Short Communication

Capacitive Sensing of Amino Acids Using Caliraxene-Coated Silicon Transducers

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Abstract
The possibility of using capacitance and flat band voltage as measurable quantities for determining amino acids that are neither electroactive nor with strong UV-vis absorption has been explored. The sensors were fabricated by immobilizing calixarene derivatives on $Si/SiO_2/Si_3N_4$ transducers. The measurements were made in sulfuric acid media of ca. pH 1 and in physiological buffer of pH 7.4. The different calixarene derivatives showed varying sensitivities to the amino acids ranging from 8 to 137 mV/decade.

Keywords: Calixarene, Amino acids, Capacitance, Silicon transducers

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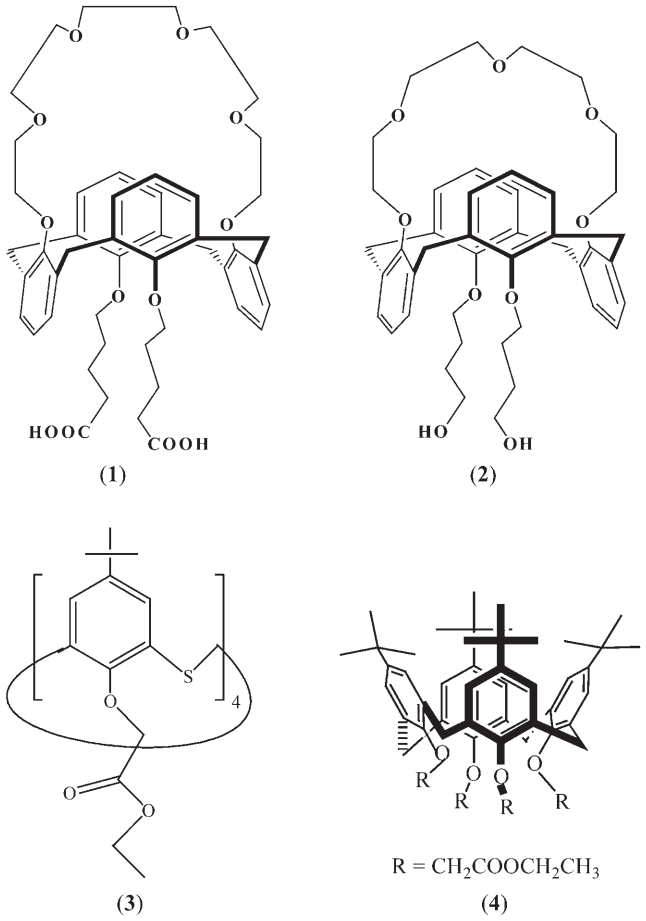
Electrochemical sensors form the largest group of chemical sensors [1, 2] but there are very few electrochemical sensors for amino acids. The determination of amino acids is of great importance in biological materials and foods. Except for phenylalanine, tryptophan and tyrosine, the others do not have sufficient UV-visible absorption for spectrophotometric determinations. Chromatographic separations followed by detection by electrooxidation at high potentials at metallic electrodes have often been carried out. Welch et al. have made use of special pulsed coulometry waveforms for detection and in situ cleaning of Au electrodes in the determination of amino acids [3], while DeMott et al. have used AgO electrode in order to lower the oxidation potentials [4]. Copper and nickel modified electrodes too have been used in alkaline media to oxidize the amino acids, wherein the reactive species like NiO(OH) and CuO(OH) formed at the electrode surface act as electrocatalysts [5 – 7]. As such electrodes do not offer selectivity, the amino acids have to be separated before, by chromatography or the signals have to be separated using chemometrics [8]. Oxidizable amino acids like cysteine, tryptophan and tyrosine have been studied in detail and several chemically modified electrodes based on electrocatalysis have been reported [9 – 12]; while in some other cases a number of enzyme based amperometric sensors [13] have been reviewed. However, use of enzyme based biosensors present some difficulties like the need to purify the required enzymes and the demanding conditions for preserving it and, often too, the pure enzyme without any cofactor is not efficient enough to catalyze the expected biochemical reaction.

Synthetic receptors like crown ethers or calixarenes, in combination with a suitable electrochemical transducer hold promise for selective recognition and determination of the nonoxidizable amino acids. Crown ethers are heterocycles that, in their simplest form, are cyclic oligomers of dioxane. The essential repeating unit of any simple crown ether is ethyleneoxy, i.e., $-CH_2CH_2O-$, which repeats twice in dioxane and six times in 18-crown-6. Calixarenes are macrocycles formed by cyclooligomerization of phenol and formaldehyde. Now they can be synthesized in a rich variety of ring sizes and substitution patterns. Their ability to complex cations, anions, and neutral molecules has made them a standard among supramolecular host molecules. There are several studies on the complexation of metal ions with such macrocyclic compounds, but fewer related to organic ions or neutral molecules and hence resulted in several sensors for metal ions based on macrocyclic compounds or macrocyclic-metal ion complex based sensors for organic molecules [14 – 19]. Complex formation between crown ethers/calixarenes and amino acids has been studied by conductometry [20], calorimetry [21], solvent extraction [22, 23] spectroscopy [24], etc. But only a few macrocycle based sensors in the form of ion selective electrodes have been reported for amines or amino acids [25 – 28]. We report, for the first time, the possibility of using calixarene derivatives as receptors for amino acids in capacitive sensors (Scheme 1).

The transducer used is $Si/SiO_2/Si_3N_4$ or the so-called EIS (electrolyte – insulator – semiconductor) structures. The capacitance measurements were done at 10 kHz frequency over the range of -500 to $+2800$ mV vs. SCE. The shift in the flat band potentials with the addition of amino acids was



Scheme 1. Calixarenic receptors:
Compound **1**: 1,3-(Diethyl-5-oxavaleric Acid)-calix[4]arene-crown-6
Compound **2**: 1,3-(Di-4-oxabutanol)-calix[4]arene-crown-5
Compound **3**: Tetraethyl *p-tert*-butylthiacalix[4]arene tetraacetate
Compound **4**: Tetraethyl *p-tert*-butylcalix[4]arene tetraacetate

indicative of the complexation phenomena and can be used quantitatively. The experiments were done in 0.05 M H_2SO_4 (ca. pH 1) and in PBS buffer (pH 7.4) media where the amino acids exist in protonated or zwitterions forms.

Calixarene based capacitive sensors for metal ions have been studied by our group in the recent past [29–31]. The same has been extended to amino acids which can exist as cations or anions or zwitterions at different pH. The $C(V)$ measurements were based on the determination of the capacitance for various polarizations of the cell, i.e., the capacitance was measured as a function of applied voltage. When ionic charges are adsorbed at the functionalized surface of IS structure, the flat band potential V_{FB} varies with the ionic concentration of the adsorbed species. Flat band potential is the potential at which there is no space-charge layer in the semiconductor. It depends on the type of semiconductor, nature and composition of electrolyte [32, 33]. The sensor sensitivity was determined by the slope of the curve giving ΔV_{FB} as a function of the concentration. Initially, the two calix-crown ethers were tested for their sensing abilities. An electrolyte of 0.05 M sulfuric acid was chosen to avoid competition from the alkali metal ions from the other commonly used salts like KCl or NaCl. This also ensured the pH to be around 1, so that the amino acids were in their protonated forms. According to previous published data, interactions between crown molecules and amino acids can be classified as a combination of H-bond formation and electrostatic interactions between the NH_3^+ group of the amino acid and the donor oxygen atoms of the ligand, which favors liberation of water molecules from the solvation shells of the reacting molecules into the solvent bulk. However, the sensitivities obtained by the calix-crown coated surfaces were very low as indicated by the sub-Nernstian slopes (Table 1) though they show stronger complexation in solution phases. Most complexation studies reported have been carried out in solution phase. However, the majority of the potential analytical applications of supramolecular receptors, like sensors, are either at gas-solid or at liquid-solid interfaces. The direct transposition of the molecular recognition properties of a given receptor from solution to the solid-liquid interface is not trivial, since non-specific interactions such as dispersion forces, orientation of the molecules and material properties such as surface morphology and layer permeability come into play. From Table 1, it is evident that the compound **1**, having carboxylic groups shows least affinity for the native amino acids, which is in agreement with the observation of Arena et al. [24]. Compound **2**, bearing hydroxyl groups shows slightly higher affinities. The different values of slopes indicate the different selectivities for a particular host – guest pair. However, both compounds are not fit to be used in physiological media containing alkali metal salts due to competition from sodium and potassium ions, and due to the rigidity of the structure. It is well known from the HSAB (Hard and Soft, Acid Base) rule that introduction of sulfur in the macrocyclic ring system reduces the affinity towards alkali/alkaline earth metal ions [34]. So, to reduce the complexation of Na and K ions, we synthesized a thiacalixarene. In addition, this macrocycle presents more flexibility than the rigid ring like structure of **1** or **2.** Also, as the work of Chang et al. [35] suggested that the amino acids can be situated in the cavity of the calixarene via tripodal hydrogen bonding interaction and ion-dipole interaction between the ammonium moiety and the oxygen atoms of ester carbonyl groups, we introduced the ester groups to make a thiacalixarene ester viz. compound **3.** Further, to verify the effect of 'sulfur' we synthesized the homologous calixarene **4** which has a methylene group instead of sulfur.

The $C(V)$ measurements were made in PBS buffer (pH 7.4) for the above representative amino acids which are neither electroactive nor fluorescent. Figure 1 shows a typical set of $C(V)$ curves at different concentrations of histidine. The capacitance curves presented some interesting phenomena. Firstly, sufficient complexation/sensitivity were observed for some of the amino acids even in presence of Na and K ions as is evident from the values of slopes in Table 1. But the sensor **3** showed higher sensitivity in sulfuric acid medium than in PBS medium. This behavior is different from that of the simple metal ions because the

Table 1. Sensor responses for different amino acids ($n = 5$ for each set of sensor and amino acid/albumin). nss: no significant shift in flat band potentials

Amino acids	Sensor 1 in H_2SO_4 Slope (mV/decade) Linear range (M)	Sensor 2 in H_2SO_4 Slope (mV/decade) Linear range (M)	Sensor 3 in PBS Slope (mV/decade) Linear range (M)	Sensor 3 in H_2SO_4 Slope (mV/decade) Linear range (M)	Sensor 4 in H_2SO_4 Slope (mV/decade) Linear range (M)
Glycine	11.9 (±2) $1.7\times10^{-2}-1.3\times10^{-4}$	13.8 (±3) $1.5\times10^{-2}-2.7\times10^{-4}$	20.3 $2.1\times10^{-2}-2.6\times10^{-4}$	35.8 (±1) $1.5\times10^{-2}-3.6\times10^{-4}$	32.9 (±1) $1.8\times10^{-2}-3.6\times10^{-4}$
Lysine	nss	9.3 (±2) $1.8\times10^{-2}-1.3\times10^{-4}$	27.5 (±2) $3.7\times10^{-2}-1.8\times10^{-4}$	43.2 (±2) $1.5\times10^{-2}-1.7\times10^{-4}$	45.7 (±3) $1.3\times10^{-2}-1.4\times10^{-4}$
Proline	nss	12.9 (±2) $2.1\times10^{-2}-5.1\times10^{-4}$	19.8 $1.7\times10^{-2}-2.6\times10^{-4}$	22.3 (±2) $1.7\times10^{-2}-1.4\times10^{-4}$	21.3 (±2) $1.7\times10^{-2}-1.4\times10^{-4}$
Histidine	nss	12.8 (±2) $2.5\times10^{-2}-2.7\times10^{-4}$	24.8 (±1) $1.5\times10^{-2}-7.8\times10^{-5}$	33.8 (±2) $1.8\times10^{-2}-1.4\times10^{-5}$	31.8 (±3) $1.8\times10^{-2}-1.4\times10^{-5}$
Asparagine	7.7 (±2) $1.5\times10^{-2}-6.8\times10^{-4}$	10.8 (±2) $1.3\times10^{-2}-1.4\times10^{-4}$	17.0 $7.7\times10^{-2}-2.3\times10^{-5}$	50.5 (±1) $1.3\times10^{-2}-1.4\times10^{-5}$	48.3 (±1) $1.3\times10^{-2}-1.4\times10^{-5}$
Aspartic acid	4.8 (±2) $5.6\times10^{-3}-6.4\times10^{-5}$	5.2 (±1) $5.1\times10^{-3}-9.4\times10^{-5}$	137.6 (±3) $7.7\times10^{-2}-6.3\times10^{-5}$	38.4 (±2) $5.5\times10^{-3}-5.4\times10^{-5}$	32.4 (±2) $5.9\times10^{-3}-5.4\times10^{-5}$
Glutamine	7.9 (±2) $1.5\times10^{-2}-2.3\times10^{-4}$	8.9 (±3) $1.1\times10^{-2}-1.4\times10^{-4}$	17.1 (±2) $8.8\times10^{-2}-4.6\times10^{-5}$	53.2 (±2) $1.6\times10^{-2}-1.4\times10^{-5}$	51.4 (±2) $1.4\times10^{-2}-1.6\times10^{-5}$
Glutamic acid	6.9 (±2) $1.4\times10^{-3}-5.2\times10^{-5}$	9.6 (±2) $1.2\times10^{-3}-1.0\times10^{-5}$	127.8 (±3) $9.3\times10^{-3}-3.4\times10^{-5}$	41.7 (±3) $4.1\times10^{-3}-5.5\times10^{-5}$	41.5 (±2) $4.2\times10^{-3}-5.5\times10^{-5}$
Albumin	nss	nss	103.2 (±3) $2.2\times10^{-3}-3.4\times10^{-7}$	11.6 (±2) $2.2\times10^{-3}-2.1\times10^{-7}$	10.6 (±2) $2.2\times10^{-3}-2.1\times10^{-7}$

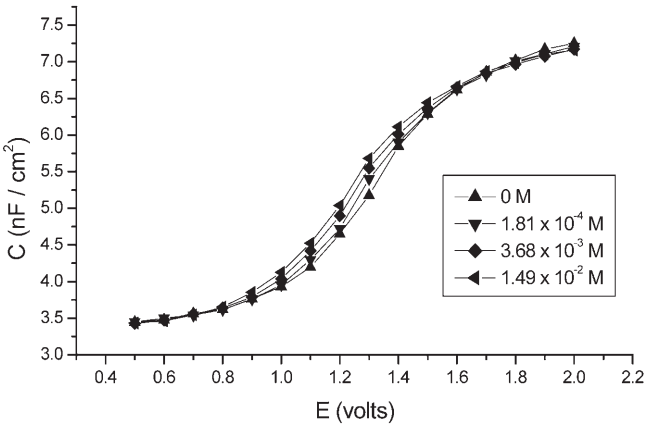


Fig. 1. A typical set of $C(V)$ curves obtained by varying histidine concentrations for the sensor **3** coated with compound **3** at pH 7.4.

amino acids exist as zwitterions at the physiological pH value of 7.4, and this complicates the complexation equilibria at the sensor surface. From Table 1, it can be seen that most of the slopes are subnernstian, and sensitivities are lesser than those encountered with metal ions. But for aspartic acid, glutamic acid (which have very low solubility in water) and albumin a supernernstian slope is obtained which is another indication that the complexation on the sensor surface do not obey a stoichiometric mechanism in certain cases. Such supernernstian responses are observed in case of polyion sensors too. These large EMF responses in the presence of physiological levels of small anions and cations have been attributed to a high value of the extraction coefficient of the polyions into the organic membrane phases of the electrodes [36, 37]. On the other hand, sensor **4** gave almost similar responses as sensor **3** in sulfuric acid but negligible or random responses in PBS solution. So, this further validates the importance of sulfur substitution in the macrocyclic ring.

Serum albumin is a midsized protein with a molecular weight of approximately 66.4 kDa and is built from 585 amino acid residues. We have attempted sensing of albumin as it is possible for some of the charged portions of the protein to bind with the calixarene molecule at the sensor surface and found that only **3** and **4**, with ester function is capable of binding to give any significant response. Further, some interference studies with binary mixtures of amino acids were done to ascertain the utility of sensor **3** in PBS medium. It was found that the presence of up to 0.3 mM of glycine, proline, asparagine, or glutamine in a solution of 1 mM glutamic or aspartic acids or albumin didn't affect their $C(V)$ curves. However, the reverse was not true. The presence of even 0.1 mM of glutamic or aspartic acid in 1 mM of glycine or proline shifted the flat band potentials by about 5 mV, which implies the suitability of sensor **3** for molecules with higher extraction into membrane phase.

This preliminary study has revealed the possibility of using the calixarene coated EIS structures for determination of nonelectroactive and non-absorbing amino acids by capacitance measurements. For the determinations in physiological media containing large amounts of Na and K ions, it is essential to use a sulfur containing ligands to prevent the overwhelming effect of alkali ions on the measurements. The method is quite reproducible as the deviations in slope are less than ±2 mV for most of the sensor-amino acid combinations (Table 1). The only common method used till now for such amino acids was chromatography or derivatization. Designing of suitable receptors can further improve the selectivity and/or sensitivity of such sensors. As the responses are nonnernstian,

further studies for other amino acids, at different pH will lead to a better understanding of the sensing phenomena and could lead to commercially useful sensors. A similar approach can serve to design synthetic receptors for recognizing peptides or even small proteins acting as antibody mimics.

Experimental

The calixarene derivatives (Scheme 1) were synthesized in the LACE of the Claude Bernard University (Lyon, France). 1,3-(Diethyl-5-oxavaleric Acid)-calix[4]arene-crown-6 (**1**) and 1,3-(di-4-oxabutanol)-calix[4]arene-crown-5 (**2**) were synthesized by the procedure described by Duta et al. [38]. These macrocycles adopt the 1,3-alternate conformation, which seems to be the most favorable for complexation. Tetraethyl *p-tert*-butylthiacalix[4]arene tetraacetate (**3**) was synthesized by the procedure described by Lamartine et al. [39]. Tetraethyl *p-tert*-butylcalix[4]arene tetraacetate (**4**) was obtained from procedure elaborated by Arnaud-Neu [40] and it adopts the cone conformation in solution.

The $Si/SiO_2/Si_3N_4$ structures purchased from LAAS (Toulouse, France). The structures were based on a p-type silicon substrate, 400-μm thickness, with 10-Ω cm resistivity, covered with 50 nm of thermally grown silicon dioxide and 100 nm of silicon nitride prepared by low pressure chemical vapor deposition (LPCVD) technique at 750 °C. The ohmic contact on reverse side of the silicon was obtained by deposition of an aluminum layer under vacuum.

The transducer surfaces were immersed in trichloroethylene followed by acetone and isopropanol with ultra sonication to degrease and clean the insulator surface. Then a treatment with sulfochromic mixture and immersion in ultra-pure water for 1 hr was made to increase the number of free active sites on the Si_3N_4 (silanol and silylamine). Finally, after the complete process, the substrate was placed in an oven at 70 °C for 10 min to remove the physisorbed water molecules. The hydrogen bonds between the OH groups of the calixarene and the active sites can improve the adhesion. Calixarene films were deposited by the humid technique spin-coating on the surface of the IS structures, at room temperature. The calixarenes were dissolved in chloroform to give a solution of 2.5×10^{-3} M and spin coated on the silicon nitride surfaces after the spreading out of a drop of 30 μL at 2000 rpm. The coated Si structures were conditioned for 1 h in the respective amino acid solutions (1×10^{-3} M) before use. They could also be reused after 3 alternate washings with 1 M H_2SO_4 and pure water followed by conditioning. When not in use, they were dried in a gentle stream of nitrogen gas and stored in airtight containers. Such stored EIS structures retained their sensor properties for 2 months.

Experiments were performed in an electrochemical cell fitted with three electrodes. The Si structure was the working electrode, platinum as the auxiliary platinum electrode and a saturated calomel electrode (SCE) as reference electrode completed the three electrode system. The electrochemical study was performed using an impedance analyzer Voltalab 40 (Radiometer Analytical, Villeurbanne, France). Direct current bias and superimposed alternating signals (10 kHz – 5 mV) were applied to the working electrode. The $C(V)$ measurements involved the determination of the capacitance for various polarizations of the cell. All measurements were performed at ambient temperature and in dark to avoid photo-induction charges in the silicon heterostructure.

The amino acids – glycine (Sigma), lysine (Sigma), asparagines (Fluka) , glutamine (Fluka), glutamic acid (Sigma), aspartic acid (Sigma), praline (Fluka) and histidine (Fluka) were the L enantiomers and had a purity of greater then 99.5 %. Albumin (bovine) was a Sigma product. 0.05 M H_2SO_4 was prepared by appropriate dilution of AR grade sulfuric acid (Fluka). The saline PBS buffer was prepared with the following constituents – 0.201 g $KCl + 8.006$ $NaCl + 0.240$ g $KH_2PO_4 + 1.44$ g Na_2HPO_4 in 1000 mL water and pH adjusted to 7.4 with KOH. Ultra pure water of Millipore grade (18 MΩ) was used for all experiments.

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