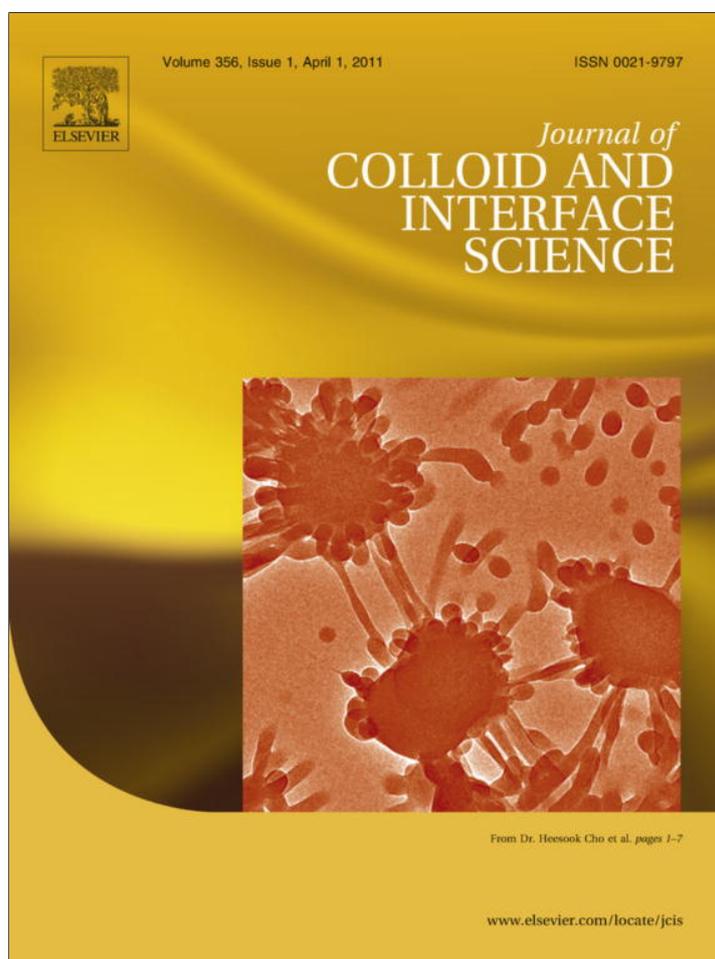


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Journal of Colloid and Interface Science

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Prolonging the lifetime and activity of silica immobilised *Cyanidium caldarium*Joanna C. Rooke^a, Bertrand Vandoorne^b, Alexandre Léonard^{a,1}, Christophe F. Meunier^a, Pierre Cambier^c, Hugo Sarmiento^d, Jean-Pierre Descy^c, Bao-Lian Su^{a,e,*}^a Laboratory of Inorganic Materials Chemistry (CMI), The University of Namur (FUNDP), 61 rue de Bruxelles, B-5000 Namur, Belgium^b Groupe de Recherche en Physiologie Végétale, Earth and Life Institute, Université Catholique de Louvain, Place Croix du Sud 5/13, 1348 Louvain-La-Neuve, Belgium^c Department of Biology, The University of Namur (FUNDP), 61 rue de Bruxelles, B-5000 Namur, Belgium^d Institut de Ciències del Mar – CMIMA, CSIC, Departament de Biologia Marina i Oceanografia, Pg Marítim de la Barceloneta 37–39, E08003 Barcelona, Spain^e State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, 122 Hongshan Luoshi Road, 430070 Wuhan, China

ARTICLE INFO

Article history:

Received 13 December 2010

Accepted 6 January 2011

Available online 11 January 2011

Keywords:

*Cyanidium caldarium*CO₂ mitigation

Cell immobilisation

Photosynthesis

Living materials

ABSTRACT

Over the past few years the idea of living photosynthetic materials has advanced from concept to reality. This work outlines the improvements made in the immobilisation of the thermotolerant acidophile *Cyanidium caldarium* (Tilden) Geitler SAG 16.91 within porous and transparent silica gels with the view to targeting photochemical materials that can be used to mitigate rising CO₂ emissions. Our results suggest that the immobilised cells are autofluorescent for at least 75 days post encapsulation and can maintain a steady oxygen production rate over a similar timeframe corroborating the viability and physiological activity of silica immobilised *C. caldarium*.

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1. Introduction

Carbon dioxide (CO₂) levels have been rising since the industrial revolution through deforestation and fossil fuel consumption. The consequences of rising CO₂ levels are alarming, such as the acidification of the oceans and the emergence of so called dead zones, where the oxygen levels are dangerously low such that no aerobic life is present [1], to increasing global temperatures through an increase in greenhouse gases [2].

Different methods for CO₂ capture, sequestration and conversion have been developed to reduce CO₂ from anthropological activities [3–5]. This work focuses on a novel strategy to reduce CO₂ emissions through photosynthesis whilst having the potential to exploit secondary benefits such as the production of high value metabolites. Living, photosynthetically active cells have been entrapped within abiotic silica frameworks to target living hybrid materials capable of CO₂ assimilation and oxygen production. Silica is advantageous in cell encapsulation owing to its tuneable porosity, optical transparency and durability to chemical and physical attack as detailed in the literature [6,7].

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Previous studies have seen the immobilisation of thylakoids, cyanobacteria and whole plant cells [8–11] within silica gel in the aim of designing photobioreactors suitable for pollution mitigation and high value product synthesis. However, flue emissions are typically warm and slightly acidic owing to the elevated levels of CO₂. These conditions may not easily be tolerated by other photosynthetic cells or isolated apparatus and thus for industrial applications it may be advisable to turn towards hot, sulfurous spring-type ecosystems for inspiration. Therefore this work develops the use of the thermotolerant acidophile *Cyanidium caldarium*, a photosynthetic red algae previously immobilised in a sodium silicate based matrix [12]. Seckbach et al. have reported that this strain can even grow vigorously with higher photosynthetic rates under streams of pure CO₂ [13] whereas Greenbaum et al. have shown how this strain interacts with flue gases revealing that, when grown in a stress reducing buffer, photosynthetic removal of CO₂ continued in an atmosphere enriched with up to 40% flue gases [14]. Furthermore studies suggest that for certain red algae, their RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) relative specificity was in general greater than for higher plants [15]. This enzyme catalyses both the carboxylation and oxygenation of ribulose-1,5-bisphosphate. Oxygenation can decrease plant productivity by up to 60% as the net amount of CO₂ fixed is reduced as the plants reoxidise the organic carbon sources produced through carboxylation [16]. Hence in terms of a CO₂ sequestration device, strong consideration must be given to this enzymatic system when selecting a photosynthetic cell.

The bioactivity of *C. caldarium* has also been exploited to reduce various ketones with good enantioselectivity [17]. In fact it has been shown that only 0.1 g (dry weight) of red algal cells is required to stereoselectively biotransform 20 mg of (+) and (–) camphorquinones compared to 15 g of plant cultured cells (*Nicotiana tabacum* and *Catharanthus roseus*). In addition the growth rate of red algae is substantially faster than plant cell cultures, which lends them to be used in environmentally benign biocatalysis, potentially via immobilisation technology to overcome separation issues.

This report details improvements made to the host matrix by reducing the by-product, sodium chloride and by replacing the precursors successfully used in previous aqueous sol–gel silica syntheses with more biocompatible alternatives [18]. Previous results had shown how precursors such as Ludox and glycerol had a negative impact on the eukaryotic cells and were incompatible with the formation of active hybrid materials [12]. This work also studies the viability, physiological activity and productivity of entrapped *C. caldarium* with time using microscopy and electrochemical techniques.

2. Materials and methods

2.1. Cell cultivation

Axenic strain, *C. caldarium* SAG 16.91 was obtained from the SAG culture collection. Details on origin and cultivation have been detailed previously in the literature. The media used was an acid media as described by the SAG collection [12].

2.2. Cell immobilisation

Algae immobilisation within silica gel was carried out via the “low-sodium” aqueous route [8]. A sodium silicate solution (1.5 M, assay 25.5–28.5%, Merck), refrigerated to 4 °C, was passed over an acid ion exchange resin (Amberlite IR 120, Acros) to obtain a metastable H_2SiO_3 sol as described elsewhere [19].

Centrifugation was employed to harvest the cells from 40 mL of suspension culture (1200 rpm, 10 min). With the supernatant discarded, the pellet was resuspended in 1 mL sterile water. Subsequently, 240 μL KOH (0.2 M) was added to 4 mL of H_2SiO_3 , obtaining a sol at pH 6 to which the resuspended cells were immediately added (Gel A). Gel formation occurred within minutes. For certain immobilisations (Gel B), 150 mg SiO_2 nanopowder, (99.5%, 5–15 nm, Aldrich) was homogenised with the silicic acid sol via sonication prior to the immobilisation procedure carried out for Gel A. This facilitated the development of silica aggregates, used to increase the overall strength of the immobilising matrix. Finally, fresh acid growth media was added on top of the gels. The work was carried out under sterile conditions using a laminar flow hood, with the non-sterile apparatus and chemicals being autoclaved prior to use. Gels were left under controlled conditions in a light chamber (Sanyo versatile environmental test chamber, 22 °C, 60% relative humidity and a 16/8 h light/dark cycle), except for the assimilation experiments with radioactive isotopes.

Transmission Electron Microscopy was performed on 3 month old samples using a Philips Technai 10 with an accelerating voltage of 80 kV. Cell fixation was carried out prior to analysis with a method described in the literature using ruthenium red to stain and identify any carbohydrates [19]. Confocal microscopy was carried out using a Leica TCS NT/SP1. The excitation wavelength of the argon ion laser was set at 488 nm and the region of detection of the fluorescent signal was between 650 and 750 nm.

CO_2 assimilation analysis was also achieved using our adapted method as previously described [9]. The radioactivity of the sample

was measured using a Beckman scintillation counter (LS 6000 SC) with the aid of an Insta-Gel Plus (Perkin Elmer) scintillation cocktail.

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC–PAD) was performed using a Dionex ICS 3000 in order to identify some selected neutral monosaccharides excreted by the encapsulated cells. Aliquots (1 mL) of media were taken from the hybrid gel-media samples 42 days post immobilisation yet 7 days after the media had been refreshed (complete replacement of supernatant on top of gels) as well as 1 mL of fresh media for comparison. These aliquots were hydrolysed in trifluoroacetic acid (1.5 mL, 2 M) at 120 °C for 2 h. The hydrolysates were then evaporated to dryness and the residues resuspended in 1 mL Milli Q grade water. Subsequently a 900 μL aliquot of each sample was centrifuged (13,000g, 15 min) prior to injection into the HPAEC system. Chromatic separation of the monosaccharides was performed on a CarboPac PA-20 column (Dionex, 3 \times 150 mm) connected to a guard column (3 \times 30 mm). 5 mM NaOH at a flow rate of 0.5 mL min^{-1} was employed under constant helium pressure as the mobile phase. A post-column derivatisation was performed by the addition of 0.75 M NaOH. The PAD sensitivity was set at 1 μA , the sample injection volume was 20 μL and column oven temperature 35 °C. The column was reconditioned with 200 mM NaOH for 10 min then re-equilibrated with the starting buffer. Pure sugars from Sigma Aldrich were used as standards and also treated to an identical hydrolysis procedure.

Oxygen production was monitored on a Chlorolab 2 system by Hansatech. A section of the hybrid gels was removed and the media refreshed every 14 days. This portion was dissected into small cubes, *ca.* $V = 3 \text{ mm}^3$, which were suspended in 800 μL aliquots of growth media within the reaction chamber of the oximeter. In rapid succession, to minimise the loss of CO_2 through neutralisation with the acid media, an excess of bicarbonate (20 μL 0.6 M NaHCO_3) was injected into the reaction chamber as the source of CO_2 and the chamber was purged with nitrogen to remove the oxygen present within the system then sealed to the atmosphere. The samples were illuminated by a collection of red LEDs centred on 650 nm with an intensity fixed at 700 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and the rate of oxygen evolution data was acquired on a PC.

Photosystem II (PSII), the first protein complex in the light dependent reactions can be analysed through pulse amplitude modulation fluorimetry (PAM). Values for ΦPSII , the PSII quantum efficiency (percentage of the light absorbed by chlorophyll used for photosynthesis) and NPQ, non-photochemical quenching (indicator of the dissipation of the incident light in heat) were obtained using a Fluorescence Monitoring System II by Hansatech Instruments on a series of identical hybrid gels that were made in a disc form within a Petri dish, with a diameter equal to the diameter of the light source. After the samples had been dark-adapted for 15 min a primary saturation pulse (18,000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) was sent to the gel followed by a constant light intensity of 600 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ over a duration of 1 min (actinic light). Finally a second saturation pulse of the same intensity of the primary pulse was sent and the various physical parameters under investigations were ascertained.

3. Results and discussion

3.1. Cell immobilisation

The immobilisation of plant cells and green algae within sodium free silica gels were used as the foundations of this study to optimise a living hybrid material based on the encapsulation of the thermotolerant acidophile *C. caldarium* [11,19,20]. Previous work has seen the immobilisation of this red algae in a matrix based

on sodium silicate and Ludox, a nanoparticulate colloidal silica [12]. However the results highlighted how this eukaryotic cell was more susceptible to the selected precursors than in the immobilisation of cyanobacteria, with reagents such as glycerol and Ludox being detrimental to the activity of the cells. It was found that glycerol hindered the Calvin cycle whereas the incorporation of both glycerol and Ludox impeded oxygen production. Hence more biocompatible precursors were required. Further to this a method to drastically reduce the amount of Na^+ , an ion that interferes with the natural osmoregulation of a cell, has been developed [8]. In this method Ludox, used widely in aqueous based silica immobilisations [9,21–23], was replaced by a silica nanopowder that tended to aggregate upon sonication with the silica sol. These aggregates would therefore play the role of a strengthening additive analogous with Ludox, yet they were too large to be internalised within a living cell unlike Ludox colloids. With the sodium concentration vastly reduced there was no longer a requirement to find a suitable osmoregulator to replace glycerol. These new hybrid gels were examined in terms of cell viability and activity using a suite of techniques as outlined below.

3.2. Cell viability

Microscopy techniques facilitated the determination of cell integrity. Transmission Electron Microscopy (TEM) studies were carried out to reveal the degree of intactness of the cell membranes 94 days post immobilisation. Fig. 1 shows a collection of images taken of the hybrid materials without silica nanopowder. The images highlight the fact that whilst some cells have badly deteriorated (i) others have been preserved beyond 3 months (ii–iv). The section studied also revealed how some cells that were in the process of division have maintained intact daughter cells within the void created during immobilisation (iii) whereas in other cases there is some degradation to the cell walls of the daughter cells inside the mother cell (iv). The dark spots found within some cells are most probably the carbohydrate stores, as Ruthenium red was used as a colorant to highlight carbohydrate production. During photosynthesis glucose is produced from the assimilated carbon dioxide. This is mainly stored in the form of starch or glucans, glucose units linked together through glycosidic bonds, within chloroplasts.

Fig. 2 reveals how the silica nanopowder (particle size 5–15 nm as sold) aggregates on addition to the metastable sol. With the aid of sonication these aggregates can be dispersed throughout the gel; they do not settle with gravity as the gel forms. These images reveal the size of the aggregates relative to the cell and the fact that they are too large to be internalised within the cell, thus highlighting the advantageous nature of silica nanopowder over Ludox. Furthermore one can see from image ii that there is little evidence of the biomineralisation process observed in the presence of Ludox based hybrid gels [12], suggesting that the silica crust seen previously is composed of nanoparticulate Ludox and it is this crust that blocked the active sites of the cell, prohibiting the photosynthetic production of oxygen. In addition, the level of silica dissolution is minimal in comparison to other immobilised aquatic photosynthetic cells [9,19], with little to no empty space evolving around the cells 3 months post encapsulation. This is a phenomenon which occurs rapidly within hours or days of immobilisation in the case of cyanobacteria and chlorophyta. This raises the question of strain selection and how the chemistry of the external cell surface plays a critical role in the choice of photosynthetic organism for immobilisation. Those cells walls that interact with the silica in such a way that causes the gel to hydrolyse may weaken the overall mechanical strength of the hybrid gel. Conversely they may create pockets within the gel that allows for cellular division and thus the propagation of life. This is one area that needs careful attention in the development of novel living materials as there is a delicate balance between the advantages and disadvantages of macrovoids within the gel.

Confocal microscopy was employed to determine whether the photosynthetic apparatus immobilised within the silica gel continued to function properly. After excitation of the hybrid material by a laser, intact chlorophyll molecules contained within the cells underwent autofluorescence, which in turn was detected by the microscope. Fig. 3 represents overlay images (optical and confocal) of the hybrid gels over time. The red spheres represent chlorophyll *a* containing cells that autofluoresce as found from confocal microscopy and by overlaying this onto the optical image one can visualise the outlines of cells (grey spheres) that have lost the ability to autofluoresce. This effect is clearly visible both after 19 and 75 days which qualitatively suggests that the number of

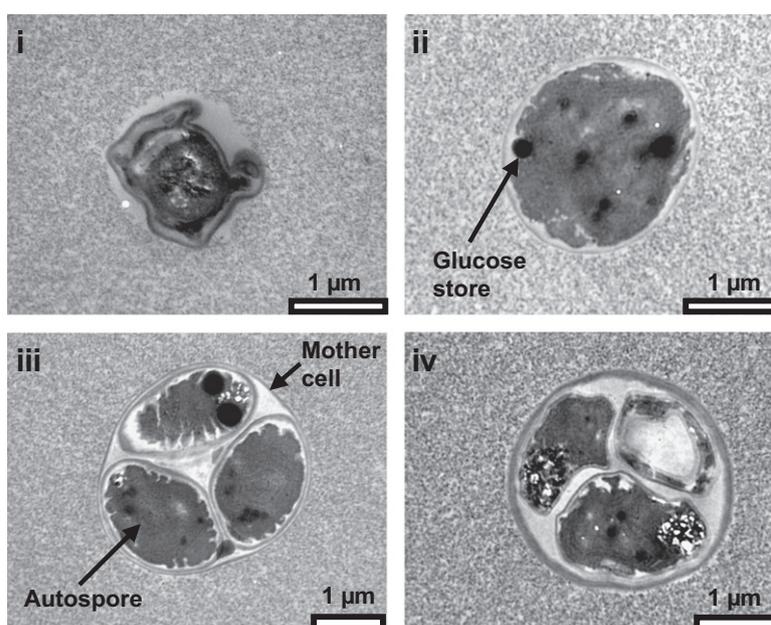


Fig. 1. TEM images of *C. caldarium* cells 94 days post immobilisation in a silica matrix obtained from a H_2SiO_3 metastable sol (Gel A).

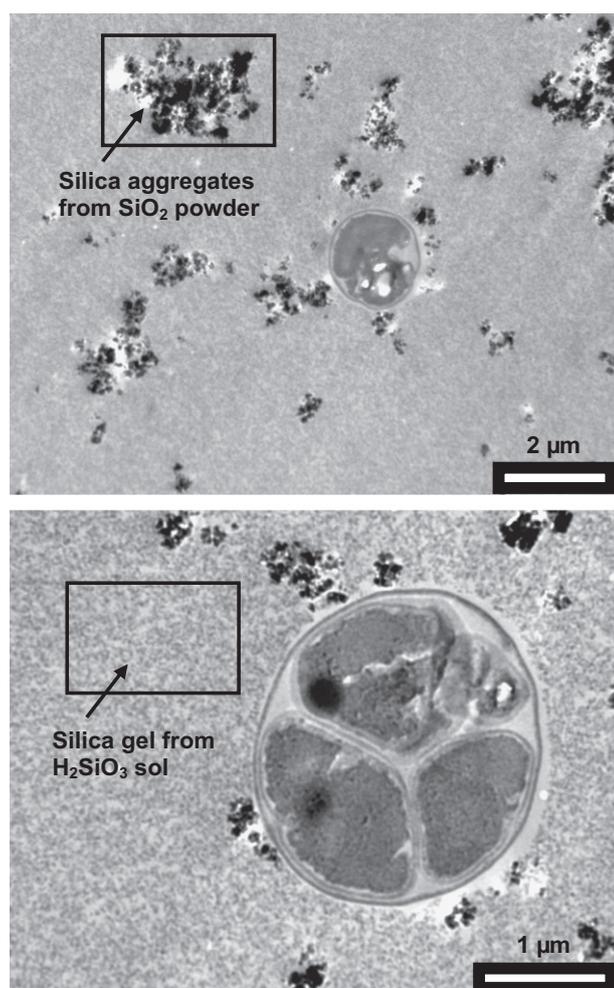


Fig. 2. TEM images of *C. caldarium* cells 94 days post-immobilisation in a silica matrix obtained from a H₂SiO₃ metastable sol fortified with a silica nanopowder (Gel B), where the rectangles identify zones of silica aggregates obtained from the SiO₂ nanopowder and silica gel obtained from the H₂SiO₃ sol.

viable cells has not diminished with time. Further to this, the presence of silica nanopowder has not affected the amount of fluorescent cells either, promoting the use of this silica source as a strengthening agent.

3.3. Cell productivity

Fluorescence alone cannot determine whether a cell is alive or dead. A cell may be dead yet the sensitive chlorophylls and accessory pigments preserved by the silica network, as previous work has shown that silica can be used in the conservation of such fragile molecules [24–26]. Conversely the cell may be in hibernation and thus the mechanism that regenerates chlorophyll has shut-down. In order to ascertain information on cell activity, experiments were undertaken to establish cell productivity from within the silica gel. CO₂ assimilation was determined by incorporating a NaH¹⁴CO₃ radiotracer into the gel precursors. The results can be found in Table 1.

These results clearly indicate the capacity of the *C. caldarium* within the living hybrid materials to assimilate CO₂ as the depletions per minute (DPM) for the hybrid gels and media far exceeds the readings taken for the blank controls. The cells for each experiment, both with and without SiO₂ nanopowder, were taken from the same culture stock within a 24 h period. As the amount of radi-

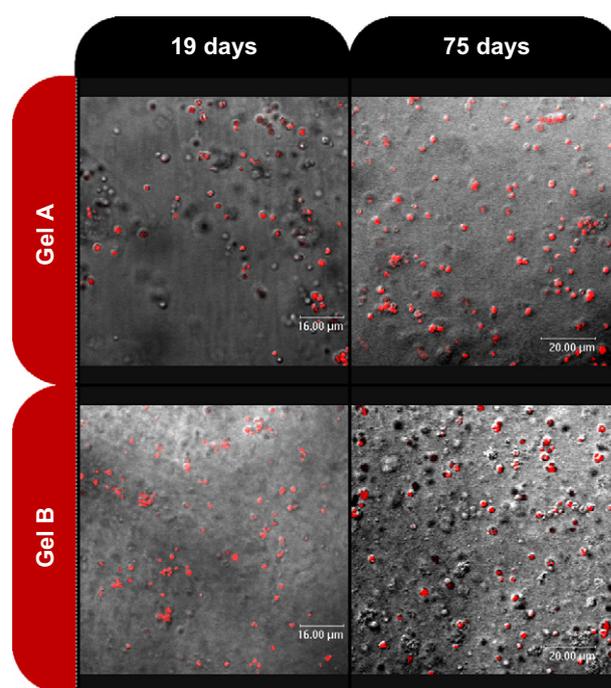


Fig. 3. Confocal microscopy images of living hybrid silica gels containing *C. caldarium* cells 19 and 75 days post-encapsulation.

Table 1

¹⁴C assimilation by *C. caldarium* immobilised in silica gel, where DPM stands for depletions per minute.

	Gel A/ ¹⁴ C DPM	Gel B/ ¹⁴ C DPM
Hybrid gel	272,000	243,000
Control gel	666	269
Hybrid media	1930	4680
Control media	219	186

otracer added to each gel was identical, these results can be compared in an approximate fashion. Here it can be seen that there is just over a 10% difference between the readings for the gels with and without nanoparticles, suggesting that the nanopowder does not significantly interfere with cell activity. Most of the assimilated carbon remains within the gel suggesting that either once assimilated it stays within the cell or that the metabolites are retained within the porous gel network and only released when the gels are crushed during this experiment. If the latter is true, it highlights the need to focus more attention on diffusion limitations. Nevertheless, some radioactivity has been excreted into the supernatant media in both samples (around 0.7% and 1.9% respectively). This would suggest that in the presence of the nanopowder, diffusion is increased, perhaps through the creation of void pockets around the silica aggregates found in close proximity to the cells.

It is possible to determine some of the building blocks of these metabolites by analysing the growth media in contact with the gels after hydrolysis with trifluoroacetic acid (TFA), using High Performance Anion Exchange Chromatography (HPAEC). In both cases the profiles of the eluting peaks were similar, with sugars such as rhamnose and galactose being detected. This is in line with studies on the cell wall of *C. caldarium* that found the walls to be rich in hemicellulose polymers, of which the hydrolysates were identified principally as galactose, glucose, mannose and xylose [27]. This may suggest that photosynthetic cells can be used to produce complex polysaccharides whose building blocks are based on these

simple sugars obtained through hydrolysis. The production of these sugars was compared to the concentration found in fresh media (origin: soil extract in media). However the results shown in Table 2 reveal how the production within hybrid gels is low. This may suggest that they do not make up the principle photosynthates of *C. Caldarium*. On careful inspection one can see that there is only a minimal increase in the values obtained from the media and Gel A, or in the case of rhamnose a reduction, within the 7 days since the media was changed which cannot really be considered a positive result. However the increase seen between the media and Gel B is more significant, with a three fold increase in rhamnose production. The HPLC analysis was carried out on the supernatant above the gels so as with the assimilation of ¹⁴C one sees how the evidence of photosynthetic activity within the media is far clearer in the gel with additional nanoparticles. Overall these results lend support to the idea that diffusion is the crux of the problem. Primary production has shown how the hybrid gels do not inhibit photosynthesis; however recovery of the photosynthates requires more thought. Currently laboratory tests are based on a static volume of supernatant on top of a hybrid gel. Work is ongoing in terms of bioreactor design. With this will bring systems in which the media is cycled through the hybrid gel or via a gravity feed, passes through the bioreactor.

Electrochemical techniques have been exploited to determine oxygen production from *C. caldarium* immobilised within silica gel. The graph in Fig. 4, representing the evolution of oxygen from the gel with time, provides evidence that the cells can continue to photosynthesise within the transparent silica gel. Furthermore, this oxygen production is sustainable for at least 11 weeks, at which the experiment was stopped as insufficient material remained. Again the results show that the presence of the SiO₂ nanopowder did not drastically affect the physiological activity of the cell, with the results for both gel systems being of a similar value at each time interval. What is also of note is that the Hansatech system is optimised on Chl *a*; aquatic photosynthetic entities usually have accessory pigments that are active at higher energies enabling light acquisition at greater sea depths. This may suggest that the results shown here are an underestimate of the capabilities of immobilised photosynthetic organisms such as *C. caldarium* as these accessory pigments cannot be activated by the red LEDs.

3.4. Photosynthetic kinetics

Photosynthetic kinetics of the immobilised cells can be ascertained using pulse amplitude modulation fluorimetry. Table 3 represents a collection of parameters captured over the course of 11 days.

These results highlight that there is still some cellular activity post immobilisation however the photosynthetic maximum efficiency, the efficiency at which the light absorbed by the PSII pigment matrix is used to drive photochemistry when the PSII centres are open, is somewhat low. This suggests that either the cells are not entirely adapted to the new environment or that a proportion of cells are inactive. Such photoinhibition stems from the fact that PSII centres are inactivated faster than they are renewed, consequently reducing the effective rate constant for PSII

Table 2

Detection of simple sugars in gel supernatants, after hydrolysis, by HPLC 7 days after media change.

	Rhamnose (ppm)	Galactose (ppm)	Glucose (ppm)	Xylose (ppm)	Mannose (ppm)
Acid media	5.98	0.700	1.15	0.397	0.615
Gel A	3.89	0.724	2.35	0.537	0.732
Gel B	20.1	1.09	3.03	1.11	0.683

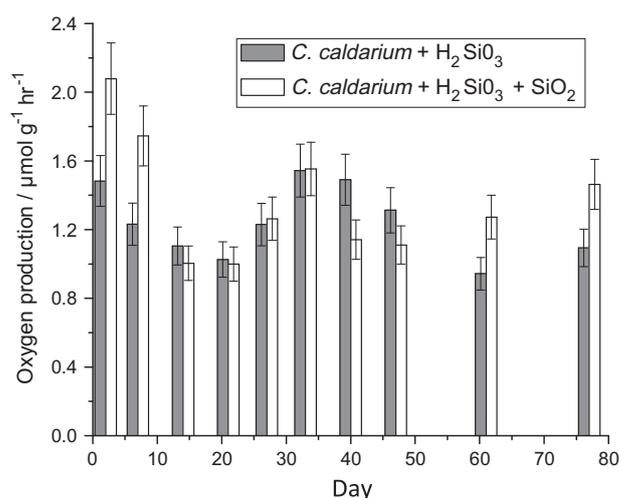


Fig. 4. Oxygen production from *C. caldarium* immobilised in silica based gels with time. Results normalised to the mass of gel used.

Table 3

Essential data on the photosynthetic efficiency of immobilised cells obtained using pulse amplitude modulation fluorimetry (PAM). Numbers in brackets represent the confidence limits within 2 sd.

Day	Fv/Fm		ΦPS _{II}		NPQ	
	Gel A	Gel B	Gel A	Gel B	Gel A	Gel B
1	0.63(2)	0.59(4)	0.4(2)	0.22(7)	0.4(2)	0.5(3)
5	0.5(1)	0.5(1)	0.4(2)	0.3(3)	0.2(3)	0.25(7)
8	0.57(9)	0.48(9)	0.38(9)	0.21(2)	0.3(5)	0.4(2)
11	–	0.64(8)	–	0.21(6)	–	0.5(3)

Fv/Fm: photosynthetic efficiency.

ΦPS_{II}: quantum yield of photosystem II.

NPQ: non-photochemical quenching.

photochemistry. The overall values for the three physical parameters quoted here are in fact different from those obtained for silica immobilised algae, whose values fell in the range expected for field observations of living leaves [19]. One difference between the synthesis procedures was the omission of culture media to the reagents prior to immobilisation. This would suggest the importance of co-immobilising nutrients within the gel and further complement the hypothesis that diffusion is limited within the matrix. However Oesterhelt et al. report that for autotrophically grown *Galdieria sulphuraria*, another acidophile in the Cyanidiaceae family, Fv/Fm is equal to (0.50 ± 0.01) which in fact is close to the values observed here for *C. caldarium* [28].

Other factors may also be in force such as the temperature of cultivation. For practical reasons studies have been carried out at ambient temperature, including cell cultivation and thus the cells may be susceptible to seasonal changes. It is clear that now the proof of concept has been established, subsequent experiments should focus on high temperature and CO₂ adapted cells working at elevated temperature environments with synthetic atmospheres similar to industrial effluents to see how these living hybrid materials respond.

4. Conclusions

This work has highlighted the potential of algae immobilisation in CO₂ remediation programmes. These results have clearly shown that *C. caldarium* can remain photosynthetically active whilst embedded within silica gel. Despite being immotile the *C. caldarium* cells can assimilate CO₂ and produce oxygen and metabolites over the course of several months providing evidence that such living

hybrid materials, containing organisms which are both acidphilic and thermotolerant, can be used in devices designed to reduce CO₂ emissions. Furthermore, the efficiency of this living hybrid material is expected to increase in elevated temperatures. All work was carried out at ambient temperature for practical reasons; however the optimal temperature for this strain is 45 °C. It is also known to exist in pH environments inferior to 1 [29].

Key improvements to the immobilising matrix in terms of minimising cytotoxic cell–matrix interactions have been achieved. TEM images have shown that the cells are able to divide into four daughter cells (autospores), yet they remain within the membrane of the mother cell or in very close proximity owing to the constricted space within the gel. Using core–shell methods it may be possible to create macrovoids within a silica gel matrix without compromising the mechanical strength of the gel. Such macrovoids should allow the cells to propagate and die much as they would in nature, ultimately prolonging the lifetime of the hybrid material.

Acknowledgments

This work was realised in the framework of “Redugaz”, an Interreg IV (France–Wallonia) project funded by the European Union and the Walloon community. The authors also thank the Interuniversity Attraction Pole (IAP-P6/17) for financially supporting “Inanomat”, a Belgian federal government project. C. Meunier and B. Vandoorne thank the Fonds National de la Recherche Scientifique (FNRS) for their Postdoctoral and Research Fellow position respectively. B.-L. Su acknowledges the Chinese Central Government for an “Expert of the State” position in the program of “Thousands Talents” and the Chinese Ministry of Education for a “Changjiang Scholar” position at the Wuhan University of Technology. H. Sarmiento benefited from a grant from the Spanish MCyl (Juan de la Cierva Fellowship JCI-2008-2727). The authors are grateful for the technical assistance of I. Virlet.

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