



Thermal lens spectrometry applied for a sensitive detection of silver-stained protein bands in polyacrylamide gels

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ABSTRACT

Proteins separated by gel electrophoresis are commonly quantified by colorimetric analysis of the protein band stain intensity. For the most sensitive stain (silver), the minimal protein amount, needed for the optical visualization of the band, is around 1 ng. This study investigates the use of thermal lens (TL) spectrometry for the quantification of subnanogram protein amounts. The developed dual laser TL sensor is set up in collinear geometry; it includes a HeNe probe beam, a 450 nm diode laser pump beam, and a gel holder. After silver staining, the polyacrylamide gel is scanned by the sensor while kept soaked in water, and in 50% (v/v) methanol/water. During scanning, the TL signal of each band is recorded in function of its position. The TL sensor can detect protein amounts as low as 0.1 ng in water, and 0.05 ng in 50% methanol/water. The limit of detection ranges from 8 to 50 pg, depending on the soaking medium and the staining strength. The theoretical results, predicting the sensitivity enhancement in methanol/water, are in agreement with the results. In conclusion, thermal lens spectrometry proves to be a valid method for the detection of subnanogram protein amounts in polyacrylamide gels, and its application can be extended to other soft gel matrices.

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1. Introduction

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a well-known technique, used in biochemistry and molecular biology, to separate proteins by their molecular weight. Once separated, the protein bands can be visualized with different staining procedures, among which Coomassie and silver stains are the most frequently used. The subsequent colorimetric quantification of the protein bands employs specific imaging systems and elaboration software. Common limits of detection are 10–25 ng protein per band by Coomassie staining, and 1 ng by silver staining. Among a variety of possible staining and detection methods, like fluorescence-based stains or UV visualization techniques [1], the colorimetric evaluation is still the most diffused on the market. With the advent of lab-on-a-chip electrophoresis, there is increasing interest in detection methods which can be integrated in a chip, and at the same time offer better sensitivity.

Thermal lens (TL) spectrometry appears to be a valuable detection approach. It is a highly sensitive analytical method for measuring sample concentrations. The TL effect is based on the absorption of light energy, and its conversion into heat. The temperature rise, occurring at the focus of the pump laser, changes locally the refractive index of the sample, and creates an optical lens. When the lens is probed by a second incoming laser beam, the measure of its spread gives direct information about the absorbance of the sample, and consequently about the sample concentration [2]. Expected benefits of TL spectrometry for gel electrophoresis scanning would be higher sensitivity compared to transmission spectrometry, and suitability for microscale detection [3–6]. Indeed, literature studies have reported that soft matrix gels are advantageous for TL measurement because they show a higher temperature coefficient of refractive index than pure liquid solvent [7–10]. Peck and Morris analyzed Coomassie-stained protein bands in polyacrylamide (PAM) gels, and they achieved limits of detection ranging from 0.3 to 1 ng protein [8]. Thompson et al. measured hemeproteins (i.e. proteins with their own absorbance in the visible range) in agarose gels with a single laser TL sensor; they reached detection limits of 180 ng [9]. Despite the promising results, these studies reported some difficulties like gel inhomogeneity, a complex staining procedure, and high background absorbance. Moreover, as they were

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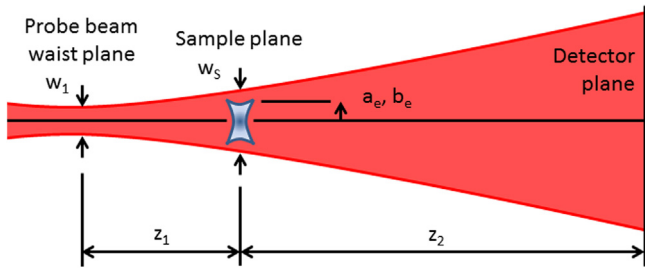


Fig. 1. Scheme representing the configuration of the geometrical parameters for the TL theoretical model. The scheme shows the probe beam (red) and the position of its waist in relation to the position of the TL generated in the sample at the waist of the pump beam. The abbreviations are listed in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

limited to measuring bands in water-soaked gels, they predicted poor enhancement by TL over transmission spectrometry [7].

The objective of this study was the measurement of silver-stained protein bands in PAM gels with a dual laser TL sensor in standard collinear setup. In particular, the study sought to detect protein quantities in the subnanogram range, thus below the detection limit of the imaging systems. To increase the sensitivity of the analysis, and overcome some of the aforementioned limitations, we focused our work especially on studying, theoretically and experimentally, the influence of using a methanol/water solution as gel-soaking medium during TL scanning. The addition of methanol to pure water was expected to improve the thermo-optical properties of the soaking medium and enhance the TL signal. Additionally, in contrast to the previous studies, this work made use of commercially available pre-cast gels and silver stain kits, which were expected to offer better quality, and reproducible properties of gels and staining. For comparison, the results obtained by the TL sensor were compared to those obtained by standard colorimetric analysis.

2. Material and methods

2.1. TL theoretical model

A theoretical model for TL, based on the Fresnel diffraction theory, was implemented to predict the signals that can be obtained from silver stain in PAM soaked in pure water, and in a 50% (v/v) solution of methanol/water. Among the water-miscible weakly polar solvents, methanol at a concentration of 50% was considered safe not to impair the transparency of PAM. Fig. 1 shows the geometrical parameters of the model, while the list of variables, symbols, and their values is given in the nomenclature in Table 1.

The temperature distribution $\Delta T(x, y, t)$, created by the absorption of the pump beam energy focused in the sample, is given by the solution of the heat problem [11]. In our case, the heat source $Q(x, y, t)$ has the form of the elliptical Gaussian pump beam, which is sinusoidally modulated:

$$Q(x, y, t) = \frac{\alpha P}{\pi a_e b_e} \exp\left(-\frac{2x^2}{a_e^2} - \frac{2y^2}{b_e^2}\right) (1 + \cos \omega t) \quad (1)$$

The resulting temperature distribution is:

$$\Delta T(x, y, t) = \frac{\alpha P}{a_e b_e k \pi^2} \int_0^t \frac{1 + \cos \omega \tau}{(t - \tau)} \frac{1}{\sqrt{\frac{a_e^2 + 8D(t-\tau)}{a_e^2 D\pi(t-\tau)}} \sqrt{\frac{b_e^2 + 8D(t-\tau)}{b_e^2 D\pi(t-\tau)}}} \exp\left[\frac{-2 \{[x - v_x(t-\tau)]^2 [b_e^2 + 8D(t-\tau)] + y^2 [a_e^2 + 8D(t-\tau)]\}}{[a_e^2 + 8D(t-\tau)] [b_e^2 + 8D(t-\tau)]}\right] d\tau \quad (2)$$

According to the mathematical TL signal model presented by Liu et al. [12], the equation of the Gaussian probe beam electric field at the sample plane (x, y, z_1) is:

$$E_1(x, y, z_1) = E_0 \cdot \left(\frac{w_1}{w_s}\right) \cdot \exp\left[-jkz_1 - \frac{jk}{2R_1}(x^2 + y^2) - \frac{k\lambda}{2\pi w_s^2}(x^2 + y^2)\right] \quad (3)$$

The TL causes a shift of the phase ($\Delta\varphi$) of the electromagnetic field according to the temperature variation ΔT :

$$\Delta\varphi = \frac{2\pi}{\lambda} \frac{\partial n}{\partial T} \Delta T(x, y, t) \quad (4)$$

Therefore, the electromagnetic field behind the TL is:

$$\begin{aligned} E'_1(x, y, z_1, t) &= E_1(x, y, z_1) \cdot \exp^{-j\Delta\varphi} \\ &= E_1(x, y, z_1) \cdot \exp\left[-j\left[\frac{2\pi}{\lambda} \frac{\partial n}{\partial T} \Delta T(x, y, t)\right]\right] \end{aligned} \quad (5)$$

and the electromagnetic field at the detector plane (x_2, y_2, z_2) can be calculated by the Fresnel integral:

$$\begin{aligned} E_2(x_2, y_2, z_1 + z_2, t) \\ = \frac{j \cdot \exp^{-jkz_2}}{2\lambda z_2} \int_{-\infty-\infty}^{+\infty+\infty} \int E'_1 \cdot \exp\left\{-\frac{jk}{2z_2} [(x_2 - x)^2 + (y_2 - y)^2]\right\} dx dy \end{aligned} \quad (6)$$

The TL signal S is defined as the change in laser intensity at the detector plane when the pump laser is switched on:

$$S = \frac{|E_{2(laser-ON)}|^2 - |E_{2(laser-OFF)}|^2}{|E_{2(laser-OFF)}|^2} \quad (7)$$

The calculations of the electromagnetic field intensity at the detector plane and of the signal S (Eq. (7)) were implemented in MATLAB (MathWorks, Natick, MA, USA). For the calculation, the sinusoidal modulation of the pump beam (see Eq. (2)) was replaced by a square wave function, which better represents the digital modulation of our experimental setup.

Table 2 shows the physical properties of 10% PAM gel soaked in water or in methanol/water 50% (v/v) in comparison to the pure solvents. We observed that soaking the gel in methanol/water caused 52% shrinkage of the gel volume, with a consequent increase in the PAM density to 21%. The estimation of the physical properties of the PAM gels soaked in methanol/water referenced (j) was done on the basis of the properties of methanol/water, and hypothesizing the same percentage variation in the parameters seen between water and 21% PAM in water, using the data of Hirata et al. [13].

The theoretical model was used firstly, to compare the signals in different soaking media at equal absorption coefficient α , and secondly to estimate the scattering component of the silver stain. For the latter, the absorption coefficient α , present in Eq. (1), was chosen to obtain from the theoretical model a signal S matching the experimental value. The coefficient α delivered by the theoretical model was then compared to the extinction coefficient α_m , acquired by spectrophotometry measurements. To obtain α_m , the extinction spectrum of a 5 ng silver-stained protein band in PAM was measured with a spectrophotometer (Synergy 2 BioTek, Winooski, VT, USA) in the visible range, in water and in the methanol/water solution. The extinction coefficient α_m was calculated from the absorbance A measured at 450 nm (pump beam wavelength), since $A = -\lg e^{-\alpha_m l}$. The extinction coefficients α_m of inferior protein loadings (1.5–0.05 ng) were calculated, as they are directly proportional to the sample concentration.

Table 1
Nomenclature.

Symbol	Variable/parameter	Value
t [s]	Time	
T [K]	Temperature	
Q [W/m ²]	Heat source	
ω [1/s]	Pump beam modulation frequency	1
k_1 [W/(m · K)]	Thermal conductivity	See Table 2
n	Refractive index	See Table 2
$\partial n/\partial T$ [1/K]	Temperature coefficient of refractive index	See Table 2
ρ [kg/m ³]	Density	
C_p [J/(kg · K)]	Specific heat	See Table 2
$D = k_1/(\rho \cdot C_p)$ [m ² /s]	Thermal diffusivity	See Table 2
α [1/m]	Absorption coefficient at 450 nm	See Table 3
P [W]	Pump beam power	0.070 ^(a)
a_e [m]	Pump beam major half axis at focus	207e ^{-6(b)}
b_e [m]	Pump beam minor half axis at focus	81e ^{-6(b)}
P_p [W]	Probe beam power	0.002 ^(a)
v_x [m/s]	Sample flow velocity	0
E_0 [V/m]	Probe beam amplitude at the waist	
w_1 [m]	Probe beam waist radius	82e ^{-6(c)}
w_s [m]	Probe beam radius at the sample plane	177e ^{-6(b)}
λ [m]	Probe beam wavelength	632e ⁻⁹
$k=2\pi/\lambda$ [1/m]	Probe beam wave number	
R_1 [m]	Curvature radius of the probe beam wavefront at the sample plane	83e ^{-3(c)}
$\Delta\varphi$ [rad]	Probe beam phase shift	
l [m]	Optical path/Gel thickness/Protein band thickness	0.001
z_1 [m]	Distance between the probe beam waist and the TL (sample plane)	0.065 ^(d)
z_2 [m]	Distance between the TL and the photodiode (detector plane)	0.393 ^(d)
S	Thermal lens signal	

(a) measured with the power meter FieldMaxII-TOP (Coherent Inc., CA, USA); (b) measured with the beam profiler Lasercam HR (Coherent Inc); (c) calculated using the Gaussian beam equation; (d) geometrical measures of the setup.

Table 2
Physical properties of the solvents and matrices in comparison.

Solvent/matrix	k_1 [W/(m · K)]	ρ [kg/m ³]	C_p [J/(kg · K)]	D [m ² /s]	n (at 20 °C)	$\partial n/\partial T$ [1/K]
Water	0.598 ^(e)	997 ^(f)	4180 ^(e)	1.431e ^{-7(e)}	1.3333 ^(e)	−0.91e ^{-4(e)}
10% PAM in water	0.550 ^(f)	1030 ^(f)	3950 ^(f)	1.350e ^{-7(f)}	1.3499 ± 0.0024 ^(g)	−2.4e ^{-4(g)}
Methanol/water 50% (v/v)	0.356 ^(h)	929 ⁽ⁱ⁾	3470 ^(k)	1.104e ^{-7(l)}	1.3424 ^(g)	−2.6e ^{-4(g)}
10% PAM in methanol/water	0.292 ^(j)	994 ^(j)	3123 ^(j)	0.940e ^{-7(j)}	1.3850 ± 0.0024 ^(g)	−3e ^{-4(g)}

(e) from ref. [14]; (f) from ref. [13]; (g) measured with a digital refractometer (Mettler Toledo, Columbus, OH, USA); (h) extrapolated from ref. [15]; (i) extrapolated from ref. [16]; (j) estimated as explained in the text; (k) calculated from water and methanol values; (l) calculated since $k=D\rho C_p$.

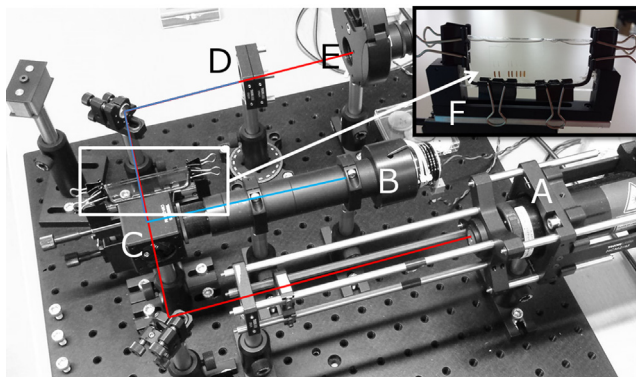


Fig. 2. The TL sensor setup: HeNe laser (A), blue diode laser (B), dichroic mirror (C), laser filter (D), pinhole (E) and zoom of the gel holder (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. TL sensor setup

The configuration of the TL setup is shown in Fig. 2. All optical components were purchased from Thorlabs (Newton, NJ, USA), if not otherwise stated. The pump laser diode (PL450 B 450 nm, nominal 80 mW, OSRAM, Munich, Germany) was driven by an electronic circuit, modulated at 1 Hz, and focused in the sample holder. The

pump laser was chosen among the available lasers for its wavelength (450 nm) close to the absorption maximum of silver stain, and for being cost-effective. The probe beam laser (HeNe 632 nm 25-LHP-121-230, 2 mW, Melles Griot, Albuquerque, NM, USA) was focused before the sample holder and was combined with the pump beam by a dichroic mirror (DMLP567R). The cavity of the HeNe laser was shielded from reflected light by an optical isolator (IO-3D-633-VLP). A construction of filter (FL632.8-10), mirrors, and absorbers allowed the blue out coming light to be filtered away without reflections to the sample. For different applications and concentration ranges, the correct pinhole diameter (from 0.6 to 2.4 mm) could be chosen using a filter wheel (LTFW6). A 2.4 mm pinhole was used for all measurements reported in this study. Probe beam spreading was measured by a photodiode (SM05PD1A). A trans-impedance amplifier (10 kΩ) converted the output current of the photodiode to a voltage. A lock-in amplifier (SR830 DSP, Stanford Research Systems, CA, USA) was used to extract and amplify the modulation signal; the time constant of the lock-in was set to 300 ms. The gel holder was mounted on an x-y stage which allowed scanning of different regions of the gel. The applied scanning velocity was 1.5 cm/min, meaning that the region corresponding to the laser spot dimensions was exposed for less than 1 s. Additionally, the long-term (0–100 s) TL signal from continuous measurement of the very same spot was analyzed. This was done to eventually detect signal variations in the time, similar to those reported by Briggs and Long [10].

2.3. SDS-PAGE, silver staining, and colorimetric analysis

All materials for SDS-PAGE were purchased from Life Technologies (Carlsbad, CA, USA) except for the bovine serum albumine (BSA, fraction V), which was from AppliChem (Darmstadt, Germany). BSA was reconstituted in distilled water and diluted to load samples from 5 to 0.05 ng. Samples were not reduced to avoid smearing caused by DTT aggregates. Electrophoresis was performed with the XCell SureLock™ mini-cell system using NuPAGE® Bis/Tris 10% gels and MOPS buffer at 200 V for 1 h. After SDS-PAGE, the gels ($n=4$) were stained using the SilverXpress® Silver Staining Kit. Standard colorimetric analysis of the stained bands was performed with the ChemiDoc™ imaging system (Bio-Rad, Hercules, CA, USA). Afterwards, the faint yellow background was bleached by incubating the gels in a solution containing 10 mM sodium thiosulfate and 10 mM potassium ferricyanide for 5–10 s. After extensive washing in distilled water, the gels were analyzed again by the imaging system.

2.4. TL measurement in water and methanol/water

After colorimetric analysis, the gels were put in the gel holder soaked in water or 50% methanol/water (v/v), and the x-y laser alignment was checked and adjusted using the two mirrors. The position of the laser focusing lenses and that of the gel holder were kept fixed for all measurements, no change in the z-direction alignment was done. The output of the lock-in amplifier was recorded and plotted against the scanned distance to visualize the peaks corresponding to the protein bands. The background was averaged before and after the peaks, and its value was subtracted. Linear fitting options were analyzed for the quantification of unknown protein loads. For the calculation of the limit of detection (LOD), the blank was measured by scanning empty gel lines after staining and bleaching.

2.5. Statistics

Measured bands in the low protein range were compared using the non-parametric Kruskal-Wallis test in SPSS (SPSS Inc., Chicago, IL, USA). Since staining and bleaching affected the absolute values, only bands within the same gel were compared.

3. Results

3.1. Theoretical TL signal in PAM soaked in water and methanol/water

According to the results of the theoretical model presented in Fig. 3, measurements taken in methanol/water delivered signals twice as strong as in water, because of the better thermo-optical properties of the soaking medium. These curves were used to calculate the absorption coefficients α (x-axis) corresponding to the experimental TL signals (y-axis).

3.2. Extinction spectra of silver stain and empty PAM

Fig. 4 shows the extinction spectra of a silver-stained 5 ng protein band and of empty PAM gels after bleaching. The gel volume shrinkage caused by incubation in methanol/water increased the apparent concentration of the band, and resulted in double-strength extinction.

The extinction coefficients (α_m) at an excitation wavelength of 450 nm, measured and calculated as described in Section 2.1, are compared in Table 3 with the absorption coefficient α obtained

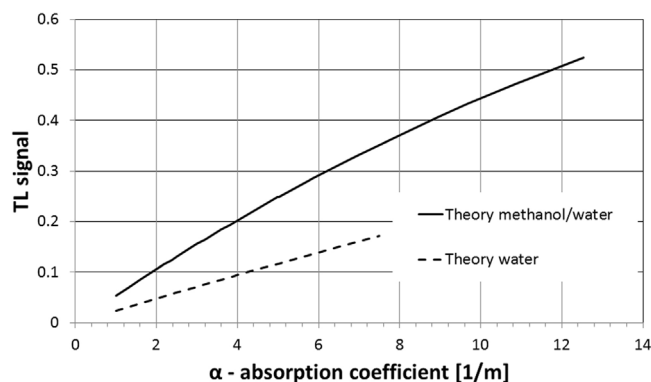


Fig. 3. Theoretical TL signal obtained in water and methanol/water in function of the absorption coefficient α .

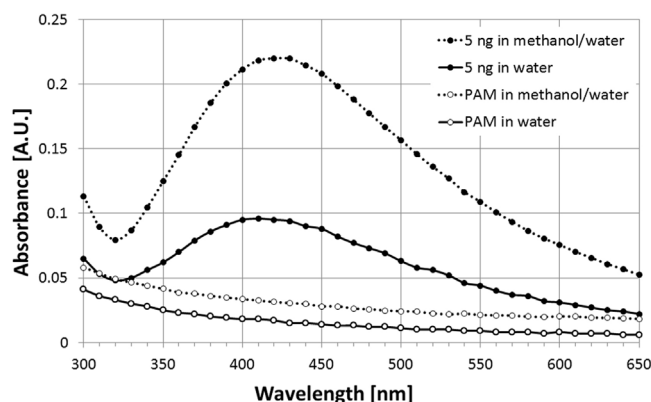


Fig. 4. Extinction spectra of a silver-stained 5 ng protein band and empty PAM gel, in water and methanol/water after bleaching.

from the theoretical model, as explained in Section 3.1. In both water and methanol/water, α_m was roughly 10 times α .

3.3. Colorimetric quantification of protein bands

Fig. 5 shows the results obtained by the imaging system before and after bleaching. The pixel grey intensity is plotted against the position corresponding to the image at the bottom; the background intensity has been subtracted. The silver staining kit could visualize protein bands down to 0.75 ng (Fig. 5a), but caused a faint yellow background which hindered TL analysis. Unfortunately, short bleaching was necessary to obtain a crystal clear background; after bleaching, both protein bands and background showed a decreased intensity (Fig. 5b).

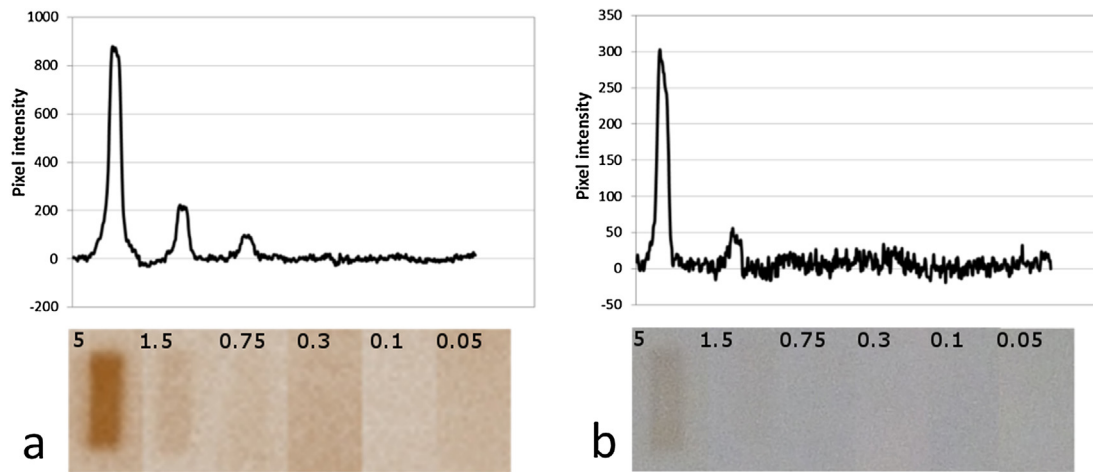
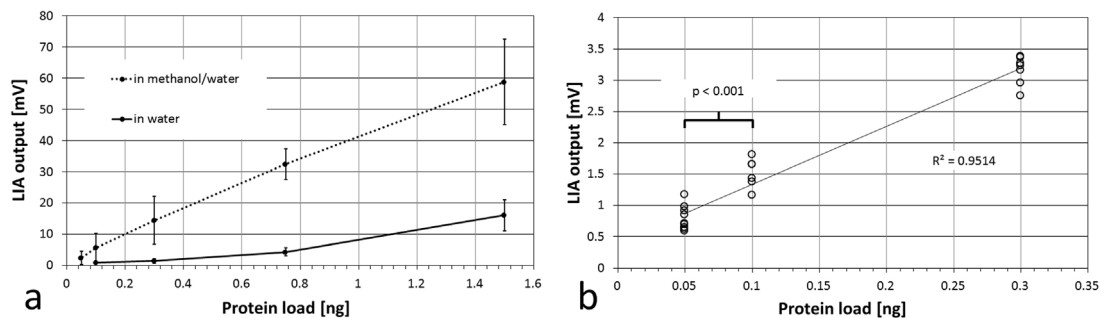
3.4. TL measurements of protein bands in water and methanol/water

The TL sensor allowed the detection of 0.05 ng protein in methanol/water-soaked gels and 0.1 ng in water-soaked gels. Fig. 6a shows the signals obtained by measuring seven bands of each protein load in four different gels. Dissimilar basal levels of staining intensity were the cause of the relatively high standard deviations among the four gels; this was especially seen when measuring in methanol/water. The different basal staining intensity was due to the difficulty in reproducing, for all the gels, the same pipetting, staining, and bleaching procedures; the difference was not eye visible, but detectable by the TL measurements. Fig. 6b shows multiple measurements of the same bands in the low protein load range;

Table 3

List of the absorbance values and extinction coefficients of PAM and of the silver stain bands in comparison to the absorption coefficients delivered by the theoretical model.

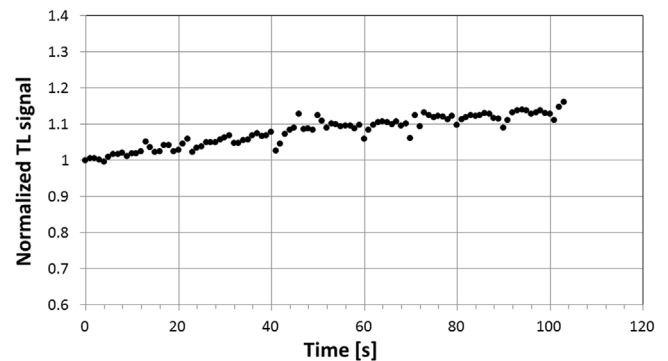
In water				In methanol/water		
Protein loading	Absorbance [A.U.]	α_m [1/m] measured	α [1/m] theory	Absorbance [A.U.]	α_m [1/m] measured	α [1/m] theory
5 ng	0.083	191.11	–	0.208	479.5	–
1.5 ng	0.0312 ^a	71.84	7.44	0.0821 ^a	189.0	–
0.75 ng	0.0201 ^a	46.28	3.45	0.0550 ^a	126.7	12.53
0.3 ng	0.01344 ^a	30.94	2.01	0.0388 ^a	89.4	9.69
0.1 ng	0.01048 ^a	24.13	–	0.0316 ^a	72.8	7.01
0.05 ng	0.00974 ^a	22.42	–	0.0298 ^a	68.6	5.86
0 ng (PAM only)	0.009	20.72	1.80	0.028	64.5	5.01

^a Calculated considering that absorbance and concentration are directly proportional.**Fig. 5.** Example of the colorimetric analysis of one gel: a) Picture of the silver stained protein bands of different loading and corresponding grey intensity lanes detected by the imaging system, before bleaching. b) The same after bleaching.**Fig. 6.** a) TL signal at the lock-in amplifier against the band protein load using water (black line) and methanol/water (dotted line) as soaking media (n = seven bands in four gels). b) Example of one gel: repeated measurements of the same bands in methanol/water at low protein load, linear fitting and statistical analysis of significance.

the inter-repetition variation is given by the gel movement during scanning.

The LOD were calculated as three times the blank standard deviation, and were 0.84 mV in methanol/water and 0.23 mV in water. In methanol/water, 0.84 mV was seen to correspond to concentrations ranging from 8 pg in strongly stained gels to 50 pg in weakly stained (Fig. 6b). In all measurements the background signal of PAM was very strong; the ratio between LOD and background ranged between 0.051 and 0.056 for both water and methanol/water.

Briggs et al. [10] reported that prolonged measurement of the same spot in agarose gel caused a variation of the TL signal over time. The long-term record (0–100 s) of the TL signal in PAM showed similar results (Fig. 7). Because of this observation, care was taken to avoid previous excitation in the region of the protein bands before scanning.

**Fig. 7.** Long-term record (0–100 s) of the TL signal trend over time by constant excitation of the same gel spot.

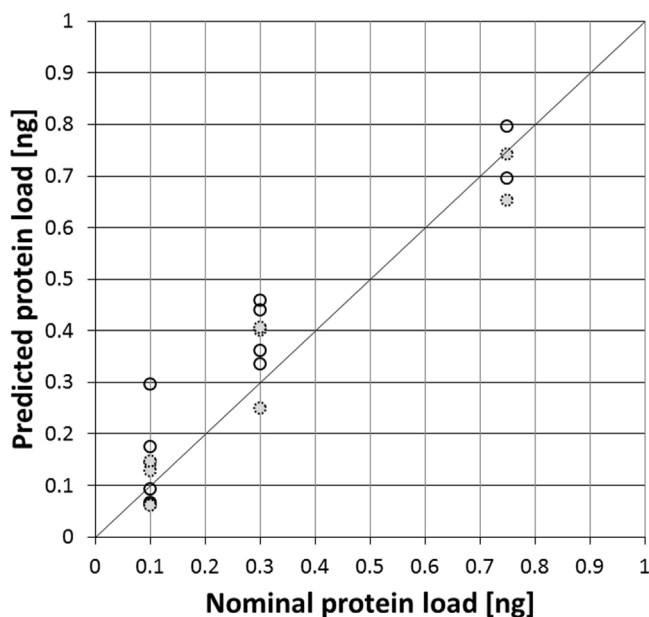


Fig. 8. Prediction of the 0.1, 0.3 and 0.75 ng loadings versus nominal values, in samples measured in methanol/water. Linear fitting was done between the 0.05 and 1.5 ng loadings (black circles) or for each value between the two neighboring loadings (grey-dotted circles).

3.5. Linear fitting

In order to predict the unknown load of protein bands below 1 ng, the performance of linear fittings was analyzed. Fig. 8 shows the nominal protein loadings (0.1, 0.3, 0.75 ng) versus the values predicted by linear fittings between the 0.05 and 1.5 ng values, and linear fittings between neighboring protein loadings within the same gel. The mean absolute errors were 0.087 and 0.057 ng for 0.05–1.5 ng fitting and neighbor fitting, respectively. The results would suggest limited linearity of the results over the investigated range; unfortunately, this is a known problem of silver stain [17]. Four standards should be used to cover the 0.05–1 ng region for accurate protein quantification.

4. Discussion and conclusions

This study set out the aim of addressing TL analysis in PAM gels stained after electrophoresis to enhance the detection of silver stain. The development of a TL sensor made possible the measurement of protein bands of subnanogram loading, achieving LOD seventy times lower than standard colorimetric analysis.

The poor thermo-optical properties of water have been a limitation for the application of TL spectrometry in the analysis of biological samples, which are commonly water-based. Consequently, we focused our work on examining the advantages of measuring in soaking media which are less polar than pure water. The increase in sensitivity seen using methanol/water has multiple reasons. Firstly, the theoretical model showed that the lower thermal conductivity and the higher thermal coefficient of refractive index produce a factor 2 increase of the output signal. Secondly, the volume shrinkage due to gel dehydration increases the protein concentration in the laser spot (a factor of 2.2, according to Fig. 3). Altogether, measurements in methanol/water are theoretically at least 4.4 times higher than in water, and experimental results have shown even higher factors. TL spectrometry has been revealed as a particularly interesting method for scanning soft matrices that are resistant to nonpolar solvents. Because of the shrinkage of the PAM gel in contact with methanol, a loss in y - resolution (i.e. the

capability of separating protein lines of slightly different molecular weights) can be expected. This problem could mainly affect complex samples, like cell lysates. Nevertheless, if the molecular weight of the target proteins is known, the right choice of PAM porosity will guarantee the needed resolution.

Although the experimental results of the TL measurements in PAM showed a clear increase in sensitivity in comparison to the colorimetric analysis, the theoretical results, if based on extinction measurements, would have predicted even higher signals. This discrepancy is probably due to the scattering properties of silver stain. The silver stain extinction spectrum is characterized by a broad peak with a maximum after 400 nm; comparing it with those of homogeneous silver nanoparticles [18], it can be assumed that the broad peak is an indication of the dimension heterogeneity of the silver particles created during staining. A factor 10 mismatch has been seen between the calculation of α_m , based on the extinction measurements, and the calculation of α , based on the theoretical model and the TL experiments. In agreement with the observations of Hlaing et al. [18], silver particles and aggregates larger than 100 nm do not contribute efficiently to the TL signal, but the high scattering cross-section dominates total extinction. The efficiency of TL detection would be increased if only monodispersed nanoparticles of small dimension (~ 10 nm) could be created during the staining reaction, but tuning the reaction parameters exceeded the objectives of this study.

The sensitivity of TL detection over transmission spectrometry can be compared, in theory, as described in [19]. Given the physical properties listed in Table 2, the TL measurement in PAM should have an enhancement factor, per unit laser power in mW, of 0.69 mW^{-1} in water and 1.62 mW^{-1} in methanol/water over transmission measurement. With a laser power of 70 mW, it results in a total enhancement of 48 and 118, respectively. Nevertheless, accounting for the scattering being 90% of total extinction, the detection efficiency should be reduced by a factor of 10. This results, lastly, in an overall enhancement of 4.8 and 11.8 of TL over transmission spectrometry.

The main limitation of this work is the relatively high background absorbance of PAM. High background increases the convective noise, and decreases the dynamic reserve, which is the ability of the method to detect small signal changes on a large background [20,21]. High background absorbance is a known issue in TL spectrometry. Although differential measurement has been proposed to solve the problem [20], unfortunately, this approach increases the complexity of the setup and its applicability to soft gel matrices is questionable.

In conclusion, in this study we have analyzed the possibility of using TL spectrometry for the analysis of silver-stained protein bands after electrophoretic separation. In particular, we have shown that TL spectrometry remains an attractive option for measurements in soft matrix gels, especially if the gel allows the use of partially nonpolar soaking media. This approach can be advantageous in applications where the gel may work as matrix for the segregation, and the concentration of the analyte.

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