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Use of β-cyclodextrin as enhancer of ascorbic acid rejection in permselective films for amperometric biosensor applications

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ABSTRACT:

Interference rejection in amperometric biosensors can be more effective introducing some modifiers during electro-deposition of permselective film. Addition of β-cyclodextrin (βCD), a cyclic oligosaccharide composed of seven glucose units, to the *ortho*-phenylendiamine (*o*PD) monomer were already demonstrated to provide an enhancement in ascorbic acid (AA) rejection. Here we evaluated the improvement in permselectivity of poly-eugenol and poly-magnolol films electro-polymerized in presence of different amounts of βCD or eugenol-βCD inclusion complex for amperometric biosensor application. Starting from Pt-Ir wire as transducer several microsensors were covered with polymeric films doped with βCD-based modifiers through constant potential amperometry. Characterization of modified polymers was achieved by scanning electron microscopy and permselectivity analysis. Poly-magnolol film in combination with βCD showed a worsening in permselectivity compared to poly-magnolol alone. In contrast, the introduction of βCD-based modifier enhanced the interference rejection toward the archetypal interferent AA, while slightly affecting permeability toward H_2O_2 compared to the poly-eugenol without modifier. The AA rejection seems to be influenced by the availability of βCD cavity as well as film performance due to concentration of βCD-Eugenol inclusion complex. A poly-eugenol film co-polymerized with 2 mM βCD-eugenol inclusion complex showed a permselectivity equal to poly-

*ortho*phenylendiamine film (PPD), with a lower permeability to AA, likely to be related with a self-blocking mechanism. Based on these results, a biosensor for glutamate was constructed with a poly-eugenol doped with βCD-eugenol as permselective layer and its permselectivity, stability and lifetime were determined.

Keywords: electro-polymerization, co-polymerization, eugenol, β-cyclodextrin, inclusion complex, self-blocking.

Biosensor is a versatile analytical technology whose fast responses and adaptability made it suitable for applications in many diverse areas: environmental monitoring, clinical diagnostics, food analysis, and industrial process control [1]. Many efforts are made worldwide in order to improve the efficiency of this technology, resulting in a growing number of publications of newly-designed biosensors [2].

The developing of a sensitive and a selective biosensor is based on the correct choice of the biological element and an efficient interference rejection. In the first generation enzymatic biosensors the oxygen-dependent activity of oxidases is exploited [3]. The electrochemical pathway leading to a measurable electrochemical signal from the presence of an analyte often involves several reactions. As an example, in a glutamate biosensor design [4] a glutamate oxidase (GluOx) through a series of redox reactions and the presence of redox cofactor flavin adenine dinucleotide (FAD) (reactions 1-2) leads to a hydrogen peroxide production:

$$\text{l-Glutamate} + H_2O + \text{GluOx/FAD} \rightarrow \alpha\text{-ketoglutarate} + NH_3 + \text{GluOx/FADH}_2 \quad (1)$$

$$\text{GluOx/FADH}_2 + O_2 \rightarrow \text{GluOx/FAD} + H_2O_2 \quad (2)$$

The H_2O_2 produced by the enzyme is proportional to glutamate concentration and can be efficiently oxidized (reaction 3) by applying a relatively high potential (approximately +700 mV *vs* Ag /AgCl) on the surface of a platinum electrode (transducer) due to electro-catalytic properties of metal oxides present on the surface [5]:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

The amperometrical detection of H_2O_2 starts at an applied potential of 0.4 V *vs* Ag/AgCl reference electrode [6]. Unfortunately, the optimum potential for H_2O_2 detection of 0.7 V is prone to interference. The electrochemical interference is due to the presence of other molecules that may undergo oxidation during the analysis, hence undermining biosensor reliability [3]. Depending on the nature of the analytical matrix and the applied potential a lot of interferents can be found (e.g., ascorbic acid, uric acid, dopamine, phenols, *etc.*). One of the most ubiquitous and abundant electrochemical interferent species for

oxidase-based biosensor is ascorbic acid (AA), that is naturally occurring in human and animal tissues and fluids, fruit and vegetables as vitamin C or as food additive (E300) [6]. Analytical performances of a glutamate biosensor can be seriously impaired by the presence of AA because glutamate often coexists in the same matrix [7]. In order to minimize interference in electrochemical biosensors various methods have been reported [8,9]. Membranes that actually improve biosensor performances prevent interferent reaching the electrode and let H_2O_2 permeate the permselective layer; the highly selective differential transport properties may depend on charge (e.g. Nafion) [10], size exclusion, polymer thickness [11] and hydrophobic/hydrophilic balance of polymeric film [12]. Numerous permselective films have been proposed for AA exclusion: among these polymers, those derived from phenylenediamines (PDs) are the most studied and effective [13].

Improving permselective properties can be achieved by modifying electro-polymerization conditions as background electrolyte [14,15], electro-polymerization potential [16,17], electro-polymerization technique [18]. Also, incorporation of modifiers into the film may influence permselectivity. A number of proteins and modifiers added to *ortho*-phenylenediamine (*o*PD) during the polymerization process led to a change in permselectivity. Among these modifiers, globular (e.g. Bovine Serum Albumine, BSA) or fibrous (e.g., caseine, gelatin) proteins, and oligo-saccharides (e.g. cyclodextrines, CD) have been reported to enhance the poly-*ortho*phenylendiamine (PPD) permselectivity [19,11].

*o*PD can well interact with βCD [20] with a defined orientation in the cavity [21]: this interaction can help electro-polymerization and contribute to permselectivity enhancement of βCD-modified PPD film [19].

βCD can help biosensors sensibility and selectivity in many ways [22,23]. The use of βCD attracted considerable attention because of its capacity to interact with a large number of molecules, forming stable inclusion complexes, helping water solubilisation of hydrophobic compound dispersion and preconcentration of the analyte. Also, βCD is virtually non toxic, inexpensive, biodegradable and easy-functionalizable. The versatility of this toroid-shaped macrocycle is due to its amphipathic nature: βCD possesses an external hydrophilic and an hydrophobic inner cavity that can guest a large number of compounds of appropriate size and functional groups [24].

A stable interaction between βCD and a monomer can lead to supramolacular recognition conferring a more defined structure to the assembling polymer [25]. As a result, some βCD molecules can be simply embedded into the growing film, without the use of other chemicals or additional steps.

Eugenol [26-29] and magnolol [16,18] are two natural compounds effective in biosensing. Taking into account the sustainable properties of βCD and the high affinity that 2-methoxy phenols have with the

cavity [30], the use of a βCD-2-methoxy phenol complex offers a new approach in designing and building-up more sustainable and performing biosensors.

In this work, βCD and βCD-eugenol inclusion complex were used as modifiers of poly-magnolol and poly-eugenol films in order to improve permselectivity and ascorbic acid (AA) rejection in oxidase based biosensors. Due to the high affinity of AA to the βCD cavity [11], the presence of the macrocycle in a biofilm could improve permselectivity towards H_2O_2.

Stability and permselectivity of modified eugenol and magnolol films were evaluated at day 1 and 8 after electro-deposition and characterized by scanning electron microscopy (SEM). In order to assess enzyme compatibility, a glutamate-based biosensor was constructed with the best performing film.

MATERIALS AND METHODS

Chemicals and solutions

All chemicals were analytical reagent grade or higher purity and dissolved in bidistilled deionized water (MilliQ®). Ascorbic acid (AA), hydrogen peroxide (H_2O_2), l-glutamate, glutamate oxidase (GluOx) EC 1.4.3.11, 200 U mL^{-1}, Yamasa Corp., Japan, bovine serum albumin (BSA), *o*-phenylenediamine (*o*PD), glutaraldehyde (GA), eugenol (>98%), sodium hydroxide (NaOH), hydrochloric acid (HCl) were purchased from Sigma-Aldrich (Milano, Italy). Magnolol was purchased from Chemos GmbH (Regenstauf, Germany). βCD (CAVAMAX7 PHARMA) was obtained from Wacker Chemie Italia (Peschiera Borromeo, Italy).

Synthesis of βCD-eugenol inclusion complex (host:guest ratio 1.5:1) and NMR measurements and description of reagents and guests were reported in Supplementary Material (SM).

The phosphate-buffered saline (PBS, 50 mM) solution was prepared using 0.15 M NaCl, 0.04 M NaH_2PO_4 and 0.04 M NaOH from Sigma-Aldrich and then adjusted to pH 7.4. GluOx solution was prepared by dissolving 200 units of enzyme in 10 µL of PBS and stored at -20 °C. The *o*PD monomer (300 mM) was dissolved in PBS, whereas eugenol (monomer, 10 mM) and magnolol (10 mM) were dissolved in NaOH (100 mM) immediately before use. Different amounts of modifier were added to monomer solution as listed in Table 1. Stock solutions of H_2O_2 (100 mM) and AA (100 mM) were prepared in water immediately before use, while stock solution of glutamate (1 M) was prepared in water. Solutions were kept at 4 °C when not in use. All *in-vitro* calibrations were performed using fresh solutions under standard conditions of pressure and temperature. GA (0.5 % w/v), and BSA (2% w/v) solutions were prepared in bidistilled water. Teflon-coated platinum (90% Pt, 10% Ir; Ø = 125 µm) was purchased from Advent Research Materials (Eynsham, England).

Platinum microsensors and glutamate biosensor construction

All the working electrodes were prepared by removing the Teflon® insulation from the platinum wire in order to expose 1 mm of bare metal. Electropolymerization and calibration were made using a four-channel equipment (eDAQ QuadStat, e-Corder 410, eDAQ, Australia), Ag/AgCl as reference electrode (RE) and a length of stainless steel needle as auxiliary electrode (AE). The electro-deposition of the polymeric layers was performed by constant potential amperometry (CPA) in 0.1 M NaOH (pH=12.85) containing 10 mM of phenol (Ciszewski, G. Milczarek 2001) [12]. *o*PD (10 mM) was dissolved in PBS (pH 7.4) as previously described in literature [9].

The applied potential for the electropolymerization of phenols (10 mM) was fixed at +263 mV for eugenol and at +170 mV for magnolol [16] and at +0.7 V for *o*PD (300 mM) [9].

Among the microsensors studied, the most promising in terms of H_2O_2 permselectivity was selected as the transducer for glutamate biosensor construction. The preparation of the glutamate biosensor consisted of dipping (5 times) a working electrode (previously electro-coated with the specific monomer and modifier) in a solution of GluOx and PEI (1%) and let it dry for 5 minutes after each dip. The final enzyme-containing net was made by dipping the biosensor in BSA (2%) and GA (0.5 %) solutions to promote the cross-linking and the immobilization of the enzyme.

Microsensor and biosensor in vitro characterization

Permselectivity studies were conducted at day 1 and 8 after construction in 20 mL PBS at room temperature. A constant potential of +0.7 V was applied and a calibration was performed after a period of stabilization. The currents generated by different concentrations of H_2O_2 (0-1000 µM) and AA (0-1000 µM) were recorded for bare Pt electrodes, microsensors (obtained with different phenols). Calibration with glutamate was performed on glutamate biosensor in order to investigate biosensor performance (K_M, V_{MAX}, linear region slope, AA blocking). Separate group of sensors were used for scanning electron microscopy (SEM) studies at day 1 after polymerization to evaluate surface changes.

Statistical analysis

H_2O_2 and AA concentrations were expressed in µM, while glutamate concentrations were given as mM. Oxidation currents (*I*) were expressed in nanoampère (nA) and given as baseline-subtracted values ± standard error of the mean (SEM). The AA ΔI value represents the difference between the current resulting from the injection of 1 mM and 0.5 mM of AA in the electrochemical cell [31]. The percent permselectivity (*S%*), Eq. (1) of H_2O_2 *versus* AA [(AA)*S%*] was calculated after calibrations by using the following equations [32]:

$$(\mathrm{AA})S\% = \frac{I\text{-AA (at 1 mM) at Pt/polymer}}{I\text{-}H_2O_2\text{ (1 mM) at Pt/polymer}} \times 100 \qquad (1)$$

Where I-AA and I-H_2O_2 means current intensity (nA) registered for AA and hydrogen peroxide. Because a variety of physicochemical parameters of the electro-deposited polymers, such as the thickness through which the molecules permeate and their corresponding partition coefficients, are unknown, *P%* is an apparent permeability and according to the published theory [17] percent apparent permeability (*P%*) was calculated as:

$$(H_2O_2)P\% = \frac{I\text{-}H_2O_2\text{ (slope)at Pt/polymer}}{I\text{-}H_2O_2\text{ (slope)at bare Pt}} \times 100 \qquad (2)$$

$$(\mathrm{AA})P\% = \frac{I\text{-AA (at 1 mM) at Pt/polymer}}{I\text{-AA (at 1 mM)at bare Pt}} \times 100 \qquad (3)$$

Statistical significance ($p < 0.05$) between groups was evaluated by calculating unpaired t-test, while differences within groups were evaluated by paired t-test.

RESULTS AND DISCUSSION

Polymerization of magnolol in the presence of βCD

Some fluorescence spectrometry studies [33] show a moderate affinity in PBS between βCD and magnolol. Several methodologies were applied in our laboratory to obtain a βCD-magnolol inclusion complex, unfortunately without success. Electro-depositions of magnolol with different concentrations of native βCD as modifier were performed. Permselectivities (AA)*S%* of poly-magnolol in the presence of 0.2 and 2 mM of βCD at day 1 (1.20 ± 0.27 and 1.28 ± 0.31, respectively) showed no statistical difference. A poly-magnolol film without modifier exhibited far better performances [(AA)*S%*=0.13 ± 0.02]. Also, the permeability toward interferent was negatively affected by the presence of modifier [0.2 mM (AA)*P%* =0.62 ± 0.18; 2 mM (AA)*P%* =0.64 ± 0.16] when compared to poly-magnolol film at day 1 [(AA)*P%*= 0.05 ± 0.01].

The high permeability of poly-magnolol polymerized with βCD to ascorbic acid suggests that this polymer has a relatively open structure compared with poly-magnolol lacking in βCD which is less permeable to the interferent.

Polymerization of eugenol with βCD or βCD-Eugenol complex

The CPA electro-polymerization was carried out using different concentrations of βCD or a βCD-eugenol inclusion complex as modifying agents (Table 1). βCD activated supramolecular recognition upon increasing its concentration (3-12 mM) and forming inclusion complexes with suitable hydrophobic compounds (e.g., eugenol) [34]. High performance was reached when βCD or a βCD-eugenol inclusion complex were used at 4 mM and 5 mM, respectively, likely due to a stoichiometry optimum for self-assembling of components.

Cyclic voltammograms of βCD did not show any peak under our experimental conditions, therefore βCD was considered as electro-chemically inert (Figure S1, Supplementary Material). The currents recorded during electrosynthesis of eugenol with or without modifiers where analyzed by GraphPad Prism software in order to asses if an exponential decay model would fit. The non-linear regression analysis found that currents collapsed in an exponential mode as reported for the non conductive polymer of oPD obtained in CPA [17] Except for protocol 1, the comparison between two different exponential decay mode (1-phase versus 2-phase) find out a 2-phase exponential decay as preferred model. In Table S-1 of supplementary material are reported two half-lives (HL), one Fast and one Slow for the 2-phase model and a single HL for 1-phase model. The slow HLs are from 30 to 85 times slower than the fast ones. Which means that the major part of monomer deposition happens during the very first second of polymerization time. As can be seen in Figure S-1 in supplementary material, polymerizations of eugenol without modifier (protocol 0) or in presence of βCD inclusion complex (protocols 5-8) show no statistical difference in HL fast, while only protocols 5 and 8 both shows an increased HL slow. *Viceversa* when native βCD was used as modifier HLs fast of protocol 2 and 4 where statistically lower than HL fast of electro-polymerization of eugenol alone. The behavior of HF fast can suggest that native βCD can influence polymerization in the very early time of polymerization process (first 0.150 sec); the effect of βCD inclusion complex happens 10 to 30 seconds later. This delay can be probably due to Host-Guest dissociation constant between βCD and the included eugenol.

^{1}H NMR spectra show the real formation of βCD-eugenol inclusion complex achieved in neutral conditions, calculated in 1.5:1 host:guest ratio; ^{1}H NMR analysis suggests that eugenol phenolic ring is included into the cavity, in a similar way for an 1:1 βCD-eugenol complex described in literature [35]. Noteworthy, polymerizations where performed in the presence of free monomer (10 mM) and in a large excess of NaOH. In such strong alkaline buffer eugenol undergoes deprotonation of phenyl-OH groups. Aiming to identify the role of βCD in the polymerization process, NMR spectra of βCD, βCD-eugenol complex and eugenol were performed in excess of NaOH. In aqueous solution, the formation of βCD complex is an equilibrium process where a free molecule of eugenol is in dynamic equilibrium with guest eugenol present in the cavity. In NMR spectra a re-organization of inclusion complex was ob-

served. During the first 7-10 minutes after the addition of a concentrate solution of NaOH, deprotonation of eugenol and contemporary interactions between NaOH with the outside cavity occur. Nevertheless, eugenol in solution would be oriented during the release from βCD cavity providing a more organized film compared to that lacking in βCD-eugenol complex.

It cannot be ruled out that the supramolecular recognition of CDs may promote the embedding of βCD into the growing film, as an increase in AA permselectivity was indeed calculated. Moreover, the high affinity of AA toward the cavity of βCD [36-38] could enhance this effect by a self-rejection mechanism. The AA-rejection may depend on the availability of βCD cavities entrapped into the film: free cavities of βCD can be saturated by ascorbic acid and then the negative charge of entrapped AA repulse other AA from the electrode (as seen for PPD) [11]. NMR shifting of external βCD protons may be attributed to a strong interaction with NaOH [39,40] and a non-inclusion complex formation with phenoxide anion [34].

Summarizing, the inclusion of βCD can affect the resulting polymeric features by two distinct behaviors that evidence a "directing" and "trapping" role of βCD, respectively. βCD can release eugenol directly to the electrode in a precise spatial orientation. After polymerization, the βCD embedded into the polymer matrix can carry out the interference rejection acting as a trap for AA. This kind of βCD-mediated interactions may affect both polymeric texture/structure and permeability of the film.

SEM study of polymeric films

The morphology of the surface of the permselective sensors at day 8 were observed under SEM (Figure 1).

Poly-eugenol (Figure 1, photo 0) exhibited a spongy and compact surface, while modified poly-eugenol films (Figure 1, photos 1-8, excluding photo 4) showed a rough and granular surface (cabbage-like) particularly pronounced upon sample 6 (Figure 1, photo 6). βCD-modified films shows a relatively flatter surface with a lower level of agglomeration (Figure 1, photos 1,3,4) in comparison to βCD-Eugenol inclusion complex modified film (Figure 1, part 5-8). The "directing" role of βCD can explain the different patterns observed in Figure 1. Most likely the phenolic ring of eugenol is enclosed into the cavity in a such a way that the allyl chain protrudes from βCD rim and can interact with Pt [12,41], properly orienting the complex. Under our experimental conditions (100 mM NaOH) NMR studies evidenced a time-dependent evolution of interactions between βCD-eugenol complex and deprotonated eugenol. During the time course of electro-polymerization, different eugenol forms may co-exist in buffer solution: deprotonated eugenol, eugenol complexed in βCD and, in a less extent, free eugenol. Different concentration of βCD-eugenol complex or free βCD added immediately before the electro-

polymerization is the main responsible for the different three-dimensional texture achieved (Figure 1, compare 1-5 to 5-8). The irregular surface in photo 8 of Figure 1 can be related to an increase in disorder of the solution due to the high βCD-eugenol inclusion complex concentration used: the high modifier concentration may have favored supramolecular recognition.

Although the small rounded-squared formations present in photos 2 and 7 in Figure 1 indicated by a red arrow have shape and size compatible with other βCD aggregates observed in aqueous solution [25, 34] our strong alkaline solution (pH >12.0) can prevent aggregate formation [39,40]. Also the poly-*o*PD (PPD) film was characterized by SEM (Figure 1, photo C) and resulted in a quite compact and smooth surface, confirming previous observations [9].

Sensor permeability and permselectivity studies at day 1

Table S-2 (Supplementary Material) summarizes the results concerning the electrochemical studies performed on day 1 on the new polymers in comparison with PPD. The parameters investigated were: permeability towards hydrogen peroxide and ascorbic acid [(H_2O_2)*P%* and (AA)*P%*] and permselectivity (AA/H_2O_2)*S%*. As can be seen in Figure 2 (part B) H_2O_2 permeabilities of poly-eugenol and modified poly-eugenol where significative lower than PPD films, likely to be related with compactness of the polymer. All the studied polymers showed a good hydrogen peroxide linearity with R^2 comprised between 0.992 and 0.999. In contrast to what was found for PPD [11,19], βCD concentration affects H_2O_2 permeability of poly-eugenol film.

Protocols 4-7 have lower permeabilities toward the reporter molecule compared to poly-eugenol (protocol 0) and protocols 1, 2, 3 and 8. It is reasonable to consider the "directing" role of βCD that would enhance compactness of the film resulting in a moderate loss in permeability. Moreover, βCD inclusion complex can exert the directing role starting even at low concentrations of βCD. In spite of the loss in H_2O_2 permeability (Figure 2, part B) protocols 2-7 are as permselective as PPD ($p>0.05$) (Figure 2, part A), among these protocol 5 has a border-line *P* value (0.069), when analysed with Mann Whitney test (a more robust test than unpaired t test) PPD(AA/H_2O_2)*S%* was better than 5 (*P* value=0.0357). The increase in permselectivity relies in the very low permeability toward ascorbic acid (Figure 2, part C) likely due to the trapping role played by the embedded βCD. Poly-eugenol modified with free βCD shows that AA permeability decreases with increasing modifier concentration (Figure 2, part C, protocols 1-4), meanwhile polymer embedded with βCD-eugenol complex shows an U behavior, with the lowest permeability at 0.2 and 2 mM of complex (respectively, protocols 6 and 7 in Figure 2). Higher AA permeabilities was observed for 0.05 and 5 mM (respectively, protocols 5 and 8). As result, AA is prevented to

reach the electrode by a self-blocking mechanism facilitated by the presence of appropriate concentration of βCD that traps AA preventing its oxidation at the electrode.

AA calibrations of the modified poly-eugenol films are reported in Figure 3: the presence of modifier increases AA rejection of poly-eugenol film (Figure 3, straight black line vs colored lines); registered currents did not increase at increasing concentrations of AA (rather decrease at higher concentrations), suggesting a self-blocking mechanism in which the interaction between AA and macrocycle embedded into the film plays a crucial role [11]. During the first injection of calibrations, available βCDs are saturated by AA and a negligible current was registered; in the subsequent injections the overall negative charge of entrapped AA can repulse other AA, resulting in constant or decreased registered currents (Figure 3). Self-blocking capability seems to be more efficient for βCD than for βCD-eugenol complex (compare part A and B of Figure 4); repulsions may depend on the availability of free cavities, which in the complex are partially occupied by eugenol or its phenoxide anion. A role played by the external surface of macrocycle is not to be excluded together with the stronger and more defined interaction between βCD-eugenol inclusion complex and the growing film.

Aging studies on the permselectivity of polymeric films

Table S-2 (Supplementary Material) and Figure 5 summarize the results from electrochemical studies with the new polymers compared to the standard PPD after 8 days.

PPD permselectivity was relatively constant (0.16 ± 0.03 on day 1; 0.24 ± 0.01 on day 8), whereas modified poly-eugenol films declined their performance. On the contrary of what happened to poly-eugenol film (which significantly improved its performance from day 1 to 8 ($p<0.05$) when modifiers where added polymeric films displayed a general loss in permselectivity ($p<0.05$ *vs* day 1), no matter if βCD or βCD-eugenol complex was used. An explanation of this involves the high three-dimensional size of modifier: the macrocycle can occupy a larger surface of transducer, decreasing the contact between monomer and transducer.

This depletion of anchoring surface can lead to less time-resistant polymeric films. Eugenol may present two interaction modes with Pt surface: with phenolic ring and with allylic side chain [12,41]. Both these parts of eugenol can be included into βCD, decreasing the anchorage between monomer and transducer. Hence, under the studied experimental conditions, the use of the modified polymers as a high performing permselective films is suitable for short-living use.

Glutamate biosensor characterization

Based on the electrochemical results, a glutamate biosensor was constructed with poly-eugenol 2 mM βCD-eugenol inclusion complex by CPA. *In vitro* sensitivity of the glutamate biosensor (Figure 5) has been determined by injecting in the electrochemical cell known amounts of glutamate (ranging from 0 to 50 mM).

The calibration curve shows a classical Michaelis-Menten kinetics, with R^2= 0.97 (n = 4), V_{max}=164 ± 5 nA and K_M = 1.4 ± 0.2 mM. The linear region slope was evaluated by considering concentrations included between 0 and 1 mM, with R^2 = 0.99 (n=4) and a slope of 63 ± 2. nA mM^{-1}. To evaluate the shielding effect of poly-eugenol modified with 2 mM βCD-Eugenol inclusion complex towards potentially interfering molecules such as ascorbic acid (AA) calibration was carried out with AA (within a 0 - 1000 μM range). Based on these calibrations a Δ*I* AA parameter was calculated as the following equation:

$$\Delta I \text{ AA} = (I\text{-AA at 1 mM}) - (I\text{-AA at 0.5 mM}) \qquad (4)$$

Where Δ*I* AA is the difference between the current produced by injection of 1 mM AA and the current produced by 0.5 mM AA. The negligible Δ*I* AA (Δ*I* AA = -0.95 nA) and the satisfactory values of V_{max} and K_M make this biosensor design suitable for glutamate sensing even at high interferent concentration.

CONCLUSIONS

Polymeric films derived from magnolol and eugenol were modified by adding βCD or βCD complex during electro-deposition in CPA. The interaction of monomer with βCD influenced the permselectivity of the modified polymer. The difficulty of magnolol to interact with βCD negatively affected the formation of a performing film. On the other hand, the favourable interaction between eugenol and βCD considerably improved permselectivity of modified poly-eugenol. The role played by βCD can be described as “directing” and “trapping”. βCD interactions activate supramolecular recognition directing the auto-assembling of the growing film into a tridimensional structure as can be seen in SEM pictures. Permselectivity studies evidenced that the trapping role played by βCD embedded into the polymer was extremely efficient in AA rejection. The very low permeability to AA and the good permeability to hydrogen peroxide of the poly-eugenol film polymerized with 2 mM βCD-Eugenol was successfully integrated in a glutamate biosensor design. Microsensors studies along with biosensor calibrations clearly show how the introduction of βCD or βCD-eugenol inclusion complex as modifier can offer a high performing and sustainable way to enhance biosensors performances for short-living use.

ASSOCIATED CONTENT

Supplementary Material

The Supplementary Material is available free of charge on the Website.

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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Figure 1. Scanning electron micrographs of the active surface of a sensor (A), bare Platinum (B), poly-phenylendiamine (C) and poly-eugenol electro-synthetized without modifier (0), with βCD (1-4) and βCD-eugenol complex (5-8).

Figure 2. Selectivity [(AA)*S%*] and permeability toward H_2O_2 [(H_2O_2)*P%*] and ascorbic acid [(AA)*P%*] for poly-eugenol sensors electro-synthetized without modifier (yellow bar), with βCD (blue bars, 1-4) and βCD-eugenol complex (green bars, 5-8) compared with values for poly-*ortho*phenylendiamine sensors (PPD, dark bar). Data are shown as value and standard error of the mean (SEM).

Figure 3. Comparison of steady-state ascorbic acid (AA) calibrations carried out amperometrically on a poly-eugenol film without modifier (black lines, 0), added with different concentration of native βCD (part A, blue lines 1-4) and βCD-Eugenol complex (part B, green lines 5-8).

Figure 4. Selectivities [(AA)*S%*] at day 1 (black bars) and at day 8 (grey bars) after polymerization for poly-eugenol sensors electro-synthetized without modifier (0), with βCD (1-4) and βCD-eugenol complex (5-8) compared with values for poly-*ortho*phenylendiamine sensors (PPD). Data are shown as values and standard error of the mean (SEM). An * means a statistically significance between day 1 and 8.

Figure 5. Michaelis-Menten non-linear regression analysis and linear region slope (inset) for glutamate biosensor design: Pt_C/poly-eugenol + β-Cyclodextrin-Eugenol/GluOx + PEI/BSA/GA.

Table 1. List of different conditions of electro-polymerization.

Entry	Monomer	Modifier	Electro-polymerization condition
0	10 mM Eugenol	-	Electro-polymerization[#]
1	10 mM Eugenol	0.1 mM βCD	Co-polymerization*
2	10 mM Eugenol	1 mM βCD	Co-polymerization*
3	10 mM Eugenol	2 mM βCD	Co-polymerization*
4	10 mM Eugenol	4 mM βCD	Co-polymerization*
5	10 mM Eugenol	0.05 mM βCD-Eugenol	Co-polymerization*
6	10 mM Eugenol	0.2 mM βCD-Eugenol	Co-polymerization*
7	10 mM Eugenol	2 mM βCD-Eugenol	Co-polymerization*
8	10 mM Eugenol	5 mM βCD-Eugenol	Co-polymerization*
PPD	300 mM *ortho*-phenylendiamine	-	Electro-polymerization[§]

[#]Electro-polymerization of eugenol in 100 mM NaOH according to Monti et al., [16], +263 mV vs Ag/AgCl;

[*]Co-polymerization of eugenol + modifier in 100 mM NaOH, +263 mV vs Ag/AgCl;

[§]Electro-polymerization of *ortho*-phenylendiamine according to Calia et al., [18], +700 mV vs Ag/AgCl.

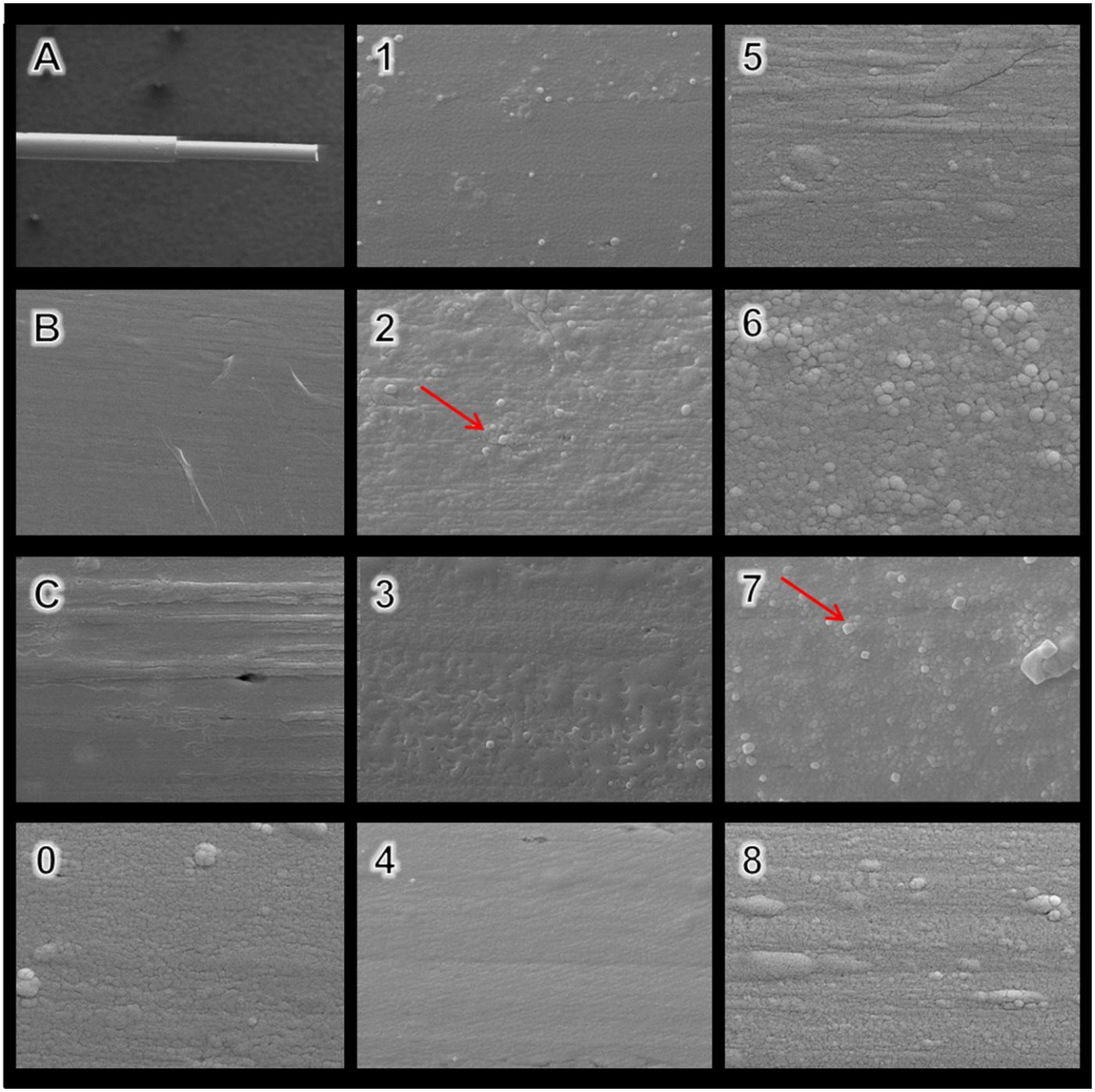
Highlights

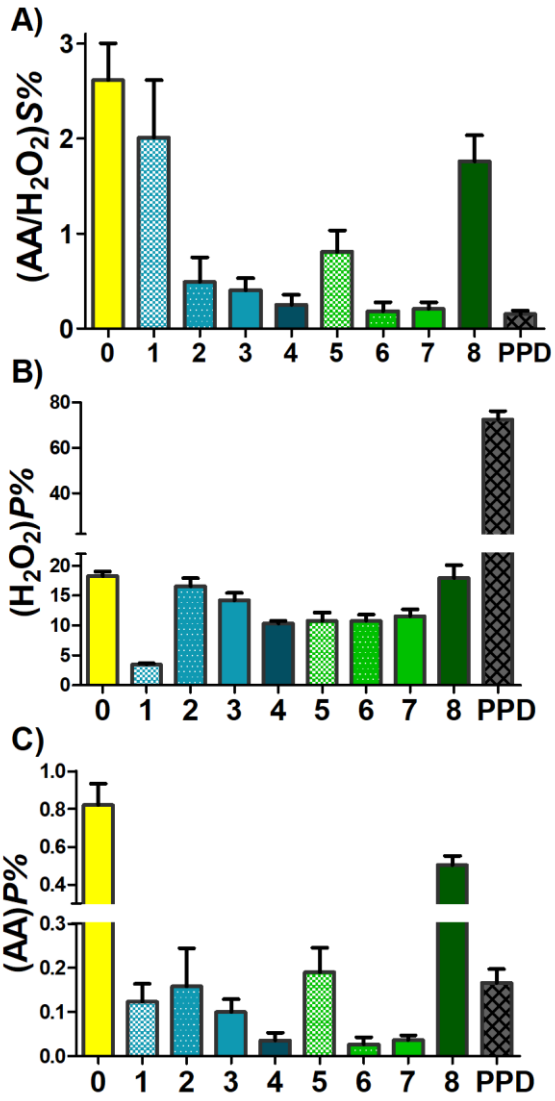
Green biosensors can be designed by using poly-eugenol as the permselective film

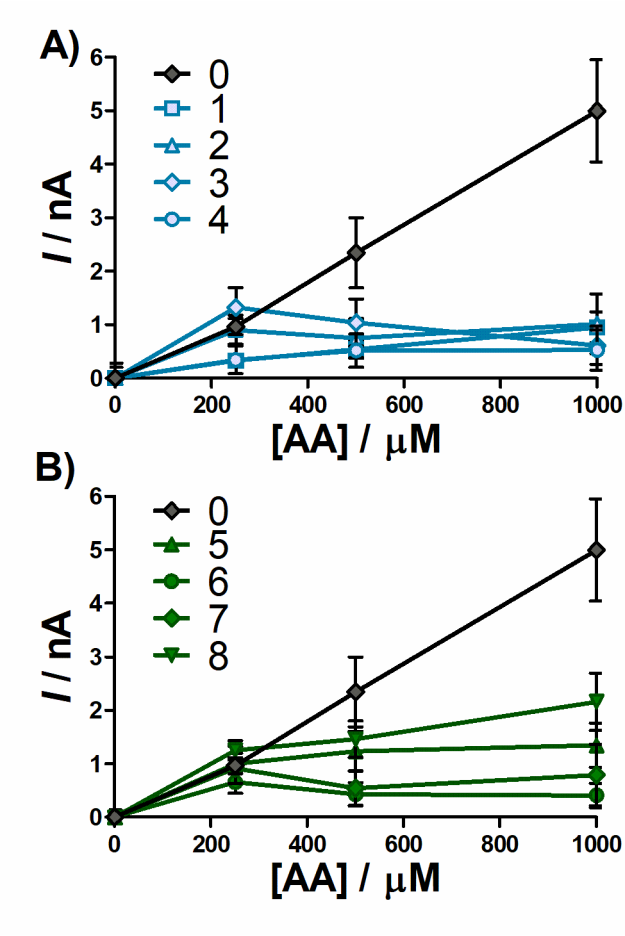
β-cyclodextrin derivatives are proposed as modifiers of poly-eugenol

Interference rejection is enhanced by β-Cyclodextrin use in eugenol polymerization

β -Cyclodextrin use enhances permselective properties of poly-eugenol film






A)
I / nA
[AA] / µM
0
1
2
3
4
B)
I / nA
[AA] / µM
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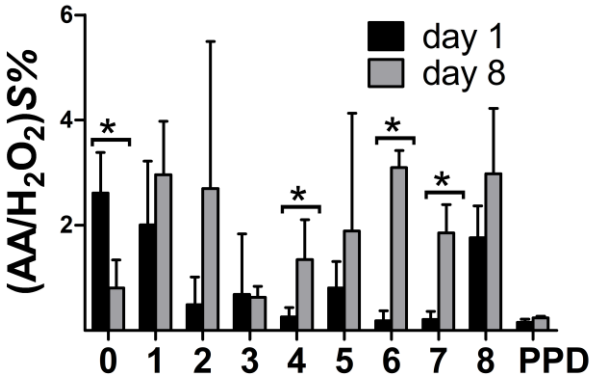
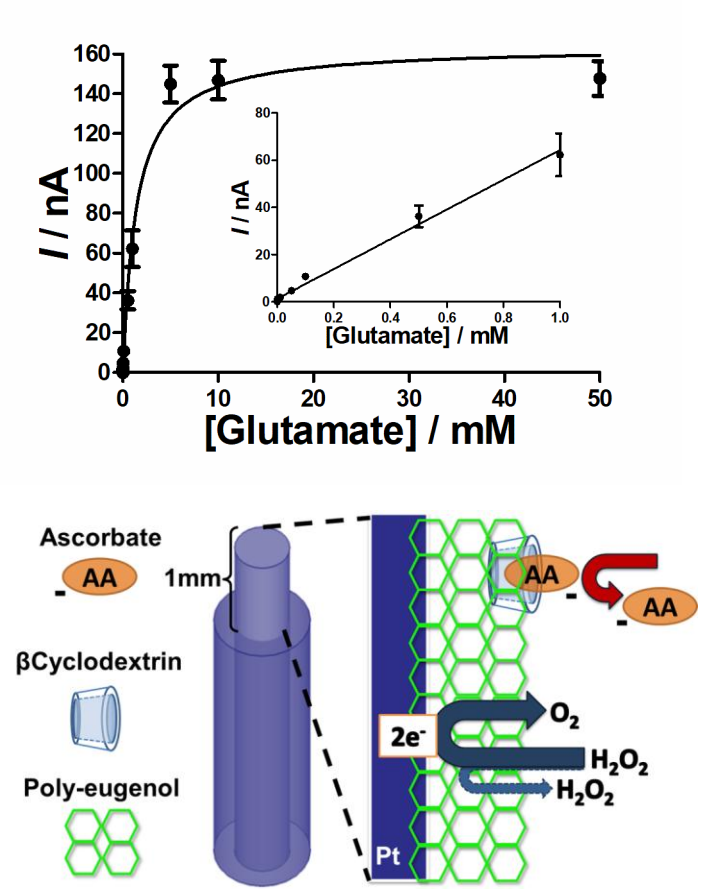
6
7
8


(AA/H2O2)S%
day 1
day 8
0 1 2 3 4 5 6 7 8 PPD



Graphical abstract