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Hybrid photosynthetic materials derived from microalgae *Cyanidium caldarium* encapsulated within silica gel

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ABSTRACT

Cyanidium caldarium (Tilden) Geitler SAG 16.91 has been encapsulated within a porous silica host structure to target novel photosynthetic hybrid materials suitable for use in solar cells or CO_2 fixation. *C. caldarium* cells are both thermophilic and acidophilic; on account of these tolerances the hybrid materials could be employed in more extreme heat conditions. TEM highlights that the external cell membrane can remain intact after encapsulation. The images reveal an alignment of silica gel around the external membrane of the cell, providing evidence that the cell wall acts as both a nucleation and polymerisation site for silica species and that the silica scaffold formed by the aggregation of colloidal particles, generates a porosity that can facilitate the transport of nutrients towards the cell. Epifluorescence microscopy and UV–visible spectroscopy have revealed the preservation of photosynthetic apparatus post-immobilisation. Productivity studies showed how the presence of silica nanoparticles within the matrix can adversely interact with the exterior cellular structures preventing the production of oxygen through photosynthesis.

1. Introduction

The photosynthetic reaction is one example of the great efficiency of nature. In principle, this reaction converts solar radiation to a more functional form, chemical energy. This is achieved by fixing carbon dioxide from the atmosphere. As a result of the conversion of carbon dioxide to metabolites, such as starches, sugars and carbohydrates, oxygen is produced as a by-product, thus it is believed photosynthesisers have changed the Earth's atmosphere over time making aerobic respiration possible [1].

There are several organisms capable of photosynthesis such as plants, cyanobacteria and rhodophyta. This report focuses on the photosynthetic eukaryote *Cyanidium caldarium*. This strain belongs to the class of rhodophyta, more commonly called red algae. *C. caldarium* is both thermotolerant and acidophilic and as such is predominately found in hot acidic springs. Acidic waters have quite a unique ecology [2,3], the absence of photosynthetic prokaryotes such as cyanobacteria in acidic thermal springs has yielded the evolution of rare eukaryotic species that are found nowhere else on Earth. The key alga of such environments is *C. caldarium*, an organism capable of surviving a pH as low as zero. This strain can also survive in temperatures of up to ca. 57 °C [4]. Therefore, the feasibility of the immobilisation of this strain has been investigated owing to the possibility of producing a photobioreactor that can be used at elevated temperatures and higher acidity.

Silica materials are an excellent chose of host structures for living cells owing to several key properties. Firstly, porosity can be introduced into the materials through the methods of fabrication [5]. A porous material is a priority in order that the nutrients required to maintain viable, living cells can be delivered direct to these cells that are found within the hybrid materials. Secondly silica materials have increased chemical stability and improved mechanical strength in comparison to organic polymers commonly used in bioencapsulation [6,7]. Silica materials are also biologically inert, non-toxic and being inorganic are not a source of food for micro-organisms [6,7]. Crucially, silica materials are optically transparent, thus light radiation can penetrate the network to reach the cells immobilised within the middle of the material thus permitting photosynthesis to continue within a hybrid material [8]. A *chimie douce* technique developed by Livage has been successfully applied to the immobilisation of living cells within silica gel [9]. The reaction pathway employs aqueous precursors and thus avoids the liberation of alcohols which occurs when silica alkoxides are used to target silica frameworks. The liberation of alcohol

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would cause the cells to die as they are sensitive to molecules such as MeOH and EtOH [10].

Although a series of works on the immobilisation of bioactive species in porous inorganic materials has been carried out [9–12] the prolongation of activity and thus the survival of these species, coupled with the ability of the cells to divide within the encapsulating media, remains a great challenge. One obstacle is the lack of knowledge about the interactions between the inorganic material and the bioactive species it encapsulates and how this interaction affects the survival and possible division of the bioactive cells. The understanding of the surface modification of the cell membranes surrounded by an inorganic hydrogel is a vital step towards successful preservation of bioactivity within porous materials, which ultimately would advance this novel domain of bioencapsulation.

The encapsulation of the thermotolerant and acidophilic photosynthetic alga *C. caldarium* within porous silica gels, to target photobioreactors capable of producing oxygen and biofuels from CO_2, H_2O and solar radiation in a facile manner, has been performed. This study explores the interfacial interaction between an external cell membrane and silica gel, the surface modification of the external cell membrane, the possible penetration of the cell by inorganic matter and the effect these phenomena have on the survival and bioactivity of the encapsulated photosynthetic algae.

2. Materials and methods

An axenic strain of *C. caldarium* SAG 16.91 was obtained from the Culture Collection of Algae (SAG[2]) at the University of Göttingen. Silica gels were synthesised using a method adapted from the literature [9,13] successfully used in previous cyanobacteria immobilisations [10,14] Briefly, 20 cm^3 of growing culture were harvested through centrifugation (1000 rpm, 10 min), the supernatant discarded and the cells resuspended in ca. 1 cm^3 glycerol (pharmaceutical grade, Merck) and 2 cm^3 LUDOX HS-40 (Aldrich). This mixture was added to 2 cm^3 sodium silicate solution (Assay 25.5–28.5%, 10× dilution, Merck) and thoroughly mixed. The gel formed within a couple of minutes on the addition of a few drops of HCl (3 M) such that the pH of the sol was between 7 and 8. Fresh growth media (pH 4) was added on top of the gels and these wet gels were left to photosynthesise under fluorescent lights indefinitely. For the cellular activity (production) studies gels without glycerol or without both glycerol and LUDOX were also prepared.

Several techniques have been employed to detect the viability and survival of *C. caldarium* cells undergoing an immobilisation process, the gels characterised were all made at least a day in advance yet were less than 1 week old. Exploiting the electronic properties of the photosynthetic pigments is an effective way to monitor whether the components of a cell can survive encapsulation. The pigments were analysed via UV–visible spectroscopy on a Perkin Elmer Lambda 35 spectrometer where the free cell suspension was measured in absorbance mode whereas the hybrid gel was analysed in reflectance mode with the aid of the solid state detector (Lambda 35 integrating sphere). Microscopy techniques have also been employed to look at the cell structures in both free and immobilised cells. Transmission electron microscopy was performed on a Philips Tecnai 10 with an accelerating voltage of 100 kV using the fixing and staining methods previously described for cyanobacteria [14]. Scanning electron microscopy images were acquired with the aid of a Philips XL 20 using the same technique as for cyanobacteria [10] and finally epifluorescent microscopy was undertaken using a Zeiss Axioskop equipped with plan Neofluar objectives and an AxioCam digital camera [14].

Cellular activity based on the light and dark photosynthetic reactions was evaluated using oximetry and primary production techniques, respectively. Oxygen evolution was monitored with a Clark electrode (Oxy-lab oxygen electrode control unit manufactured by Hansatech instruments) by disecting ca. 500 mg of monolithic gel into tiny cubes and suspending them in fresh cyanidium media supplemented with 5 μL, 0.6 M $NaHCO_3$ as CO_2 source within the chamber. Carbon assimilation was ascertained using an adapted primary production method whereby a radio-labelled $NaHCO_3$ tracer was added to the precursors prior to gel formation as described elsewhere and the radioactivity of the samples was measured using a Beckman scintillation counter (LS 6000 SC) [14].

3. Results and discussion

Scanning electron microscopy and optical microscopy have been utilised to observe the nature of the cells in free culture suspension. Fig. 1a and b shows that the cells are quasi spherical in shape. SEM has also highlighted the fragility of these cells in comparison to cyanobacteria cells from previous works [10] with several images showing ruptured cells (not shown here). This is as a result of the centrifuging step and thus it reveals how imperative it was that only a minimal amount of centrifuging was undertaken to harvest the cells for immobilisation. Algae such as *C. caldarium* are eukaryotic and have less resistant cell walls than the peptidoglycan cell walls found in bacteria such as cyanobacteria and thus are more susceptible to bursting under centrifugal forces. Fig. 1c shows the *C. caldarium* cells immobilised within the silica gel. The cells appear to have not undergone cell lysis upon encapsulation, however for a clearer idea of the integrity of the cells post-immobilisation other microscopic techniques have to be utilised.

Transmission electron microscopy has been employed to investigate the cells encapsulated within silica gel to observe the interactions between the silica network and the cells and also to monitor the structure of the cells post-immobilisation. Fig. 2 shows two TEM images of the cells encapsulated in silica gel. From these images one can ascertain that it is possible to preserve the cell after immobilisation. There is also an alignment of silica particles around the cell wall. This is in accordance with what happens in nature [15]. Previous studies have shown that the cells walls of microbes, comprised of biomolecules such as proteins and polysaccharides, have an affinity for silica [16]. The ability of amines, in particular polyamine, to catalyse the polymerisation of silicic acid in water has also been described within the literature [17].

Fig. 3 reveals in more detail the silica crust that forms around the cell wall adding evidence to previous studies suggesting that *C. caldarium* undergoes a silica biomineralisation process [15]. Electron microscopy studies have revealed that with these unicellular microbes the cell walls act as nucleation sites for silica and polymerisation sites for silicic acid. The cell walls were also found to adsorb colloidal silica. Thus in the artificial environment setup during the reaction, the interaction between the cell wall and the silica could be attributed to the adsorption of LUDOX, a colloidal suspension of nanoparticulate silica. Asada and Tazaki have suggested a model for the silification of *C. caldarium* in nature. The cell walls, being rich in proteins as well as small amounts of polysaccharides, have amine, carboxyl and hydroxyl groups as well as peptide links projecting outside of the cell wall. Hence a hydrogen bond can be formed between these groups and silicate ions. Furthermore, in this work the terminal hydroxyl groups in the silicate polymers and certain functional groups present in the cell wall can undergo a dehydration reaction, thereby allowing the silica to attach itself to the cell wall.

They develop their model further by suggesting that the cell walls act as static barriers against proton influx into the cell as

[2] SAG – Sammlung von Algenkulturen der Universität Göttingen. The SAG 16.91 culture is a freshwater strain isolated from Mt. Lawu fumaroles in Java, Indonesia. The liquid stock cultures were maintained at 25 °C under fluorescent strip lighting in a culture chamber and transferred into fresh media on a regular basis. An acid media based on a soil extract was used for cultivation as found on the EPSAG website.

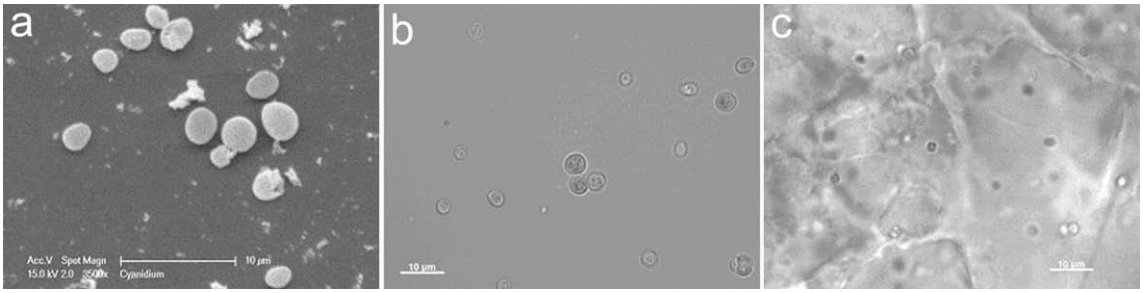

Fig. 1. (a) Scanning electron microscopy image, (b) optical microscopy image of free SAG 16.91 and (c) optical microscopy image of SAG 16.91 immobilised in silica gel, where each scale bar represents 10 µm.

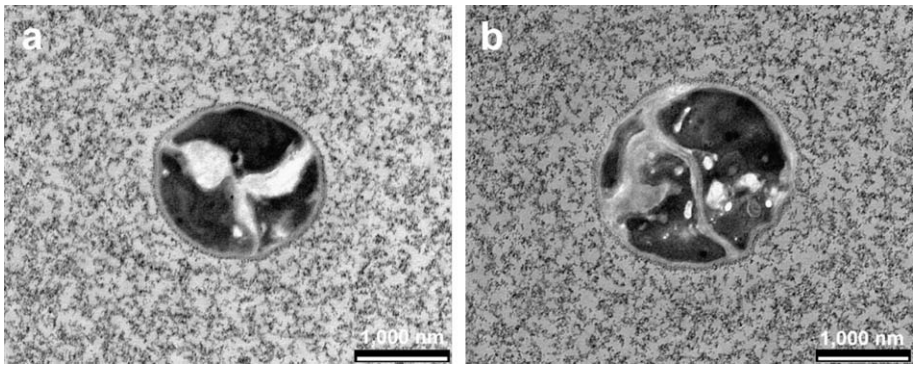

Fig. 2. Transmission electron microscopy images of SAG 16.91 immobilised within silica gel where the scale bars represent 1000 nm.

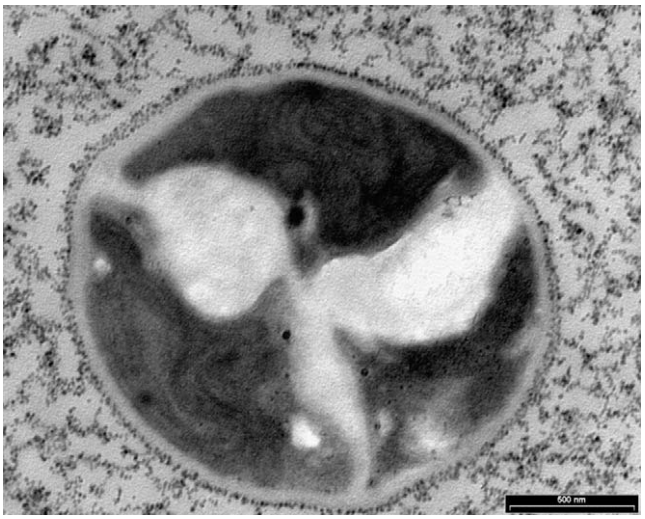

Fig. 3. Transmission electron microscopy image of cell shown in Fig. 2a at higher magnification clearly showing the alignment of silica colloids around the cell wall.

the composition of the cell wall itself absorbs a high concentration of hydrogen ions [18]. Absorption of H^+ into the interior of the cell would bring about the destruction of the chlorophyll molecules for instance and thus there is a need for a pH neutral environment within the cell itself. They suggest that the attack of the cell wall by a silicate ion generates a highly reactive silicate ion. Subsequently the polymerisation of silica increases with dehydration as other silicate ions attack the reactive ion. They believe water, generated by the dehydration step, plays an imperative role in the tolerance of *C. caldarium* to the external acidic environment.

Photosynthetic pigments, such as chlorophyll *a*, present within *C. caldarium* have the ability to autofluorescence, in effect they are fluorophores. This property can be exploited in terms of epifluorescence microscopy. Fig. 4 shows images of both free *C. caldarium* and *C. caldarium* immobilised in silica gel. The red spheres[3] in the micrographs indicate the presence of fluorescing cells within the silica gel, which in turn highlights the presence of photosynthetic pigments thus providing evidence that the cells could continue to photosynthesise post-immobilisation. The difference in background colour highlights that the cells within the gels are orientated in all directions and that as the sample is essentially in 3 dimensions there is interference from other planes in Fig. 4b, yielding a red background; it is much easier to focus a planar view with a liquid phase sample. From Fig. 4b one can see that the concentration of cells in the gels is less than those in a free culture suspension. In comparison to previous studies with cyanobacteria these cells have a slower growth rate, which is in part due to the temperature at which they are maintained. If grown in warmer temperatures then this growth rate could be accelerated. What is also of note is the arrangement of four distinct red spheres in a diamond shape which can be seen in both the liquid stock culture and also within the gel. This is the way in which the cells divide, whereby four daughter cells form within the mother cell wall. Upon rupturing the daughter cells are released [19]. These daughter cells can be seen in the TEM images, see Fig. 2, as the distinct dark areas within a mother cell. It is unlikely that this division of cells can continue post-immobilisation, owing to the confined space within the gel, unless the cells themselves can cause the silica network to disintegrate in the local vicinity creating more space. This was seen in the immobilisation of cyanobacteria within silica gel and thus it would be interesting to monitor hybrid *C. caldarium* gels over time via TEM studies to ascertain a clearer picture of cellular–silica interactions. However, the interaction between silica and the cell wall and

[3] For interpretation of color in the text, the reader is referred to the web version of this article.

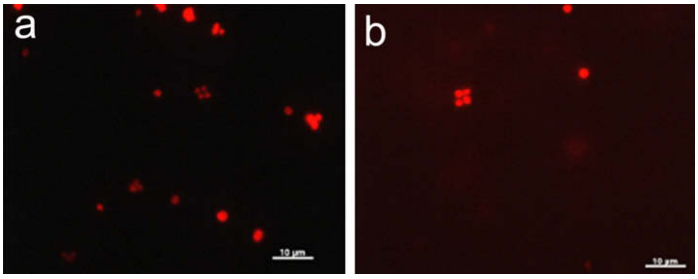

Fig. 4. Epifluorescence microscopy image of (a) free SAG 16.91 and (b) SAG 16.91 immobilised within silica gel, where the scale bars represent 10 μm.

the silica dissolution is likely to be influenced by the acidity of the media; the acidity of the cyanobacteria media is ca. pH 7 compared to pH 4 for *C. caldarium*.

Fig. 5 shows spectra from a UV–visible spectroscopy experiment taken over the visible range whereby the free suspension of cells is measured in terms of absorbance and the hybrid gels measured in terms of reflectance of visible light. These spectra show that the pigments are preserved post-immobilisation as they are essentially the same profile (allowing for the difference in data collection). The peaks around 675–680 nm are attributed to chlorophyll *a*, those at 625–630 nm phycocyanin whilst the peaks at shorter wavelength are more difficult to distinguish but are a combination of secondary chlorophyll *a* peaks and cartenoids [20]. The literature suggest that it is the light absorbed by the phycocyanin which is used most efficiently whereas there is a low efficiency in the use of light harvested by chlorophyll *a* [19]. There is a shift to higher energy for the hybrid gels which could fall within the experimental error for this technique (5%). However, a similar phenomenon was found in the study of immobilised cyanobacteria for all strains, thus there is a likelihood that the silica affects the measurements in some way.

Photosynthesis can be broken down into a series of reactions, those that require light and those that do not, the so called light and dark reactions. In simple terms the light reaction produces ATP, NADPH and O_2 whereas the dark reaction uses the ATP and NADPH to produce sugars in the Calvin cycle. A cell's ability to fix carbon can be monitored via the addition of a radio-labelled $NaH^{14}CO_3$ tracer, providing a source of CO_2 for the Calvin cycle.

The results from the uptake of the tracer are shown in Table 1. The data show that the average depletions per minute (DPM) of ^{14}C are greater for both the hybrid gel and the media used with the hybrid gels than for the respective blank samples. These results reveal that the *C. caldarium* cells are able to photosynthesise post encapsulation. The higher count rate is indicative of assimilated and excreted ^{14}C, *i.e.* organic ^{14}C, though it is clear that not all of the inorganic ^{14}C from the labelled sodium bicarbonate tracer has been removed through acidification resulting in positive, yet crucially lower, readings for the blank samples. Interestingly, these results were collected on hybrid gels made without glycerol. When glycerol was used the results (not shown) for the hybrid gels were not significantly higher than the blanks. This could mean that glycerol is not always biocompatible and that *C. caldarium* is more susceptible to its effects than cyanobacteria as it is a eukaryotic cell. Another possibility is physiological adaptations to the strain. Heterotrophic growth of *C. caldarium* has been reported in the literature so in providing an organic source (glycerol) the *C. caldarium* may well be adapt to their new environment via heterotrophic growth, no longer relying on their light harvesting mechanisms for energy.

Table 1
Primary production data from *C. caldarium* cells immobilised in silica gel.

Sample	^{14}C DPM[a]
Hybrid gel	388,651
Blank gel	1823
Hybrid media	6445
Blank media	70

[a] DPM stands for depletions per minute.

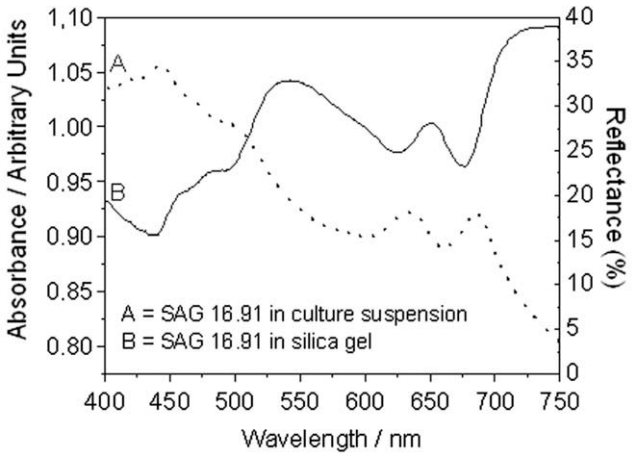

Fig. 5. Visible range spectra of (a) free SAG 16.91 and (b) SAG 16.91 immobilised within silica gel.

The light reaction can be monitored via electrochemical methods to analyse the production of oxygen in a Clark-type electrode. Measurements were taken on one day old gels and it was found that gels containing either LUDOX or LUDOX and glycerol in fact have negligible net respiration rates, whereas a gel made with sodium silicate only produces a net amount of oxygen, see Table 2. When LUDOX and glycerol were omitted the rate of oxygen production was 0.25 μmol h^{-1} g^{-1} over the first 30 min however the rate fell for the subsequent 40 min lowering the overall average. Production rates can be affected by a build up of oxygen within the Clark-type electrode as hydrogen peroxide can be formed, which inhibits photosynthesis. Normally catalase, an enzyme that catalyses the decomposition of H_2O_2, is present within cells exposed to high oxygen levels, however owing to diffusion limitations within the porous network of the gel this decomposition

Table 2
Oxygen production data from *C. caldarium* cells immobilised in silica gel.

Gel precursors	Oxygen production (μmol h^{-1} g^{-1})
Sodium silicate, LUDOX, glycerol	−0.052
Sodium silicate, LUDOX	−0.0015
Sodium silicate	0.13

may not occur with the same efficiency to the detriment of the immobilised cells.

Overall these preliminary results analysing the light and dark reactions of photosynthesis indicate how the interaction of the precursors with the cell can adversely effect the metabolism of the cell. The nanoparticles of silica seen around the exterior of the cells in TEM images may block the active sites on the eukaryotic cell wall or membrane preventing active transport processes whereas glycerol can be broken down by the cell itself into more toxic compounds [21]. Optimisation of the host scaffold of a photosynthetic material is therefore the key to efficiency and maximum metabolite production.

4. Conclusions

Aqueous pathways to silica gel have been successfully employed thus avoiding the liberation of aliphatic alcohols detrimental to algae cell viability. The polymerisation reaction of silicic acid formed *in situ* from a sodium silicate precursor has been tailored to occur within minutes via acidification of a dilute sodium silicate solution in the presence of LUDOX and glycerol. LUDOX is added as a cement to strengthen the gel via interbonding between the colloidal particles whereas the glycerol is added to protect the cells against osmotic stress from the increased concentration of sodium ions. These additions can prolong the preservation of the cells and their photosynthetic apparatus as initial observations suggest the hybrid gels remain green for at least 4 months indicating the presence of chlorophyll *a*. However it was shown that the cells were only productive in a basic sodium silicate matrix, suggesting that to encapsulate *C. caldarium* within an inert host framework nanoparticulate silica must be avoided in order to maintain the physiological function of the cells. The TEM images have clearly highlighted an interaction between the living cells and the silica hydrogel with the nanoparticulate silica aligning itself around the cell wall, forming a crust around the cell and potentially blocking the active transport sites. Furthermore owing to the small particle size the LUDOX may become internalised within the cell passing trough the cell wall and adversely interacting with the cytoplasmic membrane, a fluid phospholipid bilayer that plays a key role in endo and exocytosis processes. Moreover the data suggests that the photosynthetic pigments, responsible for light harvesting and energy conversion can be preserved which is of interest in the domains of solar cells, photobioreactors or clean energy technologies. Hybrid gels using *C. caldarium* could have different end uses to those containing cyanobacteria on account of their tolerance towards acid and towards heat, for instance as CO_2 absorbers in factory chimneys.

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