Anal Bioanal Chem
DOI 10.1007/s00216-017-0505-5

RESEARCH PAPER

Disposable electrochemical immunosensor for *Brettanomyces bruxellensis* based on nanogold-reduced graphene oxide hybrid nanomaterial

Boryana Borisova[1] · María L. Villalonga[2] · María Arévalo-Villena[3] · Abderrahmane Boujakhrout[1] · Alfredo Sánchez[1] · Concepción Parrado[1] · José M. Pingarrón[3] · Ana Briones-Pérez[4] · Reynaldo Villalonga[1]

Received: 3 May 2017 / Revised: 23 June 2017 / Accepted: 30 June 2017

Abstract The assembly of a novel disposable amperometric immunosensor for the detection of the red wine spoilage yeast *Brettanomyces bruxellensis* is reported. The nanostructured sensing interface was prepared by first coating carbon screen printed electrodes with a gold nanoparticles-reduced graphene oxide hybrid nanomaterial, which was then modified with 3-mercaptopropionic acid to further immobilize specific antibodies for *B. bruxellensis* via a carbodiimide-coupling reaction. The functionalized electrode allowed the amperometric detection of *B. bruxellensis* in buffered solutions and red wine samples in the range of 10–10^6 CFU/mL and 10^2–10^6 CFU/mL, with low detection limits of 8 CFU/mL and 56 CFU/mL, respectively. The electrochemical immunosensor also exhibited high reproducibility, selectivity, and storage stability.

Keywords *Brettanomyces bruxellensis* · Biosensor · Graphene · Screen printed electrode · Red wine

Introduction

Wine is the most popular alcoholic beverage in the world. Consequently, wine industry is a growing sector with huge socio-economic impact and multiplier effects on many other affiliated industries. According to the International Organization of Vine and Wine, the global wine production and consumption in 2016 was 267 and 242 million of hectoliters, respectively [1]. In addition, the global wine market was estimated as 104.1 million of hectoliters in terms of volume and 28.9 billion euros in terms of value [1].

In this context, red wine is one of the most appreciated and worldwide consumed wine due to its pleasant sensory properties, relevant nutritional properties and positive effect on human health [2, 3]. The most important source of spoilage of red wine is the yeast *Brettanomyces bruxellensis* (Brett) [4, 5], which can survive in wines during and after alcoholic fermentation due to its low nutrient requirements and high resistance to ethanol, SO_2 and acidic conditions [6–8]. Microbial contamination with Brett can be sourced from winemaking equipment and winery environment such as the barrels, air samples of crush, tank, and bottling lines, as well as from the surface of grape berry [9, 10].

Contamination by this yeast, even at a very low concentration of 10^3 CFU/mL, impairs unpalatable off-odors and off-flavors to red wine. Brett affect the organoleptic properties of red wines by reducing vinylphenols (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol) to ethylphenols (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol), which impart negative phenolic aroma and animal off-odor described as

Electronic supplementary material The online version of this article (doi:10.1007/s00216-017-0505-5) contains supplementary material, which is available to authorized users.

✉ Ana Briones-Pérez
ana.briones@uclm.es

✉ Reynaldo Villalonga
rvillalonga@quim.ucm.es

[1] Nanosensors and Nanomachines Group, Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain

[2] Center for Enzyme Technology, University of Matanzas, 44740 Matanzas, Cuba

[3] Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain

[4] Regional Institute of Applied Scientific Research (RIASR), Av Camilo Jose Cela S/N, Universidad de Castilla-La Mancha, Campus Universitario, 13071 Ciudad Real, Spain

Published online: 20 July 2017

"Brett character" [11, 12]. This yeast can also produce the tautomeric N-heterocyclic bases 2-ethyltetrahydropyridine and 2-acetyltetrahydropyridine, which are associated with the occurrence of mousy off-flavor in red wine. Other substances produced by Brett, like isovaleric acid, may also cause olfactory deviation of red wine even at very low concentration [12–14]. For this reason, the development of analytical strategies for the early detection of Brett in red wines receives considerable attention.

Current approaches to detect Brett contamination are mainly based on molecular biology [15–18] and classical microbiological methods [9], as well as the analytical quantification of volatiles phenols in red wine [19–21]. However, these methods are time consuming and/or need expensive and non-portable instruments. Biosensor technology could be an alternative to construct reliable, low-cost, disposable and miniaturized analytical devices for the fast and accurate detection of Brett [22]. However, little has been done in the field of electroanalytical biosensors for *B. bruxellensis*.

During the last recent years, graphene and graphene derivatives have been largely employed as building blocks for the assembly of electrochemical biosensors with improved analytical performance [23–25]. This fact has been based on the excellent electroconductive, stability and mechanical properties of graphene derivatives which allow them to act ideal transducer elements for biosensor devices [26]. In addition, rational modification of graphene with selected chemical ligands or other materials leads to new derivatives and nanohybrids with improve functional properties and capacity for the stable immobilization of biorecognition elements on the electrode surface [27–29].

In this work, we describe the facile preparation of disposable screen printed electrodes modified with a gold nanoparticles-reduced graphene oxide nanohybrid, which were employed to construct an electrochemical immunosensor for the determination of Brett.

Materials and methods

Materials

Carbon screen printed electrodes OHT-000 and graphene oxide were donated by Orion High Technologies S.L. (Spain). Polyclonal anti-Brett antibody (aBAb) was produced in rabbits by using whole *B. bruxellensis* cells as antigen (Immunostep S.L., Spain). Concanavalin A-peroxidase conjugate (ConA-HRP), $HAuCl_4$, 3-mercaptopropionic acid and (3-mercaptopropyl)trimethoxysilane (MTS) were acquired from Sigma-Aldrich Co. (USA). All other chemicals were analytical grade.

Yeasts strains used in this study were obtained from the University of Castilla-La Mancha culture collection. Strains were growth on YPD agar (1% yeast extract, 2% bacteriological peptone, 2% glucose and 2% agar–agar, *w*/*v*), incubated at 30 °C for 2–4 days and kept in slants of the same medium under refrigeration and refreshed weekly. To adjust the cell concentration in all cases, a Thoma hemocytometer cell count chamber was used.

Instruments and electrodes

Electrochemical experiments were carried out with OHT-000 screen printed electrodes, consisting of a carbon ink working electrode (4.0 mm diameter) modified with the immunosensor assembly, a carbon ink counter electrode and a Ag/AgCl ink reference electrode. Electrochemical impedance spectroscopy (EIS) experiments were performed by using a FRA2 μAutolab Type III potentiostat/galvanostat (Metrohm Autolab B.V., The Netherlands). Amperometric measurements were carried out with a dual-channel Inbea potentiostat (Inbea Biosensores S.L., Spain). Transmission electron microscopy (TEM) measurements were performed with a JEOL JEM-2000 FX microscope (JEOL Ltd., Japan). High resolution field emission scanning electron microscopy (FE-SEM) was performed with a JEOL JSM-6335F microscope (JEOL Ltd., Japan). ATR-FT-IR analysis was performed with a FT-IR Nicolet Magna 750 Series II spectrometer (Thermo Fisher Scientific, USA).

Preparation of the antibody-functionalized electrode (aBAb/Au-rGO/SPE)

Nanogold-decorated reduced graphene oxide (Au-rGO) was prepared as previously described [22]. Briefly, 50 mg of graphene oxide was dispersed in 250 mL ethanol by sonication for 1 h. The resulting dispersion was then heated at 65 °C, mixed with 25 mL of a 1% (*v*/*v*) ethanolic solution of (3-mercaptopropyl)trimethoxysilane and kept under continuous stirring at this temperature during 12 h. The mixture was cooled, filtered, and the resulting solid containing the silanized graphene derivative was exhaustively washed with ethanol and finally dried in vacuum.

To prepare the Au-rGO derivative, 50 mg of silanized graphene oxide and 150 mg $NaBH_4$ were dispersed in 40 mL milliQ water by sonication and the dispersion was mixed with 20 mL citrate-capped Au nanoparticles (20 nm), prepared according to Frens procedure [30]. The solution was stirred at room temperature for 2 h, centrifuged and the solid was exhaustively washed with ethanol and dried under vacuum at room temperature.

To provide the sensing interface with carboxylic acid groups, the working area in the OHT-000 screen printed electrode was first coated with Au-rGO by depositing two 10-μL aliquots of a 1.0 mg/mL aqueous dispersion of the nanomaterial on the electrode surface and allowing drying.

Ten microliters of 1 mM 3-mercaptopropionic acid aqueous solution was further dropped on the dried electrode and kept during 1 h at room temperature. The electrode was exhaustively washed with milliQ water and further dried with N_2.

The antibody-functionalized electrode was finally prepared by dropping 10 μL of a 1.0 mg/mL N-hydroxysuccinimide/1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride solution in 50 mM sodium phosphate buffer, pH 6.0 on the working electrode surface, and kept during 1 h at room temperature. The electrode was washed with the same buffer, dried with N_2 and then functionalized with the polyclonal antibody by dropping 10 μL of a 30 μg/mL aBAb solution in 50 mM sodium phosphate buffer, pH 7.0. The electrode was incubated during 1 h a 4 °C, then washed with cold 50 mM sodium phosphate buffer, pH 7.0 and finally stored in refrigerator until use.

Procedure for electrochemical immunosensor

The working electrode of the aBAb/Au-rGO/SPE was coated with 10 μL of Brett solution in 50 mM sodium phosphate buffer, pH 7.0 during 1 h at 4 °C. After exhaustive washing with the same cold buffer and dry, 10 μL of 10 μg/mL ConA-HRP solution in 1.0 mM $MnCl_2$, 1.0 mM $CaCl_2$, 50 mM sodium acetate buffer, pH 6.0 were dropped on the working electrode. The lectin-based biorecognition reaction was allowed to proceed at 4 °C during 1 h, and the electrodes were then washed with cold 50 mM sodium phosphate buffer, pH 7.0 and finally tested for amperometric quantification of immobilized HRP activity. To do that, 45 μL of freshly prepared 1 mM hydroquinone in 0.1 M sodium phosphate buffer, pH 6.0 were dropped on the OHT-000 SPE electrochemical cell. Changes in the cathodic current were measured at −200 mV vs pseudo Ag/AgCl after addition of 5 μL of a freshly prepared 100 mM H_2O_2 solution. Each measurement was carried out at least in triplicates, by using different antibody-modified electrodes, unless stated otherwise.

Results and discussion

Figure 1A illustrates the experimental protocol employed to assemble the antibody-functionalized electrode. The nanohybrid used as transducer element (Au-rGO) was prepared as previously described [22]. Thiol-functionalized graphene derivative was first prepared by modification of graphene oxide with (3-mercaptopropyl)trimethoxysilane and further decorated with Au nanoparticles by reaction with the reactive thiol groups on the carbon nanomaterial planar sheet. The formation of this nanohybrid was confirmed by TEM and FE-SEM analysis, as is illustrated in Fig. 2. Additional characterization was previously provided [22].

The hybrid nanomaterial was then employed to coat the working electrode surface on OHT-000 screen printed electrodes. To allow stable immobilization of the anti-Brett polyclonal antibody, the Au nanoparticles surface was provided with carboxylic acid groups by treatment with 3-mercaptopropionic acid. The presence of carboxylic acid groups in the nanohybrid was confirmed by the characteristic bands at 1700 and 1715 cm^{-1} in the corresponding ATR-FT-IR spectrum (Fig. S1 in the Electronic Supplementary Material, ESM), which can be ascribed to C = O stretch in the attached 3-mercaptopropionic acid residues and carboxylate groups at the edge of graphene oxide, respectively. This activated surface was then used as support for the

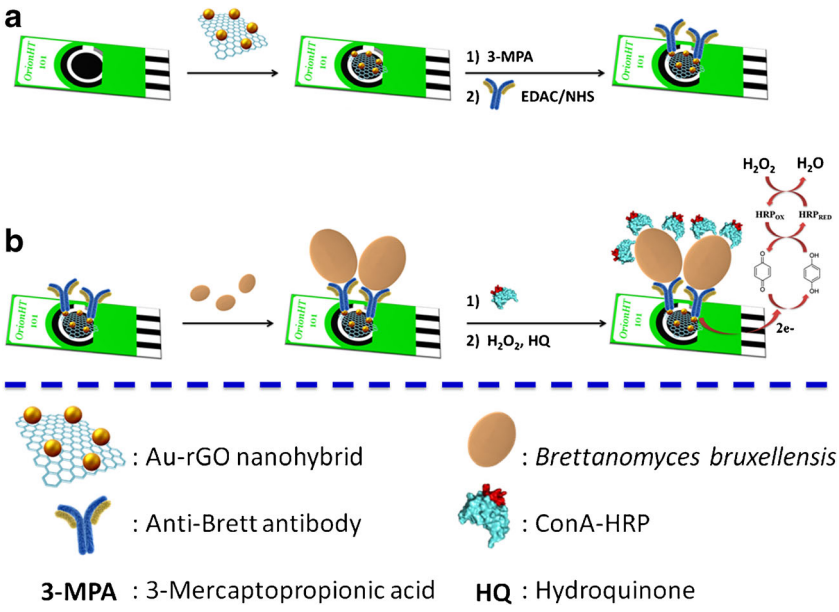

Fig. 1 Schematic display of the steps involved in the preparation (**a**) and performance (**b**) of the antibody-functionalized electrode

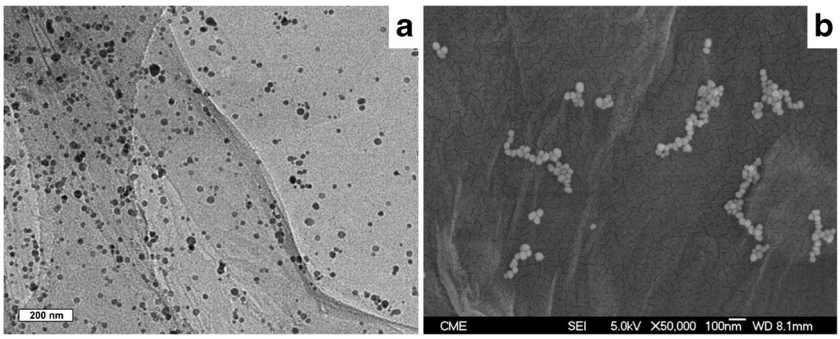

Fig. 2 Representative TEM (**a**) and FE-SEM (**b**) images of the Au-rGO hybrid nanomaterial

carbodiimide-mediated covalent immobilization of the anti-Brett polyclonal antibody.

The resulting electrode (aBAb/Au-rGO/SPE) was evaluated as sensing element for the construction of an electrochemical immunosensor for Brett. The biosensing strategy here employed was based on the assembly of sandwich-type architectures on the electrode surface by using ConA-HRP as secondary bioreceptor and signaling element (Fig. 1B). This approach was based on the ability of the lectin ConA to specifically recognize the carbohydrate moieties in the mannoproteins at the surface of the yeast cells [31]. In this regard, ConA has been previously employed to purify these mannoproteins from Brett extracts [32].

The preparation of the antibody-functionalized electrode was optimized by determining the influence of the experimental variables affecting the amperometric response of the electrode toward Brett. Figure 3A shows the effect of the Au-rGO loading on the amperometric response of the electrode after sequential incubation with 10^3 CFU/mL Brett and ConA-HRP, and using −200 mV as detection potential to quantify the immobilized peroxidase activity. The relative amperometric response of the electrode increased with the hybrid nanomaterial loading up to 20 μg. This fact could be justified by the increased amount of active carboxylate groups on the electrode surface that is suitable for the covalent immobilization of the antibody. Higher loading of the nanohybrid resulted in lower amperometric signals. This behavior could be ascribed to the formation of graphene-based multilayer structures on the electrode surface, causing steric hindrance to the antibody molecules during the immobilization process and thus reducing the amount of affinity-based bioreceptors on the sensing interface [33–35]. According to this result, 20 μg of Au-rGO was employed to modify the SPE in further experiments.

Figure 3B shows the effect of antibody loading on the amperometric response of the electrode toward 10^3 CFU/mL

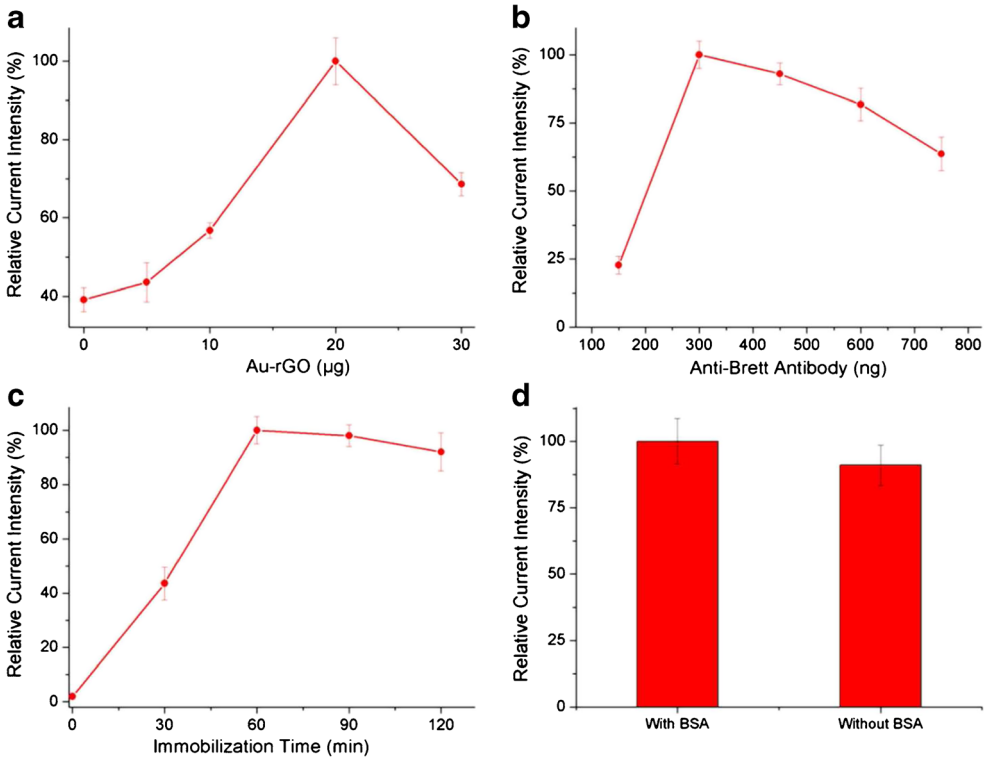

Fig. 3 Effect of the amount of nanohybrid (**a**), incubated antibody (**b**), time of immobilization (**c**) and coating with 1% BSA (**d**) on the normalized amperometric signal of the electrode toward 10^3 CFU/mL Brett. E_{app} = −200 mV, 100% relative current intensity = 460 nA

Brett. The electrochemical response of the immunosensor showed a bell-shaped behavior, reaching the highest value of cathodic current when 300 ng of antibody was immobilized on the Au-rGO-modified electrode surface. Further decrease of the analytical signal for antibody concentrations higher than 300 ng can be ascribed to the non-oriented immobilization of additional layers of antibody molecules on the electrode surface, which can also lead to the formation of undesirable antibody-antibody covalent adducts. These phenomena could cause steric hindrance and blocking of the effective biorecognition sites on the electrode surface, allowing reduction of the analytical signal toward Brett. Accordingly, further electrodes were prepared by using 300 ng as optimal amount of antibody.

The influence of the immobilization time on the immunosensor response is shown in Fig. 3C. Maximum electroanalytical signal was reached when the carbodiimide-mediated immobilization of the antibody to the electrode surface was performed during 1 h at 4 °C. Accordingly, electrodes for further experiments were assembled by using this optimum immobilization time.

Prevention of non-specific adsorption of analytes on the electrode surface is a challenge for the construction of reliable, specific, and reproducible affinity-based biosensors [36, 37]. That is why, different strategies are commonly employed to confer anti-fouling properties to the sensing interface, being coating with protein molecules one of the most used. In this work, the effect of blocking with 1% BSA (*w*/*v*) solution on the analytical signal of the immunosensor was studied. As can be observed in Fig. 3D, the amperometric response of the electrode was not significantly affected by the use of BSA as blocking agent, suggesting the hybrid nanomaterial prevent the non-specific adsorption of Brett cells on the sensing surface. For this reason, no blocking agents were employed for the assembly of further electrodes.

The assembly of the electrode under such optimal conditions was studied by EIS using $[Fe(CN)_6]^{4-/3-}$ as redox probe. All the experimental data were obtained by fitting with a conventional Randles equivalent circuit, and the resulting Nyquist plots are shown in Fig. 4. Modification of the electrode surface with the Au-rGo nanohybrid caused a noticeable reduction in the electron transfer resistance of the interface (from R_{et} = 485 to 92 Ω), as can be noticed by the large reduction in the diameter of the semicircles in the corresponding Nyquist plots. This fact suggests high conductivity properties for the hybrid nanomaterial.

An increase in the electron transfer resistance of the surface (R_{et} = 238 Ω) was noticed after coating the gold nanoparticles surface with 3-mercaptopropionic acid, but a noticeable higher value of R_{et} = 1214 Ω was measured after covalent immobilization of the antibody. This fact could be ascribed

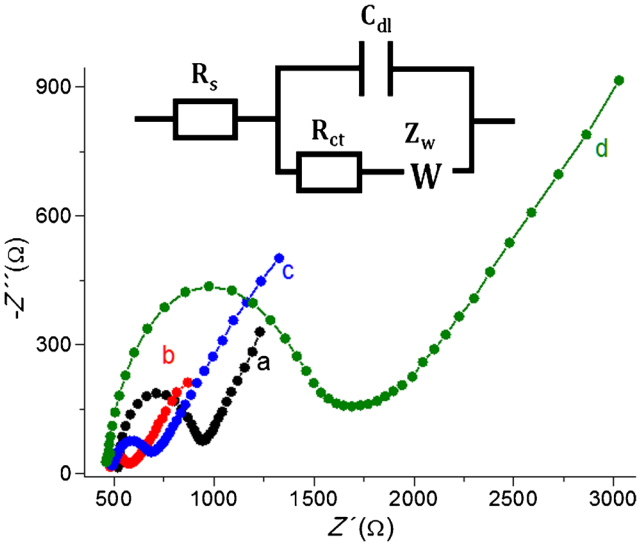

Fig. 4 Impedance plane diagram ($-Z''$ versus Z') for the EIS measurements at a OHT-000 electrode before (**a**) and after coating with Au-rGO (**b**), modification with 3-mercaptopropionic acid (**c**) and immobilization of the anti-Brett antibody (**d**) in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1)

to the non-conductive properties of the antibody protein molecules, and suggest a high protein load and successful covalent attachment of the bioreceptor on the sensing surface.

To ensure the reliable behavior of the biosensor, the optimal operational conditions for the amperometric detection of Brett were also evaluated. Figure 5A shows the effect of the incubation time of the antibody-modified electrode with the Brett samples, in buffered solution of pH 7.0 at 4 °C, on the normalized amperometric response of the device. It should be noted that higher electroanalytical signal was observed for the electrodes incubated with the yeast during 1 h. Longer incubation times caused a decrease in the analytical response of the biosensor, which could be ascribed to partial inactivation of the antibody molecules under these incubation conditions. On the other hand, the electrochemical response of the electrode showed high value when performed at 4 °C (Fig. 5B), probably by avoiding potential thermal inactivation of the antibody on the electrode surface.

The analytical performance of nanostructures immunosensor was evaluated under these optimal working conditions. Figure 6A shows the average calibration curve for 10 different experiments. The cathodic current values measured with the immunosensor exhibited a linear behavior (r = 0.989) with the logarithm of Brett concentration in the broad range of 10–10^6 CFU/mL, according to the following equation:

$$i_C(nA) = 159.1\ log\ [Brett]–49.7$$

As can be observed in Fig. 6B, the immunosensor also showed linear response toward Brett concentration in commercial and undiluted red wine samples (Ribera

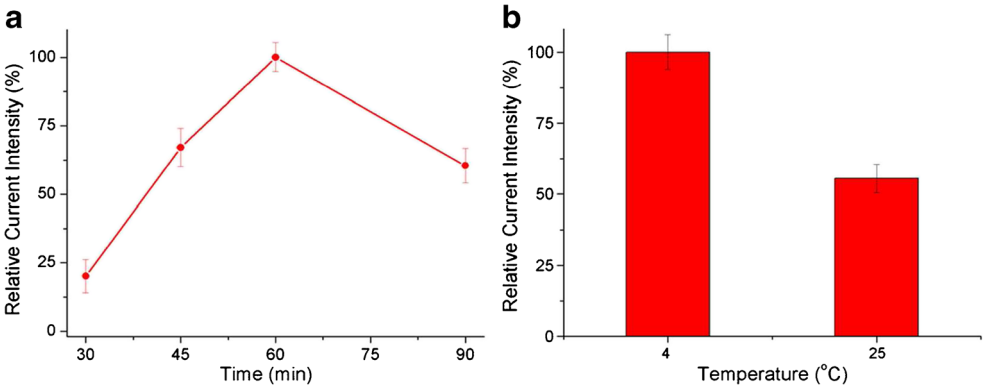

Fig. 5 Influence of the time (**a**) and temperature (**b**) of incubation of the immunosensor with 10^3 CFU/mL Brett solution on the normalized amperometric signal recorded with the electrode. E_{app} = −200 mV, 100% relative current intensity = 460 nA

del Duero, Spain), exhibiting a linear behavior (r = 0.988) in the range of 10^2–10^6 CFU/mL, according to the following equation:

$$i_C(nA) = 9.6\ log\ [Brett] + 30.0$$

As can be observed, there is a noticeable difference in the slope of these two calibration curves, demonstrating a matrix effect in the red wine sample. In addition, it should be notified that the sensitivity of the immunosensor was lower in the red wine sample, but enough to provide analytical information on the content of Brett in this media.

Low detection limit values of 8 CFU/mL and 56 CFU/mL were estimated for the amperometric immunosensors in buffered solutions and red wine samples, respectively. These values were calculated according to the $3S_b/m$ criterion, where m is the slope of the calibration curve and S_b was estimated as the standard deviation of 10 different amperometric signals recorded for the lowest Brett concentration measured in the calibration graphs (10 CFU/mL and 100 CFU/mL, respectively).

These values of detection limit are lower than those reported by direct and real-time PCR methods [38–41] and also lower that the threshold concentration of 10^3 CFU/mL at which *Brettanomyces* impairs undesirable properties to wines. These facts suggest this amperometric immunosensor can be a valuable analytical tool for wine industry.

The reproducibility of the assembly process was determined by measuring the response of 10 different electrodes prepared in the same manner, toward 10^3 CFU/mL Brett in red wine samples. The proposed assembly protocol showed good reproducibility, with a relative standard deviation of 12.3% (standard deviation/mean ×100). On the other hand, the immunosensor also showed high selectivity, yielding a very low analytical response toward other yeast commonly employed in food industry (Fig. 7).

The long-term stability of the immunosensor was estimated by storing the electrode at 4 °C under dry conditions. The electrode was periodically tested toward 10^3 CFU/mL Brett in red wine samples. As can be observed in Fig. 8, the biosensor retained more than 90% of the initial electroanalytical activity after 3 weeks of storage at 4 °C. This fact suggests successful immobilization of the antibody molecules on the nanostructured electrode surface, preserving the 3D active protein conformation and thus allowing high retention of affinity capturing properties for the immobilized antibody. It was also noticed that the stability of the biosensor was drastically reduced at longer times of storage, retaining about 53% of the initial analytical activity when stored during 4 weeks at 4 °C.

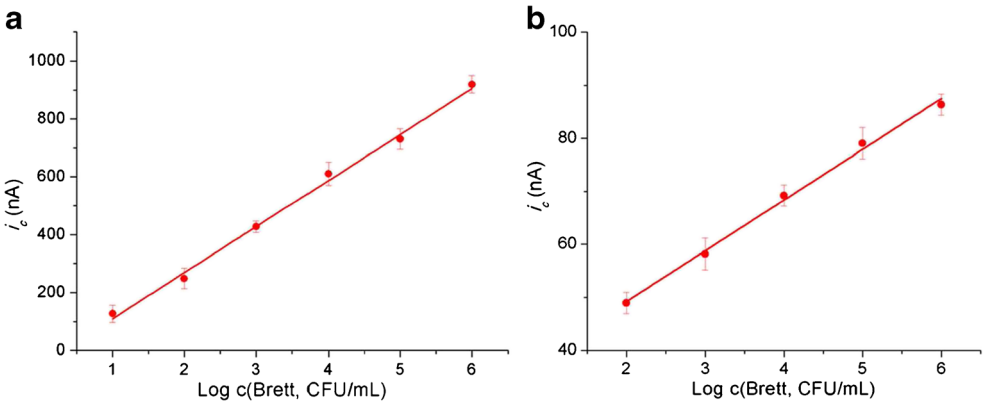

Fig. 6 Calibration curve obtained with the amperometric immunosensor toward Brett in buffered solution (**a**) and commercial red wine sample (**b**). E_{app} = −200 mV

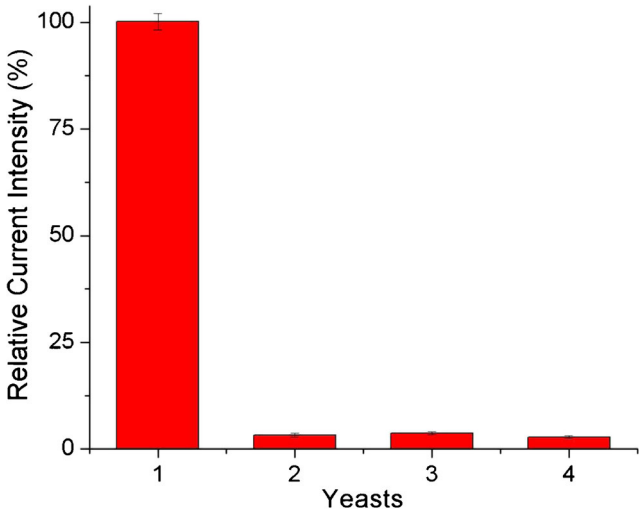

Fig. 7 Normalized electrochemical response of the immunosensor toward 10^5 CFU/mL *B. bruxellensis* (1), *S. cerevisiae* (2), thermal inactivated *S. cerevisiae* (3) and *P. pastoris* (4) in commercial and undiluted red wine samples

Conclusions

A novel disponsable electrochemical immunosensor was prepared by using carbon screen printed electrodes modified with a gold nanoparticles-graphene hybrid nanomaterial and functionalized with specific anti-Brett polyclonal antibody. The nanostructured electrode was employed to detect the yeast at very low concentration in buffered solutions and commercial red wine samples. The excelent analytical performance showed by the immunosensor suggest that screen printed electrodes modified with this nanohybrid can be employed to prepare sensitive, reliable, and disposable electrochemical devices for food analysis.

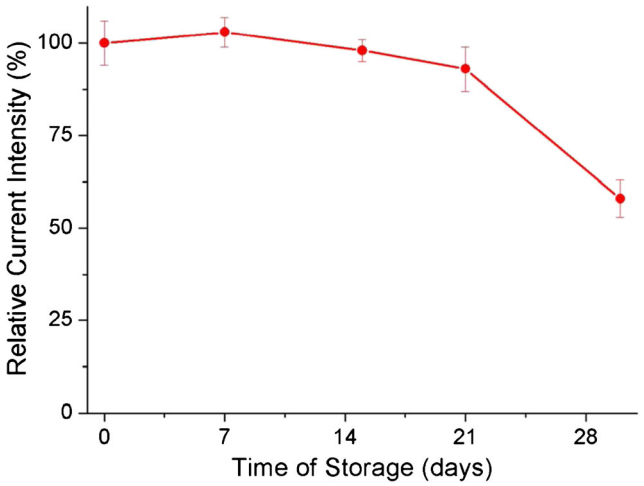

Fig. 8 Effect of time of storage on the normalized electrochemical response of the immunosensor toward 10^3 CFU/mL Brett in commercial and undiluted red wine samples

Acknowledgements Financial support from the Spanish Ministry of Economy and Competitiveness (MINECO Projects CTQ2014-58989-P and CTQ2015-71936-REDT), the Junta de Comunidades de Castilla La Mancha (JCCM Project POII-2014-011-A), and the Comunidad de Madrid, Programme NANOAVANSENS (Project S2013/MIT-3029) is gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. International Organization of Vine and Wine. State of the vitiviniculture world market. Available from: http://www.oiv.int [Accessed 2017 April 23].
2. Calabriso N, Scoditti E, Massaro M, Pellegrino M, Storelli C, Ingrosso I, et al. Multiple anti-inflammatory and anti-atherosclerotic properties of red wine polyphenolic extracts: differential role of hydroxycinnamic acids, flavonols and stilbenes on endothelial inflammatory gene expression. Eur J Nutr. 2016;55:477–89.
3. Vázquez-Fresno R, Llorach R, Perera A, Mandal R, Feliz M, Tinahones FJ, et al. Clinical phenotype clustering in cardiovascular risk patients for the identification of responsive metabotypes after red wine polyphenol intake. J Nutr Biochem. 2016;28:114–20.
4. Wedral D, Shewfelt R, Frank J. The challenge of *Brettanomyces* in wine. LWT Food Sci Technol. 2010;43:1474–9.
5. Loureiro V, Malfeito-Ferreira M. Spoilage yeasts in the wine industry. Int J Food Microbiol. 2003;86:23–50.
6. Conterno CL, Joseph TJ, Arvik T, Henick-Kling LF, Bisson LF. Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. Am J Enol Vitic. 2006;57:139–47.
7. Longin C, Degueurce C, Julliat F, Guilloux-Benatier M, Rousseaux S, Alexandre H. Efficiency of population-dependent sulfite against *Brettanomyces bruxellensis* in red wine. Food Res Int. 2016;89:620–30.
8. Smith BD, Divol B. *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. Food Microbiol. 2016;59:161–75.
9. Renouf V, Lonvaud-Funel A. Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. Microbiol Res. 2007;162:154–67.
10. Rubio P, Garijo P, Santamaría P, López R, Martínez J, Gutiérrez AR. Influence of oak origin and ageing conditions on wine spoilage by *Brettanomyces* yeasts. Food Control. 2015;54:176–80.
11. Chatonnet P, Dubourdie D, Boidron JN, Pons M. The origin of ethylphenols in wines. J Sci Food Agr. 1992;60:165–78.
12. Suárez R, Suárez-Lepe JA, Morata A, Calderón F. The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. Food Chem. 2007;102:10–21.
13. Romano A, Perello MC, Revel GD, Lonvaud-Funel A. Growth and volatile compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. J Appl Microbiol. 2008;104:1577–85.
14. Madsen MG, Edwards NK, Petersen MA, Mokwena L, Swiegers JH, Arneborg N. Influence of *Oenococcus oeni* and *Brettanomyces bruxellensis* on hydroxycinnamic acids and volatile phenols of aged wine. Am J Enol Vitic. ajev-2016.
15. Tessonnière H, Vidal S, Barnavon L, Alexandre H, Remize F. Design and performance testing of a real-time PCR assay for

sensitive and reliable direct quantification of *Brettanomyces* in wine. Int J Food Microbiol. 2009;129:237–43.
16. Willenburg E, Divol B. Quantitative PCR: an appropriate tool to detect viable but not culturable *Brettanomyces bruxellensis* in wine. Int J Food Microbiol. 2012;160:131–6.
17. Cecchini F, Lacumin L, Fontanot M, Comuzzo P, Comi G, Manzano M. Dot blot and PCR for *Brettanomyces bruxellensis* detection in red wine. Food Control. 2013;34:40–6.
18. Vendrame M, Manzano M, Comi G, Bertrand J, Lacumin L. Use of propidium monoazide for the enumeration of viable *Brettanomyces bruxellensis* in wine and beer by quantitative PCR. Food Microbiol. 2014;42:196–204.
19. Vanbeneden N, Delvaux F, Delvaux FR. Determination of hydroxycinnamic acids and volatile phenols in wort and beer by isocratic high-performance liquid chromatography using electrochemical detection. J Chromatogr A. 2006;1136:237–42.
20. Serpaggi V, Remize F, Recorbet G, Gaudot-Dumas E, Sequeira-Le Grand A, Alexandre H. Characterization of the "viable but nonculturable" (VBNC) state in the wine spoilage yeast *Brettanomyces*. Food Microbiol. 2012;30:438–47.
21. Chandra M, Madeira I, Coutinho AR, Albergaria H, Malfeito-Ferreira M. Growth and volatile phenol production by *Brettanomyces bruxellensis* in different grapevine varieties during fermentation and in finished wine. Eur Food Res Technol. 2016;242:487–94.
22. Boujakhrout A, Sánchez A, Díez P, Jiménez-Falcao S, Martínez-Ruiz P, Peña-Álvarez M, et al. Decorating graphene oxide/nanogold with dextran-based polymer brushes for the construction of ultrasensitive electrochemical enzyme biosensors. J Mater Chem B. 2015;3:3518–24.
23. Bollella P, Fusco G, Tortolini C, Sanzò G, Favero G, Gorton L, et al. Beyond graphene: electrochemical sensors and biosensors for biomarkers detection. Biosens Bioelectron. 2017;89:152–66.
24. Pandey A, Gurbuz Y, Ozguz V, Niazi JH, Qureshi A. Graphene-interfaced electrical biosensor for label-free and sensitive detection of foodborne pathogenic *E. coli* O157: H7. Biosens Bioelectron. 2017;91:225–31.
25. Wang W, Bao T, Zeng X, Xiong H, Wen W, Zhang X, et al. Ultrasensitive electrochemical DNA biosensor based on functionalized gold clusters/graphene nanohybrids coupling with exonuclease III-aided cascade target recycling. Biosens Bioelectron. 2017;91:183–9.
26. Bo X, Zhou M, Guo L. Electrochemical sensors and biosensors based on less aggregated graphene. Biosens Bioelectron. 2017;89:167–86.
27. Povedano E, Cincotto FH, Parrado C, Díez P, Sánchez A, Canevari TC, et al. Decoration of reduced graphene oxide with rhodium nanoparticles for the design of a sensitive electrochemical enzyme biosensor for 17β-estradiol. Biosens Bioelectron. 2017;89:343–51.
28. Pan LH, Kuo SH, Lin TY, Lin CW, Fang PY, Yang HW. An electrochemical biosensor to simultaneously detect VEGF and PSA for early prostate cancer diagnosis based on graphene oxide/ssDNA/PLLA nanoparticles. Biosens Bioelectron. 2017;89:598–605.
29. Chen M, Hou C, Huo D, Fa H, Zhao Y, Shen C. A sensitive electrochemical DNA biosensor based on three-dimensional nitrogen-doped graphene and Fe_3O_4 nanoparticles. Sens Actuator B Chem. 2017;239:421–9.
30. Frens G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature. 1973;241:20–2.
31. Tkacz JS, Cybulska EB, Lampen JO. Specific staining of wall mannan in yeast cells with fluorescein-conjugated concanavalin A. J Bacteriol. 1971;105:1–5.
32. Spontón PG, Spinelli R, Drago SR, Tonarelli GG, Simonetta AC. Acetylcholinesterase-inhibitor hydrolysates obtained from '*in vitro*' enzymatic hydrolysis of mannoproteins extracted from different strains of yeasts. Int J Food Sci Technol. 2016;51:300–8.
33. Aslan S, Anik U. Microbial glucose biosensors based on glassy carbon paste electrodes modified with *Gluconobacter oxydans* and graphene oxide or graphene-platinum hybrid nanoparticles. Microchim Acta. 2016;187:73–81.
34. Li H, He J, Li S, Turner APF. Electrochemical immunosensor with N-doped graphene-modified electrode for label-free detection of the breast cancer biomarker CA 15-3. Biosens Bioelectron. 2013;43:25–9.
35. Yang Y, Liu Q, Liu Y, Cui J, Liu H, Wang P, et al. A novel label-free electrochemical immunosensor based on functionalized nitrogen-doped graphene quantum dots for carcinoembryonic antigen detection. Biosens Bioelectron. 2017;90:31–8.
36. Riquelme MV, Zhao H, Srinivasaraghavan V, Pruden A, Vikesland P, Agah M. Optimizing blocking of nonspecific bacterial attachment to impedimetric biosensors. Sens Bio-Sens Res. 2016;8:47–54.
37. Wang L, Hua E, Liang M, Ma C, Liu Z, Sheng S, et al. Graphene sheets, polyaniline and AuNPs based DNA sensor for electrochemical determination of BCR/ABL fusion gene with functional hairpin probe. Biosens Bioelectron. 2014;51:201–7.
38. Delaherche A, Claisse O, Lonvaud-Funel A. Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. J Appl Microbiol. 2004;97:910–5.
39. Tofalo R, Schirone M, Corsetti A, Suzzi G. Detection of *Brettanomyces* spp. in red wines using real-time PCR. J Food Sci. 2015;77:M545–9.
40. Cocolin L, Rantsiou K, Iacumin L, Zironi R, Comi G. Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalus* in spoiled wines. Appl Environ Microbiol. 2004;70:1347–55.
41. Manzano M, Vizzini P, Jia K, Adam PM, Ionescu RE. Development of localized surface plasmon resonance biosensors for the detection of *Brettanomyces bruxellensis* in wine. Sens Actuator B Chem. 2016;223:295–300.