 V and a single 2-electron reduction step on the reverse scan (Fig. 2b). The mechanism of oxidation under these acidic conditions is shown in Fig. 2c. For analytical purposes, the species giving rise to the CV shown in Fig. 2b is preferred as it does not change over 24 h, therefore the acidic stop solution was used for all further studies. The peak separation ($-Ep = Ep_a - Ep_c$) was found to be 110 mV; this indicates that the electrochemical reaction is quasi-reversible. CV was completed on solutions containing oxidized TMB before and after adding the acidic solution.

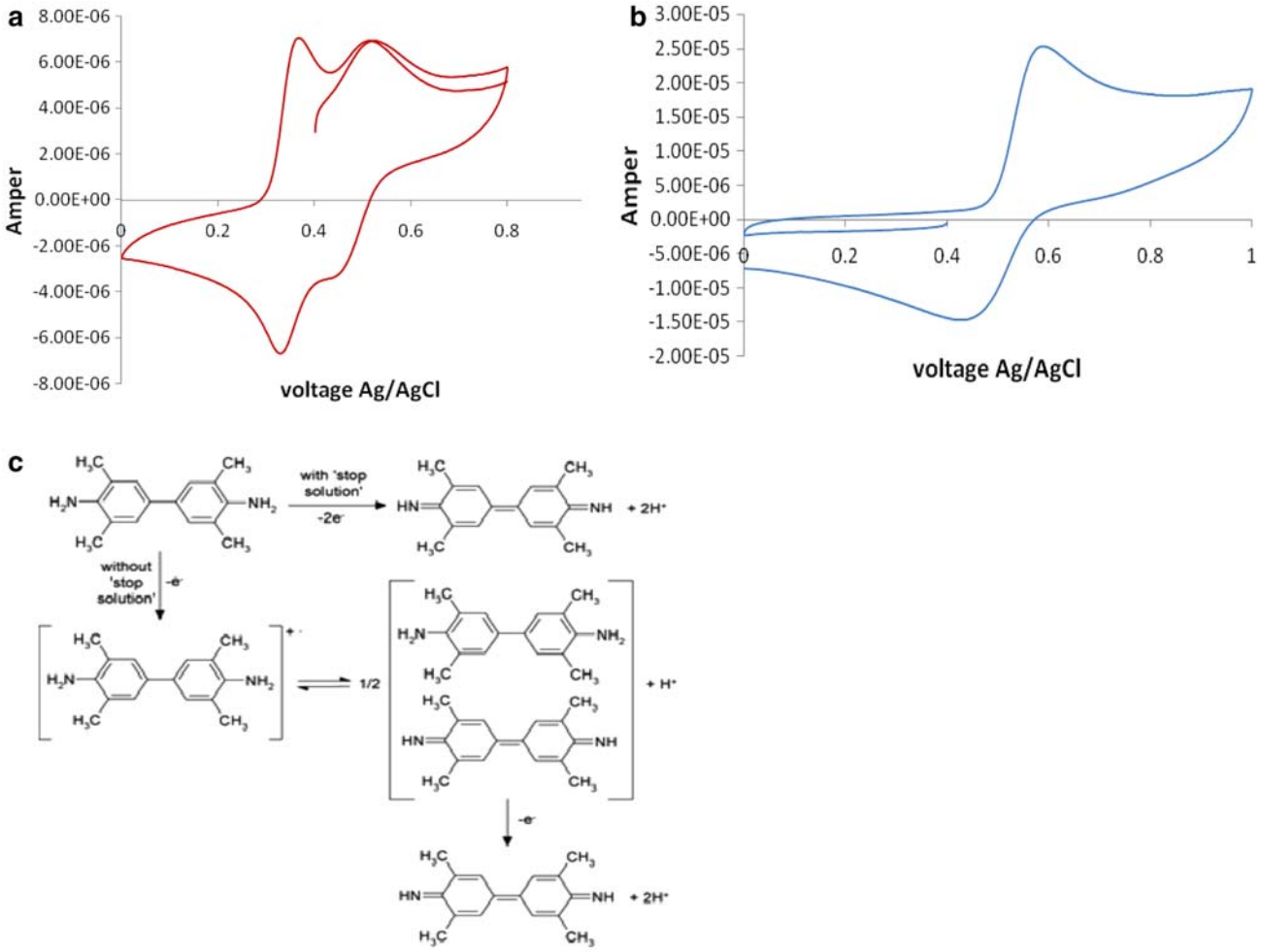


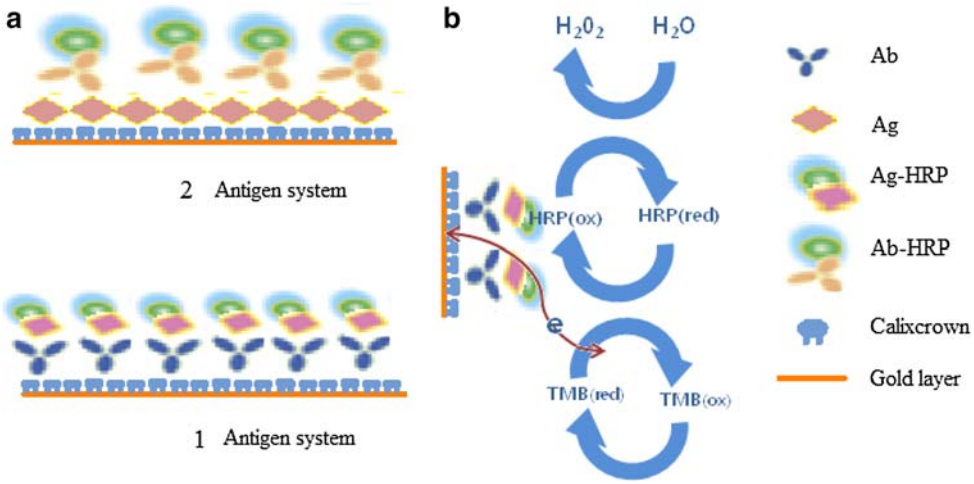
Fig. 2 Oxidation current response cyclic voltammograms for TMB **a** before and **b** after adding the acidic stop solution. **c** TMB oxidation reaction with and without of the acidic stop solution addition

ALT biosensor assembly

Two different microarray arrangements were studied. In the anti-ALT-mAb microarray, anti-ALT-mAb molecules were immobilized on the Calix[4]crown-5 ether substrate. The procedure for immobilization of the anti-ALT-mAb and antigen onto the Calix[4]crown-5 ether substrate follows: First, the wafer was incubated in anti-ALT-mAb solution for antibody microarrays (and also in at ALT antigen solution for microarrays) at 37 °C for 15 min to block on the surface. The wafer was then rinsed three times in PBS to remove excessive non-specific adsorbed then the plate was incubated with antigen and antigen HRP conjugated, at 37 °C for 15 min as optimized incubation time. In the Antigen microarray, the antigen molecules were fixed on the surface (Scheme 2). The basis of all controlled-potential techniques is the measurement of the current response related to the concentration of the target analyte to the potential applied to the working electrode. Protein immobilization is a novel technology that results in spatially oriented and spatially localized covalent coupling of proteins on Calix[4]crown-5 ether surfaces.

Fluorescence-scanning analysis

Protein immobilization on the Calixcrownchip was detected using highly sensitive Fluorescence-scanning. The 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) contains thiol groups in the lower rim and sulfide–gold covalent bonds immobilize the protein to make the Calixcrownchip. The Calixcrownchip probed with fluorescence-conjugates were scanned in a Scanarray Express HT 20-Slide Autoreader (Perkin Elmer, USA). By adjusting the laser power and contrast, the scanner was set to optimize the quality of the microarray images.



Scheme 2 **a** Schematic of an ALT biosensor microarray. *1* Anti-ALT-mAb microarray, anti-ALT anti-ALT-mAb-immobilized on Calix[4]crown-5 ether membrane; *2* Antigen microarray, Antigen HRP conjugated-immobilized on Calix[4]crown-5 ether substrate. **b** Sequence of events that occurs in the mediated system

Conjugation of fluorescence dye to protein

To label proteins fluorescence dye, 10 μL of a 1 M sodium carbonate buffer (pH 8.3) was mixed with 100 μL of the proteins (1 mg/mL in PBS). 1 μL of activated fluorescence dye (10 mg/mL) was added to the mixture and incubated at 1 h at room temperature followed by overnight incubation at 4 °C. Glycine solution was added to the mixture to quench the unreacted sites of the activated fluorescence dye. The mixture was loaded onto a Sephadex G25 chromatography column to separate the conjugates from free dye. After centrifugation of the column at 2,500 rpm for 2 min, the fluorescence conjugates were collected from the column as elutes.

Protein immobilization for fluorescence-scanning analysis

Teflon membranes with wells (diameter 1–3 mm) were attached to Calixcrownchip slides before protein samples were applied. The wells functioned as chamber barriers. The protein (anti-ALT-mAb, ALT antigen) in PBS containing 15% glycerol was spotted on the slide, and the protein chip was incubated in a 50% humidity chamber at room temperature for 2 h to allow stable molecular interaction between the Calix[4]crown-5 ether derivative and the protein. Glycerol was added to the buffer to prevent evaporation of samples during the incubation. The slide was rinsed in PBS-Tween 20 (PBST) three times for 5 min and in water once for 1 min. Water on slide was then completely removed under N_2 gas. For blocking non-specific binding, the chip was incubated with 3% BSA in PBST at room temperature for 1 h. After sequential treatment with PBST, water, and N_2 gas, the chip was probed with alexa647 (ALT antigen, anti-ALT-mAb) conjugated onto the sites of immobilized recombinant protein for 30 min in a 50% humidity chamber at room temperature in the dark. After rinsing with PBST and water, the slides were then dried in a stream of N_2 gas and scanned for quantification of fluorescence intensity.

Results and discussion

Thiol calixarene self-assembled monolayer (SAM)

In many cases, self-assembled monolayers can be bound on the surface without any complex procedures. Physical adsorption on the electrode surface is a very simple technique. Theoretically, this method could be used for any protein because the proteins are adsorbed spontaneously and irreversibly on solid surfaces [20] and after the adsorption step, the unadsorbed molecules are removed by washing. This process of protein immobilization is useful for the development of all kinds of protein chips as well as assaying methods for test proteins.

In this study cyclic voltammetry method was used for defining the best concentration to produce the best oxidation current response. The concentration range is from 0.07 Mm to 3 mM and, as shown in Fig. 3a, 2 Mm of Calix[4]crown-5 ether had been selected as the optimum concentration with 2 h incubation time.

Electrochemical Calixcrownchip based
on an anti-ALT-mAb immobilized microarray

The cyclic voltammetry oxidation current response for 10 μg/mL (15 μL) anti-ALT-mAb coating time optimization at 37 °C in Thiol Calix[4]crown-5 ether substrate is shown in Fig. 3b. For an anti-ALT-mAb immobilized microarray it was necessary to optimize the immobilized anti-ALT-mAb concentration by diluting anti-ALT-mAb in different concentrations ranging from 0 to 100 μg/mL (Fig. 4). 10 μg/mL (15 μL) anti-ALT-mAb resulted in the best signal.

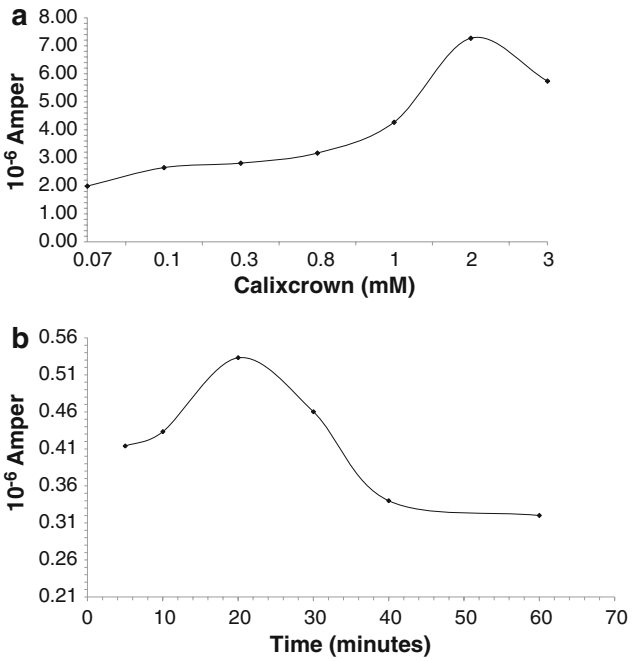


Fig. 3 **a** The effect of the coated Calix[4]crown-5 ether concentration on the oxidation current response that measured by cyclicvoltammetery method for ALT biosensor. The *points* shown are the mean of at least five measurements with a new sensing surface for each try. **b** Cyclicvoltammetery oxidation current response for 10 μg/mL ALT anti-ALT-mAb (15 μL) coating time at 37 °C in thiol Calix[4]crown-5 ether substrate the points shown are the mean of at least four measurements with a new sensing surface for each try

Electrochemical Calixcrownchip base on an antigen immobilized microarray

The optimum concentration of ALT antigen was 10 μgr/mL (15 μL). Figure 5a shows the optimization profiles of the oxidation current response and also the cyclic voltammograms (Fig. 5b). 1 μg/mL (25 mL) of antibody conjugated with HRP conjugated was selected as the optimum concentration (Fig. 5c). The antigen microarray standardized for antigen measurements (Fig. 6). In the next step the concentration of the Antigen HRP conjugated optimized and the concentration range is from 0 ngr to 20 μg/mL and in the 10 μg/mL (15 μL) of anti-ALT-mAb as it is clear in the Fig. 6b 1 μg/mL of Antigen HRP could be chosen.

Self-assembled monolayers (SAMs) have gained considerable attention recently. The high specificity of the sulfur–gold interaction allows the introduction of various functional groups which do not interfere with the adsorption process. The binding of an antigen to the appropriate anti-ALT-mAb is accompanied by only small physico-chemical changes and offers several potential advantages over the more widely used spectrophotometric/fluorescence techniques, particularly when sensitivity is desired. An increasing effort was made during the last decade to link the specificity of immunoassays with the sensitivity and the low detection limits offered by modern electrochemical techniques. The other

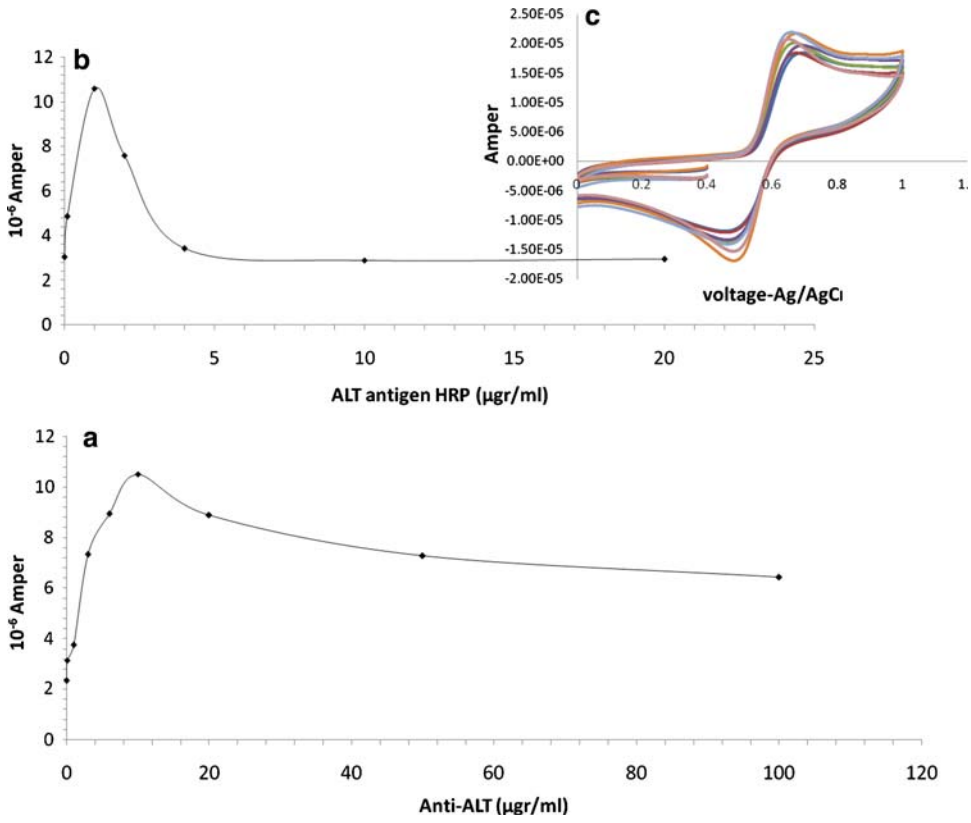


Fig. 4 Optimization profiles of the oxidation current response for anti-ALT-mAb microarray that ALT Antigen is immobilized in the Calix[4]crown-5 ether surface. Thia Calix[4]crown-5 ether coated on gold substrate optimized with increasing concentrations of **a** Antibody ALT the presence of 1 μg/mL Antigen HRP (25 μL) in PBS (pH 7.4), **b** modification profiles of oxidation current response in different concentration of Antigen HRP conjugated in presence of 10 μg/mL Antibody ALT (15 μL) in PBS (pH 7.4) and **c** cyclic voltammograms of different concentration of Antibody ALT in 1 μg/mL of AgHRP (25 μL) concentration at increasing concentrations of 15 μL of Antibody ALT: 0.1, 1, 10, 100 μg/mL in PBS (pH 7.4)

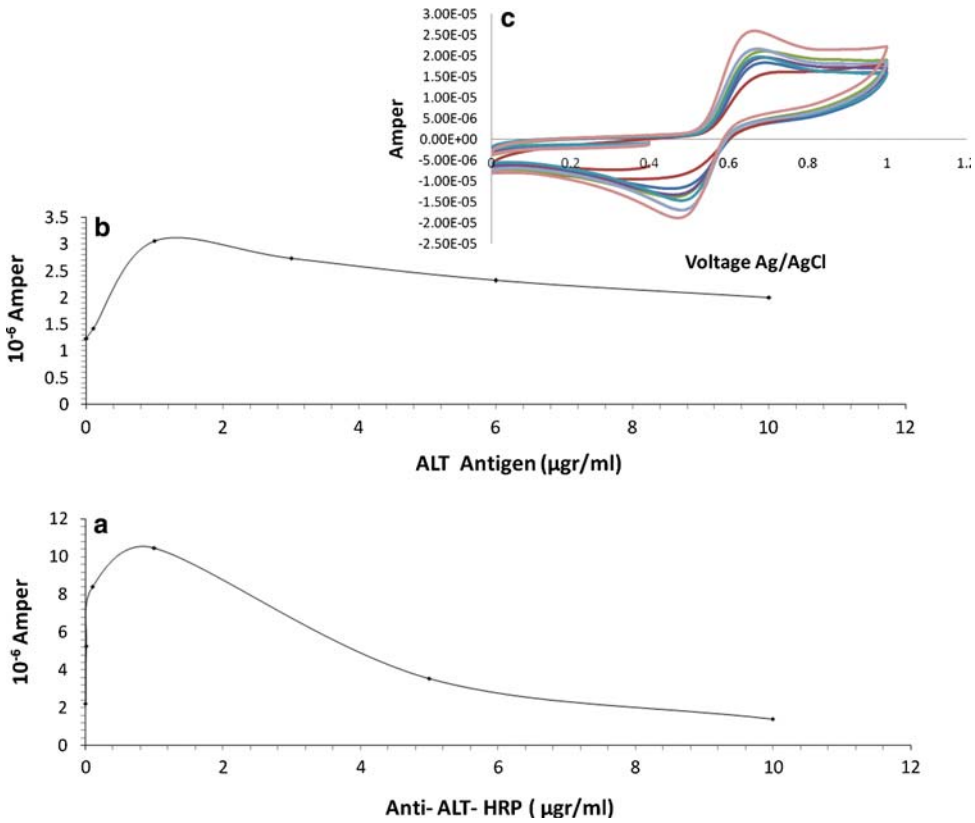


Fig. 5 Optimization profiles of the oxidation current response for Antigen microarray that ALT Antigen is immobilized in the Calix[4]crown-5 ether surface **a** for ALT immunosensor at different concentrations of Antigen in PBS (pH 7.4) in the presence of 1 µg/mL Antibody HRP conjugated (25 µL). **b** Cyclic voltammograms in the presence of 10 µg/mL Antigen that immobilized in the surface in the presence of Antibody HRP conjugated (25 µL) and at Antigen increasing concentrations: 0.1, 1, 10, 100 µg/mL. **c** The optimization profiles of oxidation current of Antibody HRP conjugated in PBS (pH 7.4) at the presence of 10 µg/mL (15 µL) Antigen immobilized

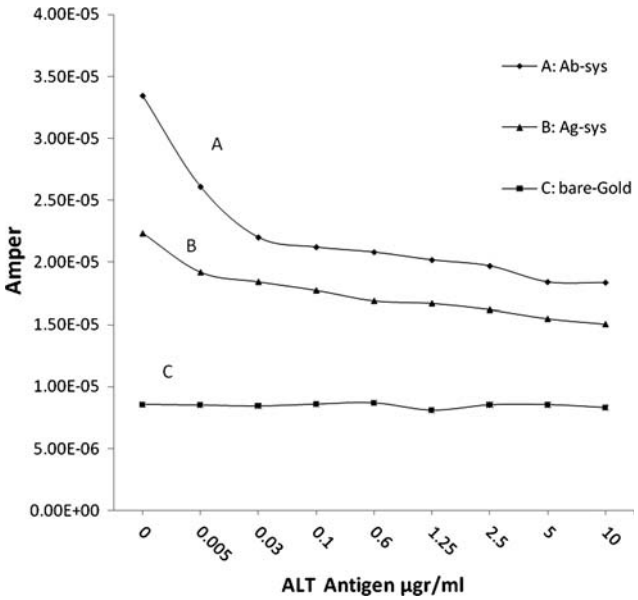


Fig. 6 Calibration profiles of the oxidation current response for an ALT biosensor. The data are presented by cyclicvoltammetry measurements in ALT biosensor microarray with Anti-ALT-mAb immobilized microarray at the surface of the Thia Calixcrown-gold substrate (A), Antigen immobilized microarray at the surface of the Thia Calixcrowngold substrate (B) and Anti-ALT-mAb immobilized microarray at the surface of the Bare Gold substrate (C). In the presence of 10 g/mL immobilized Antigen and 10 g/mL Antibody HRP conjugated for Antigen microarray (A) and 10 g/mL immobilized Anti-ALT-mAb and 1 g/mL Antigen HRP conjugated for Anti-ALT-mAb microarray (B)

important advantages of the electrochemical detection are the use of economical instrumentation which can be easily miniaturized [21].

Immunosensor microarrays are fast, economical, accurate, and easy to use. In previous study [14] used an anti-ALT-mAb-immobilized membrane, a PyO-absorbed membrane, and a gold electrode coated with SAM of hydroquinone mediator. With the optimal concentration, the ALT immunosensor demonstrated a wide dynamic range (10 pg/mL–1 mg/mL) and a low detection limit of 10 pg/mL. The sensitivity of the immunosensor was 26.3 nA/(ng/mL). In the present study with the Calixarenechip the dynamic range is 10 ng/mL–1 µg/mL with the lowest detection limit of 1 µg/mL and the observed sensitivity of the Calixcrownchip is 0.275 µA/(µg/mL). In this study the biosensors are based on immunological reactions involving the recognition of an antigen by an antibody binding site to form an antibody/antigen complex [22, 23]. Immunosensors are relatively short-lived because of poor sensitivity due to nonspecific binding. Calixarenes can be used for designing selective hosts. Their conformational properties help in ligating guests which can then be much larger than with other host molecules. Furthermore, both aqueous and non-aqueous media are accessible by choosing hydrophilic or hydrophobic groups. When the calixarene carries thiol functional groups it self-assembles as a

monolayer on Au-surfaces, allowing the protein to be immobilized as a monolayer on top of it. Protein binding to crown ether derivatives of calixarenes probably occurs via charge–dipol interactions [15, 24]. Few reviews of the use of calixarenes for biochemical recognition are available.

Fluorescence-scanning Calixcrownchip protein microarray

This study reports that anti-ALT-mAb immobilization on Calixcrownchip was much better than immobilization of ALT antigen on Calix[4]crown-5 ether biolinker. It was shown that cyclic voltammetry could be a sensitive and simple detection method. In order to confirm that the anti-ALT-mAb immobilized better than ALT antigen on Calix[4]crown-5 ether-coated slide, chip slides were analyzed with fluorescence-scanning and the result compared with bare Gold substrate. The comparison of fluorescence signals (Fig. 7a), show that the anti-ALT-mAb proteins were immobilized better than Antigen on the ProLinker^{TM} slide.

Computational molecular recognition

Recent progress in computational ability allows the study of the relatively large and complicated supramolecular system [25]. We showed that the immobilization of yeast 9Arg-GAL4 mutant protein containing extra 9Arg residues at N-terminal was much greater than that of the control GAL4 protein on ProLinker^{TM}-coated slides. Previously, the association equilibrium constants of complexes between some α-amino acids with 1,3-dimethoxy, 2,4-dithiol-Calix[4]crown-5-ether in choloroform and methanol mixed solvent (50%:50%, V: V) has been investigated by means of UV/Vis spectrophotometry titration [15]. The results suggested that Arginine and Lysine, were more shielded than the other hydrophilic charged amino acids and fit better with the ProLinker^{TM}. Arginine has the strongest complexation ability toward different Calixarene derivatives [26]. Arg forms more stable complexes with phenyl aromatic groups of Calixarene. Arg and Lys have similar structures compared with the other hydrophilic charged amino acids, so it is predicted that the structure aids in stabilizing the complexes. Therefore the theoretical study was designed for distinguishing the complexation site and esteric effects. The 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether molecule can have three possible conformations; Cone(u-u-u-u), Partial Cone (u-u-u-d) and 1,3 Alternate (d-u-d-u) conformers (Fig. 1). In Calixcrownchips the sulfur–gold covalence bonding belt the conformation in the 1,3 Alternate conformer. The initial conformations of 1,3 Alternate conformer 1,3-dimethoxy,2,4-di-thiol-Calix[4]crown-5-ether host firstly optimized with semi-empirical AM1 and PM3 energy minimization by The Hyperchem software [27] was used for initial geometry optimization and calculation of the binding energies. The gradient was estimated to be 0.05 kcal using the conjugate gradient optimization method (Polad–Ribiere algorithm). In order to reach the Global Minimum, some structural parameters of the initially optimized molecules were changed manually and the molecular structures were re-optimized. This procedure was repeated until the most stable structure was obtained. HF/6-31G optimizations of 1,3 Alternate conformers of the host was done by Gaussian 98 [28]. After optimization the conformers, B3LYP/6-311G (d, p) single point calculation (w100 h) of the final structure was done to include the effect of electron correlation and adding the polarization function as well as to reduce the basis sets superposition

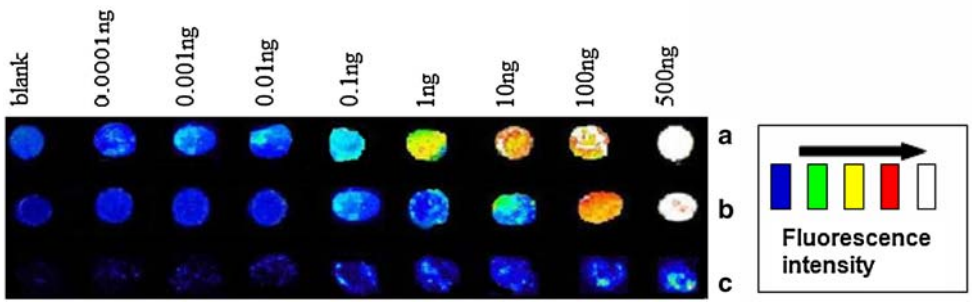


Fig. 7 ALT -1 detection. Comparison on different matrices based on fluorescence intensity as a function of ALT concentration. The same amount of **a**(anti-ALT-mAb), **b**(ALT antigen) and **c**(anti-ALT-mAb) was spotted onto matrix-coated (**a**:Calixcrownchip), (**b**: Calixcrownchip), (**c**: bare Gold) slides, and the fluorescencelabeled ALT antigen was mounted on each spot. Fluorescence-labeled ALT antigen was probed to detect labeled ALT protein after washing step. Fluorescence intensity represents the relative amount of anti-ALT-mAb immobilized on different matrix-coated Calixcrownchip slide

error (BSSE) [29]. To verify that the concluding structure was not in the local minimum point, the normal mode frequency calculation was carried out for the optimized host by using the HF/6-31G method. Each vibrational spectrum did not contain any negative frequencies; it has been shown that the optimized structure is really at the minimum point. For optimized 1,3 Alternate conformer of 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) the calculated energy is −1840367.5560 kcal/mol for the HF/6–31 g level of calculation and −1842475.6210 kcal/mol for the B3LYP/6-31+g (d, P) level of calculation.

The relative binding affinity of 1,3 Alternate conformer of 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether toward Lysine and Arginine protonated imine tautomer [30–34] was also studied by focusing on the binding site of crown ether moiety of the host molecule using the B3LYP/6-31G (d)//HF/6-31G calculation method [35]. It was shown that the primary amine ion interacting with the crown ether makes a more stable complex compared the other probable complexes and it is the same with all studied α-Amino acids in this research. The differences caused by the π electrons of the aromatic rings are significant enough to make Arg and Lys complexes more stable than the other amino acids. The complexes are more stable when three methylene carbons are between the amine groups (Fig. 8). The distance from the centroid symmetry plane that contain the crown ether function to the phenyl π electrons is 3.92342°A and the distance between the amine groups in Arginine is 3.92361°A. This matching makes the Arginine complexes more stable than lysine with a 5.10196°A distance between the amine groups. The interaction of the protonated secondary amine groups with the π electrons of the aromatic rings increases the Arginine stability energy so it is more stable than Lysine. As there are four aromatic rings against one crown ether in each host molecule so the reaction possibilities for Arginine are more probable and stable than for Lysine.

Conclusion

Using the specific affinity between an anti-ALT-mAb and its corresponding antigen, immunoassay techniques represent a promising technology and possible methodology for evaluating samples. The present research reports the design and development of amperometric immunosensors based on a competitive assay using ALT Antibody and antigen interactions. It has been compared the optimum immobilizing concentration for antibody or antigen immobilized microarrays. The protein immobilization has been studied using surface Calixarene chips formed by the thiol derivative of Calix[4]crown-5 ether molecules binding to a gold substrate. In the detection step the amount of ALT antigen was measured by competitive immunoreactions based on both anti-ALT-mAb immobilized microarrays and antigen immobilized microarrays. Amperometric measurement was made of the current resulting from the electrochemical redox reaction of an electro active species at a constant applied potential. Horseradish peroxidase (HRP) was used as the label enzyme and its activity determined by using 3,30,5,50-tetramethylbenzidine (TMB) as an electrochemical substrate based on enzyme immunoassay. High sensitivity and low detection limits makes electrochemical detection using the immunosensor an attractive method in bioanalytical chemistry. Using 1,3-dimethoxy,2,4-dithiol-Calix[4]crown-5-ether (ProLinker^{TM}) as biolinker in our previous study demonstrated that recombinant protein containing extra Arginine residues immobilized better in Calixcrownchip. A computational search for reaction sites investigating Arginine and lysine complexes with ProLinker^{TM} suggested that the difference between Arginine and Lysine complexes arise from the different distances between the protonated amine ions. When three methylene carbons separate the protonated amine ions the stabilization energy becomes slightly greater due to a more efficient overlap of the protonated secondary amine ions with the π electrons of the aromatic rings.

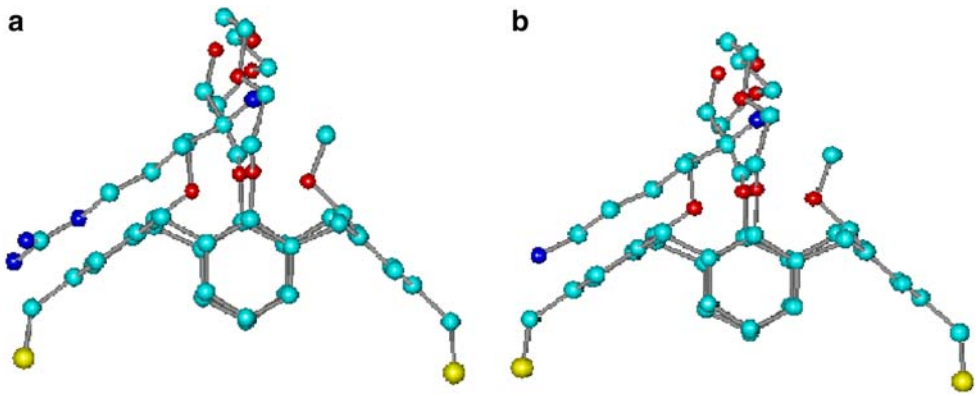


Fig. 8 Simulated configuration of Arginine-ProLinker^{TM}, Lysine-ProLinker^{TM} optimized complexes. *Cyan*, *red*, *blue* and *yellow* balls represent carbon, oxygen, nitrogen, and sulfur atoms, respectively. There are three methylene carbons separate the primary ammonium ion from the secondary ion in protonated Arginine in compare to four methylene carbons separate the primary ammonium ions in protonated Lysine

Acknowledgment This research was supported by Brain Korea 21(BK21).

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