Immobilization of urease by laser techniques: Synthesis and application to urea biosensors

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Received 8 June 2007; revised 23 November 2007; accepted 30 November 2007
Published online 22 April 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31963

Abstract: Urease thin films have been immobilized using matrix-assisted pulsed laser evaporation for biosensor applications in clinical diagnostics. The targets exposed to laser radiation were made of frozen composites that had been manufactured by dissolving urease in distilled water. An UV KrF* (λ = 248 nm, $\tau_{FWHM} \cong$ 30 ns, ν = 10 Hz) excimer source was used for the multipulse laser irradiation of the targets that were cooled down to solidification using Peltier elements. The incident laser fluence was set at 0.4 J/cm^2. The surface morphology and chemical bonding states of the laser immobilized urease thin films were investigated by atomic force microscopy and Fourier transform infrared spectroscopy. The enzymatic activity and kinetics of the immobilized urease were assayed by the Worthington method, which monitors urea hydrolysis by coupling ammonia production to a glutamate dehydrogenase reaction. Decreased absorbance was found at 340 nm and correlated with the enzymatic activity of urease. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 89A: 186–191, 2009

Key words: enzyme based sensors; urease thin films; laser techniques

INTRODUCTION

Enzyme based sensors, which belong to one of the best recognized classes of biosensors,[1–3] are the first choice as miniaturized detectors for medical diagnostics, due to their high amplification of biorecognition and selectivity.[4] Enzymes indeed are 10^8–10^{12} times more sensitive than nonbiological catalysts, which improve biodetection capability to a few ppm.[5]

Developing technologies to immobilize the biologically sensitive molecules, known as recognition elements, is the crucial factor in biosensor construction. Special attention has been paid in recent years to optimizing strategies for enzyme immobilization.[1–3,6–8] The key objective is to preserve throughout the immobilization process, the bioactivity of the molecules which only react to the presence of a definite group of materials or substances to be detected and thus ensure high selectivity.

Despite intensive research in the area of biomolecule immobilization, a generally applicable method has yet to be developed. Conventional methods are usually high cost and involve toxic chemicals and/or specific pretreated substrate materials. Instead, we investigated the possibility of using new laser techniques that could overcome these drawbacks, since they are precisely targeted and versatile and pose very low contamination risks. Rapid processing and low production cost are other expected benefits.

In particular, we focused our attention on urease, an enzyme used as a specific biorecognition element for urea ($(NH_2)_2CO$) detection. Urea is a by-product of protein metabolism whose amount in the bloodstream is checked for information on kidney function.[8–12] In addition, urea detection is also relevant

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Contract grant sponsor: Spanish Ministry of Education and Science; contract grant number: MAT2006-26534-E
Contract grant sponsor: Hungarian-Romanian Governmental Bilateral Agreement "*Biosensors design using laser radiation*" 2006–2007, Romanian Ministry for Education and Research; contract grant number: CEEX 150/2006
Contract grant sponsor: Hungarian Scientific Research Foundation OTKA; contract grant number: TS 049872

for other areas such as environmental and safety analyses[13] and the food industry.[14]

At present, laser radiation due to its highly destructive potential is not perceived as particularly useful for biomaterial processing. The targets we submitted to laser radiation in our experiments consisted of frozen composites made by dissolving or suspending biomaterials in distilled water, to minimize photochemical damage. The evaporated water molecules thus protected/shielded the biomolecules through collisions and transferred them to the substrate surface that lay plan parallel in front of the frozen target. As an additional precaution, laser pulse intensities were cut down by an order of magnitude, compared with their common level on irradiating other type of materials, e.g. inorganics. The process is known as matrix-assisted pulsed laser evaporation (MAPLE).

MAPLE has unique advantages as compared with conventional technologies used for biomaterial immobilization.[15] It allows for accurate thickness control of the growing biomaterial thin films over a wide range of thicknesses from a few nm until the micrometer range. The uniformity of the biomaterial coverage stands also among the main advantages of the technique. Multilayers of different, hybrid, materials (organics, active biomaterials, inorganics) can also be synthesized during the same technological step. Moreover, it allows for depositions using multi-component targets containing proteins and other functional materials, which ensure enhanced stability and adhesion to the substrate surface of the protein coverage.

Similar laser techniques have been applied with positive results for processing organic polymer materials[16–18] and biomolecules, insulin and horseradish peroxidise,[19] bovine serum albumin,[20] silk protein,[21,22] fibrinogen blood proteins,[23] creatinine,[24] papain,[25] or glucose oxidase.[26] It has been demonstrated that the laser processed materials preserved the composition and structure of the base materials used for target preparation. Our investigations have pioneering character as regards laser-active biomaterial interaction processes. Our intention is to elucidate, whether it would be possible to preserve, besides the structural integrity of laser processed biomolecules, their enzymatic activity. The ability to deposit by MAPLE active enzyme molecules of glucose oxidase was proved only by using as matrix material sodium dodecylsulfate.[26] This approach, however, resulted in a contaminant rich film, that contained not only the enzyme of interest but also the specific matrix material used for the target preparation. In our experiments, the evaporated water molecules will not interfere either in the protein films' growth process and their enzymatic activity.

MATERIALS AND METHODS

Urease immobilization

The biomaterial thin film growth experiments were performed in a stainless steel irradiation chamber. A pulsed UV KrF* (LLG TWINAMP, λ = 248 nm, τ_{FWHM} = 30 ns, ν = 10 Hz) laser was used for target irradiation. The composite targets were prepared by dissolving 10 wt % urease from jack beans (*Canavalia ensiformis*) in distilled water. The obtained solution was frozen to -30°C.

The laser beam was focused onto the target surface with a 30 cm FD MgF_2 lens placed outside the irradiation chamber. To avoid significant changes in the surface morphology of the targets, they were rotated during the multipulse laser irradiation with a frequency of 3 Hz. The angle between the laser beam and target surface was set to 45°. We applied 6×10^3 subsequent laser pulses to deposit each film. The laser fluence incident on the target surface was 0.4 J/cm^2.

The irradiation chamber was evacuated down to a residual pressure of 10^{-1} Pa. Thin layers for the FTIR spectroscopic measurements were deposited on KBr pellets, while those designed for morphologic studies and bioactivity tests were prepared on Si substrates lying parallel to the target 3.5 cm apart. The substrates were carefully cleaned in an ultrasonic bath using acetone and ethanol prior to introducing them into the deposition chamber. During irradiation, the substrates were kept at room temperature, while the frozen targets were cooled with Peltier elements.

The surface morphology and growth mode of the deposited urease thin films were investigated with a PicoSPM Molecular Imaging atomic force microscope (AFM) in acoustic (dynamic) configuration. The chemical composition of the obtained thin films was also studied by Fourier-transform infrared spectroscopy (FTIR) using a BIORAD FTS-65 spectrometer in the 400–4000 cm^{-1} range.

Enzymatic activity evaluation

The kinetics and enzymatic activity of urease were studied by the Worthington assay method. As reagents we used: 0.1*M* potassium in phosphate buffer, pH 7.6; 0.023*M* adenosine-5′-diphosphate (ADP) in phosphate buffer; 0.0072*M* NADH in phosphate buffer; 0.026*M* α-ketoglutarate in phosphate buffer; 1.8*M* urea in phosphate buffer; glutamate dehydrogenase 500 U/mL in phosphate buffer; and urease 25 mg/mL stock solution in phosphate buffer.

The blank containing 0.023*M* ADP, 0.0072*M* NADH, 0.026*M* α-ketoglutarate, 1.8*M* urea, glutamate dehydrogenase 500 U/mL in 0.1*M* potassium phosphate buffer solution, pH 7.6, was incubated at 25°C for 10 min to achieve temperature and absorbance rate equilibration. Then 800 μL of the blank solution was added to each urease film deposited by MAPLE.

Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamine acid. The latter compound spontaneously decomposes to generate a second molecule of ammonia and carbon dioxide. The hydrolysis of urea was measured by coupling ammonia production to a glutamate dehydrogenase reaction:

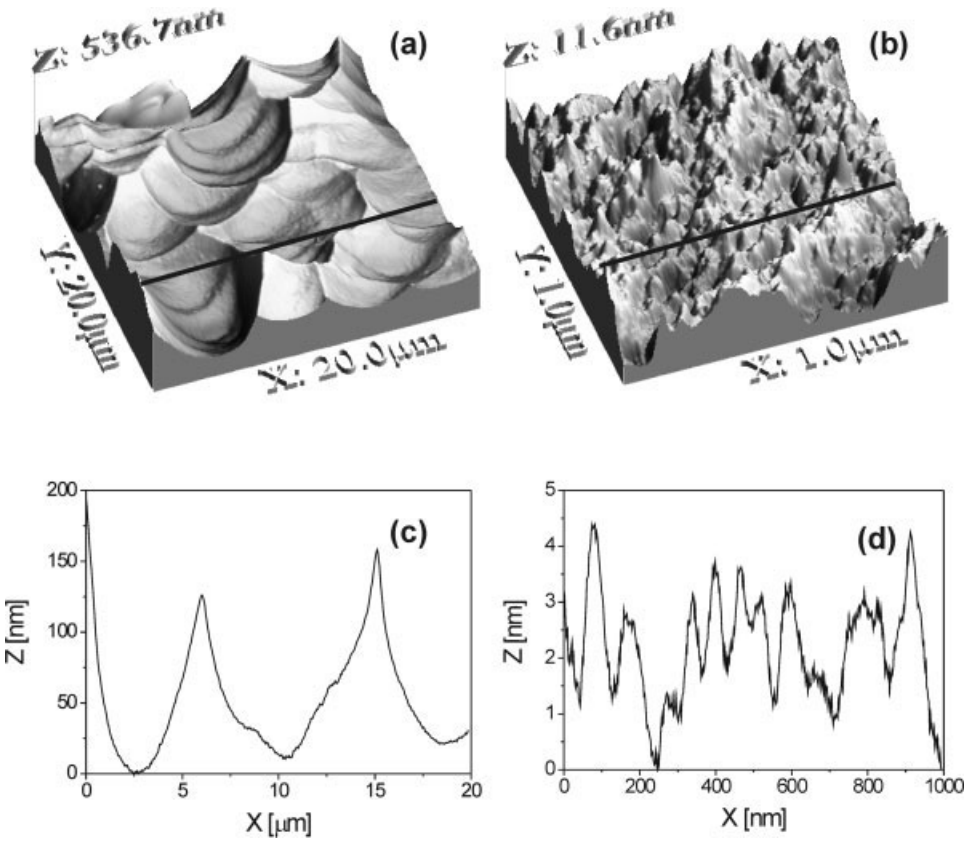

Figure 1. Tilted (a), detailed view (b) AFM micrographs, and corresponding surface profiles (c,d) of urease thin film deposited at 400 mJ/cm^2 laser fluence.

$$\text{urea} + H_2O + 2H^+ \rightarrow 2NH_4{}^+ + CO_2 \quad (1)$$

$$2NH^{4+} + 2a - \text{ketoglutarate} + 2\text{NADH} \rightarrow 2\text{glutamate} + 2NAD^+ + 2H_2O \quad (2)$$

Reaction (1) was catalyzed by urease and reaction (2) was catalyzed by another enzyme called glutamate dehydrogenase. The breakdown of one mole of urea by urease produces two moles of ammonia, which are consumed in the second reaction. Simultaneously, the NADH (reduced NAD) molecule was oxidized to NAD^+.

NADH absorbs UV light at 340 nm, whereas NAD^+ does not. NADH molecules decrease in numbers if the reaction (2) takes place. The decrease of absorbance at 340 nm was measured for different time intervals, from 0 to 25 min, with an Ultrospec 3000 spectrophotometer (Pharmacia), and correlated with the enzymatic activity of urease. Urease from jack beans was used as positive control (SIGMA).

RESULTS AND DISCUSSION

Morphological and structural characterization of immobilized urease

Figure 1(a) shows a tilted AFM image of the laser grown urease thin film. As can be noticed, the surface is characterized by an interconnected island-like morphology, with an average island height of about 240 nm. The minimum root-mean square surface roughness calculated from the AFM data was about 100 nm. This specific morphology can be considered an advantage, as it provides a considerably broader active area, compared with entirely smooth surfaces, when used as recognition element in biosensor devices.

The high resolution AFM image [Fig. 1(b)] revealed the island surfaces were granular in structure. Figure 1(c,d) show the surface topography across the lines marked in the AFM micrographs. According to the high resolution images, the size distribution of the grains was uniform in terms of both diameter and height. From the histograms in Figure 2, we estimated the average diameter was around 30 nm, whereas the average surface local height was around 5 nm. The influence of AFM tip on the size distribution measurements is more pronounced, around 10%, for the estimation of particles diameters and much less, around 3%, for their height. The deviation values were calculated using a geometric deconvolution,[27] considering a spherical tip with a radius of 5 nm. It is worth noting that the average local heights are related but do not correspond directly to the grain height. The finite size of the AFM tip leads to smaller measured values as compared to the actual surface.

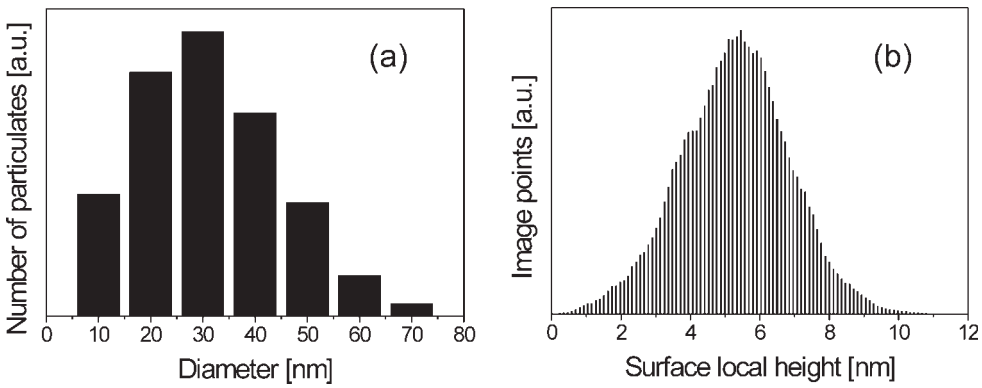

Figure 2. Histograms of (a) nanoparticle diameter and (b) surface local height as estimated from high resolution AFM micrographs.

The composition of the deposited biomolecular thin films and the chemical bonds between the elements were studied by FTIR spectroscopy (Fig. 3). Our investigations showed a good accordance between the reference spectrum of the base material of which the composite targets were made [Fig. 3(a)] and the spectrum of the MAPLE deposited thin films [Fig. 3(b)].

It is thereby confirmed that urease can be immobilized by a laser based method without any deterioration of its chemical bonds. However, despite good reproduction of the reference FTIR spectrum of the base material, some damage to the 3D structure of urease during laser irradiation, transfer, and thin film growth is not entirely ruled out.

Enzymatic activity of immobilized urease

Optimization studies

The molecular weight of urease is 480,000 Da, but the enzyme can polymerize to form six unit polymers of about 3,000,000 Da. Its catalytic activity cannot be maintained through the laser immobilization process unless its 3D structure goes unchanged, ensuring a proper conformation of the active sites for their interaction with the substrate.

We first prepared a blank solution from the reagents necessary for reactions (1) and (2) to study its kinetic rate from 0 to 60 min. The slight variation in absorbance at 340 nm during the assay interval was probably due to ammonia traces in the reagents, which were high enough for the reaction (2) to take place at a reduced kinetic rate (Fig. 4 open squares). The measurements standard errors were generally lower than ±3%.

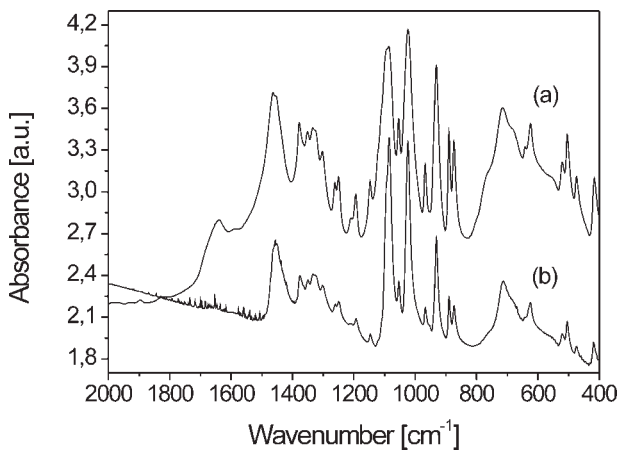

Figure 3. FTIR spectra of (a) base material used for the preparation of the composite targets and (b) urease thin film deposited at 400 mJ/cm^2 laser fluence.

Enzymatic activity evaluation

After system optimization, the MAPLE grown thin films were immersed in the solutions prepared from the reaction (1) reagents and mixed with 1.8*M* urea in 0.1*M* potassium phosphate buffer. Aliquots were taken after incubation for 5, 15, and 30 min and added to the solution prepared from the reagents for reaction (2). Kinetic analyses were performed by measuring the absorbance of the obtained solution at 340 nm over 1 h. The results are presented in Figure 4(a–c) (full squares). A linear decrease of absorbance was observed in all aliquots, regardless of the incubation time, with two different slopes, a larger one in the first time interval up to about 15 min, and another that was similar to the blank solution slopes. The faster absorbance variation within the first time interval and the increased absorbance variation with the increase in incubation time from 5 to 30 min show that the laser immobilized enzyme was active in breaking down urea. Indeed, the largest variation in absorbance was found when the enzyme was given longer time to hydrolyze urea (30 min).

These experiments strongly suggest that the laser immobilized urease preserved its 3D structure and

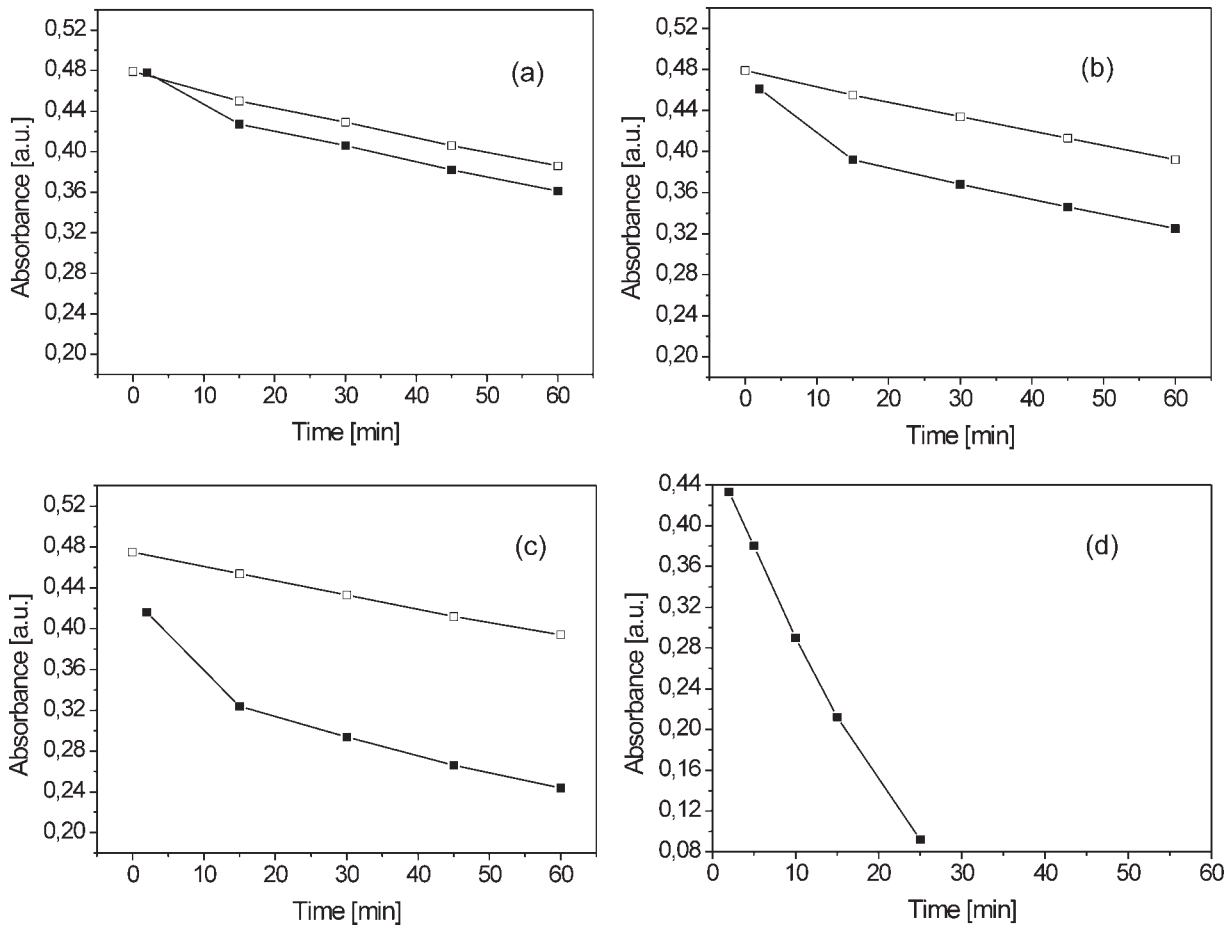

Figure 4. Kinetic assay of urease thin film deposited at 400 mJ/cm^2 laser fluence after incubation with 1.8*M* urea for (a) 5, (b) 15, and (c) 30 min; (d) kinetics of urease thin film deposited at 400 mJ/cm^2 laser fluence, incubated with the reagents necessary for reactions (1) and (2).

enzymatic activity. However, to make sure that no artefacts had interfered with our enzymatic tests, we prepared a negative control. Knowing that enzymes lose their activity after denaturation at 100°C, we boiled duplicate samples for 40 min on the water bath and then tested their kinetics in parallel with as deposited urease thin films. Both were immersed in the solutions prepared from the reagents for reactions (1) and (2) (blank solution) and mixed with 1.8*M* urea in 0.1*M* potassium phosphate buffer. The decrease of absorbance recorded for the negative control was identical to that obtained for the blank solution [open squares in Fig. 4(a–c)]. By contrast, as-deposited urease films showed a faster linear decrease of absorbance at 340 nm over 25 min [Fig. 4(d)], thus validating the functionality of the laser immobilized enzyme.

The stability of the deposited films was tested during a time interval of a few months. The samples were kept in refrigerator at 4°C when not in use. FTIR spectra and enzymatic activity were preserved until a period of about 3 months. After this time interval the onset of decomposition process was observed by FTIR spectroscopy.

CONCLUSION

Biomolecular urease thin films were grown by matrix assisted pulsed laser deposition technique. A pulsed UV KrF* (λ = 248 nm, $\tau_{FWHM} \cong$ 30 ns, ν = 10 Hz) excimer laser source was used to irradiate the frozen composite targets. Our results prove that this method can be used to grow uniform, continuous thin films, with chemical composition and molecular structure identical to those of the biomaterial originally used for target preparation. The enzymatic activity and kinetics of urease were studied using the Worthington assay method. MAPLE deposited thin films were immersed in blank solutions prepared and mixed with urea in potassium phosphate buffer. The kinetics analyses were performed by measuring the variation in solution absorbance at 340 nm over 1 h. The fast absorbance variation and

the increased absorbance variation with the increase in incubation time show that the laser immobilized enzyme was active in breaking down urea.

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