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Tyrosinase immobilized magnetic nanobeads for the amperometric assay of enzyme inhibitors: Application to the skin whitening agents

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ABSTRACT

The immobilization of tyrosinase onto glutaraldehyde activated streptavidine magnetic particles and subsequent retention onto a magnetized carbon paste electrode for the amperometric assay of tyrosinase inhibitors is described. Tyrosine was used as substrate as it is the first substrate in the melanogenesis process. The sensing mode is based on monitoring the decrease of the amperometric signal corresponding to the electrochemical reduction of dopaquinone enzymatically generated. This current decrease is due to the presence of inhibitors acting directly on the enzyme or inhibitors acting on the product of the enzymatic reaction, i.e. dopaquinone. The methodology is designed for the evaluation of the inhibitory potency of the most frequently used active substances in cosmetic marketed products against hyperpigmentation such as kojic acid, azelaic acid and benzoic acid. These compounds bind to the tyrosinase active center. Ascorbic acid is also investigated as it interrupts the synthesis pathway of melanin by reducing the melanin intermediate dopaquinone back to L-dopa. By comparing the obtained IC_{50}, under the same experimental conditions, the order of their inhibitory potency was: kojic acid ($IC_{50} = 3.7 \times 10^{-6}$ M, $K_i = 8.6 \times 10^{-7}$ M), ascorbic acid ($IC_{50} = 1.2 \times 10^{-5}$ M), benzoic acid ($IC_{50} = 7.2 \times 10^{-5}$ M, $K_i = 2.0 \times 10^{-5}$ M) and azelaic acid ($IC_{50} = 1.3 \times 10^{-4}$ M, $K_i = 4.2 \times 10^{-5}$ M) in close agreement with literature spectrophotometric inhibition data using the soluble tyrosinase.

1. Introduction

Melanin, the dark pigment produced by skin cells in the most inner layer of the epidermis, plays an important role in protecting human skin from the harmful effects of the UV sun radiations. Melanin also determines our phenotypic appearance. However, abnormal accumulation of melanin in the basal layer of the dermis is responsible for hyperpigmentation including melasma, freckles and senile lentigines [1]. These undesirable conditions of the human skin are serious esthetic problem for human beings nowadays since both appearance and quality of life are becoming more and more important. This problem is particularly prevalent in middle aged and elderly individuals [2] and this has encouraged researchers to seek for new potent inhibitors of the hyperpigmentation process. Recently, a global market demand has developed on this subject. For the past few decades, tyrosinase inhibitors have been a great concern solely due to the key role of tyrosinase in mammalian melanogenesis [3].

Hyperpigmentation is a complex process. There are many factors that influence the activity of melanocites, but it is well known and unanimously accepted that tyrosinase activity plays the main role in the pathway of melanin biosynthesis. It catalyzes the first two reactions of the melanogenesis process namely: the hydroxylation of L-tyrosine to L-dopa as monophenolase, using molecular oxygen as co-substrate, and the oxidation of L-dopa to dopaquinone as diphenolase. These steps, and mostly the first oxidizing step, are the rate-limiting steps in melanin synthesis since the subsequent reactions can proceed spontaneously at physiological pH [3].

A number of tyrosinase and melanogenesis inhibitors, from both natural and synthetic sources, have been identified [4]. However, the definition of "tyrosinase inhibitor" is sometimes misleading, the terminology being also used to refer to melanogenesis inhibitors, whose action mainly reside in some interference in melanin formation, regardless of any direct inhibitor–enzyme interaction [3]. Only specific tyrosinase inactivators and inhibitors should be regarded as "true inhibitors", which bind to the enzyme and inhibit its activity. Some skin whitening agents that act as tyrosinase inhibitors, such as kojic acid and azelaic acid, or as melanogenesis inhibitors such as hydroquinone, arbutin and ascorbic acid are the most well known to most dermatologists [5]. The interference with melanin synthesis can occur in different ways. Kojic acid is a

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chelator of the copper of the enzyme active site [6], arbutin is a pro-drug of hydroquinone that is an alternative substrate, azelaic acid blocks the tyrosine access to the active site and ascorbic acid chemically reduces the dopaquinone back to L-dopa, thus avoiding dopachrome and melanin formation [7]. Also benzoic acid, a preservative very frequently used in cosmetic and food industry [8], has been demonstrated to have inhibitory properties for tyrosinase [9].

The need for the quantification and comparative analysis at high throughput of the inhibitory activity of possible new products and of the existing ones is becoming more and more of interest for the evaluation and development of efficient inhibitors of the hyperpigmentation process.

Until now, several methods for the screening of skin-whitening agents have been proposed. The method most frequently applied exploited the spectrophotometric quantification of dopaquinone, before and after the inhibition of soluble tyrosinase [6,10]. Another method with tyrosinase in solution relied on electron spin resonance spectrometry that measured the accumulation of eumelanin radicals in melanin culture cells [11]. *In vivo* studies evaluated the inhibitors potency by photometry [12]. Usually in each of the currently developed method, the inhibitory strength of the studied compounds was compared to that of a standard inhibitor, namely kojic acid [6]. Spectrophotometry is not a primary choice analytical method for the quantification of an on going high ratio reaction which is the case of the enzymatic formation of dopaquinone due to the low reproducibility of the obtained results. The use of spectrometry has the disadvantage of the need of culture cells and the photometric one is applied to *in vivo* studies so it can be used just for substances that have been approved for human use and the implementation of such study involves ethic problems. Thus they are not suitable for rapid assays in scientific research of novel potential skin whitening agents. The use of biosensors would be an interesting and valuable alternative for the rapid screening and comparison of the inhibitory potency of the existing compounds and the novel ones due to its relative simplicity in use and the high reproducibility of the analytical data.

To the best of our knowledge there have been very few tyrosinase based biosensors fabricated for the evaluation of the inhibitory potency of kojic acid. A Clark-type oxygen electrode that monitored oxygen consumption during catechol conversion in the presence of kojic acid inhibitor was reported [13]. Another biosensor for kojic acid quantification used dopamine as substrate and it monitored the oxygen consumption with a Clark type electrode [14]. There have been a couple of tyrosinase biosensors developed for the evaluation of the inhibitory potency of benzoic acid [8,15] but none of them used L-tyrosine as enzyme substrate. Tyrosinase based biosensors usually used phenol or its derivatives as substrate with the enzyme being combined with an electrochemical transducer to sense either the oxygen consumption of the overall enzymatic reaction [14] or the enzymatic production of the electroactive quinone species [16]. There was one article reported of a tyrosinase based biosensor for quantitative analysis of phenols. Tyrosinase was immobilized to core–shell magnetic particles and subsequently attached to the surface of a carbon paste electrode with the help of a permanent magnet [17].

Tyrosinase is widely distributed in microorganisms, animals, humans and plants. The enzyme extracted from the mushroom *Agaricus bisporus* is highly homologous with the mammalian one making it well suited as a model for studies on melanogenesis. Practically all studies of inhibitors conducted so far have used the mushroom tyrosinase likely because of its readily commercial availability [3]. Yet alternative systems for testing can be obtained like mammalian tyrosinase, melanocytic cultures, co-cultures of keratinocytes and melanocytes, skin culture to finally *in vivo* application to animal skin [5,18].

Different phenolic compounds can be enzymatically oxidized by tyrosinase and several substrates have been proposed in the literature for the evaluation of the enzyme inhibitors. Dopamine was used as a substrate in the construction of an amperometric biosensor for the detection of tyrosinase inhibitors such as kojic acid, benzoic acid and SCN^- ion [14]. Catechol, p-cresol, m-cresol, phenol and p-chlorophenol were used as enzyme substrates for the determination of benzoic acid based on its inhibition properties, using an amperometric biosensor device [19–21]. L-Dopa was used as enzyme substrate in spectrophotometric evaluation of skin-whitening agents [10]. Also L-tyrosine was used as a substrate in spectrophotometric evaluation of different inhibitors [22,23]. Cellular tests with human melanocytes were used for the transformation rate study of the tyrosinase substrate L-dopa using a spectrophotometric method [24].

Recently, biosensors based on the inhibition of tyrosinase activity in the presence of a monophenol or *o*-diphenol substrate have been proposed for the determination of triazine pesticides [25], carbamate and organophosphate pesticides [16], diuron and atrazine [26], herbicides such as alachlor, diazinon and carbaryl [27], propyl gallate [28], 2,4-dichlorophenol [29], fluoride [30], benzoic acid [19], cinnamic acid and sorbic acid [31].

Besides choosing the adequate transducer and enzyme in the construction of a biosensor, an important role is played by the enzyme immobilization method. The use of magnetic particles (beads) in biomedical and pharmaceutical sciences has increased significantly in recent years [32]. The masterbeads are specially designed for allowing simple and effective immobilization of ligands such as enzyme through activation with bi-functionalized reactants [33], like glutaraldehyde. Immobilization of biomolecules onto the surface of magnetic particles can result in a number of additional functionalities: on one hand the nanostructure of the magnetic particles permits a high enzyme loading without affecting its natural structure and consequently its activity and on the other hand the beads can be attracted by a magnet and be strongly retained in close proximity to the electrode surface and readily washed away if needed [34]. The streptavidine masterbeads (NMPS) are mainly designed for immunological analyses [35] but they can very well be used for enzyme immobilization. Based on the IUPAC definition [36] such a configuration is strictly speaking not a biosensor, i.e. a self-contained integrated device, but since it has the enzyme, during the assays, in close spatial contact with the electrode we may consider it as a biosensor.

In the present work, a novel method for the evaluation of the potency of the tyrosinase and melanogenesis inhibitors used as skin whitening agents is proposed. The biosensor was fabricated using tyrosinase immobilized via glutaraldehyde activated streptavidine magnetic nanoparticles and retained onto a "magnetized" carbon paste electrode, mCPE. The interest in using a carbon paste electrode lies in its low background current, allowing for highly sensitive assay to be performed in the applied potential range close to 0.0 V versus an Ag/AgCl reference electrode. The employed tyrosinase was the mushroom tyrosinase as it is highly homology with the human enzyme. The substrate used was the L-tyrosine, the enzyme's natural substrate in the skin and the investigated inhibitors were kojic acid, azelaic acid, ascorbic acid, the most frequently used active substances in marketed products for hyperpigmentation, and benzoic acid, a well known preservative in the cosmetic industry. The obtained configuration was used to monitor the reduction current of the enzymatically generated oxidized species of L-tyrosine, i.e. the dopaquinone, in the presence of molecular oxygen (Fig. 1). The biosensor was applied to the screening of inhibitors which induced a decrease of the reduction current proportional to the inhibitor concentration and strength.

In practice, the steady state current for L-tyrosine was recorded as I_0. The biosensor response in the case of the addition of an

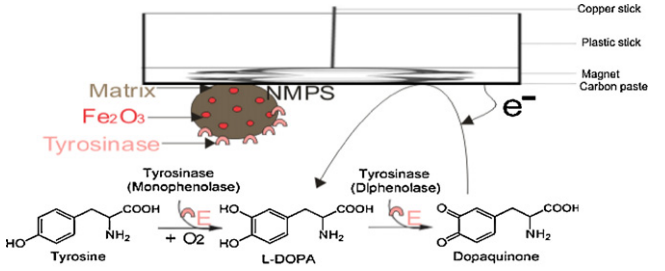

Fig. 1. Biosensor's mechanism of action.

inhibitor was recorded as I_1. The concentration of inhibitors was correlated with the percentage of inhibition (In %) which was calculated using the relationship: In (%) = $[(I_0 - I_1)/I_0] \times 100$. With I_0 and I_1 being the biosensor current signals before and after the addition of the inhibitor, respectively, I_1 was expected to be smaller comparing to I_0 as the process was inhibited.

2. Materials and methods

2.1. Material

The mushroom tyrosinase (EC 1.14.18.1) 5370 Units/mg was purchased from Sigma. L-Tyrosine and L-dopa were provided by Janssen Chimica. The Masterbeads Streptavidine (500 nm) were purchased from Ademtech France. Dopamine, glutaraldehyde 25% wt and monosodium phosphate was provided by Sigma–Aldrich and disodium phosphate was provided by Merck. The carbon paste was from Metrohm. Kojic acid was purchased from Kreglinger Europe, azelaic acid and ascorbic acid from Acros Organics and benzoic acid from Fluka.

All reagents were of analytical grade and were used as received. All solutions were prepared with distilled water and stored in the refrigerator (4 °C) when not in use. The phosphate buffer was prepared by mixing two stock solutions of monosodium phosphate 0.1 M and disodium phosphate 0.1 M to the desired pH. The stock solutions of L-tyrosine, L-dopa, kojic acid, benzoic acid, azelaic acid and ascorbic acid were prepared in phosphate buffer.

2.2. Construction of the tyrosinase-NMPS biosensor

2.2.1. Enzyme immobilization

The Streptavidin Masterbeads were monodispersed and were based on superparamagnetic particles composed of a magnetic core encapsulated by a hydrophilic polymer shell coated with streptavidin. The magnetic particles had a diameter of 500 nm (RSD max 20%), density of approx. 2.0 g/cm^3, magnetic susceptibility approx. 40 emu/g, specific surface area of 5 m^2/g, iron oxide content approx. 70% m/m and the solid content of 10 mg/mL [33].

An amount of 1 mL 10 mg/mL NMPSs was washed with 0.01 M phosphate buffer pH 6.5 (5 mL) then reacted with 1 mL of 2.5% wt glutaraldehyde solution (in 0.01 M phosphate buffer, pH 6.5) at room temperature for 30 min. After washing with 0.01 M phosphate buffer pH 6.5 (5 mL), the glutaraldehyde-treated NMPSs were reacted with 1 mL tyrosinase solution (3 mg/mL in phosphate buffer 0.01 M, pH 6.5) during 1 h at room temperature to obtain the tyrosinase-NMPSs. The resulting suspension was washed with 0.01 M phosphate buffer pH 6.5 (5 mL) and stored in 1 mL 0.01 M phosphate buffer pH 6.5 at 4 °C. During the washing steps, the beads were trapped by magnetic forces by placing the reacting Eppendorf tube close to a strong magnet and the supernatant was carefully pipetted off. The final concentration of tyrosinase-NMPSs was 10 mg/mL (referring to the amount of NMPS) [35].

A 10 mg/mL tyrosinase-bead suspension was used for L-tyrosine response optimization and an approximately 1.25 mg/mL tyrosinase-bead suspension was used in the inhibition assays. The initial 1 mL amount of the tyrosinase-NMPSs was diluted and it resulted finally in 8 mL suspension of 1.25 mg/mL tyrosinase-NMPSs that allowed performing approximately 800 inhibition assays.

Visible spectrophotometry was applied for the determination of the efficiency of the immobilization method of tyrosinase onto the NMPSs. The method consisted in determining the amount of immobilized tyrosinase by measuring the absorbance of tyrosinase in a 60 times diluted tyrosinase solution used in the immobilization process, before and after the enzyme was immobilized onto the activated NMPSs. The percentage of immobilization, calculated by referring to a calibration curve (absorbance versus tyrosinase concentration) realized in 0.01 M phosphate buffer pH 6.5 (λ = 280 nm), was found to be around 40 ± 5%. Therefore it was inferred that the inhibition assays were realized with approximately 8 Units of tyrosinase immobilized at the electrode surface.

2.2.2. Biosensor fabrication

The working carbon paste electrode (mCPE) consisted of a home made electrode: inside a plastic rod, a permanent magnet (Neody, 3 mm diameter) was inserted leaving a depression at the surface of approximately 2 mm for housing the solid carbon paste layer, and a copper wire was used as the electrical conductor.

Before each experiment the solid carbon paste was manually poured in the hole and the resulting mCPE was smoothed on a clean paper surface. The diameter of the active surface was 3 mm. A volume of 10 μL of the Tyrosinase-NMPSs suspension was spread over the surface of the electrode (in position up side down) allowing the particles to settle, being attracted within a few seconds by the magnet. Subsequently, the tyrosinase-NMPSs immobilized mCPE was oriented in the right position in a three-electrode cell. The strong attraction of the Tyrosinase-NMPSs by the magnet housed inside the working electrode allowed appropriate stirring during the amperometric experiments.

2.3. Apparatus

The experiments were performed by using as potentiostat a BASi EPSILON system with a C3 Cell Stand. Amperometry was performed in a conventional three electrodes setup with the biosensor as working, a Pt wire as auxiliary and an Ag/AgCl 3 M KCl as reference electrode, respectively. The cyclic voltammetry experiments were performed in the same conventional three electrodes setup used in amperometry. All the cyclic voltammetry experiments were recorded at 100 mV s^{-1} or 10 mV s^{-1} in a potential window comprised between −0.6 V and +1.1 V versus Ag/AgCl 3 M KCl reference electrode. The spectrophotometric assays were performed using an Agilent Hewlett Packard Diode Array UV Vis Spectrophotometer Model 8453. The pH of the solution was controlled with a Metrohm 827 pH Lab system. All experiments were performed at room temperature (23 °C).

2.4. Amperometric assay

During amperometric experiments the biosensor potential was kept at −100 mV under continuous stirring conditions with a magnetic spinbar at 300 rpm. The working potential was imposed and the background current was allowed to reach a steady state value within approx. 10 min. Different amounts of tyrosinase standard solution or inhibitor solution were added every 100 or 200 s and the current was recorded as a function of time in 10 mL 0.1 M phosphate buffer solution.

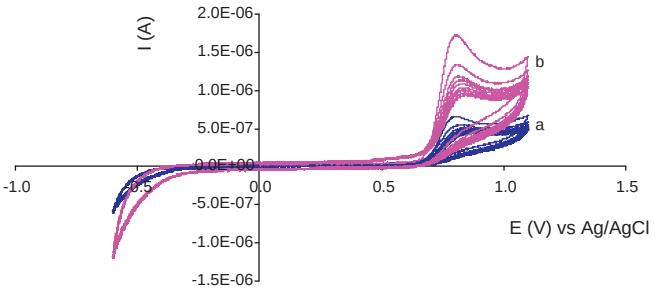

Fig. 2. Repetitive CV for L-tyrosine 4.76×10^{-5} M, 0.1 M phosphate buffer pH 6.5, mCPE, scan rate, a: 10 mV s^{-1}, b: 100 mV s^{-1}.

After each amperometric inhibition assay, the tyrosinase-NMPSs were readily removed by flushing a water burst over the biosensor surface. The surface of the mCPE was renewed after each inhibition assay.

3. Results and discussions

3.1. Working conditions

The working conditions for an optimal response to the substrate of the tyrosinase based biosensor have been tested. Since the biosensor was designed for testing inhibitors of the skin pigmentation process it was taken into account to operate as close as possible to the natural environment of the skin tyrosinase which is a transmembrane protein of melanosomes [37].

3.1.1. Selection of the applied potential

It is well known that L-tyrosine enzymatic oxidation produces dopaquinone and it was expected that this product could be detected by electroreduction at the mCPE. Likewise the electrochemical oxidation of L-tyrosine was supposed to generate dopaquinone. Cyclic voltammetry (CV) performed at 10 and 100 mV/s (from 0.0 mV to 1.1 V, and back to −0.6 V) in 0.1 M phosphate buffer pH 6.5, showed the irreversible oxidation peak of L-tyrosine (1×10^{-3} M and 4.76×10^{-5} M) since no reduction peak was observed in the investigated potential domain (Fig. 2). Same experiments were performed also for L-dopa which gave an oxidation peak at 0.550 V but still no reduction peak was observed. The absence of a reduction current and the progressive decrease of the L-tyrosine peak during multiple scanning suggested that the generated dopaquinone escaped rapidly from the electrode solution interface likely due its high reactivity giving rise to polymer-like structures fouling the electrode surface [38]. Amperometry of L-tyrosine (2.44×10^{-5} M) at the tyrosinase-NMPS mCPE, thanks to the low background current compared to CV, permitted, however, to detect a reduction current at potentials near 0 V versus Ag/AgCl likely corresponding to the electroreduction of some enzymatically generated dopaquinone remaining at the electrode solution interface. Amperometric experiments were realized at 0, −50, −100, −150, −200 and −250 mV. By decreasing the applied potential the reduction current increased but the response steady-state became less stable. An applied potential of −100 mV was considered optimal taking into account the magnitude of the reduction current, the ratio between signal and background current, the steady-state of the response plateau and the possible interfering species at the applied potential, in agreement with other tyrosinase based biosensors described in the literature [21].

3.1.2. Selection of the substrate

To the best of our knowledge there is no tyrosinase based amperometric biosensor for the evaluation of skin-whitening agents that used L-tyrosine as a substrate, reported in the literature. Moreover it has been demonstrated that the intensity of inhibition varied considerably depending on the characteristics of the substrate [19]. The biosensor response was tested comparatively both in the presence of L-tyrosine and L-dopa using 10 μL of the tyrosinase-NMPS suspension (10 mg/mL) deposited onto the surface of the mCPE. It was observed that, in the concentration range 4.98×10^{-6} M and 2.91×10^{-5} M, the sensitivity to L-dopa (-5.56×10^{-4} A/M) was almost 6 times higher comparing to L-tyrosine (-9.37×10^{-5} A/M) and the response time (the time necessary for reaching 95% of the maximum response) to L-dopa (56 s) was faster comparing to L-tyrosine (86 s). L-Tyrosine, however, was chosen as model substrate for subsequent experiments. This decision was based on the fact that, *in vivo*, L-tyrosine is the natural substrate of tyrosinase and also the starting point of melanin synthesis and its oxidation, to L-dopa, is the rate-limiting step of the whole process [3]. The sensitivity to L-tyrosine was considered high enough for assuring reliable results and the lag period of its first enzymatic oxidizing step to L-dopa was sufficiently short for further readily implementation of the testing conditions.

3.1.3. Selection of the pH

The biosensor response was studied in the pH range comprised between 5.5 and 7. It is well known that enzyme activity is highly pH dependent and that the optimum pH for an enzymatic assay must be determined empirically [39]. By decreasing the pH from 7.0 to 5.5, the amperometric signal increased but no stable steady-state plateau was obtained at pH 6.0 and 5.5. For further experiments, the pH chosen was 6.5. At this pH, the current and the steady-state of the biosensor response were considered optimal, in agreement with different literature data where the working pH of the tyrosinase based assays vary between 6.0 and 7.0. [9,14,22,23]. The selected pH corresponded to the Sigma declared tyrosinase stability pH and is also close to the normal pH range of the skin which is considered to vary between 4 and 6.5.

3.1.4. Amount of tyrosinase-NMPS spiked onto the mCPE

The amperometric response was determined as a function of L-tyrosine, in the concentration range 4.98×10^{-6} and 2.91×10^{-5} M, using different amounts (5 μL, 10 μL and 20 μL) of the 10 mg/mL tyrosinase-NMPS suspension spiked onto the surface of the mCPE. The response increased proportionally by raising the amount of immobilized enzyme onto the surface of the electrode. The sensitivities obtained were: -6.82×10^{-5} A/M, -9.37×10^{-4} A/M and -1.68×10^{-4} A/M for 5, 10 and 20 μL spiked suspension, respectively. The repeatability of 5 μL deposited beads was poor comparing to 20 μL and 10 μL deposits. The latter was selected in subsequent work since it offered good sensitivity and repeatability (RSD = 2.8%, $n = 3$) and it consumed less beads then a 20 μL deposit. For the subsequent inhibition assays it was decided to use a diluted suspension of tyrosinase-NMPS (1.25 mg/mL) since it gave a good response to L-tyrosine (3.33×10^{-4} M) and allowed to reduce substantially the cost of the assays.

3.1.5. Selection of the substrate concentration for the inhibition studies

The opinions about the substrate concentration to be considered in inhibition assays are quite contradictory. Kok et al. [40] concluded, when measuring the inhibition potency of a competitive inhibitor with an acetylcholinesterase and a choline oxidase biosensor, that the inhibition percentage increased by raising the substrate concentration. Therefore they worked in enzyme saturated substrate conditions. Shan et al. [19] demonstrated that for a tyrosinase biosensor for the determination of benzoic acid, the concentration of the catechol substrate did not affect the maximum inhibition percentage but it affected the sensitivity of the

method. It must be noted, however, that the use of a high substrate concentration would not yield sensitive inhibition responses when the quantification of a competitive inhibitor is performed by simultaneous addition of the inhibitor and the substrate. Because in competitive inhibition the substrate competes with the inhibitor for the enzyme active site, and the inhibition, especially at low inhibitor concentrations, would likely not be detected [40].

Two different L-tyrosine concentrations were studied; 3.33×10^{-4} M and 4.76×10^{-5} M, and the obtained IC_{50} for kojic acid were found to be equal within the experimental error (RSD = 2.3%). The percentage of inhibition was evaluated from the response of the uninhibited form of the enzyme versus the response of the enzyme partially inhibited. Taking into account that our device was not designed for the quantification of inhibitors but for the comparative screening and quantification of their inhibitory potency we considered that all the immobilized enzyme molecules must take part in the reaction and this could only be possible in substrate concentration corresponding to the saturation portion of the activity versus substrate curve [40]. Enzyme saturated substrate concentration, namely 3.33×10^{-4} M L-tyrosine, was then selected for further inhibition studies.

Since the concentration of substrate was quite high, it had to be established if the quantity of the dissolved oxygen in the solution was not a limiting factor for a 3.33×10^{-4} M L-tyrosine concentration. After 30 min of air bubbling into the working buffer solution the biosensor response did not change, but after 30 min of nitrogen bubbling the biosensor response decreased dramatically. The latter was also a further indirect evidence of the generation of an electroactive species by the immobilized tyrosinase. It was concluded that in the selected experimental conditions the quantity of oxygen was not a limiting factor and that the enzymatic process was saturated by the substrate.

3.1.6. Michaelis–Menten curve

A typical Michaelis–Menten plot and its linearization by the Lineweaver–Burk plot ($y(A^{-1}) = -7.91 \times 10^3 \times (M^{-1}) - 8.53 \times 10^7$, $R^2 = 1.000$, RSD slope = 3.3%, RSD intercept = 7.7%, $n = 3$) was obtained for L-tyrosine between 2.49×10^{-6} M and 4.41×10^{-4} M in the above described optimal working conditions of the tyrosinase based biosensor, namely: applied potential (E_{app}) = −100 mV, 0.1 M phosphate buffer pH 6.5, 10 μL of 1.25 mg/mL tyrosinase-NMPS suspension spiked onto the mCPE surface. The kinetic parameters K_m^{app} and I_{max} were calculated as the average of 3 consecutive determinations: $K_m^{app} = 9.7 \times 10^{-5}$ M (RSD = 3.1%, $n = 3$) and $I_{max} = -1.2 \times 10^{-8}$ A (RSD = 7.5%, $n = 3$).

The obtained K_m^{app} values (Table 2) can be compared to those reported in the literature for the enzyme in solution, i.e. $K_m^{app} = 6.2 \times 10^{-4}$ M [23] or 3.3×10^{-4} M [41] at pH 6.5, illustrating the fact that the applied immobilization technique maintained the enzyme affinity for its substrate.

3.2. Inhibitors assay

The developed biosensor was designed for testing inhibitors of the skin pigmentation process. When testing their potency, there are many factors which contribute to the final results: type of enzyme, quantity of immobilized enzyme, immobilization method, type of substrate, substrate concentration, time of contact between the enzyme, substrate and inhibitor, pH, temperature, applied potential, rate of solution stirring. In order to obtain a correct comparison of the inhibition potency of the studied compounds, it was worked under the same experimental conditions [19].

As the developed device was a novel analytical tool, in order to test its efficiency, it was applied to the study of compounds that have already demonstrated their activity in clinical practice or by using other analytical methods.

Table 1
IC_{50} for kojic acid, benzoic acid, azelaic acid and ascorbic acid.

IC_{50} (μM)	Kojic acid	Benzoic acid	Azelaic acid	Ascorbic acid
IC_{50} 1	3.66	72.0	125	12.4
IC_{50} 2	3.57	72.0	127	11.6
IC_{50} 3	3.71	72.6	128	11.0
IC_{50} 4	3.72	70.9	119	12.2
IC_{50} 5	3.63	70.5	127	11.7
$IC_{50average}$	3.66	71.6	125	11.8

The proposed biosensor was intended to test inhibitors that have two different mechanisms of action, namely: true enzyme inhibitors which bind to the active site of the enzyme and inhibitors which consume the dopaquinone intermediate of the melanin synthesis pathway.

It was checked that the NMPS mCPE (i.e. without tyrosinase) gave no response under the selected experimental conditions and in the presence of the studied compounds. It was found that L-tyrosine, kojic acid, benzoic acid, azelaic acid and ascorbic acid gave no amperometric signal.

Also blank experiments at the tyrosinase-NMPS-mCPE without addition of the L-tyrosine substrate were performed. No amperometric response was observed for the tested inhibitors.

3.2.1. "True" enzyme inhibitors

Kojic acid, benzoic acid and azelaic acid inhibit the melanin formation by competing with tyrosinase natural substrate, L-tyrosine, for the binding to the enzyme active site. As illustrated in Fig. 3a typical time-dependent response was obtained at the tyrosinase-NMPSs biosensor by L-tyrosine injection followed by stepwise additions of kojic acid. It can be seen that the biosensor baseline was reached within 600 s allowing the experiment to be performed. Once the steady state amperometric response of L-tyrosine (3.33×10^{-4} M) was observed, successive additions of inhibitor produced a diminution of the reduction current. This clearly indicated that kojic acid interfered with the production of dopaquinone at the electrode surface. The inhibition percentage (In %) increased with the inhibitor concentration. Kojic acid was tested in the concentration range 2.50×10^{-6} to 1.24×10^{-5} M where it inhibited between 33% and 88% of the response to L-tyrosine. Same experiments were performed for benzoic acid in the concentration range 3.98×10^{-5} to 1.96×10^{-4} M where it inhibited between 34% and 81% of the response and also for azelaic acid in the concentration range 9.90×10^{-5} to 4.76×10^{-4} M where it inhibited between 43% and 88% of the response.

The inhibition calibration curve of kojic acid is shown in the insert of Fig. 3. IC_{50}, the concentration of inhibitor which inhibited 50% of the L-tyrosine signal, was calculated from 5 different curves by plotting 1/In(%) versus 1/inhibitor concentration (Table 1): kojic acid $IC_{50} = 3.7 \times 10^{-6}$ M, RSD = 1.6%, $n = 5$ ($y = 4.54 \times 10^{-8}x + 7.61 \times 10^{-3}$, $R^2 = 0.9999$, RSD slope = 3.1%, RSD intercept = 4.1%), benzoic acid $IC_{50} = 7.2 \times 10^{-5}$ M, RSD = 1.2%, $n = 5$ ($y = 7.88 \times 10^{-7}x + 8.99 \times 10^{-3}$, $R^2 = 0.9990$, RSD slope = 5.1%, RSD intercept = 6.3%) and azelaic acid $IC_{50} = 1.3 \times 10^{-4}$ M, RSD = 3.0%, $n = 5$ ($y = 1.60 \times 10^{-6}x + 7.76 \times 10^{-3}$, $R^2 = 0.9996$, RSD slope = 9.3%, RSD intercept = 7.6%).

The study of the kinetics and of the mechanism of inhibition of kojic acid, benzoic acid and azelaic acid was also carried out. The response of the tyrosinase-NMPSs biosensor to L-tyrosine was studied in the absence and in the presence of different concentrations of inhibitor (Fig. 4). Table 2 data show that approximately the same maximum current was obtained but different values for the apparent Michaelis–Menten constant (K_m^{app}), determined following a Lineweaver–Burk plot, were calculated when various amounts of kojic acid was added in the initial testing solution. The same experiment was performed and the same conclusions were

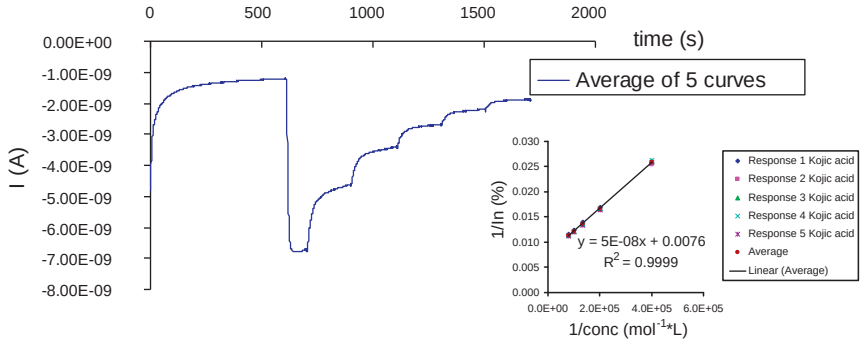

Fig. 3. Tyrosinase based biosensor amperometric response. L-Tyrosine 3.33×10^{-4} M, kojic acid inhibitor between 2.50×10^{-6} and 1.24×10^{-5} M, 0.1 M phosphate buffer pH 6.5, $E_{app} = -100$ mV, 10 μL of a 1.25 mg/mL tyrosinase-NMPS suspension spiked onto the mCPE. Insert: inhibition calibration curve.

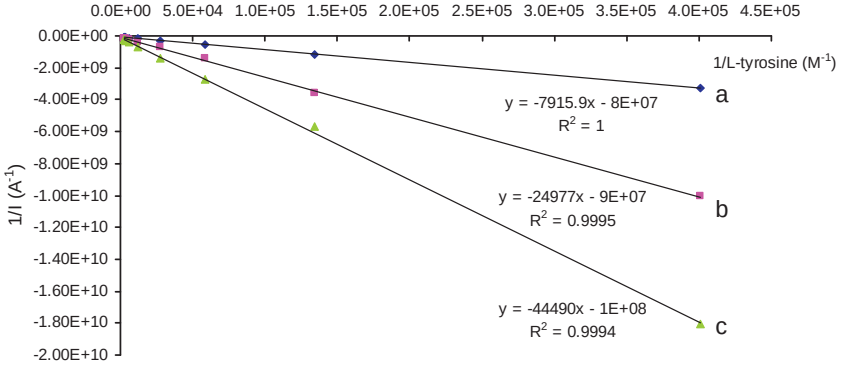

Fig. 4. Tyrosinase based biosensor amperometric data: 1/signal versus 1/[L-tyrosine]. L-Tyrosine between 2.48×10^{-6} and 4.39×10^{-4} M, a: without kojic acid, b: in the presence of 2.09×10^{-6} M kojic acid, c: in the presence of 4.18×10^{-6} M kojic acid, 0.1 M phosphate buffer pH 6.5, $E_{app} = -100$ mV, 10 μL of a 1.25 mg/mL tyrosinase-NMPS suspension deposited onto the mCPE.

dropped out also for benzoic acid and azelaic acid, data shown in Table 2. For the three inhibitors, a different value of I_{max} was obtained (-1.1×10^{-8} A, RSD = 9.2%, -1.2×10^{-8} A, RSD = 21.1% and -5.3×10^{-9} A, RSD = 5.0%, $n = 3$ for kojic acid, benzoic acid and azelaic acid respectively). This was not due to the nature of the studied molecule but to the fact that the inhibitors were tested at different periods of time after the preparation of the tyrosinase-NMPS suspension. However, as it can be seen from the stability tests, this does not influence the inhibition parameters (see below).

Since, for the same inhibitor I_{max} did not change and K_m^{app} increased proportionally with the inhibitor concentration, for kojic acid ($y(A^{-1}) = 8.13 \times 10^{+1} x(M^{-1}) + 9.93 \times 10^{-5}$, $R^2 = 0.9994$), benzoic acid ($y(A^{-1}) = 8.42x(M^{-1}) + 6.95 \times 10^{-5}$, $R^2 = 0.9941$) and azelaic acid ($y(A^{-1}) = 2.04x(M^{-1}) + 7.55 \times 10^{-5}$, $R^2 = 0.9946$), a competitive inhibition process was inferred for the three studied molecules. This conclusion is in agreement with literature data for kojic acid [14], azelaic acid [7] and benzoic acid [19]. From the primary Lineweaver–Burk data, secondary plots were generated, by plotting the slopes from the primary plots versus inhibitor concentration (Table 2) in order to determine the apparent inhibition constant, K_i (Fig. 5). The obtained K_i were 8.6×10^{-7} M, 2.0×10^{-5} M and 4.2×10^{-5} M for kojic acid, benzoic acid and azelaic acid, respectively.

3.2.2. Inhibitors of the melanogenesis process

Another inhibitor tested was ascorbic acid. Unlike kojic acid, benzoic acid or azelaic acid, ascorbic acid does not interact with the enzyme. It is known to inhibit the formation of melanin by reducing the dopaquinone back to L-dopa and thus it interrupts the melanin synthesis pathway.

The inhibition experiments were conducted in the same manner as in the case of true tyrosinase inhibitors. A time-dependent curve of the tyrosinase-NMPSs biosensor response to L-tyrosine substrate followed by successive additions of ascorbic acid was realized. The amperometric response increased as L-tyrosine was added into the electrochemical cell. Then, due to successive additions of ascorbic acid into the L-tyrosine solution (3.33×10^{-4} M) the reduction current decreased clearly indicating that ascorbic acid interfered with the reduction of dopaquinone at the electrode surface. The inhibition percentage (In %) of ascorbic acid increased by raising its

Table 2
Kojic acid, benzoic acid and azelaic acid inhibition data.

Kojic acid	Without kojic acid	2.09×10^{-6} M kojic acid	4.18×10^{-6} M kojic acid
K_m (M)	9.7×10^{-5}	2.7×10^{-4}	4.4×10^{-4}
I_{max} (A)	-1.2×10^{-8}	-1.1×10^{-8}	-9.8×10^{-9}
Benzoic acid	Without benzoic acid	3.33×10^{-5} M benzoic acid	6.67×10^{-5} benzoic acid
K_m (M)	8.2×10^{-5}	3.3×10^{-4}	6.4×10^{-4}
I_{max} (A)	-1.1×10^{-8}	-1.5×10^{-8}	-1.0×10^{-8}
Azelaic acid	Without azelaic acid	8.32×10^{-5} M azelaic acid	1.64×10^{-4} M azelaic acid
K_m (M)	8.2×10^{-5}	2.3×10^{-4}	4.2×10^{-4}
I_{max} (A)	-5.0×10^{-9}	-5.4×10^{-9}	-5.6×10^{-9}

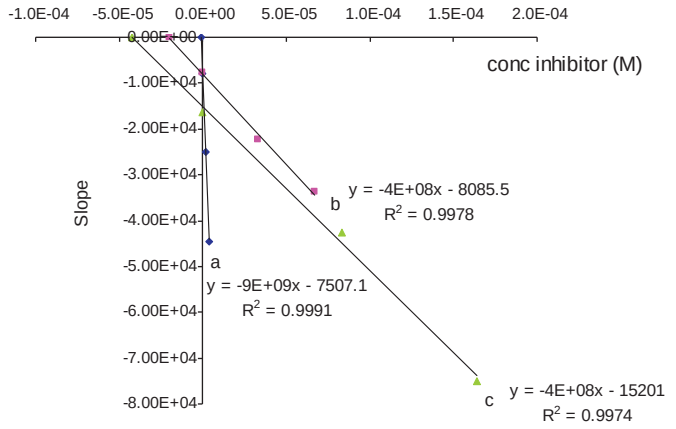

Fig. 5. Tyrosinase based biosensor data: slope of the data versus [In] (Table 2). a: kojic acid, b: benzoic acid, c: azelaic acid, 0.1 M phosphate buffer pH 6.5, $E_{app} = -100$ mV, 10 μL of a 1.25 mg/mL tyrosinase-NMPS suspension deposited onto the mCPE.

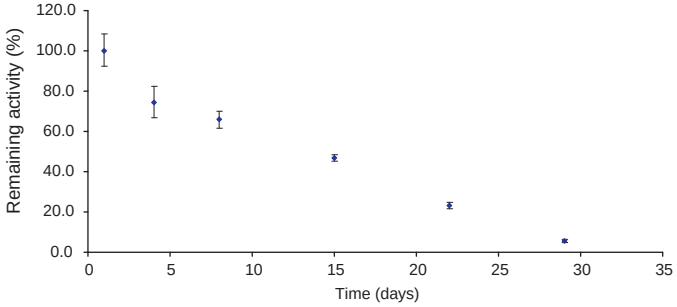

Fig. 6. Tyrosinase-NMPS stability test. Tyrosinase based biosensor, L-tyrosine substrate 3.33×10^{-4} M, 0.1 M phosphate buffer pH 6.5, $E_{app} = -100$ mV, 10 μL of a 1.25 mg/mL tyrosinase-NMPS suspension dilution deposited onto the mCPE.

concentration. Ascorbic acid was tested in the concentration range 6.62×10^{-6} to 3.23×10^{-5} M where it was found to inhibit between 33% and 82% of the biosensor response. The inhibitory potency, IC_{50}, of ascorbic acid was calculated: $IC_{50average} = 1.2 \times 10^{-5}$ M, RSD = 4.8%, $n = 5$ ($y = 1.60 \times 10^{-6}x + 7.76 \times 10^{-3}$, $R^2 = 0.9996$, RSD slope = 6.1%, RSD intercept = 7.2%) (Table 1).

By comparing the IC_{50} obtained in the same experimental conditions for all the four tested inhibitors, the order of their inhibitory potency was: kojic acid ($IC_{50} = 3.7 \times 10^{-6}$ M), ascorbic acid ($IC_{50} = 1.2 \times 10^{-5}$ M), benzoic acid ($IC_{50} = 7.2 \times 10^{-5}$ M) and azelaic acid ($IC_{50} = 1.3 \times 10^{-4}$ M).

Even if the obtained values of IC_{50} and K_i of the studied compounds cannot be directly compared with literature data due to the various factors that may contribute to the final outcomes, the obtained results are in agreement with our expectations, based on their practical utilization and on the general conclusions from the literature. Both the IC_{50} and the K_i show that kojic acid was the most potent inhibitor in comparison with ascorbic, benzoic and azelaic acid. Kojic acid is considered the standard inhibitor due to its high inhibitory potency [6] and is known as the most potent active ingredient in the commercialized skin-whitening cosmetic products. Ascorbic acid is well known for its medium inhibitory property therefore it is found as adjuvant in most of the cosmetic creams for hyperpigmentation. Azelaic acid is mainly used as an anti-acne agent but also as adjuvant in skin-whitening creams due to its inhibitory properties along with other more powerful inhibitors. Benzoic acid is above all an antibacterial agent but due to its inhibitory characteristics it is considered a preferential preservative in cosmetic creams for hyperpigmentation treatments. Benzoic acid is generally used as a tyrosinase inhibitor in food industry.

It should be outlined that the developed device allowed the quantification and the characterization of inhibitors from the point of view of their interaction with tyrosinase and the enzymatic reaction in the presence of the natural substrate L-tyrosine. In the skin pigmentation process these compounds may interference also in other biochemical steps having as final result a decrease in melanin formation. For example, azelaic acid inhibits melanin formation through other mechanism besides its interaction with the tyrosinase active site. It may also interfere with DNA synthesis and mitochondria activity in hyperactive and abnormal melanocytes [7].

3.2.3. Stability and reproducibility

The long-term stability of the immobilized tyrosinase-NMPS was evaluated by measuring the biosensor response in the presence of L-tyrosine (3.33×10^{-4} M). The biosensor retained about 66% (RSD = 1.1%, $n = 3$) of the original response after one week, 46% (RSD = 2.6%, $n = 3$) after the second week, 23% (RSD = 2.1%, $n = 3$) after the third week and 5% (RSD = 3.2%, $n = 3$) after four weeks (Fig. 6). When not in use the tyrosinase-NMPSs suspension was maintained at 4 °C. IC_{50} of kojic acid was determined at different periods of time following enzyme immobilization. Interestingly it was observed that IC_{50} was not affected by the loss of enzyme activity: the day of suspension preparation $IC_{50} = 3.8 \times 10^{-6}$ M, after one week $IC_{50} = 3.6 \times 10^{-6}$ M, after two weeks $IC_{50} = 3.7 \times 10^{-6}$ M and after three weeks $IC_{50} = 3.7 \times 10^{-6}$ M, RSD = 1.5%. After four weeks the IC_{50} was not anymore determined because the amperometric signal to L-tyrosine was considered too small, around 1 nA, to give reliable results.

The IC_{50} for 5 consecutive determinations gave RSD values of 1.6%, 1.2%, 3.0% and 4.8% for kojic acid, benzoic acid, azelaic acid and ascorbic acid, respectively. The good reproducibility of the results can be explained by the fact that the inhibition signal was normalized to the initial signal of the substrate for each curve. Thus, the final results were not influenced by the variations of substrate's signal which can occur due to the loss of the enzyme activity or due to random errors that can happen during biosensor's preparation.

4. Conclusion

A tyrosinase-streptavidine magnetic beads-magnetized carbon paste biosensor for the screening of tyrosinase "true inhibitors" and of the melanogenesis process inhibitors has been realized. The biosensor operation mode is based on monitoring the decrease of the amperometric signal of the electrochemical reduction of dopaquinone, enzymatically generated from L-tyrosine substrate, due to the presence of tyrosinase inhibitors that bind to the enzyme active site such as kojic acid, benzoic acid or azelaic acid or due to the presence of compounds that reduce the dopaquinone intermediate back to L-dopa interrupting the synthesis process like ascorbic acid. The enzyme immobilization is performed via glutaraldehyde activated streptavidine magnetic particles retained onto the surface of a magnetized carbon paste electrode. To the best of our knowledge this is the first electrochemical tyrosinase based biosensor designed for the screening of the inhibitory potency of different skin-whitening agents that uses the enzyme's skin natural substrate, L-tyrosine. The use of L-tyrosine as substrate in the design of the biosensor is the main novelty of the proposed device being the first electrochemical biosensor that mimics the two enzymatic steps of melanin formation in skin. The methodology is simple in use, suitable for rapid analysis in the evaluation of inhibitors potency.

The obtained results are in agreement with the expected ranking of the inhibitory potency of the studied skin whitening agents. The reproducibility of the results is very good and is not influenced

neither by the loss of the enzyme activity nor by other variations that can occur during the immobilization process or which can be due to electrode sensitivity fluctuation. In this respect the use of magnetic particles for tyrosinase immobilization can be regarded as an interesting approach since it allows their use "on demand" and since the sensing electrode is not affected by an enzymatic immobilization process.

Other substances acting through one of the above mentioned mechanisms could be evaluated using the developed device. Taking into consideration the relative simplicity of fabrication and use of this biosensor, it may represent an interesting and valuable analytical tool especially for the evaluation of tyrosinase – inhibitors.

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