Optical characterization of glutamate dehydrogenase monolayers chemisorbed on SiO_2

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This paper describes the formation of glutamate dehydrogenase monolayers on silicon dioxide, and their characterization by means of physical techniques, i.e., fluorescence spectroscopy and Fourier-transform infrared spectroscopy. Detailed investigations of the intrinsic stability of native proteins in solution were carried out to elucidate the occurrence of conformational changes induced by the immobilization procedure. The enzyme monolayers were deposited on SiO_2 after preexposing silicon surfaces to 3-aminopropyltriethoxysilane and reacting the silylated surfaces with glutaric dialdehyde. The optical characterization demonstrates that the immobilization does not interfere with the fold pattern of the native enzyme. In addition, fluorescence spectroscopy, thermal denaturation, and quenching studies performed on the enzyme in solution well describe the folding and unfolding properties of glutamate dehydrogenase. The photophysical studies reported here are relevant for nanobioelectronics applications requiring protein immobilization on a chip.

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I. INTRODUCTION

Proteins and enzymes are attractive for nanobiotechnology and nanoelectronics since they enable highly specific reactions, and because of their dimension in the nanometer range. In addition, they are capable of self-assembly, thus opening the attractive possibility of nanoscale surface patterning as an alternative to optical lithographic approaches. Protein-mediated electron transfer is the key phenomenon, not only in cellular processes, but also in many reactions of biotechnological interest. In recent years, much attention has been paid to developing and investigating the use of biomolecules as electron-conductive materials for bioelectronics or for the construction of amperometric devices. In addition, physiological functions of natural electron-transfer proteins can be optimized toward specific technological targets. For all these reasons, the formation and study of protein film assemblies on solid substrates are fundamental for bioelectronic applications, such as protein chips, nanobiosensors, hybrid electronic devices, etc.

Immobilization is an important aspect in the construction of nanodevices, as their functionality strongly depends on the quality of the biomolecular film. The interaction between proteins and solid substrates may lead to alterations of the native structure, with consequent loss of functionality. The different immobilization procedures generate an artificial microenvironment around the immobilized biomolecules. Physical adsorption can be obtained on metal oxides, carbon electrodes, and silica oxides, although it usually leads to protein denaturation induced by the interactions with the surface. In addition, binding of ligands might be affected and unspecific multilayers may also originate. Hence, chemical modification of the surface has to be used to increase the stability of the protein and to control the density and environment of the immobilized species. Soluble proteins carry hydrophilic groups and/or charged amino acid residues on their outer surfaces. Electrostatic interactions (including hydration) are crucial to the structure and function of proteins [1]. Thus, if the biomolecules are entrapped within a friendly hydrophilic matrix their stability is preserved, even if some properties of enzymes, such as the specific rate of the catalyzed reaction, may be affected by the immobilization in an unpredictable way. Hence, the development of enzyme-based devices requires detailed understanding of the protein immobilization processes and eventual conformational changes of the proteins. Investigation of the intrinsic stability of proteins provides a better understanding of the mechanisms affecting their shelf life and operational stability upon immobilization onto a solid surface. Structure-sensitive experiments capable of monitoring protein structure at interfaces are thus crucial for technological progress of the field. In this frame, we have investigated the structural aspects of glutamate dehydrogenase (GDH) and its binding properties to the substrate by means of optical techniques, i.e., fluorescence spectroscopy and Fourier-transform infrared (FTIR) spectroscopy.

The enzyme bovine liver glutamate dehydrogenase reversibly catalyzes the oxidative deamination of glutamate to α-ketoglutarate and ammonia, using NAD^+ or NADH as coenzymes [2]. Glutamate has widespread use as a flavor enhancing food additive, so that its determination is of interest for both the biological sciences and the food industry. Glutamate concentrations have been routinely assayed by chromatographic and potentiometric titrations, although these methods are time consuming and technically demanding. Glutamate is also reported to be a powerful neuroexcitatory amino acid involved in several behavior patterns. The importance of the pathophysiological nature of GDH-deficient neurological disorders has attracted considerable attention [3–7]. However, monitoring of glutamate release at the synaptic level is very difficult owing to its extremely low concentration and to the small size of the synapses. As a consequence, glutamate monitoring requires highly sensitive

detectors in the submicrometer range. Techniques such as high-performance liquid chromatography analysis [5] cannot provide both spatial and temporal resolution. Therefore, the characterization of the spatiotemporal nature of its release would be of particular interest. Previous work has shown that GDH can be used as a detector of glutamate release in several situations [8–12]. An amperometric biosensor was obtained by the immobilization of GDH in a carbon paste electrode, although the physical stability of the system was limited by the thermal properties of the carbon paste matrix itself [13]. In addition, a recent report demonstrated the possibility of investigating the spatiotemporal kinetics of glutamate release with a resolution of 20 μm and 500 ms integration times, demonstrating the occurrence of an extracellular wave of glutamate [14].

With the perspective of realizing a solid-state nanobiosensor, utilizing high-quality monolayers of GDH as the sensitive medium, we have carefully analyzed enzyme conformational properties in the native state and when deposited on functionalized Si/SiO_2 substrates. This may be an attractive method as an alternative to enzyme adsorption on Langmuir-Blodgett films [15].

We utilized the potential of fluorescence spectroscopy for probing the conformational and structural states that GDH molecules undergo during transfer from solution to immobilization on solid films. All proteins contain natural chromophores absorbing in the near ultraviolet, notably the aromatic residues phenylalanine, tyrosine, and tryptophan. These chromophores are also responsible for protein fluorescence. In GDH, which contains all three aromatic amino acids [16], fluorescence is dominated by the contribution of the tryptophan residues, because their quantum yield is considerably greater than those of tyrosine and phenylalanine residues [17], and because the light absorbed by other aromatic amino acids is generally transferred to tryptophan nonradiatively [18]. GDH is readily amenable to intrinsic fluorescence studies, since it contains 3 tryptophan molecules per subunit. This circumstance, however, does not facilitate the interpretation of photoluminescence data.

The aromatic amino acid tryptophan (Trp) offers an intrinsic fluorescent probe of protein conformation, dynamics, and intermolecular interactions. The emission spectrum is an important indicator of the integrity of the protein globular fold, as the fluorescence parameters of tryptophan residues are highly sensitive to the microenvironment of the fluorophores in protein structures [19–22]. For this reason, photoluminescence characteristics are widely used to study the physicochemical properties, interactions with other molecules [23], and structural transitions of protein molecules as a whole [21]. Perturbations of the native fold may lead to shifts, broadening, and loss in resolution of the spectrum.

II. MATERIALS AND METHODS

A. Chemicals

Glutamate dehydrogenase (Lot 9029-12-3, from bovine liver, EC 1.4.1.3) was purchased from Fluka (Steinheim, Germany). 3-aminopropyltriethoxysilane (99%), glutaraldehyde 50 wt % solution in water, and urea (99%) were purchased from Aldrich (Steinheim, Germany). Sodium phosphate was obtained from Carlo Erba (Milan, Italy), potassium iodide (99.5%) from Fluka (Steinheim, Germany), and acrylamide (99%) from Sigma. Electronic grade p^+-doped Si wafers with 100–200 mm thermal oxide layer were used as substrates.

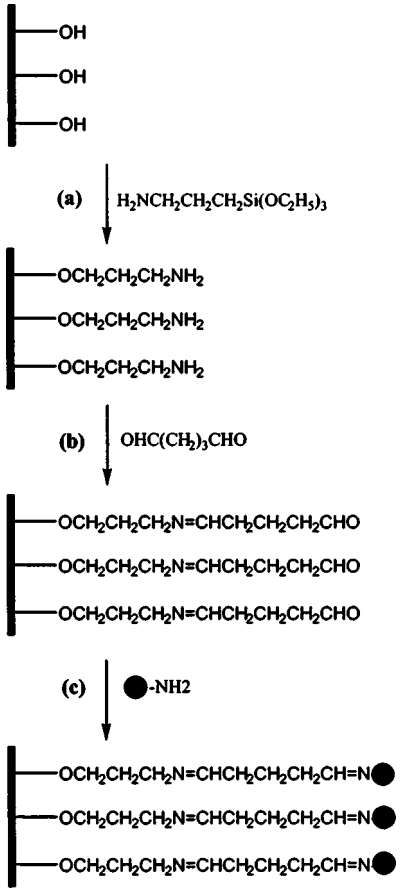

FIG. 1. Scheme of the three-step chemical reaction used for the enzyme immobilization.

Before all experiments, the enzyme was purified with an ultrafiltration cell (Amicon, Lexington, KY) with membrane YM30, under nitrogen pressure (7×10^5 Pa). The purification was carried out at 4 °C. The resulting enzyme solution shows an A_{280}/A_{260} ratio of 1.91 [2].

B. Enzyme immobilization

SiO_2 substrates (chips) of approximate dimensions 5 $\times$5 mm^2 were cut using a diamond-tipped scriber. The chips were then cleaned on a spinner (BLE, Delta 10) at 6000 rpm with trielin, acetone, methanol, and isopropylic acid for 30 s and dried with nitrogen. The surfaces of cleaned chips were modified using a three-step procedure (Fig. 1) described below.

Surface functionalization was performed by dipping the silicon chips into a freshly prepared 6.6% (v/v) solution of 3-aminopropyltriethoxysilane in $CHCl_3$ at room temperature for 3 min and then rinsed with abundant $CHCl_3$ in order to remove the unreacted silanizating agent. The alkylamine-derivatized surface was firstly exposed to a glutaraldeyde

solution (1% v/v in distilled water) for 10 min and secondly washed in distilled water and 50 mM sodium phosphate buffer (*p*H 7.0). Enzyme immobilization was achieved through Schiff base formation incubating the activated chips in a solution of glutamate dehydrogenase (2 mg ml^{-1} in 50 mM sodium phosphate buffer, *p*H 7.0) at 19 °C. After 12 h the sample was rinsed with 4.0*M* urea in 50 mM sodium phosphate (*p*H 7.0) in order to get rid of physically adsorbed molecules. After these treatments all samples were stored at 4 °C.

C. Fluorescence spectroscopy

Fluorescence emission spectra were recorded with a Perkin Elmer LS 50B spectrofluorimeter. A xenon lamp was used as the source of excitation (2 nm bandwidth), and the emitted light was observed at right angles to the excitation radiation. Photoluminescence spectra of control samples, without protein, were recorded and subtracted from the experimental samples to correct for background interference. All the emission spectra were recorded in 20 mM phosphate buffer with *p*H 7.2 at room temperature (20 °C). The optimal excitation wavelength was determined to be 292 nm, after scanning the range between 250 and 300 nm in a 3 ml cell (1 cm light path).

Photoinactivation of enzymes is a fairly well known phenomenon, which can be induced by photooxidation of susceptible amino acids, followed or not by secondary cross-linking of the protein [24]. Concerning Trp photodegradation during the acquisition time of the spectra, we found that intensity losses were negligible or less than 5% depending on the experimental conditions. This is consistent with the findings of Ghobadi *et al.* [25], who explained that excitation at 292 nm causes the least photodecomposition of the enzyme.

1. Thermal denaturation

We characterized the structural changes of the enzyme GDH upon thermal denaturation by means of intrinsic fluorescence emission. 3 ml samples of GDH (0.125 mg/ml) in phosphate buffer were heated in water baths at 100 °C for different times (incubation times). Aliquots were removed at intervals and rapidly cooled to stop the reaction by immersion in a ice-water bath.

The denaturation process was analyzed both by monitoring the shift of the peak wavelength (λ_{max}) of the emission spectrum as a function of the treatment time and by an analytical approach in which the spectrum was deconvolved by means of three Gaussians and the shifts of the three maxima ($\lambda_{max\,1}$,$\lambda_{max\,2}$,$\lambda_{max\,3}$) were analyzed. This procedure allows us to study both the integrated behavior of the denaturing enzyme and the contributions of the individual emitting species to the total redshifting spectrum.

In addition, the GDH unfolding was scanned by examining the thermally induced variations in the integrated fluorescence of the enzyme (calculated as the area under the emission curves).

2. Elastic scattering measurements

GDH self-association upon thermal denaturation at 100 °C was also monitored by the relative intensity of right-angle elastic scattering. These experiments were performed with excitation and emission set at the same wavelength (292 nm). The scattered intensity is proportional to the molecular mass of the scattering particle and hence is very sensitive to its aggregation state. The difference from that of an unaggregated protein sample in the same solvent directly probes the aggregation extent [26]. To avoid undesired scattering from polluting particles, the solutions were carefully filtered before measurements. In any case, scattered intensities were always corrected for solvent.

3. Fluorescence quenching studies

The environment of tryptophyl residues in GDH was further characterized by determining their degree of accessibility to external quenchers, such as acrylamide and KI.

The effect of I ions ($0M-0.5M$) and acrylamide ($0M-0.2M$) on the emission of GDH was tested both in native and in partially denatured conditions (1 min at 100 °C) of the enzyme (0.125 mg/ml). The initial fluorescence (F_0) of the solution (20 mM phosphate buffer, *p*H 7.2) was measured. The enzyme fluorescence from the tryptophan residues was then quenched by progressive addition of small aliquots of the stock solution of the quenchers to the fluorimetric cuvette and the fluorescence intensity (F) was measured again. After correction for filter effects arising from the light absorption by KI and acrylamide, the decrease of fluorescence was analyzed according to the Stern-Volmer and Lehrer equations [21,27].

4. Fluorescence from protein films

To monitor GDH luminescence from protein films, the exciting radiation was focused on the protein film (in phosphate buffer) held with its normal at 30° from the exciting beam direction, while the front surface emission was collected at 90° from the excitation. Scattered (reflected) light was excluded by a cutoff (high-pass) filter (CVI model CG-WG-305, transmittance greater than 305 nm) positioned between the GDH film and detection system.

D. FTIR spectroscopy

Fourier-transform infrared spectroscopy was performed as the second technique to investigate the protein films. The FTIR spectra of immobilized enzyme were measured in the transmittance mode using a 660 V FTIR spectrophotometer equipped with an IRT-30 FTIR microscope. The mapping grid was adjusted to $625\times625\ \mu m^2$ and the sampling area was scanned 100 times at 4 cm^{-1} resolution, using a mercury cadmium tellurium detector. The instrument was purged with dry nitrogen gas to reduce the interference of water vapor and CO_2 in all the FTIR measurements.

E. Statistics

For statistical considerations, the experimental results shown in Figs. 2–4 are an average of five independent measurements. The data shown in the corresponding figures in Sec. III are the mean values of the data from experiments, and the error bars indicate the standard deviations.

III. RESULTS AND DISCUSSION

In the present paper, we performed different experiments in order to evaluate the optical properties of the enzyme in solution and chemisorbed onto SiO_2 substrates to characterize different aspects of the protein structure. For this purpose, we first investigated native GDH fluorescence and thermally induced unfolding and fluorescence quenching processes and, in a second step, the emission spectra of the enzyme immobilized on silicon substrates were studied. Such basic analyses allowed us to assess the eventual structural changes caused by protein immobilization on solid films. FTIR spectroscopy completed the experimental investigation.

A. Native GDH fluorescence

Tryptophan fluorescence is widely used as a tool to get information regarding local structure and dynamics, since λ_{max} roughly correlates with the degree of solvent exposure of the chromophore. The parameters I_{max}, λ_{max}, and integrated emission intensities characterizing the photoluminescence of GDH were measured as a function of λ_{exc} from 250 to 300 nm. Excitation in the range 250–300 nm resulted in a large variation of the fluorescence quantum yield, with a distinct maximum at 292 nm [Fig. 2(a)]. Since the emission spectra exhibited no dependence on λ_{exc}, apart from the quantum yield [inset of Fig. 2(a)], it was concluded that only tryptophyl residues significantly contributed to the fluorescence emission of GDH [28]. Excitation into higher states has been found to yield less fluorescence owing to nonradiative decays and quenching processes by neighboring amino acids. This experimental evidence is in agreement with previous reports [29].

The 292 nm radiation was chosen as the exciting source in all our experiments at 20 °C (unless otherwise stated) in order to work in the maximum quantum yield regime.

The fluorescence emission spectrum of native GDH in phosphate buffer at pH 7.2, upon excitation at 292 nm, exhibits a broadband with a maximum emission (λ_{max}) at 340.5 nm. Such a λ_{max} value shows that the aromatic residues responsible for the fluorescence emission of GDH are located in a rather polar environment. The microenvironment of every residue is characterized by a particular set of physicochemical conditions (polarizability, availability of charged groups, possible specific interactions) that influence the fluorescence of the chromophore. As a consequence, the protein fluorescence results from the convolution of fluorescent contributions from individual tryptophan residues [Fig. 2(b)], which may vary over a rather wide range [30]. Figure 2(b) illustrates the deconvolution procedure: the broadband emission spectrum of GDH is deconvolved by means of three Gaussians, representing the contributions of the individual emitting species. According to the photophysical characteristics of Trp, the three different classes are identified as the *innermost, inner*, and *external* bands. This kind of analysis may lead to a deeper comprehension of the conformational changes than the basic study of the integrated behavior alone.

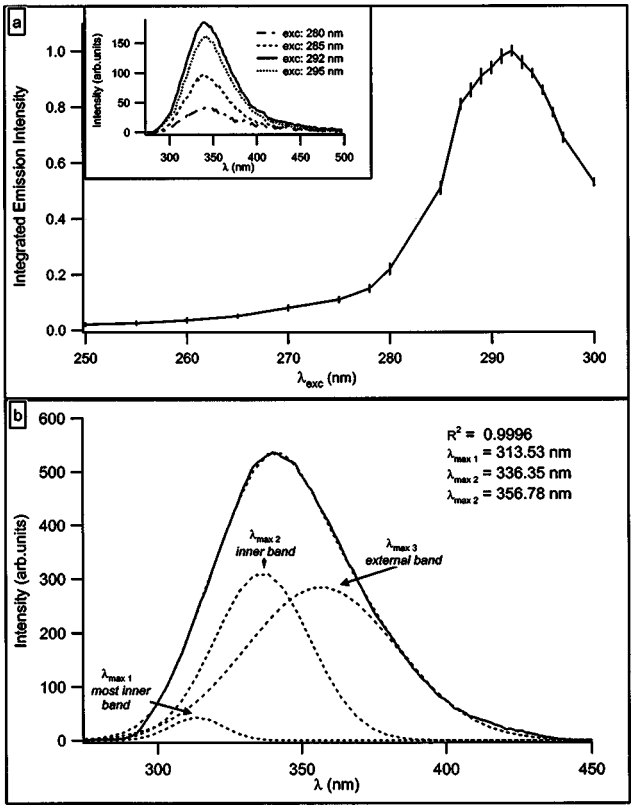

FIG. 2. GDH photoluminescence. (a) Fluorescence quantum yield: integrated emission (calculated as the area under the curves) vs excitation wavelength; inset: examples of GDH spectra at different excitation wavelengths. (b) Deconvolution analysis of the native state.

In almost all proteins ^1L_a is the fluorescing state. Quantum mechanical studies have predicted that electron density is shifted from the pyrrole ring to the benzene ring upon excitation into ^1L_a. These spectra are unstructured, because of the high degree of inhomogeneous broadening exhibited by the ^1L_a transition owing to its large dipole change (the ^1L_a state has a large dipole compared to the ground state), which usually obscures the structure [31].

B. Thermal denaturation

We have characterized the structural changes of the enzyme GDH upon thermal denaturation by measuring the changes of the intrinsic fluorescence spectra. This process may be reversible or irreversible [32]. Thermal treatment affects protein structure, exposing hydrophobic residues that were shielded in the core of the protein in its native state. Different optical responses of the enzyme reflect a different degree of exposure of hydrophobic residues in the unfolding of the protein. The increase or decrease of fluorescence caused by different amino acids indicates that externally induced alterations of the protein folding may lead to the exposure of some amino acids residues and to the shielding of others. It is not our purpose to measure specific denaturation parameters, but to use the protein intrinsic fluorescence as a probe for differences between the bulk-solution and film environments.

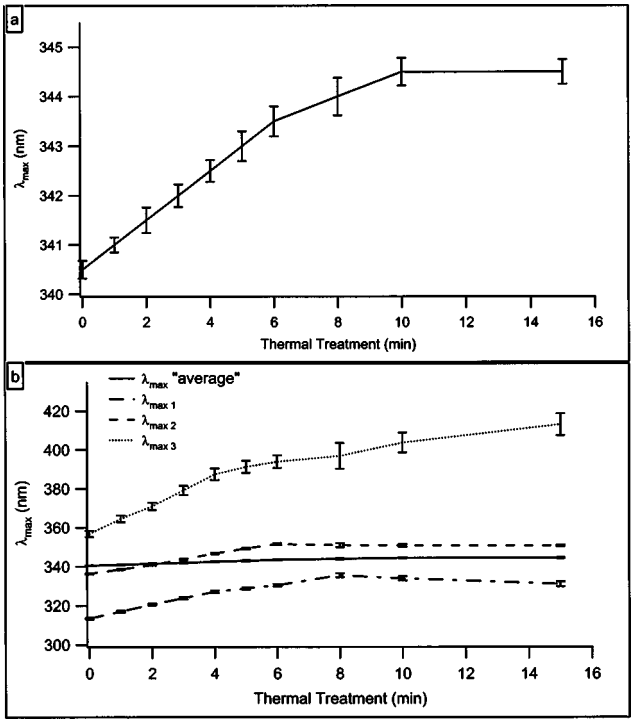

FIG. 3. Thermal denaturation. (a) Shift of λ_{max} as a function of the treatment time. (b) Deconvolution analysis: shift of λ_{max} of the three emitting bands and comparison with the ''average'' behavior.

The charge-transfer aspect of the ^1L_a transition is the source of the sensitivity to the solvent. Charged residues near the benzene or near the pyrrole end of the Trp ring will shift λ_{max}. Since these shifts are due to the electric field imposed by the protein and the solvent, they are termed the *internal* Stark effect, by analogy with the familiar shifting of energy levels via an applied (external) field [33]. Both water and protein contribute in various ratios to the shift. The large redshift observed in fluid solvents of high dielectric constant is elicited by the strong electric field (reaction field) at the solute due to the partial orientation of solvent dipoles around the large solute dipole [31]. Thus, the unbalanced intermolecular forces caused by the electronic excitation are responsible for the environmental effects of great interest in Trp spectroscopy, i.e., the fluorescence shifts that occur in different proteins.

Figure 3(a) represents the shift of the parameter λ_{max}, obtained in standard conditions, with the increase of thermal treatment at 100 °C. The incubation times range from 0 to 15 min. The emission maximum of tryptophan residues was slightly redshifted from 340.5 to 344.5 nm and a decrease in intensity at λ_{max} was also observed (not shown). Since the fluorescence of tryptophan residues primarily depends on their local environment, this alteration in the emission spectra indicates that the aromatic residues buried within the hydrophobic core of the native protein have been exposed to the aqueous solvent during unfolding [17]. Owing to the different emission properties in aqueous vs nonaqueous media, it seems that after a high-temperature treatment the structure around the chromophores has a more pronounced hydrophilic character than in the native state. Moreover, whereas conformational changes may be generally reversible, the modifications reported here are irreversible: all parameters were preserved 24 h after cooling. It should, however, be pointed out that the λ_{max} values are moderately affected in the course of this structural change. The maximum wavelength observed (344.5 nm) is slightly less than the value of 352–354 nm expected for tryptophyl side chains in a completely unfolded polypeptide chain.

It may be also significant that, in these experimental conditions, the thermal unfolding of bovine liver GDH appears as a rather gradual process. The redshifting curve steadily increases in the denaturation range, suggesting the occurrence of a more complex trend than a simple two-state transition.

The changes in the denaturation parameter λ_{max} are also presented according to the deconvolution procedure previously described [Fig. 3(b)]. The fluorescence shift of λ_{max} in the ''average'' GDH spectrum has been analyzed in relationship to the spectral variations of the three individual bands. While the ''average'' analysis would suggest a slow but regular denaturation process in which the internal tryptophan molecules possibly experience solvent exposure, a careful interpretation of data from Fig. 3(b) suggests a more complicated model, where different phenomena may account for protein unfolding.

The two ''internal'' contributions ($\lambda_{max\,1}$ and $\lambda_{max\,2}$) show a similar behavior, with a redshift only in the first part (up to 6–8 min) of the denaturation curves; afterward a blueshifting trend becomes evident. On the other hand, the ''external'' band ($\lambda_{max\,3}$) shows an upward behavior. It may be possible that, during the initial phase of the unfolding process, internal tryptophan residues become more exposed to the solvent; the thermally induced conformational changes generate aggregation processes, also which may account for the inversion of the spectral shift. In contrast, the external contribution seems to be unaffected by such aggregation phenomena. This conjecture will be discussed in more detail in the next section.

1. Elastic scattering measurements and integrated emission analysis

GDH self-association was monitored upon thermal denaturation at 100 °C by the relative intensity of right-angle elastic scattering [Fig. 4(a)]. This technique provides qualitative evidence for molecular mass increase on protein association [26].

A large enhancement of scattered light was observed after thermal treatment. This phenomenon may indicate that aggregation has occurred during such treatment. This association seems to be irreversible, like that observed for GDH after pressure-induced inactivation [34]. The conformational changes suggest that protein denaturation partially results in the formation of aggregates, such behavior indicating that irreversible thermal denaturation is due also to polymolecular processes.

It can be seen that increasing the time of thermal treatment up to 15 min proportionally affects the value of the intensity at λ_{exc} [Fig. 4(a)]. This means that the gradual and continuing aggregation phenomenon takes place simulta-

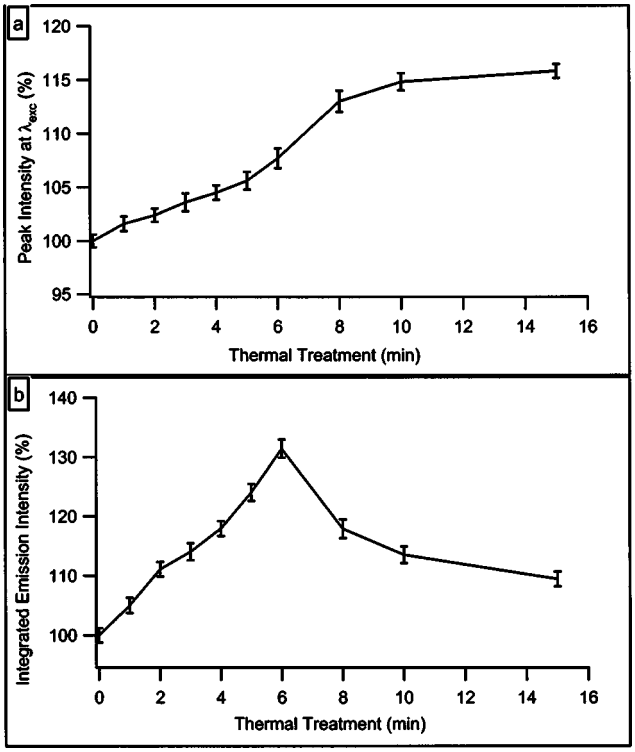

FIG. 4. Thermal denaturation: (a) intensity at λ_{exc} (elastic scattering); (b) integrated fluorescence emission (area under the curves) vs treatment time.

neously with the unfolding process. Moreover, in the final part of the plot, the self-association curve is characterized by a plateau, possibly indicating the existence of a maximum limit to aggregation caused by thermal denaturation.

Although no breaks or discontinuities in the curve of elastic scattering intensity versus treatment time can be detected, a marked variation of the slope in the region between 6 and 8 min is evident. This experimental result clearly discloses the induction of a small temperature-dependent structural transition. These assumptions, however, perfectly agree with the hypotheses previously proposed on the basis of the deconvolution approach.

On the other hand, Fig. 4(b) shows the variations of the integrated fluorescence emission of GDH incubated at 100 °C for different times. Such variations are consistent with the current discussion: In the first step of the unfolding process (up to 6 min), Trp residues increase their quantum yield and thus protein emission, owing to minor quenching efficiency by neighboring amino acids; then, in the second part, the self-association effect, due to the unfolded conformation, causes a large decrease in intensity.

C. Fluorescence quenching studies

The wavelength of maximum fluorescence intensity (λ_{max}) is commonly used as an indicator of exposure to water, i.e., as an indicator of how deeply the Trp is buried in the protein. This correlates with the effectiveness of external quenchers, and to the best of our knowledge no exceptions are documented [31]. In this regard the class of proteins with

TABLE I. Fluorescence quenching of GDH by added KI and acrylamide. Trp fluorescence was excited at 292 nm and the emission spectrum was measured over a range of added KI (0–0.5M) and acrylamide (0–0.20M) concentrations.

Quencher	Enzyme state	K_q $(M^{-1})^a$	$(f_a)_{eff}{}^b$
KI	Native	0.59	0.48
	Partially denatured	[c]	0.61
Acrylamide	Native	11.38	0.96
	Partially denatured	15.24	0.99

[a]Quenching constant calculated according to Stern-Volmer equation.
[b]Fraction of accessible chromophores calculated according to Lehrer equation.
[c]The fit was nonlinear.

λ_{max} at about 340 nm is extremely interesting because these are usually quite accessible to external quenchers.

Acrylamide is a polar, uncharged compound that has been shown to quench the fluorescence of indole derivatives predominantly by a collisional process [35], affecting both exposed and masked fluorophores [36]. Interestingly, the quenching efficiency of tryptophyl residues in proteins by acrylamide is independent of the polarity of their microenvironment and depends solely on the frequency at which they can encounter an acrylamide molecule. Thus, depending on whether or not the indole ring is sterically shielded by surrounding protein segments, the efficiency of quenching by the probe will be low or high [37].

KI was selected as the ionic quencher to quench selectively the emission of exposed Trp residues [36]. Iodide is negatively charged and therefore it is likely to be limited to quenching only surface tryptophanyl residues. Also, its ability to quench should depend on the location of neighboring charged groups.

Quenching experiments with GDH showed that fluorescence intensities decrease with increasing quencher concentrations, with no clearly apparent discontinuity. In all these experiments no shift in the emission maxima were observed. As expected, the effect of iodide quenching on GDH is less pronounced than that of acrylamide (see Table I). The ''red'' emission maximum of the native protein (340.5 nm) would suggest that the tryptophan residues of GDH are not deeply buried and inaccessible to the quencher. For the denatured enzyme, the percentages of fluorophore available to the quencher are higher, indicating that a conformational change has taken place, with a partial (at least) unfolding of the protein. For acrylamide a modified Stern-Volmer (Lehrer) plot gave a value of 96% for the Trp residues available to the quencher; in the thermally treated enzyme, under otherwise identical experimental conditions, all tryptophan residues were accessible. In spite of the negative charge on the surface of the protein, the ionic quencher KI interacts with the protein, yielding non-negligible quenching percentages (Table I). The k_q and f_a values provide a sensitive measure of the exposure of fluorescent Trp residues. When the protein matrix sterically shields a Trp residue from the solvent (re-

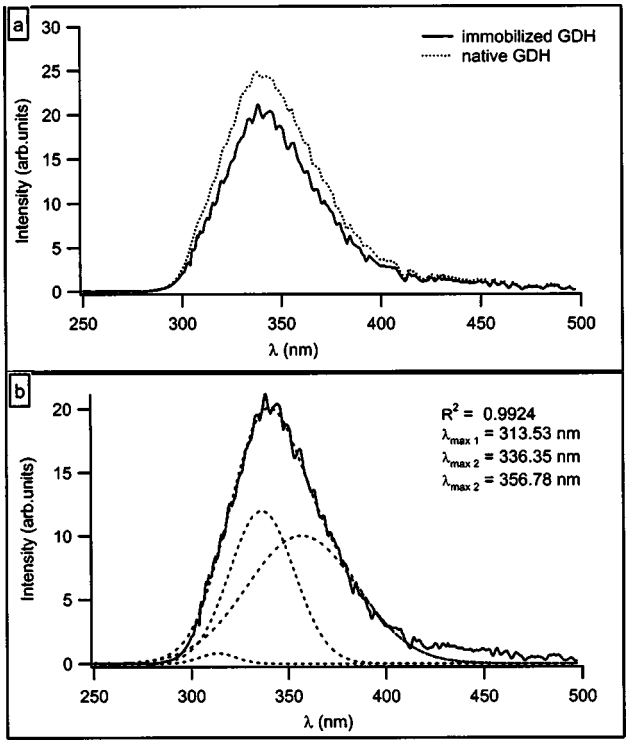

FIG. 5. Photoluminescence of GDH immobilized on Si/SiO_2 substrate: (a) comparison with the native GDH spectrum (0.125 mg/ml, rescaled by a factor of 3; however, the collection efficiencies in the two systems are different); (b) deconvolution analysis.

ducing the collisional cross section), this will be reflected in reduced k_q and f_a values. In extreme cases in which the Trp is located below the surface of the protein, penetration of the quencher into the protein matrix will be required. The penetration of the quencher will be facilitated by fluctuations in the conformation of the protein occurring on the nanosecond time scale (proteins are not rigid structures, but continually undergo stochastic fluctuations).

D. Fluorescence from protein films

The binding of small molecules to proteins is biologically fundamental because this kind of interaction (ligand binding) not only may alter the structure of proteins but also may modulate both its functions and its properties. With respect to GDH fluorescence in buffer, the spectrum of the enzyme immobilized in the film is not shifted [Fig. 5(a)], accounting for the fact that immobilization binding does not interfere with the fold pattern of the native enzyme. According to this investigation, it seems that tryptophan residues in native and immobilized proteins are found in identical locations. In addition, the deconvolution analysis [Fig. 5(b)] also confirms this experimental evidence: the three individual emitting bands maintain identical characteristics concerning their wavelength of maximum emission (λ_{max}). This indicates that the single contributions are equally influenced by the microenvironment. Furthermore, as reported in Table II, the individual contributions to the integrated enzyme fluorescence are also very similar.

TABLE II. Individual contributions to fluorescence emission as percentage of the integrated GDH photoluminescence: native state and immobilized enzyme.

	Individual contributions to fluorescence emission (%)		
Enzyme	$\lambda_{max\,1}$	$\lambda_{max\,2}$	$\lambda_{max\,3}$
Native	3.04	39.57	57.38
Immobilized	1.53	40.72	57.75

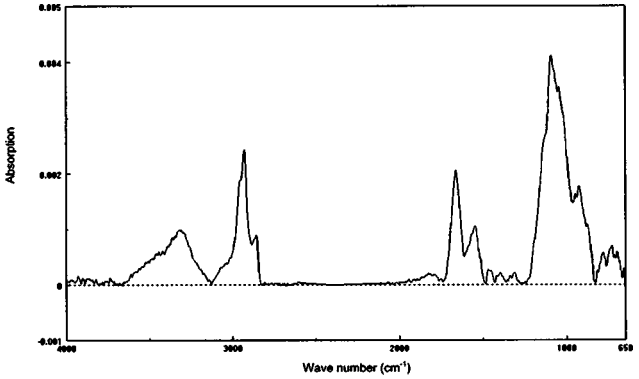

FIG. 6. FTIR spectrum of immobilized GDH enzyme.

In more detail, however, the external band ($\lambda_{max\,3}$) is characterized by an approximately equivalent value, the $\lambda_{max\,2}$ emission is slightly larger in the immobilized enzyme, while the innermost contribution is smaller in protein films than in native enzyme, possibly suggesting a weak fluorescence quenching process, merely limited to this contribution. This small effect seems to be the only consequence in GDH of the immobilization procedure. The available evidence might lead to speculations about a very weak internal rearrangement due to the interaction of the ligand binding, without affecting, however, the proper fold pattern.

In conclusion, intrinsic fluorescence spectroscopy reveals that no variations in the folding properties are present between the GDH in the native state and in the film environment.

E. FTIR spectroscopy

The spectrum shown in Fig. 6 is characterized by absorption bands arising from immobilized GDH enzyme, determined by analogy with FTIR spectra of the native enzyme. The absorption band at about 3400 cm^{-1} is attributed to the N—H stretching vibration from the amidic bond of the enzyme, while the two absorption bands at 1700–1600 cm^{-1} arise from the C═O stretching (amide I) and N—H bending (amide II) vibrations. The 2960 cm^{-1} band corresponds to the C—H stretching of CH_2 groups.

IV. CONCLUSIONS

In this study we have shown an experimental approach to forming high-quality glutamate dehydrogenase monolayers on silicon for nanobioelectronics applications. Detailed characterization by fluorescence spectroscopy and Fourier-

transform infrared spectroscopy revealed that no significant conformational changes occurred in the enzyme upon immobilization. This means that such a method may be extremely attractive for the development of alternative and stable enzyme-based nanosensors. The development of a glutamate biosensor with a spatial resolution up to a few nanometers (a nanobiosensor) [combining electron beam lithography (e-b-l) designed metal patterns with the immobilization shown here] would permit continuous monitoring of glutamate release from individual cells or subcellular structures or into the extracellular space of the central nervous system, resulting in an effective detection technique for the mapping of its concentration.

Work is in progress in our laboratories for the realization of a solid-state nanosensor utilizing the GDH monolayers as the sensitive medium.

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