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Escuela de Ciencias Químicas e Ingeniería

ALGAE ENCAPSULATION INTO SILICA MONOLITHS SYNTHESIZED BY HIGH INTERNAL PHASE EMULSIONS (HIPE)

Trabajo de titulación presentado como requisito para la obtención del título de Química

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A mi madre, por su amor y apoyo incondicional durante mi carrera. Me he convertido en una mujer fuerte e independiente gracias a los valores que ella me enseñó. Te amo mucho mami, tienes el corazón más grande que conozco. A mi padre, por creer en mis capacidades desde que era una niña e introducirme al mundo científico. Gracias a él amo la ciencia y siempre creyó que tendría un excelente futuro, ahora soy una científica. Te amo papi. A mis queridos hermanos, cada día con ustedes es diversión y alegría. Fue difícil no estar con ustedes pero sé que puedo contar con ustedes, así como ustedes pueden contar con mi apoyo. Los amo a los dos con todo mi corazón a pesar de nuestras peleas y desacuerdos.

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Nadia Priscila López Pico

Abstract

Biotechnological applications of microalgae are widespread within different important research fields, from the oldest ones focused on human nutrition until the most novel ones as a renewable energy source; however, bioremediation of CO_2 and biofuel production remains the most overwhelming application. These amazing organisms represent a substantial source of biomass for the production of high valuable metabolites as lipids and carbohydrates. Their metabolic activity is possible thanks to the photosynthesis where the chlorophyll molecule is directly involved in the beginning of a series of chemical reactions to obtain oxygen and sugars from CO_2 and water in the presence of sun light. With the purpose of fixing CO_2 through the efficient microalgae photosynthesis, this work proposes the algae encapsulation within a hierarchical porous silica monolith by using high internal phase emulsion (HIPE) as a new synthesis method to obtain bio-hybrid monoliths. To evaluate the viability and evolution of the immobilized microalgae within the monolithic structure of silica, this research was based on the fluorescent properties of their chlorophyll which was characterized by fluorescence spectroscopy and microscopy. Also, the characterization by diffuse reflectance spectroscopy in UV-Vis (DRS UV-Vis) was carried out to corroborate the qualitative analysis from fluorescence spectroscopy. The spectra from monoliths of successful encapsulation presented the relevant peaks from chlorophyll in both absorption and fluorescence modes. The novel and low cost technique developed in this work for *Chlorella vulgaris* immobilization achieved a log-term stability of at least 250 days. The photosynthetic activity was kept without constant feeding of microalgae. This bio-hybrid monolith could be applied in the future as an eco-friendly photo-bioreactor which captures CO_2 and produces high-value raw materials as well as for water depollution.

Keywords: microalgae, *Chlorella vulgaris*, immobilization, HIPE, fluorescence, silica, monolith.

Resumen

Las aplicaciones biotecnológicas de las microalgas se extienden dentro de diferentes e importantes campos de investigación, desde el más antiguo centrado en la nutrición humana hasta el más novedoso como fuente de energía renovable. Pero el área donde se desarrolla la mayor parte de la investigación es la bioremediación del aire al absorber CO_2 y la producción de biocombustibles. Estos asombrosos organismos representan una buena fuente de biomasa que proviene de la producción de metabolitos de alto valor como lípidos y carbohidratos. La actividad metabólica es posible gracias a la fotosíntesis, donde la molécula de clorofila contenida en las algas verdes está directamente involucrada en el inicio de una serie de reacciones químicas para obtener oxígeno y azúcares a partir del CO_2 y del agua en presencia de la luz solar. Con el propósito de fijar el CO_2 a través de la eficiente fotosíntesis de las microalgas, este trabajo propone la encapsulación de algas dentro de un monolito de sílice con porosidad jerarquizada mediante emulsiones con alto contenido de fase interna (HIPE) como un nuevo método de síntesis de monolitos bio-híbridos. Para evaluar la viabilidad y la evolución de las microalgas inmovilizadas dentro de los monolitos, la investigación se basó en las propiedades fluorescentes de la clorofila y se caracterizó por espectroscopia y microscopía de fluorescencia. También se llevó a cabo la caracterización espectroscopia de reflectancia difusa de UV-Vis (DRS UV-Vis) para corroborar el análisis cualitativo de la espectroscopia de fluorescencia. Los espectros de los monolitos donde la encapsulación fue exitosa presentaron los picos relevantes de la clorofila tanto en absorción como en fluorescencia. La técnica novedosa y de bajo costo desarrollada en este trabajo para la inmovilización de *Chlorella vulgaris* logró una estabilidad a largo plazo de al menos 250 días. La actividad fotosintética se mantuvo sin la alimentación constante de la microalga. Este monolito biohíbrido podría aplicarse en el futuro como un fotobiorreactor ecológico que captura CO_2 y produce materias primas de alto valor así como la descontaminación de agua.

Keywords: microalga, *Chlorella vulgaris*, immobilisación, HIPE, fluorescencia, sílice, monolito.

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Introduction

"*It's not love or money that makes the world go round, it's photosynthesis*"[1]

The United Nations declared that between 2030 and 2050 the temperature of the Earth will increase until 1.5°C causing catastrophic effects, due to the pollution originated from greenhouse gas emissions. Many scientist are aware that the impending threat of climate change has grown faster than any other moment in the history of Earth. Among the greenhouse gases emitted by the humans nowadays the carbon dioxide (CO_2) represents 68% of the total emissions.[2] The scientific world has engaged huge efforts developing technology for CO_2 fixation and renewable energy. One path to achieve both goals, on which some research has focused, are plants. These autotrophic entities, thanks to the photosynthesis, are able to capture CO_2 and produce metabolites of interest, but the efficiency of the reaction does not supply the oxygen demand actually needed and many forests have been reduced by deforestation. Some research has reported that biological CO_2 fixation appears to be the only economical and environmentally viable technology of the future.[3,4]

Among the huge variety of plants on earth, microalgae and cyanobacteria have demonstrated great advantages over terrestrial plants. To culture these aquatic organisms, there is no need to much space, feed or cares, compared to plants. The main advantage of these photosynthetic microorganisms is the efficiency with which they capture CO_2, between 10 and 50 times higher than multicellular plants.[5] All the information about the classification and biology of microalgae is well explained in Chapter 1. In order to take advantage of the different benefits of growing microalgae and the many viable applications in different fields, the present research aims at synthesizing a bio-hybrid material where *Chlorella vulgaris* will be encapsulated within a silica matrix.

Silica fulfils all the necessary features to immobilize a photosynthethic microorganism, thanks mainly, to its phototransparency. This inorganic material has been chosen for successful microalgae encapsulation and subsequent viability because of its biocompatibility, mechanical and chemical stability, and ability to form hierarchical

structures with high porosity required for the diffusion of the needed nutrients and the products that are released by the cells.[6] The immobilization in a stable matrix ensures the cell protection and allows a control of the interfacial properties of living cells. All the features associated with the silica matrix and its use for immobilization of living organisms is explained in Chapter 1 as well.

The encapsulation of photosynthetic organisms or organelles in a substrate simulates a photo-bioreactor (PBR). Most of the actual research in photo-bioreactors have demonstrated higher efficiency compared to equivalent algae suspensions.[7] To achieve the immobilization of microalgae and avoid their death, caused by any cytotoxic interaction during the synthesis, an *in situ* one–pot process is developed. This project introduces an innovative way to encapsulate the microalgae within a monolithic structure by high internal phase emulsion (HIPE). The methodology of the synthesis technique is fully detailed Chapter 2. The method employs an emulsion-polymer double template, resulting in a biomaterial with hierarchical morphology combining macro- and meso-porosity preserving high superficial area once the porosity is liberated. The soft chemistry involved in the method would keep the photosynthetic activity of the pigments from living algae. To evaluate the evolution of microalgae after encapsulation, fluorescence spectroscopy and microscopy was carried out to follow the photosynthetic activity as well as diffuse reflectance UV-Vis spectroscopy, as reviewed in Chapter 2.

The discussion of the qualitative results obtained with the monoliths for which the immobilization was successful are displayed in Chapter 3. Interactions between the matrix and the algae are analyzed along with the evolution of the algal population after the immobilization. This preliminary results empower a future quantitative analysis of the metabolic activity of the immobilized microalgae. Compiling both qualitative and quantitative information of the biomaterial allows the development of PBRs for large scale applications.

Problem statement

The encapsulation of living microalgae with long term viability and its further biotechnological application has faced several disadvantages related with the host structure and the immobilization technique. Over the years, investigations have overcome these problems when silica properties evidenced the potential use of the inorganic compound as a host for microalgae. Properties like porosity, stability, inert and non-toxic chemistry and mainly phototransparency, make silica still used for immobilization nowadays to encapsulate photosynthetic organims.[8,9] The technique usually involved in the synthesis of silica hybrids materials is sol-gel. This technique is quite useful to modify the porosity of the material which is a requirement when trapping microalgae, but when alkoxides are used as silica source the byproducts of silica condensation are cytotoxic inducing microalgae death. In spite of the toxicity of the synthesis modifications, were made to avoid contact with microalgae, like a two-step procedure or attenuation of the chemical effects with silica aqueous precursors during synthesis.

All the disadvantages mentioned above are not a problem with the newly proposed technique for microalgae immobilization devised in this thesis project. It is an easy, low cost one-pot process based on a high internal phase emulsion that can economically help Ecuadorian industry by producing it in large scale and selling it or applying it in water treatment or even improve it for application in bio-refinery. Since the Ecuadorian economy always has been based in the production of raw materials, agriculture and petroleum extraction.

General and specific objectives

- *General objective*

  Encapsulation of *Chlorella vulgaris* and alga-like plant within a hierarchical monolithic structure of silica by high internal phase emulsion (HIPE)

- *Specific objectives*

  - To grow *Chlorella vulgaris* at a lab scale to encapsulate within a hierarchical porous silica matrix

- To use *Chlorella vulgaris* and alga-like plant to synthesize the hybrid monoliths (microalgae or alga-silica monolith) by using HIPE copolymer-double template method
- To synthesize the microalga and alga-silica monoliths functionalized with copper or zinc in terms of stabilizers or micronutrients.
- To study the metals presence effect on the maintenance of the microalgae photosynthetic activity when encapsulated.
- To characterize the microalga or alga-silica monoliths by fluorescence techniques and absorption techniques.
- To evaluate the evolution of microalgae or algae-like plant within the silica monolith post-encapsulation over the time by fluorescence techniques and absorption techniques.
- To evaluate the photosynthetic activity and stability of *Chlorella vulgaris* when hybrids are in contact with the growth media.

Chapter 1: Background

1. *Microalgae*

1.1. Generalities

Through the years, microalgae have been considered a good source of biomass, mainly used as food supplement.[10] Nowadays, these aquatic plants are also exploited for different applications like dyes, pharmaceuticals, animal feed, aquaculture and cosmetics.[11] The last two decades have witnessed both prokaryotic and eukaryotic microalgae expanding into new promising applications, as shown in **Figure 1.*1***, which rise as viable solutions to the progressive decrease of fossil fuel reserves, together with the subsequent increase in oil prices, and the global warming concern.[12]

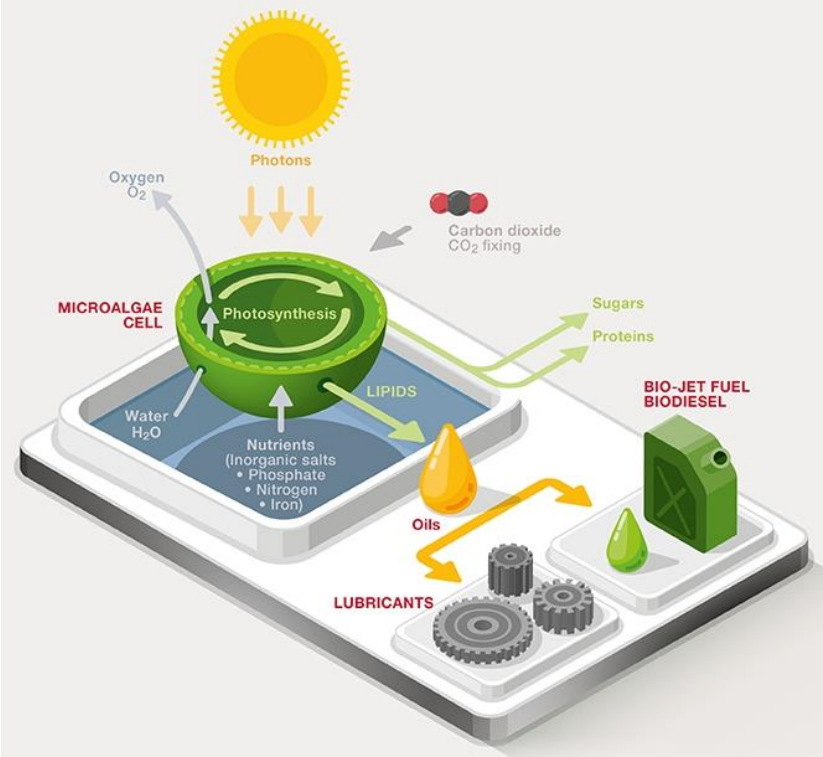

Figure 1.1 Potential pathways and applications of microalgae.[13]

The taxonomically diverse group of microalgae has been profoundly studied and nowadays most research is focused in its biotechnological applications. Phycology is the

study of algae, defining it as any organism with chlorophyll *a* and a thallus not differentiated into roots, stem and leaves.[14] Hence, when referring to the microalgae term, applied phycology alludes to the microscopic algae *sensu stricto*, which includes the oxygenic photosynthetic bacteria, that is cyanobacteria.[15] Microalgae are the world's largest group of primary photosynthetic producers in freshwater and marine ecosystems.

The photosynthetic mechanism in microalgae is similar to the one of terrestrial plants, but the unicellular or simple multicellular structure and the fact that microalgae inhabit aqueous environments allow them to have an efficient access to water, carbon dioxide and other nutrients ensuring a higher energy efficiency of photosynthesis.[16] This high photosynthesis rate makes the microalgae the principal producers of oxygen on earth.

1.2. Biology of microalgae

Algae are recognized as one of the oldest life-forms on earth.[17] As its definition says, it is a primitive plant, therefore lacking roots, stems and leaves. Algae structures are primarily dedicated to energy conversion without any development beyond cells, in consequence their simple development allows them to adapt to almost every environmental condition and prosper in the long term. In addition microalgae are able to uptake large amount of inorganic and organic nutrients because of their simple cellular structures and a large surface to volume body ratio.[18]

Referring to the ultrastructure of the diverse microalgae, prokaryotic cells (cyanobacteria) lack of membrane-bound organelles as plastids, mitochondria, nuclei, Golgi bodies, and flagella, therefore they are more analogous to bacteria rather than algae. Eukaryotic cells, which include many different types of common algae, do have these organelles that control the functions of the cell, allowing it to survive and reproduce, prevailing in most of the cases the cell division.[19]

The biomass of microalgae contains three main components: proteins, carbohydrates and lipids. Moreover the phylogenetic breadth of microalgae is reflected in an equally broad biochemical diversity of pigments, photosynthetic storage products, cell walls and mucilage,

fatty acids and lipids, oils, sterols and hydrocarbons, and bioactive compounds, including secondary metabolites.[1] Many microalgae species may accumulate extracellular polysaccharides, such as a gelatinous mass enclosing their cells, which are called envelopes, sheaths or capsules.[20]

1.3. Photosynthesis in microalgae

Photosynthesis is the key process for the survival of algae, even more it is the basis of all food chains because all the living forms on Earth depend directly or indirectly on photosynthesis as a source of energy for their metabolism and growth. It represents a unique process of sunlight energy conversion.[21] Only photoautotrophs organisms are able to achieve this process, consisting in the conversion of energy-poor inorganic substances to energy-rich organic compounds, initiated by the solar energy absorbed by photosynthetic pigments.

The internal organization of cyanobacteria corresponds to a prokaryotic cell, where there is a peripheral region (chromoplast) conformed by photosynthetic membranes.[22] While eukaryotic microalgae presents a photosynthetic apparatus organized in special organelles, called chloroplasts.[23] The chloroplasts are characterized by their green color attributed to the well-known pigments, chlorophylls, where the chemical reaction that leads to the photosynthesis arise.

The conversion of carbon dioxide and water to carbohydrates is a series of chemical reactions formerly defined as oxygenic photosynthesis.[24] It can be expressed as a redox reaction driven by light energy in which the electrons from water are transferred to carbon dioxide. Traditionally this transformation proceeds in two different stages, the so-called light reactions and dark reactions (**Figure 1.*2***).[25] The products provided by the light energy absorbed during the light reaction are the biochemical reductant nicotinamide adenine dinucleotide phosphate ($NADPH_2$), the high energy molecule adenosine triphosphate (ATP) and oxygen as byproduct. Inside the chloroplast, at the stroma, the dark reactions synthetize carbohydrates by using the $NADPH_2$ and ATP previously obtained in a sequential biochemical reduction of carbon dioxide.[17]

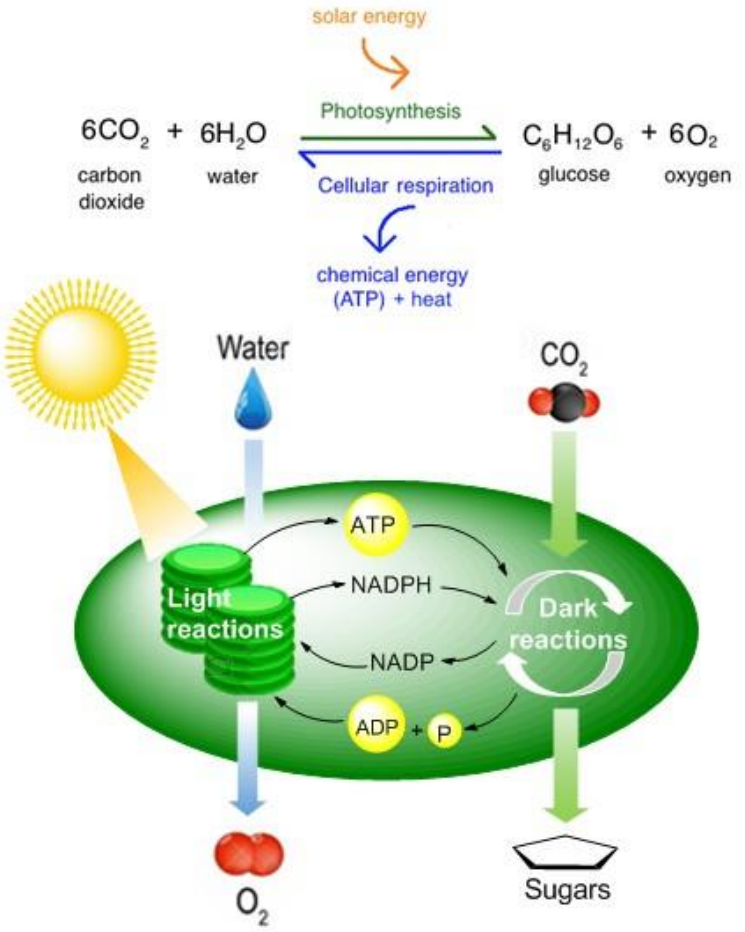

Figure 1.2 Scheme and chemical reaction of the light and dark reactions that lead to the photosynthesis inside the chloroplast.

1.3.1. Chlorophyll

Chlorophylls are green pigments involved in the photosynthetic mechanism of all plants and algae. Chlorophyll a, Chl *a*, is found in all classes of algae showing the largest fraction of their photosynthetic pigments, because it plays one of the most important functions within the photosynthetic process. Chl *a* leads the conversion of the energy from the electromagnetic radiation to the thermochemical energy needed for the subsequent electron-transport chain reactions and the dark reactions. The molecular structure of the chlorin macrocycle is responsible of the light absorption thanks to its conjugated π system.

The spectroscopic properties of chlorophylls have been widely studied and well-known. The absorption spectrum contains two major absorption bands, one at the blue near UV region and one in the near IR region. The fact that chlorophylls do not absorb the green light confers them the characteristic green or blue-green color. As seen in **Figure 1.3**, the absorption of chlorophyll presents two lowest-energy transitions called Q bands, and two higher-energy ones commonly called Soret bands. On the other hand, the emission spectrum only shows one maximum emission corresponding to the Q_x band but with a bathochromic shift.

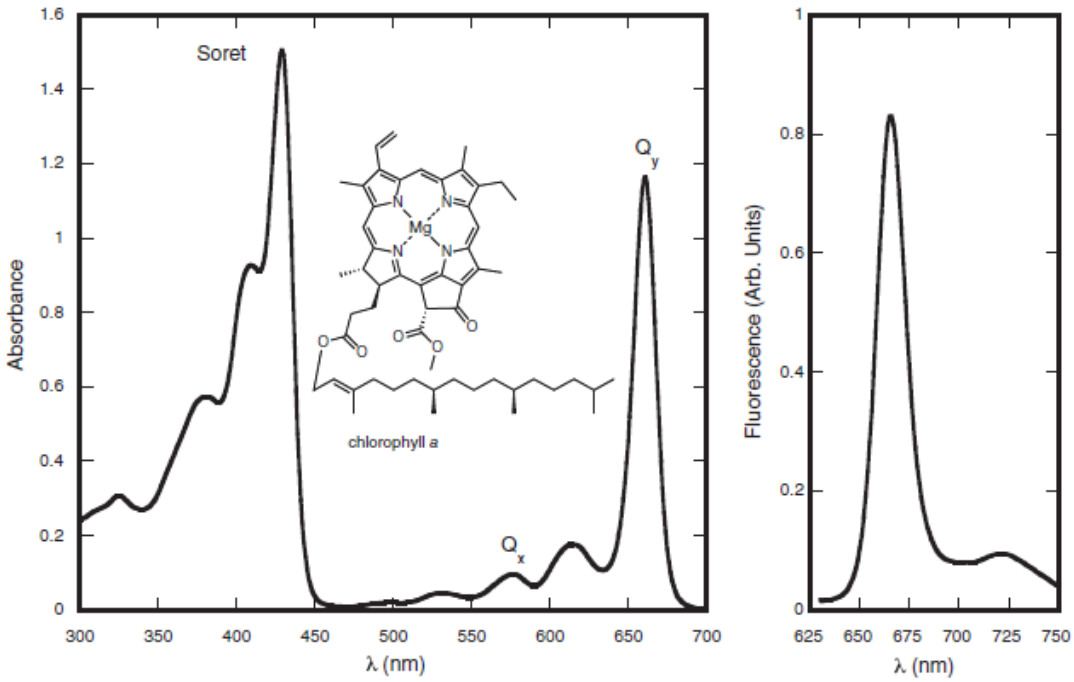

Figure 1.3 Absorption and fluorescence spectra of chlorophyll *a*.[26]

1.4. Classification of microalgae

The broad phylogenetic diversity of algae is contemplated in an equally wide range of metabolisms and biochemical properties. The phylogenetic tree of algae depicts the huge diversity of these microorganisms. They are even more diverse than either land plants or animals. Metting[27] established the classification scheme on the traditional line, where the major groups of algae are classified on the basis of pigmentation, the chemical nature of the

photosynthetic storage product, thylakoid organization and other ultrastructural features of the chloroplast, the chemistry and structure of the cell wall, the number, arrangement and ultrastructure of flagella (if any), and the occurrence of any special features.

Figure 1.*4* exhibits only four of the most important divisions of algae, in terms of abundance: blue-green algae (*Cyanophyta)*, red algae (*Rhodophyta*), green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*).

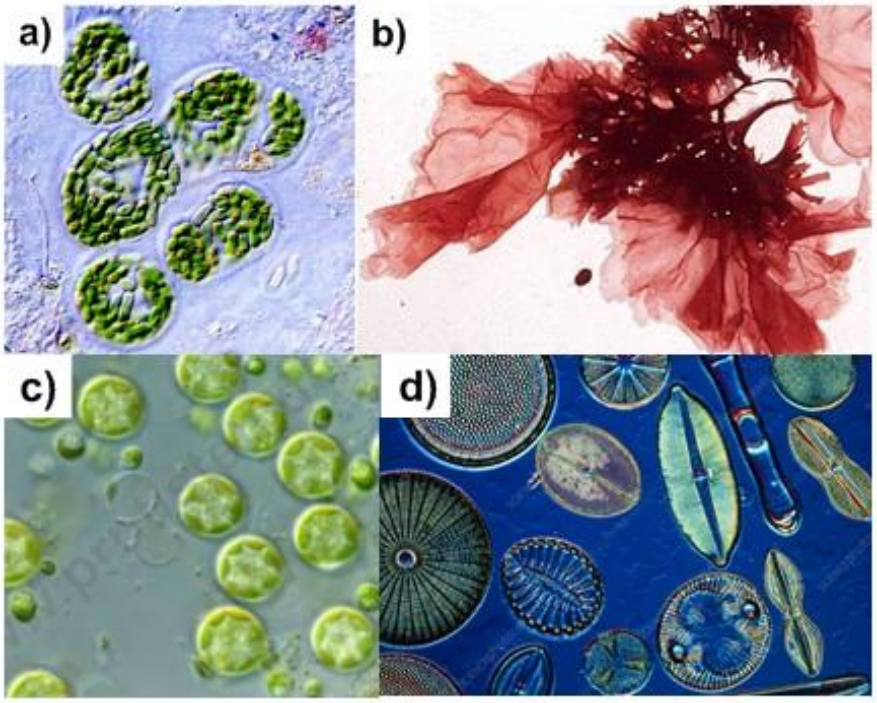

Figure 1.4 (a) Blue-green algae (cyanobacteria), (b) red algae, (c) green algae and (d) diatoms.

Some distinguishable features of each of these are presented in **Table *1***.

Table 1.1 Properties of the four most important divisions of algae

Division	Notable features	Major groups

Cyanophyta (blue-green algae)	Pigmentation: chlorophyll *a*, *d*, blue and red phycobilins, β-carotene, and several xanthophylls. Prokaryotic; Gram-negative cell walls.	Chroococcales
Rhodophyta (red algae)	Pigmentation: chlorophyll *a*; α-, β-carotenes; phycobilins; zeaxanthin, 5 minor xanthophylls. Floridean Storage product: starch. Cellulose and other cell wall materials	Bangiophycidae Floridophycidae (seaweeds only)
Chlorophyta (**green algae**)	Pigmentation: Chlorophyll *a*,*b*; α-, β-, γ-carotenes; zeaxanthin, lutein, violaxanthin, neoxanthin; several minor xanthophylls. Storage product: starch. Diverse cell wall materials, including cellulose.	Micromonadophyceae (prasinophytes) Ulvophyceae (seaweeds, filamentous microalgae) Pleurastrophyceae Chlorophyceae
Bacillariophyta (diatoms)	Pigments: chlorophylls *a*, c_1, c_2, fucoxanthin and β-carotene. Storage product: chrysolaminarin. Siliceous cell wall	Coscinodiscophyceae Fragilariophyceae Bacillariophyceae

1.4.1. Green microalgae - Chlorella.

In terms of abundance, distribution and morphology diversity, green algae covers one of the largest groups of algae that inhabits in freshwaters. For example, unicellular (e.g. *Chlamydomonas, Phacotus*) and colonial (eg *Volvox, Pyrobotrys*) flagellates, coccoid non-flagellate species (eg *ChlorelIa, Pediastrum, Ankistrodesmus, Scenedesmus*), and filamentous forms (eg *Chaetophora*, *Oedogonium*) are abundant in ponds, lakes, and streams at most latitudes.[27]

Once biotechnology emerged, there was a rapid and fascinating growth of the applications of green microalgae. Some species belonging to this algal division showed interesting characteristics which lead to their commercial exploitation. The chlorophycean genera comprises some examples of microscopic green algae utilized for commercial purposes, among these are *Chlorella*, *Dunaliella* and *Haematococcus. Dunaliella spp*. from hypersaline environments represents a good source of natural β-carotene, so an extensive study was done and is widely cultivated and commercialized.[28,29] The *Haematococcus* is a freshwater unicellular algae containing the ketocarotenoid astaxanthin accumulated in globules outside the chloroplast.[30] The astaxanthin has become a highly valuable product not only as a coloring agent for fishes and crustaceans in aquaculture but, since it shows a powerful natural antioxidant activity, it has a potential use in medical applications.[31]

1.4.1.1. *Chlorella vulgaris* features

This unicellular green eukaryotic microalga belongs to the following scientific classification: Domain: Eukaryota, Kingdom: Protista, Divison: Chlorophyta, Class: Trebouxiophyceae, Order: Chlorellales, Family: Chlorellaceae, Genus: Chlorella, Specie: *Chlorella vulgaris*.[32] Because *C. vulgaris* is considered a cosmopolitan microalga, it can be easily found in Ecuador. Guamán *et al.*[33] states that it has been found in all the lagoons located in all the Ecuadorian National Parks.

Concerning *C. vulgaris* morphology (**Figure 1.5**) it is a microscopic cell with a spherical shape of 2–10 μm diameter and it lacks motility.[34] It owns a thin rigid cell wall, conferred by a layer of glucosamine, which works as protection against invaders and harsh environmental conditions [35]. There is one cup-shaped chloroplast inside the cell in which the pyrenoid is present with high levels of ribulose-1,5-bisphosphatecarboxylase oxygenase (RuBisCO) and is the center of carbon dioxide fixation.[11,19] The accumulation of starch, composed of amylose and amylopectin, occurs inside the chloroplast, while the lipid globules are formed through nitrogen stress in the cytoplasm and chloroplast.

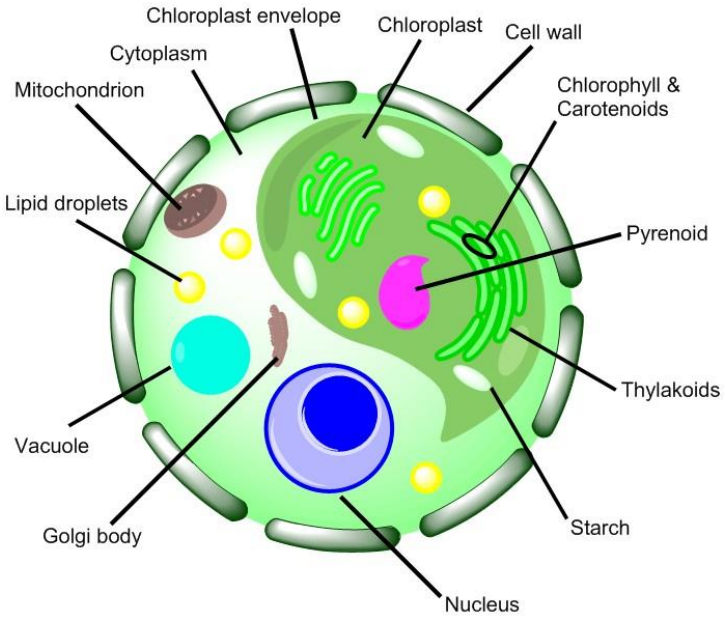

Figure 1.5 Schematic ultrastructure of *C. vulgaris* representing different organelles.

The reproduction of *C. vulgaris* is asexual and rapid through the formation of autospores (non-motile spores) of the same shape as the parent cell.[36] The fact that this microalga species grows really fast and modifies the yield of a specific component, when a growth condition has changed, makes it ideal for production. Thanks to the elevated resistance of the microalgae against harsh conditions and invaders, it is possible to realize large scale cultures.[37] *C. vulgaris* is capable of growing in autotrophic, heterotrophic and mixotrophic conditions. The autotrophic growth refers to the use of light energy through photosynthesis to obtain chemical energy (carbohydrates). In the heterotrophic growth, the microalga is fed with an external carbon source, like sugars (glucose, acetate, glycerol and glutamate), while the mixotrophic growth merge both autotrophic and heterotrophic systems by performing photosynthesis as well as consuming organic materials.[38]

1.4.1.2. Culture of *Chlorella vulgaris*

Microalgae culture guarantee many advantages over conventional agricultural systems at the moment of optimizing the utilization of solar energy. To obtain a *C. vulgaris* culture, the basic principles of microbial cultivation are applicable but, microalgae are the only microorganisms that require a light source for their growth.[39] There are physical,

chemical (abiotic factors) and biological factors (biotic factors) that might limit microalgae growth, especially for high cell density mass production cultures. ***Table 1.2*** summarizes these factors:

Table 1.2 Factors influencing the algal growth.[40]

Abiotic factors	·Light (quality, quantity) ·Temperature ·Nutrient concentration ·O_2 ·CO_2 ·pH ·Salinity ·Toxic chemicals
Biotic factors	·Pathogens (bacteria, fungi, viruses) ·Competition by other algae
Operational factors	·Shear produced by mixing ·Dilution rate ·Depth of the pond ·Harvest frequency ·Addition of bicarbonate

The most common culture mode is the batch culture system, which consists in a culture vessel (conical flask) where a determined amount of algal inoculum culture medium is placed and incubate at the adequate environmental conditions for growth. The only source of CO_2 comes from the air and it is illuminated externally by artificial light sources maintaining a temperature around 25°C.

1.4.1.3. Applications of *Chlorella vulgaris*

Since *Chlorella* was discovered in 1890 as the first microalga with a well-defined nucleus[41], the properties and characteristics of this microalga have been extensively investigated. Afterwards, it had become the most important species in microalgal industry. In the early 1990s, researchers determined that their protein content was around 55% (dry weight), and it was cultivated and sold essentially as healthy food.[42] Also the *Chlorella* culture was taken to large scale for carbon dioxide reduction in air.[43] Nowadays, Japan is the major consumer of this microalga. Besides its use as food, it has been demonstrated to be

useful for medical treatments[44,45] since it presents immune-modulating and anti-cancer properties.[46,47] Additionally, it is considered as a suitable source for biodiesel production because it is able to store important amounts of lipids and fatty acids.[48] Furthermore, the microalga has an outstanding potential of applications in bioremediation as in CO_2 mitigation and waste water treatment removing heavy metals[49], nitrogen[50] and phosphorus.[51]

The applications of the *Chlorella* genus has diversified in different fields along the time. It has a simple but efficient biochemistry and structure, together with the metabolites that is able to produce, allows the development and improvement of microalgae biotechnology. Among its different applications the most important ones are highlighted in **Table 1.*3***.

Table 1.3 Potential applications of the biomass and biochemistry of *C. vulgaris*

Biomass[52,53]	Proteins	·Human nutrition ·Animal feed ·Pharmaceuticals ·Biofilms casting ·Emulsifier ·Food additives
	Lipids	·Biodiesel ·Glycerol products ·Raw material: saturated fatty acids, poly-unsaturated fatty acids, monounsaturated fatty acids, phytosterol.
	Carbohydrates	·Bioethanol ·Animal feed ·Chemicals ·Bioplastics ·Pharmaceuticals ·Food additives
	Pigments	·Cosmetics ·Neutraceuticals ·Pharmaceuticals ·Food supplement ·Dyes and paints

	Vitamins and minerals	·Bio-fertilizers ·Antioxidants ·Functional food
Biochemistry	Biosorption	·Removal of heavy metals
	Metabolism	·Removal of nitrogen ·Removal of phosphorous
	Photosynthesis	·CO2 fixation ·H_2 production[54]

When culturing microalgae at an industrial scale some there are some problems like contamination of the cultures, population density, temperature control among others, either in open pond systems or closed reactors. Also, the applicability of microalgae biomass and its use in energy production becomes difficult when handling large microalgal culture collections, because it consumes time and therefore human and economic resources.[55] On the other hand, the principal problem faced in algal water purification systems was the harvesting of the algal biomass from the treated water for further recycling of the wastewater and to maintain the high-value algal biomass.[56] These problems were overcome when *Chlorella* was immobilized[57], besides the easy product separation, it also reduces the processing costs.

In this project the biochemical properties of living *C. vulgaris* are intended to be exploited for bioremediation due the actual concern about environmental pollution affecting water, air and soil quality. The immobilization of the microalga would help in the treatment of wastewater either by removing nitrogen and phosphorus or heavy metals while capturing CO_2 from air, acting as a bioreactor. Encapsulation of the microorganism has proven a better efficiency in the removal of the water contaminants.[58]

2. *Immobilization of microalgae*

2.1. Immobilization techniques

2.1.1. Passive immobilization

Passive immobilization relies on the natural predisposition of many microscopic organisms to attach to surfaces and grow on them.[69] This characteristic has made possible the immobilization of cells in different kinds of carriers[70], natural or synthetic. The

interaction between cell and surfaces usually is easily reversible and contamination by unstuck cells is inevitable.

2.1.2. Active immobilization

The active immobilization is differentiated by the chemical exchange between the cell and the surrounding environment. This artificial immobilization comprises the use of flocculants, chemical attachment and gel entrapment, which is the most widely used for algal immobilization. Among the gels used for the entrapment, some are synthetic (acrylamide, polyurethane, polyvinyl, etc.), others are natural polymers (collagen, agar, agarose, cellulose, alginate, carrageenan, etc.).[68] The entrapment gel must be porous to allow the diffusion of substrates and products to and from the cells. The inorganic silica matrix is one of the best options as a host structure for living cells.

2.2. Generalities

Immobilization of microorganisms within inert matrix has taken an important role in the development of new biotechnological processes and hybrid materials. This biotechnological advance has solved some problems encountered in the application of free microorganism or specific enzymes, biomolecules or organelles with relevant biological functions. When living cells are isolated, they can be very fragile or they cannot perform a particular biological process[59], after immobilization the stability increases and it is possible to scale-up the bioprocess because the cells are reusable.[60,61]

The biotechnological applications of immobilized microalgae is still being investigated, trying with a variety of matrixes and immobilization techniques. Novel living hybrid materials are continuously being designed. Referring to microalgae, these focused in the construction of biosensors and bioreactors. Immobilized algal cells are not limited to pollutant removal, these hybrid materials work as biosensors measuring toxicity[62,63], and as bioreactors producing hydrogen[64] or electricity.[65]

An immobilized cell is defined as a living cell that, by natural or artificial means, is prevented from moving independently from its original location to all parts of an aqueous

phase of a system.[66] From the latter concept, immobilized algae within a biological or inert matrix should achieve the necessary biotechnological benefits as the free algae in their medium[67]. To some extent the immobilization affects microalgae caused by the interaction between the matrix and cells. **Table 1.*4*** presents the principal requirements of a useful and efficient immobilized algal system and the properties of an ideal matrix for immobilization.[68]

Table 1.4 Basic requirements of a useful immobilized algal system and properties of an ideal matrix for immobilization.

Requirements of an useful immobilized algal system	Properties of an ideal matrix for immobilization
Retention of viability	Non-toxicity
Ability to photosynthesize	Phototransparency
High density of cells	Stability in growth medium
Continued productivity	Retention of biomass
Low leakage of cells from matrix	Resistance to disruption by cell growth

2.3. Silica matrix

The physical and chemical properties of silica face the problems related with lack of stability and uncontrolled environment of cells, presented by the aforementioned materials. Among the disadvantages of these materials are: poor thermal and mechanical stability, uncontrolled porosity and a non-fully biocompatible synthesis process.[59] However, silica is an excellent host due to (1) its porosity that is manipulated through the synthesis method[71], (2) its chemical stability and its mechanical strength that are much more superior compared to the commonly used organic polymers in bioencapsulation[72], (3) silica materials are biologically inert, non-toxic and they cannot be nutrient for external microorganisms[73], and finally, (4) silica is optically transparent allowing the penetration of light radiation which reaches the photosynthetic organisms.

2.3.1. Sol-gel encapsulation method

Most of the encapsulation processes, of enzymes or whole cells, within inorganic silica matrices have been done by the sol–gel method. The conventional method involves low-temperature hydrolysis of a proper monomeric silica precursor[74], frequently the

precursor is an alkoxide $Si(OR)_4$, where R is an alkyl group. The formation of a silicon dioxide network takes place in a two-step reaction:

- Addition of water leads to the hydrolysis of the precursor, obtaining silicic acid:

$$Si(OR)_4 + 4H_2O \rightarrow Si(OH)_4 + 4ROH$$

- Condensation proceeds spontaneously to obtain silica. Finally the overall reaction is:

$$Si(OR)_4 + 2H_2O \rightarrow SiO_2 + 4ROH$$

During the condensation reaction, there is a progressive growth of the silica particles giving rise to colloidal solutions (sols) and finally the wet gel. After gels are partially dried at room temperature (RT), the obtained solid materials consists of a porous network of hydrated silica.[75]

Unfortunately, the usual sol-gel reaction pathway with alkoxide releases alcohol as a byproduct which is not cytocompatible with microalgae, as they are sensitive to methanol and ethanol.[76] As a result new synthetic routes have to be developed to maintain the viability of sol-gel procedure in bioencapsulation, either by getting rid of the alcohol[77], using aqueous precursors[78], or two-step encapsulation based on biopolymer-silica hybrids.[8] Rooke *et al.*[79] described the entrapment of microalgae by using an aqueous precursor but, only photosynthetic pigments were preserved after encapsulation while the algal metabolism might be affected by glycerol and/or silica nanoparticles.

It is important to highlight that sol-gel process allows to modify the technique in order to tune the porosity of the silica. Depending on the synthetic approaches, the pores might be arranged and connected in very different ways. The amorphous gels prepared through this method present intrinsic micro- and mesoporosity.[80] Among the strategies to adapt the sol-gel process, according to the desired porous network, including a structure directing agent is one of them resulting in mesopore and/or macropore formation. Suitable examples of these, also called templates, are molecular species used in zeolite synthesis, low molecular weight

and block copolymer surfactants, emulsions and/or solid particles.[81] A combination of the examples mentioned before results in materials comprising multiple levels of pore sizes.

The property of controlling the porosity of the silica through the sol-gel process benefits the present research to accomplish the objective of attaining a hierarchically organized porous silica monolith, with meso- and macro porosity. Due to sol-gel procedure also allows processability in terms of shaping, the monolith form is chosen in terms of the possible applications of the hybrid material. IUPAC defines the monolith as "a shaped, fabricated, intractable article with a homogeneous microstructure that does not exhibit any structural components distinguishable by optical microscopy".[82]

The technique applied in the synthesis of the microalgae-silica monolith involves two structure directing agents (**Figure 1.6**): (1) a block copolymer, as surfactant and template to achieve mesoporosity, and (2) an emulsion (HIPE) for the formation of a macroporous level. This technique is proposed in this work for the first time with the purposes of microalgae and alga-like plant encapsulation into silica matrix.

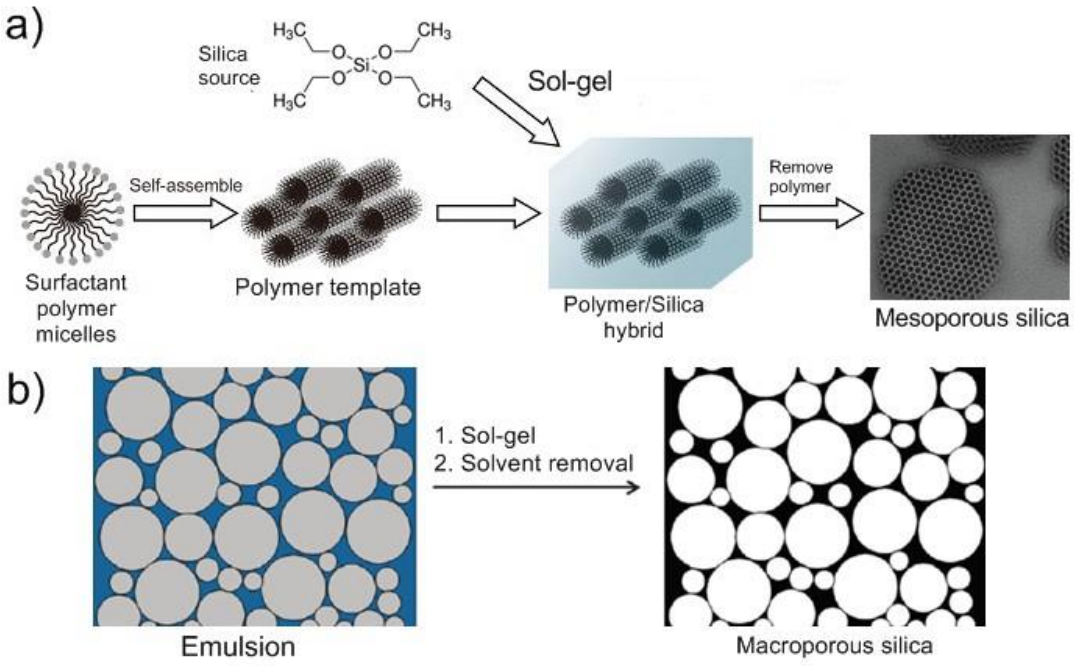

Figure 1.6 Schematic representation of the double-template in sol-gel process (a) surfactant polymer templating mechanism and (b) emulsion-templating approach to porous silica.

3. *HIPE as alga-like plant or microalgae encapsulation method*

3.1. High internal phase emulsion

A HIPE is a concentrated liquid/liquid immiscible system possessing a large volume of internal, or dispersed phase.[83] The internal phase volume ratio (ϕ) for this kind of emulsions is higher than 0.74. The value of ϕ stands for the maximum volume ratio of uniform non-deformable spheres when packed in the most efficient manner. For values above 0.74 it results in the deformation of the dispersed phase droplets into polyhedral, which are split by thin films of continuous phase.[84] Frequently large amounts of surfactant stabilize the conventional HIPEs systems consisting of an organic phase and an internal aqueous phase.

Besides applications in various areas such as food preparation, fuels, oil recovery and cosmetics, HIPEs have been really useful in materials science as templates to create highly porous structures.[85] Much of these porous materials are polymeric structures prepared by polymerizing the continuous phase of an HIPE (polyHIPE).[86] Further research is focused in replacing surfactant with colloidal particles unifying both a HIPE and a Pickering emulsion as a templating route to get highly porous materials.[87]

3.1.1. Pluronic® P123 copolymer as HIPE stabilizer

Pluronic® P123 ($EO_{20}PO_{70}EO_{20)}$ belongs to a diverse group of non-ionic triblock copolymers composed of a hydrophobic polypropylene oxide block (PO) surrounded by two hydrophilic polyethylene oxide blocks (EO).[88] PEO and PPO blocks are arranged in a basic triblock A-B-A structure and can schematically be expressed as (EO)x–(PO)y–(EO)x.[89] These different amphiphilic polymers differ by their molecular weights and variations in the EO/PO ratio. Likewise by changing the repeating units of the Poloxamer, it is feasible to modify the size, hydrophilicity and hydrophobicity of the system.[90]

The amphiphilic chemical nature of Pluronic® P123 allows it to form spherical and tubular micelles (**Figure 1.*7***), in consequence it has several applications as emulsion stabilizer[91], pharmaceutical ingredient[92], biomedical material[93], template of mesoporous

materials[94,95], among others. These applications exemplify the potential of the Pluronic triblock copolymer as a great candidate to perform the encapsulation of alga-like plant or microalgae within a silica matrix by HIPE. The three principal reasons are: (1) it will act as surfactant and emulsion stabilizer, (2) it produces well-ordered hexagonal mesoporous silica, and (3) it is biocompatible.

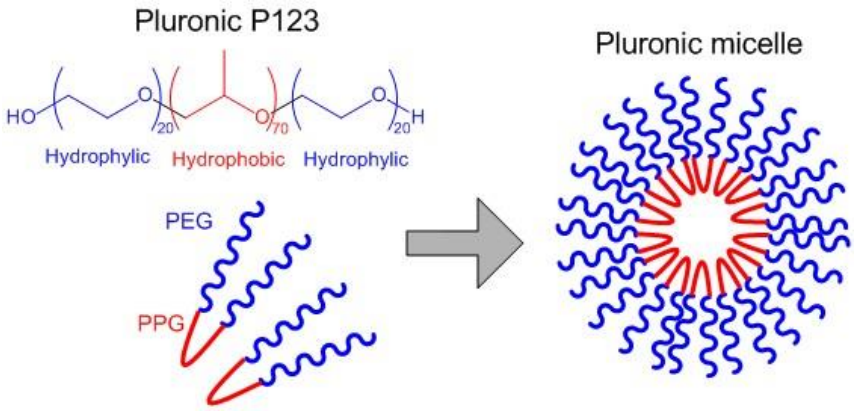

Figure 1.7 Schematic diagram showing how Pluronic micelles are formed due to the hydrophilicity of PEG block and the hydrophobicity of PPG block

3.2. HIPE-copolymer double template for microalgae or alga-like plant encapsulation

This novel one-pot technique for the immobilization of *C. vulgaris* or alga-like plant within a porous silica matrix is based in a high internal phase emulsion in which the HIPE and a copolymer are structure-directing agents in a sol-gel process[96]. This HIPE is an oil-in-water emulsion consisting of an aqueous continuous phase, which contains the surfactant Pluronic P123 in acidic solution to initiate the sol-gel process of a silica precursor (TEOS), and the oil dispersed phase (nonpolar organic solvent) in which the microalgae or alga-like plant is introduced. When the colloidal suspension of silica is generated and the alcohol has volatilized, before the gelation process starts, the oil phase is added to start the formation of the emulsion. Finally when HIPE is stabilized and it starts to dry, the oil phase evaporates while liberating the macroporosity where the algae or microalgae are immobilized. This yields a hybrid material made of microalga or alga-silica monolithic structure, as presented

in **Figure 1.*8***. Besides the role of the emulsion stabilizer, the triblock copolymer together with the templating emulsion give rise to a hierarchical, interconnected, and high meso- and macroporosity within a silica monolith, keeping also a high surface area when porosity is free.

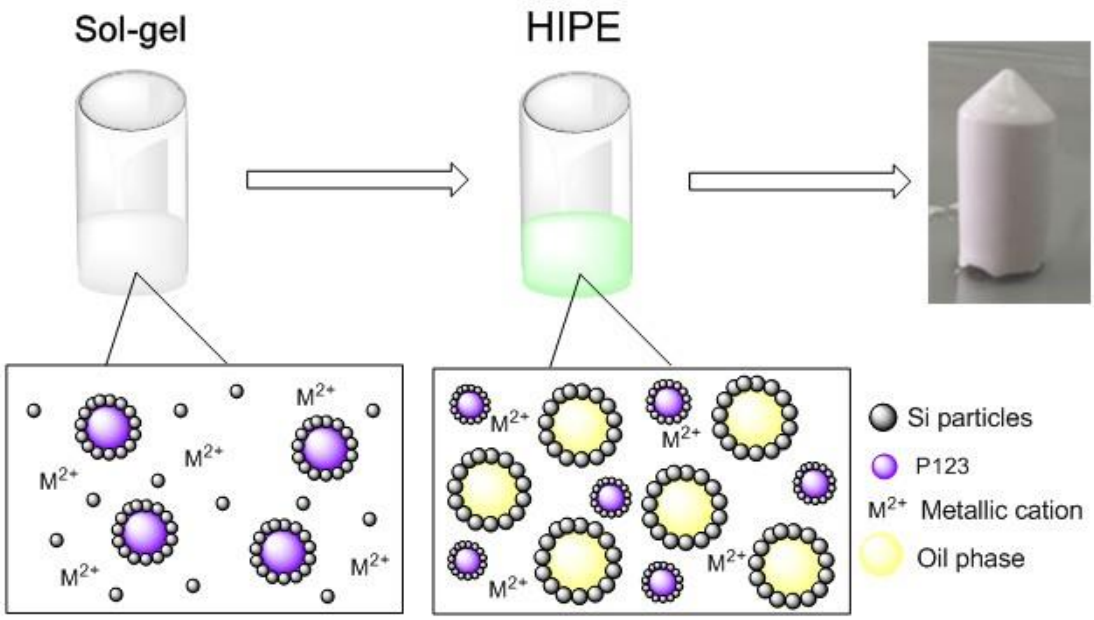

Figure 1.8 Schematic representation of the high-internal phase emulsion (HIPE) used as a template for the monolithic material.

Chapter 2: Methodology

1. *Microalgae cultivation*

1.1. Chlorella vulgaris

Chlorella vulgaris was grown at a laboratory scale by sub-culturing it in Bold's Basal medium (BBM), following the recommendations of the Standard Operating Procedure for culturing *Chlorella vulgaris* of the University of Reading. The BBM is an inorganic salts medium broadly used with free living planktonic freshwater algae, it usually contains diluted alkaline salts, phosphate and nitrates salts and transition metals salts. Cultures were maintained in 1 L Erlenmeyer flaks and aerated to aid gas exchange and to keep algal cells in suspension. Suspension cultures were incubated under illumination at 20°C until they were green enough. The growth of the microalgae culture was followed by direct measurements of a small volume of the suspension using UV-Vis spectroscopy in the visible range from day 0 until day 18.

1.2. Alga-like plant

The alga-like plant was harvested from an artificial freshwater stream used for agriculture in Urcuquí, Ecuador. Generally it gets stuck in objects that water flow cannot carry, like rocks. After a sample was taken directly from the water stream, it was thoroughly washed with water removing any visible living organism and external abiotic factors. Finally, when only the alga-like plant was visible, it was rinsed out with distilled water and kept until further use.

2. *Synthesis of hybrid microalgae or alga-like plant silica monolith by HIPE*

2.1. Materials and reagents

Tetraethyl orthosilicate (TEOS, 98%), hydrochloric acid (HCl, 37%), Pluronic® P123 ($EO_{20}PO_{70}EO_{20}$, M_n ~5800), copper (II) nitrate trihydrate ($Cu(NO_3)_2 \cdot 3H_2O$, > 98%),

zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$, > 99%), sodium fluoride (NaF, ≥ 99%), were purchased from Sigma Aldrich and used as received. The organic solvents cyclohexane (99.9%) and n-hexane (99.9%), were purchased from Fischer Chemical

2.2. Synthesis of alga-like plant and microalga-silica monolith

The encapsulation of microalgae within the pores of a silica monolith by HIPE was based on the one-pot process reported in "Reinforced silica monoliths functionalized with metal hexacyanoferrates for Cesium decontamination: A combination of one-pot procedure and skeleton calcination" [97]

The hydrolysis of 1,075 mL of the silica precursor TEOS was carried out by adding it into 2 mL of an acidic solution (pH = 2 where the rate of condensation reaches its minimum) of 20% (w/w) of Pluronic® P123 under a fixed stirring at RT. For some hybrids 9.4 mg of M(II) nitrate were mixed with the acidic solution, M(II) corresponds to Cu or Zn. When the hydrolysis of TEOS has finished after 30 min, systematically the emulsifying process began by continuously adding dropwise 5.1 mL of oil phase (cyclohexane or n-hexane), previously incorporated with *C. vulgaris* or alga-like plant biomass. To incorporate *C. vulgaris* in the oil phase 5-15 mL of the culture were centrifuged to remove the aqueous medium; this allows the emulsion stabilization and kept the oil phase/liquid phase ratio. Then the supernatant was discarded and the remaining biomass was weighed and mixed with the oil phase. It is worth to mention that preparation of the biomass should be done within a short time range before the addition of the oil phase. This recommendation will avoid algae death due to they would not have access to water until encapsulation. Silica precursor polymerization and pH regulation was enhanced by the addition of 20 µL of NaF (8 g/L) once the stability of the emulsion was reached. For the treatment of the alga-like plant, a small quantity of biomass was dried and smashed until obtaining a homogenous powder from which 5 mg were mixed with the oil phase.

The final oil-in-water emulsion was poured into a tubular plastic mold that confers the form to the monolith after the aging happens. The final step was the slow evaporation of the oil phase to liberate the macropores. The samples were placed in a desiccator at RT under

a n-hexane atmosphere for seven days. For reproducibility concerns, all the samples were fabricated in duplicate or triplicate. **Figure** shows step by step the encapsulation methodology:

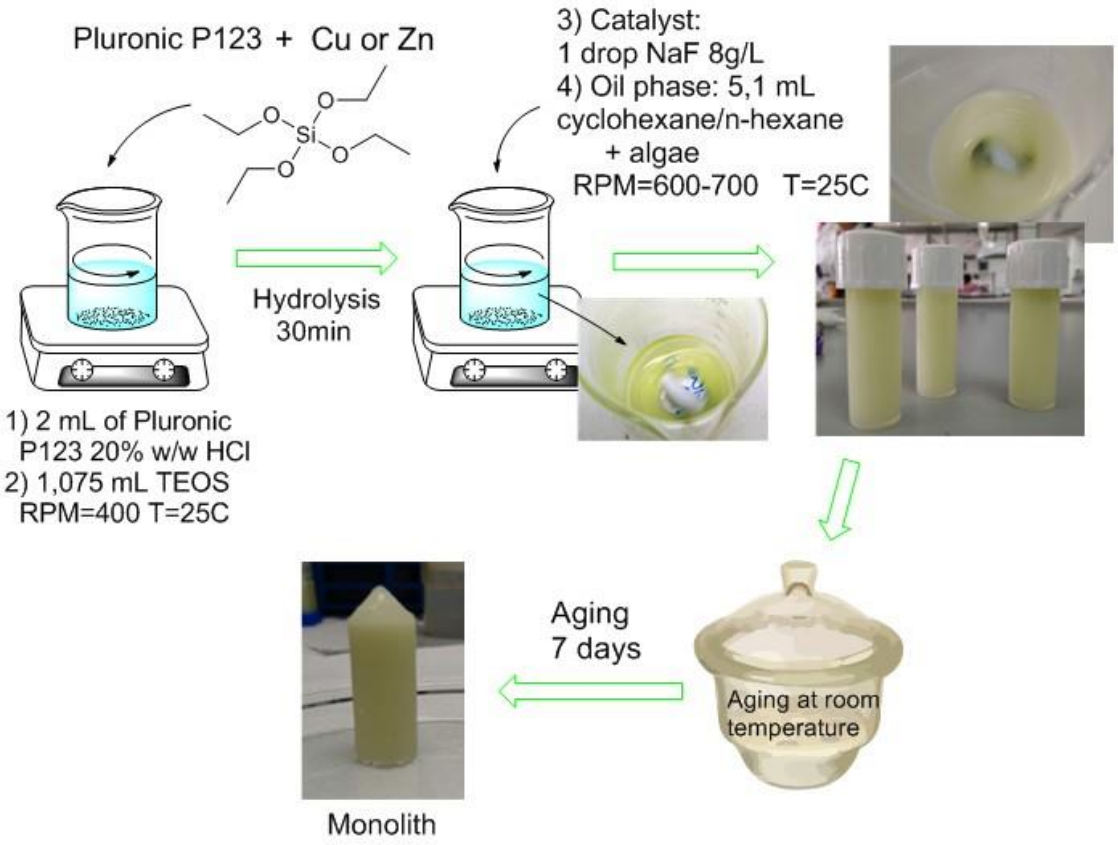

Figure 2.1 Scheme of the synthesis of algae-silica monoliths.

The most relevant samples, wherein encapsulation was successful, are shown in **Table 2.1** with their synthesis parameters.

Table 2.1 Relevant algae-silica monoliths with their synthesis parameters

Name	TEOS (ml)	Microalgae (mg)	Oil phase (ml)	Metal (mg)
Cu/Si/CV18	1.075	125 *C.vulgaris*	5.1 cyclohexane	9.54
Zn/Si/CV19	1.075	125 *C.vulgaris*	5.1 cyclohexane	9.54
Si/CV35	1.075	125 *C.vulgaris*	5.1 n-hexane	-

Si/CV36	1.075	250 *C.vulgaris*	5.1 n-hexane	-
Si/AP41	1.075	5.7 alga-like plant	5.1 n-hexane	-

One of the monoliths of each of the following samples Cu/Si/CV18 and Zn/Si/CV19, after aging process, were immersed in the BBM culture medium used to grow *C. vulgaris.*

3. Characterization of biohybrid monoliths

The characterization of the microalgae, algae-like biomass and biohybrids provides comprehensive up-to-date coverage of material's structure and properties, widely applicable in the diverse field of materials research[98]. Mainly, optical imaging and spectroscopy are commonly used in this kind of research, to have information about the morphology and spectral properties of biomaterials. Thus, UV-Vis, and diffuse reflectance UV-Vis spectroscopies, fluorescence spectroscopy and microscopy were performed in this work to characterize the samples.

3.1. Ultraviolet-visible Spectroscopy

This technique allows to keep track of microalgae growth in presence of NaF by measuring the absorbance of microalgae's chlorophyll, the intensity of the chlorophyll peaks increase meaning that the concentration of the photosynthetic pigment increasing too and therefore the microalgae population. A Lambda 1050+UV/Vis/NIR Spectrophotometer from PerkinElmer® was employed to measure an aliquot of the *C. vulgaris* culture in a quartz cuvette for liquid samples in the visible range from 400 nm to 800 nm.

3.2. Diffuse reflectance ultraviolet-visible Spectroscopy

DRS technique helps to monitor ongoing chemical reactions within solid particles or dull surfaces as powders. These kind of samples are irradiated with an electromagnetic source resulting in a collection of signals and their subsequent analysis of surface-reflected electromagnetic radiation measured as a function of the wavelenght.[99] The reflected signals are a composition of two type of reflections that can occur: the specular reflection associated to the reflection from the element's surface, where the angle of incidence equals the angle of

reflectance, and the diffuse reflection associated to a combination of interactions including absorption, transmission, and scattering properties of the illuminated element.

With the aim of following the activity of the photosynthetic pigments of microalgae within the silica matrix, data was recollected from a PerkinElmer® Lambda 1050+UV/Vis/NIR Spectrophotometer in the wavelength range from 400 nm to 800 nm implemented with a Praying Mantis module. Prior to the measurement a small piece of the monolith of about 0.5 g was pulverized and placed in the sample holder. Several measurements were made aver a long period of time every two or three weeks.

3.3. Fluorescence Spectroscopy

This method analyzes the fluorescence of a molecule by passing a beam of light through analyte containing the fluorescent specie for its identification and quantification. The technique is based on the photoluminescence produced as result of raising an electron from the ground state to the excited state followed by a quick relaxation undergoing thermal energy loss through vibrations until reaching the lowest vibrational level of the excited state where the emission of the photon occurs.[100] Then the intensity of the light emitted by the photon is detected as a function of the wavelength.

Spectra were recorded in a Cary Eclipse Fluorescence Spectrophotometer from Agilent® with an excitation at 430nm in a wavelength range from of 600 nm to 800 nm. 0.5 g of each sample were reduced to powder and placed in the sample holder without leaving holes where light could pass. This characterization was made in the Instituto de Física at the University of Puebla (BUAP), Mexico. Fluorescence spectroscopy allows to monitor the activity of the photosynthetic pigments while immobilized microalgae are alive.

3.4. Fluorescence Microscopy

This microscopy technique uses the same principle of fluorescence as the spectroscopy technique where the analyte is irradiated with high energy light and separates the fluorescent light emitted from the excitation light. Then the fluorescent light passes through the sample and a blue filter to allow the detection of the fluorescent parts.

The Olympus® BX63 Motorized Upright Microscope allows to observe autofluorescence of living microalgae and verify the evolution or not, as well as the preservation of the photosynthetic pigments. All samples were prepared before the analysis by pulverizing around 0.5 g of the hybrid monolith and placed on glass slides. Images were seen with 4X and 20X objective lenses using a black filter from 450 to 490 nm, which allows the green and red emissions

Chapter 3: Results and Discussion

Alga-like plant and microlaga-silica monolith were synthesized by a novel method, proposed by the thesis authors, which have not been used before to encapsulate living algae. These monoliths exhibit hierarchical meso- and macroporosity wherein *C. vulgaris* or an alga-like plant are encapsulated. Before encapsulation, the activity of free algae's chlorophyll was analyzed by UV-Vis spectroscopy which allows a further comparison of the activity and evolution of microalgae when encapsulated within the silica monoliths occurs. Then the biohybrid were characterized by fluorescence spectroscopy and microscopy thanks to the relevant information that these techniques can give about the chlorophyll's behavior. Finally the characterization by DRS UV-Vis supports the results from fluorescence techniques.

1. *Chlorella vulgaris growth*

Microalgae growth was followed by the increment of the intensity of the representative chlorophyll *a* bands in the visible spectra, which are Q_y and Soret, found in the red region at around 660 nm to 700nm and in the blue region between 410nm and 430 nm, respectively. The increment of the absorbance of these bands is considered as an increase of the chlorophyll concentration in the culture, based on the linear relation from Lambert Beer's law, where absorbance is directly proportional to the concentration of the analyte. Thereby an increase in the chlorophyll concentration implies that microalgae population is growing through cell division thanks to the favorable environmental culture conditions and the presence of all the nutrients required for their growth, as the ones contained in BBM.

An evident increase of the Chl *a* concentration is clearly seen starting at day 9 where the wavelengths for the maximum absorption of Q_y and Soret bands are 680 nm and 430nm, respectively, as shown in **Figure 3.1**.

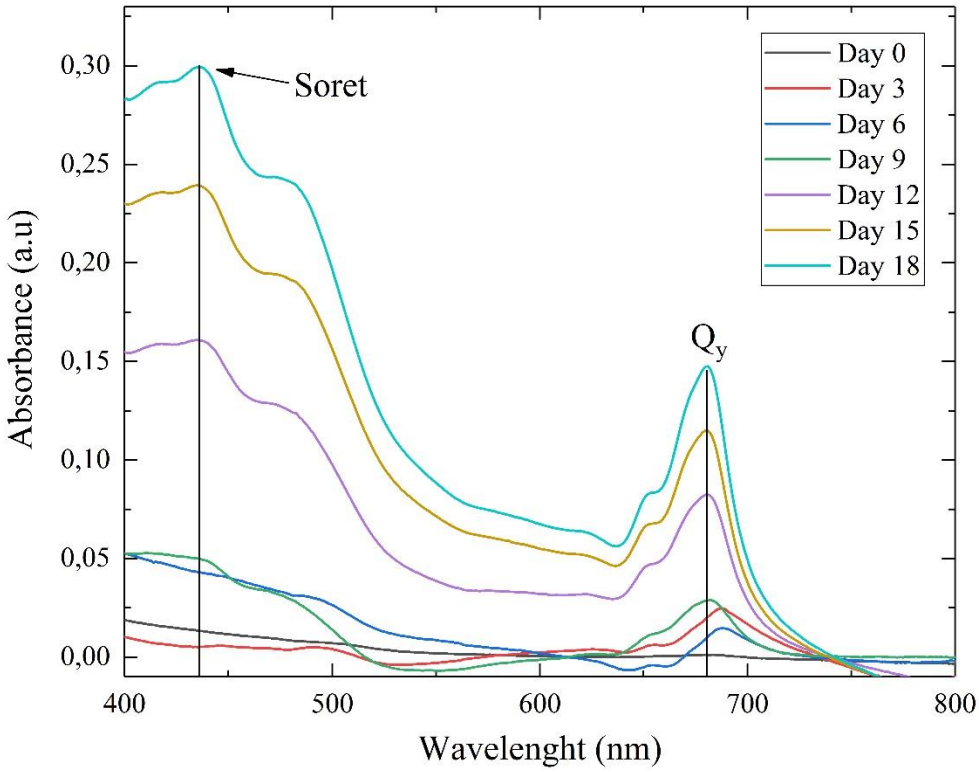

Figure 3.1 UV-Vis absorption spectra of living C. vulgaris in BBM culture media showing the characteristic bands of the chlorophyll.

The peak of Chl *a* at 680 nm is the common peak observed when algal suspension is studied as seen in **Table 3.1**. This bathochromic shift usually happens due to the Chl is not extracted but measured *in vivo* as a suspension consisting of microalgal cells grown in water which is considered a polar solvent; this causes the shift to the red region in comparison with values of the extracted chlorophyll from microalgae between 660-665 nm.

Table 3.1 Comparative UV-Vis absorption and fluorescence data of Chl *a* in microalgae

Environment	Absorption (nm)	Environment	Emission (nm)
Diethyl ether	660	Diethyl ether	668
Acetone	662	Acetone	668
Methanol	650	Methanol	674
In vivo	680	In vivo	682

The concentration of Chl *a* until the 6th day seems to be constant and the λ_{max} of the Q_y band has a small bathochromic shift. This can be explained by the lag phase that some microalgal cultures present when the culture has just begun. There two reasons why this phase might occur, either it is due to a period of physiological adjustment when there is a change in the culture conditions or the nutrient, or the presence on non-viable cells in the medium.[39] The growth lag during the first six days may be caused by the presence of the NaF solution that was added in the inoculum. The 8 g/L solution of NaF was added in the culture as a test to verify that NaF used for the synthesis of the hybrid monolith would not irreversibly damage the microalgae. Fortunately, the *C. vulgaris* growth adjusted to the change and it can normally growth as seen in **Figure 3.2** where the algal suspension acquired an intense green color making them ready for the encapsulation.

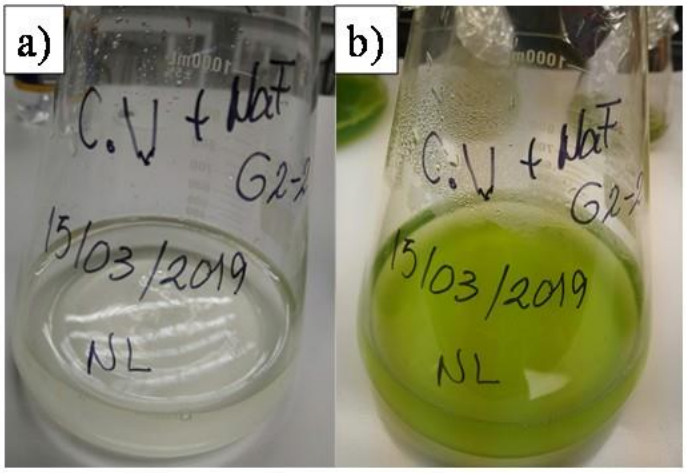

Figure 3.2 Images of the *C. vulgaris* culture at (a) day 0 and (b) day 18.

2. *Photosynthetic activity of Chlorella vulgaris within the silica monolith*

The analysis of the photosynthetic activity of encapsulated algae and microalgae is based mostly on the fluorescent properties of chlorophyll *a*. In the first instance, the influence of the presence of copper or zinc cations in the silica network is discussed by fluorescence techniques. DRS-UV-Vis results and discussion only confirm information obtained from

fluorescence and therefore a complete analysis of the photosynthetic activity of the immobilized microalgae within the hierarchical silica monolith.

2.1. Copper and zinc effect on immobilized *Chlorella vulgaris*

Transition metals, such as copper and zinc were chosen to improve the encapsulation of microalgae as a continuous source of micronutrients for long-term viability of the bio-hybrid silica monoliths as well as the bactericidal effect of copper to increase the protection for external microorganisms. Indeed, these metals are considered essential micronutrients for photosynthetic organisms, but they have severe toxic effects when their concentrations exceed 5 mg/L. During the monolith synthesis, they were introduced in the aqueous phase to form part of the silica's network when gelation occurs. Neither the metals nor the *C. vulgaris* are free within the silica matrix which allows a different interaction between the cations and the algae in contrast with their free analogs in culture media. Moreover, during the emulsion formation, it seems that the oil phase protects the microalgae biomass by creating a layer surrounding the cells. This protection avoids any direct contact with alkoxide alcoholic byproducts from TEOS and metallic cations during the synthesis and the gelation process.

2.1.1. Fluorescence spectroscopy

Both copper- and zinc-containing biohybrid monoliths present the maximum emission of Chl at around 659 nm, which corresponds to Chl *b*.[103,104] , as shown in ***Figure 3.3***

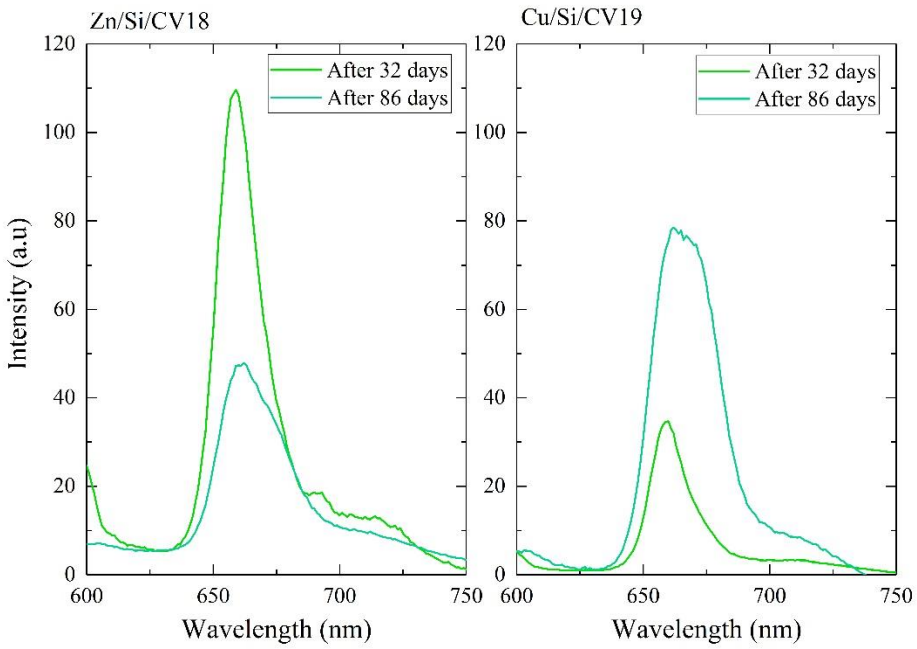

Figure 3.3 Fluorescence spectra of microalgae- silica monoliths with zinc (left) and copper (right) at different times.

The fluorescence of Chl pigments has a direct relationship with the performance of the photochemical reactions within the photosystem II, which confirms the efficacy of the encapsulation technique presented in this work in maintaining the stability of the photosynthethic pigments and, thus, the viability of the cells. The success of the Zn/Si/CV19 monolith is due to zinc that is considered as an element of life and contributes to the algae growth[109]; this explains why it is used as a trace element in the preparation of the BBM medium where *C. vulgaris* is cultured. Starting from the same conditions, it is obvious that zinc has a better impact on the viability of C. vulgaris within the monoliths, compared to copper. Indeed, the fluorescence of zinc-containing biohybrid monoliths is at least twice the one of their copper counterparts.

However, only two measurements by fluorescence spectroscopy were done during the short stay at BUAP University in Mexico. In order to confirm the observations by emission spectroscopy, the samples were studied by fluorescence microscopy.

2.1.2. Fluorescence microscopy

In **Figure 3.4**, one can see by fluorescence microscopy (taken 257 days after the biohybrids Cu/Si/CV18 and Zn/Si/CV19 were synthesized) that the presence of copper was not favorable for the microalgae and they lost their fluorescence, therefore their photosynthetic activity. However the presence of zinc was much better to preserve microalgae photosynthetic properties than copper, as shown in the same figure (b). The emitted natural red fluorescence from the chlorophyll (Chl) present in the immobilized microalgae corroborates this affirmation. This means that the photosynthetic machinery remains intact for more than 200 days since its synthesis without a constant feed of culture media. The red color is characteristic of the Chl, because it absorbs light at the UV region or blue region (like in the experiment) of the spectra and, upon excitation, it emits fluorescence at a longer wavelength than the Q bands in the red region, this shift of the Q_y band is known as the Stokes shift.[26]

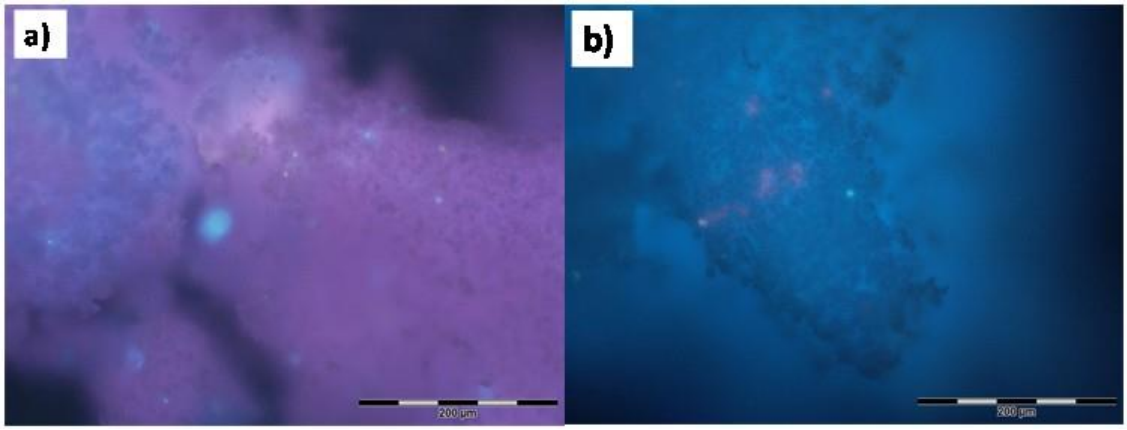

Figure 3.4 Fluorescence optical micrographs of (a) Cu/Si/CV18 and (b) Zn/Si/CV19 after 257 days of its synthesis

This characterization technique supports as well the obtained results by fluorescence spectroscopy. The light reactions undergoing within the microalgae chloroplasts still active even when entrapped into the hierarchical silica matrix. The obtained results confirm the viability of the encapsulation technique developed in this work.

2.1.3. Diffuse Reflectance UV-Vis spectroscopy

Several measurements using the DRS-UV-Vis technique were carried out at different periods of time and gave a clearer idea of the evolution of microalgae within the monolithic structure functionalized with metals, as seen in **Figure 3.**

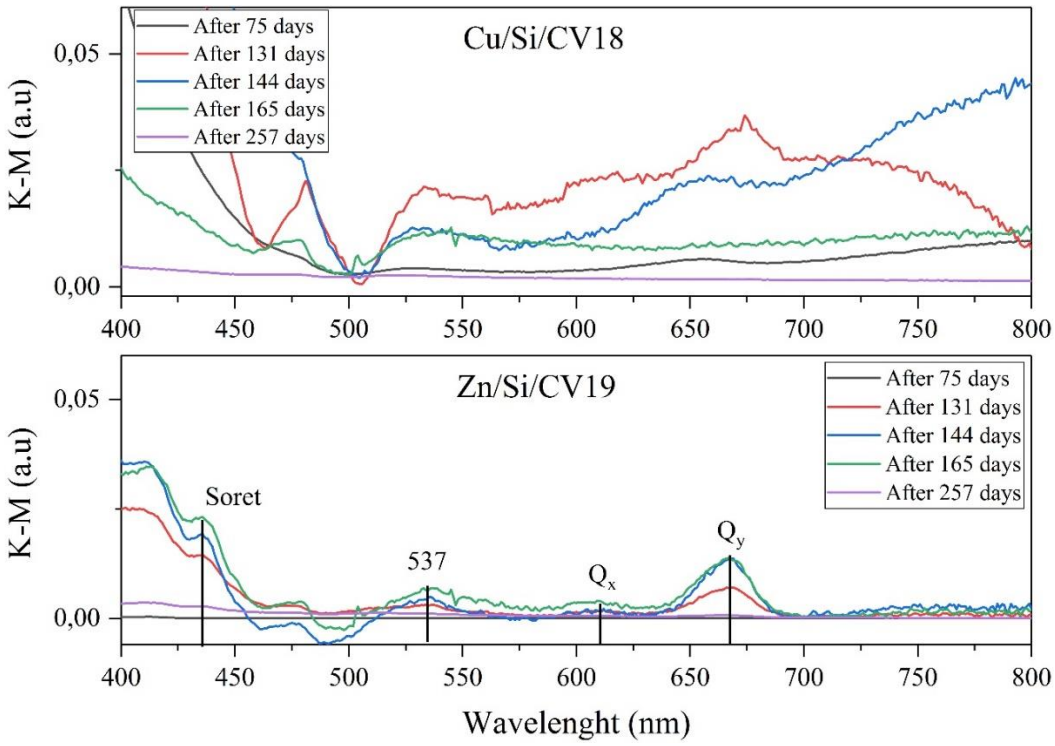

Figure 3.5 DRS UV-Vis spectra of Cu and Zn bio-hybrid monoliths measured at different periods of time and transformed to Kubelka Munk units.

DRS UV-Vis spectra demonstrates that, for the sample Cu/Si/CV18, the presence of copper within the silica matrix is harmful for microalgae. The amount of Cu added in the monolith exceeds the concentration that *C. vulgaris* can absorb without being toxic. It has been reported that at concentrations of 5 mg/L of Cu or Zn, leads to the decay of algae and the reduction of the chlorophyll content. But these high concentrations of Cu can only inhibit metabolic activity but does not necessarily induce the algal death,[107] thus the cells might not be synthesizing Chl, hence no absorption is seen. Moreover DRS UV-Vis of Zn/Si/CV19 sample reaffirms that the encapsulation of *C. vulgaris* within a silica matrix containing zinc was successful. The chlorophyll's Q_y band appears at 667 nm and the Soret band at 435 nm

which is common for *C. vulgaris*, as seen in **Table 3.1**. Additionally, another band that appears at around 537 nm corresponds to the degradation of Chl to pheophytin; this happens when the central magnesium is replaced by hydrogen. However the distinctive Q_x band from Chl is also showed at around 606 nm[108] confirming there is still chlorophyll within the immobilized microalgae and pheophytin represents the biomass of microalgae that is no longer photosynthetically active.

2.2. Test of micronutrients absorption of *Chorella vulgaris* within the silica matrix

The samples Cu/Si/CV18 and Zn/Si/CV19 were tested for the absorption of micronutrients contained in the BBM by letting them in contact with it until the last measurement.

2.2.1. Fluorescence spectroscopy

In **Figure 3.**, one can clearly see the typical emission of the chlorophyll from microalgae encapsulated within the silica monolith at 659 nm. The biohybrid monolith without constant feeding has an impressively much higher intensity of emission than the one of the monolith kept in BBM. Apparently, the presence of zinc inside the silica matrix as micronutrient might be enough for the viability of the photosynthetic activity.

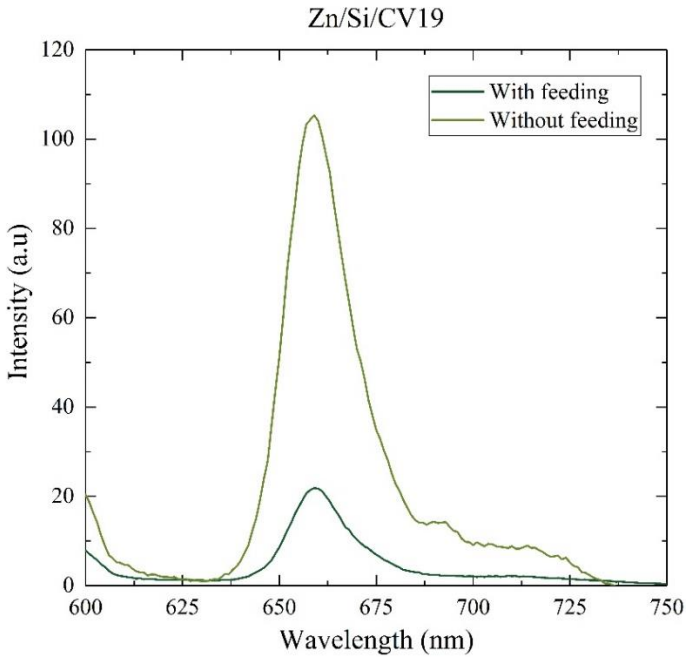

Figure 3.8 Fluorescence spectra from Zn/Si/CV19 with and without constant feeding

Regardless of the above analysis demonstrating BBM is not necessary to maintain active the chlorophyll, one measurement is not a definitive result. Hence DRS-UV-Vis data allows a better evaluation of the necessity of constant feeding or not.

2.2.2. Diffuse Reflectance UV-Vis spectroscopy

For the evaluation of the micronutrient absorption both monoliths Cu/Si/CV18 and Zn/Si/CV19 kept in BBM are evaluated trough time. In ***Figure 3.9*** the chlorophyll spectra show the common bands, Qy at 667 nm and Soret at 435 nm until they disappear through the time. The spectra were taken with the reflectance module called Praying Manthis while they were still wet (because they were immersed within the BBM). The problem of measuring wet samples is that peaks do not have a good definition.

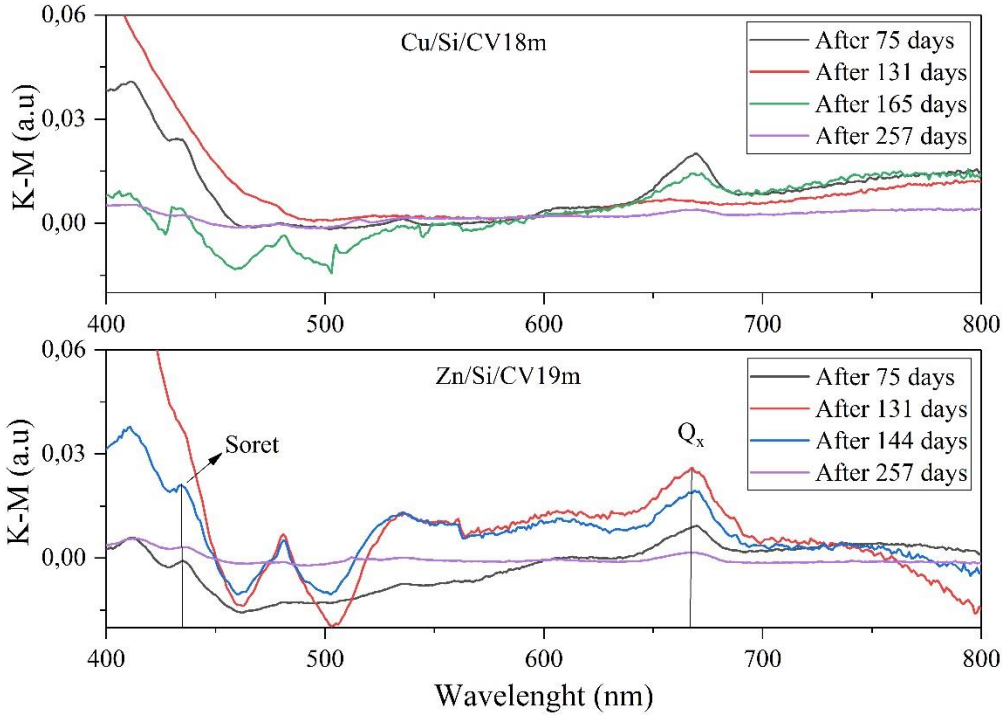

Figure 3.9 DRS-UV-Vis spectra of the zinc and copper microalgae hybrid monoliths constantly fed with the BBM.

The DRS UV-Vis spectra presents the growth of microalgae follows the pattern of the lag phase as the free algae culture seen before. The copper monolith even exhibits absorption compared with the monolith without feeding but not in a very significant way. The monolith with zinc does not improve the growth by having a constant feeding. Therefore, to improve the photosynthetic activity within the silica, it would be probably better to feed up the monolith regularly. By this encapsulation technique it is demonstrated that it is not really necessary to preserve the monoliths in contact with the BBM. However this work has also demonstrated that the purposed applications of the biohybrids like carbon dioxide fixation and wastewater treatment are achievable because wastewater could be used as source of nutrients for immobilized microalgae and they will remain intact inside the monolith.

2.3. Evolution of *Chlorella vulgaris* within the silica matrix

From the previous results, one can conclude that the fluorescence microcopy is very concordant with fluorescence spectroscopy and it can be used with the same confidence and accuracy to qualitatively characterize the biohybrid samples.

2.3.1. Fluorescence microscopy

The micrographs of samples Si/CV35 and Si/CV36 presented in **Figure 3.**, shows equally the preservation of the encapsulated microalgae because of the natural red fluorescence once excited with a blue excitation filter (450-490 nm). The fluorescence emission stills remains strong after 120 days of their synthesis. The absence of metals seems to be an environment that microalgae prefer when immobilized. Apparently, even if with zinc the fluorescence was kept over time, the quantity of this metal is still being bigger than the allowed quantity, 5 mg/L. These two samples differ one from another in the quantity of microalgae used for the synthesis, 125 mg and 250 mg, respectively.

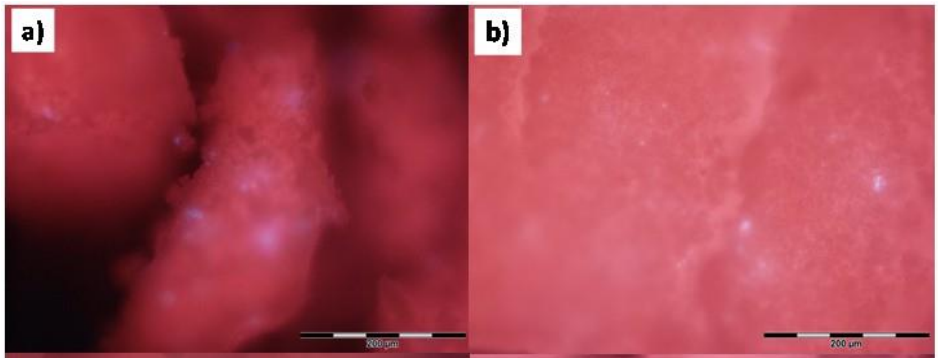

Figure 3.6 Fluorescence microscopy images from samples (a) Si/CV35 and (b) Si/CV36 after 120 days.

2.3.2. Diffuse Reflectance UV-Vis spectroscopy

An in depht study of the behavior of microalgae encapsulated over time was done by the analysis of its diffuse reflectance spectra transformed to Kubrlka-Munk units without the presence of metallic cations, **Figure 3.**.

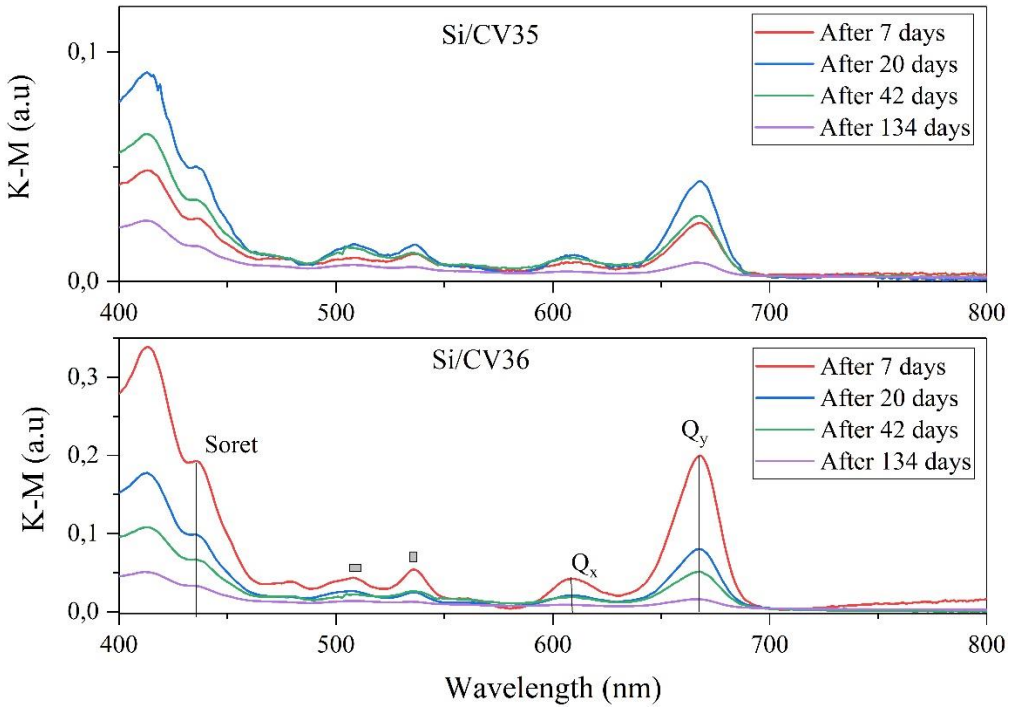

Figure 3.7 DRS-UV-Vis spectra of monoliths Si/CV35 and Si/CV36 measured at different periods of time.

The samples Si/CV35 and Si/CV36 DRS-UV-Vis exhibit spectra with the same pattern of the main peaks from chlorophyll and pheophytin. It is important to notice that here, the peaks exhibit a better definition than the ones of the biohybrid monoliths containing metallic cations which make much easier to observe the behavior of the Chl. Both hybrids absorb the light at 667-668 nm (Q_y) and 435nm (Soret) for chlorophyll. For pheophytin the corresponding typical bands are located at 505nm and 535nm. The intensity increment or decrease of the Q_y band shows how the Chl concentration increases and decreases with time. The behavior of Chl in the hybrid monoliths can be described as follows: for the sample with lower quantity of microalgae (Si/CV35), the chlorophyll content follows the same behavior as for microalgae growth in BBM where it is increasing until plateauing then started to decrease. For the sample with higher amount of microalgae (Si/CV36), the chlorophyll content starts by a huge absorption of the first measurement (7 days) and it gradually decreases as the time passes (134 days). This behavior is usually expected because when there is a larger quantity of microalgae biomass the chlorophyll content will be higher

together with a longer stability of the photosynthetic pigment. At the same time this shows that having an amount, twice the quantity of sample Si/CV35, implies that there is no more chance within the monolith to grow. Therefore, that means that now is known the maximum quantity of microalgae to put during the synthesis in order to do not affect the photosynthetic purposes of this hybrids.

3. Photosynthetic activity of algae-like plant within the silica monolith

The aim of using this species lies in the fact that the algae causing eutrophication can be encapsulated as well and create new materials for possible solutions in environments where accumulation of algae is a problem. Furthermore, due to the easiness of encapsulation of any photosynthetic organism with the HIPE copolymer-double template method to obtain hierarchical silica, one can take advantage of the native algae from Ecuador. However, further biological analysis are needed to have a better understanding of biological properties of this type of "alga" used.

3.1. Evolution of algae-like plant within the silica matrix

The evolution of the algae-like plant was also studied and compared with that of the microalgae.

3.1.1. Fluorescence microscopy

In **Figure 3.** it is observed the fluorescence emitted by the alga-like plant within the silica matrix after 121 days since its synthesis. The strong red emission produced by the excitation with a blue radiation confirms the light harvesting structure from the algae-like plant is still actively working. In contrast with the micrographs from the aforementioned microalgae it is not possible to find any difference between them. With the data obtained until now both photosynthetic organisms have displayed similar behavior.

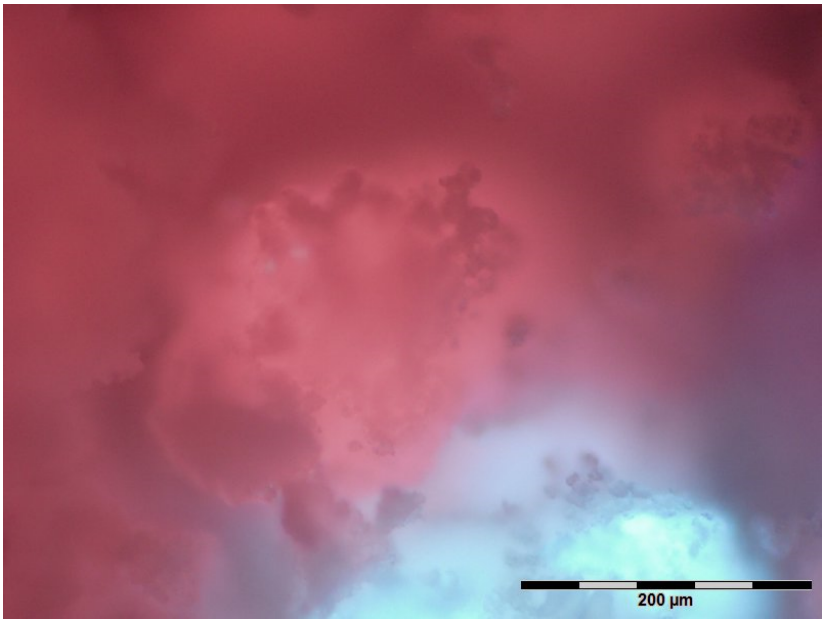

Figure 3.10 Micrograph from monolith Si/AP41.

3.1.2. Diffuse Reflectance UV-Vis Spectroscopy

Monolith Si/AP41 displays the spectra of the chlorophyll very similar to the spectra from the *C. vulgaris.* The Qy and Qx bands are present at 667 nm and 606 nm respectively, while the pheophytin peaks are at 505 nm and 534 nm, as shown in **Figure 3.**. Apparently, this algal species has the same growth behavior from the microalgae evolution where the spectra reflect a lag phase in the growth rate. These results demonstrate that the HIPE method developed in this work is very versatile regarding what could be encapsulated, since it could be any living cell or organelle. These results corroborates along with the qualitative results of fluorescence microscopy.

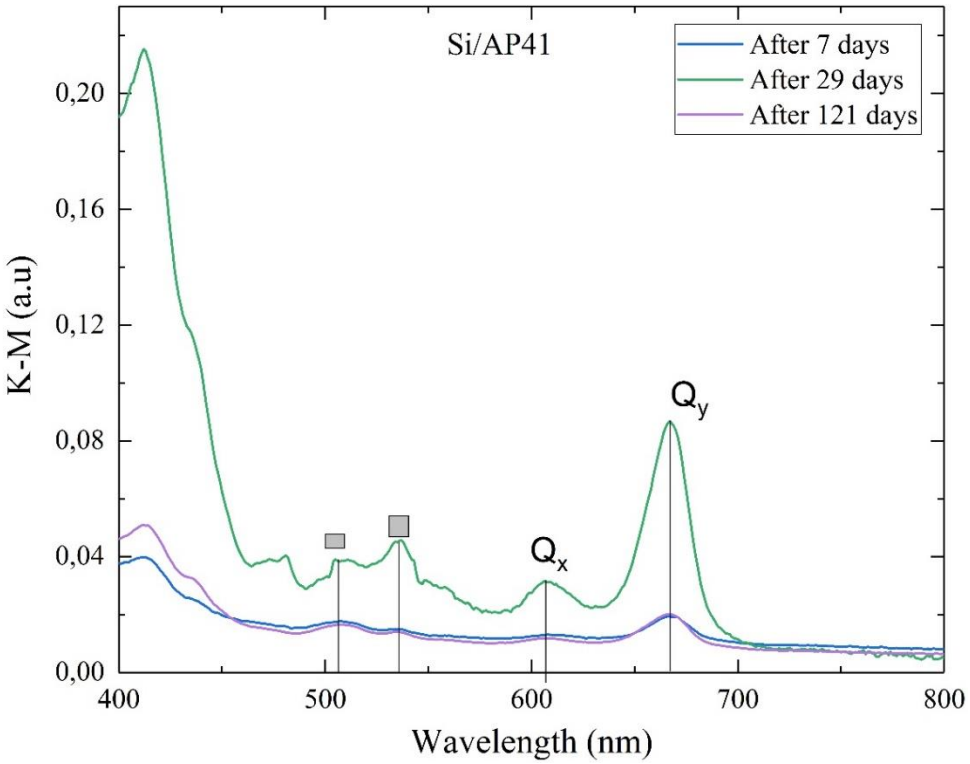

Figure 3.11 DRS-UV-Vis spectra of Si/AP41 monolith through time

Despite the fact fluorescence techniques and DRS-UV-Vis technique does not give information about the whole metabolism of the algae when encapsulated within a hierarchical silica matrix obtained by the novel method proposed in this work, one can say that this method is very promising to achieve algae and microalgae immobilization and preserve their photosynthetic activity. To ensure that the whole process of photosynthesis is working inside the entrapped algae or microalgae, this work needs quantitative techniques from oxygen or metabolites production following CO_2 absorption. The future analysis of the photosynthetic function will allow the application of the biomaterial on a large scale achieving the goal of carbon dioxide fixation and possible production of high value biomass as a photobioreactor

Chapter 4: Conclusion

- The growing of *Chlorella vulgaris* was successful and it was proved that the extra agents used for silica monoliths synthesis as NaF and n-hexane or cyclohexane did not affect the microalgae growth when cultured.
- The encapsulation of *Chlorella vulgaris* and the macro algae in a silica matrix by high internal phase emulsion is a suitable pathway to maintain their photosynthetic activity by preserving the chlorophyll pigments. The viability is demonstrated by the fluorescence emission in images obtained from fluorescence microscopy, as well as the characterization by diffuse-reflectance spectroscopy showing the evolution of the chlorophyll absorption through time.
- The synthesis of hybrid silica monoliths by HIPE is a one-pot procedure which does not require expensive reagents nor materials, it is very fast and simple and could be applied with to other living organisms or cells due to both the inorganic matrix and the synthetic process avoids any toxic interaction that might induce cell death or lysis.
- The novel technique for the synthesis of algae-silica monoliths based on an emulsion-copolymer double template seems to enhance the viability and stability of microalgae by introducing a micronutrient, such as the zinc, within the silica network. The trace metal has allowed to keep intact the photosystem II function that harvest light for at least more than 200 days without incubating the monolith in the culture media.
- The DRS-UV-Vis spectroscopy and fluorescence microscopy enable obtaining good information about the activity of the photosynthetic pigments inside the immobilized microalgae. The presence of chlorophyll and its concentration reflected in the absorption spectra measured at different periods of time gave qualitative information about the population of microalgae encapsulated within the porous silica network.

- The use of the BBM for keeping the microalgae nurtured did not improve in a significant way the stability of the photosynthetic activity of microalgae but the experiment allowed to have an evidence of their capability to be used in CO2 fixation and wastewater treatment.
- To ensure that the whole process of photosynthesis is working inside the anchored algae or microalgae, this work needs quantitative techniques from oxygen or metabolites production by CO_2 absorption. Thereby, it would be possible to achieve the application of the biomaterial on a large scale and have a possible production of high value biomass when carbon dioxide is absorbed.

Annex

Solution 0.01 M of HCl at pH=2

To prepare 100 mL of an aqueous solution of HCl, 0.084 mL were taken from the reagent bottle of HCl 37%, and poured into a 100 mL volumetric flak containing a small amount of distilled water, to avoid any hazardous reaction. Then the flask was carefully filled with distilled water until the graduation line. After measuring its pH, the as-obtained solution was correctly labelled and stored for further use.

Solution 20% (w/w) of Pluronic® P123 in HCl

To make a 100 g of the solution, 20 g of Pluronic® P123 were directly taken from the recipient and put into a flask. After that 80 g of the 0.01M HCl solution were added to dissolve the copolymer under stirring in an ice bath.

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