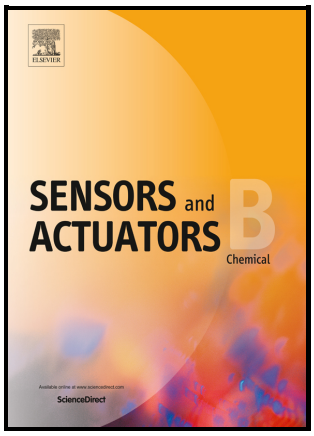

IMPROVING THE PERFORMANCE OF A BIOELECTRONIC TONGUE USING SILVER NANOWIRES. APPLICATION TO MILK ANALYSIS

Coral Salvo-Comino, Patricia Martín-Bartolomé, Jose Luis Pura, Clara Perez-Gonzalez, Fernando Martin-Pedrosa, Cristina García-Cabezón, María Luz Rodríguez-Méndez

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IMPROVING THE PERFORMANCE OF A BIOELECTRONIC TONGUE USING SILVER NANOWIRES. APPLICATION TO MILK ANALYSIS

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Highlights

- Biosensors based on combinations of AgNWs and specific enzymes have been developed
- AFM analysis demonstrates the excellent coverage of nanowires with enzymes
- AgNWs facilitate Direct Electron Transfer between the enzyme and the electrode surface
- Biosensors show low LOD and high enzymatic affinity
- A bioET has been constructed using biosensors combining enzymes and AgNWs
- The AgNWs/bioET can be successfully applied to the analysis of milks

Abstract

Recent advances in the field of electronic tongues (ET) are linked to the development of devices dedicated to a particular application. Following this idea, we have developed a voltammetric bioelectronic tongue (bioET) specifically dedicated to analyze milk. The performance of the multisensor system has been improved by incorporating biosensors combining specific enzymes for the detection of sugars present in milk (β-galactosidase, glucose oxidase and galactose oxidase) with silver nanomaterials. It has been demonstrated that silver nanowires (AgNWs) provide a more effective platform for the immobilization of biomolecules than silver nanoparticles (AgNPs), inducing unique performance characteristics in terms of sensitivity and detection limits. Two multisensor systems have been developed; one based on combinations of AgNWs and enzymes (AgNW/bioET) and a second based on combinations of AgNPs and enzymes (AgNP/bioET). Principal component analysis (PCA) demonstrates that the bioET based on combinations of AgNWs and enzymes (AgNW/bioET) can discriminate 9 classes of milk with different fat content (skimmed, semi-skimmed and whole), as well as different nutritional compositions (classic, calcium-enriched and lactose-free), with a higher capacity than the bioET based on combinations of AgNPs and enzymes (AgNP/bioET). Support vector machine (SVMR) models show excellent correlation coefficients between the responses of the bioETs and physicochemical parameters commonly used to evaluate the quality of milk (acidity, density, fat, proteins, lactose, total dry matter and non-fat dry matter). The good results obtained support the dairy industry's interest in dedicated bioETs, not only for classification purposes but also to

obtain information concerning several physicochemical parameters in a single measurement.

Keywords: electronic tongue, bioET, electrochemical sensor, milk

1. Introduction

The implementation of new methods to evaluate the quality and safety of milk is of great importance to the dairy industry [1]. Electrochemical sensors offer several advantages, such as high sensitivity and fast response time. For this reason, a wide range of sensors and biosensors for the quality control of milk has been developed [2].

Electrochemical sensors can be combined to obtain multisensor systems that, coupled to a pattern recognition system, form the so-called electronic tongues (ET). ETs have been widely used to analyze foods and beverages offering global information about the sample [3-5]. They have been employed to analyze dairy products and applied in many aspects of quality control, including the evaluation of taste, freshness, detection of adulterations, or recognition of the origin [6]. These general systems consist of potentiometric sensors based on polymeric membranes [7-9] or voltammetric sensors based on metallic electrodes [10-14]. Unfortunately, only few attempts to use e-tongues to assess the chemical composition of milk have been reported [9, 15-16] The analysis of milk using ETs is not a completely solved problem and new improvements in the field are required.

ETs can adopt some of the important advances achieved in the field of electrochemical sensors, whose analytical performance can be improved by incorporating nanomaterials that enhance sensitivity thanks to their excellent electrocatalytic properties [17-19]. Only a few examples of ETs dedicated to the dairy industry have used nanomaterials. These include a voltammetric ET based on nanostructured Layer-by-Layer films [16]; a potentiometric ET using sensors modified with nanoparticles [20]; and an impedimetric ET with electrodes modified with electrospun nanofibers [18].

The performance of ETs could be improved by developing systems dedicated to a specific application. This is why the development of bioelectronic tongues (bioETs) combining unspecific sensors with biosensors has been an important innovation: bioETs simultaneously provide overall information about the sample (as classical ETs do) plus information about specific compounds provided by the biosensors included in the array [21-27]. In the case of dairy products, the presence of biosensors could provide quantitative information about sugars (lactose, glucose or galactose) and this will be an important advantage in the field. The efficiency of electrochemical biosensors can be fostered by combining enzymes with such nanomaterials as nanocarbons or nanoparticles. Such nanomaterials are efficient electron mediators and are excellent immobilization platforms. In addition, nanomaterials can be used to obtain third generation biosensors based on Direct Electron Tranfer (DET) [28]. Only a few examples of voltammetric bioETs using combinations of enzymes and nanomaterials have been reported. For instance, a bioET containing phenol oxidases and glucose oxidase combined with nanoparticles has been successfully used to analyze grapes and musts [29, 30]. The development and study of the capabilities of multisensory systems containing different combinations of enzymes and nanomaterials is a new field of research.

Our purpose here is to design a bioET specifically dedicated to milk by developing biosensors formed by the combination of enzymes specific to the sugars present in milk and nanomaterials. Milk is rich in lactose, glucose and galactose and their concentration is directly related to the quality and type of milk [31]. Several examples of individual biosensors dedicated to the detection of lactose, glucose and galactose, where β-galactosidase (β-Gal), glucose oxidase (GOx) and galactose oxidase (GaOx) have been co-immobilized with nanomaterials (graphene, CNTs, AuNPs), have been published [28,32-35]. Silver nanoparticles (AgNPs) can be an excellent alternative to enhance the performance of biosensors due to their electrochemical properties, their high electrical conductivity (6.301×10^7 $S \cdot m^{-1}$), and their ability to amplify bioelectrochemical signals [36-39]. Recently, it has been evidenced that the morphology of the nanomaterial has a strong influence on the electrochemical response of biosensors. One dimensional (1D) metallic nanomaterials (nanowires or nanotubes) have much higher electron mobility and density of active sites, as well as a higher specific surface area than their 0D counterparts [40]. Despite this interest, only a few examples of biosensors combining AgNWs with enzymes have been reported. These include one example of a tyrosinase biosensor [41], three examples of glucose biosensors [42-44], and one example of a cholesterol biosensor [45]. Until now, the benefits of incorporating AgNWs based biosensors in a bioET have not been evaluated.

As mentioned before, the objective of this work is to design a bioET dedicated to analysing milk. The system will be used for discrimation and classification purposes. In addition, in order to obtain quantitative information, the system will include three enzymes (β-Gal, GOx and GaOx) specific to compounds present in milk (lactose, glucose and galactose). To enhance the performance of the biosensors, enzymes were combined with silver nanowires that have been used as immobilization supports. The bioET based on AgNWs and enzymes (AgNW/bioET) was applied to the analysis of 63 UHT milk samples with different fat content and nutritional composition. The performance was compared to that of a similar bioET based on combinations of nanoparticles and enzymes (AgNP/bioET). The capability to discriminate and classify has been evaluated using unsupervised (PCA) and supervised (LDA) multivariate methods. SVMR was also used to create mathematical models to correlate the physicochemical parameters commonly used in the milk industry to evaluate the quality of milk (acidity, density, lactose, fat and protein content and total dry matter and non-fat dry matter) with the electrochemical responses of the bioETs. The benefits of using Ag nanowires (AgNWs) instead of Ag nanoparticles (AgNPs) have been established by comparing the capabilities of the bioET based on AgNWs with that of a similar device based on AgNPs.

2. Materials and methods

2.1. Chemicals

Silver nitrate ($AgNO_3$), polyvinylpyrrolidone (PVP, Mw = 55,000), ethylene glycol (EG) anhydrous (99.8%), phosphate buffer, acetone, ethanol, Nafion® 117 solution, galactose oxidase (GaOx) (from *Dactylium dendroides*, 3000 ud/g solid), β-galactosidase (β-Gal), (from *Aspergillus oryzae*, 10,9 ud/mg solid) and glucose oxidase (GOx) (from *Aspergillus niger*, 168400 ud/mg solid) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Deionized water from MilliQ (Millipore-Sigma Aldrich, Darmstadt, Germany) (resistivity 18.2 MΩ·cm) was used in all experiments. Potassium chloride (KCl) was provided by PanReac AppliChem (Barcelona, Spain).

Boron Doped diamond substrates were provided by NEOCOAT (La Chaux-de-Fonds, Switzerland). $NaBH_4$ 98% was purchased from Alfa Aesar (Haverhill, MA, USA).

2.2. Instruments

The synthesis of the AgNWs was carried out using a Perimax peristaltic pump from Spetec and a Sorvall ST 8 Centrifuge (Thermo Sci-entific, Walthman, USA). UV–Vis characterization of silver nanomaterials was performed in a UV-2600 device (Shimadzu, Duisburg, Germany). Electrochemical measurements were carried out using a PGSTAT128 potentiostat/galvanostat (Autolab Metrohm, Utrecht, The Netherlands) in a three-electrode electrochemical cell, using a BDD sensor as the working electrode. Ag|AgCl/KCl was used as the reference electrode and a platinum sheet (2 cm^2) as the counter electrode.
Atomic force microscopy (AFM) was used to determine the topography of the films deposited on the BDD substrates in a Cypher ES Environmental AFM device (Oxford Instruments, Asylum Research, Wiesbaden, Germany) operated in tapping mode with blueDrive photothermal excitation technology. The tip used was AC160TSA-R3 (Oxford Instruments, Asylum Research, Wiesbaden, Germany).

2.3. Milk samples

A set of 63 UHT milk samples corresponding to 9 classes of milk (7 replicas per class) with different fat content and different nutritional composition were used in the study. The milk categories included were: Classic Skimmed (CS), Classic Semi-Skimmed (CSS), Classic Whole (CW), Calcium Skimmed (CaS), Calcium Semi-Skimmed (CaSS), Calcium Whole (CaW), Lactose-Free Skimmed (LFS), Lactose-Free Semi-Skimmed (LFSS) and Lactose-Free Whole (LFW).
Physicochemical parameters were obtained by classical standard methods: acidity (Tritration method ISO 22113:2012), density (Hydrometer method ISO 2449:1974), fat (Gravimetry Röse-Gottlieb method ISO 1211:2010), proteins (Kjeldahl method ISO 8968-1:2014), lactose content (HPLC ISO 22662:2007). Total dry matter (TDM) and non-fat dry matter (NFDM) were also analysed (ISO 6731:2010) [46]. Results are collected in Table S.1.

2.4. Preparation of the biosensors. Bioelectronic tongue

AgNWs were synthesized following a modification of the polyol method described in a previous work [41]. AgNPs were synthesized following the Creighton method [47]. The silver nanomaterials were deposited onto BDD substrates (1 cm^2) by adding 25 μL of the corresponding silver nanomaterial suspension (2 g/L AgNWs or AgNPs in ethanol) by drop-casting. After drying, 25 μL of the enzyme (5 g/L GOx, GaOx and β-Gal in phosphate buffer 0.01 M, pH 7.0) were drop-casted onto the electrode surface. After drying, 25 μL of Nafion diluted in ethanol 1:3 was drop-casted and left to dry overnight at room temperature. Finally, the biosensors were washed with a phosphate buffer 0.01 M solution.

2.5. Bioelectronic tongues (bioETs)

The biosensors thus obtained were used to construct two bioETs. The first, denoted as AgNW/bioET, was based on combinations of enzymes with AgNWs (β-Gal/AgNWs, GOx/AgNWs and GaOx/AgNWs). The second, denoted as AgNP/bioET, was based on combinations of enzymes and AgNPs (β-Gal/AgNPs, GOx/AgNPs and GaOx/AgNPs and). Both bioETs were used to analyze milks with different nutritional characteristics. The electrochemical responses were probed by cyclic voltammetry (CV) in milk diluted 1:2 in 0.1 M KCl solution. The area of the BDD exposed to milk was 0.07 cm^2 limited by an O-ring seal.

The measurements were registered over a potential range of -1 V to 1 V, a potential step of 10 mV, and at a scan rate of 100 mV/s. It is important to remark that the optimum pH of the enzymes GOx, GaOx and β-Gal are 7.5, 7 and 6 respectively. All three enzyme enzymes are active at pHs between 5.5 and 9 that corresponds to the pH of diluted milk.

The multivariate data analysis was performed by using MATLAB R2021a (The Mathworks Inc., Natick, MA, USA). For statistical purposes, milk samples were measured by septuplicate. To reduce the high dimensionality of the voltammetric signals, a feature extraction tool, based on "bell-shaped-windowing" curves called "kernels", was used [48,49]. Using this method, eight coefficients per voltammogram were obtained. These coefficients were then used as the input variables for the Principal Component Analysis (PCA) and the Linear Discriminant Analysis (LDA), used for the recognition of sample patterns and dis(similarities) between varieties of milk. Mathematical correlations between the results obtained using the voltammograms registered by the bioET and the physicochemical analysis were established using Support Vector Machine Regression (SVMR) models. Radial Basis Function was chosen as the core function because of its ability to avoid overfitting and its capability to establish non-linear correlations between the data sets. All the classification models were subjected to leave one out validation.

3. Results and discussion

3.1.Structural characterization of the biosensors

AFM images of the BDD electrodes covered with AgNPs, AgNWs and combinations of nanomaterials with enzymes are illustrated in Figure 1. AgNPs appeared as spherical structures with an average diameter of 80±10 nm. The images of AgNWs displayed large and homogeneous wires with an average diameter of 550±43 nm and length of 10±2 μm. When the enzymes were immobilized on the nanomaterial surface, an increase in the size of the nanostructures was observed. This is illustrated in Figures 1.c.-1.f, where the topographic images of β-Gal deposited onto AgNPs or AgNWs are shown. As observed in the figure, the enzymes appeared homogeneously distributed on the nanomaterial surface, producing a clear increase in the diameter of the nanostructures (diameters of 180±28 nm for β-Gal/AgNPs and 750±81 nm for β-Gal/AgNWs). Similar results were obtained with GaOx (GaOx/AgNPs diameter 173 ±23 nm; GaOx/AgNWs diameter 717 ±74 nm;) and GOx (GOx/AgNPs diameter 160 ±19 nm; GOx/AgNWs diameter 692 ±73 nm

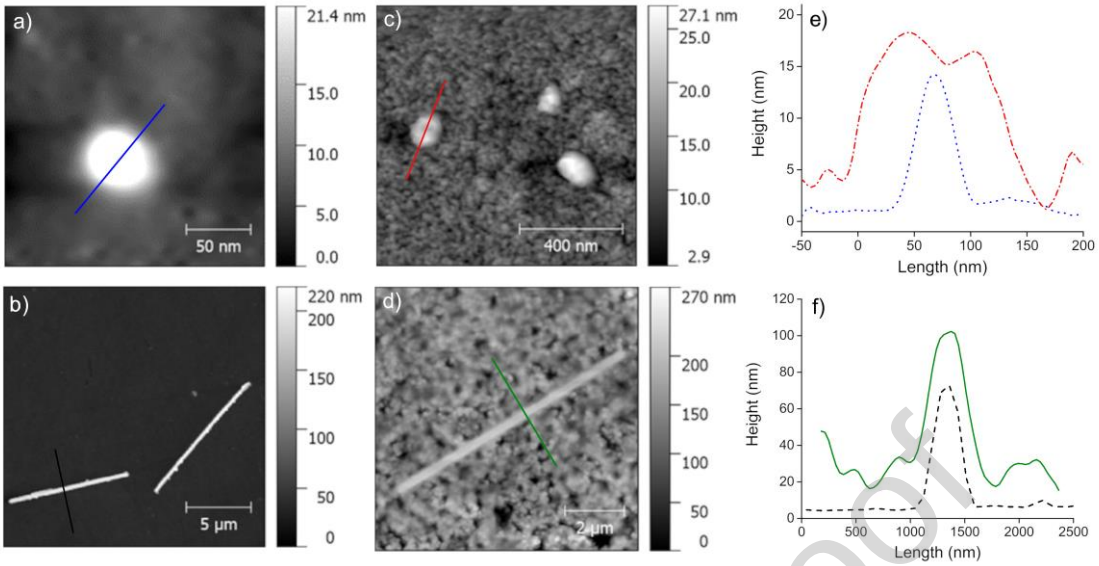


Figure 1. AFM topography of (a) AgNPs, (b) AgNWs, (c) β-Gal/AgNPs, (d) β-Gal /AgNWs. AFM profile of (d) AgNPs (blue dotted line) and β-Gal/AgNPs (red dashed-dotted line) and (f) AgNWs (black dashed line) and β-Gal/AgNWs (green solid line).

3.2. Electrochemical impedance spectroscopy (EIS)

Using electrochemical impedance spectroscopy (EIS), the interface properties of the sensors were investigated. This approach determines the electron transfer resistance (R_{et}), from the diameter value of the semicircle obtained from the Nyquist diagram. EIS experiments were carried out on a bare BDD substrate, BDDs modified with AgNPs or AgNWs, BDDs modified with enzymes, and BDDs modified with enzyme/AgNPs or enzyme/AgNWs composites, immersed in 5mmolL^{-1} $Fe(CN)_6^{3-/4-}$ in KCl 0.1M. The results obtained are collected in Table S.2. The Nyquist plot of the electrodes analyzed showed similar trends and this is illustrated in (Figure 2). As observed, the R_{et} of the BDD bare electrode was estimated to be 65.1±3.6 Ω/cm^2. This value decreased to 47.1±1.8 Ω/cm^2 when AgNPs were deposited on the electrode surface and to 14.3±0.4 Ω/cm^2 when AgNWs were used (Figure 2.a). Taking into account the fact that the masses of the AgNPs and AgNWs deposited onto the BDD electrode were similar, this result confirms that the conductivity and the electron transfer resistance of the modified electrode is affected by the shape and size of the silver nanomaterial. The higher aspect ratio of AgNWs provides higher electrical conductivity and electron transfer capabilities than AgNPs. The Nyquist plot of the electrodes modified with enzymes is illustrated in Figure 2.b for β-Gal. As observed in the figure, the value of the R_{et} for a BDD electrode modified with β-Gal was found to be 139.6±5.7 Ω/cm^2. The resistance decreased in the presence of nanomaterials, being 43.2±1.4 Ω/cm^2 for β-Gal/AgNPs and 36.2±0.7 Ω/cm^2 for β-Gal/AgNWs. The same trends were observed in biosensors modified with GOx and GaOx.

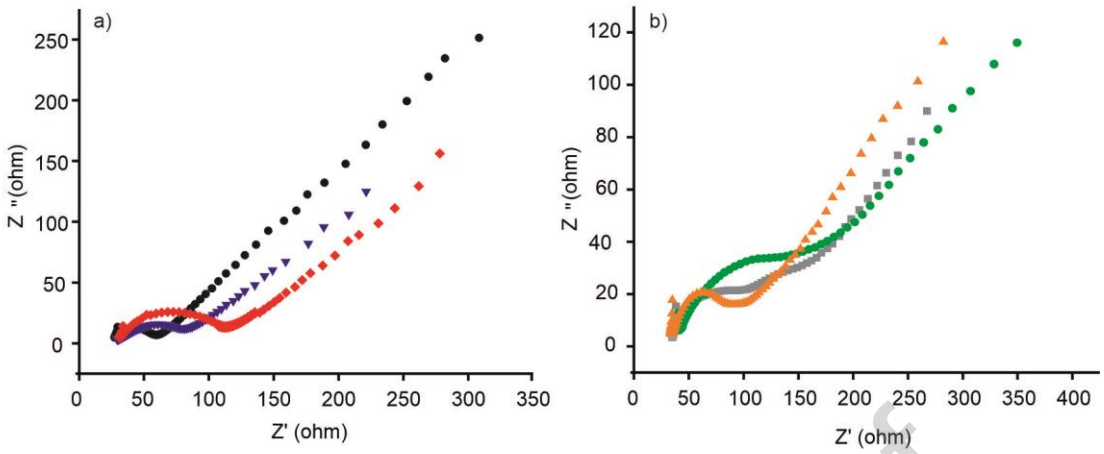


Figure 2. Electrochemical impedance spectroscopy (EIS) of (a) bare BDD (red squares), AgNP (blue triangles) and AgNW (black circles) sensors and (b) β-Gal (green circles), β-Gal/AgNW (orange triangles) and β-Gal/AgNP (grey squares) biosensors in the presence of 5mmolL^{-1} $Fe(CN)_6^{3-/4-}$ in KCl 0.1M.

3.3.Electrochemical properties

The electrochemical characteristics of the sensing devices were analyzed by studying the responses towards standard solutions of the target analytes (10^{-4} mol·L^{-1} glucose, galactose or lactose in 0.01 mol·L^{-1} phosphate buffer pH 7.00) (Figures 3, S1 and S2,).
The electrochemical responses of BDD electrodes modified with AgNPs or AgNWs showed the typical Ag^+/Ag redox couple at ca. ~200 mV (cathodic wave) and ~400 mV (anodic wave) [41] (Figure S3).
When GOx or GaOx were deposited on a bare BDD electrode, very small response was observed, apart from a wide cathodic peak at -900 mV corresponding to the formation of H_2O_2 according to reaction 1 linked with the oxidation of the enzyme.

$$O_2 + 2H^+ + 2\,e^- \leftrightarrow H_2O_2 \qquad \text{(reaction 1)}$$

The responses of the GOx/AgNPs biosensor showed redox peaks at E_{an}= -30 mV and at E_{cat}= -250 mV, and GOx/AgNWs biosensor showed peaks at E_{an}= 70 mV and at E_{cat}= -200 mV, for GOx/AgNWs. These peaks are attributed to the transference of two protons and two electrons from glucose to GOx through the electroactive enzyme cofactor FAD to produce $FADH_2$ (reaction 2).

$$GOx/FAD + 2e^- + 2H^+ \leftrightarrow GOx/FADH_2 \qquad \text{(reaction 2)}$$

This result confirmed the occurrence of Direct Electron Transfer (DET) at the silver nanomaterial surface. This is an interesting result because DET is quite difficult to achieve, since the redox-active centers are deeply embedded in the enzyme structure. It has been reported that DET can be facilitated by depositing GOx or GaOx on top of such nanostructures as carbon nanotubes (CNTs), graphene or carbon nanospheres, among others [28, 50-52]. The results displayed here are an example of DET using

silver nanomaterials as support. It is worth noting that the intensity of the peaks was higher when AgNWs were used as the enzymatic support over spherical nanoparticles.

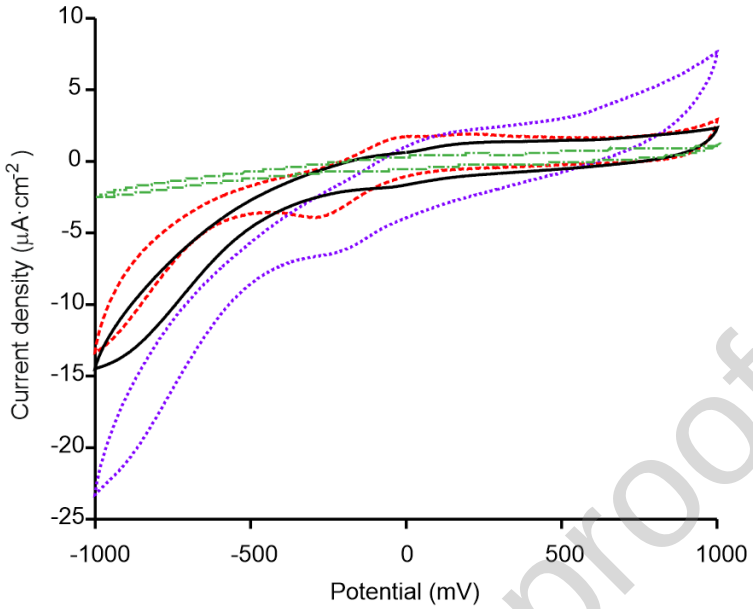
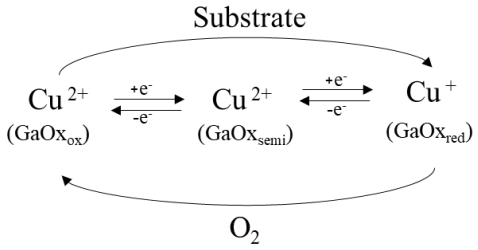


Figure 3. Cyclic voltammograms obtained for GOx (black solid line), GOx/AgNPs (red dashed line) and GOx/AgNWs (blue dotted line) electrodes in the presence of glucose 10^{-4} mol·L^{-1} in 0.01 mol·L^{-1} phosphate buffer pH 7.00 and for GOx/AgNWs (green dash-dotted line) in the absence of glucose in 0.01 mol·L-1 phosphate buffer pH 7.00.

The electrochemical response of galactose oxidase biosensors (GaOx/AgNPs and GaOx/AgNWs) also involved the transfer of two electrons and two protons to catalyze the oxidation of galactose through the oxidation and reduction of its copper center (reaction 3) [53]. It was characterized by an anodic peak at ~200 mV and a cathodic peak at ~100mV, characteristic of the Cu(II)/Cu(I) redox couple of the enzyme. Very small peaks were observed when GaOx was deposited on unmodified BDD electrodes because the redox center is not accessible.



(reaction 3)

In the case of biosensors based on β-Gal, the cathodic wave observed at ~-850 mV was due to the variations of ion concentrations produced by the lactose hydrolysis involving proton transfer through the nafion membrane [54]. In the absence of redox processes, DET is not applicable.

The voltammetric responses obtained at different scan rates (from 10 to 1000 mV·s^{-1}) were employed to study the dynamics of the electron transfer process of the biosensors.

In the case of GOx and GaOx, the studies were carried out using the cathodic peak corresponding to the DET process in the 200-250 mV region. In the cases of β-Gal, the studies were carried out in the cathodic peak at ca. -850 mV. All the biosensors showed a linear relationship with the square root of the scan rate, in the range of 100 – 1000 $mV \cdot s^{-1}$. This is typical of a diffusion-controlled process. In the range of 10-100 $mV \cdot s^{-1}$, the cathodic current was linearly dependent on the scan rate, indicating that, within this range, the limiting step was a surface-controlled process. The obtained slopes and the correlation coefficients are listed in Table 1

The studies carried out at increasing scan rates were also used to calculate the active area A and the surface coverage (Γ) to determine the molecular active sites of the biosensors and to evaluate the benefits of using AgNWs or AgNPs. For this purpose, the Randles-Sevick equation was used to estimate the electrochemical

active surface area (A) of the developed biosensors in the high scan rate range, following equation 1:

$$Ip = 0.4463nFA\left(\frac{nF}{RT}\right)^{1/2} D^{1/2}v^{1/2}\, C \qquad \text{(eq. 1)}$$

where n is the electron transfer number, A is the active area of the electrode surface, D is the diffusion coefficient for the oxidized species at 298.15K ($D_{glucose}$= $6.7 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$, $D_{galactose}$=$7.0 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$ and $D_{lactose}$=$5.6 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$ [55]), C is the concentration of the solution, υ is the scan rate and other symbols have their usual meanings. The area A was calculated from the slope of the curve obtained by representing the cathodic peak current versus the square root of the scan rate. Then, the electrode surface coverage (Γ) was calculated from the slope of the curve obtained by representing the cathodic peak current versus the scan rate, using the Laviron equation (eq. 2) [56]:

$$I_p = \frac{n^2F^2A\Gamma v}{4RT} \qquad \text{(eq. 2)}$$

The product A*Γ was used to calculate the number of molecular active sites, which is a useful parameter to evaluate the influence of the shape of the silver nanostructures. As shown in Table 1, the number of active sites increased in the presence of metallic nanomaterials. Remarkably, the higher values were always obtained when AgNWs were used as enzymatic support instead of AgNPs. For instance, the number of active sites was 5.20 μmol for GOx (in the absence of nanomaterial) and increased to 6.01 μmol (GOx/AgNPs), and to 6.62 μmol (GOx/AgNWs). The same tendency was observed for GaOx biosensors, where the number of active sites was 5.20 μmol in the absence of nanomaterial, 6.96 μmol for GaOx deposited on AgNPs, and 8.87 μmol for GaOx immobilized on AgNWs. This increase can be attributed to conformational changes induced by the interaction between the enzymes and the nanomaterials, which facilitate the DET between the enzymatic active site and the electrode [57, 58]. The effect is more intense in biosensors containing AgNWs because nanowires offer a higher contact surface to the enzymes and show a larger capacity for protein loading than nanoparticles. The increase in the number of active sites was even stronger in the case of β-Gal (2.56 μmol for β-Gal; 2.88 μmol for β-Gal/AgNPs and 4.70 μmol for β-Gal/AgNWs).

The direct electron rate constants (k_s) and the charge transference coefficient α associated to the DET phenomenon for GOx and GaOx biosensors were calculated at a scan rate value of 1000 mV/s following equation 3[56]:

$$Logks = \alpha log(1-\alpha)+(1-\alpha)log\alpha - log\ (RT/nF\upsilon\ ) - \alpha(1-\alpha)(nF\Delta Ep/2.3RT) \quad \text{(eq. 3)}$$

The k_s and values for the GOx/AgNWs and GOx/AgNPs biosensors were 1.05 s^{-1} and 0.28 s^{-1} and 1.49 s^{-1} and 1.31s^{-1} respectively. The α value could also be easily calculated from the slope of I_{pc} vs. log ν. The values obtained were 0.56 for GOx/AgNWs, 0.69 for GOx/AgNPs, 0.57 for GaOx/AgNWs and 0.37 for GaOx/AgNPs biosensors. The calculated values were higher when AgNWs were used. These results demonstrate that the presence of nanowires accelerates the DET process with a fast direct electron transfer process.

The LOD and sensitivity were obtained from the calibration curves from the Differential Pulse Voltammograms at increasing concentrations of glucose, galactose or lactose. The results are listed in Table 1. The LODs were calculated using the 3σ/m criterion, where σ is the standard deviation for 5 measures of the blank and m is the slope of the calibration plot for the cathodic peak. The LODs obtained were lower or comparable to previously reported results for GOx, GaOx and β-gal immobilized on nanomaterials [58-62]. In addition, the LODs were lower when AgNWs were used as support, reaching values in the range of 10^{-10}M, which is one order of magnitude lower than in the LODs obtained in the presence of AgNPs. This considerable enhancement confirms that AgNWs improve the efficiency of the enzymatic immobilization, increasing the number of active sites and promoting electron transfer. The sensitivity calculated from the slope of the linear part of the calibration plot was higher in the presence of AgNWs. This is due to the large specific surface area and the higher number of active sites provided by nanowires that facilitate the electron transfer. Finally, the linear ranges observed were higher or at least comparable to other published enzymatic glucose oxidase, galactose oxidase or β-galactosidase biosensors [58-62].

In order to determine the enzyme-substrate affinity, the apparent Michaelis-Menten constant (K_M^{app}) was calculated using a variation of the Lineweaver–Burk approach (eq. 4) [63]:

$$\frac{1}{I} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}\cdot[S]} \quad \text{(eq. 4)}$$

where I and I_{max} are the cathodic currents detected under the steady-state and saturated conditions, respectively, and [S] is the concentration of the analyte. The K_M^{app} values were found to be lower than the values previously reported and obtained under the same pH and temperature condition [64] (Table 1), confirming the excellent affinity between the immobilized enzyme and the substrate.

Table 1. Results obtained from studies at increasing scan rates and increasing concentrations of the corresponding substrate.

	Biosensor	Slope I vs. V 10-100 $mV.s^{-1}$	Slope I vs $V^{1/2}$ 100-1000 $mV.s^{-1}$	Active sites (µmol)	|Sensitivity| ($A \cdot M^{-1}$)	|LOD| (nM)	|LOD| (%m)	Linear range (nM)	K_M^{app} (pM)
Response to lactose	β-Gal	9.61±1.27 (R^2=0.989)	29.55±4.62 (R^2=0.984)	2.56±0.33	18.44 ±2.47 (R^2=0.904)	9.83±1.31	0.33±0.01	2.2-66	8.45±0.48
	β-Gal/AgNPs	10.83±1.02 (R^2=0.958)	29.61± 3.72 (R^2=0.987)	2.88±0.27	19.70 ± 1.87 (R^2=0.986)	2.71±0.25	0.09±0.01	2.2-66	517.3±25.8
	β-Gal/AgNWs	17.66±1.36 (R^2=0.988)	29.94± 3.64 (R^2=0.988)	4.70±0.36	71.41 ±3.88 (R^2=0.999)	0.14±0.01	0.04±0.01	2.2-66	139±5.9
	GOx	21.07±1.96 (R^2=0.951)	28.72±4.54 (R^2=0.983)	5.20±0.64	79.13 ±6.87 (R^2=0.885)	3.46±0.30	0.12±0.01	2.2-31	37.3±2.1
Response to glucose	GOx/AgNPs	22.082±1.42 (R^2=0.994)	33.04± 2.65 (R^2=0.981)	6.01±0.52	64.81 ± 4.66 (R^2=0.888)	0.97±0.07	0.03±0.01	2.2-31	69.4±3.3
	GOx/AgNWs	24.90±1.39 (R^2=0.985)	26.23± 2.10 (R^2=0.988)	6.62±0.41	83.03±4.21 (R^2=0.952)	0.31±0.01	0.010±0.01	2.2-31	53.2±2.2
	GaOx	19.50±2.08 (R^2=0.927)	21.68±3.03 (R^2=0.969)	5.20±0.38	32.24 ± 3.65 (R^2=0.984)	1.98±0.22	0.07±0.021	2.2-11	2.1±0.1
Response to galactose	GaOx/AgNPs	26.17±1.83 (R^2=0.979)	20.43±2.14 (R^2=0.994)	6.96±0.33	27.20 ± 2.18 (R^2=0.964)	3.12±0.24	0.11±0.01	2.2-36	28.0±1.4
	GaOx/AgNWs	33.20±2.15 (R^2=0.977)	27.16±2.22 (R^2=0.979)	8.87±0.39	92.63±6.04 (R^2=0.929)	0.23±0.01	0.01±0.02	2.2-33	123±6.4

3.4.Analysis of milk using bioETs

Once the individual sensors had been characterized, two biosensor arrays (bioETs) were constructed. The first was based on combinations of enzymes and nanowires, (GOx/AgNWs, GaOx/AgNWs and β-Gal/AgNWs) and the second on combinations of enzymes and nanoparticles (β-Gal/AgNPs, GOx/AgNPs and GaOx/AgNPs). Both systems were used to analyze milk and the performance of both bioETs was compared. The electrochemical responses of the electrodes were registered using cyclic

voltammetry in milk diluted 50% in 0.1 M KCl. The measurements were registered from -1 V to 1 V, using a potential step of 10 mV, and at a scan rate of 100 mV/s.

The voltammetric responses showed broad peaks, whose positions and intensities depended on the shape of the silver nanomaterial, on the nature of the enzyme and on the composition of the milk analyzed. This is illustrated in Figure 4, where the responses of different biosensors to Classical Skimmed (CS) milk and Lactose Free Skimmed (LFS) milk are shown. It is worth noting that the responses provided by the biosensors based on nanowires were more intense than the responses obtained in the presence of nanoparticles. This can be explained by the higher surface-to-volume ratio of the one-dimensional AgNWs that provide more surface-active sites, affording a better anchorage for enzyme immobilization than silver nanoparticles. This demonstrates the fundamental role that the morphology of the nanomaterial plays in the development of biosensors in terms of effectiveness and sensitivity. In order to illustrate the importance of the enzymatic activity, Figure S4 compares the voltammetric responses of sensors based on β-Gal to classical and lactose-free milk.

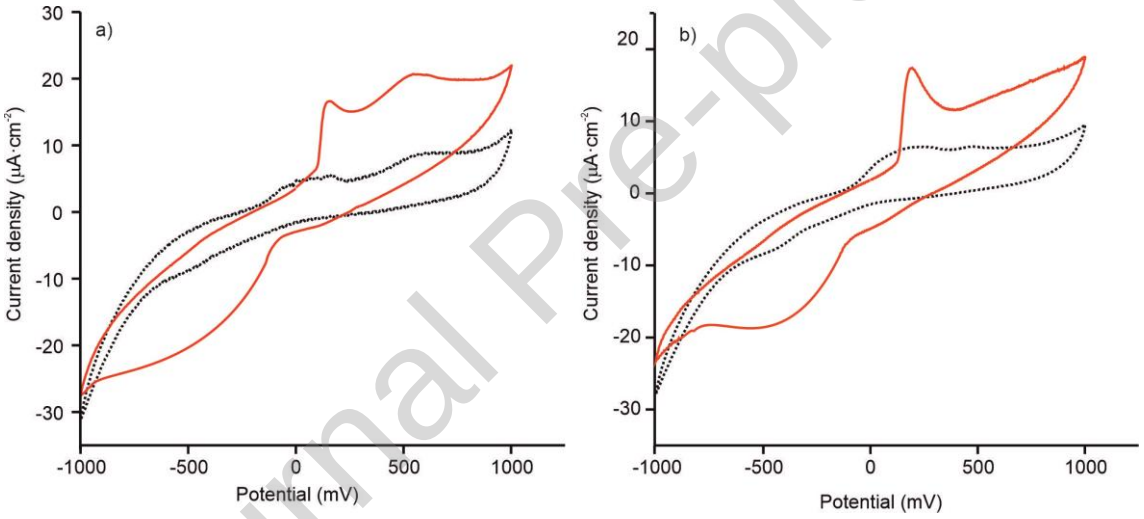


Figure 4. Electrochemical response of the individual sensors based on GaOx/AgNPs (black dotted line) and GaOx/AgNWs (red solid line) in a) 50% diluted Classical Skimmed milk and b) Lactose-free Skimmed milk in 0.1 M KCl solution.

Both bioETs provided distinct responses towards each type of milk. and these signals were used as the input variables for multivariate analysis. Signals were preprocessed using a mathematical reduction technique based on kernel functions [48]. Then, the discriminative capability was evaluated using principal components analysis (PCA). The PCA score plot and the PCA loading plot obtained from both bioETs are shown in Figure 5.

As shown in Figure 5a, the explained variance for the AgNW/bioET was 77.9%, distributed in 34.2% (PC1), 31.5% (PC2) and 12.2% (PC3). The discrimination between milk samples was dominated by the fat content. Skimmed milk appeared in the front part of the diagram (pinkish bullets negative PC3). Whole milk appeared in the back and top part (greenish bullets) and semi-skimmed milk appeared in the region in between the whole and skimmed milk (blueish bullets).

The explained variance for the AgNP/bioET was 79.2% (Figure 5b) distributed in 35.6% (PC1), 27.1% (PC2) and 16.4% (PC3). In this case, discrimination was also achieved, but the milks were not clearly grouped according to the fat content or their nutritional composition.

In order to elucidate the contribution of the electrodes to the discrimination capabilities of the bioETs, the loading plot of the principal component analysis was studied (Figures 5c and 5d). The loading plots offered by the AgNW/bioETs showed a homogeneous distribution of the variables located within the correlation circle of the plot. This distribution of variables is the result of the complementarity of the biosensors selected and confirms that all the variables are significant to obtain a good classification model. The distribution of variables obtained from the AgNP/bioET was not so homogeneous, pointing to a lower complementarity and this can be the reason for the lower capability of discrimination showed by the AgNP/bioET.

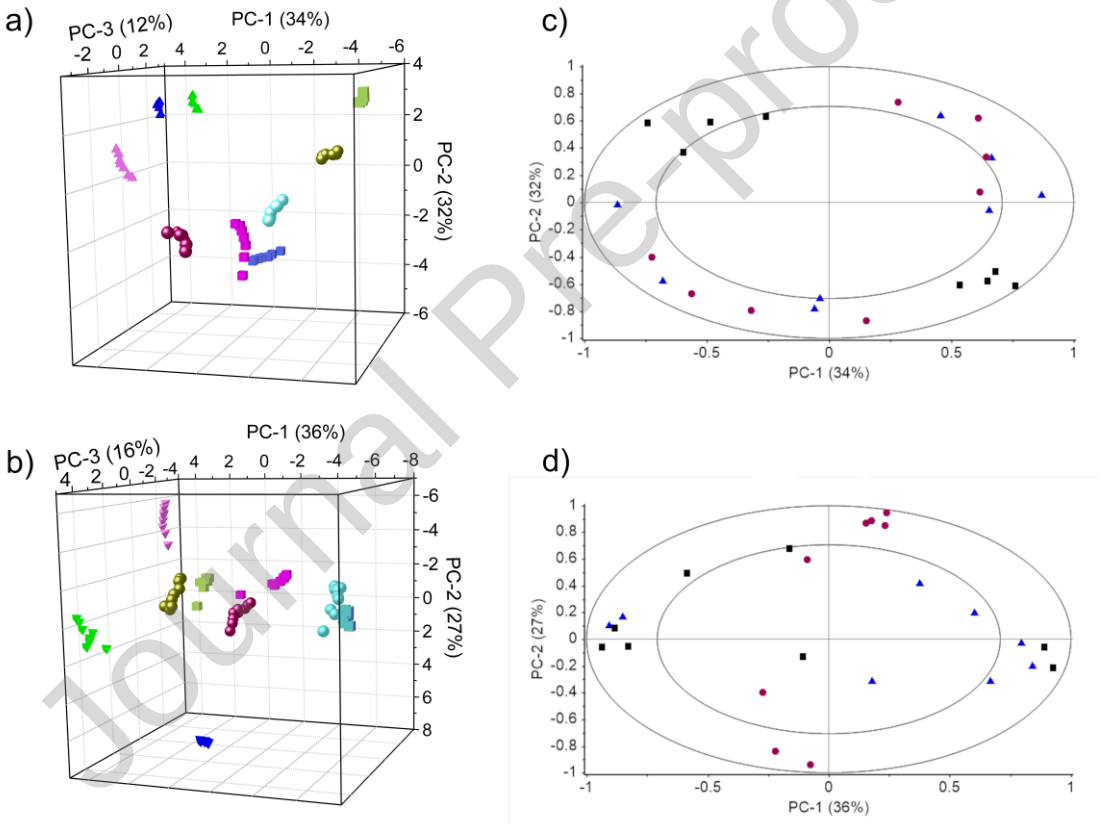


Figure 5. Left: PCA score-plots of a) AgNW/bioET and b) AgNP/bioET. Right PCA loading plots of c) AgNW/bioET and d) AgNP/bioET. The obtained results are from milk with different fat contents: [Skimmed (pinkish colors), Semi-Skimmed (blueish colors) and Whole (greenish colors)]; or nutritional characteristics: [Classic milk (circles), milk enriched with Calcium (triangles), Lactose-Free milk (squares)]. In the loading plots, the biosensor arrays were represented by β-Gal/AgNWs or β-Gal/AgNPs (black squares), GaOx/AgNWs or GaOx/AgNPs (red circles), and GOx/AgNWs or GOx/AgNPs (blue triangles).

Linear discriminant analysis (LDA) was applied to develop a predictive classification model to categorize milk samples in three groups: classic milk, calcium enriched milk and lactose free milk. A classification model was built for each one of the bioETs developed. The model was validated by leave-one-out methodology. The results are presented in Table 2. The LDA model calculated from data registered using the AgNW/bioET resulted in a recognition accuracy of 100% for all milk samples (error rate 0%) for both training and validation. LDA was also applied to the outputs obtained using the AgNP/bioET. In this case, 100% of the samples were correctly classified into one of the three classes (classic, calcium enriched milk), while during the leave one out cross validation, a sample of lactose free milk was classified incorrectly (validation error rate 4.75%). This result confirms again the important role of the shape of the nanomaterial in the performance of the biosensors.

Table 2. Results of the calibration and validation of LDA classification models for both bioETs

AgNW/bioET				
Training		Calcium	Classic	Lactose free
	Calcium	21 (100%)	0	0
	Classic	0	21 (100%)	0
	Lactose free	0	0	21 (100%)
Validation		Calcium	Classic	Lactose free
	Calcium	21 (100%)	0	0
	Classic	0	20 (100%)	0
	Lactose free	0	1 (4.75%)	20 (95.25%)
AgNP/bioET				
Training		Calcium	Classic	Lactose free
	Calcium	21 (100%)	0	0
	Classic	0	21 (100%)	0
	Lactose free	0	0	21 (100%)
Validation		Calcium	Classic	Lactose free
	Calcium	21 (100%)	0	0
	Classic	0	21 (100%)	0
	Lactose free	0	1(4.75%)	20 (95.25%)

Finally, Support Vector Machine Regression (SVMR) was used to establish correlation models between the electrochemical responses of the bioETs and physicochemical parameters classically used to evaluate the quality of milk (acidity, density, % fat, % proteins, % lactose, % TDM and %NFDM) (Table 3). Radial Basis Function was chosen as the core function due to its ability to avoid overfitting and its capability to handle non-linear interactions between sensor inputs and target characteristics [65]. The regularization parameters (c) and kernel parameter (γ) were optimized by applying a grid search method, where approaches to the model were made using log10c and log2γ, varying from [10, 10] for an epsilon of 0.1. The best validation accuracy for each of the systems developed was achieved when γ=0.01 combined with c=100 and c=35.94, for AgNW/bioET and AgNP/bioET, respectively.

Good correlation coefficients (R^2) and low residual errors were obtained in both calibration and prediction for all the parameters analyzed. Excellent correlations were obtained for protein concentration and acidity, which are among the parameters most commonly used in the dairy industry to evaluate the quality of milk. The high correlation with acidity can be explained by the strong influence of the pH in enzymatic activity. The good correlation found with lactose is due to the enzymes included in the array, whose targets were lactose and the two components of lactose (galactose and glucose). It is also remarkable how, in the case of lactose concentration, the AgNW/bioET achieved a higher correlation coefficient of 0.988 compared to 0.974 for the AgNP/bioET. This could be explained by an enhanced enzymatic response of the biosensors in the presence of AgNWs, since the enzymes in the bioET were selected specifically to detect changes in lactose content.

Table 3. Results of the calibration and validation of SVMR for both bioETs

	AgNW/bioET						
	Acidity (ºD)	**Density (g/ml)**	**Fat (%m)**	**Proteins (%m)**	**Lactose (%m)**	**TDM (%m)**	**NFDM (%m)**
R^2c	0.984	0.987	0.995	0.984	0.988	0.979	0.986
RMSEc	0.137	0.348	0.137	0.025	0.252	0.171	0.061
R^2p	0.975	0.977	0.953	0.975	0.966	0.945	0.975
RMSEp	0.210	0.403	0.292	0.037	0.428	0.347	0.088
	AgNP/bioET						
	Acidity (ºD)	**Density (g/ml)**	**Fat (%m)**	**Proteins (%m)**	**Lactose (%m)**	**TDM (%m)**	**NFDM (%m)**
R^2c	0.973	0.981	0.992	0.973	0.974	0.976	0.983
RMSEc	0.148	0.353	0.121	0.026	0.381	0.175	0.061
R^2p	0.978	0.977	0.988	0.973	0.951	0.968	0.978
RMSEp	0.191	0.402	0.149	0.033	0.518	0.218	0.076

$RMSE_c$: "root mean square of calibration error". R^2_c correlation coefficient in calibration $RMSE_p$: "root means square of prediction error". R^2_p correlation coefficient in prediction.

Thus, using the AgNW/bioET could be a better choice to assess simultaneously in a single measurement.

3.5.Repeatability, reproducibility and lifetime

The repeatability was analyzed by measuring the decrease in intensity after 5 consecutive cycles. In all cases the coefficient of variation was lower than 4.9% in standard solutions and lower than 6.3% in milk. The reproducibility was calculated from the responses of two identical sensors. Values were lower than 3.9% for sensors immersed in standard solutions and lower than 4.3 % in sensors immersed in milk samples, confirming the good level of precision achieved in the fabrication of the biosensors (Figure S5). Biosensors could be cycled repeatedly (up to 50 cycles) without considerable losses of intensity (lower than 8%). However, once the sensors were withdrawn from the milk, a decrease in the intensity of the peaks was perceived (due to

the adhesion of a layer of fat). For this reason, biosensors were considered as disposable devices and each milk was analyzed with a new set of sensors.

4. Conclusions

In this work, different strategies were followed to obtain a bioelectronic tongue dedicated to the analysis of milk with improved performance. On the one hand, the array included biosensors based on enzymes able to detect sugars present in milk (β-galactosidase, galactose oxidase and glucose oxidase). On the other hand, the performance of the biosensors was improved by combining the enzymes with silver nanomaterials that induce unique performance characteristics in terms of sensitivity. It has been demonstrated that the aspect ratio of the nanomaterial is of great importance. Silver nanowires (AgNWs) provide a more effective platform for the immobilization of biomolecules than silver nanoparticles (AgNPs). The LODs were lower when AgNWs were used as support, reaching values in the range of 10^{-10} M, which was one order of magnitude lower than in the LODs obtained in the presence of AgNPs. These results demonstrate that the presence of nanowires accelerates the DET process with a fast, direct electron transfer process.

Principal component analysis (PCA) demonstrated that the bioET based on combinations of AgNWs and enzymes could discriminate 9 commercial types of milk according to their nutritional composition, with a higher capacity than the bioET based on combinations of AgNPs and enzymes. The PCA loading plot confirmed the excellent complementarity of the biosensors.

Excellent correlations with the chemical parameters commonly used in the quality control of milk were found using Support Vector Machines. This result confirms that the developed system shows the benefits of biosensors (specificity), nanomaterials (promoting the enzymatic activity) and the advantages of multisensory systems that provide global information about the sample and, at the same time, can be used to give quantitative information of several physicochemical parameters in a single measurement. Although both bioETs work appropriately, the AgNW/bioET-NWs could be a better choice than the AgNP/bioET due to the larger specific surface area and the higher number of active sites provided by nanowires.

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Author Contributions.

Conceptualization, M.L.R. and C.G.C; Methodology, C.S.C., Software, F.M.P.; Experimental work, C.S.C, P.M.B, J.L.P and C.P.; Writing – Original Draft Preparation,

C.S.C.; Writing – Review & Editing, M.L.R.; Project Administration, M.L.R..; Funding Acquisition, M.L.R.

Conflicts of Interest.

The authors declare no conflict of interest.

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Credit authors statement

As the corresponding author of this paper, I declare that the contribution sof the authors have been the following:

Author Contributions.

Conceptualization, M.L.R. and C.G.C; Methodology, C.S.C., Software, F.M.P.; Experimental work, C.S.C, P.M.B, J.L.P and C.P.; Writing – Original Draft Preparation, C.S.C.; Writing – Review & Editing, M.L.R.; Project Administration, M.L.R..; Funding Acquisition, M.L.R.

Declaration of Competing Interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights

- Biosensors based on combinations of AgNWs and specific enzymes have been developed
- AFM analysis demonstrates the excellent coverage of nanowires with enzymes
- AgNWs facilitate Direct Electron Transfer between the enzyme and the electrode surface
- Biosensors show low LOD and high enzymatic affinity
- A bioET has been constructed using biosensors combining enzymes and AgNWs
- The AgNWs/bioET can be successfully applied to the analysis of milks