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Supramolecular Chemistry of Cyclodextrins in Cuba

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Supramolecular Chemistry of Cyclodextrins in Cuba

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Cyclodextrins (CDs) are a class of cyclic oligosaccharides composed of α(1 → 4)-linked D-glucopyranose units in the $^4C_1$ chair conformation. The overall form of the molecules is a truncated cone with an essentially hydrophobic cavity. Such a structure allows them to form stable inclusion complexes with a wide variety of guests. That is the reason for which CDs constitute an indivisible part of supramolecular chemistry, a field of chemistry in constant growth and development all around the world.

Here we present a survey of the different types of supramolecular compound that CDs and their derivatives can form and the applications we are targeting in Cuba. This consists mainly of the following.

- **Pharmaceutical formulations of copper(II) complexes insoluble in water, solubilized by their inclusion in βCD and in one of its dimers.**
- **Cu,Zn-SOD enzyme mimetics based on copper(II) complexes of CD derivatives with ammonium salts included in the CD cavity.**
- **Enzymes conjugated, by both chemical and enzymatic methods, to CDs with increased thermostability and catalytic activity.**
- **Sensors based on the self-assembly of CD derivatives on metal electrodes and nanoparticles, which permit selective differentiation between species with very similar electrochemical properties.**

Keywords: Cyclodextrin; SOD-like activity; Copper complex; Protein conjugation; Sensor

INTRODUCTION

Cyclodextrins (CDs) are a class of cyclic oligosaccharides composed of α(1 → 4)-linked D-glucopyranose units in the $^4C_1$ chair conformation. The overall form of the molecules is a truncated cone with an essentially hydrophobic cavity. Such a structure allows them to form stable inclusion complexes with a wide variety of guests. That is the reason for which CDs constitute an indivisible part of supramolecular chemistry, a field of chemistry in constant growth and development all around the world. It is interesting to point out that supramolecular compounds of CDs were reported [1] about ten years before Pedersen's communication on crown ethers.

The fact that CDs are becoming cheaper from year to year makes them an attractive field for developing countries, such as Cuba. Supramolecular chemistry of cyclodextrins began in Cuba only in the early nineties, mainly with a biological profile. Initially it was studied only at the Laboratory of Bioinorganic Chemistry (LBI), Faculty of Chemistry, University of Havana under the direction of Prof. Roberto Cao. Some years later, former students and coworkers of this lab (R. Villalonga and E. Almirall) started to work in this area at their own universities (Matanzas and Pedagogical Institute of Pinar del Rio, respectively). In the central region of Cuba, at the Central University of Santa Clara, some attempts were made at working with βCD for the stabilization of new drugs. 1999 was an important year when Alex Fragoso (LBI) received his PhD degree. This thesis was considered the best of the year in both Chemistry and Natural Sciences in Cuba [2].

In this paper, we present a survey of the different types of supramolecular compound that CDs and their derivatives can form and the applications we are targeting in Cuba. The main focus is on pharmaceutical formulations of copper(II) complexes insoluble in water, Cu,Zn-superoxide

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dismutase (Cu,Zn-SOD) mimics, CDs conjugated to enzymes to increase their stability, and sensors based on the self-assembly of CD derivatives on metal electrodes.

PHARMACEUTICAL FORMULATIONS

CDs have been widely used in pharmaceutical formulations of drugs to increase their water solubility (e.g. itraconazole) and stability (e.g. PGE_1), to mask odor (garlic oil) and flavor, to perform a drug release control (loteprednol) and to favor their absorption (e.g. piroxicam) [3]. In most cases such formulations are based on unmodified CDs mixed with the corresponding drug. When a large increase in water solubility is desired sometimes dimethyl- or hydroxypropyl-βCD is used [3].

In our group, bis(morpholyldithiocarbamato)copper(II), $Cu(MorDTC)_2$ has been studied as a scavenger of superoxide anion [4] and nitric oxide, both reactive radicals that can cause negative effects *in vivo*. This neutral copper(II) complex is very insoluble in water, with a K_s of less than 10^{-15}, which causes problems in experimental studies in aqueous solutions. This limitation was initially overcome by preparing the complex in the presence of an excess of βCD. The highly hydrophobic copper(II) compound, $Cu(MorDTC)_2$, forms a stable 2:1 CD/$Cu(MorDTC)_2$ complex with βCD, but not with αCD for steric reasons, as observed by electronic spectroscopy. From these data the inclusion constant was determined using the LETAGROP program and a value of $\log K_{21} = 7.47 \pm 0.04$ was obtained.

The inclusion of $Zn(MorDTC)_2$ [isostructural with the Cu(II) complex] in βCD was also studied using the PM3 semiempirical method. A stable 2:1 complex was predicted, with a separation between the secondary rims of both βCDs of about 5.4 Å. According to the performed PM3 calculations, the copper(II) center is not included in the βCD cavity, as depicted in Fig. 1.

Aqueous solutions of $Cu(MorDTC)_2$ included in βCD with concentrations up to 10^{-4} M were achieved but solid formulations were unsatisfactory. Therefore, we decided to obtain a low-molecular weight, water-soluble βCD polymer using epichlorohydrin as the cross-linker [5]. Different syntheses were performed in order to obtain a product that could increase the water solubility of $Cu(MorDTC)_2$. This corresponded to a βCD dimer of 2600 g/mol of average molecular weight, according to the MALDI mass spectrum. The presence of monomers with an average molecular weight of 1300 was also detected, corresponding to mixtures of βCDs substituted with 2–3 units of 2,3-propanediol.

With $Cu(MorDTC)_2$ included in the βCD dimer its water solubility increased by an additional order of magnitude, up to 10^{-3} M. The resulting system was stable for weeks and could be evaporated repeatedly to dryness and dissolved by simple addition of water. The reactivity of $Cu(MorDTC)_2$ towards $O_2^{\bullet -}$ was not affected by its inclusion in either βCD or βCD dimer, since copper(II) remains outside the cavities, according to the performed PM3 calculations (Fig. 1). The calculated distance between the secondary rims of both βCD (5.4 Å) permits a small species such as the superoxide radical, to easily approach the copper(II) center without any steric hindrance.

The SOD-like activity of the copper(II) complex under different conditions was determined. The obtained IC_{50} values (the concentration necessary to dismutate 50% of $O_2^{\bullet -}$ generated) were: 3.2 μM for $Cu(MorDTC)_2$ [4], 0.47 μM for $Cu(MorDTC)_2$ included in βCD and 1.0 μM when it was included in the βCD dimer. The higher activity of the second system can be attributed to the positive effect of H-bond formation between the OH groups of βCD and the substrate, as will be analyzed in more detail further on. In the βCD dimer the activity may have slightly dropped due to the observed increase in the viscosity of the system which can affect the diffusion of the substrate. Nevertheless, the activity of the copper(II) compound in this latter case is of the same order of magnitude as the free compound, something very important in the proposed formulation.

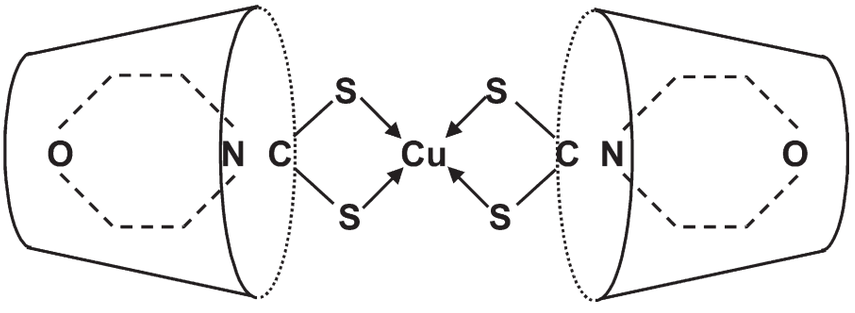

FIGURE 1 Schematic representation of the inclusion of bis(morpholyldithiocarbamato)copper(II) in the βCD cavity.

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To the best of our knowledge this constitutes the first report on a radical scavenger compound, which when included in a βCD dimer maintains its scavenging property.

Cu,Zn-SOD Mimetics

Cu,Zn-SOD is a highly efficient metalloenzyme ($k = 2 \times 10^9\ \mathrm{mol^{-1}\,l\,s^{-1}}$) in its dismutation of superoxide radical [6] [Eq. (1)]

$$2O_2^{\bullet -} + 2H^+ = H_2O_2 + O_2 \qquad (1)$$

This overall reaction involves a cyclic two-step process in which the Cu(II) center is consecutively reduced and reoxidized to form the products. However, beyond this apparently simple mechanism, several structural and kinetic features are manifested, resulting in a nearly perfect natural catalytic system. For example, the Arg-141 residue, located in the vicinity of the active site [7], is assumed to play two important roles. Its positive charge electrostatically attracts the substrate toward the active site and, once superoxide radical is coordinated to Cu(II), it stabilizes it through H-bond formation with the guanidinium residue [8]. Theoretical studies [9] as well as chemical modification of Arg-141 [10] have demonstrated that the positive charge of Arg-141 is responsible for the enhancement of the activity of the wild metalloenzyme by about 30–90%.

Modeling enzyme–substrate interactions with artificial systems is a great challenge for chemists and has been the subject of extensive study [11]. There are several reports on Cu(II) complexes with SOD-like activity [12–15] but systematic studies of the enzyme–substrate recognition events in Cu,Zn-SOD using models are scarce [16–18].

We selected mimetics of Cu,Zn-SOD based on CD derivatives with dithiocarbamate (DTC) moieties to act as ligands in the formation of the corresponding copper(II) complexes. From our previous experience with dithiocarbamato complexes, we expected acceptable results with such a system [4,13]. We took into consideration that they form highly stable square-planar neutral copper(II) complexes of formula $Cu(DTC)_2$ and, as such, their activity is independent of pH and ionic strength. The hypothetical mimic is represented in Fig. 2.

In this model, the H-bonds are formed by the OH groups of CD. Since the secondary OH groups are more acidic, substitutions on the secondary rim of the CD with the dithiocarbamate group gave a higher incidence of SOD-like activity. The electrostatic attraction over the anionic substrate is exerted by quaternary ammonium salts (QAS) consisting of trimethyl- and triethyl-ammonium salts derived from *p*-toluidine and cyclohexylamine. When the quaternary ammonium salts were included, no variation in the geometry of the Cu(II) complex was observed by EPR spectroscopy. Under such conditions the SOD-like activity increased by 30–80% with respect to the uncomplexed CD model. This activity was assumed to be affected by the ionic strength of the medium. As expected, an increase in the ionic strength reduced the effect of such attraction due mainly to partial screening of the positive charge of the QAS. The higher the values of the inclusion constants and the positive charges over the nitrogen (ammonium) atom (calculated by the AM1 semiempirical method), the greater the effect observed for a given increase in the ionic strength.

In summary, in the mimetic system a cooperative effect was obtained between H-bond formation and electrostatic attraction over the anionic substrate, $O_2^{\bullet -}$, as depicted in Fig. 2.

CDs CONJUGATED TO ENZYMES

The increase of the thermostability of peptides and proteins (enzymes) has been achieved through chemical conjugation [19–21], immobilization on solid supports [22], and inclusion in polymer matrixes [23]. Particularly when chemical conjugation is used, a decrease in the enzymatic activity is generally observed. The use of CDs to protect

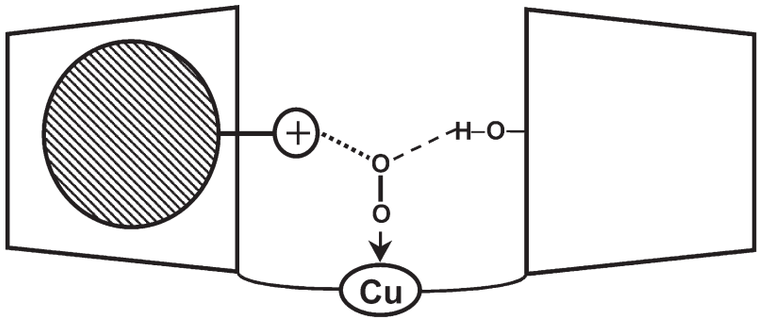

FIGURE 2 Hypothetical mimic of Cu,Zn-SOD based on βCD derivatives [18].

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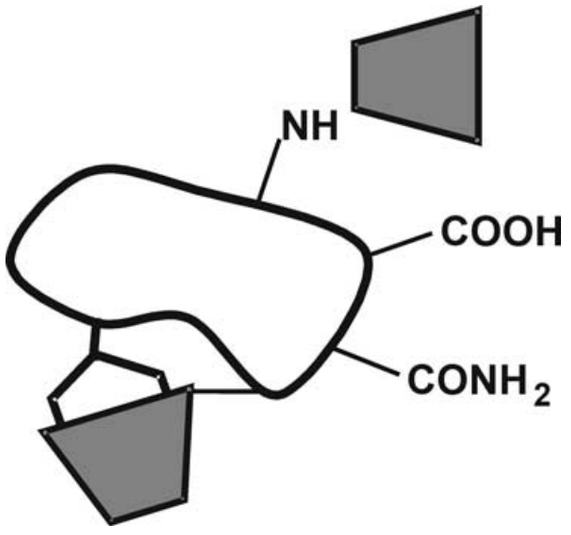

FIGURE 3 Schematic representation of the intramolecular inclusion between a CD and an amino acid moiety in CD-containing enzymes.

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proteins from denaturation has been an uncommon practice, although amino acid moieties such as phenylalanine and tyrosine should be capable of being included in CD cavities.

Some biomedically important peptides and proteins have been reported to increase their stability in the presence of CDs [24]. This is the case for erithropoetin, a glycoprotein hormone which induces an increase in red cell mass. When this protein was complexed to hydroxypropyl-βCD, 100% of its activity was maintained for 10 days compared to only 50% of the activity in the absence of CD [25]. The few reports on the interaction of CDs with proteins or peptides concern only the undefined inclusion of the former in the CD cavity. To our knowledge, CDs conjugated to proteins have not been reported. We chose this novel approach in order to increase the necessary stability of different enzymes for their practical use. The hypothesis was to obtain stable forms of the enzymes that would be easy to handle, without significantly spoiling their activity.

It is important to conjugate an enzyme under very mild conditions in order to not affect its active conformation. If such a requirement can be accomplished, amino acid residues, such as phenylalanine, can be included in the cavity of the conjugated CD (Fig. 3). This latter process is intramolecular and has no possibility to affect the protein's conformation since the inclusion, as a reversible and spontaneous equilibrium, will only take place between amino acid residues and the neighboring conjugated CDs that are sterically accessible. On the other hand, the conjugation of CDs must provoke an increase in the hydrophilicity on the protein surface, which reduces the energetically unfavorable interactions between the hydrophobic amino acid residues and the surrounding water molecules Therefore, this latter factor also protects the protein's stability.

Bovine pancreatic trypsin (EC 3.4.21.4), a serine protease that has important industrial and biomedical applications [26], was selected to be modified by α-, β- and γ-cyclodextrin derivatives. Only CDs mono-substituted on the primary rim were studied. This way the wider secondary rim would be sterically unaffected and, therefore, more accessible to potential guests. Ammonium, 1,2-ethylenediamine, 1,3-propylenediamine and 1,4-butylenediamine were used to achieve mono-substitution of the CDs. Thus, mono-6-amino-6-deoxy-βCD ($\beta CDNH_2$), mono-6-ethylenediamino-6-deoxy-βCD (βCDEN), mono-6-propylenediamino-6-deoxy-βCD (βCDPN) and mono-6-butylenediamino-6-deoxy-βCD (βCDBN), in addition to the mono-6-amino-6-deoxy derivatives of α- and γCDs, $\alpha CDNH_2$ and $\gamma CDNH_2$, were prepared.

The conjugation of trypsin with the above mentioned CD derivatives was achieved by two

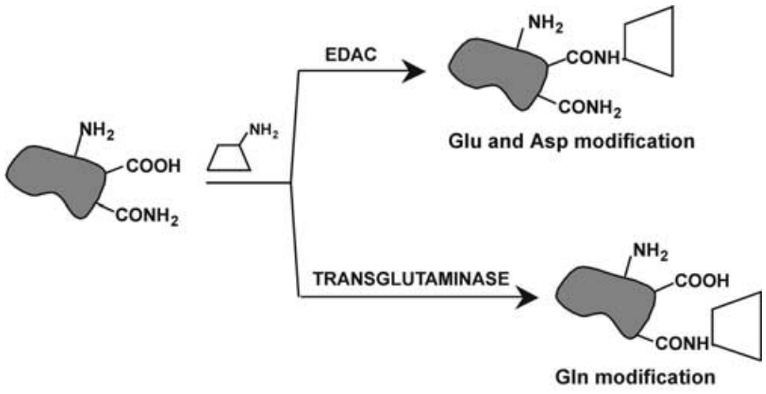

FIGURE 4 Methods used to conjugate CD derivatives to trypsin.

different ways, as depicted in Fig. 4: (1) chemically, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) as coupling agent, and (2) through an enzyme-catalyzed reaction.

It was expected that the use of both methods would permit a comparison of the obtained conjugated trypsins, with the CD derivatives bonded in two different positions. At the same time, the use of two different mild methods of conjugation would serve to compare the results obtained.

TABLE I Half-life times of native and trypsin-CD conjugates at different temperatures

	Half-life (min)			
Enzyme	45°C	50°C	55°C	60°C
Native	117 ± 6	17 ± 1	8.3 ± 0.1	2.3 ± 0.2
Trypsin–βCDNH$_2$	112 ± 2	57 ± 7	50 ± 4	37 ± 1
Trypsin–βCDEN	268 ± 10	42 ± 2	33 ± 3	28 ± 1
Trypsin–βCDPN	39 ± 1	19 ± 1	5.3 ± 0.5	5.2 ± 0.3
Trypsin–βCDBN	408 ± 4	128 ± 11	47 ± 3	27 ± 1

CHEMICAL CONJUGATION

Chemical conjugation was carried out through the condensation reaction between the CD amino derivatives and the carboxylic acid groups of the protein (glutamic and aspartic moieties), with the formation of an amide bond.

The amino acid sequence of bovine pancreatic trypsin reveals that the enzyme contains five glutamic and four aspartic acid residues [27]. The chemically obtained trypsin–CD conjugates contained about 2 molar equivalents of CD per trypsin [28]. According to this information, the degree of modification of the enzyme was calculated as only 20% in the prepared conjugates. This result was not surprising, taking into account that two aspartic acid residues are less exposed and involved in the catalytic site of trypsin (Asp-102 and Asp-189) [29], and also that two glutamic acid residues (Glu-70 and Glu-80) are bonded to calcium ions [30]. The conjugated CDs were located at Asp-153 and Glu-186 residues, as revealed by mass spectrometry, neither of which participates in the active site of the enzyme [29]. Actually, the active site is formed by the Asp-102–His-57–Ser-195 triad [29].

The thermal stability of trypsin was significantly improved by the conjugation of the CD derivatives, except for the case of βCDPN. This effect was reflected either by the increased T_{50} values, the temperature at which 50% of the initial activity was retained, as well as by the greater half-life at different temperatures shown by the prepared adducts (Table I). The thermostability of the enzyme was increased by 2.4–14.5°C after modification. Trypsin, as a protease, can be involved in autolytic processes, and in its unfolded form becomes more susceptible [31]. The autolysis of the trypsin–CD conjugates was noticeably lower than that of the native form. In this sense, the half-lives of the enzyme modified by the α-, β- and γCDNH$_2$ derivatives were 6.0, 5.5 and 8.2 times higher than that corresponding to the non-modified trypsin, respectively.

Chemical conjugation of an enzyme practically always leads to a decrease in its catalytic activity due to an almost unavoidable modification of the conformation of the protein. This situation was to be expected in the trypsin-CD conjugates. Surprisingly, not only was the activity unaffected but an increase was observed, except again for βCDPN, as depicted in Fig. 5. In the latter case the activity decreased by 15% with respect to the native enzyme.

A decrease in the K_m values indicates that the CDs conjugated to trypsin can interact with the substrate BAEE (*N*-α-benzoyl-L-arginine ethyl ester). In fact, we determined by ^{1}H NMR that the inclusion constants of BAEE in αCDNH$_2$, βCDNH$_2$ and γCDNH$_2$ in D$_2$O at pD 8.0 and 25°C were 420, 180

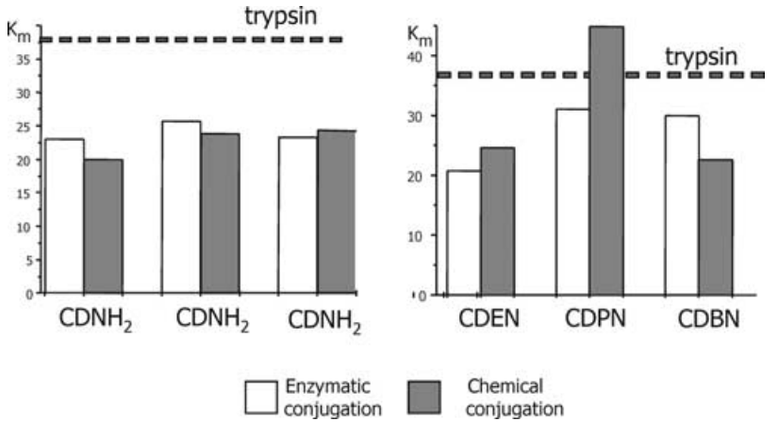

FIGURE 5 K_m values of trypsin conjugated to different CD derivatives by chemical and enzymatic methods [28,35].

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and $100\,M^{-1}$, respectively. The formation of such stable inclusion complexes should increase the concentration of BAEE in the microenvironment of the active site, shifting the equilibrium to the formation of the Michaelis complex. We took into consideration that the modified Asp-153 residue is located very close to the active site of the enzyme [32]. The relationship that exists between the inclusion constants of the CD–BAEE complexes and the decrease in the Michaelis constant (K_m) of the conjugates prepared supports this interpretation.

The only case in which the value of K_m increased was for the chemical conjugation carried out with βCDPN. Apparently, in this latter case the relative position of the conjugated CD is inadequate for such a purpose. This consideration could also explain the observed decrease in its thermostability.

The esterolytic activity of $\alpha CDNH_2$, $\beta CDNH_2$ and $\gamma CDNH_2$ conjugates represented about 140%, 145% and 160% of the enzymatic activity of the native trypsin, respectively. In addition, an increase in the esterolytic activity was also observed for the βCDBN conjugate.

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ENZYMATIC CONJUGATION

The conjugation of CDs by an enzymatic method was carried out using transglutaminase (TGase, EC 2.3.2.13), as depicted in Fig. 4. TGase produces either intra- or intermolecular isopeptide bonds by using the γ-carboxamide group of endoprotein glutamine residues as an acyl donor substrate and the ε-amino groups of endoprotein lysine residues as an acyl acceptor [33]. Moreover, reactive lysines may be substituted by several low molecular weight compounds containing primary amino groups, giving rise to a variety of protein-(γ-glutamyl) derivatives [34]. This latest procedure was selected by us, using the CD amino derivatives.

The oligosaccharide content in all the trypsin–CD conjugates enzymatically obtained was determined to be 3 mol CD per mol of protein. According to this result, a degree of modification of about 30% was estimated for the enzyme–CD conjugates [35].

The thermostability, expressed as T_{50}, increased from 49°C to about 65°C after trypsin modification. Furthermore, the modified trypsin forms were markedly more resistant to incubation at different temperatures ranging from 45 to 70°C.

The CD–trypsin conjugates were markedly less sensitive to the autolytic degradation in comparison with the native protein. In this sense, the half-life of the modified enzyme forms of trypsin was six times higher than the corresponding native protein. The observed stabilization against autolysis could be due to the steric hindrance of autodigestion sites of trypsin with the bulky oligosaccharide moieties, since several glutamine residues are located very close to the possible sites of trypsin autolytic cleavages, i.e. Gln-47–Arg49, Gln-199–Lys-200 and Gln-218–Lys-217 [27].

The enzymatic attachment of CD moieties to the protein surface of trypsin was also found to improve the catalytic properties of the enzyme. The specific esterolytic activity of the modified forms of trypsin was increased to about 110%, 128% and 124% for the $\alpha CDNH_2$, $\beta CDNH_2$ and $\gamma CDNH_2$ conjugates, respectively, compared to the native enzyme. In addition, both the affinity of the trypsin toward BAEE and k_{cat} were improved after modification. Consequently, the catalytic efficiency (k_{cat}/K_m) of the three CD–trypsin complexes proved to be about 2, 2.7 and 2.5 times higher than that of the corresponding native form of trypsin.

Trypsin contains 10 glutamine residues in its polypeptide chain [27], and only one of these residues (Gln-30) is significantly buried inside the protein structure [36]. It should also be pointed out that none of these glutamine residues participates in the active site of the enzyme [29]. According to the tridimensional structure of trypsin, one of the most exposed glutamine residues is located at position 192, covering the entrance to the specific binding pocket of the enzyme. In fact, it has been postulated that Gln-192 might pack against the substrate once it has formed the enzyme–substrate complex [36]. Therefore, it was expected that the conjugation of a CD derivative at that position would permit an increase in the stability of the Michaelis complex, corresponding to a decrease in the value of K_m, as was obtained.

The results obtained for trypsin conjugated to CDs using both chemical and enzymatic methods were very similar. This indicates that the presence of CDs, no matter to which moiety of the protein they are conjugated, can increase its thermostability. If the transformation takes place under mild conditions the activity of the enzyme should not be affected.

The importance of observing very mild conditions can be emphasized by a negative result obtained by us. We additionally performed the conjugation of trypsin through the condensation of a mono-aldehyde of βCD (mono-6-formyl-βCD) to the accessible (36%) lysine moieties of trypsin, and its further reduction with $NaBH_4$ [37]. The thermostability of this conjugate was enhanced by 10.5°C but it retained only 60% of the esterolytic activity of the native enzyme. In this case, the use of $NaBH_4$ as reducing agent did not guarantee sufficiently mild conjugation conditions not to affect the protein's conformation and activity.

According to our results, conjugation of CDs to enzymes may stabilize their necessary folding, leaving the active centers unaltered. The substrate

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can be complexed by the conjugated CD. This gives a new approach to the stabilization of enzymes, something of importance for their practical application.

SENSORS BASED ON THE SELF-ASSEMBLY OF CD DERIVATIVES ON METAL ELECTRODES

Several metal electrodes, mainly gold, have been used to chemisorb thiolate derivatives of βCDs on their surfaces [38–42]. These sulfur-containing CD derivatives can form SAM on electrode surfaces. If the remaining free surface of the electrode is chemically sealed the electroactive probes can only access the metal through the CD cavity. Such restriction imparts steric selectivity to the modified electrodes. Nevertheless, this approach has been insufficiently explored up to now [43].

We selected a polysubstituted CD (poly-6-deoxy-CD-dithiocarbamate) to form a SAM on silver rods to be used as selective electrodes for the identification of isomers [43]. In order to prepare such electrodes it was initially necessary to confirm the chemisorption of dithiocarbamates on silver electrodes. This test was carried out on a silver electrode chemisorbed with morpholyldithiocarbamate (MorDTC), prepared by immersing the bare silver electrode in a solution of MorDTC overnight. Once complete chemisorption of MorDTC was confirmed, the bare electrode was immersed overnight in a solution of poly-6-deoxy-CD-dithiocarbamate. The electrode was then submitted to the same process but using a MorDTC solution in order to seal the free spaces. Since morDTC is ionic, it cannot be included in the CD cavity, leaving that area of the electrode free.

When the double modified electrode was immersed in a solution of $K_3[Fe(CN)_6]$ containing cyclohexanol or adamantane the peaks of the iron(III) electroactive probe disappeared, due to the competing inclusion process. This experiment demonstrates the SAM properties of the double modified electrode. This electrode was used over several weeks with good reproducibility. Similar results were obtained from one electrode to another.

The molecular recognition properties of the SAM electrode were studied and used to discriminate between the positional isomers of the nitrobenzoate anion and nitrophenol. For these nitro compounds the *ortho* isomer does not present any reduction peak attributed to the nitro group, whereas the *meta* and *para* isomers do. In the former isomer both functional groups are next to each other provoking a steric effect great enough to avoid the orientation of the nitro group towards the silver surface of the SAM electrode (Fig. 6). Both *meta* and *para* isomers can be included in the β-CD cavity in such a way that the nitro group is susceptible to interaction with the silver surface. According to AM1 semiempirical calculations, the other groups (COO^-, –OH) can form H-bonds with the secondary hydroxy groups of β-CD.

With poly-6-deoxy-αCD-dithiocarbamate results similar to those of the βCD derivative were obtained. The corresponding γCD derivative was not selective to the different positional isomers.

These experiments demonstrate that the obtained double modified silver electrode, chemisorbed with poly-6-deoxy-βCD-dithiocarbamate, behaves as a SAM electrode with molecular recognition properties that allow discrimination between the positional isomers of the nitro compounds. It might be expected that this type of SAM electrode can also recognize positional isomers of other electroactive aromatic and cyclic compounds. Work is in progress in that direction in our laboratory.

After obtaining positive results on the use of SAM electrodes of polysubstituted sulfur-containing CDs we decided to combine these electrodes with proteins conjugated with CDs or conjugated with molecules that are typical guests of CDs, such as adamantane. The idea was to develop devices such as those depicted in Fig. 7.

The immobilization of proteins on electrode surfaces is of theoretical and practical importance [44]. In this relatively new field of research, different strategies have been applied: covalent attachment, physical absorption and film deposition [45–47]. The first two methods require pre-activation of the electrode surface, commonly by forming a SAM of

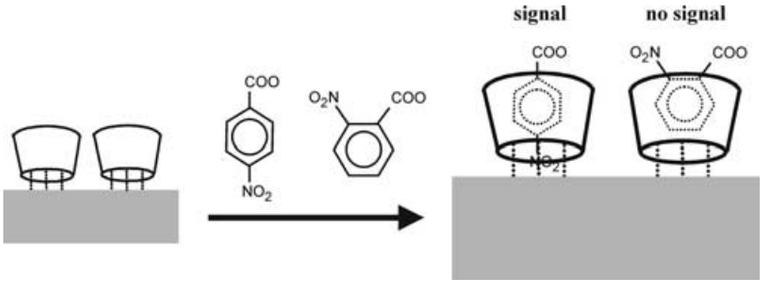

FIGURE 6 Molecular recognition of *p*- and *o*-nitrobenzoate anion by a CD-modified electrode [43].

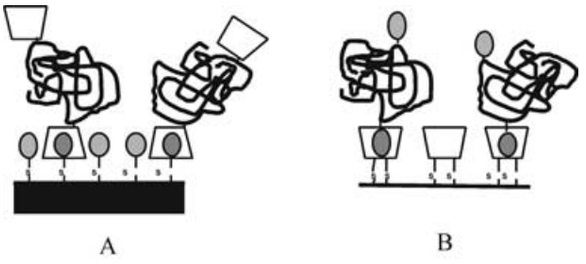

FIGURE 7 Strategies for protein immobilization on electrodes through complementary host–guest interactions: (A) CD–protein conjugates self-assembled on electrodes modified with hydrophobic guests. (B) Guest-modified protein self-assembled on electrodes modified with CD hosts.

a thiocarboxylic acid or cysteamine, depending on the nature of the protein.

Covalent attachment provides stable protein layers and requires chemical activation of the support before protein coupling. Physical adsorption is easy to accomplish and widely applicable but is greatly sensitive to ionic strength and substrate concentration. Finally, protein deposition on redox, electroactive, or ion-exchange polymers or on surfactant films to form bi- or multilayers is also a widely studied alternative.

When a protein is attached to the electrode surface it is important to select an adequate spacer group, which protects against the denaturation of the protein by the electric current. If the free end of the spacer is a sulfur-containing group a SAM can be formed on the electrode surface. To the best of our knowledge little or no attention has been paid to the use of supramolecular chemistry for the formation of SAM of modified proteins on an electrode surface. These supramolecular interactions can be easily tuned by the selection of appropriate host and guest molecules.

The study started by conjugating adamantane to cytochrome c (Cyt c) and then forming an electrode as represented in Fig. 7B. This figure depicts a self-assembled bilayer, where the first monolayer corresponds to polythiolated βCD chemisorbed onto a silver electrode, while the second bilayer comprises Cyt c conjugated with adamantane (Cyt c-A) included in the cyclodextrin cavities. Adamantane constitutes an excellent guest for βCD, with an inclusion constant of about $10^4\,M^{-1}$ [48], which indicates the likelihood of a stable second monolayer.

It must be pointed out that the interfacial inclusion constants of 1-adamantylammonium and 1-adamantanecarboxylate ions at gold electrodes modified with a monolayer of mono-6-(3-mercaptopropionamido)-6-deoxy-β-cyclodextrin have already been reported. *K* values of 1.3×10^4 and $2.4 \times 10^3\,M^{-1}$, respectively, were obtained using impedance spectroscopy [49].

Cyt c, containing a heme group, is a well known electrochemical probe. When it interacts with a metal electrode the protein is rapidly denatured and no signal is observed. However, in the presence of 4,4′-bipyridine or its analogs, reversible signals are observed [50–52]. Our intention was to obtain electrochemically stable Cyt c through conjugation to adamantane (A) able to form the second

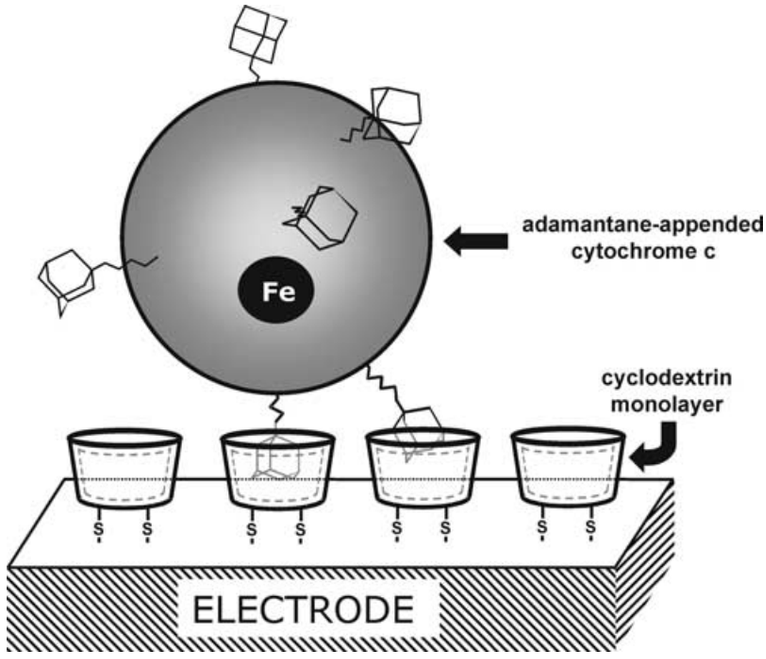

FIGURE 8 An example of the situation represented in Fig. 7b. Immobilization of Cyt c-A on a silver electrode modified with CD hosts [53].

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monolayer. Under such conditions the SAM of Cyt c-A conjugate could perform an efficient long-range ET with the electrode, avoiding its direct contact with the surface, and therefore, protecting the protein.

Cyt c modification with adamantane units was carried out at pH 6 by allowing it to react with an excess of sodium 1-adamantanecarboxylate in the presence of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDAC) as a coupling agent [53]. This method afforded a Cyt c modified through the lysine residues located at the protein surface. The average degree of substitution of Cyt c was estimated as 8.9 by measuring the remaining number of free amino groups using the trinitrobenzenesulfonate method. That means that Cyt c-A contains about 9 adamantane units per protein molecule (47% substitution of Lys residues). As pointed out above, the fact of obtaining 9 mol of adamantane per Cyt c-A should favor an efficient ET due to variations in the donor–acceptor distance.

Cyt c-A was immobilized on the surface of a silver electrode chemisorbed with polythiolated β-cyclodextrin (CDSH) through the formation of host–guest complexes between conjugated adamantane units and the β-CD cavities, as depicted in Fig. 8. The voltammetric response of immobilized Cyt c-A is quasi-reversible with $E_{1/2} \sim 0.18\,V$ (vs. Ag/AgCl). The signal is reproducible and very stable with time and its intensity does not depend on the concentration of Cyt c-A. In fact, when the electrode was immersed in a buffered solution of the conjugate for several minutes, removed, rinsed with buffer and voltammetrically tested, the response was totally reproduced. Addition of competitive guests, such as 1-adamantanol, results in the disappearance of the signal, but only after overnight exposure. These findings suggest that Cyt c-A is strongly included in the CD cavity, self-assembled on the electrode surface, without losing its electroactive properties in a long-range ET.

The positive results obtained with Cyt c-A included on a SAM silver electrode with polythiolated β-CD lead us to expect further applications of similar systems to immobilize electroactive proteins. As has been seen, this novel strategy implies the formation of a self-assembled bilayer on an electrode surface, where the second layer is an electroactive protein. This system permits stabilization of proteins and may allow the use of such devices as biosensors.

CONCLUSIONS

The results presented in this paper give assurance that there remains a wide field for the search of new applications of CD derivatives. New varieties of inclusion device can be developed by modifying CDs, where their polymers seem to play an important role. Especially promising is the conjugation of peptides and proteins to CDs, both for improving their biochemical properties and for their use in the development of new biosensors. This consideration is based not only on the reported results, but also on the fact that peptides, proteins, monoclonal agents, etc. are rapidly playing a pivotal role in the development of new drugs.

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