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Rhodamine B doped silica encapsulation matrices for the protection of photosynthetic organisms

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ABSTRACT

An advanced encapsulation matrix that efficiently protects microalgae from harmful UV light without causing toxicity to the entrapped culture is developed based on the electrostatic adsorption of the dye Rhodamine B on silica preformed particles during sol-gel synthesis. The three microalgae (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*) were previously immobilized in alginate following the Two-step procedure. Once entrapped in the silica gel, Rhodamine B act as an inner cut-off filter, protecting the encapsulated organisms from UV radiation. This matrix allows the sterilization of encapsulation devices without affecting the viability of the entrapped microalgae cells. The condensation of Si(IV) in the presence of silica particles with adsorbed dye generates silica matrices with good mechanical stability. Furthermore; no appreciable differences in microstructure, as assessed by SAXS (Small Angle X-ray Scattering), are caused by the addition of the dye.

1. Introduction

Cyanobacteria are important for several established biotechnologies such as the production of pharmaceutical compounds, food additives and pigments. In addition, they provide inexpensive systems for on-line and *in situ* pollution monitoring. Encapsulation of living cyanobacteria into a porous silica gel matrix is a recent approach that may dramatically improve the efficiency of certain production processes in bioreactor applications or provide with portable, fast and economical tools for bioremediation or toxic compounds' detection in the environment (Dickson and Ely, 2013; Ramachandran et al., 2009; Depagne et al., 2011; Léonard et al., 2011). The application of the sol–gel technology to develop host materials for cell-based biotechnological devices has been continuously explored over the last decades (Meunier et al., 2010). In the recent years, efforts have been mainly focused on the diversification of encapsulated cell types as well as the chemical nature and structure of the inorganic or hybrid matrices. As a result, the range of potential applications of these "living materials" has enormously increased (Blondeau and Coradin, 2012).

The two-step procedure (CA Patent, 1997; Perullini et al., 2005; Perullini et al., 2012), which includes a pre-encapsulation of the biological guest in Ca(II)-alginate matrix, allows to protect living cells from cytotoxic synthesis precursors and extreme pH conditions (Perullini et al., 2011; Perullini et al., 2014) while providing with an extension of cellular viability and preserved biological activity in the long-term (Duarte et al., 2012; Perullini et al., 2008, 2007). Along with continuous efforts to adapt materials chemistry to the conditions of life, developments to improve matrix properties and functions are currently creating materials that fulfill the requirements of different applications. In particular, since higher energy photons belonging to the UV range decompose biomolecules and can strongly impact on cell viability (Malanga et al., 1997a), intrinsic UV filtering properties would be highly desirable. The inclusion of UV absorptive CeO_2 nanoparticles within the inorganic matrix can act as an inner cut-off filter (Sicard et al., 2011). The addition of dye molecules in the encapsulation matrix, though being a simpler and more economic procedure, has been regarded as less effective due to the possible bleaching and/or leaking of molecules. However, recent works demonstrate that the fluorescent quantum yield can even be enhanced by the silica matrix host and that these dye-doped silica present high photostability and low toxicity (Liang et al., 2013).

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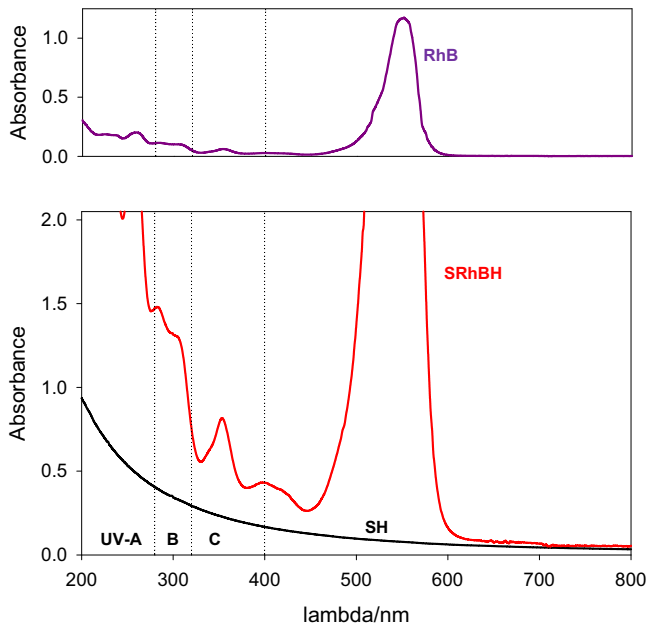

Fig. 1. Upper: UV–vis absorbance spectra of Rhodamine B 10^{-5} M in aqueous solution (RhB); lower: UV–vis absorbance spectra of 1 mm-thick silica hydrogel (SH, black line) and Rhodamine B loaded silica hydrogel (SRhBH, red line). From right to left, dotted vertical line denotes the lower energy limits for UV-A, B and C radiations ranges, respectively.

We propose herein the use of Rhodamine B as synthesis additive during the sol-gel synthesis of silica matrices and demonstrate that it remains retained within the silica matrix, has no toxic effects on three encapsulated microalgae (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*) and confers effective protection, preventing microalgae deterioration under harmful UV doses. Moreover, this synthesis additive allows external sterilization of encapsulation devices with different applications such as biosensors or modular bioreactors.

2. Experimental

2.1. Algae growth

Chlorella vulgaris (CV), *Pseudokirchneriella subcapitata* (PS) and *Chlamydomonas reinhardtii* (CR) algal strains were purchased from The Culture Collection of Algae and Protozoa at Cumbria, United Kingdom. CV and PS were grown in the Lefebvre-Czarda medium (AFNOR, 1980) whereas CR 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 5000 lux and 8 h of darkness. Viability is evaluated by the plate counting technique in semisolid media.

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 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, 1000 μl of the algal suspension in Na(I)-alginate was poured into an individual mold and the 0.1 M $CaCl_2$ solution was added in the form of a mist by means of a nebulizer machine.

The second step of the immobilization procedure consists of a silicate (sodium silicate, Riedel-de Haën; NaOH 10%, SiO_2 27%) sol–gel process in the presence of commercial silica nanoparticles (Ludox HS-40®, 40% in water, obtained from Aldrich), leading to a nanoporous monolithic structure. Monoliths were prepared at room temperature by mixing volumes of the different precursor solutions to obtain a SiO_2 water molar relation of 3.8% with a fixed proportion of polymeric to particulate silica precursors (1:4) at constant pH 7.0, adjusted with HCl.

When indicated, Ludox HS-40® preformed silica particles were left in contact with Rhodamine B (1.0×10^{-3} M water solution), previous to use in silica sol–gel synthesis. Final concentration in silica gel was 2.5×10^{-4} M.

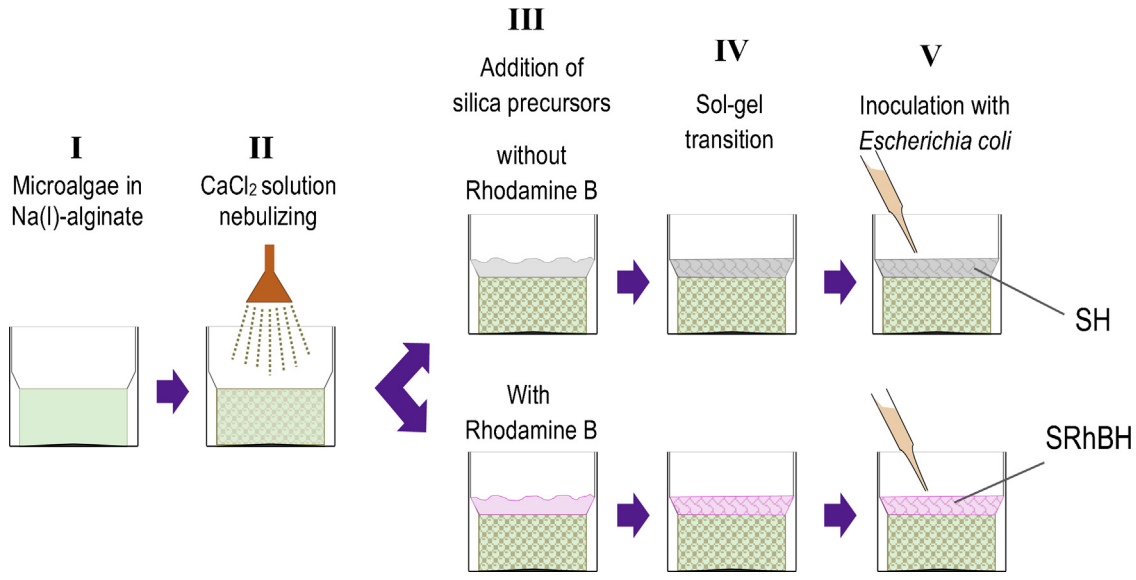

Fig. 2. Schematic representation of the immobilization of microalgae in silica hydrogel (SH) and Rhodamine B doped silica hydrogel (SRhBH) matrices. (I) microalgal cells in culture media added with 1.0% Na(I)-alginate are poured into a small acrylic mold; (II) $CaCl_2$ solution is added as a mist, to form a Ca(II)-alginate encapsulation of microalgae culture. (III–IV) Synthesis of the inorganic matrix above the Ca(II)-alginate pre-encapsulation matrix, leading to a silica nanoporous layer of ~1 mm thickness (generating a SRhBH sample if the Ludox HS-40® used as precursor is pre-stabilized with Rhodamine B, or a "SH" sample if Rhodamine B is not added). (VI) Inoculation with *Escherichia coli* in isotonic solution previous to UV-irradiation experiment (Fig. 3).

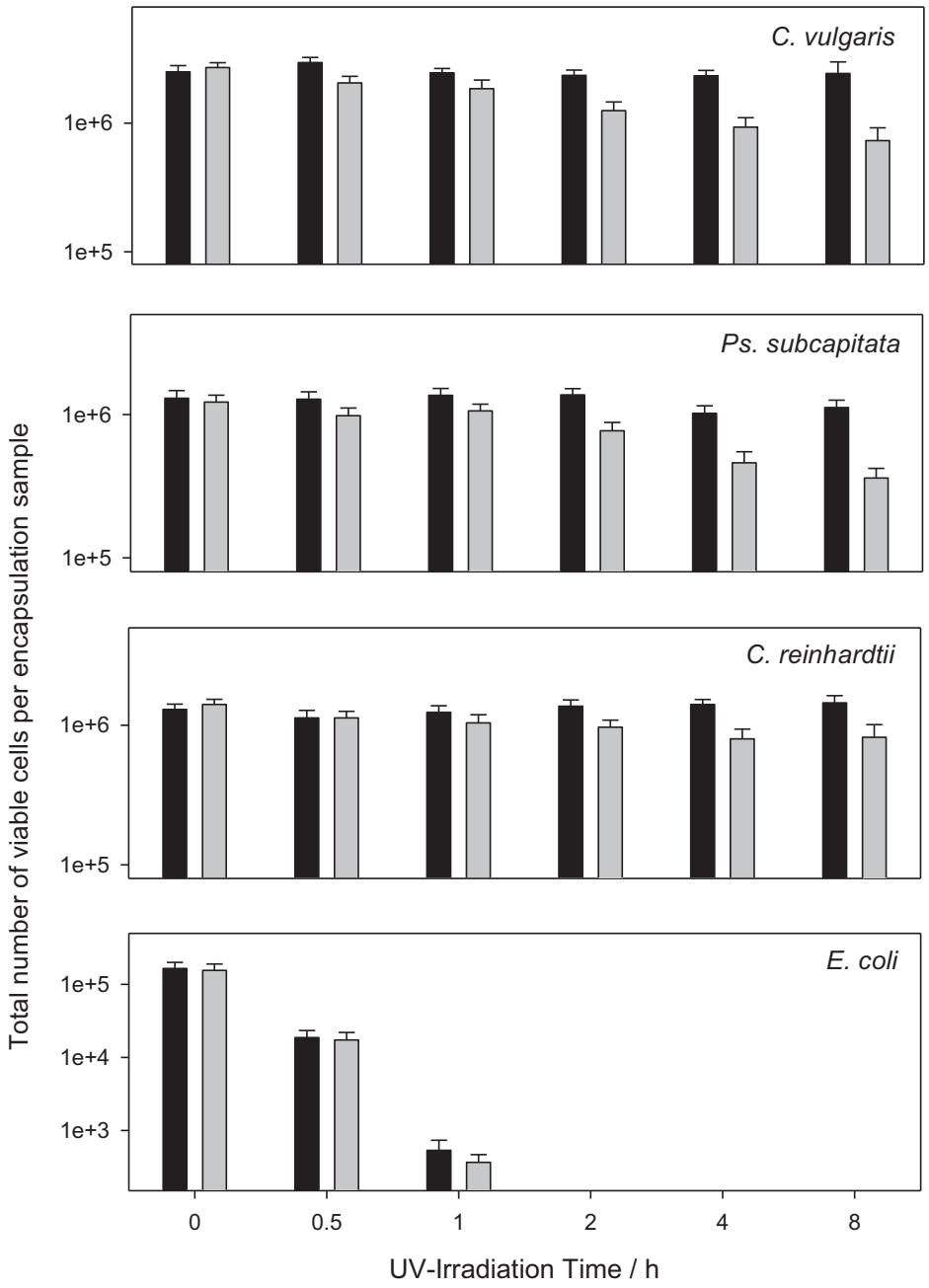

Fig. 3. Rhodamine B doped silica hydrogel (black column) protection of microalgae immobilized cells (*C. vulgaris*, *Ps. subcapitata* and *C. reinhardtii*, as indicated). For each treatment, silica hydrogel without Rhodamine B (grey column) is used as control. The sterilization efficiency (*E. coli*) is also evaluated as a function of UV irradiation time. The values of *E. coli* cell counting for each irradiation time correspond to the average of all samples (external surface), since they presented no significant differences.

2.3. Adsorption isotherms

For dye-adsorption experiments, samples of wet gels are left in contact with aliquots of 2000 μL of Rhodamine B aqueous solution in different concentrations (1–50 μM, previously adjusted to pH 7.0). To quantify the concentration of Rhodamine B (RhB), the UV–vis spectrometer was calibrated to a concentration range between 0.1 and 1.0 μM at a wavelength of 554 nm, which corresponds to the absorption maximum of RhB. The concentration of dye in solution is measured spectrophotometrically at $\lambda = 554$ nm. The fraction of dye adsorbed on the silica matrix is calculated from the difference between the concentration in solution at $t = 0$ and after the systems had reach the equilibrium. Experimental data are fitted to the Freundlich model:

$$S = FC_e^n \quad (1)$$

where S is the mass of adsorbate by mass of adsorbent, F is the Freundlich constant of adsorption, C_e is the dye concentration in solution at equilibrium and n is a constant that varies from 0 to 1. This empirical model takes into account the heterogeneity of the different adsorption sites on the silica surface and thus it can be applied to many systems, especially to those that contain solutes in a solid/liquid interface (Rudzinski and Panczyk, 2000; Hamdaoui et al., 2008).

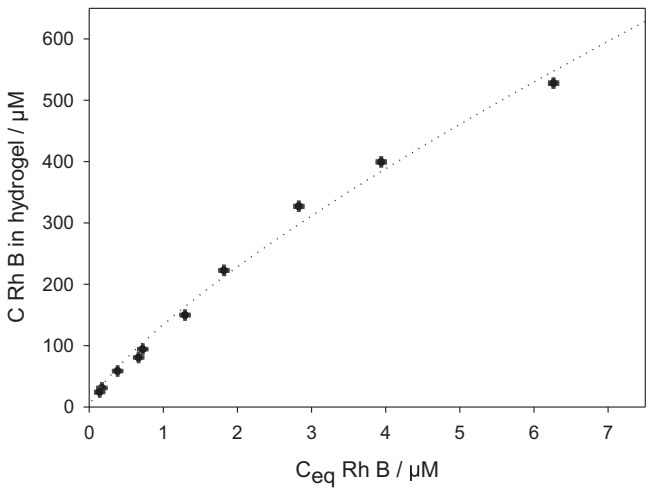

Fig. 4. Adsorption isotherm at 25 °C of Rhodamine B (RhB) on silica hydrogel synthesized in the absence of the dye: Adsorbed dye (expressed as µmol of RhB per liter of hydrogel) vs. C_{eq} is the µM concentration of the dye in solution in the equilibrium. The dotted line corresponds to the fitting to the Freundlich model (fitting parameters: $F = 141.5$; $n = 0.698$).

2.4. Microstructure characterization of samples

The microstructure characterization was performed at the LNLS SAXS2 beamline in Campinas, Brazil, working at a wave vector (q) range: $0.09\ nm^{-1} < q < 2.2\ nm^{-1}$, a wavelength ($\lambda$) = 0.1488 nm, and a sample stage in vacuum with mica windows (standard for liquids) (Cavalcanti et al., 2004). Data analysis was performed with SASfit program.

2.5. Evaluation of toxicity towards microalgae

Tests were carried out on free algae and concern measurements of esterase activity (EA) and the intensity of chlorophyll fluorescence (CF).

For EA tests: 100 µL of algae suspensions were cultured in the presence of 10 mg of silica hydrogel (SH) or silica hydrogel with the addition of Rhodamine B (SRhBH). After 48 h of contact with the silica matrix, 20 µM of Fluorescein Diacetate (FDA; Sigma) was used as substrate by the algal esterase and degraded into fluorescein, a fluorescent product (excitation 480 nm, emission 538 nm) measured as a function of time using a microplate reader (FLUOstar OPTIMA®) in fluorescence intensity mode. Results are expressed as percentage of EA with respect to a control sample consisting of 100 µL of algae suspensions cultured 48 h in the absence of silica matrix.

For CF tests: The intensity of chlorophyll fluorescence of 2000 µL of microalgae culture was measured in the presence of 200 mg of SH or SRhBH sample. The emission at 682 nm under a 469 nm excitation light was measured with a spectrofluorimeter (FLUOstar, BMG®). The toxicity caused by the silica matrix samples was determined by the fluorescence enhancement (E):

$$E = \frac{F_a - F_b}{F_b} \times 100$$

where F_b is the fluorescence for a test batch before contact with the silica matrix and F_a is the fluorescence for the same test batch after the exposure (2 h of contact with a sample consisting of 200 mg of SH or 200 mg of SRhBH).

Both assays were undertaken with the following cellular concentrations: $[CV] \approx 2.5 \times 10^6$ cells/mL; $[PS] \approx 1.3 \times 10^6$ cells/mL; $[CR] \approx 1.3 \times 10^6$ cells/mL.

2.6. Efficiency of sterilization and UV-protection

Escherichia coli K 12 was cultured aerobically in Luria Bertani broth (LB) at 37 °C by 18 h. After this time, the culture was diluted with isotonic solution to achieve an inoculum of 1.6×10^6 CFU (Colony Forming Units)/mL.

Encapsulation Modules where inoculated with 100 µL of *E. coli* inoculum and then exposed to the UV-germicidal lamp of a laminar flow (30 min of exposure/cycle). The efficiency of sterilization as well as the microalgae viability was assessed by CFU counting on LB agar and algal cell counting on a Neubauer chamber, respectively. To collect the total amount of viable *E. coli* in an immobilization device, the silica matrix layer was removed, mechanically disrupted and re-suspended in sterile isotonic solution.

3. Results

Fig. 1 presents the absorption spectra of both the pure silica hydrogel (SH) and a Rhodamine B-loaded one (SRhBH). Both hydrogels were molded inside quartz cuvettes with 1 mm optical path. In the visible region, the absorption is negligible for SH and presents a maximum at 554 nm for SRhBH due to Rhodamine B absorption. However, in the UV range, the SRhBH sample presents a strong absorption characteristic of RhB mounted over the silica scattering background, providing an extra significant filter for UV-C radiation while practically cuts off the radiation belonging to UV-B or higher energies, that are deleterious for the majority of organisms, including microalgae (Malanga et al., 1997b).

Although not critical for biosensors' developments, the absorption band in the visible region (500–590 nm) within the photosynthetic active range could be detrimental for applications that demand actively growing cells. To further assess this hypothesis, we evaluated the growth of CV encapsulated in SH and SRhBH matrices submitted to different light treatments (see Supplementary Information). The obtained results support previous published work in which the spectral shifting by Rhodamine B has been reported to enhance algae growth in a double tubular reactor (algae inside and dye solution outside) (Prokop et al., 1984) since the emission of the dye coincides with the range of highest production rate and energy conversion efficiency of most green algal species (for CV: 510–610 nm) (Kubín et al., 1983).

SH and SRhBH matrices were molded inside acrylic casts containing microalgae culture entrapped in calcium-alginate gel (Fig. 2). Many replicates were prepared of the different algae species: *Chlorella vulgaris* (CV), *Pseudokirchneriella subcapitata* (PS) and *Chlamydomonas reinhardtii* (CR). The external surface of all immobilization devices was inoculated with Escherichia coli (EC) and exposed to a UV source immediately after. Three samples of each treatment were withdrawn every 30 min of irradiance. The number of bacterial cells on the surface of encapsulation modules as well as the immobilized microalgae cells was assessed as a function of irradiation time up to a total time of 8 h (Fig. 3).

After 1 h of irradiance and regardless of the evaluated sample, *E. coli* counting was non-detectable, proving the efficiency of sterilization. The counting of microalgae cells immobilized in SH showed a significant decrease after 2 h of irradiance, while the cell counting of those entrapped in SRhBH remained unaltered. After a cumulative exposure time of 8 h, SH entrapped algae presented a viability of 30% (CV), 32% (PS) and 56% (CR), while SRhBH-immobilized algae cell counting remained almost unaltered, demonstrating the effectiveness of the UV barrier. Additional essays were performed to ensure viability of encapsulated algae cells, since direct observation of cells under the microscope alone bears no direct relation to the photooxidative death of the algal cells as there are several mechanisms involved in the complex response of microalgae to

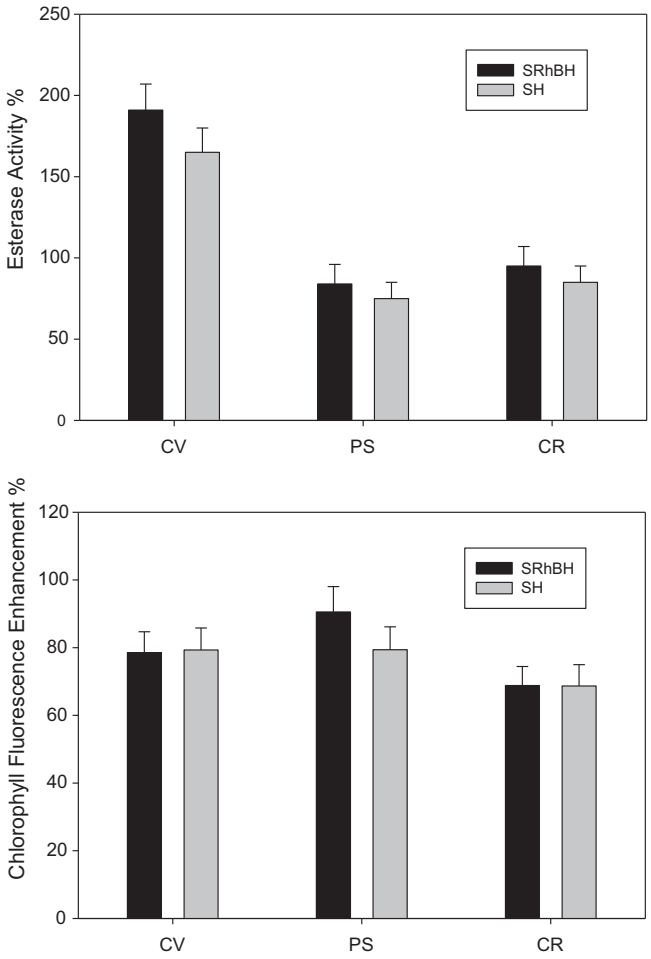

Fig. 5. Percentage of esterase activity (up) and Chlorophyll Fluorescence (down) referred to control microalgae in M4 culture medium from *C. vulgaris* (CV), *Ps. subcapitata* (PS) and *C. reinhardtii* (CR) in contact with a Silica Hydrogel (SH) or Rhodamine B doped Silica Hydrogel (SRhBH) sample.

light stress. In all cases, immobilization matrices remained stable and the entrapped cultures remained viable for more than 1 month.

An important issue to take into account is the intrinsic toxicity of the dye in contact with the immobilized biological guest. Being a cationic dye RhB is expected to adsorb on silica surface, living a low concentration of the dye bio-available in solution. Fig. 4 shows the experimental adsorption isotherm of RhB in SH matrix. The parameter F from the fitting to the Freundlich model (eq. (1)), estimates the partition coefficient between the dye adsorbed on silica and the dye in solution ($F = 141.5$). Thus, the dye remains attached to the immobilization matrix, ensuring efficient UV-filtering protection and its bio-available concentration is lower than 2×10^{-6} M.

The intrinsic toxicity of RhB was assessed by comparing the Esterase Activity (EA) and the Chlorophyll Fluorescence (CF) of microalgae in contact with SRhBH and SH matrices. As can be observed in Fig. 5, the EA is perturbed by the presence of the silica matrix, but no significant difference in EA can be attributed to the addition of the dye in the silica hydrogel formulation. Similar results are obtained for CF: the enhancement of fluorescence experienced by cells in the presence of SH is similar to that observed for SRhBH.

Furthermore, the microstructure and mechanical stability of the hydrogel was not affected by the addition of RhB as synthesis additive. Both, the Young modulus of the hydrogels, used as an indicator of their mechanical stability, and the scattering function obtained from SAXS experiments, resulting from the hydrogel microstructure, were identical for SH and SRhBH samples (see Electronic Supplementary Material).

4. Conclusion

The design of cell encapsulation devices relies on a delicate balance between the requirements of the material, in terms of chemical or mechanical stability, optical transparency, porosity, among others, and the constraints imposed by the preservation of cell viability (i.e. chemical toxicity, pH, salinity). Furthermore, for particular applications and especially when devices are exposed to particular stresses during normal operation conditions, specific properties of the encapsulation matrix are desirable or even necessary. Our data demonstrate that it is possible to confer UV-filtering properties to silica hydrogels without impacting on the survival of encapsulated microalgae cells by means of dye used as synthesis additive. In this context, Rhodamine B is particularly well-suited as it operates at a very low concentration (ca. 0.01%, w/w) and does not alter the silica hydrogel microstructure or mechanical stability.

Apart from being useful for applications were microalgae encapsulations are exposed to UV harmful radiation, this procedure can be also highly valuable for external sterilization procedures to ensure no involuntary contamination occurs during the manufacturing process of encapsulation devices for industrial applications. It is worth underlining that the modification of the synthesis is not time consuming, cheap, and easy to scale up.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.05.013.

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