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# Sol–gel silica platforms for microalgae-based optical biosensors

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ABSTRACT

An advanced hybrid biosensing platform with improved optical quality is developed based on the acidic encapsulation of microalgi in silica matrices synthesized by TAFR (tetraethoxysilane derived alcohol free route). The three microalgi (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*) were previously immobilized in alginate following the two-step procedure. Tuning the alginate protecting function with the aid of Tris–HCl buffer, the sol–gel synthesis was conducted at pH 4.0 well below the tolerance limit imposed by the encapsulated microalgi. The acidic condensation of Si(IV) generates silica matrices with outstanding optical properties that suit the requirements of biosensors based on optical detection methods.



## 1. Introduction

During the last decades, there has been a growing interest in the design of biosensors as portable, fast and economical tools for toxic compounds detection in the environment (Rechnitz and Ho, 1990). Microalgae cells provide inexpensive systems for on-line and *in situ* pollution monitoring, based on enzyme activity inhibition by specific pollutants at low concentration levels (Singh and Mittal, 2012). In particular, *Chlorella vulgaris* esterase activity (EA) is known to be mainly inhibited by pesticides (Chouteau et al., 2005).

The limiting step in the development of these biosensors is the immobilization of the algal cells in a biocompatible matrix without alteration of cell metabolism. Most of the immobilization techniques rely on the use of synthetic polymers (acrylamide, polyurethanes), proteins (gelatine, collagen), or natural polysaccharides (agar, carrageenan or alginates), which are highly biocompatible (Moreno-Garrido, 2008). However, other desirable qualities of the immobilization matrix include the ability to prevent leaking of the encapsulated cells and the long-term stability in natural environments. The sol–gel process (Brinker and Scherer, 1990) provides a biocompatible synthetic route for whole-cell entrapment within inorganic silica matrices that exhibit good mechanical and chemical stability in order to produce easy-to-handle operative units (Livage and Coradin, 2006). The possibility to construct an optical sensor with these bio-functional materials has recently been addressed, with promising yet moderate success (Depagne et al., 2011).

Aqueous silicates and preformed silica particles have also been explored for sensitive-cell entrapment. The main constraint for the development of biosensors based on silica encapsulation of algal cells is the low optical quality of matrices synthesised by the usual silicate-based route (Ngyyen-Ngoc and Tran-Minh, 2007). In spite of the mild conditions of sol–gel synthesis, routes based on alkoxides, mainly due to the alcohol by products, evidenced cytotoxicity (Coiffier et al., 2001) in particular to eukaryotic cells (Kuncova et al., 2004). An alternative to produce a biomaterial with good macroscopic properties is to enhance encapsulated cells viability by means of milder alkoxide based sol–gel procedures with previous removal of the cytotoxic alcohol generated during the synthesis (Ferrer et al., 2002). These alcohol-free routes include a previous hydrolysis in acid media and the controlled low pressure evaporation of the alcohol that results as a byproduct of the hydrolysis and condensation of alkoxide precursors immediately before the addition of living cells. Using this strategy, a long-term encapsulation of *C. vulgaris* in an alkoxide-derived silica matrix has recently been achieved (Sicard et al., 2011).

However, even following the alkoxide route, to obtain really transparent silica matrices, it is necessary to work with high concentration of precursors and to set the pH of the condensation at

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values less than the region tolerated by *C. vulgaris* cells (Perullini et al., 2011a). By means of a two-step synthesis strategy based on a pre-encapsulation in Ca(II)-crosslinked alginate polymer (CA Patent, 1997), the stress and cellular death suffered by sol–gel entrapped microorganisms can be effectively reduced (Perullini et al., 2008). Moreover, it is possible to enhance the protective function of the alginate cover by further reinforcement of the pre-encapsulation matrix (Perullini et al., 2011b).

In this work we propose the design and construction of silica-based algal biosensors for future applications in monitoring of polluted water by optical detection methods, based on a two-step encapsulation procedure. Reinforcing the alginate matrix with Tris–HCl buffer during the acid sol–gel synthesis of the silica matrix we achieved a high initial viability for different algal species (*C. vulgaris*, *P. subcapitata* and *C. reinhardtii*) entrapped in alkoxide-derived silica hydrogels synthesized at pH 4.0. The effect of the gradient of pH established in the silica–alginate interface on the sol–gel synthesis is further evaluated by small angle X-ray scattering (SAXS) measurements, since it is known that slight variations in pH may cause a significant change in the microstructure of the host matrix. [13]. The enhanced optical properties of the matrix were assessed in terms of the attenuation of fluorescence of a fluorescent dye, fluorescein, and the inhibition to algal growth inside these biomaterials.

In order to dimensionalize the prototype biosensor based on fluorescence emission detection of algal esterase activity, *C. vulgaris* encapsulation devices with different silica hydrogel widths were prepared. The fluorescence emission produced by EA when fluorescein di-acetate (FdA) was used as substrate is an indication of the transport of this substrate within the matrix.

## 2. Materials and methods

### 2.1. Algae growth

*C. vulgaris*, *P. subcapitata* and *C. reinhardtii* were used. Algal strains were purchased from The Muséum histoires naturelles in Paris *C. vulgaris* and *P. subcapitata* were grown in the Lefebvre-Czarda medium (AFNOR, 1980) whereas *C. reinhardtii* was grown in tris acetate phosphate (TAP) medium (Gorman and Levine, 1965) and were transplanted weekly under sterile condition (autoclaving 20 min, 130 °C, 1.3 bars). Algae were maintained in a nycthemeral cycle of 16 h of illumination at 10,000 lux and 8 h of darkness. Culture growth was evaluated by Malassez cell counter.

### 2.2. Two-step encapsulation

The pre-encapsulation in alginate is performed by stirring 1 volume of cells suspended in culture media with 1 volume of Tris–HCl buffer (10 mM, pH = 7.5) and 2 volumes of 2% Na(I)-alginate (Fluka BioChemica). Formation of alginate beads was performed by dropwise addition of this cell suspension in a 0.1 M $CaCl_2$ solution. After 10 min stirring, beads of about 3 mm diameter were easily collected by filtration. Alternatively, 100 μL of the algal suspension in Na(I)-alginate was poured into each plate well and the 0.1 M $CaCl_2$ solution was added in the form of a mist by means of a nebulizer machine.

Silica sol is obtained as previously described, [11] by mixing 20 mL of tetraethoxysilane (TEOS 98%), 6.25 mL of $H_2O$ and 0.72 mL of HCl 0.6 M. The sol was vigorously stirred for 24 h. To obtain the aqueous sol, the hydrolyzed silica sol was diluted with an equal volume of $H_2O$, before removing ethanol under vacuum (48 °C, 30 mbar) until a weight loss that corresponds to quantitative removal of the ethanol generated by the hydrolysis reaction (solution A).

The biomaterials synthesized by the TEOS derived alcohol free route (TAFR) are obtained by dropping a mixture of 0.600 mL of solution A and 0.300 mL of KOH to adjust the pH to the specified value in adequate moulds containing the algae-Ca(II)alginate pre-encapsulation.

For esterase activity and algal growth inhibition experiments, 2 h after addition of silica precursors, biomaterials were washed with Tris–HCl buffer to equilibrate the pH of the silica matrix to the working pH = 7.5.

### 2.3. pH monitoring during gelation

To monitor the pH inside the alginate beads during the sol–gel synthesis, universal indicator is added to cell-free alginate prepared as described in 2.2, except for the incorporation of cells. To improve the pH gradient observation, the Na(I)-alginate solution was introduced in a sample holder (UV–vis couvette) and alginate cross-linking was achieved by nebulization of a 0.1 M $CaCl_2$ solution followed by immersion in the same solution for 10 min.

After this, $CaCl_2$ solution is removed and sol–gel synthesis is performed as previously described, except that the condensation of silica is performed at pH 2.5 to evaluate the protection effect of the alginate matrix at extreme conditions. Samples are immediately placed on a digital scanner in order to acquire images of the evolution of the pH indicator color by sequential scans. The pH value as a function of time and distance from silica–alginate interface is estimated by image analysis with ImageJ free software (rsb, in press).

### 2.4. Silica microstructure

The microstructure characterization was performed at the LNLS SAXS2 beamline in Campinas, Brazil. The measured intensity is displayed as a function of the reciprocal space momentum transfer modulus $q = 4\pi \sin(\theta)/\lambda$, where $2\theta$ is the scattering angle, and $\lambda = 0.1488$ nm is the radiation wavelength. The typical q range was from 0.09 to 2.2 $nm^{-1}$. Data analysis was done with SASfit program. (SANSSoft, in press) The microstructure of the sample was evaluated as a function of distance from silica–alginate interface. Samples were prepared as described in Section 2.3, except for the addition of pH indicator and cut in 0.5 mm width slices in the direction perpendicular to pH gradient.

Field emission scanning electron microscopy (FESEM) inspection was performed at the Centro de Microscopías Avanzadas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina, using Zeiss Gemini-FESEM microscope.

### 2.5. Attenuation of fluorescence

The optical quality of silica matrices synthesized by TAFR was evaluated from the attenuation of the fluorescein fluorescence emission (538 nm wavelength) under excitation light (480 nm wavelength). Different concentrations of aqueous solutions of the fluorescent dye were incorporated during the sol–gel synthesis, which was performed as described in Section 2.2, except that the condensation of silica was done at different pH (2.5 to 7.0) in order to evaluate the enhancement of optical properties as a function of synthesis pH. A calibration curve was done with the same fluorescent dye concentrations free in solution. The attenuation of silica matrices synthesized by TAFR was compared to that caused by silica matrices of equal total $SiO_2$ content obtained by the silicate-LUDOX® aqueous synthesis route. All samples were evaluated at the same time using a microplate reader (FLUOstar OPTIMA®) in fluorescence intensity mode.

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### 2.6. Growth of algae entrapped within TAFR-based hydrogels

To evaluate the degree in which the development of algae is affected by the encapsulation in alginate and silica, the algal growth was measured. The algal growth inside the voids was studied for individual cavities after 3 days of culture in LC liquid medium. At the initial time, calcium alginate beads with a content of $10^4$ cells/mL were dispensed into appropriate moulds, and the silica encapsulation procedure was performed as described above. The volume of silica precursor solution was set in order to obtain a silica layer of 4.0 mm on top of the microalgi-alginate bead. After 3 days, the silica hydrogel was removed and samples were exposed to 0.05% potassium citrate to solubilize the Ca(II)-alginate beads. The total number of cells inside individual cavities was determined by counting cells in a Mallassez counting chamber. To analyse cellular growth, the percentage of inhibition (I) is calculated using the equation:

$$I = \frac{N_c - N_i}{N_c} 100$$

where $N_i$ is the growth for test batch $i$ (entrapped cells) and $N_c$ is the mean growth for the control batch (free cells). Test and control batches were run in triplicate.

### 2.7. Esterase activity

The esterase activity (EA) is peaked during the exponential growth phase, it then decreases rapidly. Thus esterase activity tests were performed 5 days after transplantation. In order to determine EA we used fluorescein di-acetate (FdA) as substrate. The reaction product fluorescein is fluorescent and EA can easily be measured from the fluorescein fluorescence emission (538 nm wavelength) under excitation light (480 nm wavelength) when the FdA is brought into contact with algal cells. This can be done by adding the FdA solution into the microplate wells containing free *C. vulgaris* algae in solution, in 1% Na(I)-alginate solution, encapsulated in 1% Ca(II)-alginate and encapsulated in Ca(II)-alginate–silica hydrogels prepared as previously described in Section 2.2. TAFR hydrogels were synthesized at pH 4.0 and the volumes of silica precursors were adjusted to obtain devices with different silica widths (between 0.5 and 5.0 mm). At initial time, 10 μL of FdA solution 30 μM was added to algal cells in suspension or seeded on top of the different encapsulation treatments and the fluorescence emission at 480 nm was measured as a function of time using a microplate reader (FLUOstar OPTIMA®) in fluorescence intensity mode.

## 3. Results

### 3.1. pH gradient in the Ca(II) alginate matrix and evaluation of initial viability

In the design of biosensors with encapsulated photosynthetic cells, as well as for the design of devices based on optical detection systems it is mandatory to minimize the attenuance of the host matrix in the visible region (Nguyen-Ngoc et al., 2009). Optical properties of TEOS based silica hosts obtained via the alcohol-free route can be improved by decreasing the pH of the condensation reaction. [13]

Since the main difference in terms of biocompatibility introduced by the proposed encapsulation procedure is the acidic synthesis of the silica hydrogel, microalgae viability is associated to the changes of pH in the Ca(II)-alginate pre-encapsulation matrix. Fig. 1a shows a photograph of the sample prepared to evaluate the evolution of the Ca(II)-alginate matrix pH as a function of the distance to the silica interface (synthetized at pH = 2.5). As can be appreciated from the color of universal pH indicator, the pH within the Ca(II)-alginate is almost unchanged, even for the extreme conditions imposed by a condensation at pH 2.5, not only due to the higher proton concentration, but also to a higher gelation time (12 h). The Ca(II)-alginate pH as obtained from digital image analysis (see Supplementary information) is plotted as a function of the distance to the silica interface (Fig. 1b). As observed, at a distance of 0.3 mm from the silica interface, the pH is above 4.0 which is well tolerated by *C. vulgaris* (Rachlin and Grosso, 1991).

To further assess the protection function of the buffer-reinforced alginate matrix, the initial viability of encapsulated *C. vulgaris* (CV) cells was evaluated and compared to One-pot encapsulation and two-step encapsulation with Ca(II)-alginate without addition of Tris–HCl buffer. In all cases, the pH of silica matrix synthesis was set to 2.5 and for the 2-step procedures, Ca(II)-alginate beads of 3 mm were used for pre-encapsulation. The proposed method employing Tris–HCl buffer-reinforced alginate beads showed an initial viability of (97 ± 2)%, while the 2 step encapsulation with non-reinforced alginate beads and the one-pot procedure presented a markedly decreased viability of (55 ± 5)% and (23 ± 4)%, respectively.

### 3.2. Algal growth inhibition

To assess the optical quality of the silica matrices, we analyzed the degree to which the scattering of visible light affected the encapsulated algae growth rate, counting the number of *C. vulgaris* cells developed as a function of gel thickness. Following the same protocol employed in previous studies, at the initial time, calcium alginate beads with a content of $10^4$ cells/mL were dispensed into acrylic molds, and TAFR silica encapsulation (condensation reaction at pH 4.0) was performed to obtain a gel thickness of 4.0 mm. The algal growth inside the voids was studied for individual cavities after 3 days of culture in AFNOR liquid medium. CV and PS growth rate were unaffected (growth inhibition < 2%) while CR showed a slight growth inhibition (5 ± 2%).

In a previous study of the scattering of visible light by silica hydrogels based on silicate and pre-formed silica nanoparticles (LUDOX®), [12]It was found that CV growth was affected by both the attenuance of the matrix (tuned from its LUDOX® content) and the silica layer thickness. However, for the matrix with lower LUDOX® content (i.e. higher optical quality), the growth rate was unaffected, even for gel thicknesses up to 4.0 mm. On the other hand, regardless of the optical quality of the silicate-LUDOX® matrix, no detriment in CV growth rate was found for a silica thickness of 1.9 mm or thinner. Recently, it was found that even using the optimized ratio of silicate-LUDOX® (i.e. 1:3 in Si molar relation) and a thin layer (1.9 mm) of silica hydrogel, for the encapsulation of *C. reinhardtii* (CR) and *P. subcapitata* (PS), the algal growth inhibition was 6 ± 3% and 13 ± 4% for PS and CR, respectively. 7−b

The fact that even for a silica width as thick as 4.0 mm, all microalgae species present an almost unaffected growth rate, confirm the enhanced optical quality of the proposed TAFR encapsulation matrix with respect to aqueous silicate-LUDOX® based hydrogels.

### 3.3. Characterization of silica microstructure and optical properties

The attenuation of fluorescein fluorescence caused by different formulations of silica matrices is shown in Fig. 2. As can be seen, for TAFR matrices, the attenuation of fluorescence decreases with the pH of synthesis and the TAFR matrix synthesized at pH 4.0 produces almost no attenuation of fluorescence; improvement of the optical qualities for matrices synthesized at lower pH is negligible. On the other hand, the silicate-LUDOX based matrix presents a high attenuation of fluorescence resulting in a significant decrease of signal (attenuation by a factor of 2.5). This goes

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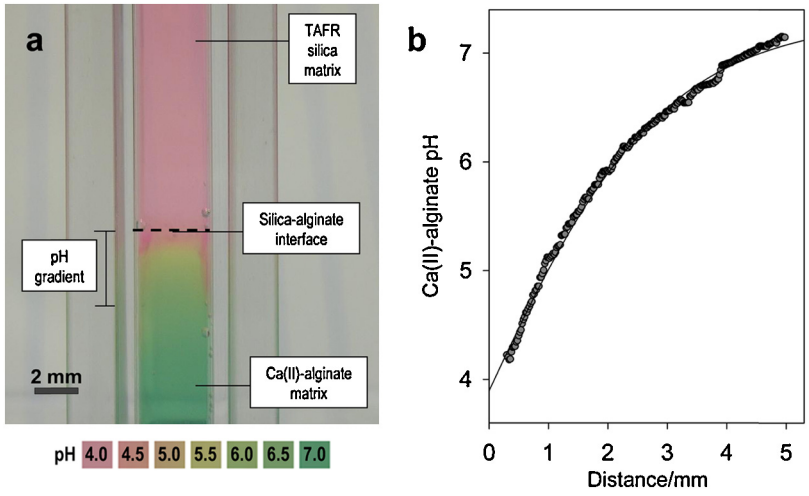


**Fig. 1.** (a) Photograph of the sample prepared to evaluate the evolution of the Ca(II)-alginate matrix pH in contact with TAFR silica matrix synthesized at pH 2.5 (the pH is evaluated using a universal pH indicator) and (b) Ca(II)-alginate matrix pH as a function of the distance to the silica interface as obtained from digital image analysis.

to show that a synthesis at pH = 4 is optimal to obtain a good compromise between biocompatibility and optical quality.

The effect of the gradient of pH established in the silica–alginate interface on the sol–gel synthesis is further evaluated, since it is known that slight variations in pH may cause a significant change in the microstructure of the host matrix. [13]As discussed above, by means of the two-step procedure and due to the protection provided by the Ca(II)-alginate pre-encapsulation, the inorganic matrix synthesis can be attempted under more cytotoxic conditions, [16]Allowing to conduct the synthesis at a lower pH than possible in one-pot encapsulation procedures. The HCl-buffer reinforced alginate confers protection to the biological host, while at the same time can affect the sol–gel synthesis pH conditions. Then, another important point to be addressed is the possible perturbation of the microstructure of the silica matrix due to slight variations in the pH near the silica–alginate interface.

The backbone of silica hydrogel is formed by fractal clusters resulting from condensation and particle-aggregation processes (Zarzycki, 1987). Given the small size of elementary particles and primary clusters composing the structure, a detailed characterization of the microstructure based on electron microscopy is not possible. However, FESEM images of the samples support the results obtained by SAXS analysis (see Electronic Supplementary Information). SAXS curves were interpreted in terms of a mass fractal model, in which silica clusters are described by their radius of gyration ($R$) and their fractal dimension ($D$) (Mandelbrot, 1983). From the analysis of the scattering function, $I(q)$, obtained from SAXS experiments for successive layers from the silica hydrogel taken at different distances from silica–alginate interface (at regular intervals of 0.5 mm), the changes in its microstructure can be evaluated. As shown in Fig. 3, subtle gradual changes in microstructure are observed with no discontinuities in fractal dimension or in the radius of gyration of clusters. At the alginate–silica interface, the silica backbone is similar to that obtained at a fixed synthesis pH = 4.5, but at a distance as short as 2.0 mm from the interface the microstructure resembles that of a TAFR hydrogel synthesized at pH = 2.5. This goes to show that the buffered alginate environment has short range influence and that there seems to be no discontinuities in the silica microstructure.

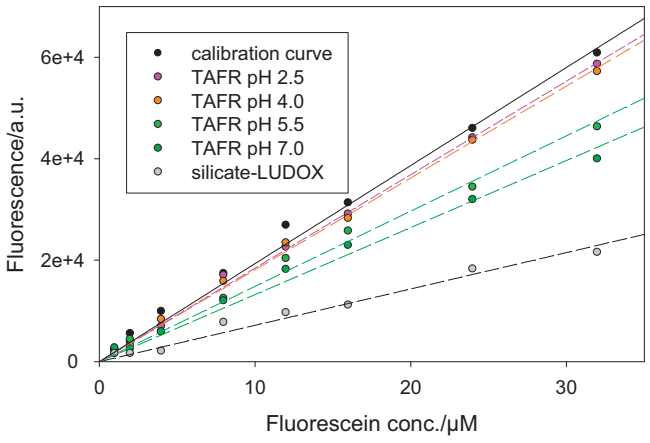


**Fig. 2.** Attenuation of the fluorescein emission at 538 nm under excitation light of 480 nm caused by different formulations of silica matrices (silicate-LUDOX® matrix and TAFR matrices synthesized at pH between 2.5 and 7.0).

### 3.4. *Chlorella vulgaris* esterase activity and transport of the substrate through the matrix

The esterase activity (EA) of *C. vulgaris* was measured using fluorescein di-acetate (FdA) as substrate and detecting the reaction product (Fluorescein) emission as a function of time for different encapsulation treatments consisting of Ca(II)-alginate–silica hydrogels with variable silica widths. A schematic representation of the experiment is shown in Fig. 4B. The kinetics of this reaction for each sample were compared with that obtained for several controls: free *C. vulgaris* algae in aqueous and in 1% Na(I)-alginate solution suspension and encapsulated in 1% Ca(II)-alginate matrix. Considering that FdA is negatively charged at the working pH (7.5), it is not expected to be adsorbed onto silica surface (isoelectric point ~ 2), so it is supposed to be retarded only by diffusion through the silica matrix pores. The retardation observed for the different silica widths are in good agreement with the reported diffusion coefficients of anionic species in similar matrices ($\sim 3.10^{-10}$ m$^2$ s$^{-1}$) (Dickson et al., 2013).

To evaluate de diffusion retardation independently of the attenuation effects, the detection of fluorescence is done from the bottom, so that the loss of fluorescent signal observed in the prototype biosensors is not due to attenuation caused by the silica matrix itself. On the other hand, diffusion is fast enough to allow a kinetic behavior similar to free algal cells for thin silica layers (0.5 mm width). This demonstrates that in devices with silica paths between 0.5 and 1.0 mm the diffusion of substrate through the encapsulation matrix is not limiting signal detection.

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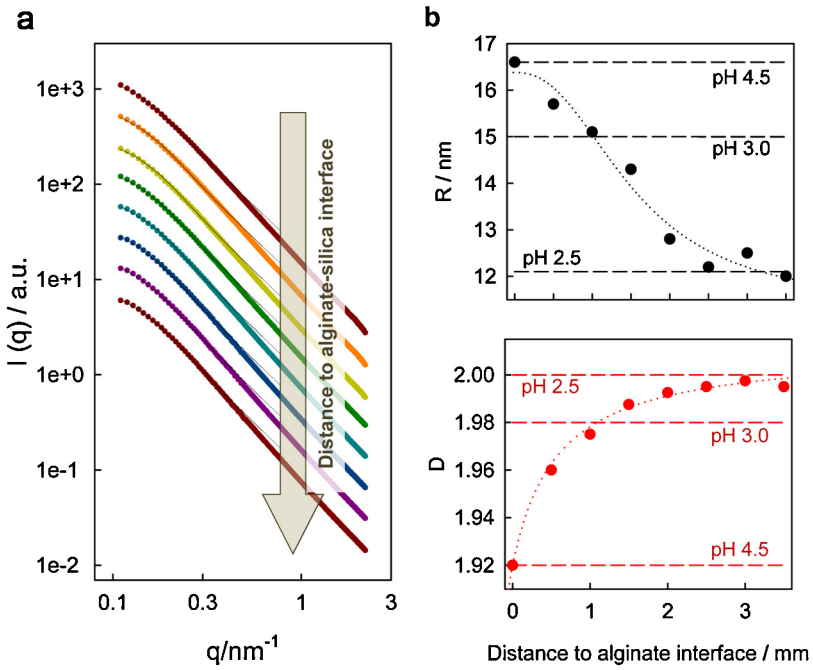


**Fig. 3.** (a) Log–log SAXS intensity plots of TAFR hydrogel layers taken at different distances from the alginate–silica interface (0.0–3.5 mm). Black-lines are fittings of the mass fractal approach, from which the radius of gyration of the clusters ($R$) and the fractal dimension ($D$) are derived. The curves were shifted vertically by different factors for clarity. (b) Microstructure parameters ($R$ and $D$) fitted from the SAXS curves as a function of distance from the alginate–silica interface. Dotted lines are a guide to the eye. Dashed lines indicate the values of microstructure parameters obtained for TAFR hydrogels synthesized at the indicated fixed pH (2.5, 3.0 and 4.5).

## 4. Discussion

The microstructure of the silica hydrogel is determined by the synthesis parameters, and so the properties of the matrix can be tuned to satisfy the needs from particular applications. In the design of biosensors based on encapsulation of algal cells in TAFR hydrogels, there is a compromise between many aspects which are relevant for biosensor performance. The optical quality and the transport properties of the matrix are of crucial importance in these devices. The samples synthesized at pH < 4.0 resulted almost translucent and regarding transport properties, samples synthesized at higher pH values (in the pH range 4.0–6.0) showed higher diffusion coefficients. The selected pH value (4.0) results from a compromise between optical and transport properties desired in

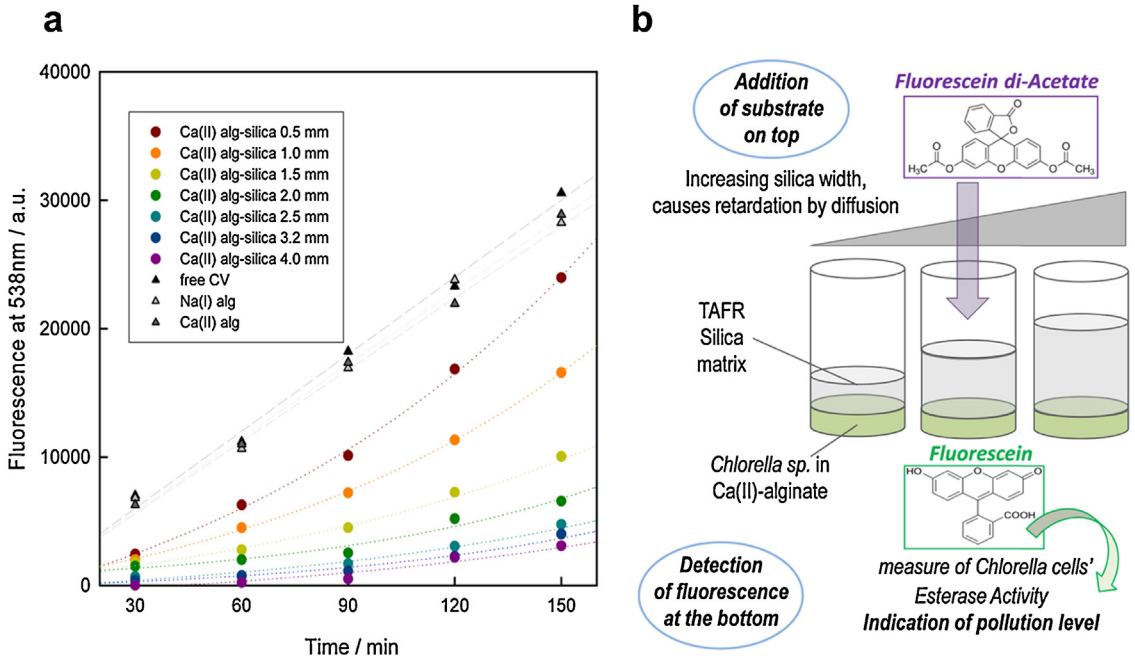


**Fig. 4.** (a) Detection of *Fluorescein* fluorescence (emission at 538 nm) caused by the Esterase Activity of *C. vulgaris* (CV) on fluorescein di-acetate substrate. CV cells were encapsulated on Ca(II)alginate–silica devices with different silica widths (between 0.5 and 5.0 mm, indicated on each curve) and controls consist of CV free cells, CV in 1% Na(I)-alginate solution and immobilized in 1% Ca(II)-alginate matrix. The measurements were done as a function of the time elapsed from the addition of substrate. Dotted-curves are a guide to the eye. (b) Schematic representation of the experiment.

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the final material. The proposed two-step procedure reinforced by buffer showed to be effective in the protection of the encapsulated algal cells during the synthesis. This enhanced encapsulation matrix highly extents the range of possible applications of these functional biomaterials.

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