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Nanoparticle modified QCM-based sensor for lipase activity determination

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A highly sensitive lipase activity sensor was developed and tested. It is based on the application of SiO_2 nanoparticle loaded olive oil as a lipase substrate, deposited on a QCM crystal. The heavier nanoparticles' release during the substrate enzymatic degradation causes a QCM frequency response enhancement proportional to the nanoparticle/substrate mass ratio.

Lipases (EC 3.1.1.3 triacylglycerol acylhydrolase) are pancreatic enzymes of the hydrolases group, primarily involved in fat degradation.[1,2] Lipases are also produced at an industrial scale using microorganisms.[3] Their significance as a diagnostic tool for pancreatic diseases,[4] and their technological importance[5–11] as catalysts in the food, detergent, pharmaceutical, leather, textile, cosmetic, paper, and biofuel production industries call for the development of appropriate methods for the evaluation of their activity. Nevertheless, even though a range of volumetry-, spectro-photometry-, radioactivity-, immunoassay-, conductimetry-, chromatography-, and biosensor-based techniques for lipase quantification have been suggested,[12–18] until now none of them entirely satisfy the analytical requirements. Thus, new methods for lipase activity determination, overcoming the drawbacks of the currently applied methods, such as tedious and time-consuming procedures, and the use of non-specific substrates, radioactive materials, or expensive reagents and laboratory equipment, have to be put into practice. Quantifying enzyme activity using a quartz crystal microbalance (QCM), which provides real time information and enables sensitive measurements, could be considered as a promising alternative. In many biological applications, *e.g.* for the detection of nucleic acids and proteins, QCM signal amplification and sensitivity increase were reached using nanoparticles (NP) *via* sandwich hybridization of the surface capture probe, target, and nanoparticle modified probe (probe/target/probe-NP),[19–22] or *via* target labeling with nanoparticles by means of ligands such as biotin.[23–25] Au nanoparticles were used almost exclusively for signal enhancement, because of their structural features and biocompatibility.

In this work, a QCM modified with SiO_2 nanoparticle loaded olive oil as a lipase substrate was used for rapid and sensitive enzyme activity evaluation by real time monitoring of the enzymatic substrate degradation. Nanoparticle removal along with the evolution of the olive oil lipolysis resulted in a significant sensitivity increase, because of the higher nanoparticle specific mass compared with the specific mass of the substrate. The suggested approach, the subject of the present work, is applied for the first time for lipase activity quantification. In contrast to the techniques for QCM signal amplification mentioned above, in this study signal enhancement is achieved due to the nanoparticles' release. Higher specific mass ratio (nanoparticles to substrate) resulted in higher sensitivity enhancement.

Lipase from *Rhizopus arrhizus* ($\sim$10 U mg^{-1}), purchased from Sigma, was employed in all the experiments. The enzyme activity was verified applying the European Pharmacopoeia recommended standard lipase activity assay. The lipase unit was defined as the amount of enzyme that liberates 1 μmol of fatty acid from olive oil per minute under the assay conditions. The data obtained (10.4 U mg^{-1}) under different storage conditions were consistent with the values established by the manufacturer. Commercial extra virgin olive oil (Bertolli, Italy) was used as an enzyme substrate. As known, triolein is a specific lipase substrate.[18] However, olive oil is most suitable in lipase activity assays, because of its lower cost. Triolein represents 4–30% of olive oil.[26]

All the other chemicals were analytical reagent grade and were used without further purification. The experiments were performed in a phosphate buffer solution (0.1 M, pH 8), obtained by dissolving appropriate amounts of K_2HPO_4 and KH_2PO_4 in distilled water. The same buffer was used to prepare the enzyme solutions.

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Silicon oxide nanoparticles (SiO_2, 99.8%, 10–20 nm), surface modified with single layer organic chains, were purchased from SkySpring Nanomaterials, Inc., USA (product # 6864HN). The nanoparticle modification, giving them super hydrophobic and oleophilic properties, allowed them to be dispersible in non-polar and weak polar organic solvents. They were designed for applications with lubricating greases, resins, adhesives, elastomers, inks, sealants, wax, leather, and waterproof and anti-corrosive coatings.

The olive oil–SiO_2 dispersion was obtained by dissolving 1.2 mg of oil into 5 mL of chloroform and adding 6 mg of SiO_2 nanoparticles.

A homogeneous and transparent olive oil–SiO_2 gel was prepared by hand mixing the olive oil and silicon oxide nanoparticles in 15 : 1 ratio. The gel was stable for several months. The suggested simple technique for oil gel fabrication without application of additional agents such as proteins for emulsion stabilization, and without subsequent treatment procedures for their conversion into gels could be of interest for the food, cosmetics, and pharmaceutical industries.

Dispersion and gel characterization was performed by optical microscopy employing an XJP-H100 microscope, equipped with a 9MP MU900 digital camera, connected to a PC running AmScope ToupView software, and by scanning electron microscopy using a JEOL JSM-840 microscope. The obtained images are presented in Fig. 1 and 2.

After chloroform evaporation, the dispersion formed a thin white film similar to a porous solid. No evidence of oil leakage was found.

According to Adelmann *et al.*,[27] a network of silica, percolating throughout the oil matrix is formed. The percolating nature of the silica nanoparticles is also responsible for the gelation, while the low volume fraction associated with their low size gives the gel a transparent appearance.[27] The SEM micrographs demonstrated the formation of homogeneous smooth layers with a uniform topology.

The QCM measurements were accomplished using a model CHI400A series electrochemical quartz crystal microbalance. The model includes a potentiostat/galvanostat CHI440A, an external box with oscillator circuitry, and a QCM cell. The density of the gold coated quartz crystals (13.7 mm crystal diameter, and 5.11 mm diameter of the gold disk) was 2.648 g cm^{-3}, and the shear modulus was 2.947×10^{11} g cm^{-1} s^{-2}. For the 8 MHz crystal, the mass change is 0.14 ng for 0.1 Hz frequency change.

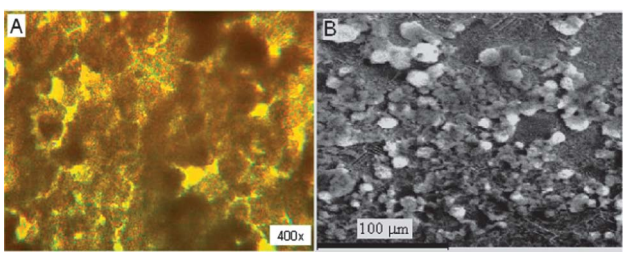

Fig. 1 Optical (A) and SEM (B) micrographs of the olive oil–SiO_2 dispersion after $CHCl_3$ evaporation. (Dispersion content: oil: 1.2 mg; SiO_2: 6 mg; $CHCl_3$: 5 mL.)

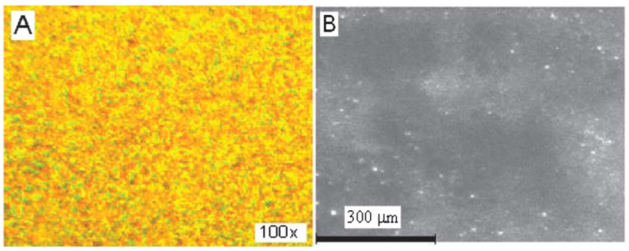

Fig. 2 Optical (A) and SEM (B) micrographs of the olive oil–SiO_2 gel (oil and silicon oxide nanoparticles mixed in 15 : 1 ratio).

First, a crystal cleaning procedure was performed including the following steps: immersing of the crystals into chloroform and treating them in an ultrasonic bath for 1 min, rinsing with deionized water, immersing for 5 min into a 1 : 1 : 5 solution of H_2O_2 (30%), NH_3 (25%), and deionized water heated to a temperature of 75 °C, rinsing with deionized water, and drying.

Three types of QCM sensors were prepared for the objectives of this work. Sensor type 1 was obtained by QCM quartz crystal modification by drop-coating using pure olive oil (the lipase substrate); for sensor type 2 preparation, the crystals were modified by nanoparticle loaded olive oil deposited also by drop-coating. Sensor type 3 was fabricated by spin-coating onto the Au disc of the quartz crystals of the oil–SiO_2 gel prepared according to the procedure described above.

For the sensors of type 1 and 2 the drop-coating procedure consisted of the following: 10 μL of chloroform solution, containing 1.2 mg, 2.4 mg, or 4.8 mg olive oil dissolved in 5 mL of $CHCl_3$ (for type 1), or 10 μL of oil–SiO_2 dispersion into chloroform (for type 2), was deposited onto the gold disk of the QCM crystal by a micropipette. The oil–chloroform solution covered the entire available surface of the quartz crystal limited by the O-rings, because of the low viscosity; thus, a reproducible surface and reproducible results were reached for every determination. After chloroform evaporation, a thin oil film (for type 1 sensor) or a thin nanoparticle loaded oil film (for type 2 sensor) was achieved.

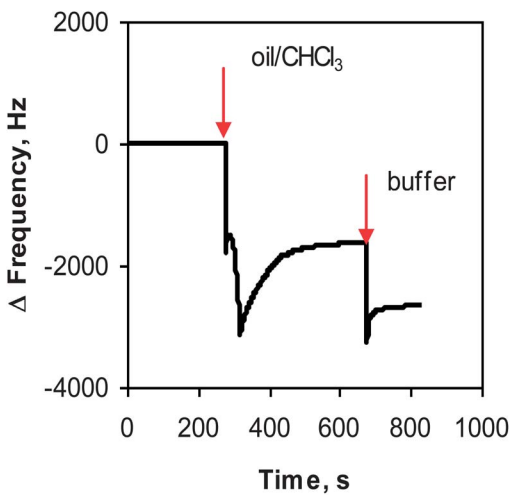

Fig. 3 Transient QCM sensor response to oil–$CHCl_3$ and buffer additions.

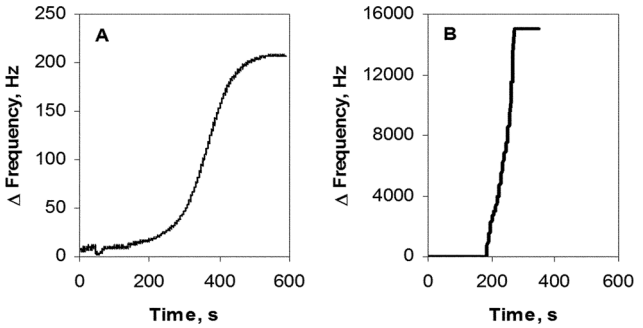

Fig. 4 Sensor responses to 100 μg mL^{-1} lipase: (A) sensor 1 response; (B) sensor 3 response. PBS 0.1 M, pH 8; 500 rpm; 25 °C.

Table 1 Comparison of the sensor 3 and sensor 1 responses

Lipase concentration, μg mL^{-1}	Signal amplification ($\Delta f_2/\Delta f_1$)
0.01	100-fold
0.1	95-fold
1	97-fold
10	99-fold
100	98-fold
1000	99-fold

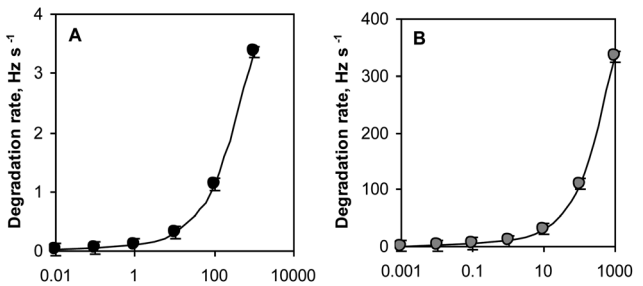

Fig. 5 Calibration curves for the lipase activity determination obtained using: (A) sensor 1; (B) sensor 3. PBS 0.1 M, pH 8; 500 rpm; 25 °C.

Table 2 Limit of detection (LOD) of the key methods applied for lipase activity determination

Method	LOD, U
Titrimetric assay using olive oil as a substrate[38]	10^{-2}
Colorimetric assay using the copper soap method[38]	10^{-1}
Spectrophotometric assay with *p*-nitrophenyl laurate[38]	10^{-4}
The proposed QCM assay without nanoparticles	10^{-5}
The proposed QCM assay with nanoparticles	10^{-6}

The drop-coating process was monitored in real time by recording the transient QCM sensor response. As demonstrated in Fig. 3, the QCM frequency decreases after dropping of the oil–chloroform solution, due to the crystal's total mass increase. Then, the chloroform evaporation provoked a frequency increase up to a plateau appearance, corresponding to a thin oil layer formation onto the Au disk surface of the QCM crystal, after complete $CHCl_3$ evaporation. Next, the addition of a buffer solution led to a frequency drop, typical for the air to water transition of the coated crystals.[28] The study of the transient QCM sensor response has been largely used to predict volatile organic compounds.[29,30] Lipid coated quartz crystals in particular are sensitive to chloroform.[30]

The lipase activity assays were based on the evaluation of the change of the olive oil degradation rate as a function of lipase concentration. The lipase substrate degradation rate was determined by the slope of the linear part of the obtained curves in coordinates of frequency change *vs.* time, applying the least squares linear regression analysis, using the software GraphPad Prism, registered mark of GraphPad Software, Inc.

The enzyme activity was correlated to a frequency change using the Sauerbrey equation:[31] $\Delta f_1 = -K\Delta m_S$. Taking into consideration that: $\Delta m_S = M\Delta n_S$, and $U = \Delta n_S/\Delta\tau$, one can obtain: $U = -\Delta f_1/KM\Delta\tau$.

When the nanoparticle loaded olive oil deposited on the QCM crystal is employed as a lipase substrate, the heavier nanoparticles leave the substrate layer simultaneously with the products of the enzymatic degradation of the olive oil, causing a greater decrease of the total QCM crystal mass compared with the case of non-charged substrate application. The nanoparticles' release was attributed only to the enzymatic olive oil layer degradation and the occurrence of concurrent reactions was excluded.[32]

The enzyme activity and QCM frequency change with time recorded applying the afterward approach, were correlated using the following modified Sauerbrey equation, including the mass change due to the nanoparticles' release: $\Delta f_2 = -K(\Delta m_{NP} + \Delta m_S)$.

Given that: $\Delta m_{NP} + \Delta m_S = A\Delta n_{NP} + M\Delta n_S$ and $U = \Delta n_S/\Delta\tau$, it results that: $U = -(\Delta f_2/KM\Delta\tau) - (A\Delta n_{NP}/M\Delta\tau)$.

Therefore, the nanoparticle loaded substrate employment for enzyme activity determination by QCM causes a sensitivity increase, due to the QCM signal amplification, quantitatively expressed by the following equation:

$$\Delta f_2/\Delta f_1 = 1 + \Delta m_{NP}/\Delta m_S.$$

The symbols used in these equations are: f_i – frequency, τ – time, m_{NP} – mass of the nanoparticles, m_S – mass of the enzymatic substrate, A – molecular mass of the nanoparticles, M – molecular mass of the substrate, n_{NP} – number of micromoles of nanoparticles, n_S – number of micromoles of substrate, U – enzyme activity unit, and K – a constant, depending on the resonant frequency of the fundamental mode of the crystal f_o, the area B of the gold disk coated onto the crystal, the shear modulus μ of quartz, and the density of the crystal ρ ($K = -2f_0^2/B\sqrt{\mu\rho}$).

The nanoparticles' release was confirmed by spectrophotometric assays, by monitoring the lipolysis of olive oil, deposited onto a disposable transparent PET strip covered by Fe_2O_3 nanoparticles, which played the role of a light barrier. Nanoparticle removal with the degradation products resulted in light absorption diminution.[33] Additionally, the incorporation of

ferromagnetic nanoparticles like Co, Ni, Fe_3O_4 or Fe_2O_3 into the sensitive layer allows their rapid collection by a magnet, which leads to an increase of the rate of the enzymatic reaction due to the reduction of the diffusion constraints.[34]

Degradation experiments for lipase activity evaluation were performed in accordance with the following protocol: installing the modified quartz crystal into the QCM cell, filling the cell with 180 μL PBS, stirring for 10 minutes at 500 rpm for frequency stabilization resulting from the equilibrium establishment at the interface of the coated crystal in contact with the PBS buffer; adding drop wise 20 μL lipase solution with defined concentration (activity), and finally recording the frequency change with time. The quantification could be affected by pH and temperature changes, as well as by the sensitive layer composition and thickness. Thus, to guarantee the reliability of the obtained results, all the experiments were carried out in a phosphate buffer solution with pH corresponding to the optimum pH of the selected enzyme (pH 8), and at a constant temperature of 25 °C, considered as a standard ambient temperature, keeping the sensitive layer composition and thickness constant.

The experiments performed employing the type 1 sensor, modified by the deposition of 10 μL oil solutions containing 1.2 mg, 2.4 mg, and 4.8 mg olive oil respectively, dissolved into 5 mL of $CHCl_3$, demonstrated an increase of the plateau height with the oil mass decrease. The slope of the curves: frequency change *vs.* time however did not vary with the deposited oil quantities. This slope depends only on the oil interface contact surface with the enzyme containing solution, and the deposition of a greater oil quantity resulted in QCM damping only.

The QCM sensor frequency responses to 100 μg mL^{-1} lipase, obtained applying sensor type 1 and sensor type 3, are shown in Fig. 4A and B, respectively. Sensor type 1 and sensor type 3 were fabricated as described above. For the preparation of sensor type 1 a solution containing 1.2 mg of olive oil dissolved in 5 mL of $CHCl_3$ was used. The signal amplification reached using sensor type 2 was inferior to those obtained using sensor type 3. Hence, the further experiments were carried out with sensor type 3 and the results obtained were compared with those acquired with sensor type 1.

The degradation process initially involves enzyme molecule adsorption on the substrate layer, the beginning of the enzyme reaction, and additional film swelling, resulting in a frequency decrease. At higher activities, the frequency decrease is insignificant (Fig. 4), because of the very rapid film degradation. The dissolution of the products led to a frequency increase. Finally, complete substrate layer degradation is achieved, indicated by the observed almost no-change in the QCM frequency. Such a degradation model was used to interpret the features exhibited by peptide cross-linked dextran hydrogels, as well as cellulose and triolein films submitted to the action of elastase,[35] cellulase,[36] and lipase,[37] respectively, investigated using the QCM and QCM-D techniques.

In the presence of nanoparticles, almost a 100-fold signal amplification was achieved, as data presented in Table 1 demonstrate. These results were obtained as the ratio of the experimentally established olive oil degradation rates in the presence and absence of nanoparticles.

Taking into consideration that for the 8 MHz crystal used, the mass change is 0.14 ng for a 0.1 Hz frequency change, and that the degradation rate of the olive oil layer evaluated by applying sensor type 1 and sensor type 3 was respectively 1.12 Hz min^{-1} and 110.17 Hz min^{-1} in the presence of 100 μg mL^{-1} lipase, it was found that Δm_S and Δm_{NP} are 1.57 ng min^{-1} and 152.67 ng min^{-1}, respectively. Thus, the greater mass of the nanoparticles released, resulted in about a 100-fold response increase of the QCM during the enzymatic degradation of the lipase substrate.

The constructed calibration curves for the lipase activity evaluation in the range of 1.04×10^{-5} U mL^{-1} (0.001 μg mL^{-1}) up to 10.4 U mL^{-1} (1000 μg mL^{-1}), are presented in Fig. 5. The reproducibility of the determinations, estimated by measuring the QCM response to 100 μg mL^{-1} lipase, was <3% ($n = 4$).

The amplification of the signal by the loss of nanoparticles together with the olive oil layer degradation led to a sensitivity increase, and allowed us to reach a limit of detection as low as 1.04×10^{-6} U mL^{-1} (0.0001 μg mL^{-1}) lipase, compared with 1.04×10^{-5} U mL^{-1} (0.001 μg mL^{-1}) obtained in the absence of nanoparticles. The limit of detection was determined by the lowest enzyme concentration (μg mL^{-1}) that produces a measurable frequency change with a signal to noise ratio of 3 under the conditions employed. Enzyme concentration was associated with enzyme activity.

As data in Table 2 demonstrate, the developed method for lipase activity determination is the most sensitive reported until now. Moreover, the method is practically reagentless, since prefabricated crystals mounted on sticks with a previously deposited sensitive layer could be used. Therefore, the suggested technique allows lipase activity determination more easily and faster than the established techniques.

The suggested approach based on the use of a nanoparticle modified QCM sensor, namely sensor type 3, was applied for evaluating the lipase activity in a crude lipase extract obtained from defatted rice bran. The extraction was accomplished as reported in the literature.[39] The lipase activity was also assayed by volumetric titration, employing olive oil as an enzyme substrate.[39] The percentage error of the determination was found to be less than 5%, thus confirming the accuracy of the proposed new method.

Conclusion

In this work, a new simple, rapid, and highly sensitive QCM-based method for lipase activity determination is proposed. The QCM quartz crystal was modified by deposition of SiO_2 nanoparticle loaded olive oil, employed as an enzyme substrate. The heavier nanoparticles' release, following the substrate lipolysis, resulted in a QCM frequency response enhancement proportional to the nanoparticle/substrate mass ratio. A ten-fold LOD improvement was achieved in the lipase activity determination applying a nanoparticle loaded substrate compared with the non-loaded one.

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