Novel photosynthetic CO_2 bioconvertor based on green algae entrapped in low-sodium silica gels

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A photosynthetic bioreactor for CO_2 assimilation has been designed using silica sol–gel immobilisation technologies with the chlorophyta *Botryococcus braunii* (Kützing) and *Chlorella vulgaris* (Beijerinck). The living hybrid gels formed revealed a mesoporosity that enabled diffusion of nutrients and gases, promoting the light and dark photosynthetic reactions from within the bulk of the material. To determine the efficiency of the photosynthetic bioreactor in terms of CO_2 remediation, the activity and viability of the encapsulated cells have been monitored through oximetry, ^{14}C assimilation, pulse amplitude modulation fluorimetry and confocal microscopy, revealing a long term productivity of living hybrid materials capable of photosynthetic processes for at least 80 days. Structural and textural properties of the gels were established through ^{29}Si MASNMR and N_2 physisorption respectively.

Introduction

Projects to combat the rise in atmospheric carbon dioxide (CO_2) have been popularised through necessity, as the reduction of anthropologically generated CO_2 is a priority in today's society. This greenhouse gas is one of the major factors in the current rise in average global temperatures.[1] The solution is far from easy, many different approaches are being considered from carbon storage[2] and CO_2 adsorption[3] to artificial photosynthesis.[4]

On a global scale, photosynthesis is an effective mechanism against greenhouse gas accumulation in the atmosphere as it fixes CO_2. In essence, photosynthetic systems harvest their energy from solar radiation and through a complex electron transfer mechanism, convert CO_2 and water into chemical energy, releasing O_2 as a by-product. This elegant mechanism has proved difficult to replicate *in vitro*, with the light harvesting pigments being very sensitive to external conditions. It thus seems logical to take advantage of the entire living system, the photosynthetic cell.

In nature, the photosynthesis of microalgae is by far more efficient than terrestrial plants. In algae, nearly all of the biomass is photosynthetic. In contrast, the major components of terrestrial plant biomass are roots and stems, which respire but generally do not photosynthesise. Although primary producers in the ocean (mostly microalgae) are responsible for nearly half of the primary production of the biosphere (approximately 60 Gt of C per year), they represent only 0.2% of global primary producer biomass.[5]

This paper focuses on the exploitation of microalgae to create living hybrid materials capable of CO_2 capture and conversion. These hybrid materials can then potentially be used in devices to remove CO_2 from the waste generated during industrial processes. The objective is to take living photosynthetic cells and to encapsulate them in a biocompatible host structure that can immobilise them, separating them from the gases and/or liquids in need of scrubbing, whilst offering some protection against the foreign environment.

The strains selected, *Chlorella vulgaris* and *Botryococcus braunii* were chosen based on the metabolites they produce and their prior applications. *C. vulgaris* has acquired attention due to the secondary metabolites it can produce, such as anti-tumour promoting glyceroglycolipids.[6] Furthermore, alginate immobilised *C. vulgaris* has proven to be effective in the biosorption and biodegradation of contaminants such as tributyltin,[7] nickel and copper.[8] However *C. vulgaris* and *Chlorella sp*. have predominantly been used in liquid phase photobioreactors for CO_2 fixation[9–12] owing to the greater photosynthetic capacity microalgae have over terrestrial plants and their ability to turn flue gases into biomass suitable for animal feed, rich in proteins.[11] In addition *C. vulgaris* is of great interest in the domain of CO_2 removal owing to its tolerance towards high levels of CO_2 coupled with SO*x*, NO*x* and VOCs, which are all commonly found in effluent gases. CO_2 elimination capacities of this strain in various bioreactors have been cited in the literature such as 260 mg L^{-1} hr^{-1} in a membrane photobioreactor with 1% CO_2[13] or 63.9 g m^{-3} hr^{-1} using a flow-through photobioreactor exposed to an air stream with over 1850 ppm CO_2.[12] Other researchers have suggested the production of biomass[10] and biodiesel[14] from *Chlorella sp*., grown through carbon sequestration. In a similar vein, *B. braunii* attracts interest through its production of lipids from photosynthetic CO_2 assimilation.[15–18] One study revealed

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a CO_2 fixation rate of 497 mg L^{-1} day^{-1} for the same cultivated strain, SAG 30.81.[18] On manipulation of *B. braunii*, high yields of hydrocarbons can be obtained which are suitable for hydrocracking, forming biofuels suitable to power various forms of transportation.[19] These strains have been used before on a large scale as cultures in suspension (*e.g.* for biofuel production), however these algae farms have certain inconveniences arising from being "one-shot" cultures, such as filtration problems in separating the biomass from the culture medium and generating residual waters. Hybrid materials could be an alternative to avoid some of these problems.

Several key properties have made silica an ideal choice as an immobilising matrix.[20] It is often porous, allowing nutrient media and gases to permeate throughout the support. It is also optically transparent, which enables light energy to penetrate the bulk of the hybrid material allowing photosynthesis to continue within the core of the gel. Silica is essentially chemically inert and has great mechanical strength in comparison with organic polymers previously used in cell encapsulation.[21] It can also be used for *in situ* encapsulation through biocompatible techniques.[22] The use of silica gel as a whole cell immobilising matrix has evolved from the use of TMOS and TEOS alkoxide precursors,[23] which release toxic aliphatic molecules, to an aqueous route based on the acidification of sodium silicate, water glass.[24] Previous projects have seen the successful encapsulation of fungi,[25] *E. coli*,[26] cyanobacteria,[27,28] rhodophyta[29] and whole plant cells[30] within porous silica gel *via* aqueous methods. However, the use of sodium silicate precursors results in the release of sodium chloride which can induce osmotic shock in cells not adapted to high extracellular sodium concentrations. Therefore the protocol has been modified to form the metastable intermediate in the aqueous route to silica gel (silicic acid; H_2SiO_3) prior to *in situ* encapsulation, as first seen in the immobilisation of thylakoids.[31]

This work concentrates on the immobilisation of unicellular green algae, from the division chlorophyta, owing to the relationship between green algae and higher plants. Green algae are eukaryotic organisms that are generally aquatic, having chloroplasts that contain chlorophyll *a* and *b* as well as beto-carotene and xanthophylls and store food as starch in their plastids. One school of thought is that higher plants emerged from green algae.[32] Unicellular green algae may be advantageously more resistant to immobilisation, as they are independent discrete entities not relying on a higher infrastructure of cells. As such they could be more easily adaptable to the new environment, the separation of cells by the porous framework of the gel, ultimately yielding gels that are active for a much longer duration.

Both *B. braunii* and *C. vulgaris* have been successfully embedded in sol–gel ceramics (biocers) by Soltmann and Böttcher.[33] They employed dip coating techniques to good effect, revealing that an 80–100% cell survival rate can be achieved 12 and 25 days post-immobilisation respectively for cells stored at 4 °C in the dark. This promotes the idea that silica sol–gel technology should enhance the properties of hybrid materials based on *C. vulgaris* and *B. braunii*. Furthermore it has been shown that *C. vulgaris* can survive immobilisation using sodium silicate type matrices with the eventual goal of designing novel optical biosensors to detect PSII inhibitory herbicides.[34,35] Finally it has recently been revealed that sol–gel technology can be successfully employed to immobilise the chlorophyta *Haematococcus pluvialis*.[36] The cells are of interest owing to their ability to produce astaxanthin, however the glycol ether solvents required to extract this compound are cytotoxic. Co-immobilising glycerol, a protecting additive, into the sol–gel layers has prolonged cell viability beyond 40 days.

The vast bank of evidence in the literature that suggests microalgae have great capacities to sequestrate anthropologically generated CO_2, coupled with previous immobilisation studies, advocate a promising future for silica encapsulated green algae in the domain of CO_2 mitigation technologies.

Experimental

Chlorophyta strains

Axenic algal strains, *Chlorella vulgaris* (SAG 211-11b) and *Botryococcus braunii* (SAG 30.81), were obtained from the SAG culture collection (*Sammlung von Algenkulturen der Universität Göttingen*) and cultivated in ESP media.†

Immobilisation methodology

Algae immobilisation within silica gel was carried out *via* a "low-sodium" aqueous route, previously used in the entrapment of thylakoids.[31] A H_2SiO_3 metastable sol was obtained by passing a sodium silicate solution (1.5 M, assay 25.5–28.5%, Merck), refrigerated to 4 °C, over an acid ion exchange resin (Amberlite IR 120, Acros). The resin had previously been rinsed with chilled acidified water (pH 2, acidified with HCl). The resin-silicate mix was vigorously agitated for 10 min to increase contact then the "low-sodium" sol was recuperated *via* filtration. The precursor was sterilised by passing it through a mechanical filter (0.22 μm).

Cells were harvested from a suspension culture (20 mL, *ca.* 4 weeks since inoculation) through centrifugation (1,200 rpm, 10 min), the supernatant discarded and the pellet resuspended in ESP growth media (4X concentrate). Meanwhile, 240 μL KOH (0.2 M) was added to 4 mL of H_2SiO_3 to obtain a sol at pH 6 to which the resuspended cells were immediately added. Gels formed within a few minutes. In certain cases 150 mg SiO_2 nanopowder (99.5%, 5–15 nm, Aldrich), was homogenised with the silicic acid sol *via* sonication for 5–10 min prior to basification, to form agglomerates that would strengthen the resultant silica matrix. Finally, fresh ESP media was added on top of the preformed 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.

† SAG 211-11b was isolated from a eutrophic pond near Delft, Netherlands and SAG 30.81 was isolated from Laguna Huaypo, Dpto. Cuzco, Peru. The stock cell suspension cultures were maintained at 25 °C under fluorescent strip lighting and transferred into fresh basal medium with 0.1% Proteose-Peptone (ESP – Erddekokt + Salze + Peptone), details of which can be found on the EPSAG website.

Characterisation techniques

Porosimetry. Nitrogen adsorption–desorption experiments were performed at −196 °C using a Micromeritics TRISTAR 3000. The samples were dehydrated in ethanol then supercritically dried with carbon dioxide to preserve the porous structure of the wet gels. The pieces of aerogel obtained were then degassed under vacuum at 60 °C overnight prior to performing the measurements. Specific surface areas were calculated using the BET (Brunauer–Emmett–Teller) equations and the porosities were characterised by the BJH (Barrett–Joyner–Halenda) method to determine the average pore diameter and the pore size distributions.

Solid state nuclear magnetic resonance. ^{29}Si magic angle spinning nuclear magnetic resonance (MASNMR) spectra were recorded on a Bruker MSL 400 MHz spectrometer.

Transmisson electron microscopy. Transmission electron microscopy (TEM) was performed on a Philips Technai 10 with an accelerating voltage of 80 kV. Cell fixation was carried out prior to analysis using the following method: *ca.* 1 mm^3 pieces of gel were placed in a fixative (2.5% gluteraldehyde in sodium cacodylate buffer; 0.1 M, pH 7.4) coloured with a ruthenium red stain and left at 4 °C. After 3–4 h the samples were rinsed in 0.2 M sodium cacodylate buffer at pH 7.4 then left overnight in a 1% OsO_4 solution, again buffered with 0.1 M sodium cacodylate to which the ruthenium red stain had previously been added, for post-fixation. After further rinsing in sodium cacodylate buffer, the samples were dehydrated through a graded ethanol series with the final steps in propylene oxide. Finally the samples were set in epoxy resin, LX112 and then cut using an ultramicrotome.

Confocal microscopy. The autofluorescence of the photosynthetic pigments within the immobilised chlorophyta was analysed 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 650–750 nm.

Carbon dioxide assimilation. Carbon dioxide assimilation by immobilised chlorophyta was verified *via* the ^{14}C incorporation method.[27,29] A radioactive tracer, $NaH^{14}CO_3$, was added to the 4X concentrate ESP media and the immobilisation carried out as above. After an incubation period of 72 h the gels were broken down with 1 M HCl to neutralise the excess bicarbonate. The samples were subsequently bubbled with air overnight, to dissipate the inorganic $^{14}CO_2$, leaving only organic ^{14}C assimilated and excreted by the cells. An Insta-Gel Plus (Perkin Elmer) scintillation cocktail was added and mixed with the aid of a vortex. Radioactivity was measured using a Beckman scintillation counter (LS 6000 SC) and the external standard method for quench correction.

Oxygen production. A Clark type polarographic sensor comprised of a central platinum cathode and a concentric silver anode bridged with a potassium chloride saturated thin paper wick was employed in a Chlorolab 2 system by Hansatech to detect oxygen evolution from the hybrid gels. A portion of the gel was taken for destructive analysis every 7 days and the media of the parent sample was refreshed. The samples were cut into 3 mm^3 sized die and suspended in 800 μL aliquots of ESP media supplemented with 4 μL 0.6 M $NaHCO_3$. The system was purged of dissolved oxygen and closed to the atmosphere. The samples were illuminated by a collection of red LEDs centred on 650 nm with an intensity fixed at 700 μmol m^{-2} s^{-1} and the rate of oxygen evolution data was acquired on a PC.

Pulse amplitude modulation fluorimetry. A pulse modulated chlorophyll fluorometer (FMS 2 by Hansatech) was employed to determine pertinent data, such as the quantum yield of photosystem II, photosynthetic efficiency and non-photochemical quenching, of cells 1 day and 1 month post immobilisation. The gels were made in a disc form within a Petri dish, with a diameter equal to the diameter of the light source. The samples were kept in the dark for 15 min prior to analysis and inbetween analyses media was added to the Petri dish.

Results and discussion

Photosynthetic bioreactor design

Immobilisations of the chlorophyta *Chlorella vulgaris* and *Botryococcus braunii* were based on a combination of previous studies with cyanobacteria[27,37] and with plant cells.[30] The chlorophyta (green algae) have similarities with both types of organisms. Whilst the chlorophyta chosen are unicellular aquatic photosynthetic organisms, similar to *Synecococcus* cyanobacteria used previously, they in fact belong to the plantae and not the bacteria kingdom and are as such eukaryotic cells, with the potential to be more susceptible to external factors than cyanobacteria.

For reasons of biocompatibility, whole cell immobilisation is often performed using aqueous sol–gel techniques, thus avoiding the release of alcohol arising from the hydrolysis and polycondensation of alkoxide silica sources.[24] As both *C. vulgaris* and *B. braunii* come from freshwater sources† it would seem logical that their tolerance to saline environments is limited. For this reason a "low-sodium" silica gel was selected as the host scaffold. In such cases the sodium silicate aqueous precursor was passed over an acid ion exchange resin, allowing the replacement of sodium by hydrogen, facilitating the formation of the metastable intermediate sol in the aqueous route to silica gel, H_2SiO_3, prior to *in situ* cell encapsulation.[31]

Initial trials were carried out at pH 8, a pH previously optimised for cyanobacteria immobilisations in sodium silicate based gels.[27] However the rehydrolysis of the silica gel was rapid, occurring between a week and a month post encapsulation. This problem was resolved by working at pH 6. This could be a direct result of the variance in gel formation at different pH levels. Under basic catalysis conditions highly branched clusters normally form whereas under acidic catalysis primarily linear and randomly branched polymers form. This may lead to greater interconnectivity between the silica species, strengthening the network towards rehydrolysis.[38] One other possible hypothesis is the role Na^+ plays in strengthening or cross-linking the gels. Liquefaction was rarely a problem in sodium silicate based cyanobacteria immobilisations yet when

H_2SiO_3 was employed, diluted by growth media, there was a tendency for gels to liquefy that were fabricated with media of lower Na^+ concentration.[37]

Whilst being too acidic for cyanobacterial cells, pH 6 is in fact similar to the pH used in the immobilisation of whole plant cells.[30] Having common ancestry with higher plants means that green algae have many similarities to more complex plant cells and as such they have a higher tolerance towards more acidic environments such that this adjustment in pH did not inhibit photosynthetic activity.

Structural characterisation of living hybrid gels

For purposes of clarity the gels discussed have been encoded as seen in Table 1. These hybrid gels were characterised by solid state ^{29}Si MASNMR to determine the degree of condensation within the abiotic support, thus alluding to the extent at which the matrix contracted on formation (Fig. 1).

These results show not only that in all cases the major share of silica is found in the Q_4 environment (SiO_4, $\delta = -111$ ppm) but also that there is a significant contribution from Q_3 (SiO_3OH, $\delta = -101$ ppm) indicative of terminal silanol groups on the surface of the gel. This contribution is far greater than found in the case of cyanobacteria immobilised in sodium silicate based gels,[24] which can be explained by the vast increase in surface area as found from the adsorption-desorption of nitrogen, (Table 1).

The supercritical drying process preserves the porous network by avoiding the collapse of pores through capillary action. Adsorption-desorption measurements carried out on gels dried at 60 °C without pre-treatment grossly underestimated the pore dimensions and the surface areas too were slightly reduced. Analysis of the textural properties of the gels reveals a slight decrease in surface area on the inclusion of cells within silica gel for all cases and the mesopore diameters were between 11–17 nm. The surface areas were very high compared to sodium silicate based gels.[27] This vast increase in surface area may suggest a great improvement to the porous network, aiding the diffusion of nutrients and gases throughout the bulk of the hybrid material.

One interesting observation of these hybrid gels is their tendency to liquefy, especially those containing *B. braunii*, when disturbed. When the gels were regularly cut up for destructive analysis such as oximetry measurements, the remaining gel became weaker, liquefying within 5–8 weeks. Generally, this was not the case for gels left within their mould. Therefore physical as well as physiological interactions previously discussed[27,29–31] may play a role in the disintegration of living biomaterials.

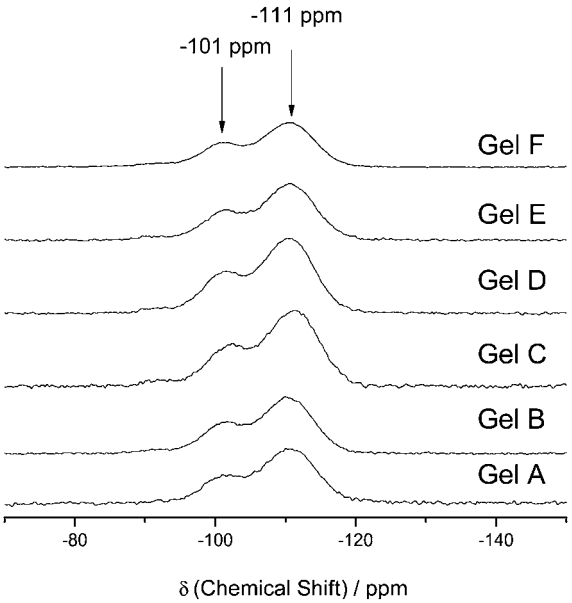

Fig. 1 ^{29}Si MASNMR spectra of silica gels obtained from H_2SiO_3 and dried at 60 °C; where (A) and (B) are control gels with no cells; (C) and (D) contain *C. vulgaris*; (E) and (F) contain *B. braunii*. Gels (B), (D) and (F) have been reinforced with silica nanoparticles.

Viability of living hybrid gels

Images A–H in Fig. 2 show how cell preservation is possible at least one month post encapsulation for *C. vulgaris*. The amount of silica dissolution increases with time, although it is less pronounced than with certain cyanobacterial strains in silica gel.[27] This dissolution can be seen in the voids that appear around the cell as time progresses, image D *cf.* image A. This would suggest that the interactions between silica and the cell walls of algae differ from that of cyanobacteria. When looking at the cells present in the 1 month ultramicrotome cut of Gel C, there are many examples of deformities from the spherical form of the chlorophyta compared to the other gels but this may not be a true reflection on the sample as a whole considering the activity of the cells is comparable with a gel fortified with SiO_2, as revealed in the following section.

The images in Fig. 2 for Gels D and F show how the silica nanopowder has aggregated to form clusters large enough to not interfere with the active sites of the algae cells. Within Gel D there are several examples of cellular division 1 week post encapsulation, however as the room to grow within the void is limited there is much competition for space. This fight for space

Table 1 Composition and physical properties of the hybrid silica gels and the blank controls

	Gel A	Gel B	Gel C	Gel D	Gel E	Gel F
Cells	NO	NO	*C. vulgaris*	*C. vulgaris*	*B. braunii*	*B. braunii*
H_2SiO_3/mol dm^{-3}	1.5	1.5	1.5	1.5	1.5	1.5
SiO_2 nanopowder (5–15 nm)/mg	0	150	0	150	0	150
Surface area (BET)/m^2 g^{-1}	795	661	755	618	728	603
Average pore diameter (BJH)/nm	20.0	12.5	13.0	14.7	11.2	16.7
Average pore volume (BJH)/cm^3 g^{-1}	4.10	1.7	2.3	2.0	1.8	2.2

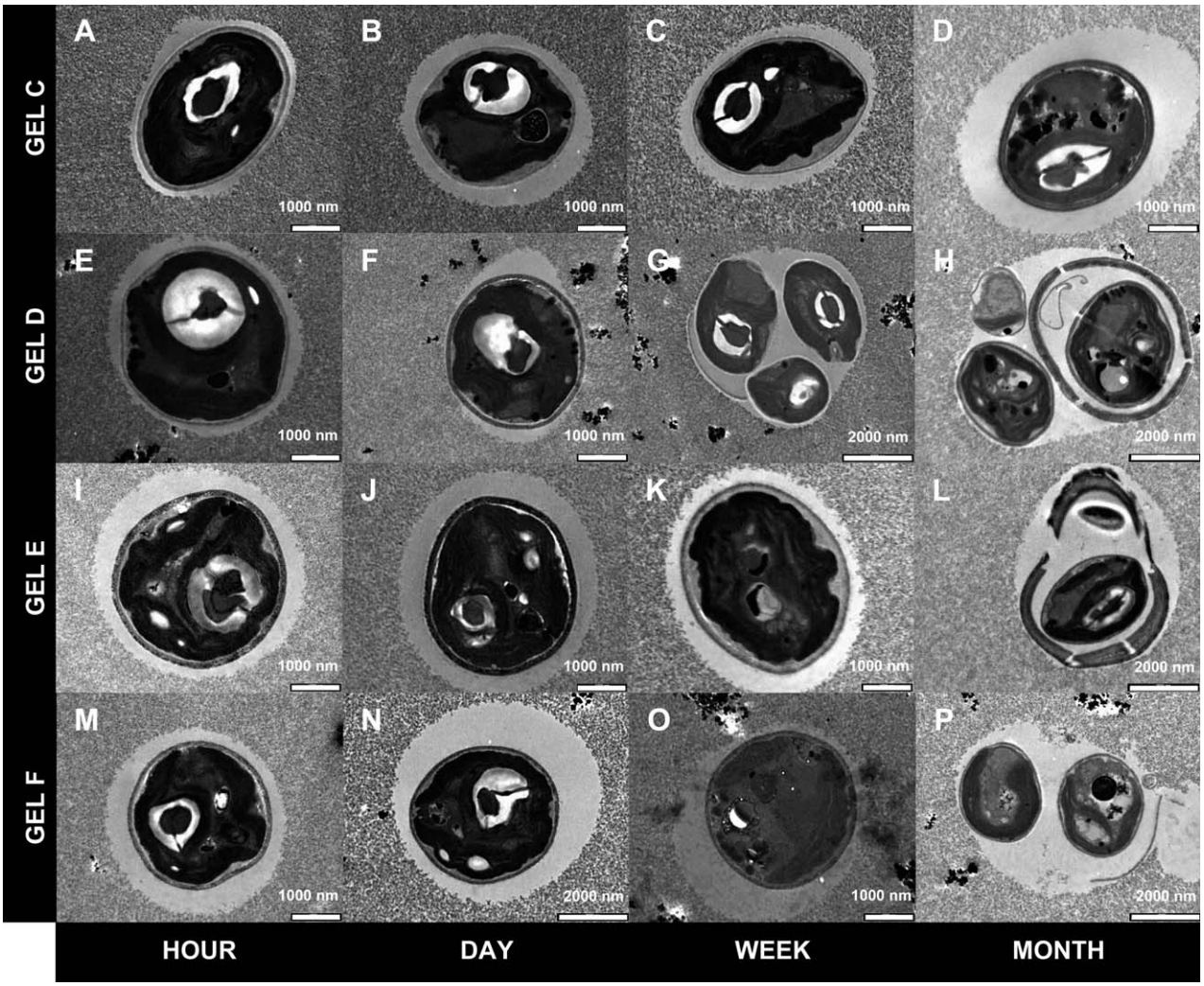

Fig. 2 TEM images of living hybrid gel C and D containing *C. vulgaris* and gel E and F containing *B. braunii* taken 1 hour, 1 day, 1 week and 1 month post encapsulation.

is best shown in image H, which reveals that not all daughter cells survive from the division of the parent cell. However cellular division post encapsulation is common to both *C. vulgaris* and *B. braunii* as images G and P reveal. Images I–P highlight that cell wall integrity is also preserved post encapsulation for *B. braunii* over the course of one month and that cellular division is possible within the gel, yet not all cells can survive in the limited space. Image L reveals the presence of the cell wall of the parent cell that has burst, releasing the next generation of daughter cells; however within the plane of the ultramicrotome cut we see only one cell that is intact with a much smaller entity that has no apparent nucleus or photosynthetic membrane above. This is strong evidence that the growth of silica encapsulated cells is problematic, yet if the ability of the surviving generation of cells to assimilate CO_2 can be preserved then in turn the activity of the living hybrid material could be prolonged. For all gels there was no evidence of cellular division in the cuts made 1 h after immobilisation, suggesting that the divisions really did occur post encapsulation. Although to observe this cellular division is an advance in the domain of photosynthetic cell immobilisation, the lack of space could have a detrimental effect on the new cells in close proximity if the dying cells were to transmit signals to trigger cell death in the surviving cells, in other words, work must continue on the optimisation of void space within the gel to target a truly living functional material.

These TEM images have revealed the nuclei, starch stores and photosynthetic membranes of the immobilised cells as expected. However, a cationic dye, ruthenium red, was employed that specifically highlights polysaccharides in order to monitor any extracellular secretions. As was the case in the work of Livage[39] in the immobilisation of *C. fusiformis* in silica gel, the ruthenium red has revealed some evidence of excreted compounds into the voids around the cell, as seen in the magnified image in Fig. 3. This may suggest that the cells are able to biotransform CO_2 within the gel into more useful compounds.

The preservation of photosynthetic apparata was monitored using confocal microscopy (Fig. 4 and 5). These results suggest that the pigments can be preserved at least 118 days post encapsulation, providing evidence of the cells' photosynthetic activity in silica gel. Furthermore there is a high concentration of autofluorecent cells over time, indicating a high percentage of cells that maintain chlorophyll *a* regeneration within the gel, thus

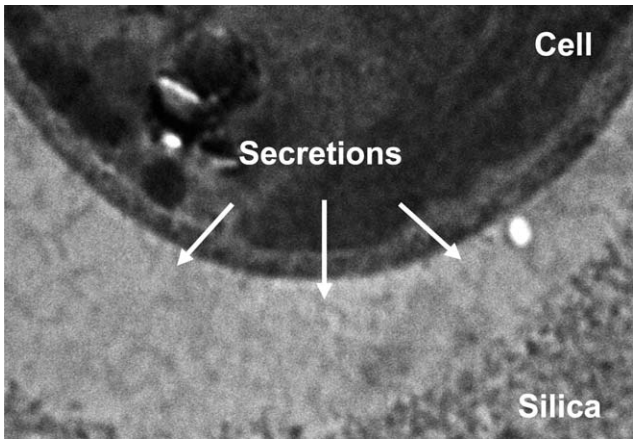

Fig. 3 Magnified zone of Fig. 2O; Gel F containing *B. braunii* taken 1 week post encapsulation, identifying the cell, silica support and excreted polymers within the void.

yielding cells with the potential to assimilate CO_2 within the silica itself.

In the case of *B. Braunii* the gel had liquefied prior to the measurements taken at day 86 and beyond and in such cases the cells can migrate to the growth media phase. There are subsequently no longer immobile. The images often reveal the presence of clusters of cells, either formed during harvesting *via* centrifugation or that the strains form colonies when grown in suspension. Such aggregations of cells may permit greater porous voids to be formed, allowing more space for cellular replacement, with cells growing and dividing whilst in the gel. In each case there is evidence of either senescence or cell death by the presence of grey, non-fluorescing cells.

CO_2 assimilation and long term productivity

The photosynthetic activity, in particular the Calvin cycle, of the cells immobilised within silica gels has been evaluated. To ensure the validity of this technique, used previously in the immobilisation of cyanobacteria[27] and rhodophyta,[29] an immobilisation of *C. vulgaris* was carried out in the light as well as in the dark and the results then compared (Table 2).

From Table 2 we can see that there is a vast difference in the radioactivity measured from samples kept in the dark as opposed to those left in the light. Therefore little assimilation of $^{14}CO_2$ occurs during the fabrication of the gels pre-encapsulation, most is assimilated upon immobilisation confirming that the Calvin cycle is not interrupted post encapsulation. The same experiment was repeated, in the light, for gels C–F and the results can be found in Table 3.

These results highlight that a substantial uptake of $^{14}CO_2$, obtained from $NaH^{14}CO_3$, continues within the living hybrid gels in all cases, revealing that all materials are photosynthetically active. No comparison can be made between strains or about the inclusion of nanoparticulate silica as the concentration of cells per gram of gel is unknown. Long term productivity studies were performed by oximetry measurements to determine the oxygen production rate with time for gels C–F. Results are shown in Fig. 6.

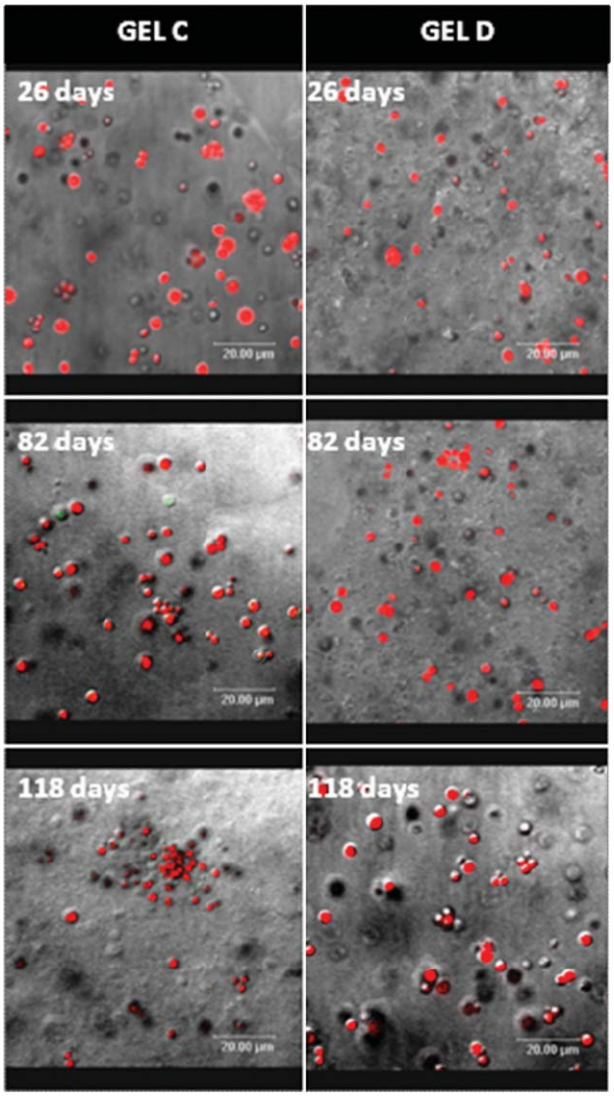

Fig. 4 Confocal microscopy images overlaid onto optical microscopy images of *C. vulgaris* immobilised in silica gel with time (26, 82 and 118 days post encapsulation). Gel D corresponds to a gel fortified with silica nanoparticles.

Considering experimental errors, the upper chart (*C. vulgaris* immobilised in silica gel) shows that oxygen production can be sustained for over 50 days with small fluctuations from the reading taken on the first day. Conversely the lower chart (*B. Braunii* immobilised in silica gel) shows a steady decrease in oxygen production over time. Analysis was stopped much sooner for Gel E owing to liquefaction. Furthermore, activity has been detected beyond 80 days in certain cases. These results reveal that the inclusion of a silica nanopowder does not hinder the photosynthetic capacity of the immobilised cells, unlike the use of colloidal nanoparticulate silica, Ludox, in the immobilisation of the red algae *Cyanidium caldarium*.[29] In fact it plays a vital role in preventing liquefaction, imparting robustness to the gel.

The results in Table 4 highlight the decrease in photosynthetic activity with time, most probably due to the stresses exerted on the cells isolated from their natural environment, or from oxygen saturation within the gel. An oxygen rich environment can pose problems through the generation of oxygen radicals which in

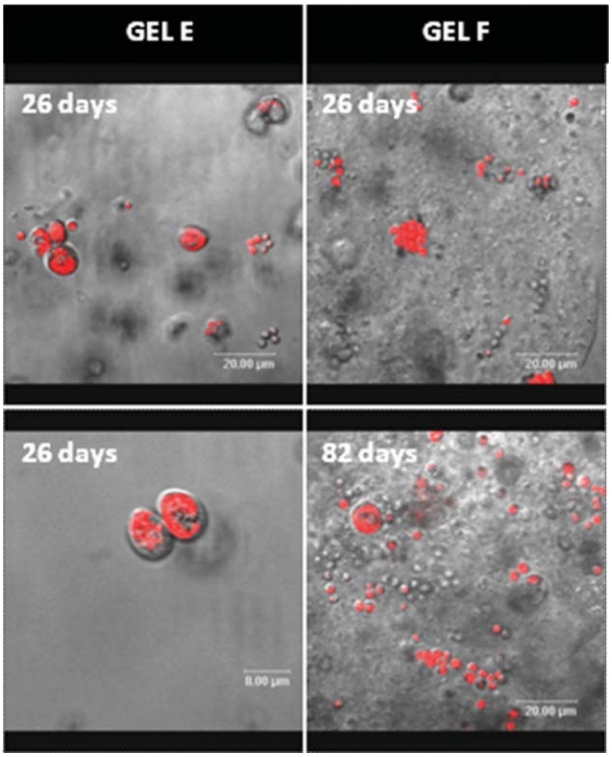

Fig. 5 Confocal microscopy images overlaid onto optical microscopy images of *B. braunii* immobilised in silica gel with time (either 26 or 82 days post encapsulation). Gel F corresponds to a gel fortified with silica nanoparticles.

Table 2 Primary production data from *C. vulgaris* immobilised in silica gel, in each case the result is an average of three samples (3SF).[a]

	^{14}C DPM in light	^{14}C DPM in dark
Hybrid gel	668000	34300
Blank gel	1200	452

[a] DPM stands for depletions per minute.

Table 3 Primary production data from *C. vulgaris* and *B. braunii* immobilised in silica gel, in each case the result is an average of three samples (3SF).[a]

	Gel C	Gel D	Gel E	Gel F
Hybrid gel/^{14}C DPM	235000	183000	157000	176000
Blank gel/^{14}C DPM	289	452	355	427

[a] DPM stands for depletions per minute.

turn attack the cells. However there is still some significant activity one month post encapsulation which is promising for the long term viability of sol–gel encapsulated whole cells. A decrease in Fv/Fm can be an indicator of nutrient stress until the cells become acclimatised to the stressed conditions.[40] If this is the case then either the diffusion needs to be improved, the media replenished or the cells need to be acclimatised prior to immobilisation by restricting growth nutrients. More in depth studies

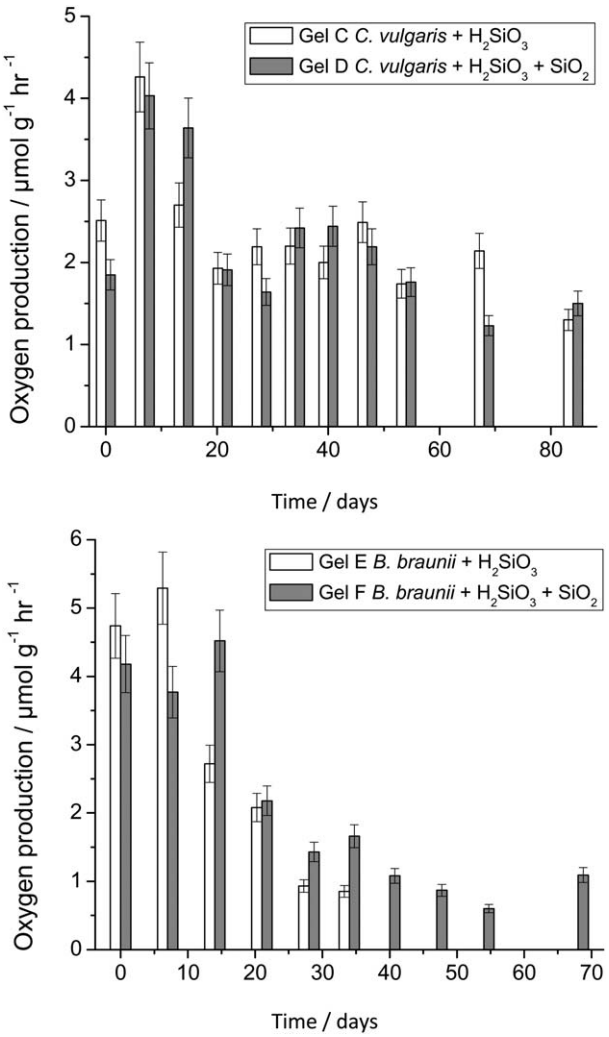

Fig. 6 Normalised oxygen production rate of hybrid gels C–F with time; (upper) *C. vulgaris*; (lower) *B. braunii*.

on the photosynthetic kinetics of cells now needs to be performed for a deeper understanding of the effect encapsulation has on the living cells.

Overall this research suggests that the living hybrid materials fabricated from *C. vulgaris* were more durable in terms of both cell viability and consistency of the matrix; although it was possible to maintain an intact *B. braunii* gel for several months if it was left undisturbed. As the gels were dissected for analysis purposes they became weaker, accelerating the effect of liquefaction and thus cell leaching. The onset of liquefaction was delayed in gels fortified with silica nanopowder, suggesting that the aggregations formed played a major role in increasing the mechanical strength of the hybrid gel. Furthermore, the silica nanopowder did not significantly interfere with the active sites of the cells as the materials continued to photosynthesise, producing oxygen for extended periods. The potential of these immobilised cells to photosynthesise was confirmed through a kinetics study, although their ability is significantly reduced over time as the cells senesce. This long-term viability and activity, revealed through microscopy and oximetry, holds much

Table 4 Essential data on the photosynthetic efficiency of immobilised cells obtained using Pulse amplitude modulation fluorimetry (PAM)[abc]

	Fv/Fm		ΦPS_{II}		NPQ	
	Day	Month	Day	Month	Day	Month
Gel C: *C. vulgaris*	0.850(8)	0.62(5)	0.69(2)	0.33(4)	0.17(5)	0.16(3)
Gel D: *C. vulgaris* with SiO_2	0.811(1)	0.65(1)	0.60(2)	0.35(3)	0.24(6)	0.12(5)
Gel E: *B. braunii*	0.85(1)	0.75(4)	0.72(2)	0.46(4)	0.22(4)	0.19(2)
Gel F: *B. braunii* with SiO_2	0.82(1)	0.70(2)	0.584(7)	0.42(3)	0.26(2)	0.19(6)

[a] Fv/Fm Photosynthetic efficiency. [b] ΦPS_{II} Quantum yield of photosystem II. [c] NPQ Non photochemical quenching.

promise for photobioreactors based on silica immobilised cells that target continued CO_2 assimilation.

Conclusions

These results confirm that the use of silica sol–gel encapsulation technologies can be extended to the immobilisation of green algae. The use of a low-sodium matrix should be inherently better at preserving the cells than a matrix derived from sodium silicate directly as the cells originate from fresh water sources, and as such the ESP growth medium has a very low sodium concentration in comparison to the BG-11 and ASN III media used in the cultivation of cyanobacteria in previous immobilisations. However, careful attention needs to be paid to the pH during encapsulation. Gels formed at pH 8 had a greater tendency to liquefy regardless of the strain employed.

These results show how the photosynthetic activity of chlorophyta can be preserved after immobilisation within a 3D silica gel structure, with both light and dark cycles, O_2 production and CO_2 assimilation respectively, continuing post-encapsulation. The photosynthetic apparatus of *Botryococcus braunii* and *Chlorella vulgaris* are capable of autofluorescence 82 and 118 days after entrapment respectively, with liquefaction being the limiting factor to preservation in the case of *B. braunii*. Upon liquefaction the cells retain the green colour imparted by chlorophyllous pigments and can even begin to proliferate the growth media and dissolved silica mix highlighting that even though encapsulation inhibits cell division for extended periods of time, the growth cycle can begin again upon liberation.

One potential optimisation in the domain of CO_2 mitigation by microalgae is to use genetically modified cells, selected for increased efficiency and productivity. The advantage living hybrid materials then hold is that the cells are imprisoned and separated from wild strains. The release of genetically modified strains may have devastating effects on the environment as they invade and colonise regions populated by wild strains, subsequently competing for vital nutrients and ultimately could disturb natural ecosystems.

These living hybrid materials should find uses in CO_2 sequestration technologies in cases where cell separation and recuperation are ideal. Photobioreactors based on such materials could play a key role in CO_2 management projects to reduce anthropological CO_2 output from factories, transportation and domestic heating systems in a convenient and efficient fashion. It may also be used to oxygenate environments or to promote the production of secondary metabolites that are formed as a direct result of external cellular stresses.

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