Author's Accepted Manuscript

Low electro-synthesis potentials improve permselectivity of polymerized natural phenols in biosensor applications

Patrizia Monti, Giammario Calia, Salvatore Marceddu, Maria A. Dettori, Davide Fabbri, Samir Jaoua, Robert D. O'Neill, Quirico Migheli, Giovanna Delogu, Pier A. Serra

Cite this article as: Patrizia Monti, Giammario Calia, Salvatore Marceddu, Maria A. Dettori, Davide Fabbri, Samir Jaoua, Robert D. O'Neill, Quirico Migheli, Giovanna Delogu and Pier A. Serra, Low electro-synthesis potentials improve permselectivity of polymerized natural phenols in biosensor applications, *Talanta,* http://dx.doi.org/10.1016/j.talanta.2016.10.019

Low electro-synthesis potentials improve permselectivity of polymerized natural phenols in biosensor applications

Patrizia Monti,[a,c] Giammario Calia,[a,b] Salvatore Marceddu,[d] Maria A. Dettori,[c] Davide Fabbri,[c] Samir Jaoua,[e] Robert D. O'Neill,[f] Quirico Migheli,[a] Giovanna Delogu,[c] and Pier A. Serra,[*b]

[a] Dipartimento di Agraria and Unità di Ricerca Istituto Nazionale di Biostrutture e Biosistemi, Università degli Studi di Sassari, Viale Italia 39, I-07100 Sassari, Italy.

[b] Dipartimento di Medicina Clinica e Sperimentale, Università degli Studi di Sassari, Viale S. Pietro 43/b, I-07100 Sassari, Italy.

[c] Istituto CNR di Chimica Biomolecolare - UOS Sassari - Traversa La Crucca 3, I-07100, Sassari, Italy.

[d] Istituto CNR di Scienze delle Produzioni Alimentari - UOS Sassari - Traversa La Crucca 3, I-07100, Sassari, Italy.

[e] Department of Biological & Environmental Sciences, College of Arts and Sciences, Qatar University, P.O. Box: 2713, Doha, Qatar.

[f] UCD School of Chemistry, University College Dublin, Belfield, Dublin 4, Ireland.
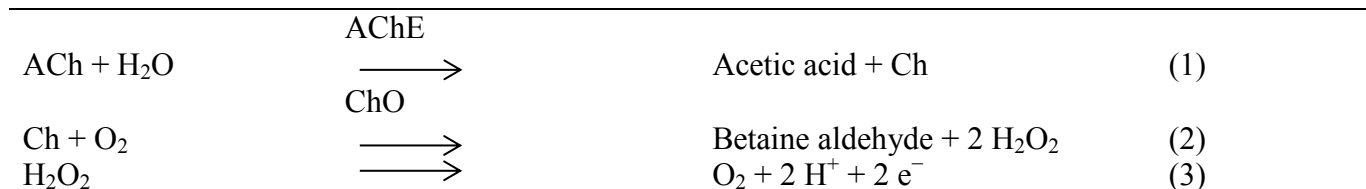
[*]Corresponding Author. paserra@uniss.it

ABSTRACT

First-generation amperometric biosensors are often based on the electro-oxidation of oxidase-generated H_2O_2. At the applied potential used in most studies, other molecules such as ascorbic acid or dopamine can be oxidized. Phenylenediamines are commonly used to avoid this problem: when these compounds are electro-deposited onto the transducer surface in the form of poly-phenylenediamine, a highly selective membrane is formed. Although there is no evidence of toxicity of the resulting polymer, phenylenediamine monomers are considered carcinogenic. An aim of this work was to evaluate the suitability of natural phenols as non-toxic alternatives to the *ortho* isomer of phenylenediamine. Electrosynthesis over Pt-Ir electrodes of 2-methoxy phenols (guaiacol, eugenol and *iso*eugenol), and hydroxylated biphenyls (dehydrodieugenol and magnolol) was achieved. The potentials used in the present study are significantly lower than values commonly applied during electro-polymerization. Polymers were obtained by means of constant potential amperometry, instead of cyclic voltammetry, in order to achieve multiple polymerizations, hence decreasing the time of realization and variability. Permselective properties of natural phenols were significantly improved at low polymerization potentials. Among the tested compounds, *iso*eugenol and magnolol, polymerized respectively at +25 mV and +170 mV against Ag/AgCl reference electrode, proved as permselective as poly-*ortho*-phenylenediamine and may be considered as effective polymeric alternatives. The natural phenol-coated electrodes were stable and responsive throughout 14 days. A biosensor prototype based on acetylcholine esterase and choline oxidase was electro-coated with poly-magnolol in order to evaluate the interference-rejecting properties of the electrosynthesized film in an amperometric biosensor; a moderate decrease in ascorbic acid rejection was observed during *in vitro* calibration of biosensors.

KEY WORDS: amperometry, guaiacol, eugenol, isoeugenol, dehydrodieugenol, magnolol.

INTRODUCTION

Biosensor technology provides easy-to-use, effective devices for medical, environmental and agro-food analysis [1]. These tools can avoid some disadvantages related to conventional analytical methods, such as sample preparation [2-3], high cost, and need for qualified personnel [4]. Amperometric biosensors are often characterized by a simple design and fast kinetics, and because of their very fast response times [5] they can provide essentially real-time analysis. In a first-generation amperometric biosensor the sensing element is often an oxidase, and the signal transduction pathway involves an analyte-related production of H_2O_2 as reporter molecule. For instance, an amperometric biosensor for acetylcholine (ACh) detection is based on two different enzymes: acetylcholine esterase (AChE) and choline oxidase (ChO) [6]. These enzymes catalyze ACh hydrolysis and choline (Ch) oxidation with subsequent production of H_2O_2 (reactions 1 and 2) [7]; the resulting biosensor signal is often due to H_2O_2 electro-oxidation occurring at a platinum surface (reaction 3) at a relatively high applied potential, say +0.7 V versus Ag/AgCl reference electrode (RE).

$$ACh + H_2O \xrightarrow{\text{AChE}} \text{Acetic acid} + Ch \quad (1)$$

$$Ch + O_2 \xrightarrow{\text{ChO}} \text{Betaine aldehyde} + 2\ H_2O_2 \quad (2)$$

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

Since the optimal oxidation of H_2O_2 occurs at relative high potential (>0.4 V versus Ag/AgCl reference electrode [8]), numerous substances may be oxidized on the transducer surface. Electrochemical interference due to the presence of easily oxidized molecules [such as ascorbic acid (AA), uric acid (UA) and dopamine (DA)] can be avoided by the direct electrodeposition of polymeric films on the transducer surface [9-11]. The function of such films is to prevent interference molecules reaching the transducer surface [12]. Many polymeric films are available for AA rejection [13]. Among these polymers, those derived from phenylenediamines (PDs) are the most studied. Several efforts have been reported in order to improve the electrodeposited poly-phenylenediamine (p-PD) films. For example, among the three isomers of PD (*ortho*, *meta* and *para*), the *ortho*-phenylenediamine-derived film has the best permselective performance for brain monitoring [12].

The background electrolyte in which electropolymerization occurs is one factor that can influence permselectivity [14-15]. Another variable that can strongly influence polymerization and permselectivity of electrosynthesized polymers is the applied polymerization potential, as observed for p-PD [16].

In a previous work [17], the naturally occurring compounds eugenol, *iso*eugenol , dehydrodieugenol and magnolol demonstrated their abilities to electropolymerize on a platinum surface by means of relatively high potential (2 V *versus* Ag/AgCl). Direct comparison between a p-PD film obtained by electrodeposition of *ortho*-PD and natural polymers highlighted the need to improve permselective properties of these polyphenols.

As observed by Ciszewski and Milczarek [18] the upper limit potential applied during the electropolymerization of eugenol by means of cyclic voltammetry (CV) can strongly determine the permselectivity properties of the polymer. Molecular size exclusion, ionic interaction and hydrophobic/hydrophilic features of the permeating molecule drive the transport properties of the electro-coated polymer [19]. When eugenol is polymerized (p-eugenol) by CV, analyte/interference selectivity is governed by hydrophobicity of the permeating molecule [18]. Small and non-charged molecules, as H_2O_2 [19-18], NO [18,20] and O_2 [21] can easily permeate p-eugenol. On the other hand, the transit of anionic molecules of approximately the same size such as NO_2^- and larger AA [18-19,21] and UA [18,20] anions is strongly hindered. Cationic DA, a larger molecule compared to H_2O_2 or O_2, can permeate throughout the p-eugenol thanks to its relative hydrophobicity and its difference in charge. Interestingly, DA permeability increases when polymerization potential is extended from 0.8 to 2.0 V 18. The presence of oxidative-generated groups on p-eugenol, attributed to carbonyl and carboxylic type by FT-IR analysis, was suggested to decrease the hydrophobicity of the p-eugenol coating [18]. Beside a hydrophobic interaction also a electrostatic rejection mechanism can be hypothesized: carboxylic acid groups can be ionized, generating negative charges which can reject molecules of the same sign, like AA. Thus p-eugenol showed promising AA-rejection properties for sensor application polymerized by CV [20-25] both at extremely high oxidative potentials (from 0 to 2 V) [17-18] or when a lower range (from 0 until +0.7 V) was applied [26].

Despite the p-eugenol showed promising characteristics for sensor application, it was not as performative as p-PD when a direct comparison was carried out [17]. At the same time, other natural phenols (e.g., magnolol) demonstrated a better performance than p-eugenol [17].

Although the *o*PD is considered the best permselective film for AA rejection it has the disadvantage of being an *in vitro* mutagen [27] and an *in vivo* carcinogen agent in rats and mice [28]. Although there is no scientific evidence of its carcinogenicity in human, this monomer could be considered potentially dangerous for health.

In order to offer a natural and equally effective alternative to the *o*PD this work aimed to improve perm-selectivity of phenols derived-films by applying lower electropolymerization potentials. A direct comparison was performed in order to asses which polymers derived from natural phenols have a better permselectivity compared to *o*PD. Taking into account that the potential applied can greatly influence permselectivity of phenol-derived films, the present investigation explored the effect of lower applied potentials in the electropolymerization of the 2-methoxylphenols guaiacol, *iso*eugenol and eugenol, and the symmetric dimers dehydrodieugenol and magnolol, the latter two being hydroxylated biphenyls. All these molecules, belonging to the natural pool, are electro-polymerizable monomers that electro-deposit on platinum by CV or constant potential amperometry (CPA) [17]. These naturally occurring compounds are less toxic than *ortho*-PD and exert hydrophobic interactions; moreover, the biphenyl structure of dehydrodieugenol and magnolol can facilitate the interaction between the film and the biological part of biosensor [29]. We decided to achieve polymerization by means of CPA, in order to apply a unique, well-defined fixed potential during polymerization. This technique also permits the polymerization of four electrodes at once, hence saving time, materials and increasing reproducibility. Additionally, the best performing coating was used as the permselective film in the construction of a ACh biosensor based on AChE and ChO.

MATERIAL AND METHODS

Chemicals and solutions
All chemicals were analytical reagent grade or higher purity and dissolved in bidistilled deionized water (MilliQ®). Acetylcholine (Ach), choline (Ch), AA, DA, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), polyethyleneimine (PEI), guaiacol, acetone, eugenol (>98%), sodium hydroxide (NaOH), hydrochloric acid (HCl) and *iso*eugenol (*cis-trans* mixture) were purchased from Sigma-Aldrich (Milano, Italy). Acetylcholinesterase (AChE,) type VI–S from *Electrophorus electricus*, Eletric eel (EC: 3.1.1.7), and choline oxidase (ChO) from *Alcaligenes species* (EC: 1.1.3.17) were diluted in PBS until 10 U/ml and 1 U/ml, respectively, and stored at -20°C when not in use. Magnolol was purchased from Chemos GmbH (Regenstauf, Germany). The naturally occurring compound dehydrodieugenol was synthesized as described in literature [17]. 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. Guaiacol, eugenol and *iso*eugenol (phenol monomers, 10 mM) and magnolol and dehydrodieugenol (phenol dimers, 10 mM) were dissolved in NaOH (100 mM) immediately before use. Stock solutions of DA (100 mM), H_2O_2 (100 mM) and AA (100 mM) were prepared, respectively, in: bidistilled water, 0.01 M HCl and 0.01 M *ortho*-phosforic acid immediately before use. 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. Teflon-coated platinum (90% Pt, 10% Ir; Ø = 125 μm) and silver wires (Ø = 250 μm) were purchased from Advent Research Materials (Eynsham, England).

Platinum microsensor and AChE/ChO biosensor construction
All the working electrodes were prepared removing the Teflon® insulation from the platinum wires in order to expose 1 mm of bare metal.
CV was performed in 10 mM of the phenols in 0.1 M NaOH (pH=12.85) in order to determine their first oxidation peak, in the potential of 0 to 2 V for 10 cycles at a scan rate of 100 mV s^{-1}. The first oxidation peaks ($1^{st}E_{Ox}$) obtained by CV of the phenols are reported in Figure 1. We applied four different polymerization potentials (applied potential, E_{App}) for each phenol. The relative E_{App} was calculated by setting $1^{st}E_{Ox}$ as 0. In this way we applied a potential lower of 50 mV, and higher of +100 mV and +500 mV, with respect to $1^{st}E_{Ox}$; also an E_{App} of zero corresponding to $1^{st}E_{Ox}$ was included. The corresponding E_{App} referred to Ag/AgCl reference electrode (RE) can be seen in Figure 1. The electro-deposition of the polymeric layers was performed by means of CPA using the same background electrolyte of CV for 15 min.

Electropolymerization and calibration were made using the four-channel equipment (eDAQ QuadStat, e-Corder 410, eDAQ, Australia), Ag/AgCl as RE and a stainless steel wire as auxiliary electrode (AE). The best performing polymer was included in the design of a biosensor based on the two enzymes AChE and ChO. The AChE/ChO biosensor construction started with the adsorption of enzymes on the electropolymerized electrodes by simple dipping. The enzyme mix was obtained from 10 μl of ChO solution (1 U/μL) and 10 μl of AChE (10 U/μL) solution. After few seconds, electrodes were quickly dipped in a 1% w/v solution of polyethyleneimine (PEI). Enzymes-PEI dipping was repeated for 5 times with a drying time of 5 minutes between dips. Then electrodes were dipped into a 2% bovine serum albumin (BSA) solution and a quick dip into a 1% glutaraldehyde solution. The immobilization occurred by placing the electrode at 37°C for 15 minutes. Biosensors were stored at +4°C partially immersed in PBS until use.

Microsensor and biosensor *in vitro* characterization

Permselectivity studies were conducted at day 1 and repeated at day 14 after polymerization in 20 mL PBS (pH 7.4) 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 DA (0-100 μM), H_2O_2 (0-1000 μM) and AA (0-1000 μM) were recorded for bare Pt electrodes and for microsensors coated with different permselective films (obtained by applying different polymerization potentials to the phenolic monomers). Separate group of sensors were used for scanning electron microscopy (SEM) studies at day 14 after polymerization.

Two different sets of AChE/ChO biosensors covered with a permselective film from PD or magnolol were calibrated against ACh (0-2500 μM), Ch (0-500 μM) and H_2O_2 (0-500 μM) in the same experimental conditions as reported above.

Statistical analysis

DA, H_2O_2 and AA concentrations were expressed in μM. Oxidation currents were expressed in nano-ampere (nA) and given as baseline-subtracted values ± standard error of the mean.

According to the theory [16] percent apparent permeability (P%) was calculated as:

$$P_{H_2O_2}\% = \frac{\text{I-}H_2O_2 \text{ (slope)at Pt/polymer}}{\text{I-}H_2O_2 \text{ (slope)at bare Pt}} \times 100 \qquad (4)$$

$$P_{AA}\% = \frac{\text{I-AA (1 mM)at Pt/polymer}}{\text{I-AA (1 mM)at bare Pt}} \times 100 \qquad (5)$$

$$P_{DA}\% = \frac{\text{I-DA (0.1 mM)at Pt/polymer}}{\text{I-DA (0.1 mM)at bare Pt}} \times 100 \qquad (6)$$

Where I-H_2O_2, I-AA and I-DA means current intensity (nA) registered for, respectively, hydrogen peroxide, AA and DA. 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.

The percent permselectivity (S%), Eqs. (7) and (8) of H_2O_2 versus AA ($S_{AA}\%$) or DA ($S_{DA}\%$) was calculated after calibrations by using the following equations [30]:

$$S_{AA}\% = \frac{\text{I-AA (1 mM)at Pt/polymer}}{\text{I-}H_2O_2 \text{ (1 mM)at Pt/polymer}} \times 100 \quad (7)$$

$$S_{DA}\% = \frac{\text{I-DA (1 mM)at Pt/polymer}}{\text{I-}H_2O_2 \text{ (1 mM)at Pt/polymer}} \times 100 \quad (8)$$

AA rejection on biosensor was evaluated calculating ΔI-AA which is the difference between the current produced by injection of 1 mM AA and the current produced by 0.5 mM.

AChE/ChO biosensors were calibrated with Ach (0-2500 μM), and a nonlinear fitting (Michaelis−Menten equation) was performed on the entire concentration range in order to evaluate V_{max} and apparent K_M. A further calibration with Ch and H_2O_2 (0-500 μM) was performed on the same biosensors. After calibrations, biosensor currents were plotted versus Ach, Ch or H_2O_2 concentrations and linear regressions (slope) were calculated in a range comprised between 0 and 500 μM.

Linear slopes were used to obtain biosensor efficiency (BE%) for ACh (Eq. (9)) and Ch (Eq. (10)) calculated as reported in a previous study [5]:

$$BE_{ACh}\% = \frac{slope\ (ACh)}{slope\ (H_2O_2)} \times 100 \quad (9)$$

$$BE_{Ch}\% = \frac{slope\ (Ch)}{slope\ (H_2O_2)} \times 100 \quad (10)$$

Total Area and Plateau current can be obtained from polymerization curve. Total Area is the area under the curve during polymerization time, and represents a measure of the total electronic exchange (nC); plateau current is the current (nA) registered at the end of polymerization, giving a measure of the monomer permeability throughout the polymeric film.

Statistical significance ($p<0.05$) between groups was evaluated by calculating unpaired t-test. Correlation was evaluated calculating Pearson's coefficient.

RESULTS AND DISCUSSION

Electropolymerization of phenols at high applied potentials
In order to establish whether a low potential during electro-polymerization can improve permselectivity suitable for sensor application each phenol was deposited onto the sensor surface at low and high potentials. The high E_{App} group was obtained as reported by Calia *et al.*, [17] and a further molecule was tested in addition: guaiacol. This molecule is the simplest of the entire phenols set: it contains only the guaiacyl unit, lacking any other substituents on the phenolic ring (Figure 1).

Its electropolymerization has been reported only once [19]. In Table 1 p-guaiacol permselectivities are compared with those of the other phenols, polymerization conditions were applied as described [17]. The permselective properties of phenol itself has been described previously [31] and appears to be unsuitable for first-generation biosensors because of low permeability to the reporter molecule hydrogen peroxide. The $S_{AA}\%$ values for p-guaiacol electro-deposited using CV were significantly better than for p-*iso*eugenol and p-dehydrodieugenol, and in order to avoid the dramatic effect of a highly positive relative E_{App} during CPA, guaiacol has been selected for polymerization at lower potentials.

Electropolymerization of phenols at low applied potentials
As described in the experimental section, we chose the E_{App} in relation to the individual $^{1st}E_{Ox}$ of each phenol and expressed E_{App} relative to $^{1st}E_{Ox}$ rather than the reference electrode (Figure 1). Our purpose was to choose an E_{App} that would reflect the ease of oxidation of each phenol. In fact there was a 238 mV span between the lowest oxidation peak of *iso*eugenol and the highest peak of eugenol (Figure 1). At the relative E_{App} of −50 mV oxidation and polymerization were slow, while at a relative E_{App} of 100 mV reactions were faster, and at +500 mV a further oxidation of polymers can occur. During CPA, polymerization currents showed a very rapid decay; the amount of oxidation current generated by the oxidation of monomers decreased during polymerization due to electrode passivation; at the end of polymerization time a stable, negligible oxidation current (plateau) was reached. Oxidation currents decay indicates a non-conductive polymer deposition for almost all phenols. Interestingly, during polymerization at a relative E_{App} of +500 mV for dehydrodieugenol and eugenol, the initial current decay is followed by an increase in oxidation current; differently from other polymerization a plateau current is not reached (Figure S-1, Supplementary Material).

Effect of lower applied potentials on permeability and permselectivity at day 1

Permselectivity values obtained at high relative E_{App} reported in Table 1 can be compared with Table 2, showing that the the best results were obtained for phenolic molecules by applying lower relative E_{App} values. Permselectivity values were statistically lower (p<0.05) when lower potentials were used, hence significantly improving polymers' performance in AA and DA rejection (Table 1 and Table 2). At low E_{App} permselective membranes that are more suitable for amperometric sensor use can be obtained. Table 2 also highlights better permselectivity performance of phenol-derived film compared with values obtained with p-PD. A low E_{App} the p-magnol coating has the same capacity as the p-PD in rejecting the interfering AA and, at the same time, it has the advantage of a better DA rejection.

Beside a general improvement of permselectivities using low potentials, some other performances can be observed. Notably, the best $S_{DA}\%$ values were obtained at the lowest relative E_{app}; $S_{DA}\%$ deteriorates at increasing potential for guaiacyl-containing molecules such as guaiacol, eugenol, *iso*eugenol and dehydrodieugenol. The permeabilities studies at day one (Figure 2) suggest that the lack of permselectivity towards DA depends to a larger extent on increased apparent permeability for DA ($P_{DA}\%$) than on a decrease in apparent permeability towards H_2O_2. The increase in $P_{DA}\%$ may be due to an increase of the polymer oxidation indicated by the higher Total Area registered during electropolymerization.

In fact, Total Area and $P_{DA}\%$ strongly correlate (Table S-2, Supplementary Material). Ciszewski and Milczarek [18] proposed for p-guaiacol and p-eugenol permeabilities a model based on hydrophobic interactions. The films are able to concentrate the relatively hydrophobic non-protonated DA. Magnolol films do not seem to be influenced by this phenomenon, maintaining quite stable $P_{DA}\%$ (Figure 2). This feature may be due to the different structure of magnolol, bearing a phenolic ring in place of a guaiacyl unit, shared by the other phenols.

The $S_{AA}\%$ behavior seems not so strongly dependent on the relative E_{app}, except for p-eugenol which will be discussed later. Generally, permselectivities towards AA are quite low, indicating a good selective exclusion for this interference species. The most impermeable film towards AA was p-magnolol, having the lowest $P_{AA}\%$ (0.051±0.007) and the best $S_{AA}\%$ at -50 mV. Noteworthy, these values are not significantly different from p-PD (p>0.05). Also at -50 mV p-dehydrodieugenol showed a very good $S_{AA}\%$ (Table 2). Differently from eugenol, *iso*eugenol and guaiacol, when dissolved in a basic medium, dehydrodieugenol does not form a phenoxyl radical in alkaline solution at pH >9 due to high hemolytic bond dissociation energies (BDE) of the phenolic O-H bond [32] comparable to that of magnolol [33]. Although a phenoxyl radical derived from magnolol can be hypothesized, there is no experimental evidence of this reaction. The phenoxyl radical is an oxidation product that may represent the starter of polymerization. The high pH of the background electrolyte may induce a phenoxyl radical formation for eugenol, *iso*eugenol and guaiacol, that readily triggers polymerization; on the other hand, oxidizing dehydrodieugenol and magnolol into their corresponding radicals involves extra time and may lessen a part of oxidative power needed for electropolymerization. The peculiar monomer reactivities, the increase in hydrophobicity and spatial disposition of the aromatic rings due to biphenylic structure could be responsible for the diverse formation of polymers at the different relative E_{App} used.

Electrostatic repulsion does not seem to be the only reason for the significant $S_{AA}\%$ improvement at low electropolymerization potentials. Since AA in neutral pH is deprotonated, an overoxidation should improve the permselectivity but the best $S_{AA}\%$ of the two dimers p-dehydrodieugenol and p-magnolol was obtained with the less oxidating (lowest) relative potential applied (-50 mV).

Most likely, permselectivity towards AA depends on many factors like size exclusion and different conformational features of the electropolymerized film. At -50 mV under the $1^{st}E_{Ox}$, polymerization rates are slowed. This could help the spatial disposition of assembling monomer, especially for molecules provided with a free C-C rotation bond like the two studied hydroxylated biphenlys. A tighter net, added to a hydrophobic effect, excludes the larger AA but allows the smaller H_2O_2 to move throughout the polymer and reach the transducer.

The influence of relative E_{App} on permselective properties of eugenol deserves a separate discussion. At a relative E_{App} of 0 mV, p-eugenol seems to be an impermeable membrane. In fact, all permeabilities

6

towards AA, DA and H_2O_2 reach the minimum when eugenol is electropolymerized nears its $1^{st}E_{Ox}$ (Figure 2, part B). Furthermore, p-eugenol is the only monomer that has a negative correlation between $P_{AA}\%$ versus plateau and Total Area.

The reason lies in the different chemical species involved in the polymerization mechanism. In alkaline solution (pH>9.5) eugenol and *iso*eugenol easily form their corresponding phenoxyl radicals that improve solubility of these hydrophobic compounds [32] and activate different reactions. A highly reactive quinone methide stabilization was proposed for eugenol, whereas *iso*eugenol can stabilize into a benzyl radical [34]. Also a pathway involving O-demethylation in *ortho* position of phenolic ring has been proposed for these two isomers leading to a *ortho*quinone radical formation [35]. The different experimental condition used here can differently affect radicals and intermediate involved in polymerization. Radicals from eugenol might be polymerized into a very tight net, almost impenetrable at relative E_{App} equivalent to its $^{1st}E_{Ox}$, whereas at a lower potential (-50 mV) radical formation is slowed, leading to short chain oligomers that may lead to a loose net. This allows molecules to assume a favorable tridimensional orientation on the transducer surface during electro-deposition and, in time, within polymer matrix. At higher electropolymerization potentials, a further oxidation may lead to different radicals and loss of methyl group, gaining also a charge exclusion property. Furthermore, it is generally acknowledged that eugenol can be adsorbed on Pt electrode involving the allyl chain [18]: this may help eugenol to deposit on the surface, giving rise to an impermeable film. The p-eugenol obtained at relative E_{App} of 0 and +100 shows too low permeability to the reporter molecule H_2O_2 for sensing applications. Nevertheless, when polymerized at E_{App} of -50 and +500 mV, eugenol improves permeability towards H_2O_2, reaching satisfactory permselectivity towards DA (at a relative E_{App} of -50 mV) and AA (at a relative E_{App} of +500 mV). These results highlight how a suitable relative E_{App} selection for electropolymerization is crucial for obtaining highly permselective films.

Effect of CPA applied potential on permeability and permselectivity at day 14

Aging studies revealed for each polymer that the best results in $S_{AA}\%$ were obtained for a relative E_{App} of -50 mV (Table 2). The $S_{DA}\%$ for p-dehydrodieugenol for a relative E_{app} of 0 mV is not statistically different (p>0.05) for $S_{DA}\%$ at a relative E_{App} of -50 mV (Table S-1, Supplementary Material). The lowest electropolymerization potential generated a polymer whose properties were actually improved by aging.

It is possible that a lower polymerization leads to the formation of oligomers that can graft on the transducer and adapt their spatial disposition over time. At higher polymerization rates, triggered by a higher E_{App}, oligomers can be readily oxidized and radicals generated react in a longer, hindered and randomized chain. Unlike p-magnolol, p-dehydrodieugenol degenerated over time, although at a different rate. In fact, p-magnolol permselectivity decreased only for DA, whereas improved permselectivity was observed for AA, reaching a $S_{AA}\%$ of 0.1 ± 0.01. p-dehydrodieugenol permselectivity decreased significantly for DA (0.4 ± 0.1 at day 1, 7.2 ± 0.03 at day 14) and to a lesser extent for AA (0.4 ± 0.04 at day 1, 1.2 ± 0.03 at day 14). In this case, the hindering due to the *ortho* methoxylic groups on the phenolic ring might prevent molecules from stabilizing into a homogeneous film over time.

As previously mentioned, p-magnolol and p-*iso*eugenol electro-deposited at a relative E_{App} of −50 mV, improved their permselectivities, even reaching the excellent performance of *o*PD. This improvement was mainly due to an increase in their $P_{H_2O_2}\%$ values (compare Figure 2 with Figure S-2, Supplementary Material). In general, natural phenols polymerized at lower potentials are stable for 14 days - if not even more permselective than day 1- and can be used in place of the p-PD when a shielding ability to AA and DA is required for sensing application up to 14 days.

SEM microphotography

In a previous work [17] electropolymerization seemed to produce a rough and granular surface particularly clear for CPA electrodeposition using dimers. Similar features could not be distinguished for polymers obtained at lower applied potentials (Figure S-1, Supplementary Material).

SEM analysis at day one showed a smooth and compact surface, clearly different from the surface of Pt-Ir. Orientation of hindered phenols like magnolol or dehydrodieugenol did not produce any particular three-dimensional texture. As a representative group, Figure 3 shows SEM images from phenols pol-

ymerized at a relative E_{App} of 0 mV. Although at this E_{App} value, p-eugenol was characterized by very peculiar values in permselectivity and permeability (Figure 2), these features cannot be distinguished by observing the superficial aspect of the film. A p-eugenol film shown in Figure 3 part B seems to be quite similar to the film obtained from its symmetric dimer dehydrodieugenol (Figure 3, part D).

Design of a AChE/COx biosensor prototype

The p-magnolol permeselective properties were validated in a biosensor design based on ChO/AChE enzymes. A p-magnolol film was electrosynthesized using CPA at +170 mV *versus* Ag/AgCl (-50 mV relative E_{App}). (Figure 4).

Four biosensors were constructed and *in vitro* calibrations were performed on day 1 after polymerization. *In vitro* sensitivity of the AChE/COx biosensor (Figure 4) was determined by injecting known amounts of acetylcholine (ranging from 0 to 2500 µM) into the electrochemical cell. The calibration curve shows a classical Michaelis-Menten kinetics, with R^2= 0.961 (n = 4), V_{max}= 338 ± 13 nA and K_M = 635 ± 61 µM, The linear region slope is between 0 and 500 µM, presenting a slope of 0.336 ± 0.007 nA µM^{-1} with R^2 = 0.994 (n = 4). Further calibrations against Ch and H_2O_2 were performed in order to calculate the biosensor efficiencies (BE_{ACh}% and BE_{Ch}% respectively). The magnolol-coated biosensor showed a BE_{ACh}% of 40.3 ± 1.7% and a BE_{Ch}% of 49.5 ± 2.3%. These values are lower than the empirically maximum values of 60% reported in a previous study for biosensor having the same geometry and similar designs [5]. As observed by other authors [36], the values of BE% show a conversion of Ch to H_2O_2 higher than ACh. For what concern the p-PD-coated biosensor, BE_{ACh}% was 32.01 ± 1.3% and BE_{Ch}% 38.4 ± 3.1%, values significantly lower than those detected in the presence of the magnolol film. A possible explanation to this phenomenon could be that a more favorable interaction between the film and the biological and non-biological components of biosensor might enhance enzyme activity [29] and improve biosensor efficiency.

Regarding the shielding properties of the film, the only significant difference involves a decrease in AA rejection. ΔI-AA obtained for the biosensor was approximately six times higher than the enzyme-free p-magnolol sensor (biosensor ΔI-AA = 0.90 ± 0.05 nA versus sensor ΔI-AA = 0.14 ± 0.02 nA). This difference may be attributed to the introduction of PEI in the biosensor design. PEI, a polycation used as enzyme stabilizer, may be responsible of an electrostatic attraction of AA; in fact, a similar interaction was found in a p-PD-shielded glutamate biosensor [37]. PEI removal from the biosensor design determined a moderate increase of ΔI-AA of around twice that of the p-magnolol sensor (ΔI-AA of biosensor without PEI was 0.25 ± 0.07 nA).Unfortunately, PEI has been shown to be indispensable for enhancing biosensor activity; indeed the analytical performance of the AChE/ChO biosensor lacking PEI deteriorated dramatically (data not show). While the permeability of the film to AA and DA remained stable for a period of two weeks, we observed a decrease of V_{max} (215 ± 25 nA) and an increase of K_M (1.20 ± 0.19 mM) at day 14.

CONCLUSIONS

Lowering electropolymerization applied potentials led to a substantial improvement in permselectivity for each phenol analyzed in this study. Although mechanisms governing permselectivity deserve a deeper analysis, this study shows for the first time that lower potentials contribute to achieve a higher performance compared to higher potential. Permselectivities values obtained are satisfactory for a use in a first-generation amperometric biosensor. The reasons why lower polymerization potentials can help monomer forming a highly permselective film may lie in their structural and physico-chemical properties, reduced polymerization rate and prevention of overoxidation. While *in vitro* calibration underlined significant differences in permselective films, SEM microimages showed a quite similar compact film for all phenols. Noteworthy, the excellent results were obtained by CPA, a technique that saves time, materials and provides more reproducible results compared to CV.

From day 1, polymers derived from natural phenols displayed values comparable with those of p-PD, a suspect carcinogenic agent to humans, and provided interference-rejection properties useful for biosen-

8

sor application. Particularly, magnolol polymerized by CPA at 170 mV *versus* Ag/AgCl showed a not significantly different (p>0.05) $S_{AA}\%$ and a 36 times better $S_{DA}\%$ when compared to p-PD at day 1. The p-magnolol proved to be a good permselective film when incorporated into an AChE/ChO-based biosensor despite a reasonable decrease in AA-rejection can be due to an interaction between the film and biosensor components.

Aging studies, carried out for CPA-generated films at low applied potentials, confirmed for the first time that p-magnolol and p-*iso*eugenol can be regarded as effective and a healthier alternatives to p-PD film for AA and DA rejection in biosensor applications.

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.

REFERENCES
[1] A.P.F. Turner, Biosensors: sense and sensibility, Chem. Soc. Rev. 42 (2013) 3175–3648.
[2] G. Calia, G. Rocchitta, R. Migheli, G. Puggioni, Y. Spissu, G. Bazzu, V. Mazzarello, J.P. Lowry, R.D. O'Neill, M.S. Desole, P.A. Serra, Biotelemetric Monitoring of Brain Neurochemistry in Conscious Rats Using Microsensors and Biosensors, Sensors (Basel) 9 (2009) 2511-2523.
[3] A. Rasooly, K.E. Herold, Biosensors for the analysis of food- and waterborne pathogens and their toxins. J. AOAC Int. 89 (2006) 873-883.
[4] A. Kaushik, S.K. Arya, A. Vasudev, S. Bhansali, Recent Advances in Detection of Ochratoxin-A, Crit. Rev. Biotechnol. 2 (2013) 1-11.
[5] R.D. O'Neill, J.P. Lowry, G. Rocchitta, C.P. McMahon, P.A. Serra, Designing sensitive and selective polymer/enzyme composite biosensors for brain monitoring in vivo, Trends Anal. Chem. 27 (2008) 78–88.
[6] K.M. Mitchell, Acetylcholine and choline amperometric enzyme sensors characterized in vitro and in vivo Anal. Chem. 76 (2004) 1098-1106.
[7] I.B. Rejeb, F. Arduini, A. Arvinte, A. Amine, M. Gargouri, L. Micheli, C. Bala, D. Moscone, G. Palleschi, Development of a bio-electrochemical assay for AFB1 detection in olive oil, Biosens. Bioelectron. 24 (2009) 1962–1968.
[8] Y.Q. Dai, K.K. Shiu, Highly Sensitive Amperometric Glucose Biosensor Based on Glassy Carbon Electrode with Copper/Palladium, Electroanalysis 16 (2004) 1806–1813.
[9] G. Rocchitta, O. Secchi, M.D. Alvau, D. Farina, G. Bazzu, G. Calia, R. Migheli, M.S. Desole, R.D. O'Neill, P.A. Serra, Simultaneous telemetric monitoring of brain glucose and lactate and motion in freely moving rats, Anal. Chem. 85 (2013) 10282-10288.
[10] H. Matsuhisa, M. Tsuchiya, Y. Hasebe, Protein and polysaccharide-composite sol–gel silicate film for an interference-free amperometric glucose biosensor, Colloids Surf. B Biointerfaces 111 (2013) 523-529
[11] S. Vaddiraju, D.J. Burgess, F.C. Jain, F. Papadimitrakopoulos, The role of H_2O_2 outer diffusion on the performance of implantable glucose sensors, Biosens. Bioelectron. 24 (2009) 1557-1562.

[12] S.J. Killoran, R.D. O'Neill, Characterization of permselective coatings electrosynthesized on Pt-Ir from the three phenylenediamine isomers for biosensor applications, Electrochim. Acta 53 (2008) 7303-7312.

[13] A. Malinauskas, R. Garjonytė, R. Mažeikienė, I.Jurevičiūtė, Electrochemical response of ascorbic acid at conducting and electrogenerated polymer modified electrodes for electroanalytical applications: a review, Talanta 64 (2004) 121-129.

[14] S.A. Rothwell, S.J. Killoran, R.D. O'Neill, Enzyme immobilization strategies and electropolymerization conditions to control sensitivity and selectivity parameters of a polymer-enzyme composite glucose biosensor, Sensors 10 (2010) 6439-6462.

[15] S.A Rothwell, S.J Killoran, E.M. Neville, A.M. Crotty, R.D. O'Neill, Poly(o-phenylenediamine) electrosynthesized in the absence of added background electrolyte provides a new permselectivity benchmark for biosensor applications, Electrochem. Communications 10 (2008) 1078-1081.

[16] S.A. Rothwell, C.P. McMahon R.D. O'Neill, Effects of polymerization potential on the permselectivity of poly(o-phenylendiamine) coatings deposited on Pt-Ir electrodes for biosensor applications, Electrochim. Acta 55 (2010) 1051-1060.

[17] G. Calia, P. Monti, S. Marceddu, M.A. Dettori, D. Fabbri, S. Jaoua, R.D. O'Neill, P.A. Serra, G. Delogu, Q. Migheli, Electropolymerized phenol derivatives as permselective polymers for biosensor applications Analyst 140 (2015) 3607-15.

[18] A. Ciszewski, G. Milczarek, Preparation and general properties of chemically modified electrodes based on electrosynthesized thin polymeric films derived from eugenol Electroanalysis 13 (2001) 860-867.

[19] G Milczarek, A. Ciszewski, Permselective Properties of Electropolymerized Guaiacol Derivatives, Electroanalysis 15 (2003) 529-532.

[20] A. Ciszewski, G. Milczarek, A new Nafion-free bipolymeric sensor for selective and sensitive detection of nitric oxide, Electroanalysis 10 (1998) 791-793.

[21] D.W. Paul, I. Prajapati, M.L. Reed, Electropolymerized eugenol: evaluation as a protective film for oxygen sensing, Sens. Actuators B Chem. 183 (2013) 129-135.

[22] G. Milczarek, A. Ciszewski, Functionalized gold nanoparticles and films stabilized by in situ formed polyeugenol, Colloids Surf B Biointerfaces 90 (2012) 53-57.

[23] L.L. Okumura, N.R. Stradiotto, N.V. Rees, R.G. Compton Modifying glassy carbon (GC) electrodes to confer selectivity for the voltammetric detection of L-cysteine in the presence of DH-homocysteine and glutathione, Electroanalysis 20 (2008) 916-918.

[24] B.A. Patel, M. Arundell, K.H. Parker, M.S. Yeoman, D. O'Hare, Detection of nitric oxide release from single neurons in the pond snail, Lymnaea stagnalis, Anal. Chem. 78 (2006) 7643-7648.

[25] A. Ciszewski, G. Milczarek, Polyeugenol-modified platinum electrode for selective detection of dopamine in the presence of ascorbic acid, Anal Chem 71 (1999) 1055-1061.

[26] R. Toniolo, N. Dossi, A. Pizzariello, S. Susmel, G. Bontempelli, Simultaneous detection of ascorbic acid and hydrogen peroxide by flow-injection analysis with a thin layer dual-electrode detector Electroanalysis 23 (2011) 628-636.

[27] T. Watanabe, T. Hirayama, S. Fukui, Phenazine derivatives as the mutagenic reaction product from o- or m-phenylendimine derivatives with hydrogen peroxide, Mutat. Res. 227 (1989) 135-145.

[28] M. Matsumoto, M. Suzuki, H. Kano, S. Aiso, K. Yamazaki, S.Fukushima, Carcinogenicity of ortho-phenylendiamine dihydrochloride in rats and mice by two-year drinking water treatment, Arch. Toxicol. 86 (2012) 791-804.

[29] P.J. Hajduk, M. Bures, J. Praestgaard, S.W. Fesik, Privileged molecules for protein binding identified from NMR-based screening, J. Med. Chem. 43 (2000) 3443-3447

[30] S.A. Rothwell, R.D. O'Neill, Effects of applied potential on the mass of non-conducting poly(ortho-phenylenediamine) electro-deposited on eqcm electrodes: Comparison with biosensor selectivity parameters, Phys. Chem. Chem. Phys. 13 (2011) 5413-5421.

[31] J.D. Craig R.D. O'Neill Electrosynthesis and permselective characterization of phenol-based polymers for biosensor application, Anal. Chim. Acta 495 (2003) 33–43.

10

[32] S. Fujisawa, T. Atsumi, Y. Kadoma, H. Sakagami, Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity, Toxicology 177 (2002) 39-54.

[33] R. Amorati, J. Zotova, A. Baschieri, L. Valgimigli, Antioxidant Activity of Magnolol and Honokiol: Kinetic and Mechanistic Investigations of Their Reaction with Peroxyl Radicals, J. Org. Chem. 80 (2015) 10651−10659.

[34] T. Atsumi, S. Fujisawa, K. Tonosaki, A comparative study of the antioxidant/prooxidant activities of eugenol and isoeugenolo with various concentrations and oxidant conditions, Toxicol. in vitro 19 (2005) 1025-1033.

[35] F. Bertrand, D.A. Basketter, D.W. Roberts, J.P. Lepoittevin, Skin sensitization to eugenol and isoeugenol in mice: possible metabolic pathways involving ortho-quinone and quinone methide inter-mediates, Chem. Res. Toxicol. 10 (1997) 335-343.

[36] A. Khan, S. Ab Ghani, Multienzyme microbiosensor based on electropolymerized *o*-phenylenediamine for simultaneous in vitro determination of acetylcholine and choline, Biosens. Bioe-lectron. 31 (2012) 433-438.

[37] C.P. McMahon, G. Rocchitta, P.A. Serra, S.M. Kirwan, J.P. Lowry, R.D. O'Neill, The efficiency of immobilised glutamate oxidase decreases with surface enzyme loading: an electrostatic effect, and re-versal by a polycation significantly enhances biosensor sensitivity, Analyst 131 (2006) 68-72.

11

Figure and Table captions:

(in order of appearance)

Figure 1. First oxidation peak potentials ($^{1st}E_{Ox}$) and potential referred to Ag/AgCl reference electrode are reported in the table. The graphic shows the CV first cycle of each phenol setting the $^{1st}E_{Ox}$ as 0, only the forward part is reported. Structures of studied phenols are on the right.

Figure 2. Selectivities (S%) and apparent permeabilities (P%) of phenol-derived polymeric film versus relative polymerization potential (relative E_{app}) at day 1; n=4.

Figure 3. SEM microphographs at 5000x magnification of phenol polymerized at a relative E_{app} of 0 mV. A: p-guaiacol (p-GU); B: p-eugenol (p-EU); C: p-*iso*eugenol (p-*i*EU); D: p-dehydrodieugenol (p-DEU); E: p-magnolol (p-MA); F: time course of polymerizations of phenols at relative E_{app} of 0 mV.

Figure 4. *In vitro* calibration of a Cox/ACE biosensor, incorporating a p-magnolol permeselective film, showing Michaelis-Menten kinetics and linear regression (inset); n=4.

Table 1. Permselectivities (S%) of different phenols compared to guaiacol on day 1.

		p-guaiacol	p-eugenol	p-*iso*eugenol	p-dehydrodieugenol	p-magnolol
$S_{AA}\%$	CV	2 ± 0.1	1.0 ± 0.2	5 ± 0.6	7 ± 0.8	1 ± 0.1
	CPA	149 ± 5	7 ± 0.8	149 ± 16	24 ± 3	53 ± 6
$S_{DA}\%$	CV	25 ± 1	14 ± 1	31 ± 4	18 ± 2	4 ± 0.4
	CPA	55 ± 6	26 ± 3	70 ± 8	19 ± 2	15 ± 2

CV: cyclic voltammetry; CPA: constant potential amperometry; polymerization conditions are the same as [17]; n=4.
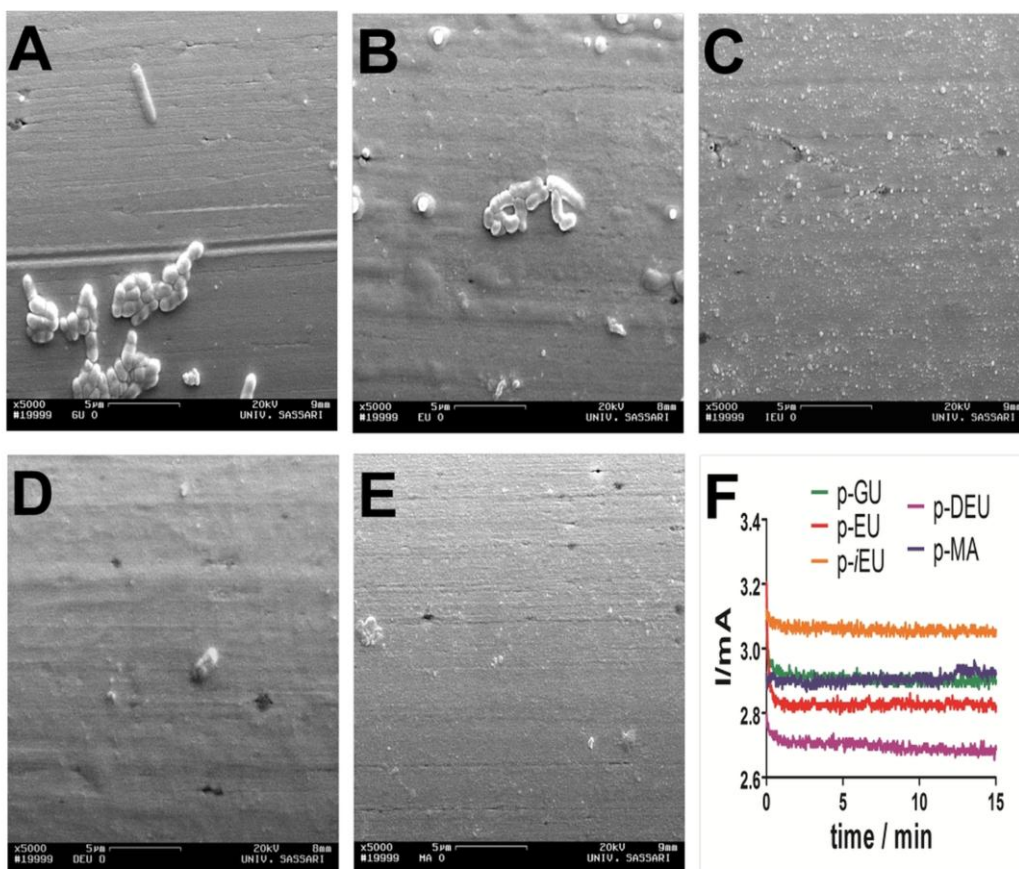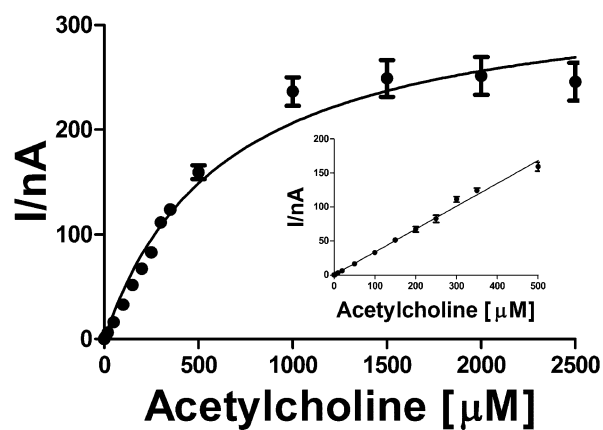
Table 2. The electropolymerization potential (relative E_{App}) with the best permselectivity values at day 1 and 14 of phenols in comparison with p-PD.
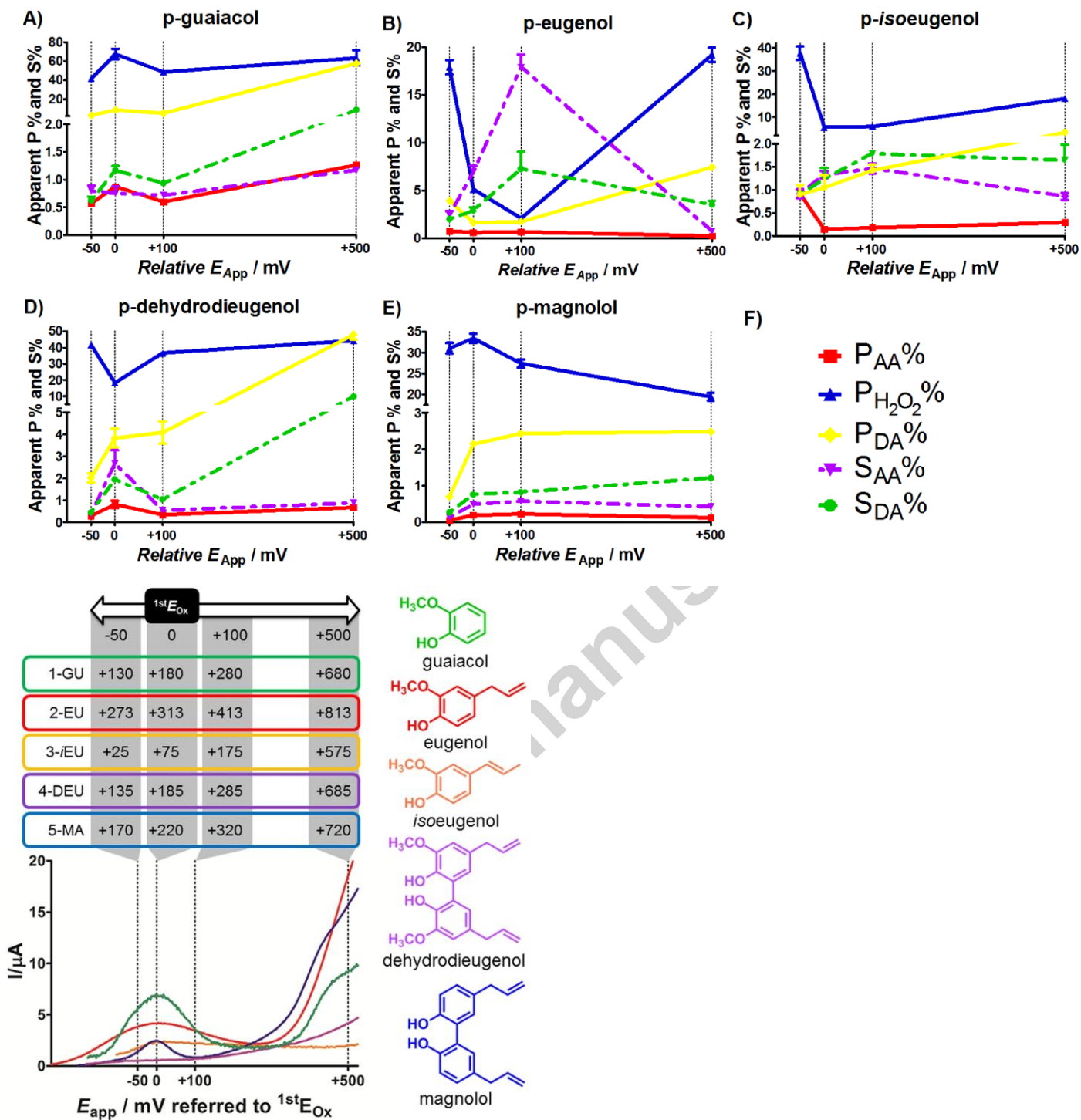
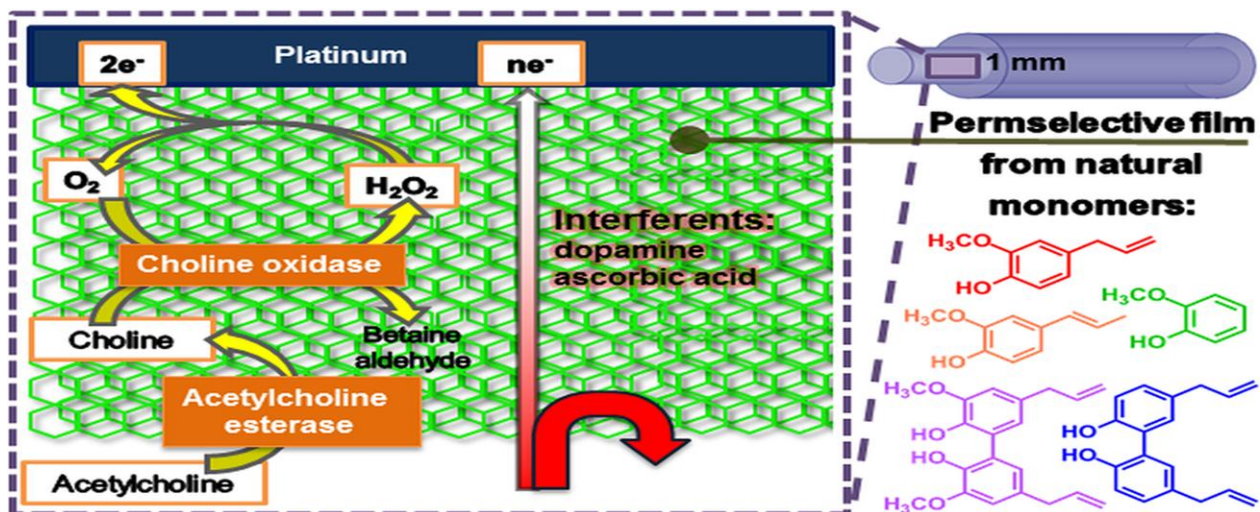|	p-PD	p-guaiacol	p-eugenol	p-*iso*eugenol	p-dehydrodieugenol	p-magnolol
DAY 1						
$S_{AA}\%$	0.2 ± 0.1	0.7 ± 0.1*	0.7 ± 0.2*	0.9 ± 0.1*	0.4 ± 0.4*	0.1 ± 0.02
Potential group	as from Calia et al. 2015 [17]	+100	+500	+500	-50	-50
$S_{DA}\%$	10 ± 1	0.6 ± 0.1*	2.0 ± 0.3*	0.9 ± 0.2*	0.4 ± 0.1*	0.3 ± 0.01*
Potential group	as from Calia et al. 2015 [17]	-50	-50	-50	-50	-50
DAY 14						
$S_{AA}\%$	0.2 ± 0.02	0.8 ± 0.2*	0.8 ± 0.3	0.06 ± 0.01*	1.2 ± 0.03*	0.1 ± 0.01*
Potential Group	as from Calia et al. 2015 [17]	-50	-50	-50	-50	-50
$S_{DA}\%$	1.1 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	7.2 ± 0.03*	0.7 ± 0.06*
Potential group	as from Calia et al. 2015 [17]	-50	-50	-50	0	-50

* significantly different from p-PD, unpaired t-test on n=4.

Highlights

1. Permselective polymers from natural phenols are proposed for biosensor application.

2. Low applied potentials significantly improve film permselectivity.

3. Magnolol and *iso*eugenol electro-deposition leads to the best performing films.