Entrapment of chlorophyll from *Chlorella vulgaris* and *Chlorella protothecoides* into microporous silica synthesized by a sol-gel method

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Abstract

This study presents results of the preparation, absorption and emission characterizations of chlorophyll samples extracted from green microalgae, based on the importance of this pigment and its optical properties. In this work, we characterized extracts containing chlorophyll from green microalgae of the genus *Chlorella* (*C. vulgaris* and *C. protothecoides*) via absorption and emission spectroscopies. The present results were compared with those of chlorophyll extracted from grass, previously reported. Microalgal chlorophyll samples were covalently bonded to the pore surface of organo-modified silica xerogels for a major spectroscopic analysis. Our findings indicate that the composition of green microalgal extracts corresponds mainly to chlorophyll *a* with a minimal amount of *β*-carotene. UV-VIS, IR absorption and UV-VIS emission spectra of the samples displayed the typical bands of chlorophyll *a*, and some other bands ascribed to *β*-carotene. Both the obtained absorption and emission spectra identify the algae chlorophyll bands and are unique. An effect of a radiative energy transfer mechanism between the silica matrix and the microalgal chlorophyll was observed as reabsorption bands in the emission spectra, and this effect was more evident in the *C. vulgaris* sample with respect to *C. protothecoides*.

Introduction

Since several years ago, the silica nets have been used as supporting matrix for ions and molecules given their spectroscopic properties of minimum or null intensity at the UV-VIS-IR region (300 to 800 nm). In the case of silica xerogels, which are relatively easy to synthesize, they have been able to trap several molecules, thus allowing the characterization of their optical properties. In turn, potting ensures the integrity of the supported molecule until its potential application, when, under special conditions, the molecule desorption is carried out in the site where its activity is required. Under these circumstances, the optical properties of the potted molecule remain unaltered.

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Among green microalgae, *Chlorella vulgaris* and *Chlorella protothecoides* are considered promising candidates as a feedstock for a variety of biotechnological processes, based on their relatively high photosynthetic efficiency, high production rates of biomass and cell composition. These processes include the production of biofuels (Heredia-Arroyo et al. 2010, Meng et al. 2020, Monjed et al. 2021), nutraceuticals (Matos et al. 2019), biofertilizers (Ferreira et al. 2021), wastewater treatment (Akao et al. 2020, Petrini et al. 2020) and CO2 mitigation (Saﬁ et al. 2014a). Regarding the cell composition of the above mentioned microalgal species, this primarily includes proteins, lipids, carbohydrates, pigments, some minerals and vitamins. Proteins from *C. vulgaris* have been highly valorized by food and cosmetics industries (Ursu et al. 2014). With respect to pigments, the most abundant is chlorophyll (located in the thylakoids) (Saﬁ et al. 2014b). The first application of chlorophyll comes from the 1850’s, when Alexandre Edmond Becquerel used it as a photo-stabilizer, because of its photochemical properties (Kephart 1955). Chlorophyll is a family of substances serving as photoreceptors in the photosynthesis process. Applications of chlorophyll by food, cosmetic and pharmaceutical industries drive the interest to culture microalgae with high chlorophyll contents, due to the anti-odorant, antioxidant and antimutagenic properties of these compounds, and of some of their derivatives (Hosikian et al. 2010, Halim and Danquah 2013, D’Alessandro and Antoniosi Filho 2016). The basic structure of this set of substances contains a porphyrin ring coordinated to a magnesium atom. There are six different types of chlorophylls, each one identified by a letter (a, b, c, d, e and f), however, their ascribed signals are not always observed in the spectra when performing a spectroscopic characterization: in the best case only chlorophylls a and b bands are present, and in minor proportion chlorophylls c1 and c2 could be identified (figure 1). Chlorophylls a and b possess a similar structure: both contain a phytol chain attached to the magnesium porphyrin. The difference between these two chlorophyll types resides in the side C3 group: a methyl group in chlorophyll a and a formyl group in chlorophyll b (ﬁgures 1(a) and (b)). This structural difference is responsible for the position of the maximum absorbance in the corresponding absorption spectra: at 660–665 nm for chlorophyll a, and 642–652 nm for chlorophyll b (Hosikian et al. 2010, Halim and Danquah 2013). Chlorophyll c is a subset of some other structures, c1 and c2 structures are shown in ﬁgures 1(c) and (d), respectively.

Cell growth rate, and the total amount and relative concentrations of chlorophylls in microalgal cells depend on culture conditions and their life cycles (Seyfabadi et al. 2011, Halim and Danquah 2013, Benavente-Valdés et al. 2016, Nam et al. 2017). For instance, the amount ratio of chlorophyll a to chlorophyll b in green microalgae varies in the range of 0.64 to 5, while the range for higher plants is much narrower, going from 1 to 1.4 (Halim and Danquah 2013). Chlorophylls a and b are main components of the pigments that have been found in C.
Table 1. Gelling mixtures of chlorophyll from microalgae of C. vulgaris and C. protothecoides for the synthesis of translucent monolithic organo-functionalized silica xerogels.

| Sample          | HCl:TEOS (ml) | H₂O (ml) | Chlorella/ethanol (ml) | OSA (ml)* | V₁ (ml)* |
|-----------------|--------------|----------|-----------------------|-----------|---------|
| Blank           | 5            | 0.26     | 0.65                  | 0         | 6.0     |
| Chlorella-OSA   | 5            | 0.26     | 0.64                  | 0.1       | 6.0     |

* OSA = Organo Substituted Alkoxide, i.e.: Allyl-trimethoxysilane (Ally-TEOS), and phenyltriethoxysilane (Ph-TEOS). V₁ = Total mixture volume.

C. vulgaris and C. protothecoides. In the former, the content of chlorophyll a ranges from 250 to 9630 µg g⁻¹, while the concentration of chlorophyll b is in the 72–5770 µg g⁻¹ range (González and Bashan 2000, Safi et al 2014b). Moreover, the content of chlorophyll could also be modified via mutagenesis of strains (e.g. in the study of Shin et al 2016). Other pigments with multiple therapeutic properties, such as β-carotene, astaxanthin, canthaxanthin, lutein, pheophytin a, pheophytin b and violoxanthin, can also be found, whose concentrations are also dependent on growing conditions (Safi et al 2014b). Furthermore, it is important to bear in mind that the amount of extracted bioactive compounds of interest, such as chlorophyll, depends on the processing conditions of the biomass (Safi et al 2014a, Stramarkou et al 2017).

The absorption process is due to an excitation of the atomic electrons, which are induced to undergo a transition between basal and excited states. Absorption and emission spectroscopies constitute a set of the analytical techniques used for identification of photosynthetic pigments. Chlorophyll a, from plants, presents characteristic absorption bands at wavelength values of 420 nm and 660 nm in organic solvents (Er et al 2015). In turn, chlorophyll b present in higher plants and green algae, has absorption main peaks at 453 and 643 nm. Chlorophyll c presents absorption bands at wavelength values of 445 nm and 625 nm, and is usually found in brown algae (Rabinowitch and Govindjee 1969, Songaila et al 2013, Calogero et al 2015).

In this study, we followed a previously developed process to evidence and characterize the bonding of chlorophyll from microalgae to the pore walls of modified silica synthesized from organo-substituted alkoxides, and tetraethoxysilane by a sol-gel method (Serratos et al 2013, García-Sánchez et al 2016). Chlorophyll extracted from C. vulgaris and C. protothecoides was covalently bonded to the surface of the pores of the organo-modified silica xerogels. This study provides new insights and spectroscopic information on the extracts containing chlorophyll from grass (Poa vulgaris) and from green microalgae of the genus Chlorella (C. vulgaris and C. protothecoides).

Materials and methods

Reagents
Tetraethoxysilane (TEOS), 3-aminopropyl-triethoxysilane (APTES), allyl-trimethoxysilane (Ally-TEOS), phenyl-triethoxysilane (Ph-TEOS) and solid reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents were purchased from Fluka (Sigma-Aldrich branch).

Microalgal strains and culture conditions
Both strains of microalgal, C. vulgaris (UTEX 259) and C. protothecoides (UTEX 256), were acquired from the Culture Collection of Algae at the University of Texas at Austin. They were cultured on a sterile protease peptone-agar medium in test tubes at room temperature, under illumination from cool-white fluorescent lamps with an intensity of 400 lux and light-dark cycles of 12 h:12 h. When the strains had reached maximum growth, ~4 × 10⁻³ g samples were collected with an inoculating loop.

Trapping chlorophylls from C. vulgaris and C. protothecoides into organo-modified silica xerogels
Chlorophylls from microalgal samples were extracted with ethanol. To obtain the fluorescence characteristics of the extracts containing chlorophylls, the samples were processed as described previously (Serratos et al 2013, García-Sánchez et al 2016). The chlorophylls from microalgae were bound inside the pores of monolithic, translucent organo-modified silica xerogels synthesized from tetraethoxysilane (TEOS), and in two organo-modified silica alkoxides (OSA): allyl-trimethoxysilane (Ally-TEOS) and phenyl-triethoxysilane (Ph-TEOS). In order to improve the dispersion and the adherence to the silica network, chlorophyll was functionalized in an ethanol solution with 0.40 ml of 3-aminopropyl-triethoxysilane (APTES), maintaining continuous magnetic stirring and a temperature of 70 °C for 24 h. The functionalization reaction was monitored through NIR absorption. The volumes of reagents used to prepare a blank and each chlorophyll containing sample are indicated in table 1. Samples were prepared in duplicate. Each sample was placed in a ~6 ml PS cells covered with parafilm, and kept under dark conditions while being monitored by UV spectrophotometry until the
emerging gel was separated from the container walls and no remaining solvent was left. As soon as the gel contraction step ended, the resultant samples were characterized by UV absorption, NIR, fluorescence spectroscopies, N\textsubscript{2} adsorption and Electron Dispersion x-Ray Scattering (EDX).

**UV-VIS-NIR absorption spectroscopies**
All absorption spectra were carried out in a Cary–Varian 5E model spectrophotometer, in the 200 to 800 nm (UV-VIS), and from 1250 nm to 2500 nm (NIR range). The Fourier transform infrared (FTIR) spectra were obtained from a Perkin-Elmer GXFTIR spectrometer.

**Photoluminescence spectroscopy**
Emission spectra were obtained at room temperature, in the 300 to 800 nm range, using a Perkin-Elmer model 650–1050 spectrofluorometer equipped with a 150 W Xenon lamp. The photoluminescence measurements were also carried out with a Perkin-Elmer fluorescence spectrophotometer to obtain the emission and excitation spectra. The light source was a 10 W pulsed Xenon lamp with a width at half peak intensity of 0.01 ms. The excitation wavelength for measurements were set at 280 and 300 nm for the emission and excitation spectra, respectively (Lozano et al 2015).

**N\textsubscript{2} adsorption-desorption**
Textural properties of samples were determined by nitrogen adsorption. The measurements were performed with a Micromeritics ASAP 2020 system at liquid nitrogen temperature (77 K). Before measurement, samples were degassed at 373 K for 12 h. Pore size distribution curves were computed by using the non-local density functional theory (NLDFT) method (Ravikovitch and Neimark 2001) applied to the N\textsubscript{2} desorption boundary branch of the isotherms.

**Electron dispersion x-ray scattering (EDX)**
SEM images were obtained with a jeol model 7600F microscope, equipped with an Oxford instruments model X-MAX 20 mm\textsuperscript{2} detector (SDD) for mapping. Electron Dispersion X-ray Scattering (EDX) measurements were employed to identify the presence and visualize the distribution of organic groups and chlorophyll from C. vulgaris and C. protothecoides, and some chemical elements forming part of the SiO\textsubscript{2} inorganic matrix. The elemental analysis data were normalized, and the results were obtained from the average of nine spectra taken over the surface of each sample.

**Cell culture**
HeLa-L cells were grown in Dulbecco-MEM medium (Gibco; Rockville, MD, USA) supplemented with 10% fetal bovine serum and incubated under an atmosphere of 95% air/5% CO\textsubscript{2} at 37 °C. Genotyping of the HeLa parental and transformed cell lines at INMEGEN (Mexico) indicated that they shared 15 of 16 canonic allelic markers with the original HeLa clone from the ATCC.

**Determination of growth inhibition potency by chlorophyll**
Chlorophyll was dissolved in DMSO and sterilized by filtration; control cultures were determined in the presence of vehicle. To determine the chlorophyll inhibitory potency on cancer cells growth, HeLa cells were grown in 70 mm petri dishes at \(10^{5}\) cells/well. After 24 h, chlorophyll was added (0.1, 1, 10 \(\mu\)g) and cells were cultured for additional 96 h. Cancer cell proliferation was determined by counting cells with trypan blue staining. The total number of alive cells were counted in a Neu Bauer chamber.

**Results and discussion**

**UV-VIS-NIR spectroscopic analysis**
The absorption spectra of C. vulgaris and C. protothecoides were compared with the spectrum of chlorophyll from Poo vulgaris (Chl. Poo vulgaris) containing 0.0% OSA was measured and taken as reference; all of them are shown in figures 2(a) and (b), for Ally-TEOS alkoxide and \(\beta\)-TEOS, respectively. Figure 2(a) shows that, for both C. vulgaris and C. protothecoides, there is a narrow band at 285 nm. From 350 nm to 800 nm, typical absorption bands for chlorophyll were observed; sin embargo, aquí se observan algunos picos de absorción que no se presentan la de Chl. Poo vulgaris in the presence of organo-substituted silicon alkoxides (Serratos et al 2013). Among the details observed in the wide band (400–500 nm) the prominent peaks found at 405, 435 and 468 can be attributed to: a) the absorption for chlorophyll \(a\) (which exhibits a maximum intensity at 415 nm) (Rabinowitch and Govindjee 1969, and b) \(\beta\)-carotene, found in the region from 450 to 490 nm (Cîntă Pinzaru et al 2015). Another band is observed at 664 nm, which is attributed to the \(Q_{1}\) band inherent to this kind of
porphyrins. Figure 2(b) shows the absorption spectra for three types of chlorophyll embedded into SiO$_2$ monolith using Φ-TEOS as organo-substituted alkoxide. The 'band gap' effect can be observed at ∼300 nm in the ultraviolet range. Similarly, in the region from 400 to 500 nm, there is a broad band composed fundamentally by the chlorophyll $a$ and β-carotene signals.

**Photoluminescence spectroscopic analysis**

For *C. protothecoides* samples in both alcoxides, the fluorescence spectra of the same samples (obtained in the range 350–800 nm) were similar at both excitation wavelengths $\lambda_{\text{exc}} = 280$ nm and $\lambda_{\text{exc}} = 300$ nm (figures 3(a) and (b)). In the red and near infrared regions, some typical chlorophyll emission bands, with maximum intensities at 649, 666, 711 and 764 nm, are distinguished. An overlap of the emission band of the matrix and the absorption band of *C. protothecoides* and *C. vulgaris*, suggests a mechanism of energy transfer from the matrix to the chlorella’s molecules.

Figures 4(a) and (b) depict the absorption and photoluminescence spectra, measured at wavelengths of 280 nm and 300 nm for *C. vulgaris*, in the UV-VIS range. The emission spectrum of pure SiO$_2$ matrix is included too. The graph clearly shows a band of reabsorption for the emission spectra of SiO$_2$ monoliths with samples from *C. vulgaris* in the region of 350 nm to 450 nm. This indicates that there are a kind of mechanism of radiative energy transfer, and, as judging by its appearance, this effect is more evident in the sample of *C. vulgaris* with the alkoxide Φ-TEOS.

Figures 5(a) and (b) compare the emission spectra of the *C. protothecoides* and *C. vulgaris* samples for the functionalized Ally-TEOS and Φ-TEOS alkoxide monoliths, respectively. A difference in the fluorescence between *C. protothecoides* and *C. vulgaris* is observed possibly due to differences in the chemical structures of the chlorophylls.
The diagram levels obtained from absorption spectrum are presented in figure 6 in order to understand the optical behavior of this material. Upper arrows indicate the absorption at wavelengths of 285, 405, 435, 468 and 664 nm; bottom dot-line arrows represent the non-radiative decay mechanism, whereas the bottom continuous arrows correspond to the emission bands at 457, 470, 648 and 686 nm observed in the fluorescence spectra.
In addition, assessments of the half-life of the fluorescence band in 664 nm of chlorella bound to the silica matrix pores modified with the alkoxides Ally-TEOS y Φ-TEOS were carried out (table 2). Our results show slight variations at the decay time, thus suggesting the need of further experiments evaluating the decay time to define the specific mechanism of energy transfer.

### Infrared spectroscopic analysis

The absorption spectra in the near infrared region (1250 nm to 2500 nm) showed well-defined bands at 1414, 1640, 1903 and 2263 nm, which were associated with the presence of chlorophyll incorporated in the silica monolith, and are consistent with the absorption bands (in the same region) of Chl. Poa vulgaris, for both Ally-TEOS and Φ-TEOS reported by García-Sánchez et al 2016. Although chlorophyll from C. protothecoides concentration is low in the monolith, the characteristic absorption bands in the IR absorption spectra are well-defined (see figure 7). On the other hand, C. vulgaris chlorophyll samples did not show this behavior, since C. vulgaris chlorophyll did not absorb in this region. This result constitutes another indication that chlorophyll of C. vulgaris has a different chemical structure compared to chlorophyll from C. protothecoides.

### Adsorption isotherms and pore size distributions

The nitrogen adsorption isotherms (relative pressure $p/p^0$ versus adsorbed volume in cm$^3$, at standard pressure and temperature conditions) are shown in figure 8(a). The pore size distributions are included in figure 8(b). Pore size distributions were calculated from desorption curves by the non-local density functional theory (NLDFT). A three-modal distribution for the Φ-TEOS C. vulgaris sample, with a pore size local maxima around 15.6, 21.1 and 25.8 Å, and a bimodal distribution with local maxima around 15.6 and 25.8 Å for the Ally-TEOS C. vulgaris sample were observed, which is consistent with those obtained in our previous work (Serratos et al 2013). This three modal distribution is mainly due to the difference in the size of the molecules of phenyl and allyl radicals in the organo-substituted alkoxide, thus determining the positional geometry of the functionalized chlorophyll molecule inside the SO$_2$ cavities. The form of the adsorption isotherms (type I, according to IUPAC) and the dimension size of the evaluated pores, confirm the microporosity of the samples with BET areas around 558 and 583 m$^2$ g$^{-1}$ for samples Φ-TEOS C. vulgaris and Ally-TEOS C. vulgaris, respectively.

### Scanning electron microscopy

In figures 9(a) and (c), SEM images of the surface of monoliths corresponding to Ally-TEOS and Φ-TEOS C. vulgaris samples are shown. Graphics 9b and 9d depict the X-Ray spectra obtained by the EDX method, indicating the elements contained in these materials. The same elements are detected in both alkoxide samples.
Ally-TEOS and Φ-TEOS; however, their composition differs. The presence of chlorophyll was not detected through the identification of magnesium atoms, since its amount was very likely under the detection limit of this technique.

The composition of Ally-TEOS C. vulgaris and Φ-TEOS C. vulgaris samples was estimated from EDX spectra obtained from 7 different regions. The mean composition (weight percent) of the Ally-TEOS C. vulgaris is the following: 23.8 ± 8.9% C, 50 ± 5.4% O, 26 ± 3.4% Si, 0.09 ± 0.02 % S, and 1.4 ± 0.2% Cl. For the Φ-TEOS C. vulgaris sample, results are as follows: 24 ± 1.6 % C, 38 ± 1.5% O, 37 ± 1.3% Si, and 0.5 ± 0.04 % Cl. The presence of carbon in both kinds of samples indicates the presence of chlorophyll and β-carotene, the latter revealed by its chemical structure, which can be also observed in the absorption spectra. In addition, some differences in the content of the elements estimated, such as O and Si, are shown. These differences were already
noted in the fluorescence spectra. The presence of magnesium was under the detection limit of this technique, due to the relatively low concentration of chlorophyll in these samples. The magnesium content in sample chlorophyll a Poa vulgaris was around 2.7%, close to the already reported value by García-Sánchez et al (2016).

Chlorophyll prevents cellular growth in HeLa cancer cells
To demonstrate the effect of Chlorophyll on cancer cells’ growth, HeLa cells were seeded in the presence of different concentrations of Chlorophyll (0.1, 1 and 10 μg) and the viability was determined with trypan blue in a Neu Bauer chamber after 96 h of treatment. The density of HeLa cells was affected with an inhibition of 30% at the lowest concentration with 0.1 μg ml⁻¹. The growth was more affected in cells treated with 1 μg ml⁻¹ (50%), while with 1 μg the growth was inhibited in 90%. The effect in cell density was not different when the Chlorophyll was exposed to red light for 30 min (data not shown). As a result, the Chlorophyll extracted from Chlorella vulgaris might be suggested as a potential anticancer agent.

Conclusions
Incorporating any kind of chlorophyll (from grass or microalgae) into a SiO₂ net allows a clear identification via absorption, IR absorption and UV-VIS emission spectroscopies. Through the absorption spectra, we identified that both extracts from Chlorella contained chlorophylls as well as β-carotene. By binding chlorophyll to alkoxides like Φ-TEOS and Ally-TEOS, it was possible to identify the bands corresponding to chlorophyll a and β-carotene in an efficient manner since, in the corresponding spectra, the inner absorption did not interfere with the matrix. This efficiency has already been demonstrated in previous reports for Poa vulgaris, for which Ally-TEOS and Φ-TEOS permitted the observation of the optical response inherent to the molecule of chlorophyll from microalgae. It is noteworthy, the methodology employed here could serve to improve the characterization of the properties of novel Si-based materials and brings a promise for a new landmark in the development of cancer therapy. Of recent interest, both chlorophyll and Chlorella are currently being encapsulated in nanospheres to be tested in cancer cell cultures by our group (experiments in progress). The relevance of these ongoing studies lies in the therapeutic potential that these molecules represent for treatment of cancer and others degenerative disorders.

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Data availability statement
All data that support the findings of this study are included within the article (and any supplementary files).

Author contributions
IN Serratos and R. Sosa performed the synthesis of the hybrid materials, the fluorescent characterization, data analysis, writing and correction of manuscript. A. Santamaría was involved in writing and corrections of the manuscript. HJ Ávila-Paredes, P Ruiz-Sánchez, G Saucedo-Castañeda were involved in the acquisition, maintenance and culture of both strains of microalgae, C. vulgaris UTEX 259 and C. protothecoides UTEX 256, which were obtained from the Culture Collection of Algae at the University of Texas at Austin; H.J. Ávila-Paredes also contributed to the writing of this paper I Hernández-Reséndiz carried out the infrared studies, cell culture and determination of growth inhibition potency by Chlorophyll. J.M. Esparza-Schulz and V. Bustos-Terrones performed the N₂ sorption experiments and discussions. A. Arrieta obtained the SEM images and EDX data analysis.
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