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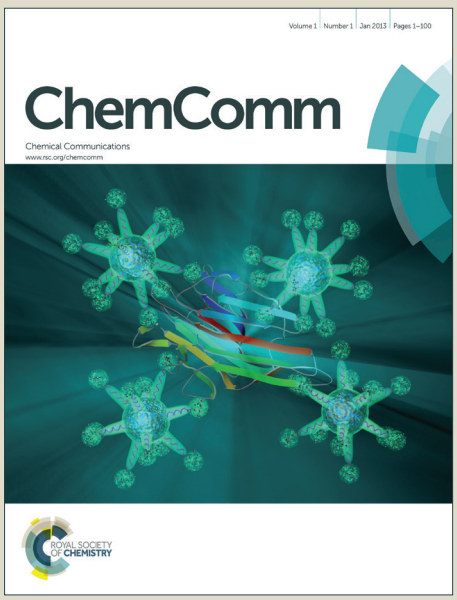

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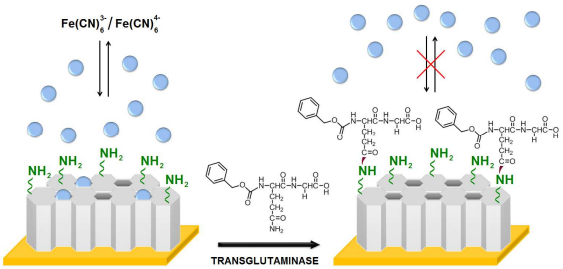

A novel nanochannel-based electrochemical approach to determine transglutaminase activity by using mesoporous silica thin films-coated electrodes.

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ARTICLE TYPE

Nanochannel-based electrochemical assay for transglutaminase activity

Iñigo Fernández,[a] Alfredo Sánchez,[a] Paula Díez,[a] Paloma Martínez-Ruiz,[b] Prospero Di Pierro,[c] Raffaele Porta,[c] Reynaldo Villalonga,[*a, d] José M. Pingarrón[*a,d]

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A novel electrochemical assay to quantify tranglutaminase activity is reported. The assay is based on the enzyme-controlled diffusion of $Fe(CN)_6^{3-/4-}$ through amino-functionalized nanochannels of a mesoporous silica thin film on a Au surface in the presence of N-benzyloxycarbonyl-L-glutaminylglycine.

The establishment of rapid and reliable analytical strategies for enzyme activity quantification is a subject of broad interest due to the wide use of enzymes in clinical, industrial, basic research, chemical and environmental applications[1]. In this context, transglutaminases (TGases, protein-glutamine γ-glutamyl transferases, EC 2.3.2.13) are particularly interesting enzymes due to their ability to cross-link protein substrates. TGases are widely distributed in different organisms such as bacteria, plants, invertebrates and vertebrates.[2] In humans, TGases play important biological functions in the protection and prevention of body injury, tissue assembly and repair, and are also involved in the pathophysiological development of many diseases.[3] From an industrial point of view, TGases are relevant for food processing and for the preparation of edible films and neoglycoconjugates.[2,4]

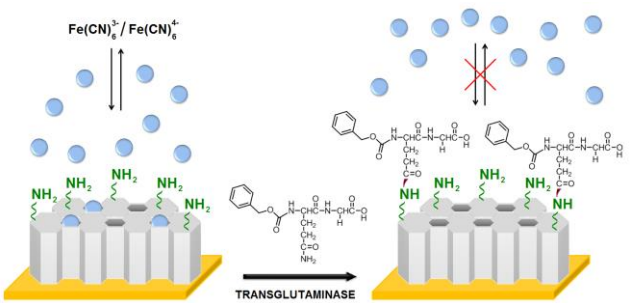

Fig. 1 Schematic display of the processes involved in the electroanalytical determination of TGase activity.

TGases are commonly assayed through complicated optical and radioactive protocols,[5] difficult to be automated. Since electroanalytical methods are easily automated, miniaturized and integrated into portable and hand-held devices, they constitute ideal candidates to face up the problem of enzyme activity quantification. In this context, we recently described a novel voltammetric strategy to quantify TGase activity by using cyclodextrin-patterned Au surfaces.[6] However, this approach implied a time consuming protocol for the synthesis of the oligosaccharide derivative and the sequential assembly of the sensing surface.

This communication describes for the first time a nanomaterial-based electrochemical assay for the quantification of TGase activity exhibiting remarkable advantages with respect to previous approaches. The rational of the strategy is illustrated in Fig. 1. It is based on the gold surface patterning with an array of well-ordered nanosized channels prepared by electrodeposition of a mesoporous silica thin film.[7] Subsequently, the outer film surface was functionalized with N^1-(3-trimethoxysilylpropyl) diethylenetriamine in order to provide the nanomaterial with amino-donor groups for TGase. The nanopores at the amino-enriched silica thin film acted as conductive channels for the diffusion of an electroactive probe to the electrode surface, thus providing a suitable electroanalytical signal. Then, the nanochannels were selectively gated by the catalytic action of TGase in the presence of the glutamine-donor substrate N-benzyloxycarbonyl-L-glutaminylglycine (CBZ). This produced a decrease in the accessibility of the electrochemical probe to the modified electrode surface and, accordingly, in the recorded voltammetric signal, by means of an enzyme-controlled diffusion mechanism.

The assembling of the electrode surface involved the former coating of the gold disk electrode (3.0 mm diameter) with a mesoporous silica thin film with vertically-aligned nanochannels by electrochemically-induced polycondensation of hydrolysed tetraethyl orthosilicate solution using the cationic surfactant cetyltrimethylammonium bromide as porogen specie[7] (see Supplementary Information for details). The prepared silica films exhibited large surface area with vertically-aligned nanochannels of uniform pore size and arranged in a honeycomb structure, as revealed by TEM analysis (Fig. 2). Selected area electron diffraction analysis (SAED) showed a well-defined diffraction pattern with the typical hexagonal symmetry form of mesoporous silica. FE-SEM analysis revealed the formation of a large 2D structure over the whole surface and showed that the used procedure avoided the formation of a large number of undesired nanoparticulated forms on the electrode surface (Fig. 3A). Cross-sectional analysis of the nanomaterial by FE-SEM in COMPO mode (Fig. 3B) confirmed the nanometric thinness of this planar structure. Similar results were obtained by AFM analysis (Fig. 3C), revealing an average thinness of about 40 nm for the mesoporous thin film (Fig. 3D).

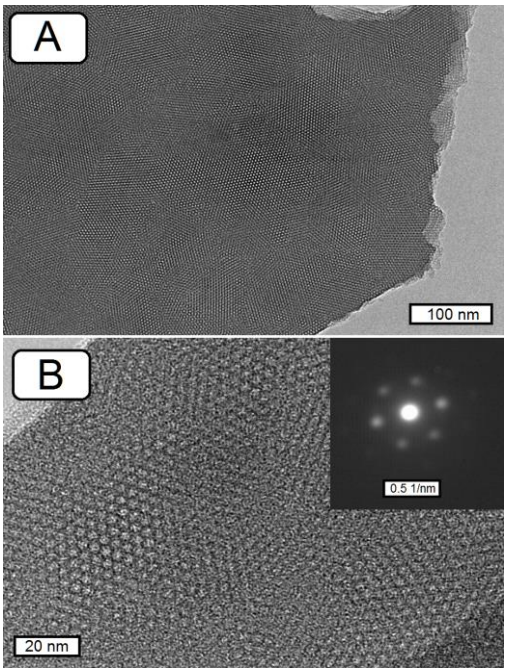

Fig. 2 TEM images of the mesoporous silica thin film at low (A) and high (B) magnification. Inset: SAED analysis of the film.

The film-coated electrode was provided with primary amino groups to be recognized as amino-donor substrates for TGase, by dipping the nanostructured surface into a 280 mM ethanolic solution of N^1-(3-trimethoxysilylpropyl) diethylenetriamine for 3 h followed by exhaustive washing with ethanol and water.

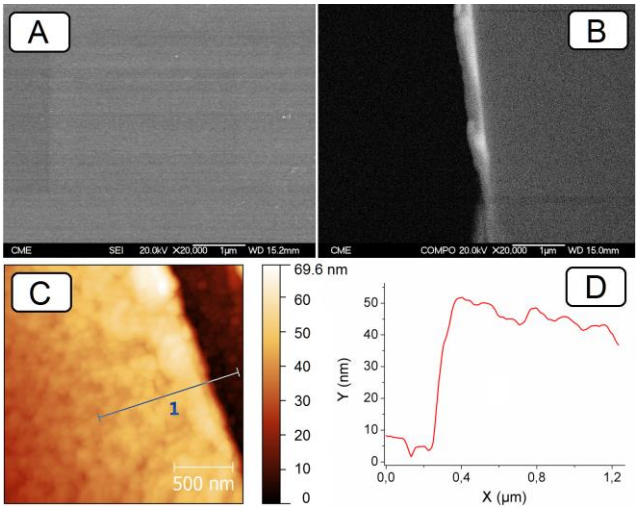

Fig. 3 Top-view (A) and cross-sectional (B) FE-SEM images of the film. AFM (C) and sectional analysis (D) of the film.

The interfacial changes occurring during the assembly of the amino-functionalized mesoporous silica film on the Au electrode surface were examined by cyclic voltammetry and electrochemical impedance spectroscopy using $[Fe(CN)_6]^{4-/3-}$ as redox probe (Fig. 4). The electrodeposition of the surfactant-templated silica film on the Au electrode led to the complete suppression of the redox probe voltammetric response indicating full coverage of the electrode surface. However, washing with ethanolic HCl solution to remove the surfactant included into the nanochanels and the subsequent amine-functionalization with N^1-(3-trimethoxysilylpropyl)diethylenetriamine allowed the redox probe voltammetric peaks to appear which suggested the pores opening and the occurrence of electrostatic interactions between the negative charged electrochemical probe and the amino groups at the film surface, favouring the diffusion of the $[Fe(CN)_6]^{4-/3-}$ ions to the electrode.[8]

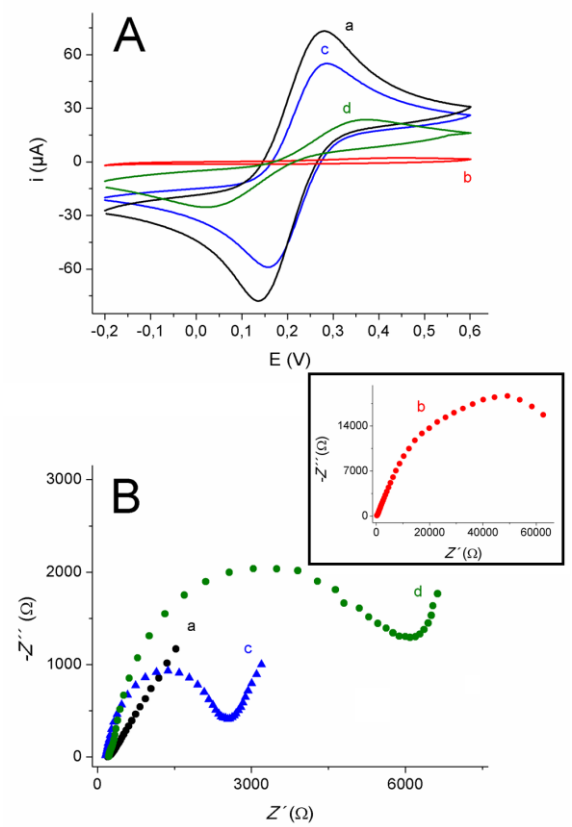

Fig. 4 Cyclic voltammograms (A) and Nyquist plots (B) recorded with the Au electrode in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) before (a) and after modification with the mesoporous silica thin film (b), N-[3-(trimethoxysilyl)propyl] ethylenediamine (c), and incubation with 15 mM CBZ and 40 µU/mL TGase for 1 h (d).

Similar conclusions were drawn from electrochemical impedance spectroscopy (EIS) measurements. Coating of the gold electrode with the surfactant-templated silica film produced a significant increase in the electron transfer resistance (from 136 Ω to 90.2 kΩ), indicating high coverage of the electrode surface with the nanomaterial. A significant reduction of the electron transfer resistance was further observed after washing and treatment with the silane derivative (R_{ct} = 2.2 kΩ).

The ability of the nanostructured electrode to be recognized as amino-donor substrate for TGase was also evaluated by EIS and cyclic voltammetry. In order to do that, the amino-enriched surface was incubated for 1 h in a 15 mM CBZ solution in the presence of 40 µU/mL *Streptoverticillium* sp. TGase. CBZ is a suitable γ-glutamyl donor substrate for TGase and, therefore, it was expected that the product of the TGase-catalyzed reaction, was attached to the electrode surface through ε-(γ-glutamyl)-lysine isopeptide linkages. This enzyme-catalyzed formation of the isopeptide derivative on the outer face of the mesoporous silica film was anticipated to provoke a gating effect in the

nanochannels, causing steric hindrance for the diffusion of the redox probe to the Au surface.

Curves d in Fig. 4 display the Nyquist plot and cyclic voltammogram recorded with the film-coated electrode after 1 h incubation with TGase. As can be observed, a dramatic increase in the interfacial electron transfer resistance (R_{ct} = 5.8 kΩ) was produced, as well as a noticeable decrease in the voltammetric peak currents and a broader peak potentials separation. In this sense, it is important to point out that both impedimetric and voltammetric profiles were unaffected when no CBZ or TGase were present in the incubation media as well as by using a heat inactivated TGase. These findings support that the diffusion of the redox probe to the electrode surface was restricted by an enzyme-controlled mechanism.

According to these results, the amino-enriched thin film coated electrode was employed to design a voltammetric sensing assay for TGase activity. As an example, the time-dependent voltammetric behavior of the amino-enriched electrode in the presence of 15 mM CBZ and 4 µU/mL TGase is shown in Fig. 1S-A (Supplementary Information). The anodic peak current values (i_a) decreased progressively with the incubation time and such decrease exhibited a linear behavior as expected for an enzyme-catalyzed reaction (Fig. 1S-B). The slope of these linear plots ($\Delta i/\Delta t$) can be considered as the relative initial velocity of the enzyme-catalyzed reaction by assuming a Michaelis-Menten kinetics. The slope values showed a linear dependence (r^2 = 0.995) with active enzyme concentration in the 0.4 - 185 µU/mL range, with a sensitivity of 6.3 mA mL U^{-1} s^{-1} (Fig. 2S in Supplementary Information). The repeatability of the electrochemical assay for 4 µU/mL TGase concentration was evaluated by assembling five different electrodes. A R.S.D. value for the slope values of the resulting i_a vs. *t* plots of 5.1%. was obtained. It is important to emphasize that the proposed assay improves considerably the analytical performance with respect to that described previously using a gold surface patterned with cyclodextrin-based molecular nanopores in terms of a wider linear range at low enzyme concentrations (0.4 - 185 µU/mL *vs* 1.9 - 37 mU/mL) and a much higher sensitivity (6.3 mA mL U^{-1} s^{-1} *vs* 1.4x10^{-9} mA mL U^{-1} s^{-1}).[6]

The electrochemical assay showed also the potentiality to be used for the determination of Pb^{2+} in water due to the irreversible inhibition of TGases by this ion[9] as a consequence of the presence of an active thiol group at the active site of these enzymes[10]. The assay was performed by comparing the time-dependent voltammetric behavior of the nanostructured electrode in the presence of 15 mM CBZ and 100 µU/mL TGase with and without addition of $Pb(NO_3)_2$ at different concentrations. The inhibition percentage (%Inh) was calculated from the following equation for various Pb^{2+} concentrations:

$$\%Inh = (S_0 - S_I)x100/S_0$$

where S_0 and S_I were the slope vales of the i_p *vs* t plots without and with addition of Pb^{2+}. The electrode response was found to be linear over the 4.0 – 500 µM Pb^{2+} concentration range (r^2=0.997) with a sensitivity of 0.19 % M^{-1} (Fig. 3S in Supplementary Information).

Conclusions

In summary, we have described here a novel voltammetric method for measuring TGase activity, based on the enzyme-controlled diffusion of an electrochemical probe through the nanochannels of an amino-enriched mesoporous silica thin film assembled on a gold surface. This approach could be extended to other non-redox enzymes able to alter access of electrochemical probes to the nanopores through enzyme-catalyzed reactions, and opens new possibilities to design portable and automated sensor devices for this purpose and even for the determination of potential inhibitors.

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Notes and references

[a] *Departments of Analytical Chemistry and* [b]*Organic Chemistry I, Faculty of Chemistry, Complutense University of Madrid, 28040-Madrid, Spain. Tel: +34 913944315; E-mail: pingarro@quim.ucm.es; rvillalonga@quim.ucm.es*

[c] *Department of Chemical Sciences, University of Naples "Federico II", 80126 Naples, Italy*

[d] *IMDEA Nanoscience, Cantoblanco, 28049-Madrid, Spain*

† Electronic Supplementary Information (ESI) available: Preparation of the nanostructured electrode and analytical methods.

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