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Journal:	<i>Analytical Methods</i>
Manuscript ID	AY-ART-06-2018-001376.R1
Article Type:	Paper
Date Submitted by the Author:	n/a
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Article type: Full paper



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ARTICLE

Bionanocomposite-modified glassy carbon electrode for the determination of 4,4'-methylene diphenyl diamine

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Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A nanosensor based on a glassy carbon electrode modified with the biopolymer chitosan, multi-wall carbon nanotubes, and gold nanoparticles (MWCNTs-CS-Au/GCE) was developed for the determination of 4,4'-diaminodiphenyl diamine (MDA). Cyclic voltammetry (CV) was used to investigate the electrochemical behavior of the sensor in the presence of MDA. MDA displayed a well-expressed oxidation peak at 0.54 V (versus Ag/AgCl) in Britton–Robinson (B–R) universal buffer solution (pH = 10). The transfer coefficient, α , and the overall number of electrons (n) involved in the catalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface were also determined by CV. The reactivity of spiked MDA was strongly dependent on the pH of the supporting electrolyte, with a pH dependence of the MDA oxidation quantified in 27.576 mV pH⁻¹. Through chronoamperometry, the diffusion coefficient (D) of MDA was calculated as 9.49×10⁻⁵ cm² s⁻¹. A limit of detection of MDA was estimated to be ~ 20 nM through amperometry experiments, while three linear ranges were found for MDA, i.e. 0.49–10.14 μM, 10.14–94.9 μM, and 94.9–261.18 μM. Real sample tests enabled emphasizing the potential involved in this nanocomposite-modified electrode as a new analytical tool for the determination of MDA.

1. Introduction

Polyurethane (PU) adhesives are obtained through the polymerization of isocyanate monomers and polyols. PU adhesives are the most commonly used adhesive systems by converters to obtain multilayer packaging systems composed of two (or more) layers joined by a tie layer. Unreacted isocyanate monomers will generate primary aromatic amines (PAAs) as soon as they come into contact with water (1). PAAs are toxic compounds and/or suspected human carcinogens. The combination of a potentially high exposure and high toxicity explains why the use of these chemicals is extensively regulated, by both national and European Union (EU) legislation (2). The specific migration limit of PAAs to foods set by the EU in order to protect the consumers' health is 10 ng for total PAAs (expressed as aniline-equivalent) per gram of food or food simulant.

Recently, various approaches have been used in order to quantify low concentrations of PAAs that migrated from the packaging material into the foodstuff. To achieve a reliable and

selective determination of PAAs that possibly migrated from the packaging materials into the food, an update of the analytical tools is essential for the food industry. In particular, the widely used spectrophotometric/colorimetric method (as equivalent to aniline) does not seem to provide accurate results compared to, for example, chromatography techniques, which are, however, more complicated and expensive, thereby less willingly adopted by most converters (3).

In recent years, electrochemical methods have attracted a lot of attention as new analytical methods for determining various analytes in very low amounts. Electrochemical sensors are one of the most popular subclasses of chemical sensors. A chemical sensor is a small device that consists of a transduction element covered by a chemical or biological recognition layer in order to quantify the analyte in the sample matrix. In the case of electrochemical sensors, the electrode is used as the transduction element (4). A sensing electrode (as the transduction element), a counter-reference electrode, and an electrolyte are the main parts of typical electrochemical sensors. The analytical information is obtained from the electrical signal that results from the interaction of the target analyte and the recognition layer at the sensing electrode (5).

When using bare electrodes as sensing electrodes, a fouling phenomenon could take place on the electrode's surface. Detection limit, sensitivity, overall reliability, and reproducibility are the most important parameters that can be negatively affected by this phenomenon. Most antifouling strategies foresee the use of a modified electrode with greater fouling resistance than an unmodified electrode. There are two main groups of nanoparticles that can be used to modify working electrodes to control fouling: i) carbon-based nanomaterials, such as carbon nanotubes or graphene (these are important

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Electronic Supplementary Information (ESI) available: cyclic voltammograms at the MWCNTs-CS-AuNPs/GCE surface at different pH values and at different scan rates (5–200 mV/s). See DOI:

due to their electrocatalytic properties, large surface area, and fouling resistance) and ii) metallic nanoparticles, which have antifouling properties as well as high electrical conductivity and important electrocatalytic properties (6).

In this study, we describe the fabrication of a new bionanocomposite-modified electrode based on multi-wall carbon nanotubes (MWCNTs), chitosan (CS), and gold nanoparticles (AuNPs) for the electrocatalytic determination of 4,4'-methylene diphenyl diamine (MDA), which is one of the most important PAAs that can migrate from PU-based packaging materials to the food. The analytical performance of the sensor was investigated, and the results showed that a combination of MWCNTs, CS, and AuNPs remarkably improved the current response. Based on our findings, the modified electrode exhibited clear functional advantages compared to other analytical methods in terms of repeatability, stability, reproducibility, and technical feasibility for the MDA determination. Full electrochemical characterization of the modified electrode was carried out and its electrochemical properties described.

2. Experimental

2.1. Chemicals and apparatus

MDA (analytical grade 98%, molar mass 198.26 g mol⁻¹), AuNPs (5 nm diameter), MWCNTs (\geq 98% carbon basis), CS (deacetylation \geq 75%), phosphoric acid (85-90%), boric acid (99.999% trace metals basis), and acetic acid (99%) were purchased from Sigma Aldrich (Milan, Italy). Ethylene glycol monomethyl ether (EGMe) was bought from Merck (Milan, Italy), whereas alumina powder (type DX, 0.05 μ m average size) was bought from EMS (Hatfield, PA, U.S.). Britton–Robinson (B–R) universal buffer (0.04 M boric acid, 0.04 M acetic acid and 0.04 M phosphoric acid) was prepared in deionized water and was tested as the supporting electrolyte.

All electrochemical experiments were carried out using an Autolab Potentiostat PGSTAT 302N potentiostat (Metrohm, Herisau, Switzerland) equipped with Nova 2.1 software. Before the experiments, the instrument was corrected for bias by a calibration procedure operated by the factory. A conventional three-electrode electrochemical cell was used throughout the experiments, using a platinum electrode and an Ag/AgCl (sat.), KCl (3 M) as counter and reference electrodes, respectively. The working electrode was a glassy carbon electrode modified using a multi-wall carbon nanotubes-chitosan and gold nanoparticles (MWCNTs-CS-AuNPs/GCE) composite coating. All electrochemical experiments were run at 25 \pm 2.5 °C under ambient conditions. The pH measurements were performed with a BASIC 20+ pH meter (Crison Instruments, S.A. Barcelona, Spain).

2.2. Preparation of modified electrodes

The MWCNT dispersion was prepared by adding 0.5 mg of MWCNTs into 1 g of EGMe and ultrasonication by means of a UP400S (powermax = 400 W; frequency = 24 kHz) ultrasonic

device (Hielscher, Teltow, Germany) equipped with a cone frustum titanium sonotrode (mod. H3, tip \varnothing 3 mm, amplitudemax = 210 μ m; acoustic power density = 460 W cm⁻²) under the following conditions: 0.5 cycle and 50% amplitude, for a period of 10 min. Subsequently, 600 μ L of the MWCNTs-EGMe dispersion was mixed with 150 μ L of a CS solution (1 wt% in acidic water) and again ultrasonicated for 3 min to achieve a completely homogeneous MWCNTs-CS suspension. Eventually, 500 μ L of the MWCNTs-CS suspension was mixed with 200 μ L of AuNPs (16.5 \times 10¹² particles/mL), and the solution was ultrasonicated for 3 min to make the suspension homogeneous.

Before applying the bionanocomposite modifier, the glassy carbon electrode (GCE) surface was polished with alumina abrasive slurry (mean particle size 0.05 μ m) on a polishing cloth and then rinsed with double distilled water. To prepare the final MWCNTs-CS-Au/GC electrode, 15 μ L of MWCNTs-CS-Au dispersion was placed directly onto the GCE surface and dried with an infrared lamp (type B, 1440 W) (Helios Italquartz srl, Cambiago, Italy) at a distance of 40 cm for 10 min. The MWCNTs-CS/GCE was made by dropping 15 μ L of the MWCNTs-CS dispersion on the surface of the GCE and drying for 10 min.

2.3. Morphological characterization of electrode surface

The surface morphology of the electrode was captured using a field emission scanning electron microscope (FE-SEM) (Hitachi S-4800, Schaumburg, IL) at an acceleration voltage of 1–5 kV and an electrode current of 10 μ A. MWCNTs-coated, MWCNTs/CS-coated, and MWCNTs-CS-AuNPs-coated GCE specimens were mounted with carbon tape on stubs and their surfaces observed after sputtering with Pt/Pd (60/40) under argon for 20 s at a current of 80 mA.

2.4. Real sample analysis

Thermo-sealed bags of 1 dm² of surface area per side were prepared using a multilayer packaging material (Castagna Univel Spa, Guardamiglio, Italy) consisting of polyethylene terephthalate (PET, 12 μ m thick), polyvinylidene chloride coating (PVDC, 6 μ m thick), and low-density polyethylene (LDPE, 50 μ m thick), whereby a PU adhesive was used to join the PET layer to the remaining part of the film. The potential migration of PAAs from the PU-based multilayer packaging material was assessed under the worst-case condition. For this purpose, each bag was filled with 100 mL of food simulant B (i.e., acetic acid water solution, 3 w/v %) (7,8). The test was conducted at 121 °C for 20 min in an autoclave (Asal 760, Steroglass srl, Perugia, Italy). After this time, 10 mL of the simulant B was diluted with 10 mL of the B–R buffer, followed by the addition of specific amounts of MDA monitored by amperometry. From the quantitative determination of MDA, the final recovery (%) was determined.

3. Results and discussion

3.1. Morphological characterization of modified GCEs

The surface morphology of the different electrodes was investigated by FE-SEM, as shown in Figure 1. MWCNTs of approximately 12 nm average diameter and 15–20 μm in length were uniformly distributed on the surface of the GCE to form a three-dimensional network that increased the specific surface area of the electrode (Figure 1a). When the MWCNTs were loaded in the main polymer phase (Figure 1b), they were no longer clearly visible, although the rough surface of the CS-based modified GCE confirmed their presence. Figure 1 (c) shows a flat surface made of MWCNTs, CS, and AuNPs, the latter hardly visible at the magnification of the analysis and most likely encased within the main biopolymer network.

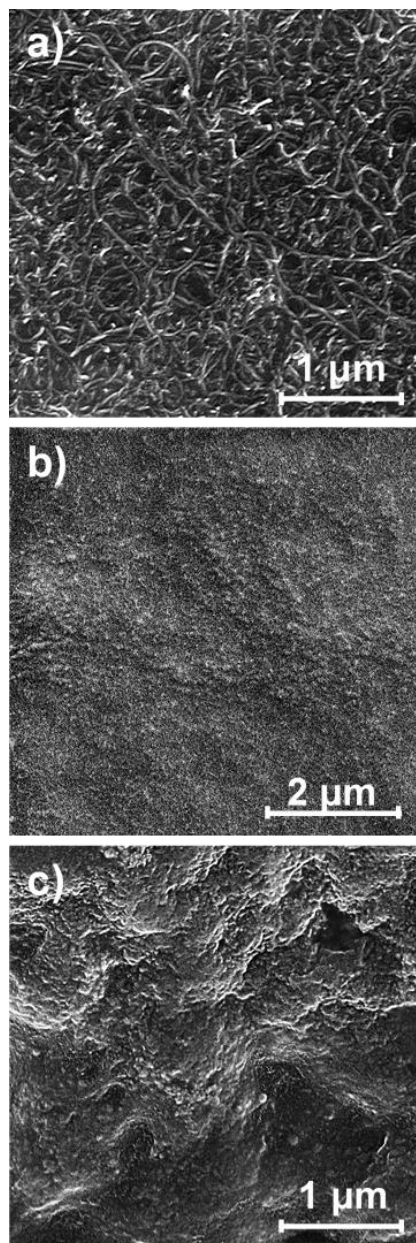


Figure 1. FE-SEM images of (a) MWCNTs, (b) MWCNTs-CS, and (c) MWCNTs-CS-AuNPs-modified GCE.

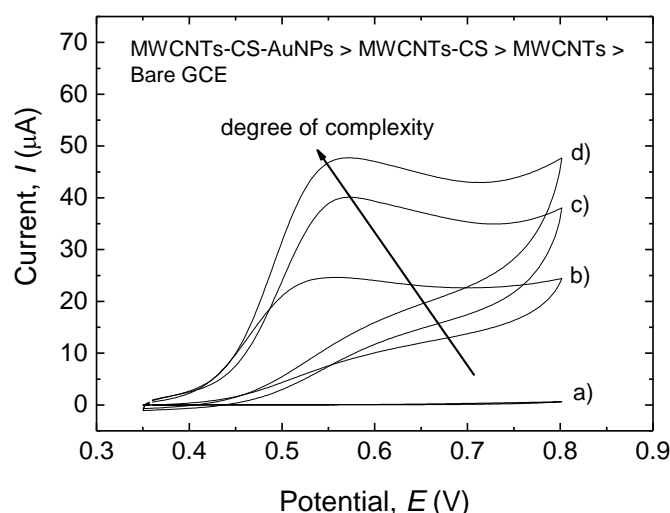


Figure 2. Cyclic voltammograms in B–R buffer (0.1 M, pH 10) at a scan rate of 50 mV/s in the presence of 500 μM of MDA: (a) bare GCE, (b) MWCNTs/GCE, (c) MWCNTs-CS/GCE, and (d) MWCNTs-CS-AuNPs/GCE.

3.2. Electrochemical behavior of MDA on electrode surface

The electrochemical behavior of the modified electrodes was examined using cyclic voltammetry (CV).

Figure 2 shows the voltammograms of the bare GCE, MWCNTs/GCE, MWCNTs-CS/GCE, and MWCNTs-CS-AuNPs/GCE. The experiments were carried out in a B–R universal buffer solution (pH 10) containing 500 μM MDA at 50 mV/s from 0.35 V to 0.8 V. The bare GCE showed a weak oxidation peak with extremely low current (trace a, Figure 2). According to trace b of Figure 2, the oxidation peak current increased remarkably after modification with MWCNTs, suggesting that MWCNTs could enhance the electron transfer rate while increasing the electrochemical surface area of the electrode (9).

In order to improve the performance of the electrode, a mixture of CS and MWCNTs was used in the next step. As shown in Figure 2, trace c, the peak current increased dramatically compared to the MWCNTs-modified electrode. It was inferred that CS could effectively improve the performance of MWCNTs through its film-forming ability and good adhesion, resulting in the amplification of the current response (10). Ultimately, in an attempt to upsurge the sensitivity of the sensor, the MWCNT-CS solution was mixed with AuNPs and the electrode was modified with the MWCNT-CS-AuNPs bionanocomposite solution. The final voltammogram showed that the peak current increased further compared to the MWCNT-CS/GCE, plausibly due to the increase of the electrode's conductivity owing to the excellent inherent electronic properties of the AuNPs, eventually acting as an electron wire. The electrocatalytic oxidation characteristics of MDA on different modified electrodes are summarized in Table 1.

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Table 1. Comparison of the electrocatalytic oxidation peak current (I_p) of MDA (500 μ M) on various electrode surfaces at pH 10.0.

Electrode	Oxidation peak current (μ A)	Modifier
Bare GCE	0.01	-
MWCNTs/GCE	14.66	15 μ L MWCNTs
MWCNTs-CS/GCE	23.47	15 μ L MWCNTs-CS
MWCNTs-CS-AuNPs/GCE	27.50	15 μ L MWCNTs-CS/AuNPs

3.3. Effect of pH value

The effect of the pH on the electrochemical behavior of MDA was studied by CV at the MWCNTs-CS-AuNPs/GCE surface at different pH values (from 2 to 11) in a 0.1 M B-R buffer solution containing 500 μ M of MDA (Figure S1 of ESI).

When the pH value increased from 2 to 10, the oxidation peak current increased, reaching its maximum at pH 10, after which an apparent steady condition was achieved (Figure 3). Moreover, the oxidation peak potential, E_{pa} , shifted to negative values when the pH increased, indicating that protons were involved in the oxidation reaction (11). This was further confirmed by the linear relationship between the oxidation peak potential and the pH and expressed as: $E_{pa} = -27.576\text{pH} + 820.24$ ($R^2 = 0.985$). The potential negatively shifted by 27.576 mV per pH unit. According to the Nernst equation, a slope approaching 0.0592/2 indicates an electron-to-proton proportion equal to 2:1 (12), that is, one proton per two electrons was involved in the electrochemical oxidation of MDA.

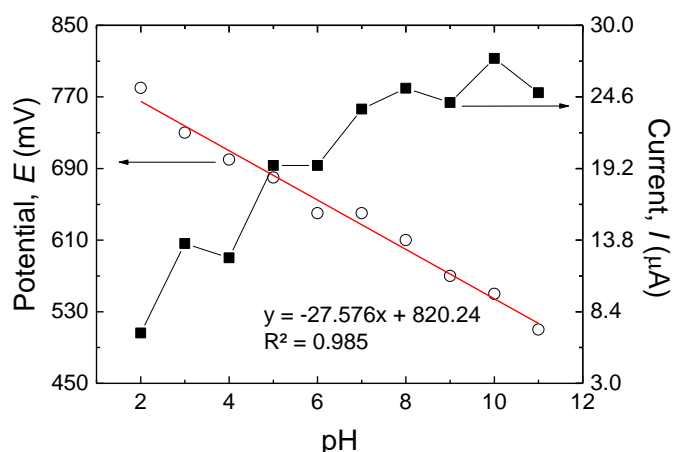


Figure 3. Effect of the pH on the oxidation peak current and oxidation peak potential in a 500 μ M of MDA (B-R solution 0.1 M) on the surface of the MWCNTs-CS-AuNPs/GCE.

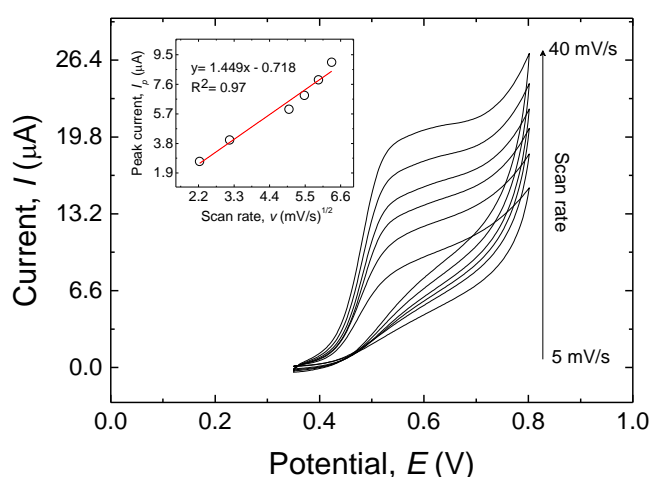


Figure 4. Cyclic voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μ M MDA at different scan rates (5–40 mV/s). The electrocatalytic peak current (I_p) variation as a function of the square root of sweep rate is shown in the inset.

3.4. Electrocatalytic oxidation of MDA at the MWCNTs-CS AuNPs/GCE surface

The effect of scan rate (ν) on the electrocatalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface was investigated by CV within a range of 5–200 mV/s (Figure S2 of ESI) at an MDA concentration of 500 μ M. For clarity, the range 5–40 mV/s is shown in Figure 4. Figure 4 (inset) shows the dependence of the oxidation peak current (I_p) drawn from each scan rate against the square root of the potential scan rate ($\nu^{1/2}$). The linearity of the ensuing plot suggests that the catalytic reaction of the analyte at the electrode's surface is diffusion-limited; that is, no absorption of the analyte within the nanocomposite polymer network occurs before oxidation (13).

Figure 5 displays the linear sweep voltammograms obtained applying different scan rates (25–40 mV/s) to obtain information on the rate-determining step of the electro-oxidation of MDA (14). The inset of Figure 5 shows the Tafel regions obtained from the linear sweep voltammograms. The slope of each region was determined according to equation 1 (15):

$$\text{Tafel}_{\text{slope}} = \frac{(1-\alpha)n_a F}{2.3RT} \quad (1)$$

where α is the electron transfer coefficient, n_a is the number of electrons transferred until the end of the rate-determining step (up to and including the rate-determining step), F is Faraday's constant, R is gas constant (8.314 J mol⁻¹ K⁻¹), and T the temperature (K). An average slope of 11.8 V⁻¹ was eventually calculated. If the rate-determining step of the electrode process includes one electron transfer, the charge transfer coefficient (α) is 0.50.

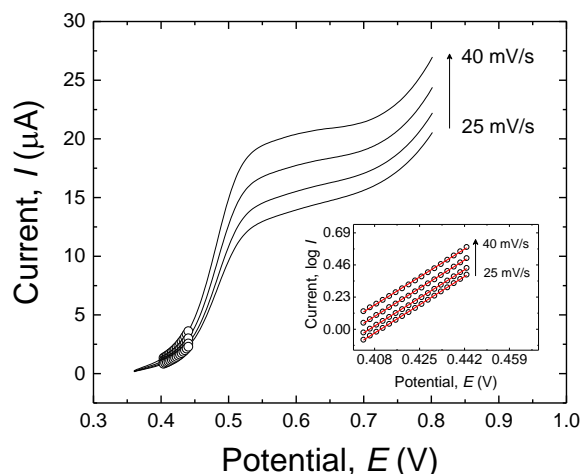


Figure 5. Linear sweep voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μM MDA at 25–40 mV/s. The Tafel plot derived from the linear sweep voltammograms is shown in the inset.

Using CV, the number of electrons (n_α) involved in the rate-determining step of the reaction was calculated according to the following equation:

$$|E_p - E_{p/2}| = \frac{47.7}{\alpha n_\alpha} \quad (2)$$

where E_p is the peak potential (oxidation in our case) of a selected CV trace (800 mV/s in this study) and $E_{p/2}$ is the half-peak potential (i.e., the potential [V] at the half-peak current [$I_p/2$]). $|E_p - E_{p/2}|$ value was calculated to be 68 mV, from which a value of 0.701 was calculated for αn_α (16). Because for a totally irreversible reaction, the electron transfer coefficient (α) is assumed to be 0.50, a final n_α value of ca. $1.39 \approx 1$ for the oxidation of MDA was calculated, which is slightly higher than the theoretical value of ≈ 1 assumed by eq. 1.

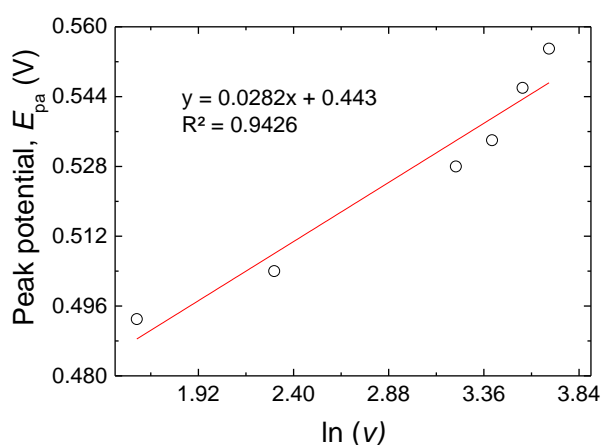


Figure 6. Experimental data (black dots) and linear regression of anodic peak potential (E_{pa}) versus the natural logarithm of the scan rate [$\ln(v)$].

The total number of electrons (n) involved in the overall catalytic reaction was gathered according to Laviron's theory, which is used to investigate the kinetic mechanism of the electrode toward the analyte. Accordingly, for a totally irreversible electrode process, the anodic peak potential (E_{pa}) and the natural logarithm of the scan rate $\ln(v)$ can be defined by the following equation (17):

$$E_{pa} = E^\theta + \left(\frac{RT}{\alpha nF}\right) \ln\left(\frac{RTK^\theta}{\alpha nF}\right) + \left(\frac{RT}{\alpha nF}\right) \ln v \quad (3)$$

where α is the transfer coefficient, K^θ is the standard rate constant of the reaction, n is the electron transfer number, v is the scanning rate, E^θ is the formal redox potential, R is the gas constant, T is the absolute temperature, and F is the Faraday constant. Using the raw voltammograms as reported in Figure 4, a linear relationship between E_{pa} and $\ln(v)$ was obtained as expressed by the equation E_p (V) = $0.0282 \ln v$ (mV/s) + 0.443 (Figure 6). According to Laviron's equation, the electron transfer number (n) can be calculated from the slope of E_{pa} versus $\ln(v)$, that is, $RT/\alpha nF$. After appropriate substitutions, a final value of n $1.83 \approx 2$ was obtained, indicating that the electrochemical oxidation of MDA at the MWCNTs-CS-AuNPs composite-modified GCE is a two-electron transfer process.

3.5. Chronoamperometric measurements

The electrochemical oxidation of MDA at the surface of MWCNTs-CS-Au/GCE was studied through chronoamperometry at a potential of 700 mV and varying the concentration of MDA from 0.005 mM to 0.4 mM (Figure 7). For an electroactive material with a diffusion coefficient D , the current response under diffusion control is described by the Cottrell equation (15):

$$I = \frac{nFAD^{1/2}C_b}{\pi^{1/2}t^{1/2}} \quad (4)$$

where n is the number of electrons exchanged per reactant molecule, F is the Faraday constant ($9.648 \times 10^4 \text{ C mol}^{-1}$), A is the geometric area of the electrode (0.0314 cm^2), C_b is the bulk concentration of the analyte (mol cm^{-3}), and D ($\text{cm}^2 \text{ s}^{-1}$) is the diffusion coefficient of the analyte.

From the raw chronoamperometric traces, a linear curve was obtained for the different concentrations of MDA by plotting I against $t^{1/2}$ (Figure 7, inset a). By plotting the slope of each individual straight line, the overall slope of the best-fit line (Figure 7, inset b) can be defined from equation 4 as:

$$It^{1/2} = \frac{nFAD^{1/2}C_b}{\pi^{1/2}} \quad (5)$$

D can thus be drawn from:

$$D = \frac{(\text{slope})^2 \pi}{(nFAC_b)^2} \quad (6)$$

The overall slope ($33.32 \mu\text{A s}^{1/2} \text{ mM}^{-1}$) was used within the Cottrell equation to eventually obtain a diffusion coefficient of $9.49 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$.

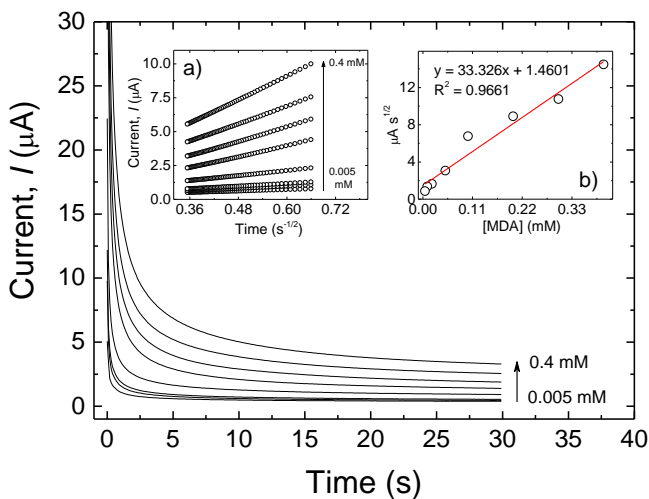


Figure 7. Current response over time of the MWCNTs-CS-AuNPs/GCE in B–R buffer solution (0.1 M, pH 10.0) containing different concentrations of MDA (0.005 – 0.4 mM) by chronoamperometric measurements (potential 700 mV). Numbers 1–8 correspond to the different MDA concentrations. Insets: (a) chronoamperograms of the intensity (*I*) as a function of the reciprocal square root of time ($t^{-1/2}$); (b) linear plot of the slopes of the eight straight lines in the inset (a) against the MDA concentration.

3.6. Amperometric studies of electrocatalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface

The sensitivity of the MWCNTs-CS-AuNPs-modified electrode to MDA was investigated by amperometry, which has been shown to be a more effective method than voltammetry due to the stirring conditions during the experiment (4). Figure 8 shows the amperogram obtained from a rotating MWCNTs-CS-AuNPs/GCE at a fixed potential of 700 mV in a 0.1 M B–R buffer solution (pH 10.0) containing different concentrations of MDA.

During successive dropwise additions of MDA, a well-defined stairs-like current versus time plot was obtained. The calibration plot was linear in three concentration ranges (i.e., 0.49–10.14 μM [Figure 8, inset a], 10.14–94.9 μM [Figure 8, inset b], and 94.9–261.18 μM [Figure 8, inset c]) of MDA. The sensitivity of the modified electrode to MDA, calculated as the slope of the lower linear range in the amperometric plot, was 0.1509 $\mu\text{A} (\mu\text{M})^{-1}$, from which the lower limit of detection (LOD) and the lower limit of quantification were calculated according to the following equations (18,19):

$$LOD = \frac{3 \times S_{bl}}{m}$$
 (7)

$$LOQ = \frac{10 \times S_{bl}}{m}$$
 (8)

where S_{bl} is the standard deviation of the blank response (μA) obtained from 10 replicates of the blank solution (0.001) and m is the slope of the aforementioned lower linear range in the amperometric plot. A final LOD and LOQ values of 20 nM

and 66 nM were eventually calculated for the MWCNTs-CS-AuNPs/GC electrode.

The operational stability of the MWCNTs-CS-AuNPs/GCE composite-modified electrode was further examined by amperometry, and the results are shown in the inset (d) of Figure 8. The amperometric current response of MDA was almost unchanged when continuously run up to 3310 s in 5 μM MDA containing B–R buffer. This observation shows that throughout the monitored time span, there was no inhibition effect due to the adsorption of MDA and MDA's oxidation products on the modified electrode surface.

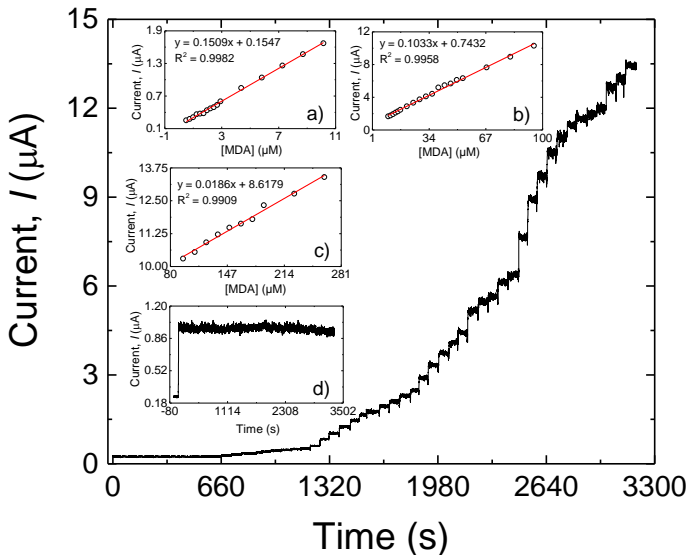


Figure 8. Amperometric responses at a rotating MWCNTs-CS-AuNPs/GCE held at 700 mV in different concentrations of 0.49 to 261.18 μM of MDA. The variation of the amperometric current against the MDA concentration is shown in insets (a) (0.49–10.14 μM), (b) (10.14–94.9 μM), and (c) (94.9–261.18 μM). Inset (d) shows the stability of the response of the MWCNTs-CS-AuNPs/GCE during 3310 s (MDA concentration of 5 μM).

Table 2. Amount of added (spiked) and measured MDA at the MWCNTs-CS-AuNPs/GCE surface, with the resulting recovery percentage after the migration test using the acidic food simulant (simulant B) under typical sterilization conditions (121 $^{\circ}\text{C}$ for 20 min).

Sample	Spiked (μM)	Found (μM)	Recovery (%)
	0	-	-
Sample 1	3.5	3.54	101.1
	5	4.93	98.6
	5.5	5.4	98.1

The accuracy was estimated as the recovery from known concentrations of MDA (Table 2). The recovery ranged from 98.1 to 101.1%. These results show the adequate accuracy of the method and suggest that the modified electrode as prepared in this work is well-suited for the quantification of MDA possibly

migrating from food packaging materials exposed to extensive thermal treatments (e.g., sous-vide cooking, pasteurization, and sterilization).

Finally, the precision (repeatability) of the electrochemical method was assessed preparing independently three different MWCNTs-CS-AuNPs/GCE sensors. For each sensor, subsequent injections ($n = 15$) of MDA standard solutions at three different concentrations (5, 10, and 15 μM) were analyzed. The relative standard deviation (RSD) was $3.13 \pm 0.25\%$, which indicates the satisfactory repeatability and good reproducibility of the proposed electrode.

All the relevant analytical parameters of the MWCNTs-CS-AuNPs/GC electrochemical sensor used for the detection of MDA are summarized in Table 3.

Table 3. Analytical parameters of the MWCNTs-CS-AuNPs/GC electrochemical sensor used for the MDA determination.

Parameter	Value
	0.49-10.14 μM
Linearity (ranges)	10.14-94.9 μM
	94.9-261.18 μM
Sensitivity	0.1509 $\mu\text{A} (\mu\text{M})^{-1}$
LOD	20 nM
LOQ	66 nM
Recovery (%)	99.27 ± 1.60
RSD (%)	3.13 ± 0.25

3.7. Real sample analysis

In this experiment, we have attempted to determine the MDA concentration that had possibly migrated from the PU-based food packaging material into the food simulant through a migration test carried out under typical sterilization conditions (121 $^{\circ}\text{C}$ for 20 min). The standard addition method was used to evaluate the potential application of the MWCNTs-CS-AuNPs/GCE as an analytical device for MDA determination in real samples. To achieve this goal, the recovery of MDA was determined by amperometry after the addition of MDA in the buffered food simulant B (acetic acid water solution, 3 w/v %) (Table 2).

Conclusions

A new electrochemical sensor with high sensitivity and a low limit of detection was developed for the quantification of the MDA, which is one of the two most representative PAAs possibly migrating from multilayer packaging materials made of PU adhesives into foodstuffs. The sensor was developed using a MWCNTs-CS-AuNPs bionanocomposite to modify the surface of the electrode. The synergistic effect between MWCNTs, CS, and AuNPs toward the electro-oxidation of MDA was recognized as the main factor that dictates the excellent electrocatalytic performance of the sensor in terms of sensitivity, detection limit, and stability over a reasonably extended temporal window (no fouling on the surface of the electrode). The tests

conducted on real samples indicated that the modified sensor has the potential to be used as an analytical device for the quantification of MDA.

Conflicts of interest

There are no conflicts to declare.

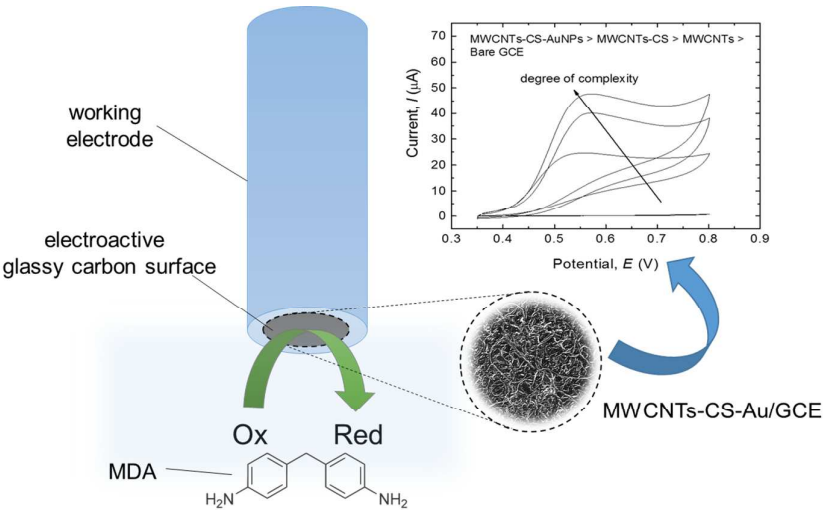
Acknowledgments

We are thankful to Dr. K. Morozova for precious technical support.

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Graphical Abstract



The analytical performance of a modified-GCE for the determination of MDA was increased with the degree of complexity of the bionanocomposte modification.

Bionanocomposite-modified glassy carbon electrode for the determination of 4,4'-methylene diphenyl diamine

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Electronic Supplementary Information

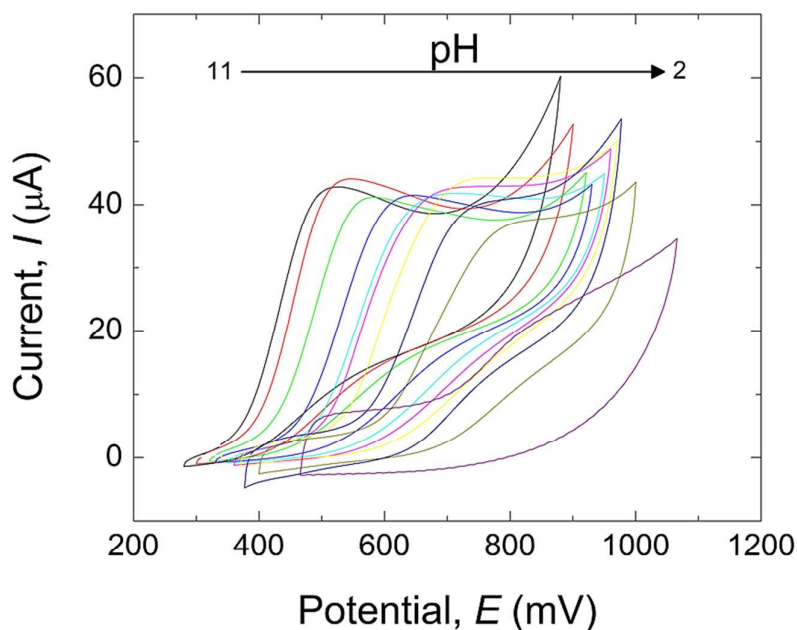


Figure S1. Cyclic voltammograms at the MWCNTs-CS-AuNPs/GCE surface at different pH values (from 2 to 11) in a 0.1 M B-R buffer solution containing 500 μM of MDA.

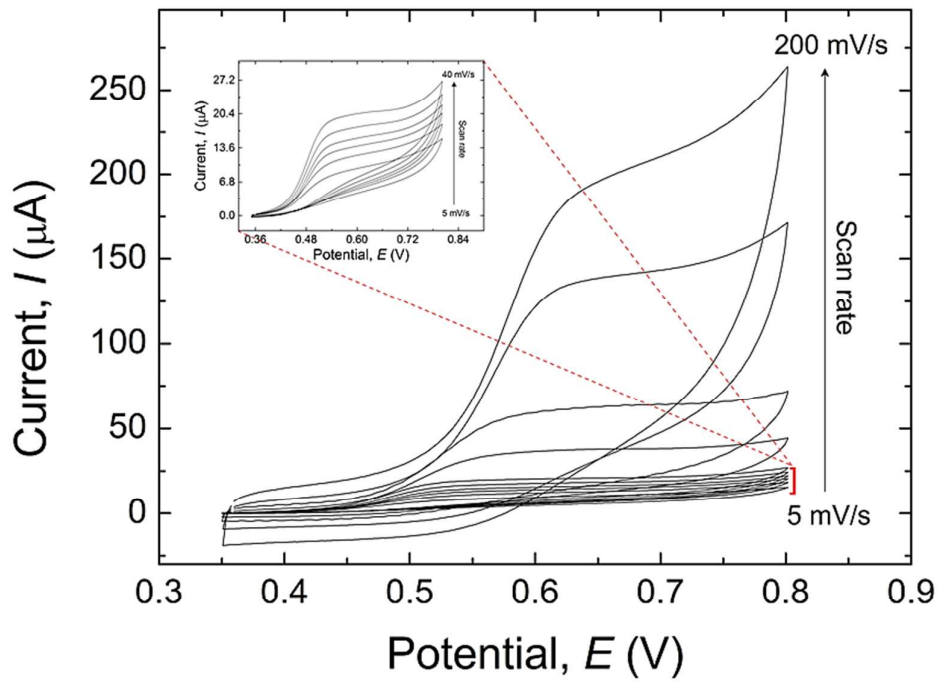


Figure S2. Cyclic voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μM MDA at different scan rates (5–200 mV/s). For clarity, the 5–40 mV/s scan rate range is shown in the inset.



UNIVERSITÀ DEGLI STUDI DI MILANO

DeFENS, Department of Food, Environmental and Nutritional Sciences
National Consortium of Materials Science and Technology (INSTM)

Milan, 24 July 2018

Jill Venton
Associate Editor
Analytical Methods

Subject: Manuscript submission

Dear Professor Venton,

please find enclosed the revised version of the manuscript entitled '*Bionanocomposite-modified glassy carbon electrode for the determination of 4,4'-methylene diphenyl diamine*' that we are submitting for possible publication in Analytical Methods as a research article.

The manuscript has been thoroughly revised according with the reviewers' comments and suggestions. We would like to thank the reviewers for their excellent input. We believe that the manuscript was considerably improved because of their valuable comments. Moreover, new analyses have been performed to substantiate the claims on our previous version.

An itemized list of the responses to each point raised by the reviewers has been included in this resubmission.

We assure that this work, or its contents in some other form, has not been published previously by any of the authors and/or is not under consideration for publication in another journal at this time.

Finally, all co-authors have read and approved the manuscript in its final form.

Thank you for your consideration of this work.

Yours faithfully,

Stefano Farris (corresponding author)

A handwritten signature in blue ink, reading 'Stefano Farris', written over a horizontal line.

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Journal Name

ARTICLE

Bionanocomposite-modified glassy carbon electrode for the determination of 4,4'-methylene diphenyl diamine

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A nanosensor based on a glassy carbon electrode modified with the biopolymer chitosan, multi-wall carbon nanotubes, and gold nanoparticles (MWCNTs-CS-Au/GCE) was developed for the determination of 4,4'-diaminodiphenyl diamine (MDA). Cyclic voltammetry (CV) was used to investigate the electrochemical behavior of the sensor in the presence of MDA. MDA displayed a well-expressed oxidation peak at 0.54 V (versus Ag/AgCl) in Britton–Robinson (B–R) universal buffer solution (pH = 10). The transfer coefficient, α , and the overall number of electrons (n) involved in the catalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface were also determined by CV. The reactivity of spiked MDA was strongly dependent on the pH of the supporting electrolyte, with a pH dependence of the MDA oxidation quantified in 27.576 mV pH⁻¹. Through chronoamperometry, the diffusion coefficient (D) of MDA was calculated as 9.49×10^{-5} cm² s⁻¹. A limit of detection of MDA was estimated to be ~20 nM through amperometry experiments, while three linear ranges were found for MDA, i.e. 0.49–10.14 μ M, 10.14–94.9 μ M, and 94.9–261.18 μ M. Real sample tests enabled emphasizing the potential involved in this nanocomposite-modified electrode as a new analytical tool for the determination of MDA.

1. Introduction

Polyurethane (PU) adhesives are obtained through the polymerization of isocyanate monomers and polyols. PU adhesives are the most commonly used adhesive systems by converters to obtain multilayer packaging systems composed of two (or more) layers joined by a tie layer. Unreacted isocyanate monomers will generate primary aromatic amines (PAAs) as soon as they come into contact with water (1). PAAs are toxic compounds and/or suspected human carcinogens. The combination of a potentially high exposure and high toxicity explains why the use of these chemicals is extensively regulated, by both national and European Union (EU) legislation (2). The specific migration limit of PAAs to foods set by the EU in order to protect the consumers' health is 10 ng for total PAAs (expressed as aniline-equivalent) per gram of food or food simulant.

Recently, various approaches have been used in order to quantify low concentrations of PAAs that migrated from the

packaging material into the foodstuff. To achieve a reliable and selective determination of PAAs that possibly migrated from the packaging materials into the food, an update of the analytical tools is essential for the food industry. In particular, the widely used spectrophotometric/colorimetric method (as equivalent to aniline) does not seem to provide accurate results compared to, for example, chromatography techniques, which are, however, more complicated and expensive, thereby less willingly adopted by most converters (3).

In recent years, electrochemical methods have attracted a lot of attention as new analytical methods for determining various analytes in very low amounts. Electrochemical sensors are one of the most popular subclasses of chemical sensors. A chemical sensor is a small device that consists of a transduction element covered by a chemical or biological recognition layer in order to quantify the analyte in the sample matrix. In the case of electrochemical sensors, the electrode is used as the transduction element (4). A sensing electrode (as the transduction element), a counter-reference electrode, and an electrolyte are the main parts of typical electrochemical sensors. The analytical information is obtained from the electrical signal that results from the interaction of the target analyte and the recognition layer at the sensing electrode (5).

When using bare electrodes as sensing electrodes, a fouling phenomenon could take place on the electrode's surface. Detection limit, sensitivity, overall reliability, and reproducibility are the most important parameters that can be negatively affected by this phenomenon. Most antifouling strategies foresee the use of a modified electrode with greater fouling resistance than an unmodified electrode. There are

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Electronic Supplementary Information (ESI) available: cyclic voltammograms at the MWCNTs-CS-AuNPs/GCE surface at different pH values and at different scan rates (5–200 mV/s). See DOI:

two main groups of nanoparticles that can be used to modify working electrodes to control fouling: i) carbon-based nanomaterials, such as carbon nanotubes or graphene (these are important due to their electrocatalytic properties, large surface area, and fouling resistance) and ii) metallic nanoparticles, which have antifouling properties as well as high electrical conductivity and important electrocatalytic properties (6).

In this study, we describe the fabrication of a new bionanocomposite-modified electrode based on multi-wall carbon nanotubes (MWCNTs), chitosan (CS), and gold nanoparticles (AuNPs) for the electrocatalytic determination of 4,4'-methylene diphenyl diamine (MDA), which is one of the most important PAAs that can migrate from PU-based packaging materials to the food. The analytical performance of the sensor was investigated, and the results showed that a combination of MWCNTs, CS, and AuNPs remarkably improved the current response. Based on our findings, the modified electrode exhibited clear functional advantages compared to other analytical methods in terms of repeatability, stability, reproducibility, and technical feasibility for the MDA determination. Full electrochemical characterization of the modified electrode was carried out and its electrochemical properties described.

2. Experimental

2.1. Chemicals and apparatus

MDA (analytical grade 98%, molar mass $198.26 \text{ g mol}^{-1}$), AuNPs (5 nm diameter), MWCNTs ($\geq 98\%$ carbon basis), CS (deacetylation $\geq 75\%$), phosphoric acid (85–90%), boric acid (99.999% trace metals basis), and acetic acid (99%) were purchased from Sigma Aldrich (Milan, Italy). Ethylene glycol monomethyl ether (EGMe) was bought from Merck (Milan, Italy), whereas alumina powder (type DX, $0.05 \mu\text{m}$ average size) was bought from EMS (Hatfield, PA, U.S.). Britton–Robinson (B–R) universal buffer (0.04 M boric acid, 0.04 M acetic acid and 0.04 M phosphoric acid) was prepared in deionized water and was tested as the supporting electrolyte.

All electrochemical experiments were carried out using an Autolab Potentiostat PGSTAT 302N potentiostat (Metrohm, Herisau, Switzerland) equipped with Nova 2.1 software. Before the experiments, the instrument was corrected for bias by a calibration procedure operated by the factory. A conventional three-electrode electrochemical cell was used throughout the experiments, using a platinum electrode and an Ag/AgCl (sat.), KCl (3 M) as counter and reference electrodes, respectively. The working electrode was a glassy carbon electrode modified using a multi-wall carbon nanotubes-chitosan and gold nanoparticles (MWCNTs-CS-AuNPs/GCE) composite coating. All electrochemical experiments were run at $25 \pm 2.5^\circ\text{C}$ under ambient conditions. The pH measurements were performed with a BASIC 20+ pH meter (Crison Instruments, S.A. Barcelona, Spain).

2.2. Preparation of modified electrodes

The MWCNT dispersion was prepared by adding 0.5 mg of MWCNTs into 1 g of EGMe and ultrasonicated by means of a UP400S (powermax = 400 W; frequency = 24 kHz) ultrasonic device (Hielscher, Teltow, Germany) equipped with a cone frustum titanium sonotrode (mod. H3, tip \varnothing 3 mm, amplitudemax = 210 μm ; acoustic power density = 460 W cm^{-2}) under the following conditions: 0.5 cycle and 50% amplitude, for a period of 10 min. Subsequently, 600 μL of the MWCNTs-EGMe dispersion was mixed with 150 μL of a CS solution (1 wt% in acidic water) and again ultrasonicated for 3 min to achieve a completely homogeneous MWCNTs-CS suspension. Eventually, 500 μL of the MWCNTs-CS suspension was mixed with 200 μL of AuNPs (16.5×10^{12} particles/mL), and the solution was ultrasonicated for 3 min to make the suspension homogeneous.

Before applying the bionanocomposite modifier, the glassy carbon electrode (GCE) surface was polished with alumina abrasive slurry (mean particle size $0.05 \mu\text{m}$) on a polishing cloth and then rinsed with double distilled water. To prepare the final MWCNTs-CS-Au/GC electrode, 15 μL of MWCNTs-CS-Au dispersion was placed directly onto the GCE surface and dried with an infrared lamp (type B, 1440 W) (Helios Italquartz srl, Cambiago, Italy) at a distance of 40 cm for 10 min. The MWCNTs-CS/GCE was made by dropping 15 μL of the MWCNTs-CS dispersion on the surface of the GCE and drying for 10 min.

2.3. Morphological characterization of electrode surface

The surface morphology of the electrode was captured using a field emission scanning electron microscope (FE-SEM) (Hitachi S-4800, Schaumburg, IL) at an acceleration voltage of 1–5 kV and an electrode current of 10 μA . MWCNTs-coated, MWCNTs-CS-coated, and MWCNTs-CS-AuNPs-coated GCE specimens were mounted with carbon tape on stubs and their surfaces observed after sputtering with Pt/Pd (60/40) under argon for 20 s at a current of 80 mA.

2.4. Real sample analysis

Thermo-sealed bags of 1 dm^2 of surface area per side were prepared using a multilayer packaging material (Castagna Univel Spa, Guardamiglio, Italy) consisting of polyethylene terephthalate (PET, 12 μm thick), polyvinylidene chloride coating (PVDC, 6 μm thick), and low-density polyethylene (LDPE, 50 μm thick), whereby a PU adhesive was used to join the PET layer to the remaining part of the film. The potential migration of PAAs from the PU-based multilayer packaging material was assessed under the worst-case condition. For this purpose, each bag was filled with 100 mL of food simulant B (i.e., acetic acid water solution, 3 w/v %) (7,8). The test was conducted at 121°C for 20 min in an autoclave (Asal 760, Steroglass srl, Perugia, Italy). After this time, 10 mL of the simulant B was diluted with 10 mL of the B–R buffer, followed by the addition of specific amounts of MDA monitored by amperometry. From the quantitative determination of MDA, the final recovery (%) was determined.

3. Results and discussion

3.1. Morphological characterization of modified GCEs

The surface morphology of the different electrodes was investigated by FE-SEM, as shown in Figure 1. MWCNTs of approximately 12 nm average diameter and 15–20 μm in length were uniformly distributed on the surface of the GCE to form a three-dimensional network that increased the specific surface area of the electrode (Figure 1a). When the MWCNTs were loaded in the main polymer phase (Figure 1b), they were no longer clearly visible, although the rough surface of the CS-based modified GCE confirmed their presence. Figure 1 (c) shows a flat surface made of MWCNTs, CS, and AuNPs, the latter hardly visible at the magnification of the analysis and most likely encased within the main biopolymer network.

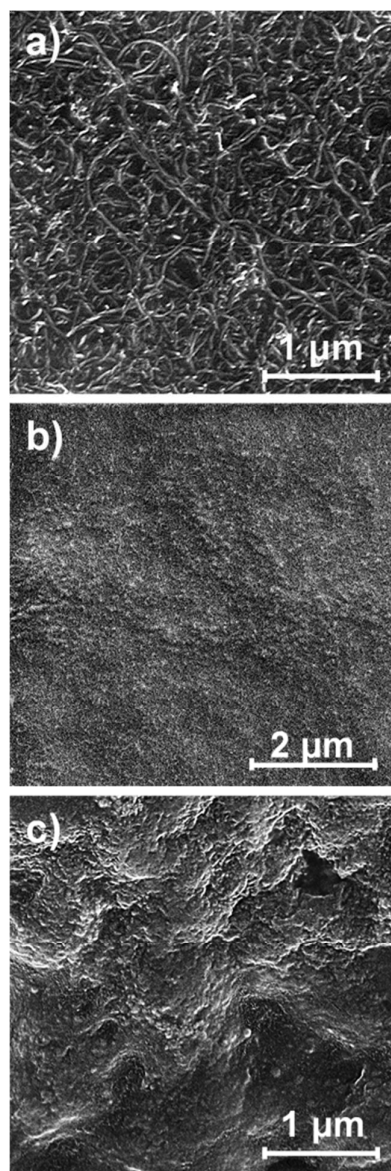


Figure 1. FE-SEM images of (a) MWCNTs, (b) MWCNTs-CS, and (c) MWCNTs-CS-AuNPs-modified GCE.

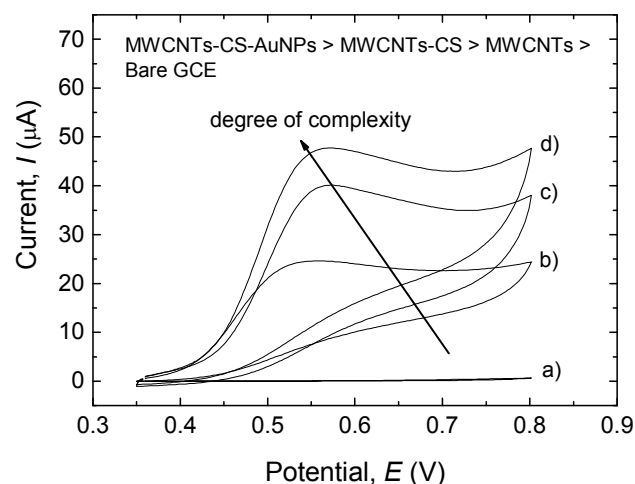


Figure 2. Cyclic voltammograms in B-R buffer (0.1 M, pH 10) at a scan rate of 50 mV/s in the presence of 500 μM of MDA: (a) bare GCE, (b) MWCNTs/GCE, (c) MWCNTs-CS/GCE, and (d) MWCNTs-CS-AuNPs/GCE.

3.2. Electrochemical behavior of MDA on electrode surface

The electrochemical behavior of the modified electrodes was examined using cyclic voltammetry (CV).

Figure 2 shows the voltammograms of the bare GCE, MWCNTs/GCE, MWCNTs-CS/GCE, and MWCNTs-CS-AuNPs/GCE. The experiments were carried out in a B-R universal buffer solution (pH 10) containing 500 μM MDA at 50 mV/s from 0.35 V to 0.8 V. The bare GCE showed a weak oxidation peak with extremely low current (trace a, Figure 2). According to trace b of Figure 2, the oxidation peak current increased remarkably after modification with MWCNTs, suggesting that MWCNTs could enhance the electron transfer rate while increasing the electrochemical surface area of the electrode (9).

In order to improve the performance of the electrode, a mixture of CS and MWCNTs was used in the next step. As shown in Figure 2, trace c, the peak current increased dramatically compared to the MWCNTs-modified electrode. It was inferred that CS could effectively improve the performance of MWCNTs through its film-forming ability and good adhesion, resulting in the amplification of the current response (10). Ultimately, in an attempt to upsurge the sensitivity of the sensor, the MWCNT-CS solution was mixed with AuNPs and the electrode was modified with the MWCNT-CS-AuNPs bionanocomposite solution. The final voltammogram showed that the peak current increased further compared to the MWCNT-CS/GCE, plausibly due to the increase of the electrode's conductivity owing to the excellent inherent electronic properties of the AuNPs, eventually acting as an electron wire. The electrocatalytic oxidation

characteristics of MDA on different modified electrodes are summarized in Table 1.

Table 1. Comparison of the electrocatalytic oxidation peak current (I_p) of MDA (500 μ M) on various electrode surfaces at pH 10.0.

Electrode	Oxidation peak current (μ A)	Modifier
Bare GCE	0.01	-
MWCNTs/GCE	14.66	15 μ L MWCNTs
MWCNTs-CS/GCE	23.47	15 μ L MWCNTs-CS
MWCNTs-CS-AuNPs/GCE	27.50	15 μ L MWCNTs-CS/AuNPs

3.3. Effect of pH value

The effect of the pH on the electrochemical behavior of MDA was studied by CV at the MWCNTs-CS-AuNPs/GCE surface at different pH values (from 2 to 11) in a 0.1 M B-R buffer solution containing 500 μ M of MDA (Figure S1 of ESI).

When the pH value increased from 2 to 10, the oxidation peak current increased, reaching its maximum at pH 10, after which an apparent steady condition was achieved (Figure 3). Moreover, the oxidation peak potential, E_{pa} , shifted to negative values when the pH increased, indicating that protons were involved in the oxidation reaction (11). This was further confirmed by the linear relationship between the oxidation peak potential and the pH and expressed as: $E_{pa} = -27.576\text{pH} + 820.24$ ($R^2 = 0.985$). The potential negatively shifted by 27.576 mV per pH unit. According to the Nernst equation, a slope approaching 0.0592/2 indicates an electron-to-proton proportion equal to 2:1 (12), that is, one proton per two electrons was involved in the electrochemical oxidation of MDA.

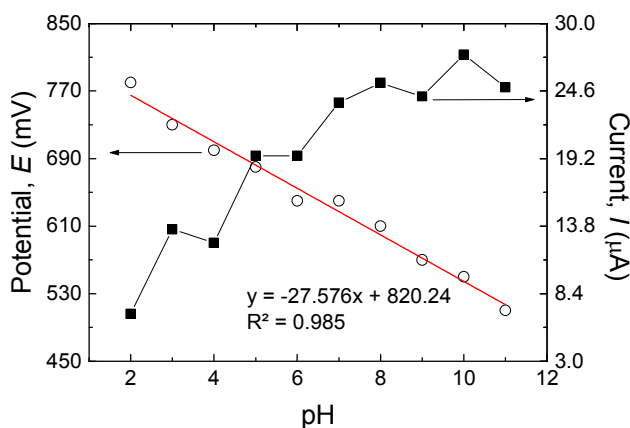


Figure 3. Effect of the pH on the oxidation peak current and oxidation peak potential in a 500 μ M of MDA (B-R solution 0.1 M) on the surface of the MWCNTs-CS-AuNPs/GCE.

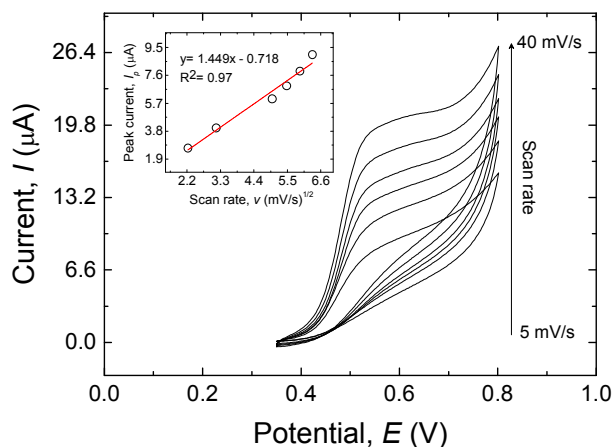


Figure 4. Cyclic voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μ M MDA at different scan rates (5–40 mV/s). The electrocatalytic peak current (I_p) variation as a function of the square root of sweep rate is shown in the inset.

3.4. Electrocatalytic oxidation of MDA at the MWCNTs-CS AuNPs/GCE surface

The effect of scan rate (ν) on the electrocatalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface was investigated by CV within a range of 5–200 mV/s (Figure S2 of ESI) at an MDA concentration of 500 μ M. For clarity, the range 5–40 mV/s is shown in Figure 4. Figure 4 (inset) shows the dependence of the oxidation peak current (I_p) drawn from each scan rate against the square root of the potential scan rate ($\nu^{1/2}$). The linearity of the ensuing plot suggests that the catalytic reaction of the analyte at the electrode's surface is diffusion-limited; that is, no absorption of the analyte within the nanocomposite polymer network occurs before oxidation (13).

Figure 5 displays the linear sweep voltammograms obtained applying different scan rates (25–40 mV/s) to obtain information on the rate-determining step of the electro-oxidation of MDA (14). The inset of Figure 5 shows the Tafel regions obtained from the linear sweep voltammograms. The slope of each region was determined according to equation 1 (15):

$$\text{Tafel}_{\text{slope}} = \frac{(1-\alpha)n_a F}{2.3RT} \quad (1)$$

where α is the electron transfer coefficient, $n\alpha$ is the number of electrons transferred until the end of the rate-determining step (up to and including the rate-determining step), F is Faraday's constant, R is gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), and T the temperature (K). An average slope of 11.8 V^{-1} was eventually calculated. If the rate-determining step of the electrode process includes one electron transfer, the charge transfer coefficient (α) is 0.50.

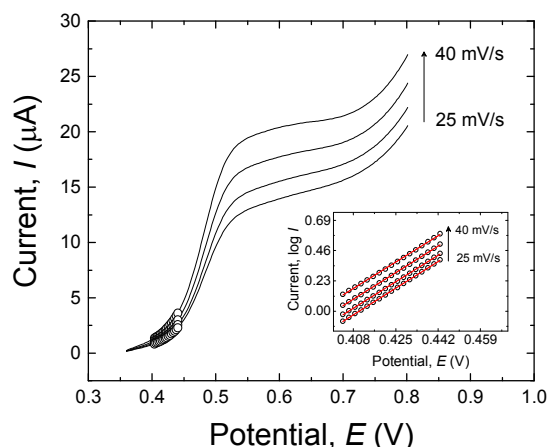
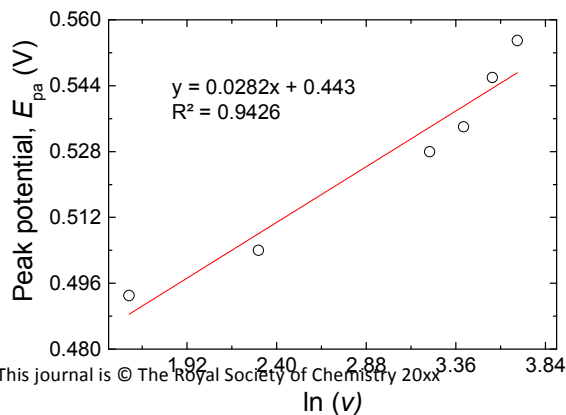


Figure 5. Linear sweep voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μM MDA at 25–40 mV/s. The Tafel plot derived from the linear sweep voltammograms is shown in the inset.

Using CV, the number of electrons (n_α) involved in the rate-determining step of the reaction was calculated according to the following equation:

$$|E_p - E_{p/2}| = \frac{47.7}{\alpha n_\alpha} \quad (2)$$

where E_p is the peak potential (oxidation in our case) of a selected CV trace (800 mV/s in this study) and $E_{p/2}$ is the half-peak potential (i.e., the potential [V] at the half-peak current [$I_p/2$]). $|E_p - E_{p/2}|$ value was calculated to be 68 mV, from which a value of 0.701 was calculated for αn_α (16). Because for a totally irreversible reaction, the electron transfer coefficient (α) is assumed to be 0.50, a final n_α value of ca. $1.39 \approx 1$ for the oxidation of MDA was calculated, which is slightly higher than the theoretical value of ≈ 1 assumed by eq. 1.



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Figure 6. Experimental data (black dots) and linear regression of anodic peak potential (E_{pa}) versus the natural logarithm of the scan rate [$\ln(v)$].

The total number of electrons (n) involved in the overall catalytic reaction was gathered according to Laviron's theory, which is used to investigate the kinetic mechanism of the electrode toward the analyte. Accordingly, for a totally irreversible electrode process, the anodic peak potential (E_{pa}) and the natural logarithm of the scan rate $\ln(v)$ can be defined by the following equation (17):

$$E_{pa} = E^\theta + \left(\frac{RT}{\alpha nF}\right) \ln\left(\frac{RTK^\theta}{\alpha nF}\right) + \left(\frac{RT}{\alpha nF}\right) \ln v \quad (3)$$

where α is the transfer coefficient, K^θ is the standard rate constant of the reaction, n is the electron transfer number, v is the scanning rate, E^θ is the formal redox potential, R is the gas constant, T is the absolute temperature, and F is the Faraday constant. Using the raw voltammograms as reported in Figure 4, a linear relationship between E_{pa} and $\ln(v)$ was obtained as expressed by the equation $E_p \text{ (V)} = 0.0282 \ln v \text{ (mV/s)} + 0.443$ (Figure 6). According to Laviron's equation, the electron transfer number (n) can be calculated from the slope of E_{pa} versus $\ln(v)$, that is, $RT/\alpha nF$. After appropriate substitutions, a final value of $n \approx 1.83 \approx 2$ was obtained, indicating that the electrochemical oxidation of MDA at the MWCNTs-CS-AuNPs composite-modified GCE is a two-electron transfer process.

3.5. Chronoamperometric measurements

The electrochemical oxidation of MDA at the surface of MWCNTs-CS-Au/GCE was studied through chronoamperometry at a potential of 700 mV and varying the concentration of MDA from 0.005 mM to 0.4 mM (Figure 7). For an electroactive material with a diffusion coefficient D , the current response under diffusion control is described by the Cottrell equation (15):

$$I = \frac{nFAD^{1/2}C_b}{\pi^{1/2}t^{1/2}} \quad (4)$$

where n is the number of electrons exchanged per reactant molecule, F is the Faraday constant ($9.648 \times 10^4 \text{ C mol}^{-1}$), A is the geometric area of the electrode (0.0314 cm^2), C_b is the bulk concentration of the analyte (mol cm^{-3}), and D ($\text{cm}^2 \text{ s}^{-1}$) is the diffusion coefficient of the analyte.

From the raw chronoamperometric traces, a linear curve was obtained for the different concentrations of MDA by plotting I against $t^{-1/2}$ (Figure 7, inset a). By plotting the slope of each individual straight line, the overall slope of the best-fit line (Figure 7, inset b) can be defined from equation 4 as:

$$It^{1/2} = \frac{nFAD^{1/2}C_b}{\pi^{1/2}} \quad (5)$$

D can thus be drawn from:

$$D = \frac{(\text{slope})^2 \pi}{(nFAC_b)^2} \quad (6)$$

The overall slope ($33.32 \mu\text{A s}^{1/2} \text{mM}^{-1}$) was used within the Cottrell equation to eventually obtain a diffusion coefficient of $9.49 \times 10^{-5} \text{cm}^2 \text{s}^{-1}$.

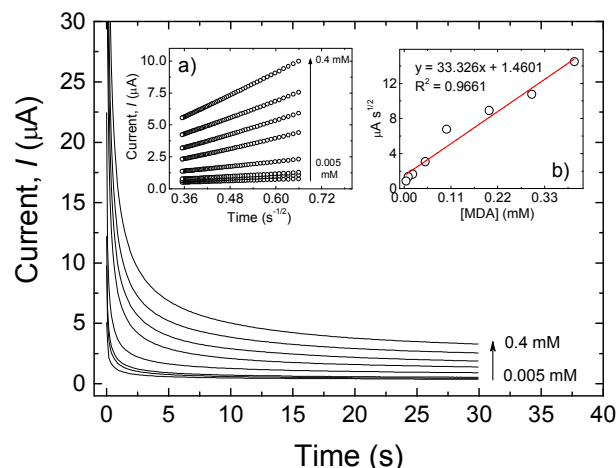


Figure 7. Current response over time of the MWCNTs-CS-AuNPs/GCE in B-R buffer solution (0.1 M, pH 10.0) containing different concentrations of MDA (0.005 – 0.4 mM) by chronoamperometric measurements (potential 700 mV). Numbers 1–8 correspond to the different MDA concentrations. Insets: (a) chronoamperograms of the intensity (I) as a function of the reciprocal square root of time ($t^{-1/2}$); (b) linear plot of the slopes of the eight straight lines in the inset (a) against the MDA concentration.

3.6. Amperometric studies of electrocatalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface

The sensitivity of the MWCNTs-CS-AuNPs-modified electrode to MDA was investigated by amperometry, which has been shown to be a more effective method than voltammetry due to the stirring conditions during the experiment (4). Figure 8 shows the amperogram obtained from a rotating MWCNTs-CS-AuNPs/GCE at a fixed potential of 700 mV in a 0.1 M B-R buffer solution (pH 10.0) containing different concentrations of MDA.

During successive dropwise additions of MDA, a well-defined stairs-like current versus time plot was obtained. The calibration plot was linear in three concentration ranges (i.e., 0.49–10.14 μM [Figure 8, inset a], 10.14–94.9 μM [Figure 8, inset b], and 94.9–261.18 μM [Figure 8, inset c]) of MDA. The sensitivity of the modified electrode to MDA, calculated as the slope of the lower linear range in the amperometric plot, was $0.1509 \mu\text{A} (\mu\text{M})^{-1}$, from which the lower limit of detection (LOD) and the lower limit of quantification were calculated according to the following equations (18,19):

$$LOD = \frac{3 \times S_{bl}}{m} \quad (7)$$

$$LOQ = \frac{10 \times S_{bl}}{m} \quad (8)$$

where S_{bl} is the standard deviation of the blank response (μA) obtained from 10 replicates of the blank solution (0.001) and m is the slope of the aforementioned lower linear range in the amperometric plot. A final LOD and LOQ values of 20 nM and 66 nM were eventually calculated for the MWCNTs-CS-AuNPs/GCE electrode.

The operational stability of the MWCNTs-CS-AuNPs/GCE composite-modified electrode was further examined by amperometry, and the results are shown in the inset (d) of Figure 8. The amperometric current response of MDA was almost unchanged when continuously run up to 3310 s in 5 μM MDA containing B-R buffer. This observation shows that throughout the monitored time span, there was no inhibition effect due to the adsorption of MDA and MDA's oxidation products on the modified electrode surface.

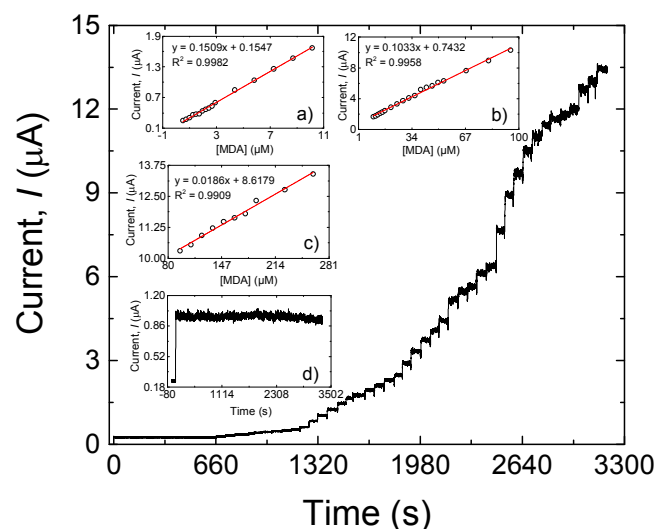


Figure 8. Amperometric responses at a rotating MWCNTs-CS-AuNPs/GCE held at 700 mV in different concentrations of 0.49 to 261.18 μM of MDA. The variation of the amperometric current against the MDA concentration is shown in insets (a) (0.49–10.14 μM), (b) (10.14–94.9 μM), and (c) (94.9–261.18 μM). Inset (d) shows the stability of the response of the MWCNTs-CS-AuNPs/GCE during 3310 s (MDA concentration of 5 μM).

Table 2. Amount of added (spiked) and measured MDA at the MWCNTs-CS-AuNPs/GCE surface, with the resulting recovery percentage after the migration test using the acidic food simulant (simulant B) under typical sterilization conditions (121 °C for 20 min).

Sample	Spiked (μM)	Found (μM)	Recovery (%)
Sample 1	0	-	-
	3.5	3.54	101.1
	5	4.93	98.6

	5.5	5.4	98.1
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The accuracy was estimated as the recovery from known concentrations of MDA (Table 2). The recovery ranged from 98.1 to 101.1%. These results show the adequate accuracy of the method and suggest that the modified electrode as prepared in this work is well-suited for the quantification of MDA possibly migrating from food packaging materials exposed to extensive thermal treatments (e.g., sous-vide cooking, pasteurization, and sterilization).

Finally, the precision (repeatability) of the electrochemical method was assessed preparing independently three different MWCNTs-CS-AuNPs/GCE sensors. For each sensor, subsequent injections ($n = 15$) of MDA standard solutions at three different concentrations (5, 10, and 15 μM) were analyzed. The relative standard deviation (RSD) was $3.13 \pm 0.25\%$, which indicates the satisfactory repeatability and good reproducibility of the proposed electrode.

All the relevant analytical parameters of the MWCNTs-CS-AuNPs/GC electrochemical sensor used for the detection of MDA are summarized in Table 3.

Table 3. Analytical parameters of the MWCNTs-CS-AuNPs/GC electrochemical sensor used for the MDA determination.

Parameter	Value
	0.49-10.14 μM
Linearity (ranges)	10.14-94.9 μM 94.9-261.18 μM
Sensitivity	$0.1509 \mu\text{A} (\mu\text{M})^{-1}$
LOD	20 nM
LOQ	66 nM
Recovery (%)	99.27 ± 1.60
RSD (%)	3.13 ± 0.25

3.7. Real sample analysis

In this experiment, we have attempted to determine the MDA concentration that had possibly migrated from the PU-based food packaging material into the food simulant through a migration test carried out under typical sterilization conditions (121 $^{\circ}\text{C}$ for 20 min). The standard addition method was used to evaluate the potential application of the MWCNTs-CS-AuNPs/GCE as an analytical device for MDA determination in real samples. To achieve this goal, the recovery of MDA was determined by amperometry after the addition of MDA in the buffered food simulant B (acetic acid water solution, 3 w/v %) (Table 2).

Conclusions

A new electrochemical sensor with high sensitivity and a low limit of detection was developed for the quantification of the MDA, which is one of the two most representative PAAs possibly migrating from multilayer packaging materials made

of PU adhesives into foodstuffs. The sensor was developed using a MWCNTs-CS-AuNPs bionanocomposite to modify the surface of the electrode. The synergistic effect between MWCNTs, CS, and AuNPs toward the electro-oxidation of MDA was recognized as the main factor that dictates the excellent electrocatalytic performance of the sensor in terms of sensitivity, detection limit, and stability over a reasonably extended temporal window (no fouling on the surface of the electrode). The tests conducted on real samples indicated that the modified sensor has the potential to be used as an analytical device for the quantification of MDA.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

We are thankful to Dr. K. Morozova for precious technical support.

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Journal Name

ARTICLE

Bionanocomposite-modified glassy carbon electrode for the determination of 4,4'-methylene diphenyl diamine

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Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A nanosensor based on a glassy carbon electrode modified with the biopolymer chitosan, multi-wall carbon nanotubes, and gold nanoparticles (MWCNTs-CS-Au/GCE) was developed for the determination of 4,4'-diaminodiphenyl diamine (MDA). Cyclic voltammetry (CV) was used to investigate the electrochemical behavior of the sensor in the presence of MDA. MDA displayed a well-expressed oxidation peak at 0.54 V (versus Ag/AgCl) in Britton–Robinson (B–R) universal buffer solution (pH = 10). The transfer coefficient, α , and the overall number of electrons (n) involved in the catalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface were also determined by CV. The reactivity of spiked MDA was strongly dependent on the pH of the supporting electrolyte, with a pH dependence of the MDA oxidation quantified in 27.576 mV pH⁻¹. Through chronoamperometry, the diffusion coefficient (D) of MDA was calculated as 9.49×10^{-5} cm² s⁻¹. A limit of detection of MDA was estimated to be ~20 nM through amperometry experiments, while three linear ranges were found for MDA, i.e. 0.49–10.14 μ M, 10.14–94.9 μ M, and 94.9–261.18 μ M. Real sample tests enabled emphasizing the potential involved in this nanocomposite-modified electrode as a new analytical tool for the determination of MDA.

1. Introduction

Polyurethane (PU) adhesives are obtained through the polymerization of isocyanate monomers and polyols. PU adhesives are the most commonly used adhesive systems by converters to obtain multilayer packaging systems composed of two (or more) layers joined by a tie layer. Unreacted isocyanate monomers will generate primary aromatic amines (PAAs) as soon as they come into contact with water (1). PAAs are toxic compounds and/or suspected human carcinogens. The combination of a potentially high exposure and high toxicity explains why the use of these chemicals is extensively regulated, by both national and European Union (EU) legislation (2). The specific migration limit of PAAs to foods set by the EU in order to protect the consumers' health is 10 ng for total PAAs (expressed as aniline-equivalent) per gram of food or food simulant.

Recently, various approaches have been used in order to quantify low concentrations of PAAs that migrated from the

packaging material into the foodstuff. To achieve a reliable and selective determination of PAAs that possibly migrated from the packaging materials into the food, an update of the analytical tools is essential for the food industry. In particular, the widely used spectrophotometric/colorimetric method (as equivalent to aniline) does not seem to provide accurate results compared to, for example, chromatography techniques, which are, however, more complicated and expensive, thereby less willingly adopted by most converters (3).

In recent years, electrochemical methods have attracted a lot of attention as new analytical methods for determining various analytes in very low amounts. Electrochemical sensors are one of the most popular subclasses of chemical sensors. A chemical sensor is a small device that consists of a transduction element covered by a chemical or biological recognition layer in order to quantify the analyte in the sample matrix. In the case of electrochemical sensors, the electrode is used as the transduction element (4). A sensing electrode (as the transduction element), a counter-reference electrode, and an electrolyte are the main parts of typical electrochemical sensors. The analytical information is obtained from the electrical signal that results from the interaction of the target analyte and the recognition layer at the sensing electrode (5).

When using bare electrodes as sensing electrodes, a fouling phenomenon could take place on the electrode's surface. Detection limit, sensitivity, overall reliability, and reproducibility are the most important parameters that can be negatively affected by this phenomenon. Most antifouling strategies foresee the use of a modified electrode with greater fouling resistance than an unmodified electrode. There are

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Electronic Supplementary Information (ESI) available: cyclic voltammograms at the MWCNTs-CS-AuNPs/GCE surface at different pH values and at different scan rates (5–200 mV/s). See DOI: 10.1039/x0xx00000x

two main groups of nanoparticles that can be used to modify working electrodes to control fouling: i) carbon-based nanomaterials, such as carbon nanotubes or graphene (these are important due to their electrocatalytic properties, large surface area, and fouling resistance) and ii) metallic nanoparticles, which have antifouling properties as well as high electrical conductivity and important electrocatalytic properties (6).

In this study, we describe the fabrication of a new bionanocomposite-modified electrode based on multi-wall carbon nanotubes (MWCNTs), chitosan (CS), and gold nanoparticles (AuNPs) for the electrocatalytic determination of 4,4'-methylene diphenyl diamine (MDA), which is one of the most important PAAs that can migrate from PU-based packaging materials to the food. The analytical performance of the sensor was investigated, and the results showed that a combination of MWCNTs, CS, and AuNPs remarkably improved the current response. Based on our findings, the modified electrode exhibited clear functional advantages compared to other analytical methods in terms of repeatability, stability, reproducibility, and technical feasibility for the MDA determination. Full electrochemical characterization of the modified electrode was carried out and its electrochemical properties described.

2. Experimental

2.1. Chemicals and apparatus

MDA (analytical grade 98%, molar mass $198.26 \text{ g mol}^{-1}$), AuNPs (5 nm diameter), MWCNTs ($\geq 98\%$ carbon basis), CS (deacetylation $\geq 75\%$), phosphoric acid (85–90%), boric acid (99.999% trace metals basis), acetic acid (99%), and aniline ($\geq 99.5\%$) were purchased from Sigma Aldrich (Milan, Italy). Ethylene glycol monomethyl ether (EGMe) was bought from Merck (Milan, Italy), whereas alumina powder (type DX, $0.05 \mu\text{m}$ average size) was bought from EMS (Hatfield, PA, U.S.). Britton–Robinson (B–R) universal buffer (0.04 M boric acid, 0.04 M acetic acid and 0.04 M phosphoric acid) was prepared in deionized water and was tested as the supporting electrolyte.

All electrochemical experiments were carried out using an Autolab Potentiostat PGSTAT 302N potentiostat (Metrohm, Herisau, Switzerland) equipped with Nova 2.1 software. Before the experiments, the instrument was corrected for bias by a calibration procedure operated by the factory. A conventional three-electrode electrochemical cell was used throughout the experiments, using a platinum electrode and an Ag/AgCl (sat.), KCl (3 M) as counter and reference electrodes, respectively. The working electrode was a glassy carbon electrode modified using a multi-wall carbon nanotubes-chitosan and gold nanoparticles (MWCNTs-CS-AuNPs/GCE) composite coating. All electrochemical experiments were run at $25 \pm 2.5^\circ\text{C}$ under ambient conditions. The pH measurements were performed with a BASIC 20+ pH meter (Crison Instruments, S.A. Barcelona, Spain).

2.2. Preparation of modified electrodes

The MWCNT dispersion was prepared by adding 0.5 mg of MWCNTs into 1 g of EGMe and ultrasonicated by means of a UP400S (powermax = 400 W; frequency = 24 kHz) ultrasonic device (Hielscher, Teltow, Germany) equipped with a cone frustum titanium sonotrode (mod. H3, tip \varnothing 3 mm, amplitudemax = 210 μm ; acoustic power density = 460 W cm^{-2}) under the following conditions: 0.5 cycle and 50% amplitude, for a period of 10 min. Subsequently, 600 μL of the MWCNTs-EGMe dispersion was mixed with 150 μL of a CS solution (1 wt% in acidic water) and again ultrasonicated for 3 min to achieve a completely homogeneous MWCNTs-CS suspension. Eventually, 500 μL of the MWCNTs-CS suspension was mixed with 200 μL of AuNPs (16.5×10^{12} particles/mL), and the solution was ultrasonicated for 3 min to make the suspension homogeneous.

Before applying the bionanocomposite modifier, the glassy carbon electrode (GCE) surface was polished with alumina abrasive slurry (mean particle size $0.05 \mu\text{m}$) on a polishing cloth and then rinsed with double distilled water. To prepare the final MWCNTs-CS-Au/GC electrode, 15 μL of MWCNTs-CS-Au dispersion was placed directly onto the GCE surface and dried with an infrared lamp (type B, 1440 W) (Helios Italquartz srl, Cambiago, Italy) at a distance of 40 cm for 10 min. The MWCNTs-CS/GCE was made by dropping 15 μL of the MWCNTs-CS dispersion on the surface of the GCE and drying for 10 min.

2.3. Morphological characterization of electrode surface

The surface morphology of the electrode was captured using a field emission scanning electron microscope (FE-SEM) (Hitachi S-4800, Schaumburg, IL) at an acceleration voltage of 1–5 kV and an electrode current of 10 μA . MWCNTs-coated, MWCNTs-CS-coated, and MWCNTs-CS-AuNPs-coated GCE specimens were mounted with carbon tape on stubs and their surfaces observed after sputtering with Pt/Pd (60/40) under argon for 20 s at a current of 80 mA.

2.4. Real sample analysis

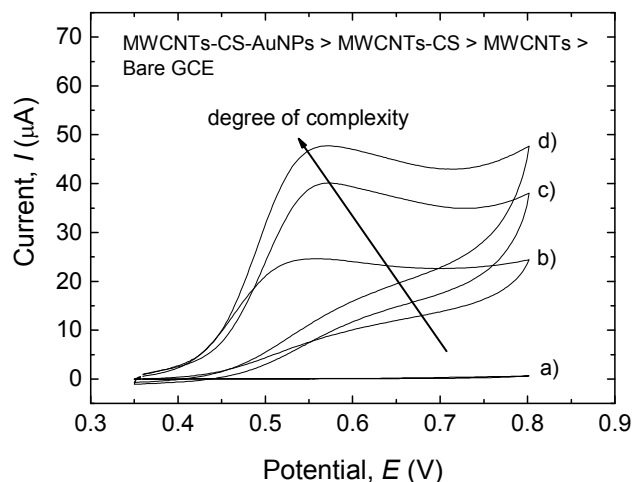
Thermo-sealed bags of 1 dm^2 of surface area per side were prepared using a multilayer packaging material (Castagna Univel Spa, Guardamiglio, Italy) consisting of polyethylene terephthalate (PET, 12 μm thick), polyvinylidene chloride coating (PVDC, 6 μm thick), and low-density polyethylene (LDPE, 50 μm thick), whereby a PU adhesive was used to join the PET layer to the remaining part of the film. The potential migration of PAAs from the PU-based multilayer packaging material was assessed under the worst-case condition. For this purpose, each bag was filled with 100 mL of food simulant B (i.e., acetic acid water solution, 3 w/v %) (7,8). The test was conducted at 121°C for 20 min in an autoclave (Asal 760, Steroglass srl, Perugia, Italy). After this time, 10 mL of the simulant B was diluted with 10 mL of the B–R buffer, followed by the addition of specific amounts of MDA monitored by

amperometry. From the quantitative determination of MDA, the final recovery (%) was determined.

3. Results and discussion

3.1. Morphological characterization of modified GCEs

The surface morphology of the different electrodes was investigated by FE-SEM, as shown in Figure 1. MWCNTs of approximately 12 nm average diameter and 15–20 μm in length were uniformly distributed on the surface of the GCE to form a three-dimensional network that increased the specific surface area of the electrode (Figure 1a). When the MWCNTs were loaded in the main polymer phase (Figure 1b), they were no longer clearly visible, although the rough surface of the CS-based modified GCE confirmed their presence. Figure 1 (c) shows a flat surface made of MWCNTs, CS, and AuNPs, the latter hardly visible at the magnification of the analysis and most likely encased within the main biopolymer network.



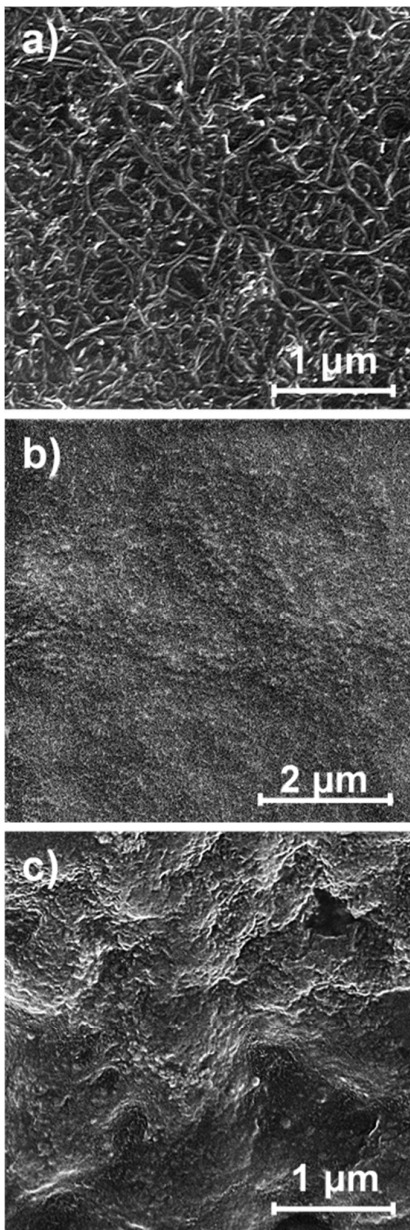


Figure 1. FE-SEM images of (a) MWCNTs, (b) MWCNTs-CS, and (c) MWCNTs-CS-AuNPs-modified GCE.
Figure 2. Cyclic voltammograms in B–R buffer (0.1 M, pH 10) at a scan rate of 50 mV/s in the presence of 500 μM of MDA: (a) bare GCE, (b) MWCNTs/GCE, (c) MWCNTs-CS/GCE, and (d) MWCNTs-CS-AuNPs/GCE.

3.2. Electrochemical behavior of MDA on electrode surface

The electrochemical behavior of the modified electrodes was examined using cyclic voltammetry (CV).
Figure 2 shows the voltammograms of the bare GCE, MWCNTs/GCE, MWCNTs-CS/GCE, and MWCNTs-CS-AuNPs/GCE. The experiments were carried out in a B–R universal buffer solution (pH 10) containing 500 μM MDA at 50

mV/s from 0.35 V to 0.8 V. The bare GCE showed a weak oxidation peak with extremely low current (trace a, Figure 2). According to trace b of Figure 2, the oxidation peak current increased remarkably after modification with MWCNTs, suggesting that MWCNTs could enhance the electron transfer rate while increasing the electrochemical surface area of the electrode (9).
In order to improve the performance of the electrode, a mixture of CS and MWCNTs was used in the next step. As shown in Figure 2, trace c, the peak current increased dramatically compared to the MWCNTs-modified electrode. It was inferred that CS could effectively improve the performance of MWCNTs through its film-forming ability and good adhesion, resulting in the amplification of the current response (10). Ultimately, in an attempt to upsurge the sensitivity of the sensor, the MWCNT-CS solution was mixed with AuNPs and the electrode was modified with the MWCNT-CS-AuNPs bionanocomposite solution. The final voltammogram showed that the peak current increased further compared to the MWCNT-CS/GCE, plausibly due to the increase of the electrode’s conductivity owing to the excellent inherent electronic properties of the AuNPs, eventually acting as an electron wire. The electrocatalytic oxidation characteristics of MDA on different modified electrodes are summarized in Table 1.

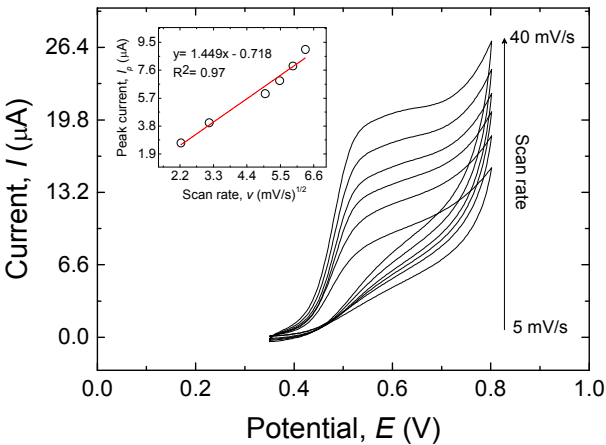


Table 1. Comparison of the electrocatalytic oxidation peak current (I_p) of MDA (500 μM) on various electrode surfaces at pH 10.0.

Electrode	Oxidation peak current (μA)	Modifier
Bare GCE	0.01	-
MWCNTs/GCE	14.66	15 μL MWCNTs
MWCNTs-CS/GCE	23.47	15 μL MWCNTs-CS
MWCNTs-CS-AuNPs/GCE	27.50	15 μL MWCNTs-

3.3. Effect of pH value

The effect of the pH on the electrochemical behavior of MDA was studied by CV at the MWCNTs-CS-AuNPs/GCE surface at different pH values (from 2 to 11) in a 0.1 M B-R buffer solution containing 500 μM of MDA (Figure S1 of ESI-3).

When the pH value increased from 2 to 10, the oxidation peak current increased, reaching its maximum at pH 10, after which an apparent steady condition was achieved (Figure 3). Moreover, the oxidation peak potential, E_{pa} , shifted to negative values when the pH increased, indicating that protons were involved in the oxidation reaction (11). This was further confirmed by the linear relationship between the oxidation peak potential and the pH and expressed as: $E_{pa} = -27.576\text{pH} + 820.24$ ($R^2 = 0.985$). The potential negatively shifted by 27.576 mV per pH unit. According to the Nernst equation, a slope approaching 0.0592/2 indicates an electron-to-proton proportion equal to 2:1 (12), that is, one proton per two electrons was involved in the electrochemical oxidation of MDA.

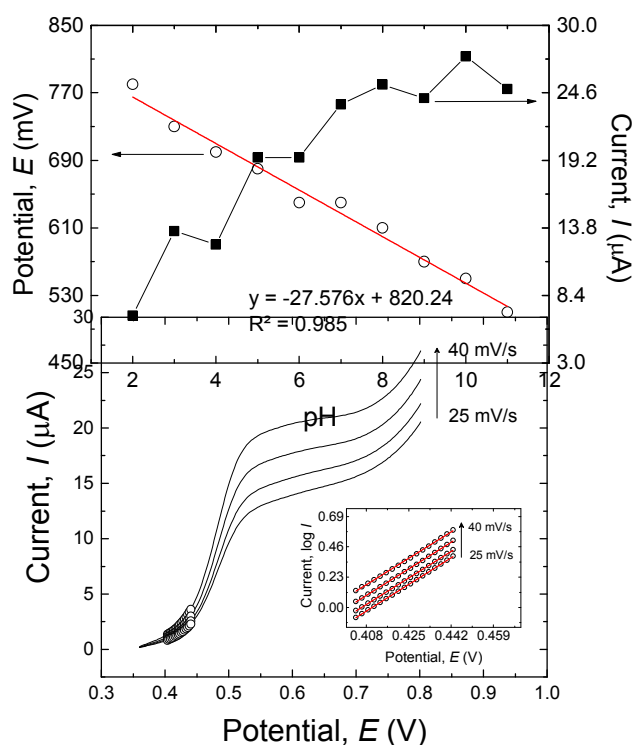


Figure 3. Effect of the pH on the oxidation peak current and oxidation peak potential in a 500 μM of MDA (B-R solution 0.1 M) on the surface of the MWCNTs-CS-AuNPs/GCE.

Figure 4. Cyclic voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μM MDA at different scan rates (5–40 mV/s). The electrocatalytic peak current (I_p) variation as a function of the square root of sweep rate is shown in the inset.

3.4. Electrocatalytic oxidation of MDA at the MWCNTs-CS AuNPs/GCE surface

The effect of scan rate (ν) on the electrocatalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface was investigated by CV within a range of 5–200 mV/s (Figure S1 of ESI) at an MDA concentration of 500 μM . For clarity, the range of 5–40 mV/s at an MDA concentration of 500 μM is shown in Figure 4. Figure 4 (inset) shows the dependence of the oxidation peak current (I_p) drawn from each scan rate against the square root of the potential scan rate ($\nu^{1/2}$). The linearity of the ensuing plot suggests that the catalytic reaction of the analyte at the electrode's surface is diffusion-limited; that is, no absorption of the analyte within the nanocomposite polymer network occurs before oxidation (13).

Figure 5 displays the linear sweep voltammograms obtained applying different scan rates (25–40 mV/s) to obtain information on the rate-determining step of the electro-oxidation of MDA (14). The inset of Figure 5 shows the Tafel regions obtained from the linear sweep voltammograms. The slope of each region was determined according to equation 1 (15):

$$\text{Tafel}_{\text{slope}} = \frac{(1-\alpha)n_{\alpha}F}{2.3RT} \quad (1)$$

where α is the electron transfer coefficient, n_{α} is the number of electrons transferred until the end of the rate-determining step (up to and including the rate-determining step), F is Faraday's constant, R is gas constant (8.314 J mol⁻¹ K⁻¹), and T the temperature (K). An average slope of 11.8 V⁻¹ was eventually calculated. If the rate-determining step of the electrode process includes one electron transfer, the charge transfer coefficient (α) is 0.50.

Figure 5. Linear sweep voltammograms of MWCNTs-CS-AuNPs/GCE in a B-R buffer solution (0.1 M, pH 10.0) containing 500 μM MDA at 25–40 mV/s. The Tafel plot derived from the linear sweep voltammograms is shown in the inset.

Using CV, the number of electrons (n_{α}) involved in the rate-determining step of the reaction was calculated according to the following equation:

$$|E_p - E_{p/2}| = \frac{47.7}{\alpha n_{\alpha}} \quad (2)$$

where E_p is the peak potential (oxidation in our case) of a selected CV trace (800 mV/s in this study) and $E_{p/2}$ is the half-peak potential (i.e., the potential [V] at the half-peak current [$I_p/2$]). $|E_p - E_{p/2}|$ value was calculated to be 68 mV, from which a value of 0.701 was calculated for αn_α (16). Because for a totally irreversible reaction, the electron transfer coefficient (α) is assumed to be 0.50, a final n_α value of ca. $1.39 \approx 1$ for the oxidation of MDA was calculated, which is slightly higher than the theoretical value of ≈ 1 assumed by eq. 1.

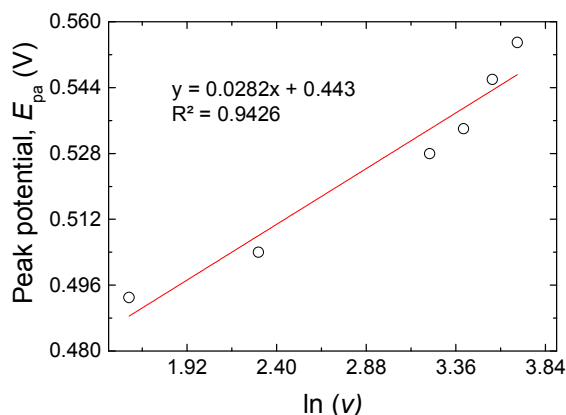
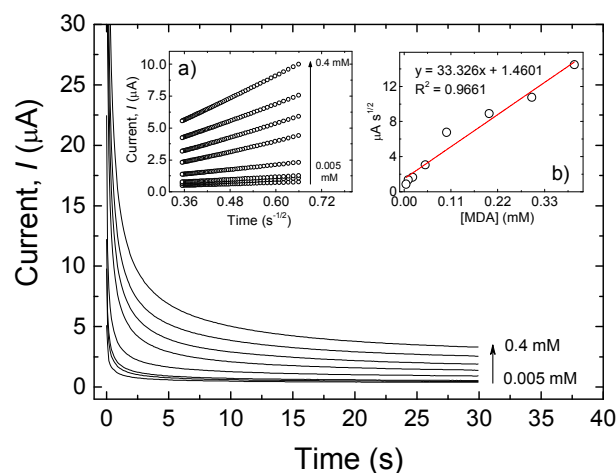


Figure 6. Experimental data (black dots) and linear regression of anodic peak potential (E_{pa}) versus the natural logarithm of the scan rate [$\ln(v)$].



The total number of electrons (n) involved in the overall catalytic reaction was gathered according to Laviron's theory, which is used to investigate the kinetic mechanism of the electrode toward the analyte. Accordingly, for a totally irreversible electrode process, the anodic peak potential (E_{pa}) and the natural logarithm of the scan rate $\ln(v)$ can be defined by the following equation (17):

$$E_{pa} = E^\theta + \left(\frac{RT}{\alpha nF}\right) \ln\left(\frac{RTK^\theta}{\alpha nF}\right) + \left(\frac{RT}{\alpha nF}\right) \ln v \quad (3)$$

where α is the transfer coefficient, K^θ is the standard rate constant of the reaction, n is the electron transfer number, v is

the scanning rate, E^θ is the formal redox potential, R is the gas constant, T is the absolute temperature, and F is the Faraday constant. Using the raw voltammograms as reported in Figure 4, a linear relationship between E_{pa} and $\ln(v)$ was obtained as expressed by the equation E_p (V) = $0.0282 \ln v$ (mV/s) + 0.443 (Figure 6). According to Laviron's equation, the electron transfer number (n) can be calculated from the slope of E_{pa} versus $\ln(v)$, that is, $RT/\alpha nF$. After appropriate substitutions, a final value of n $1.83 \approx 2$ was obtained, indicating that the electrochemical oxidation of MDA at the MWCNTs-CS-AuNPs composite-modified GCE is a two-electron transfer process.

3.5. Chronoamperometric measurements

The electrochemical oxidation of MDA at the surface of MWCNTs-CS-Au/GCE was studied through chronoamperometry at a potential of 700 mV and varying the concentration of MDA from 0.005 mM to 0.4 mM (Figure 7). For an electroactive material with a diffusion coefficient D , the current response under diffusion control is described by the Cottrell equation (15):

$$I = \frac{nFAD^{1/2}C_b}{\pi^{1/2}t^{1/2}} \quad (4)$$

where n is the number of electrons exchanged per reactant molecule, F is the Faraday constant (9.648×10^4 C mol $^{-1}$), A is the geometric area of the electrode (0.0314 cm 2), C_b is the bulk concentration of the analyte (mol cm $^{-3}$), and D (cm 2 s $^{-1}$) is the diffusion coefficient of the analyte.

From the raw chronoamperometric traces, a linear curve was obtained for the different concentrations of MDA by plotting I against $t^{-1/2}$ (Figure 7, inset a). By plotting the slope of each individual straight line, the overall slope of the best-fit line (Figure 7, inset b) can be defined from equation 4 as:

$$It^{1/2} = \frac{nFAD^{1/2}C_b}{\pi^{1/2}} \quad (5)$$

D can thus be drawn from:

$$D = \frac{(\text{slope})^2 \pi}{(nFAC_b)^2} \quad (6)$$

The overall slope ($33.32 \mu\text{A s}^{1/2} \text{mM}^{-1}$) was used within the Cottrell equation to eventually obtain a diffusion coefficient of $9.49 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$.

Figure 7. Current response over time of the MWCNTs-CS-AuNPs/GCE in B-R buffer solution (0.1 M, pH 10.0) containing different concentrations of MDA (0.005 – 0.4 mM) by chronoamperometric measurements (potential 700 mV). Numbers 1–8 correspond to the different MDA concentrations. Insets: (a) chronoamperograms of the intensity (I) as a function of the reciprocal square root of time ($t^{-1/2}$); (b) linear plot of the slopes of the eight straight lines in the inset (a) against the MDA concentration.

3.6. Amperometric studies of electrocatalytic oxidation of MDA at the MWCNTs-CS-AuNPs/GCE surface

The sensitivity of the MWCNTs-CS-AuNPs-modified electrode to MDA was investigated by amperometry, which has been shown to be a more effective method than voltammetry due to the stirring conditions during the experiment (4). Figure 8 shows the amperogram obtained from a rotating MWCNTs-CS-AuNPs/GCE at a fixed potential of 700 mV in a 0.1 M B-R buffer solution (pH 10.0) containing different concentrations of MDA.

During successive dropwise additions of MDA, a well-defined stairs-like current versus time plot was obtained. The calibration plot was linear in three concentration ranges (i.e., 0.49–10.14 μM [Figure 8, inset a], 10.14–94.9 μM [Figure 8, inset b], and 94.9–261.18 μM [Figure 8, inset c]) of MDA. The sensitivity of the modified electrode to MDA, calculated as the slope of the lower linear range in the amperometric plot, was $0.1509 \mu\text{A} (\mu\text{M})^{-1}$, from which the lower limit of detection (LOD) and the lower limit of quantification were calculated according to the following equations (18,19):

$$LOD = \frac{3 \times S_{bl}}{m} \quad (7)$$

$$LOQ = \frac{10 \times S_{bl}}{m} \quad (8)$$

where S_{bl} is the standard deviation of the blank response (μA) obtained from 10 replicates of the blank solution (0.001) and m is the slope of the aforementioned lower linear range in the amperometric plot. A final LOD and LOQ values of 20 nM and 66 nM were eventually calculated for the MWCNTs-CS-AuNPs/GC electrode.

The operational stability of the MWCNTs-CS-AuNPs/GCE composite-modified electrode was further examined by amperometry, and the results are shown in the inset (d) of Figure 8. The amperometric current response of MDA was almost unchanged when continuously run up to 3310 s in 5 μM MDA containing B-R buffer. This observation shows that throughout the monitored time span, there was no inhibition effect due to the adsorption of MDA and MDA's oxidation products on the modified electrode surface.

Figure 8. Amperometric responses at a rotating MWCNTs-CS-AuNPs/GCE held at 700 mV in different concentrations of 0.49 to 261.18 μM of MDA. The variation of the amperometric current against the MDA concentration is shown in insets (a) (0.49–10.14 μM), (b) (10.14–94.9 μM), and (c) (94.90–261.18 μM). Inset (d) shows the stability of the response of the MWCNTs-CS-AuNPs/GCE during 3310 s (MDA concentration of 5 μM).

Table 2. Amount of added (spiked) and measured MDA at the MWCNTs-CS-AuNPs/GCE surface, with the resulting recovery percentage after the migration test using the acidic food simulant (simulant B) under typical sterilization conditions (121 °C for 20 min).

Sample	Spiked (μM)	Found (μM)	Recovery (%)
	0	-	-
Sample 1	3.5	3.54	101.1
	5	4.93	98.6
	5.5	5.4	98.1

The accuracy was estimated as the recovery from known concentrations of MDA (Table 2). The recovery ranged from 98.1 to 101.1%. These results show the adequate accuracy of the method and suggest that the modified electrode as prepared in this work is well-suited for the quantification of MDA possibly migrating from food packaging materials exposed to extensive thermal treatments (e.g., sous-vide cooking, pasteurization, and sterilization).

Finally, the precision (repeatability) of the electrochemical method was assessed preparing independently three different MWCNTs-CS-AuNPs/GCE sensors. For each sensor, subsequent injections ($n = 15$) of MDA standard solutions at three different concentrations (5, 10, and 15 μM) were analyzed. The relative standard deviation (RSD) was $3.13 \pm 0.25\%$, which indicates the satisfactory repeatability and good reproducibility of the proposed electrode.

All the relevant analytical parameters of the MWCNTs-CS-AuNPs/GC electrochemical sensor used for the detection of MDA are summarized in Table 3.

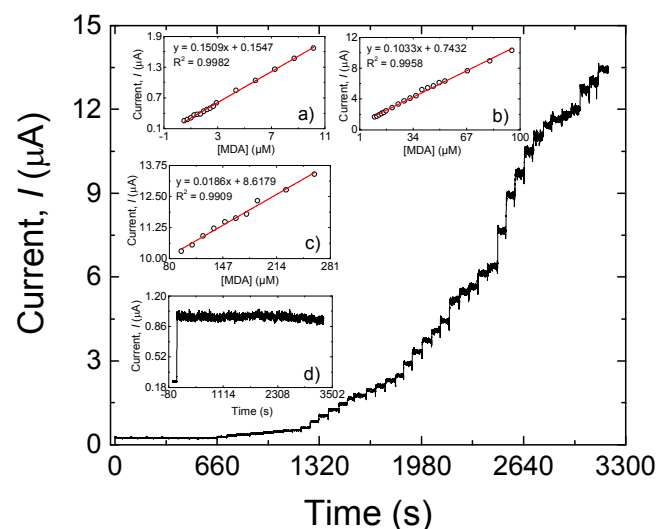


Table 3. Analytical parameters of the MWCNTs-CS-AuNPs/GC electrochemical sensor used for the MDA determination.

Parameter	Value
	0.49-10.14 μM
Linearity (ranges)	10.14-94.9 μM 94.9-261.18 μM
Sensitivity	0.1509 $\mu\text{A} (\mu\text{M})^{-1}$
LOD	20 nM
LOQ	66 nM
Recovery (%)	99.27 \pm 1.60
RSD (%)	3.13 \pm 0.25

3.7. Real sample analysis

In this experiment, we have attempted to determine the MDA concentration that had possibly migrated from the PU-based food packaging material into the food simulant through a migration test carried out under typical sterilization conditions (121 °C for 20 min). The standard addition method was used to evaluate the potential application of the MWCNTs-CS-AuNPs/GCE as an analytical device for MDA determination in real samples. To achieve this goal, the recovery of MDA was determined by amperometry after the addition of MDA in the buffered food simulant B (acetic acid water solution, 3 w/v %) (Table 2).

Conclusions

A new electrochemical sensor with high sensitivity and a low limit of detection was developed for the quantification of the MDA, which is one of the two most representative PAAs possibly migrating from multilayer packaging materials made of PU adhesives into foodstuffs. The sensor was developed using a MWCNTs-CS-AuNPs bionanocomposite to modify the surface of the electrode. The synergistic effect between MWCNTs, CS, and AuNPs toward the electro-oxidation of MDA was recognized as the main factor that dictates the excellent electrocatalytic performance of the sensor in terms of sensitivity, detection limit, and stability over a reasonably extended temporal window (no fouling on the surface of the electrode). The tests conducted on real samples indicated that the modified sensor has the potential to be used as an analytical device for the quantification of MDA.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

We are thankful to Dr. K. Morozova for precious technical support.

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Reviewer(s)' Comments to Author:

Reviewer #1:

What is the aim of aniline?

Answer:

We apologize for the oversight. Indeed, aniline was used in a previous study for the 'interference' experiments.

Can authors provide TEM images of the samples? It is easier to get and approve nanoparticles size with this techniques. Some other techniques? XRD, AFM...

Answer:

We are not sure to have gotten this point. If the reviewer refers to the TEM of the modified electrode, it is not possible due to the size of the electrode (too much hindrance to fit into the sample compartment). We could have performed AFM, but it does not seem to provide additional information over the SEM micrographs.

Figure 3. It is better to show voltammograms and these dependences as inset figure

Answer:

According to the reviewer's suggestion, the Figure 3 has been modified and a new panel showing the raw voltammograms has been added as Electronic Supplementary Information (Figure S1).

What about higher scan rates? It seems like two peaks? See 40 mV/s!

How authors can explain this? Please provide voltammograms for scan rates up to 200 mV/s

Answer:

The presence of a subtle second peak could be due to the fouling occurring on the electrode's surface after putting the electrode in contact with the analyte for a while.

According to the reviewer's suggestion, the voltammograms up to 200 mV/s have been included as Electronic Supplementary Information (Figure S2).

Proposed mechanism?

Answer:

The proposed mechanism is shown in the following: $\text{MDA} \longrightarrow \text{MDA}^{2+} + 1\text{H}^+ + 2\text{e}^-$



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Why this potential (700 mV)?

Answer:

Because this is the minimum potential to allow the oxidation of MDA to occur (is the potential after the oxidation peak).

Three linear ranges is very interesting! How authors can explain this!??

Answer:

A possible explanation for the occurrence of three linear ranges is the saturation of the working electrode caused by the interaction between the MDA and the nanocomposite film, as also postulated by Castro et al. in their very recent work on the detection of trinitrotoluene using a graphene oxide/carbon nanotube nanocomposite sensor (Analytica Chimica Acta 2018, in press. DOI: 10.1016/j.aca.2018.06.055).

What about interferences studies?

Answer:

We have first to point out that the only interference for this kind of materials can arise from other compounds in the material and not from the surrounding medium. Indeed, the PAAs detection should be done on the multi-layer material after its manufacturing (e.g., after lamination) and possibly under the worst foreseeable conditions (e.g., pasteurization or sterilization for packages intended for retorting operations). In any case, the material must be placed in a simulant (e.g., acidic water), so not in a complex medium. We have performed some interference studies using some additives commonly added in master-batch to the polymer film, such as Irganox[®] 1010 and Irgafos[®] 168, which are two additives widely used as antioxidant and processing stabilizers of polyolefins, respectively. The addition of these two additives did not yield any electrochemical signal; that is, their addition did not affect the overall behavior of the AuNPs/MWCNTs-CS/GC electrode toward MDA.

We are thankful to the reviewer for his advices and his positive comments.

Reviewer #2:



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DEFENS

This manuscript is well written and very interesting. However, it needs some additional explanations, data and tables. Validation parameters are missing such as linearity, range, LOD, LOQ, accuracy, precision, recovery studies, sensitivity etc. The RSD and Bias% values should be calculated for showing the precision and accuracy studies and added to the related part of the text with their explanations. These data should be given as table/s.

Answer:

Following the reviewer's comment, a new table that includes all the suggested statistical parameters has been added (Table 3 in the new version). Accordingly, new relevant parts have been added in the new version of the manuscript.

We would like to thank the reviewer for his comments and suggestions, which improved the manuscript in its final form.

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Statement of societal impact

The societal impact of this work is inherently linked to the risk associated to the primary aromatic amines (PAAs) exposure by consumers. PAAs are toxic (carcinogenic) compounds that can possibly migrate from multilayer packaging materials including polyurethane adhesive to the food. At the moment, an ‘in-line’ assessment of PAAs is lacking within most companies. This is mainly due to the expensive, time consuming, and tough analytical methods (especially chromatographic methods) that are available for PAAs determination, which is thus carried out especially by specialized laboratories. Providing alternative methods that are sensitive, selective, cheap, fast, and user-friendly can be of relevance to make PAAs assessment a routine control in the packaging lines, therefore preventing consumers’ exposure.