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Amino β-cyclodextrins immobilized on gold surfaces: Effect of substituents on host-guest interactions

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ABSTRACT

In the present paper we describe a simple method to immobilize amino cyclodextrins (CDs) and methylated-amino cyclodextrins on gold surfaces. We also report on the effect that the presence of methyl groups in the broader rim of the cyclodextrin causes on the interaction with the guest molecule bentazon. By means of electrochemical measurements, X-ray photoelectron spectroscopy and contact angle experiments we have demonstrated that the CDs attach covalently to the gold surfaces by amide bond formation and that the CD cavity is oriented opposite to the gold surface. We have shown that methylated-CD/Au modified surfaces are more sensitive towards the recognition of the herbicide bentazon than the non-methylated variants. The association constants for the corresponding interactions of the immobilized CD with the guest molecule have been determined from surface plasmon resonance experiments. The magnitudes of these constants ($30.8 \pm 1.0\ M^{-1}$ and $80.5 \pm 4.2\ M^{-1}$ for amino-CD and methylated-amino cyclodextrins, respectively) are consistent with the change of hydrophobicity caused by methyl groups. The results demonstrate the feasibility of using CD-gold modified surfaces to encapsulate herbicides such as bentazon within the macrocyclic receptor without necessity of carrying out the experiments in solution.

1. Introduction

Cyclodextrins (CDs) have been proposed as macrocyclic receptors with adequate recognition properties based on host-guest interactions. They can be used to develop less expensive and robust sensors as an alternative to biological molecular systems.

Moreover, it is known that the chemical functionalization of the broader rim of CDs increases the binding properties observed in solution [1]. It has been demonstrated that chemically modified CDs on the broader rim provide additional factors that contribute to form a more stable inclusion complex due to additional interactions with the guest [2,3]. Several functional groups (neutral, apolar, polar, charged groups) have been used to produce these chemically modified CDs on the broader rim: methyl-, ethyl-, hydroxypropyl-, sulfobutyl-, etc. The polarity or the charge of the functional groups can enhance the encapsulation ability depending on the characteristics of the guest molecule. Pérez and Escandar [4] studied inclusion complexes in solution using different functionalized CDs finding stronger interaction with the guest molecules when methyl, ethyl or hydroxypropyl-βCD were used as compared with natural or charged βCDs. Within this context, we have determined different association constants using charged CD (sulfobutylether-βCD) in solution and have compared them with those shown by native βCDs [5].

Although a large number of studies about host-guest interactions in solution have been reported, in the field of sensor designing only a few studies have been performed out of solution, that is, with the CDs immobilized on a surface, in order to take advantage of the additional interactions provided by the functionalization of the broader rim. Sometimes these studies report conflicting or partial results respect to those carried out in solution making comparisons difficult. For example, a thiolated methyl-βCD [6,7] immobilized on gold has been tested for ibuprofen [8] using ferrocene as electroactive marker; however the authors concluded

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that the native βCD is analytically favorable as compared to the methylated receptor. The same authors have used thiolated 2,3 di-*O*-methylβCD for measuring H_2O_2 and the protein Lacasse with methylene blue included in its cavity as an active component of the monolayer [9]. However, their discussion is focused mainly on the improvement of the reproducibility of the monolayer and there is no comparison with the native β-CD or with the CDs functionalized with other groups.

Although the most common strategy to immobilize CDs is the chemisorption of thiolated CDs on a gold surface in order to produce a self-assembled monolayer [10–17], and strategies using amino-βCD have been informed [18–22], other different anchoring groups are being tested (Hinge et al. [23], Tang et al. [24]) to overcome limitations related with packing, mobility, orientation and/or electronic transfer as pointed by Huskens et al. [25]. However, the synthesis of CDs with a specific anchoring group could become a limiting step. We have shown that the building of a platform via bottom-up approach using surfaces modified with functional groups able to link the CDs is a strategy that might be used for sensor design [22].

The present work is aimed at evaluating the effect that the presence of methyl groups on the broader rim of βCDs immobilized through a bottom-up approach on gold surfaces have on the host-guest interactions towards the herbicide bentazon as compared with those provided by non-methylated CDs immobilized in an identical way. This post-emergence herbicide is widely used in modern agriculture to control broad-leaved and grassy weeds in rice and corn, among others. Bentazon is not readily adsorbed by soil particles and is commonly found in both the surface and the groundwater in rice-growing areas and, therefore, it constitutes a health risk because of its toxicity [26]. Besides its practical importance in agriculture, bentazon (Fig. 1) was chosen as electrochemical probe since it has been shown that binds with both βCD and Methyl-βCD in solution showing different association constants [27]. In the context of the present paper, an effective difference in behavior when using surface immobilized functionalized CDs can give important information (as compared with the results obtained in solution) in order to produce systems that enhance the selectivity in the molecular recognition of a specific analyte target by means of its encapsulation within a macrocyclic receptor. The modified surfaces were first characterized using different techniques to verify both the effective anchorage of the macrocyclic receptor as well as the appropriate orientation of its cavity. Electrochemical and SPR techniques were used to monitor the host-guest interactions.

1.1. Reagents

Some reagents were of analytic grade: 4-mercapto benzoic acid (4-MBA), N-hydroxysulfosuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), ethanolamine (EtOH-NH_2) and bentazon (BTz) were from Sigma-Aldrich. The amino-β-cyclodextrins (amino-β-CDs) used (AraChem, Netherlands) were heptakis-(6-amino-6-deoxy)-β-cyclodextrin (**β-CD$_7$**, MW = 1128 g/molfree base) and heptakis-(2,3-di-O-methyl-6-amino-6-deoxy)-β-Cyclodextrin (**β-CD$_7$Me**, MW = 1325 g/molfree base). Both contain 7 amine terminal groups at the narrow edge of their structure (at each C_6). The β-CD_7Me contains 14 additional methyl groups in the broad edge of its structure. Other chemicals were of reagent grade and were used without further purification. All solutions were prepared with ultrapure water (18 MΩ cm) from a MilliQ system.

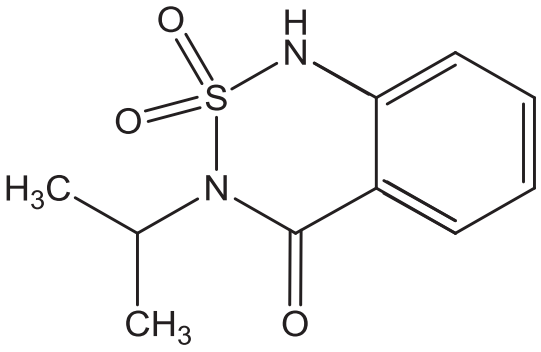

Fig. 1. Molecular Structure of bentazon.

1.2. Apparatus

All electrochemical experiments were conducted on a CHI-Instruments 440D electrochemical Workstation, equipped with a three electrodes system. The working electrode was an amino-βCDs surface-modified gold disk with a geometric area of 0.0314 cm^2 (CH-Instruments, CHI 101). A Pt wire was used as counter electrode and an Ag/AgCl/1 M KCl electrode (CH-Instruments, CHI 111) was used as reference electrode. The measurements were performed at room temperature (25 °C, approx.); the pH adjustment was monitored using an Oakton microprocessor pH meter (model 700).

1.3. Modification and Characterization of the surface of the gold electrode

The amino-β-cyclodextrins immobilization protocol was performed according to the method previously reported by our research group [22]. Briefly, the amino-β-CDs were immobilized on gold by amide bond formation with the carboxylic groups of a self-assembled monolayer of 4-mercapto benzoic acid (4-MBA). As a preliminary step to the modification of the gold electrode, the surface was cleaned by three sequential treatments: (i) mechanical, (ii) chemical and (iii) electrochemical. The real surface area was determined from the reduction charge of the anodically formed oxide. This charge was measured from 0.65 to 1.15 V (vs Ag/AgCl) in the negative sweep and compared with the charge associated with the oxide monolayer reduction for a polycrystalline gold electrode equal to 482 μC cm^{-2}, reported by Oeschand Janata [28]. The roughness factor was obtained from the ratio of the real to geometric surface area. The roughness factor used in this work was 2.6 ± 0.2.

The determination of the surface elemental composition and the characterization of the various functional groups present on the modified surfaces were carried out by X-ray photoelectron spectroscopy (XPS). The XPS spectra were recorded with a PHOIBOS-150 (SPECS) hemispherical electron energy analyzer under a base pressure of $5 \cdot 10^{-10}$ torr using Mg Kα radiation. Constant pass energies of 100 and 20 eV were used to record the wide scan and narrow scan spectra respectively. The energy scale was referenced to the binding energy (BE) of the Au $4f_{7/2}$ core level at 84.0 eV.

The hydrophilicity and hydrophobicity of the modified surfaces were evaluated by means of contact angle measurements in a Ramé-Hart goniometer, model 200, equipped with a CCD camera and DROPstandard image software. The gold surfaces used were bare SPR sensor disks which were modified with amino-β-CDs using the procedure described previously (see section 1.3 Modification and Characterization of the surface of the gold electrode). The contact angle was determined using the sessile drop method, that is, by depositing on the gold surface a drop of 2 μL of ultrapure water or phosphate buffer, as appropriate.

The modification of the gold electrodes with the amino-β-CDs

was monitored by cyclic voltammetry (CV) at 0.100 Vs^{-1}. We used a 1.0×10^{-3} M $K_3[Fe(CN)_6]$ solution prepared in 0.1 M phosphate buffer at pH 8.0 was used as redox probe as we described in our previous work [22]. The electrochemical measurements were performed in triplicate, using independent electrodes, all modified at the same time.

1.4. Electrochemical behavior of bentazon on modified surfaces

The electrochemical behavior of 5.0×10^{-4} M bentazon prepared in 0.1 M phosphate buffer at pH 6.0 was evaluated by differential pulse voltammetry (DPV). Various accumulation times were tested and no significant differences were found for accumulation times longer than 15 min. Therefore, we used an accumulation time of 20 min to ensure constant current values in the differential pulse voltammograms. The potential scan rate, the pulse amplitude and the pulse width used were 20 mV s^{-1}, 50 mV and 50 ms, respectively.

1.5. SPR experiments

All experiments were carried out on a dual channel SPR 7500DC (Reichert) using gold chips functionalized with a 4-Mercaptobenzoic acid (4-MBA) self-assembled monolayer (SAM) at 25 °C. This surface functionalization was performed *ex situ* following the same procedure that we reported previously [22], i.e. by immersion of the chip during 30 min in a 10 mM ethanolic solution of 4-MBA. At the end of the adsorption step the substrate was exhaustively cleaned with ethanol and dried under nitrogen flow. The chips were cleaned before each experiment by washing them exhaustively with ethanol and ultrapure water and dried with N_2 flow.

Immobilization of CD procedure: The optimized immobilization protocol has been described in detail previously [22] so the same conditions of concentrations and pH were used in the present work. However, since in the current experiments a dual channel SPR with a flow cell system was used, the experiment times were adjusted accordingly. Thus, in a first step, the surface was stabilized with phosphate buffer at pH 7.4 (PBS) at a flow rate of 20 μL min^{-1}(25 °C) until a constant refractive index was obtained. Then, an EDC (0.05 M):NHS (0.05 M) mixture in PBS was injected over both channels (working and reference channels) for 7 min at a flow of 20 μL min^{-1} to achieve the transformation of the surface carboxylic acid groups in ester groups (surface activation). Then, a 3×10^{-3} M cyclodextrin solution in PBS was injected only through the working channel for 40 min at 5 μL min^{-1}, to promote amide bond formation. Finally, the excess esters were deactivated by flowing a 1 M ethanolamine solution over both channels during 7 min at 20 μL min^{-1}. Therefore, a β-cyclodextrin surface modified was obtained in one channel (working channel) while the other (unmodified) channel was used as a reference.

1.6. Determination by SPR of the association constant of the amino-β-CDs/BTz inclusion complex

Binding experiments: A bentazon solution at different concentrations (3, 5, 7, 9, 11, 13, 15 and 17 mM) prepared in 0.1 M phosphate buffer at pH 6.0 with 5% DMSO was injected into the cell over both channels at a flow of 5 μL min^{-1}. Due to the low solubility of bentazon in water (500 mg/L), the addition of a co-solvent was necessary to reach a larger range of concentrations similarly to what has been reported for other analogous systems [29]. The experimental data were corrected for instrumental bulk artifacts by double referencing, a control sensor chip surface (reference channel) and buffer injections using integrated SPRAutolink (Reichert, USA). Data analysis was performed with TraceDrawer software. The binding experiment was carried out at 5 μL min^{-1} with 17 min for association bentazon/β-cyclodextrin and 5 min for the dissociation of the inclusion complex, using a 0.1 M phosphate buffer solution at pH 6.0 with 5% DMSO as running buffer. The association time of 17 min was determined injecting a 17 mM bentazon solution and letting it flow until equilibrium was reached, i.e., until the SPR response remained constant. In such a way, it was possible to set the minimum time required. To allow the system to reach the equilibrium (in the interaction bentazon/β-CD) a delay time to register the response is also necessary. Too short times do not allow reaching the equilibrium (and then the Langmuir model cannot be applied) while long times would unnecessarily lengthen the experimental time.

2. Results and discussion

2.1. Characterization of modified surfaces

2.1.1. XPS measurements

The wide scan XPS spectra recorded from the different samples were all very similar. A characteristic example is given in Fig. 2A which corresponds to the Au/4-MBA/β-CD_7 sample. The spectrum is dominated by the lines corresponding to the gold substrate. Minor contributions from oxygen, carbon, nitrogen and sulphur are clearly observed (see labels in Fig. 2A). The spectrum shows some small contributions corresponding to P (around 135 eV) and K (295 eV) which must correspond to a minor contamination coming from the phosphate buffer solution which was not completely removed by washing.

Fig. 2 also shows the high resolution S 2p, N 1s and C 1s spectra recorded from the same sample (the spectra of the rest of samples were also very similar). Fig. 2B shows the S 2p spectrum recorded from the above mentioned sample. Two contributions are observed: one at 161.9 eV (76%) and a second at 164.0 eV (24%). These values are similar to those reported by authors as Pensa and Beulen et al. [30,31]. The lowest BE component corresponds to thiolate groups bound to the gold surface, R-S-Au, while the component appearing at higher BE values is attributed to R-SH species that are unbound. It is important to note that there are no traces of elemental sulfur impurities in our spectra, which would give a signal at BE ≈ 161 eV. The data indicate that the procedure has been successful in immobilizing 4-MBA on the gold surface.

The C 1s spectrum (Fig. 2C) contains four contributions at 285.0, 285.9, 287.1 and 288.7 eV which have been reported to be characteristic of β-CD films [25,32]. The assignment of the different peaks to functional groups is given as an inset in Fig. 2C. Fig. 2D depicts the N 1s spectrum which contains a main, major contribution at 400.3 eV and a much smaller, minor contribution at 403.5 eV. There is a large, general consensus in the scientific literature [33] that identify the contribution at 400.3 eV with the presence of amide bonds and that state that the amine-type bonds would appear at smaller binding energy (around 399.0 eV). The contribution appearing at larger binding energy would correspond to quaternary nitrogen. This latter species has been reported [32] to be present in the spectra recorded from β-CD films and has no clear origin. In any case it represents a small part of the N 1s spectral area (about 8%). Taken together, the XPS results indicate that the β-CDs compounds have been effectively immobilized on the gold surface and that the condensation reaction of the amine groups present in the original cyclodextrin with carboxylic acid has generated amide groups. So, it can be concluded that the CDs have been anchored covalently to the gold surface via amide bond formation with the carboxylic groups of 4-MBA previously immobilized on the surface.

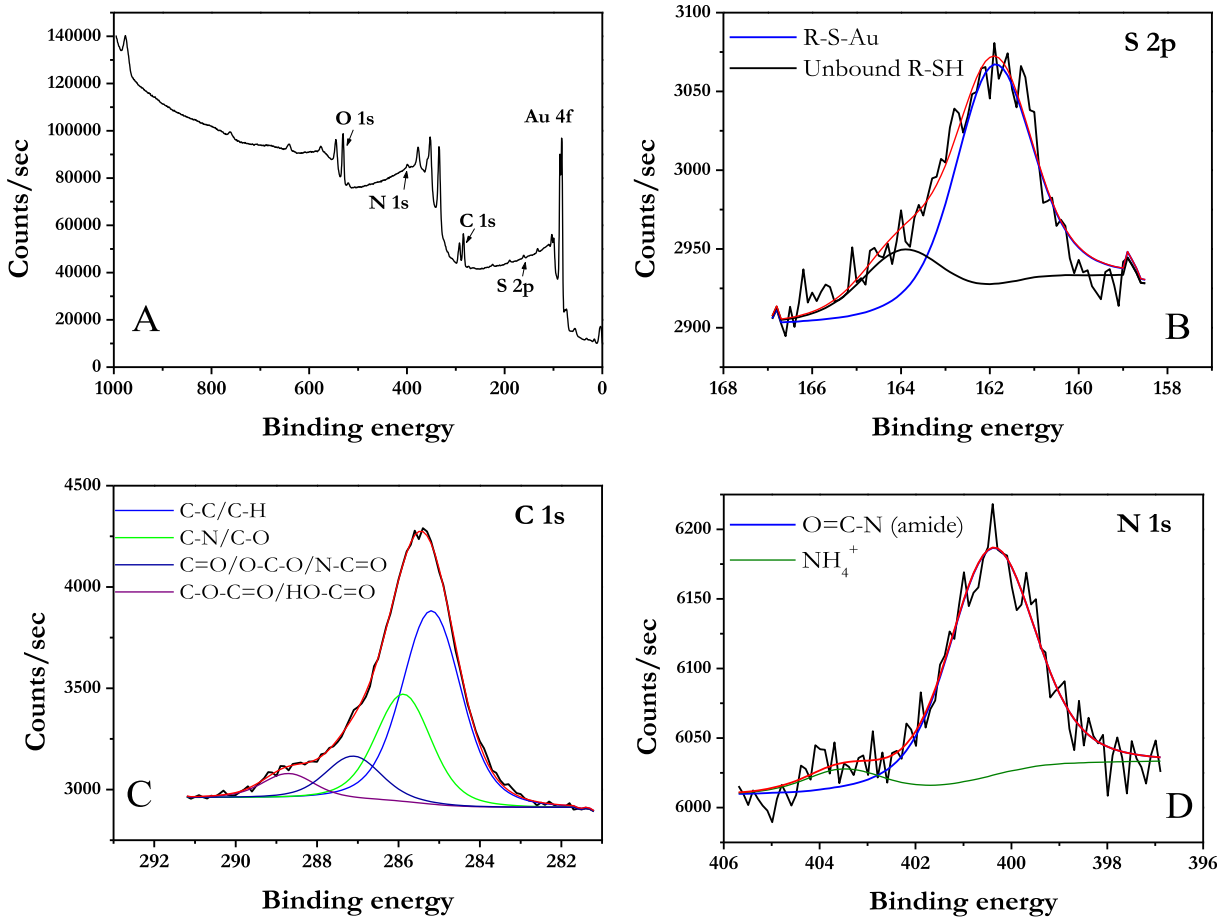

Fig. 2. **(A)** Wide XPS spectrum recorded from Au/4-MBA/β-CD_7, **(B)** S 2p, **(C)** C 1s and **(D)** N 1s spectra recorded from Au/4-MBA/β-CD_7.

2.1.2. Contact angle measurements

Contact angle measurements have been performed to determine the hydrophobicity of the surface in order to obtain information related to the composition and structure of the different surfaces. Static contact angles of water drop both at a bare gold surface and at a gold modified surface were measured.

The Au surface was firstly modified with 4-MBA (Au/4-MBA). As described in the experimental section (and as it was corroborated by the XPS results, see above), the CD derivatives were covalently attached to Au/4-MBA in a phosphate buffer solution at pH 7.4 where the carboxylic groups exposed to the solution were activated using EDC + NHS [34].

Table 1 shows the contact angle values obtained for the 4-MBA gold-modified surface by depositing on it a drop of a 0.05 M phosphate buffer solution with different pH values. The contact angles remain about constant in the pH range 5.0–7.4 and then decrease abruptly at pH = 8.0 in agreement with the apparent surface pKa of carboxylic groups previously reported [35–37]. At pHs close to 8.0, the surface is negatively charged providing electrostatic interactions that facilitate the reaction with EDC and NHS, with the subsequent immobilization of the CD derivatives.

Table 1
Contact angle values measured on the Au/4-MBA surface by depositing a drop of 2 μL of a 0.05 M phosphate buffer solution with different pH values.

pH	θ (°)
5.0	58.25 ± 0.07
6.0	62.20 ± 2.40
7.4	57.47 ± 1.56
8.0	28.23 ± 1.98

Fig. 3 shows the drop profiles obtained from the bare gold and cyclodextrin modified-gold electrodes. The static contact angle at the bare gold surface was 88.70 ± 0.17° in good agreement with previously reported values [38] while the contact angle for Au/MBA/β-CD_7 was 36.36 ± 0.06° indicating an increase of the hydrophilicity and wettability of the modified surface with respect to that of bare gold.

This is surely related with the presence of secondary hydroxyls (which are highly hydrophilic) on the broader rim of the cyclodextrin structure. When amino-β-CD_7Me is immobilized on the surface (Au/MBA/β-CD_7Me) the contact angle value changes to 66.97 ± 0.06°, what can be explained by the presence of the methyl groups located at the broader rim of the cyclodextrin structure, that bring about an increase in the hydrophobic nature of the surface with respect to that of the non-methylated CD [25,31]. The contact angle results in both CDs modified surfaces with respect to those shown by the bare gold surfaces is an indication that the amino-CD molecules lay on the surface in such a way that the broader rim is oriented to the solution.

2.1.3. Electrochemical characterization of the modified electrodes

2.1.3.1. Reductive desorption. According to the well-known behavior of thiolated modified gold electrodes [39], the reductive desorption in basic media allows determining the monolayer

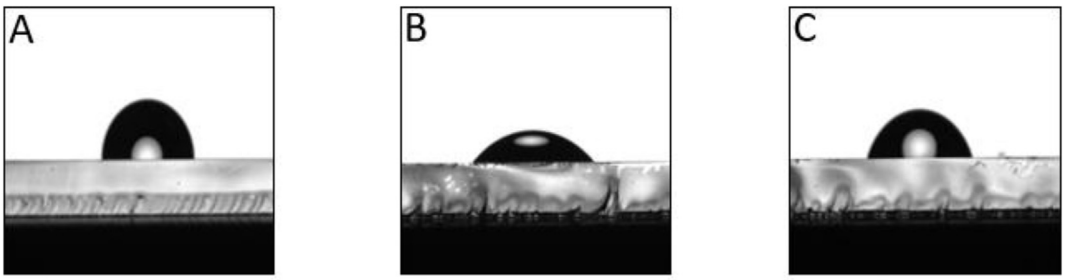

Fig. 3. Characterization of the amino-β-CDs-modified gold surface by contact angle measurements. Images of a 2 μL water droplet in contact with a gold bare substrate (**A**), gold substrate modified with amino-β-CD_7 (**B**) and (**C**) with amino-β-CD_7Me.

coverage. As a preliminary step to the immobilization of the amino-β-CDs on gold, the coverage of Au/4-MBA was determined through reductive electroadsorption.

Cyclic voltammograms of the Au/4-MBA electrodes were recorded applying a sweep of potential towards negative values (from −0.2 V to −1.4 V) at 0.1 V s^{-1} in 0.1 M NaOH solution. Only one reduction peak at −0.9 V corresponding to reductive desorption of Au-S was observed (Fig. 4). The surface coverage was determined from the integration of the desorption peak area corresponding to the Au-S bond using the real area of the gold electrode ($0.107 \pm 8.2 \times 10^{-3}$ cm^2) calculated on the basis of the procedure reported in Ref. [28]. The value obtained was $9.13 \times 10^{-10} \pm 0.59$ mol cm^{-2} which is in good agreement with the data reported by other authors [40,41] for a compact monolayer.

2.1.3.2. Cyclic Voltammetry. The immobilization of the amino-β-CDs derivatives on the gold electrodes was characterized by cyclic voltammetry using $K_3[Fe(CN)_6]$ as the redox probe. According to previous reports, ferrocyanide does not form inclusion complexes with the amino-β-CDs, therefore the electronic transfer between the ferrocyanide and the gold surface is inhibited if the cyclodextrin derivatives are immobilized on the latter. Therefore, the study of this electron transfer inhibition can be used to gain insight on the effective immobilization of the amino-β-CDs on the gold surface [42,43]. The cyclic voltammograms of 1.0×10^{-3} M $[Fe(CN)_6]^{3-}$ in 0.1 M phosphate buffer solution at pH 8.0 on both bare and modified gold electrodes are shown in Fig. 5. A reversible electron transfer process with a cathodic (Epc) and anodic (Epa) peak

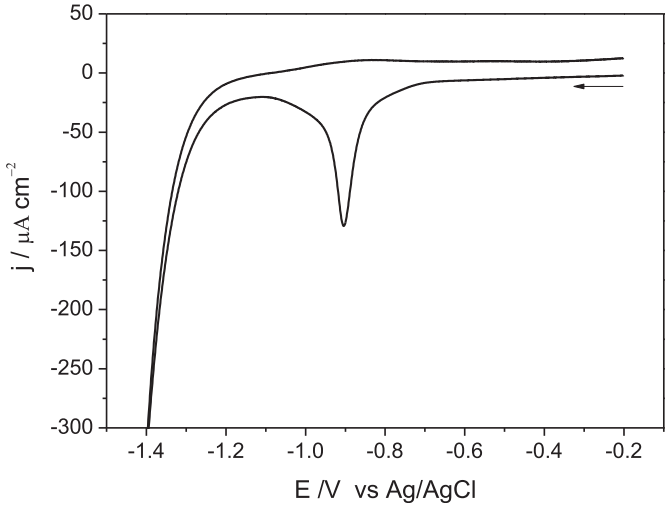

Fig. 4. Cyclic voltammogram of stripping of the 4-MBA SAM on a polycrystalline gold surface obtained in 0.1 M NaOH solution. Scan rate = 0.100 V s^{-1}.

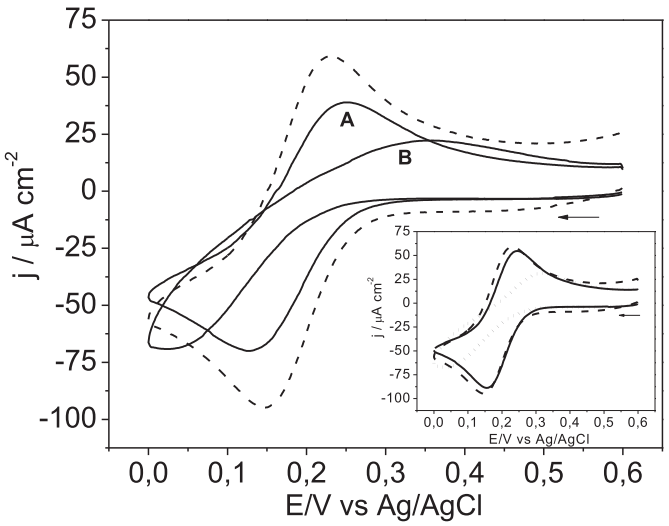

Fig. 5. Cyclic voltammograms obtained in 1.0×10^{-3} M $K_3[Fe(CN)_6]$ in 0.1 M phosphate buffer solution pH 8.0 on bare gold **(dashed line)**; Au/4-MBA/β-CD_7 **(A)** and Au/4-MBA/β-CD_7Me **(B)**. v = 0.100 V s^{-1}. Current density is normalized by the real area. **Inset**: Cyclic voltammograms obtained in 1.0×10^{-3} M $K_3[Fe(CN)_6]$ in 0.1 M phosphate buffer solution pH 8.0 on bare gold **(dashed line);** Au/4-MBA **(dotted line)** and Au/4-MBA/EDC + NHS **(solid line).** v = 0.100 V s^{-1}.

separation, ΔEp= (68 ± 3) mV is observed in the voltammogram of the bare Au electrode (dashed line). The formal potential ($E^{0\prime}$), taken as the average of Epc and Epa, was 200 mV. This Au electrode was modified with 4-MBA and then with EDC + NHS to promote the immobilization of the amino-βCDs. These steps have been already studied [22] and are shown in the inset in Fig. 5. As pointed out previously, a decrease in current density and a higher ΔEp are observed at Au/4-MBA (dotted line), as compared with the bare gold electrode (dashed line), due to the electrostatic repulsions between the highly negatively charged ferricyanide and the deprotonated carboxylic terminal group of 4-MBA. The subsequent voltammogram at Au/4-MBA/EDC + NHS (solid line) is very similar to that obtained on the bare electrode because the activation of the carboxylic groups with EDC + NHS neutralizes the charge on the surface.

When the amino-β-CD_7 is immobilized on the prepared gold electrode (Au/4-MBA/β-CD_7), a decrease of the current density together with a loss of the reversibility of the redox process is observed (Fig. 5, curve A). At this modified surface, ΔEp is increased to 120 ± 5 mV, $E^{0\prime}$ is shifted to 190 mV, and ipa and ipc decrease by about 25 and 30%, respectively. An even more significant change in reversibility is observed in the case of the methylated-cyclodextrin sample Au/4-MBA/β-CD_7Me in whose voltammogram (Fig. 5, curve B) a much larger ΔEp value is observed (330 mV). Besides, ipa

decreases by about 25% while ipc decreases by around 40%.

From the analysis of the reduction peak currents measured on the bare and modified Au electrodes, we can determine the hindrance (B) as an estimation of the CD layer density, as we have previously reported for this kind of immobilizations [42]. The B values obtained for Au/4-MBA/β-CD_7 and Au/4-MBA/β-CD_7Me were very similar: 0.25 and 0.27, respectively, suggesting that the layer density is similar in both samples. Both derivatives, β-CD_7 and β-CD_7Me, have the same number of amino groups located on the narrow edge of their structure, therefore, it is expected that a similar immobilization can be reached in both cases since all of these amino groups are capable to bond with the carboxylic group of the 4-MBA molecules present on the surface. Although it has been informed that the secondary hydroxyl groups (located at the C_2 and C_3, i.e. at the broader rim) can facilitate the formation of intermolecular hydrogen bonds between neighboring CDs [44], resulting in a more rigid network in the amino-β-CD_7 case, this seems to have a very minor effect on the layer density. Therefore, it is reasonable to infer that the change in reversibility of the electron transfer processes can be mainly attributed to the greater hydrophobicity presented by β-CD_7Me, due to the exposed methyl groups located in the broad edge of its structure.

According to the characterization results described above, it can be concluded that the CD derivatives are effectively chemisorbed on the surface through the 4-MBA molecule and that the CD cavities are exposed towards the solution.

2.2. Electrochemical and SPR studies of modified surfaces

The host-guest interactions of CD derivatives immobilized on gold surfaces have been studied using bentazon (BTz) as a guest. This herbicide has a keto-enol tautomerism, is a weak acid with a pK_a of 3.3 and, therefore, its oxidation is independent of the pH providing this is above 3.5. The formation of inclusion complexes with different β-CDs has been previously studied and the corresponding association constants in solution have been determined [27,45]. Therefore, bentazon was used in this study as a model molecule to test the interaction with the immobilized cyclodextrins on gold surfaces as well as to evaluate the effect of the hydrophobicity of the methyl groups on the broader rim of the cyclodextrins in this interaction. The formation of an inclusion complex of BTz/β-CD has been previously studied at pH 6.0 and the corresponding association constant being determined at pH 6.0 by differential pulse voltammetry [2]. Since the pK_a is 3.3, BTz predominantly exists in its anionic form in phosphate buffer at pH 6.0. Besides, it shows a definite and reproducible oxidation signal on glassy carbon and gold at this pH [46]. Therefore, we decided to use the same pH conditions in the present study.

The electrochemical behavior of BTz on both bare gold and amino-β-CDs-modified gold surfaces was studied using differential pulse voltammetry (DPV) with the aim of obtaining well defined oxidation peaks. Fig. 6 shows DPVs performed in 0.1 M phosphate buffer at pH 6.0 using an accumulation time of 20 min in order to promote the inclusion of BTz. On the bare gold surface (dashed line) an oxidation process is observed with a peak potential $E_p = 916 \pm 4$ mV and a current density $I_{pa} = 94 \pm 6\ \mu A\ cm^{-2}$. Rahemi et al. have proposed that the oxidation process of BTz occurs in the nitrogen of the tertiary amine, since the high electron density caused by the free electron pair of nitrogen makes it susceptible to oxidation [46]. The oxidation of BTz on the Au/4-MBA/β-CD_7 surface (Fig. 6, curve A) is observed at $E_p = 984 \pm 4$ mV, with a displacement of 68 ± 4 mV to more positive potentials together with a peak broadening and an increase in the current density of 28% ($I_{pa} = 121 \pm 8$) $\mu A\ cm^{-2}$, as compared with that observed in the bare gold electrode. In the case of Au/4-MBA/β-CD_7Me (Fig. 6, curve

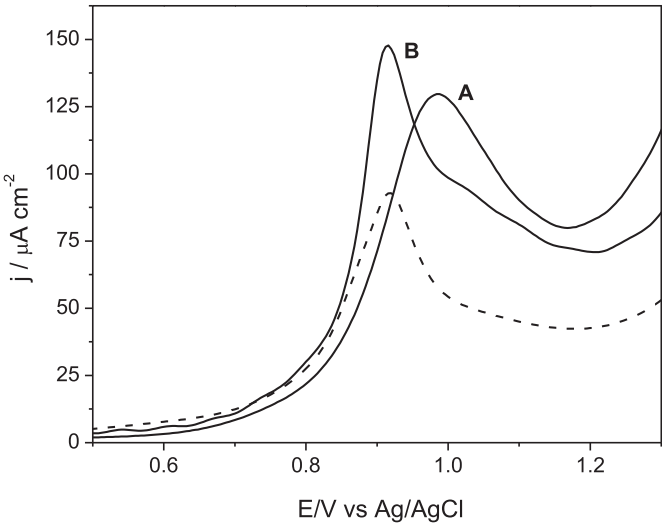

Fig. 6. Differential pulse voltammetry curves for 5.0×10^{-4} M bentazon on bare gold electrode (dashed line), **(A)** Au/4-MBA/β-CD_7 and **(B)** Au/4-MBA/β-CD_7Me in 0.1 M phosphate buffer pH 6.0 using an accumulation time of 20 min.

B) the oxidation of BTz takes place at the same potential value than in the bare gold surface but showing an increase in current density of 50% ($I_{pa} = 141 \pm 6$) $\mu A\ cm^{-2}$. Both modified gold electrodes show a current variation coefficient close to 5.0%. The increase in current intensity can be explained on the basis of the formation of an inclusion complex between the amino-β-CDs and BTz. The increase in peak current is larger in the Au/4-MBA/β-CD_7Me surface due to the larger hydrophobicity that the methyl groups located on the broader rim of its structure confer to this cyclodextrin.

While the increase in current is observed in both modified surfaces, only in the sample Au/4-MBA/β-CD_7 a change in peak potential is observed. This can be explained taking into account that, as it has been previously suggested [3,27], an inclusion complex having the benzene ring inside the cavity of the cyclodextrin can have been formed in this case. Therefore, since the electroactive moiety of the BTz molecule is located outside the cavity, there would be a host-guest complexation processes, coupled to an electron transfer reaction, which would require a short additional potential. This effect is no observed in Au/4-MBA/β-CD_7Me which might suggest that a higher association would be occurring due either to presence of methyl groups in the broad rim of the cyclodextrin or to the formation of an inclusion complex having a different structure than in the non-methylated case. This latter assumption, however, would be unlikely taking into account the results of previous investigations carried out in solution [3] where an inclusion complex with a 1:1 stoichiometry was formed but no changes in its structure were found for different β-CD derivatives (sulfobutylether-, methyl-, dimethyl-CD, etc) [2,3].

These variations on the peak potential could be explained taking into account two possible effects. On one hand, a double layer effect can be considered: when the Au surface is chemically modified, the electric field felt by the guest molecule is probably smaller than on the unmodified Au surface, where for the same potential drop across the interface the outer Helmholtz plane can be closer to the metal surface. On the other hand, a thermodynamic effect could be also playing a role: there is an increase of the activity of the guest molecule due to its complexation with the CDs and a shift in the equilibrium potential is produced.

It is reasonable to think that the double layer effect is similar in both methylated and non-methylated CDs, resulting in a positive shift, respect to the unmodified surface, due to the smaller electric

field. However, the higher activity of the guest molecule due to the complexation with both CDs (methylated and non-methylated) produces a shift of the equilibrium potential in the negative direction. Due to the higher affinity, the negative shift is larger in the methylated CD and, therefore, counteracts the positive shift due to the double layer effect. A similar compensation of effects has been discussed by Cuesta et al. [47].

Therefore, in order to evaluate the effect of methyl groups, SPR experiments were carried out to obtain the association constants of the interaction between BTz and the different immobilized amino-β-CDs on gold surfaces. A dual channel SPR was used (which allows a double correction of blank), with a flow cell system that allows transporting the BTz solutions towards the immobilized CDs. β-CD_7 and β-CD_7Me were immobilized on SPR sensors following the same protocol of chemisorption on a 4-MBA self-assembled monolayer, using the amine coupling procedure by means of the above described EDC/NHS reaction.

The SPR spectrometer measures changes in the refractive index of the solution close to the surface, which can be related with interaction processes between the analyte in solution and the molecules adsorbed on the electrode surface. Fig. 7 shows a schematic representation of the experimental curves related to association and dissociation processes. First, the modified surface is stabilized with a 0.1 M phosphate buffer solution at pH 6.0 with 5% DMSO (baseline). Then, the interaction of BTz molecules with the β-CDs immobilized on the surface is evaluated following the increase of the refractive index (RIU) until a maximum is reached once the equilibrium is achieved and the refractive index remains constant (association). At this point, each β-CD available on the surface binds to a BTz guest molecule, considering a 1:1 stoichiometry. Then, the guest molecules contained in the flow begin to be replaced by water molecules and/or phosphate buffer. Within this dynamics, BTz molecules start being located outside the hydrophobic cavity of the β-CDs consequently causing a decrease in the response in units of refractive index (dissociation). Before starting each injection of the BTz solutions, the surface is regenerated, stabilizing the baseline as previously described, with a waiting time of 10 min between each injection to obtain a newly available surface for the BTz/β- CDs interaction.

Each curve observed in Fig. 8A and B corresponds to the injection of BTz at different concentrations. The asterisk in the graphs denotes the beginning of the association and dissociation phases. For both sensorgrams the saturation is not reached in the BTz concentrations range analyzed.

Fig. 8C and D show the equilibrium response vs. BTz concentration curves. A plateau in the response is reached for the interaction of BTz with Au/4-MBA/β-CD_7Me (Fig. 8 D) while this is not observed for Au/4-MBA/β-CD_7 (Fig. 8C). The $1/R_{eq}$ vs. 1/[BTz] plots shown as insets in these figures follow a clear linear trend. Measurements in equilibrium are not affected by mass transport, which makes possible the use of a high cyclodextrin concentration and low flow rates [48].

The association constant was determined considering the Langmuir isotherm model. In this analysis a non-lineal global fitting (taking into account all concentrations employed) was used according to the equation:

$$R_{eq} = \frac{B_{max}\,[BTz]}{([BTz] + K_D)};$$

where K_D is the association constant, B_{max} is the maximal response, R_{eq} is the equilibrium response to a given concentration and [BTz] is the bentazon concentration.

The Langmuir model was applied considering that: i) the inclusion complex corresponds to a 1:1 stoichiometry (bentazon:cyclodextrin), ii) Inclusion only involves the cyclodextrin localized on the surface and iii) the saturation coverage corresponds to the complete occupancy of the exposed cyclodextrins. Finally, no interaction between neighboring adsorbed molecules or atoms is assumed.

The association constant values obtained for BTz with Au/4-MBA/β-CD_7 and Au/4-MBA/β-CD_7Me are $30.8 \pm 1.0\ M^{-1}$ and $80.5 \pm 4.2\ M^{-1}$, respectively. It is worth noting that the reproducibility of the values is good in spite of the low magnitude of the observed response. The substitution of the cyclodextrin secondary alcohol groups by methyl groups modifies the host-guest interactions, increasing the inclusion constant of the BTz by around 2.5 times.

This methylation effect could be explained on the basis of a different orientation of the guest molecule within the cavity of the methylated CD with respect to that adopted within the non-methylated host. Bethanis et al. [49] reported such a difference in guest orientation in the case of tri-methylated-βCD and βCD although, as a consequence, a smaller penetration of the guest was observed in the tri-methylated-βCD. However, as mentioned before, this assumption would be unlikely taking into account the present electrochemical results.

The larger value measured for the association constant in the

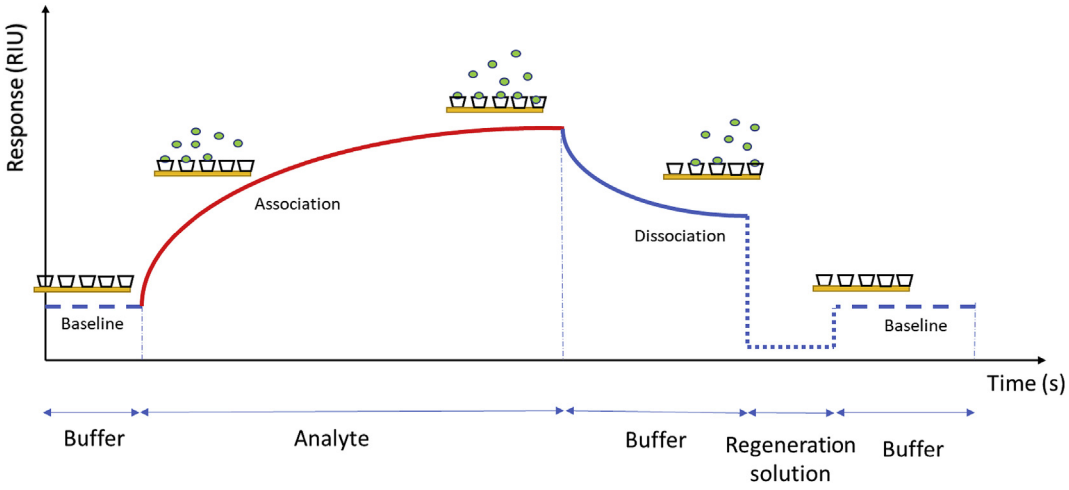

Fig. 7. Schematic representation of typical sensorgram.

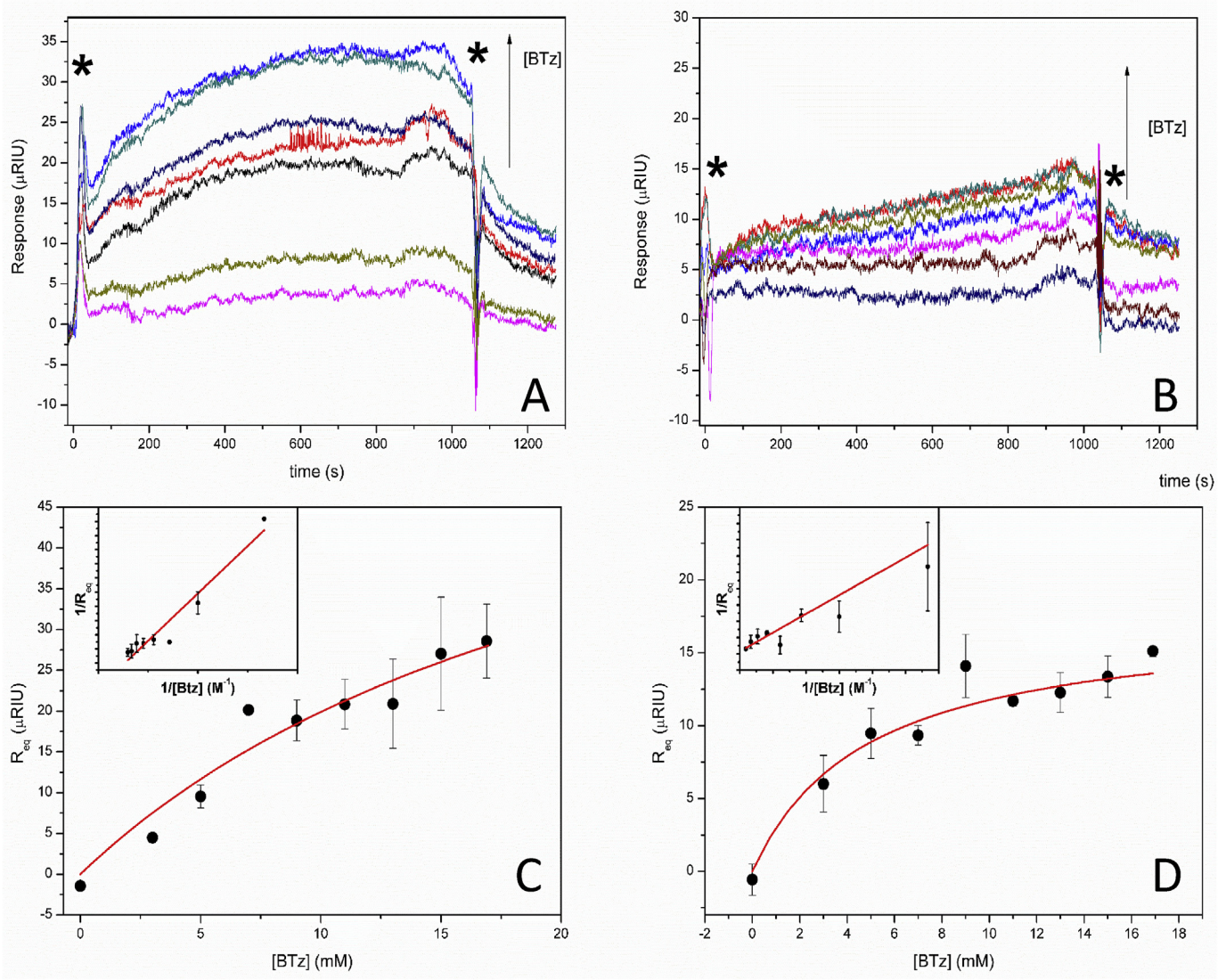

Fig. 8. Interaction sensorgrams of bentazon with Au/4-MBA/β-CD_7 (A) and Au/4-MBA/β-CD_7Me (B). The arrow marked '[BTz]' indicates the direction of increasing bentazon concentration from 3 to 17 mM. Equilibrium response of the interaction at different bentazon concentrations for Au/4-MBA/β-CD_7 (C) and Au/4-MBA/β-CD_7Me (D). Inset in C and D: The $1/R_{eq}$ vs. 1/[BTz] plots.

methylated CD could be attributed to the hydrophobic character of the host. Biswas et al. [50] have reported that the association constant determined for a DMO/DIMEB inclusion complex (where DIMEB is a methyl derivative-βCD) is larger than for a DMO/βCD inclusion complex. The larger wavelength shift and the higher quantum yield that they observe in the fluorescence results obtained from the DMO/DIMEB inclusion complex can be rationalized on the basis of the more hydrophobic character of the cavity of the methyl derivative as compared with that of the native βCD, what agrees with our contact angle results. Therefore, bentazon could be better accommodated within the methylated-βCD since the non-polar benzene ring would find a more comfortable inclusion in this hydrophobic host cavity. An alternative additional effect could be related with the methoxy groups that could form slightly stronger H bonds with water molecules than the βCD hydroxyl groups [50]. Those water molecules could play a role in the stabilization of the negative charge associated to the part of the bentazon molecule that remains outside of the host cavity.

Since our method is based on a flow system, a direct comparison with previous association constants reported in the literature could be complicated although still useful [51]. Besides, variations in Ka values reported in the literature depend, among other factors, on the method used for their determination [45]. Our association constants values are coherent with those determined using fluorometric measurements [27] for the inclusion complexes of BTz with β-CD and Methyl-β-CD in solution, although those found in solution are higher, 105 ± 8 and $160 \pm 14\ M^{-1}$ respectively, than those measured in the present investigation. Lower association constants obtained by SPR than those measured in solution were reported for the complexation of steroids with β-CD [52]. The difference has been attributed to space constraints due to the immobilization of cyclodextrin on a surface together with the effects introduced by the flow system used for their determination by SPR.

The use of different techniques for the determination of association constants usually results in different values for these constants. So, as we have reported recently for bentazon/β-cyclodextrin complexes in solution, values such as 118 ± 20, 140 ± 7 and 102 ± 5 have been determined by differential pulse voltammetry, UV–Vis and fluorescence techniques, respectively [45].

Sometimes the differences found are very large. For example in the case of hydroxypropyl-βcyclodextrin, association constant values of 244 ± 19 and 103 ± 30 were determined by differential pulse voltammetry and fluorescence, respectively [45]. In consequence, the results of the present paper indicate the effect produced by the derivatization of CDs can be indeed evaluated by SPR beyond the specific values of the association constants.

Thus, the tendency observed in this work follows that reported in solution. An increase in the hydrophobicity of the broader rim favors the inclusion equilibrium for the organic molecule bentazon. The advantages of the procedure reported here are clear because knowing the effect of specific groups on the broader rim, the behavior of the modified surface can be controlled.

3. Conclusions

Amino-cyclodextrins can be anchored covalently to gold electrodes employing simple methods via amide bond formation with the carboxylic groups of 4-mercaptobenzoic acid previously immobilized on the gold surface. The results show that using this procedure the CD cavity is oriented opposite to the gold surface. In such a configuration, the host-guest interactions can be enhanced for the encapsulation of certain analytes within the macrocyclic receptor cavity. In particular, we have proved the sensitivity of the modified surfaces for the encapsulation of the herbicide bentazon. This sensitivity is higher when methyl groups are present in the broad rim of the CD because of their large hydrophobic character.

The results show the feasibility of using the modified surfaces for molecular recognition as an alternative route to other biological or enzymatic approaches. The procedure reported here can represent, however, a much simpler and more advantageous approach than those.

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