# UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

**Escuela de Ciencias Químicas e Ingeniería**

## TÍTULO: Encapsulation of photosynthetic plant cells within hierarchical silica monolith enriched with chlorophyll a by high internal phase emulsion (HIPE) for $CO_2$ adsorption

Trabajo de integración curricular presentado como requisito para la obtención del título de
Ingeniera en Polímeros

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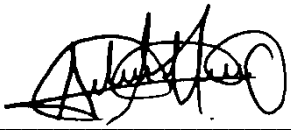

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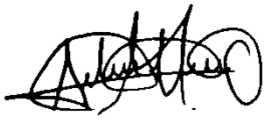

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# Dedication

I would like to dedicate my thesis to my beloved parents and close friends for offer me their unconditional love and have always been there for me. Thank you so much.

Andrea Vaca Oviedo

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Foremost, I would like to express my sincere gratitude to my parents, Jane and César and my brother Cristian for their love and invaluable support, either morally, spiritually, and physically throughout my life.

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Andrea Vaca Oviedo

# Resumen

Actualmente, la deficiente disposición de los desechos de jardinería urbana generados durante la poda de pasto se ha convertido en un problema ambiental considerable. En lugar de dar una aplicación útil a estos desechos, simplemente se tiran en basureros comunes generando contaminantes y patógenos. En este proyecto estos residuos son aprovechados para extraer sus cloroplastos, con el objetivo de desarrollar posteriormente biorreactores que adsorban dióxido de carbono. Primero, los cloroplastos de la espinaca (unos de los más estudiados por varios autores) se extrajeron y analizaron para ser encapsulados en una matriz de sílice sintetizada por el método de emulsión de alta fase interna (HIPE), usado por primera vez, obteniendo así monolitos híbridos Chl/Si enriquecidos con clorofila a. Luego, los cloroplastos obtenidos a partir del pasto se compararon con los de espinaca, mostrando tener características similares. Debido a que el procedimiento no involucra una química agresiva para los cloroplastos, se pudo mejorar la encapsulación de las bioentidades en un proceso in situ, prolongando su actividad fotoquímica. Los cloroplastos extraídos se analizaron por microscopía óptica y confocal, así como también por espectroscopía UV-Vis para analizar su integridad y contenido de clorofila. Los materiales híbridos obtenidos se caracterizaron por microscopía confocal y por espectroscopía de fluorescencia y de reflectancia difusa de UV-Vis para monitorear durante el tiempo al complejo de captación de luz. Finalmente, se observó que los cloroplastos seguían teniendo una actividad fotoquímica de al menos 90 días.



**Palabras Clave:** encapsulación, cloroplastos, monolitos híbridos, HIPE.

# Abstract

Currently, the deficient disposition of urban gardening waste generated during the operations of pruning-cutting grass has been converted into a considerable environmental problem. Contaminants and pathogens are generated since it is discarded in common garbage dumps instead of using these residues to give them a useful application. This project takes advantage of these residues to isolate their chloroplasts, with the aim of subsequently developing bioreactors that adsorb carbon dioxide. First, chloroplasts from spinach (ones of the most studied by several authors) were extracted and analysed for encapsulation into silica matrix synthesized by high internal phase emulsion (HIPE) method, it was used, for the first time, for obtaining Chl/Si hybrid monoliths enriched with chlorophyll a. Then, chloroplasts from grass were compared to those from spinach showing to possess almost the same features. Due to the soft chemistry involved by HIPE, one could enhance the encapsulation of the bio-entities in one-pot process, prolonging their photochemical activity. The isolated chloroplasts were analyzed by optical and confocal microscopy as well as UV-Vis spectroscopy to see their intactness and chlorophyll content. The obtained hybrids materials were characterized by confocal microscopy and fluorescence and diffuse reflectance UV-Vis spectroscopies for monitoring their light-harvesting complex showing to have an activity up to at least 90 days.



**Key Words:** encapsulation, chloroplasts, Chl/Si hybrid monoliths, HIPE.

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## 1 INTRODUCTION

Living organisms are able to accomplish complex chemical reactions with efficiency, functionality, and selectivity. The ability to harness this natural efficiency is why this topic is so central to modern industrial organic chemistry[1]. Mainly, chloroplasts are amazing photosynthetic organisms inside the cells of the mesophyll in plant leaves with the capacity to perform the photosynthesis process. In higher plants, chlorophyll *a (*Chl *a)*, chlorophyll *b (*Chl *b),* and carotenoids are the photosynthetic pigments, located in chloroplasts, responsible for carrying out the main steps of the photosynthesis. This process aims to convert large quantities of light energy into chemical energy through the synthesis of organic compounds[2]. This process is detailed in section 2 of this document.

Isolating the key component in charge of photosynthesis (chloroplasts or thylakoids) has been the main goal of many investigations[3–5]. However, they are fragile and unstable when they are isolated from their medium. Immobilizing these biological organisms to an inert and insoluble matrix can increase their operational stability[6]. There are some methods to immobilize biological organisms in an inert matrix, such as covalent binding, adsorption and encapsulation of the living organisms. The latter appears to be the most promising way to preserve their bioactivity[7]. In this way, some materials have been used such as agar, alginates and silicates[8,9]. The latter offers one of the easiest ways of cell encapsulation due to its inherent properties. In fact, the existence of silica hosts in nature for photosynthetic organisms like diatoms[9] is a hint of developing "cell/silica" materials. Additionally, silica monoliths can have a hierarchical and ordered structure, which allows an efficient diffusion of the products released by themselves as well as the $CO_2$ adsorption[10]. One way to obtain a leaf-like material has been sol-gel method, but it suffers from some disadvantages, e.g., the long-time synthesis and also from the considerable amount of sodium ions released that induce an osmotic stress to the cells[11]. In this sense, this project presents for the first time the encapsulation of chloroplasts by HIPE for obtaining a hybrid monolith/plant cell, enriched with chlorophyll *a* in one-pot process. The objectives and problem statement are presented in sections 3 and 4, respectively. The synthesis process, chloroplasts extraction

and characterization techniques are described in detail in sections 5 and 6, respectively. This work is also focused in finding the proper conditions of synthesis to prolong their photochemical activity; thus, some influence factors such as the presence of metals and the concentration of silica used are studied by different characterization techniques and discussed in section 7.

## 2 BACKGROUND

### 2.1 PHOTOSYNTHETIC PIGMENTS

The photosynthetic pigments are responsible for carrying out the main steps of the photosynthesis process. Without them, light cannot be absorbed, and therefore energy cannot be stored. There are different photosynthetic pigments present in plants: chlorophyll *a (*Chl *a)*, chlorophyll *b (*Chl *b)* and carotenoids, whose structures are shown in **Figure 2.1**. Chl *a* is a primary pigment that allows photosynthesis because it is an intermediate in the electron transport chain. Chl *b* is an accessory pigment, whose molecular structure is similar to that of Chl *a*, except that a formyl group replaces the methyl group. This difference changes the maximum absorption to shorter wavelengths. Carotenoids are also accessory pigments that are involved in many functions, among them: absorption of the light, transferring energy, photoprotection, and xanthophyll cycle processes[12].

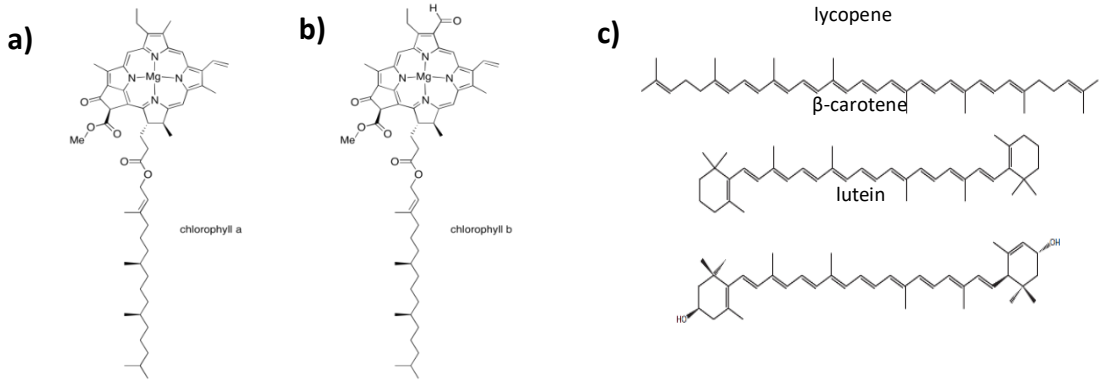


**Figure 2.1** Chemical structures of a) chlorophyll a, b) chlorophyll b, and c) several carotenoids. *Source:* Blankenship*, et al.*[12]

In order to study chlorophylls behaviours, their light absorption and/or emission spectra are commonly used. The peak intensities and their wavelengths give information about the properties of the reaction center complex and the electron transport chain. On the one hand, all chlorophylls have two major absorption bands, one in the blue or near UV region and one in the red or near IR region[13]. The blue absorption band produces a second excited state, which is the lowest excited state; this state is used for electron transfer and energy storage in photosynthesis. These absorption bands correspond to $\pi \rightarrow \pi*$ transitions, which

involve the electrons in the conjugated $\pi$ system of the chlorin macrocycle. The electronic transitions have transition dipole moments with different strengths and orientations. The lowest-energy transitions are called the Q bands, and the higher-energy transitions are known as Soret bands. They are also commonly called Soret bands. For Chl *a* the $Q_y$ transition is strongly polarized along the y molecular axis and the weaker $Q_x$ transition is partially polarized along the x molecular axis. The Soret band referred to a mixed polarization[14]. On the other hand, the peaks in the fluorescence spectrum are in slightly higher wavelength values in comparison with the absorption. The fluorescence emission is polarized along the *y* molecular axis, as it is emitted from the $Q_y$ transition. This spectrum is like the mirror image of the absorption spectrum because the ground and excited state have similar shapes[12]. **Figure 2.1** represents the absorption and fluorescence spectra of Chl *a* where Q-bands represent the transitions with the lowest energy (662nm and 578nm), and Soret band are the higher energy ones (430nm).

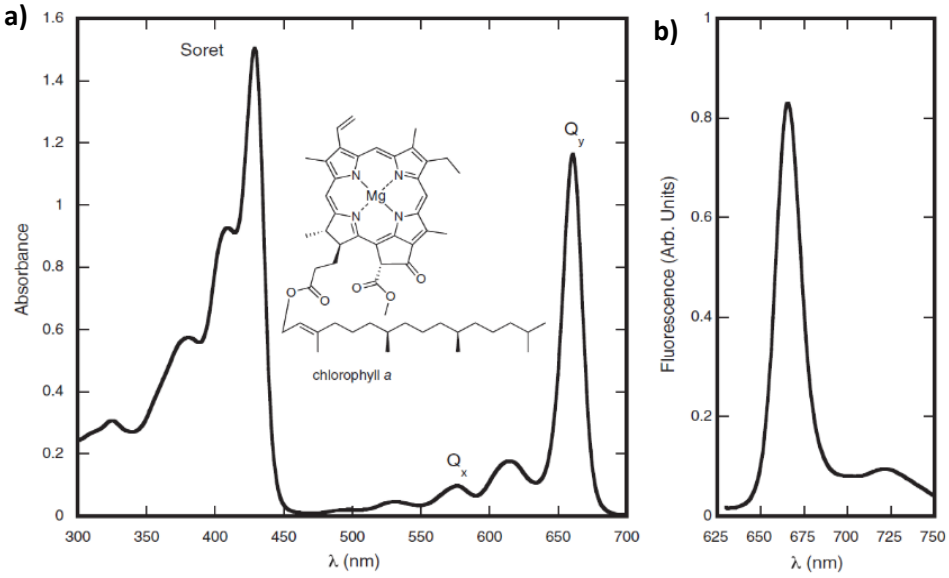


**Figure 2.2.** Spectra a) absorption and b) fluorescence of Chl *a* in diethyl ether. *Source*: Blankenship, *et al.*[12]

This spectrum is obtained in this research to analyse the estimate the amount of chlorophyll, among others. The photosynthetic pigments present in higher plants are hydrophobic organic pigments located in the thylakoid membrane, which in turn are found in chloroplasts.

## 2.2 CHLOROPLASTS

In eukaryotic photosynthetic cells, photosynthesis takes place in subcellular structures, called chloroplasts. Chloroplasts are subcellular structures inside the cells of the mesophyll in plant leaves and green algae. They are of a size up to 10µm long and up to 2µm thick[15], and they are constituted by chloroplast envelope, stroma, and thylakoid system, as represented in **Figure 2.3**.

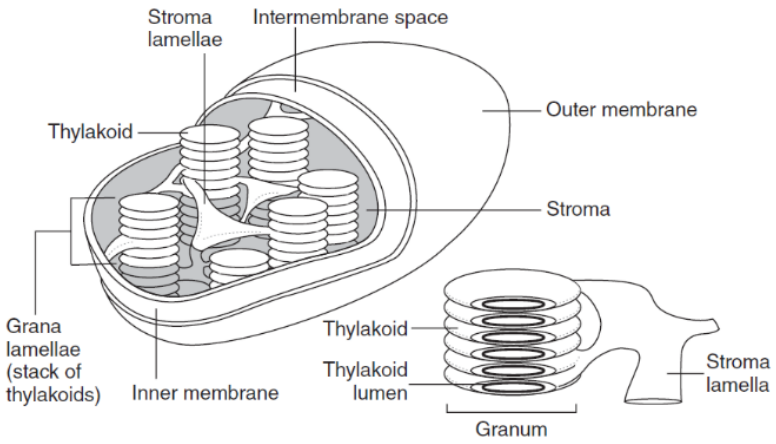


**Figure 2.3.** Schematic diagram of the morphology of a chloroplast. *Source*: Blankenship *et al.* [12]

The envelope is constituted by two layers separated by an intermembrane space and has the function of letting small molecules and ions pass into the chloroplasts. Also, the inner membrane can synthesize fatty acids, lipids, and carotenoids. The alkaline aqueous fluid surrounding the thylakoids is known as stroma and contains soluble enzymes, especially those that carry out the carbon fixation. Thylakoids consist of a thylakoid membrane surrounding a thylakoid lumen. A staked group of thylakoids closely associated are called grana thylakoid membranes, each chloroplast has around 10-100 grana. Those that are not stacked are known as stroma thylakoid membranes[12,15].

Once the chloroplasts are isolated from their medium, they become very vulnerable to many different factors. One way to determine the developmental state of the photosynthetic apparatus is knowing the chlorophyll concentration and the percentage of intact chloroplasts. On the one hand, the chlorophyll concentration gives an estimation of the photosynthetic capacity per unit area of the leaf that determines the rate of photosynthesis. One of the first methods that allow estimating this quantity was proposed

by Arnon[16]; using the absorption of light by aqueous acetone (80%) extracts of chlorophyll. Based on this method, there have been some modifications that take into account more factors to have more accuracy, such as the method reported by Sheikh *et al.* [17] which is detailed in **Appendix 1**. On the other hand, the reaction reported by Hill *et al.*[18] , characteristic for the photosynthesis process, is generally used to determine the percentage of intact chloroplasts contained in isolation chloroplasts preparations. It has been demonstrated that isolated chloroplasts from green plants can also liberate molecular oxygen under the presence of light and a suitable hydrogen acceptor (Hill oxidant)[19]. This essay is based on the inability of the ferricyanide to cross the outer envelope of the chloroplasts when they are intact, but it reacts with the soluble protein contained in the stromal compartment if they are broken. So, if there are broken chloroplasts present, and a decrease in the absorbance at ~410 nm (absorption maxima) will be observed using spectrophotometric measurement. This decrement corresponds to the reduction of the $Fe^{3+}$ to $Fe^{2+}$, when the chloroplasts are broken[20]. Therefore, the intactness is measured following two reactions. One is inducing an osmotic shock to the chloroplasts to broke them; this reduction reaction rate represents one hundred percent of broken chloroplasts (reference value). The other one is following the reaction rate without osmotic shock, which corresponds to the percentage of broken chloroplasts in the suspension (real value). So, comparing these values, it is possible to calculate the percentage of intact chloroplasts presents in chloroplasts suspension. This procedure is detailed in **Appendix 2**.

### 2.3 PHOTOSYNTHESIS

Photosynthesis is a biological process in which light energy is captured, stored, and converted into chemical energy to drive other cellular processes. This process involves chlorophyll pigments and carries out electron transfer. The sunlight irradiates spectrum radiation from gamma rays to radio waves, but only between 400 to 700 nm is the wavelength range in which the photosynthetic organisms are active. This range represents 45% of the total solar irradiance that reaches the Earth's surface that can be used to bioenergy applications[12].

The main step of this process is carried out by a multiprotein complex, known as photosystem, which is divided as Photosystem II (PSII) and Photosystem I (PSI), shown in **Figure 2.4**. Both have the same structure: a light-harvesting complex that serves as a support to an antenna system that has the function of collecting the light. Then, it is delivered to the reaction center where the photochemistry takes place. The molecules of Chl *a* contained in the reaction center have the particularity to convert the light into an excited electron. This excited electron passes to a primary electron acceptor; this lack of negative charge is replaced by another electron. In PSII, the electron comes from the splitting of water, which releases oxygen as a waste product. In PSI, the electron comes from the chloroplast electron transport chain, reducing the nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH[21]. All these steps promote an electrochemical gradient in the thylakoid lumen that is used to synthase adenosine triphosphate (ATP)[15]. The hydrogen, stored as NADPH, is combined with carbon dioxide from the atmosphere to make sugars and some inorganic molecules in the dark reactions[22]. Then, in eukaryotic photosynthetic organisms, phosphorylated sugars, synthesized in the stroma, are transported to the outer part of chloroplasts, called cytosol, and converted into sucrose[23].

The overall equation of photosynthesis is:

$$2nCO_2 + 2nH_2O + photons \rightarrow 2(CH_2O)_n + 2NO_2 \qquad (2.1)$$

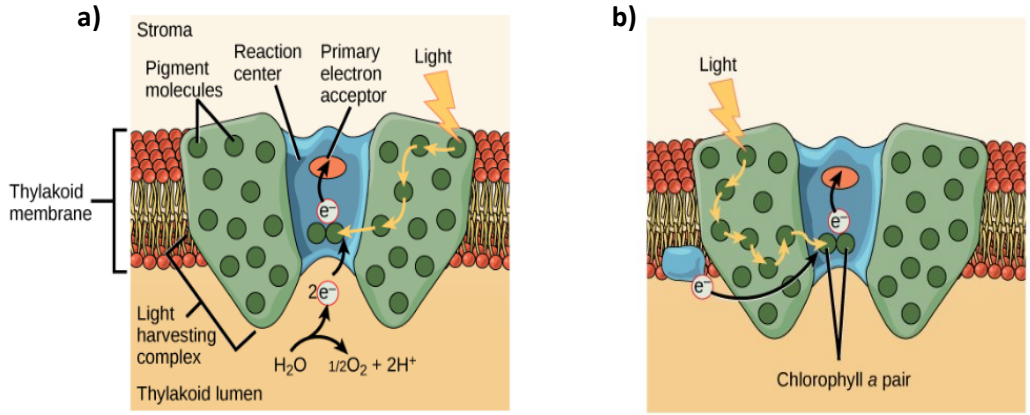


**Figure 2.4.** Schematic representation of energy conversion in a) Photosystem II(P680) and b) Photosystem I (P700). *Source:* Clark *et al*.[15]

Many attempts to mimic this process and try to replicate the nanostructures found in photosynthetic systems have been carried out by researchers interested in designing processes that can efficiently transfer energy and capture $CO_2$[24,25]. However, only limited results have been obtained due to some facts such as the high cost of synthesis because of their complex chemistry involved[26]. Based on that, another promising approach has been to isolate the photosynthetic materials from nature as opposed to artificially synthesizing the light-harvesting apparatus, and to use a host material to immobilize them. In this way, a wealth of bio-mimetic and bio-inspired hybrid materials have been created as "living materials"; this strategy has already been realized in the design of biocatalysts, bioreactors, and biosensors[27].

### 2.4 PLANT CELL IMMOBILIZATION METHODS IN DIFFERENT MATRICES

The vast interest of research to use the potential of photosynthetic organisms to convert $CO_2$ into useful products has been increasing due to the increasing atmospheric $CO_2$ concentrations and their effects over time[28]. So, researches have been widely interested in finding a suitable way to immobilize these organisms into some different matrices, creating a leaf-like photo-bioreactor. Using only the key component involved in the photosynthesis (chloroplasts or thylakoids) of the whole plant, the function of the mitochondria can be avoided. Thus, the efficiency of this pure photosynthetic material is higher than the whole-cell plants[29]. However, the photochemical activity of free chloroplasts suspensions declines very quickly and disappears after around three days when they are isolated from its medium[4,30]. For this reason, some immobilization methods to provide them protection have been studied in order to find the most suitable way of synthesis, which depends on physicochemical characteristics the precursors and the procedure carried out. Unlike ponds or suspension bioreactor systems, these biohybrids allow the continuous harnessing of photosynthetic organisms in a non-destructive way, avoiding their leaching and protecting them against external reagents. Moreover, this solid bioreactor permits to have control over the growth conditions to provide them a favourable environment and prolong their activity.

There are three ways to immobilize a biomaterial[31]: adsorption, covalent binding, and entrapment, as indicated in **Figure 2.5**. The method based on adsorption is simple, mild, and it allows the reuse of the biomaterial and substrate because the molecule is adsorbed physically on the matrix. Some pre-formed substrates used for this purpose are sponges, fibers, sheets, and foams. However, the easy leaking of the adsorbed molecule due to the weak physical interaction with the support can lead to the desorption of molecules, resulting from changes in pH, temperature, bio-entity type, or ionic strength[7].

In the second method that based on covalent binding, the bioentities are bound to the substrate by covalent links. The active bioentities are attached to supports, such as nanopores, nanoparticles, or nanofibers. However, complex reaction steps that involved this technique are not economical, and during covalent binding can occur an alteration of the conformational structure, resulting in loss of activity[31].

The last one, also called the encapsulation method, is the most promising way to preserve the bioactivity of the biological entities because it does not damage them and provides some advantages, such as easy synthesis process, good mechanical strength and stability, tunable porosity, and good resistance to microbial attack[4].

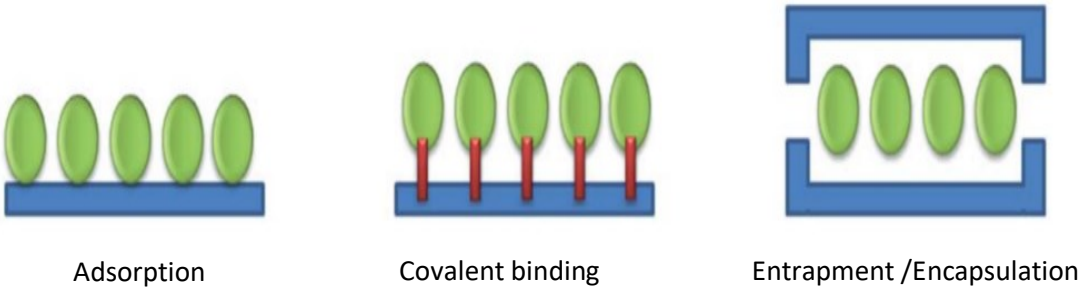


**Figure 2.5.**Three different ways to immobilize a bio-material. *Source*: Yang *et al.*[31]

One of the most used materials for the bioentities entrapment are the polysaccharides, such as agar and alginate gels. Simó *et al.*[32] reported a review with different cell immobilization techniques using alginate gels. The alginate is a polymer of mannuric acid and guluronic [3] that can be used to create capsules by mixing aqueous solutions of sodium alginate and solutions with divalent cations, like $CaCl_2$[3]. The gelification is performed when it is carried out the ionic cross-linking of negatively charged carboxyl groups of the alginate

chain and divalent metal ions with opposite charges [33]. Recent studies in order to improve the effectiveness have been trying cross-linking coating techniques. However, Simó *et al.* [32] concluded that it is still a challenge to avoid the toxic effect produced by the metabolic effects by this polysaccharide itself (polyelectrolyte) and the high calcium content.

Porous silica due to its surface properties appears to offers some properties that possess natural organic supports with more stability and less toxicity[4].

### 2.5 SILICA AS BIOMATERIAL

Inspired on diatoms, researchers demonstrated that silica is the most promising matrix to encapsulate living organisms. Diatoms are eukaryotic algae found in the oceans, waterways, and soils of the world. They are surrounded by a silica cell wall, called flustule, providing them mechanical protection, a source that allows the exchange of nutrients and protection from high intensity solar light[34]. Indeed, Meunier *et al.* [29] reported that the silica matrix could be tailored to preserve the activity of the thylakoids and chloroplasts. It is because this kind of material has many promising properties to be a suitable matrix, such as an inexpensive way of synthesis, hydrophilic nature, chemically inert, transparency, stability, and resistance.

Some studies have been developed using silica monoliths by the sol-gel method[35–39]. To create a stable hybrid silica matrix by this method, a precursor of silica (such as alkoxysilanes or sodium silicates) is necessary. Then occurs the hydrolysis and condensation of the precursor in water. Finally, the silica polymerizes around the cells. However, using the sol-gel method entails the high release of alcohol and sodium ions, which induces an osmotic shock to cells[11]. Logically, this process only works when the contact of the by-products with cells is avoided, by eliminating them before the cell culture is added[4]. Moreover, the size and shape of the pores obtained can produce stress to the organisms, because of the contraction phenomena. It is produced by the inevitable evaporation of water and condensation of silanol groups that produce tensile capillary stress in the pores [40].

Consequently, the principal difficulty has been to find a suitable pathway to avoid these problems. A promising option is to carry out the synthesis throughout the High Internal phase emulsion (HIPE) method reported for the first time in 2004 by Carn *et al.* [41]. Using an

oil phase as the second template could provide the bioentities a soft medium avoiding contact with $Na^+$ ions and alcohol groups. Moreover, due to the double template, the capillary stress decrease, avoiding the shrinkage of the pores that affects to the chloroplast survival.

## 2.6 HIGH INTERNAL PHASE EMULSION METHOD (HIPE)

HIPE is a method to synthesize hierarchal inorganic porous monoliths with a high control degree over the size and shape of the porous[41]. Taking into account the size of the pores, the materials can be classified as microporous (pore size below 2 nm), mesoporous (2–50 nm), and macroporous (exceeding 50 nm) solids[42]. Using this method is possible to obtain monoliths with a hierarchal structure with a double template: direct emulsion at the macroscale and micellar templates at the mesoscale. It is used sol-gel process to create a system, through an emulsion step, where the inorganic material is deposited around the oil droplets and the surfactant, in which gives the macroporosity and mesoporosity, respectively.

An emulsion is a mixture of immiscible fluids, in which one of the phases is dispersed in the form of droplets. To form an emulsion, it is necessary to use a surfactant to stabilize the two phases. A precursor sol should be added to the aqueous phase (first template), and the solidification is generated by the change of the conditions, such as use of a catalyst and change in temperature[43]. If a second template is required, another structure-directing agent is added to the ceramic precursor sol before the solidification, obtaining a material with double porosity. It must be considered that the templates should be removed by solvent extraction or calcination to release the porosity [44].

In **Figure 2.6** it can be observed a scheme of the distribution of the droplets obtained as a result of performed emulsions with different methods. **Figure 2.6a** represents the type of water in oil (W/O) emulsion, in which the droplet phase is water, and the fluid phase is the organic solvent, while **Figure 2.6b** shows oil in water (O/W) emulsion, in which the droplet phase is an organic solvent while the continuous phase is water. In **Figure 2.6c**, it can be observed that the emulsion obtained with the method called high internal phase emulsion (HIPE) is a kind of O/W emulsion, but the principal different characteristic is that the volume

percentage of the oily droplets dispersed in the aqueous phase is higher than in a classical emulsion. It has been reported that the percentage of droplet volume fraction can exceed the close-packing limit of 74% [38].

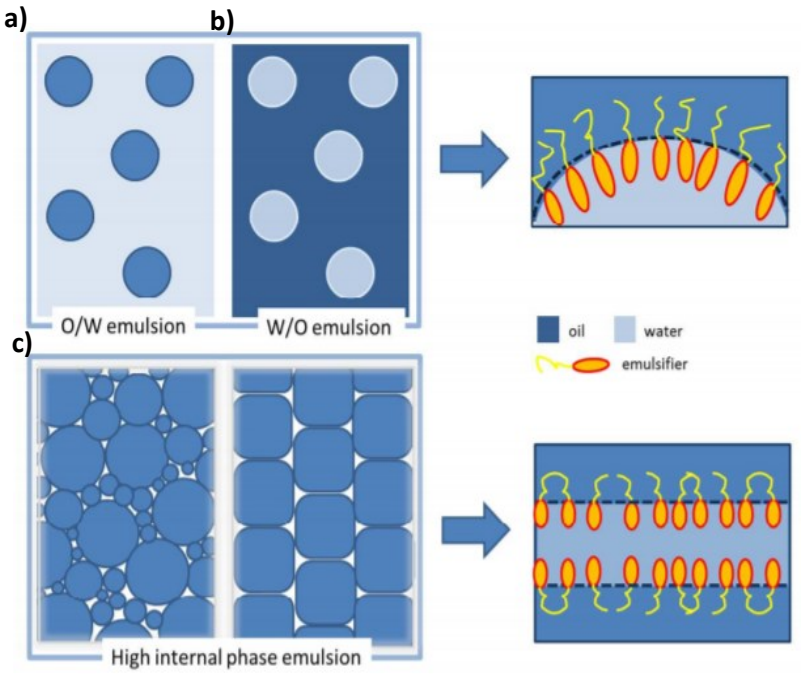


**Figure 2.6.**Schematic diagram of oil droplets distribution in a) oil in water emulsion, b) water in oil emulsion, and c) high internal phase emulsion. *Source:* Feinle *et al.* [38]

The group of Bakouv[41] prepared this system from an oil-in-water high internal phase emulsion (HIPE) based on tetraethoxy-orthosilicate (TEOS) in water as the aqueous phase, tetradecyltrimethylammonium bromide (TTAB) as a surfactant and using dodecane as oil phase. As a result, they got hierarchically interconnected structures with macro-mesoporosity with an average mesoporosity of 800 $m^2g^{-1}$. In their research, they compared the results monoliths textures when the pH parameter is changed, concluding it is a key influential factor. Also, they could determine that the oil volume fraction influences the final texture.

In the procedure for obtaining hierarchical porous material, the first step is the hydrolysis of the silica precursor and occurs when the oxygen present in the water attack nucleophilically the silicon atom producing alkoxy ligands (equation 2.2). Then, the condensation occurs at the oil-water interface, forming Si-O-Si- bonds (equation 2.3).

Hydrolysis $\equiv Si\text{-}OR + HOH \rightarrow \equiv Si\text{-}OH + ROH$ (2.2)

Condensation $\equiv Si\text{-}OR + HO\text{-}Si\equiv \rightarrow \equiv Si\text{-}O\text{-}Si\equiv + ROH$ (2.3)

The overall reaction can be written as:

$$Si(OR)_4 + 2H_2O \rightarrow SiO_2 + 4ROH \quad (2.4)$$

This route of synthesis was used by Sommer-Marquez *et al.* [45] applying tetraethyl orthosilicate (TEOS) as silica precursor, cyclohexane as oil phase because of its volatile properties, Pluronic P123 as a molecular surfactant which is also biocompatible, non-toxic and widely used for pharmaceutical applications[46]. Their research was focused on the development of silica monoliths for selective entrapment of Cs, so they functionalized these monoliths with metal (Co, Zn, and Cu) hexacyanoferrate (MHCF) particles. Also, they used calcination to liberate the porosity and to increase mechanical strength. Finally, they got macro/mesoporous silica monoliths with high specific surface area (around 571$m^2$ $g^{-1}$) before the impregnation with MHCF.

Therefore, based on the beneficial interaction between silica and living organisms and the advantages of encapsulation procedures that the authors mentioned before[45]. Also, considering the advantages of the HIPE to got macro/mesoporous silica monoliths with high specific surface. This work reports for the first time the use of HIPE to encapsulate chloroplasts with Pluronic P123 as the surfactant and hexanes as the oil phase. It is consider the use of Pluronic P123 as surfactant instead of the TTAB used by Bakouv *et al.* [41] due to that the ammonium is toxic to the cells.

## 3 PROBLEM STATEMENT

In Ecuador, the legislation concerning environmental management is very recent, and it is not very strict, being inapplicable in most cases[47]. The growing of common grass in the Imbabura province, mainly in Urcuquí, one of the places in Ecuador whose principal activity is the agriculture[48] and the growth of grass is considerable, and that is why it has to be prune very often. However, this pruned grass is thrown away into the common dumps, which are, most of the time, open ones. Consequently, the deficient disposition of these residues has been converted into a potential problem, especially in developing countries such as Ecuador. The lack of adequate management of these residues generates bad odors, leachate production, and greenhouse gas emission into the atmosphere that cause a negative impact on the environment and significant losses of resources in terms of energy, nutrients, and organic matter. This project takes advantage of these residues to extract their chloroplasts, with the objective of developing photobioreactors that could adsorb $CO_2$, by using for the first time HIPE method, that could absorb $CO_2$. In that sense, that agriculture waste could acquire a precious economical value, and renders effective its valorisation. Interfacing inorganic materials and biological systems to develop biosensors and bioreactors are of high significance in the photo-biochemistry field. And, at the same time, finding a suitable matrix and synthesis route to achieve it remain a challenge.

# 4 OBJECTIVES

## GENERAL

Encapsulation of isolated chloroplasts from spinach and grass leaves into a hierarchical silica monolith synthesized by High Internal Phase Emulsion method.

## SPECIFICS

i. To extract intact chloroplasts from spinach and grass leaves.

ii. To estimate the percentage of intact chloroplasts after the extraction procedure.

iii. To evaluate the possible use of chloroplasts obtained from pruning waste residues estimating the total amount of chlorophyll after the extraction method.

iv. To encapsulate *in situ* the chloroplasts into silica matrix enriched with chlorophyll *a* using the High Internal Phase Emulsion method.

v. To study the influence of 1) HIPE method as a procedure of encapsulation 2) the presence of Zn-Cu, and 3) the amount of silica used in order to prolong the photochemical activity of the chloroplasts over time.

# 5 METHODOLOGY

## 5.1 REAGENTS

The chemicals to make the isolation medium: sodium chloride (NaCl), Tris-HCl, chloride magnesium ($MgCl_2$), ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) were acquired from Sigma-Aldrich with analytical grade. Percoll® (pH 8.5-9.5 at 25 °C), TEOS (purity 98%), Pluronic P123 (purity 99%), copper nitrate trihydrate ($Cu(NO_3)_2 \cdot 3\ H_2O$, purity 99%), zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6\ H_2O$, purity 98.5%), hexanes (purity 98.5%) and sodium fluoride (NaF, purity 99%), were acquired from Sigma-Aldrich and used without further purification.

## 5.2 ISOLATION OF INTACT CHLOROPLASTS

This procedure was based on the protocol reported by Sigma Aldrich[49] and Robinson[50]. Spinach leaves (from which stalks and midribs had been removed) and grass leaves have been used as a source to extract the chloroplasts.

30 g of plant leaves were washed with distilled water. It should be considered that all steps were made at 4°C in darkness to avoid starch accumulation, which can rupture the chloroplast envelope during centrifugation. The leaves were blended with 120 mL of an ice-cooled isolation medium (0.33 M Sorbitol, 0.3 M NaCl, 0.1 M Tris-HCl pH 7.6 at 25 °C, 5 mM $MgCl_2$, 2 mM EDTA, 0,1 mM BSA). The mixture was filtered through a single layer of cotton and centrifuged for 3 min at 1200 RPM. This step has the objective of discarding tissue debris, which appear like a white pellet at the bottom of the tube. The supernatant was transferred to new tubes, and they were centrifuged for 7 min at 3500 RPM. Then, the newly obtained supernatant was discarded, and the green pellet of each tube was resuspended with 1-2 mL of the isolation medium by gently pipetting up and down. After that, all suspended pellets were poured into one tube. Later, the purification of intact chloroplasts was made through the Percoll gradient. It consisted of preparing 40% Percoll by mixing 4 mL Percoll with 6 mL of BSA solution. Once it was done, 6 mL of chloroplast suspension was covered with 10 mL of 40% Percoll and centrifuged for 12 min at 3500 RPM. The upper phases were removed, and the green pellet at the bottom with intact

chloroplasts was resuspended in 1 mL of the isolation medium and kept at 4 °C until its use. **Figure 5.1** is a summarized scheme of the procedure described.

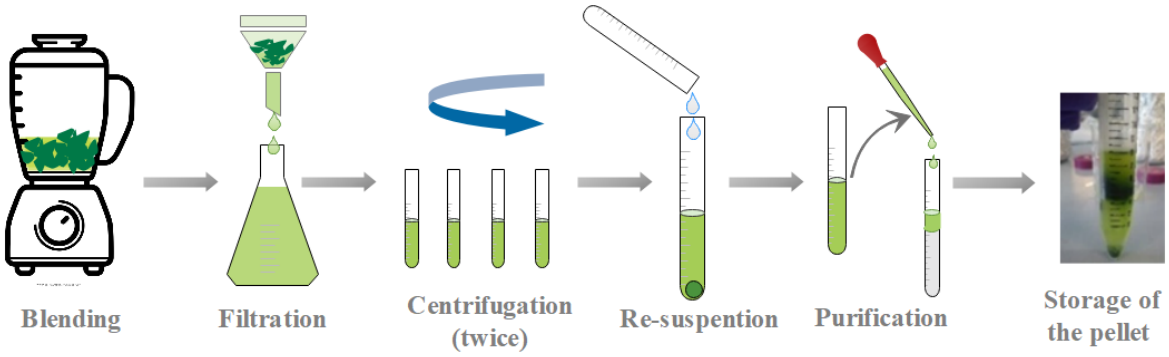


**Figure 5.1.** Scheme of the procedure to isolate the chloroplast from spinach leaves. For grass, the Percoll and BSA medium was not used due to the impossibility to acquire them at University.

### 5.3 SYNTHESIS OF SILICA-MONO/CHLX HYBRID MONOLITH BY HIPE

This procedure was based on the method reported by Sommer-Marquez, *et al.*[45] to prepare one hybrid monolith. Hydrolysis of the silica precursor, tetraethyl orthosilicate (TEOS), was carried out in an acidic aqueous solution pH=2, of 2 mL of 20% (w/w) tri-block copolymer (P123), 0.45 g of copper nitrate, and 0.45 g of zinc nitrate. The emulsion was performed by the slow addition of the oil phase (hexanes) previously mixed with chloroplasts suspension. Silica precursor polymerization was enhanced by the addition of 20 µL of NaF (8 g/L) before carrying out the emulsification procedure. The final product was poured into plastic molds. In **Figure 5.2** is represented the whole procedure, with the phenomena that occur in each step, to prepare one hybrid monolith.

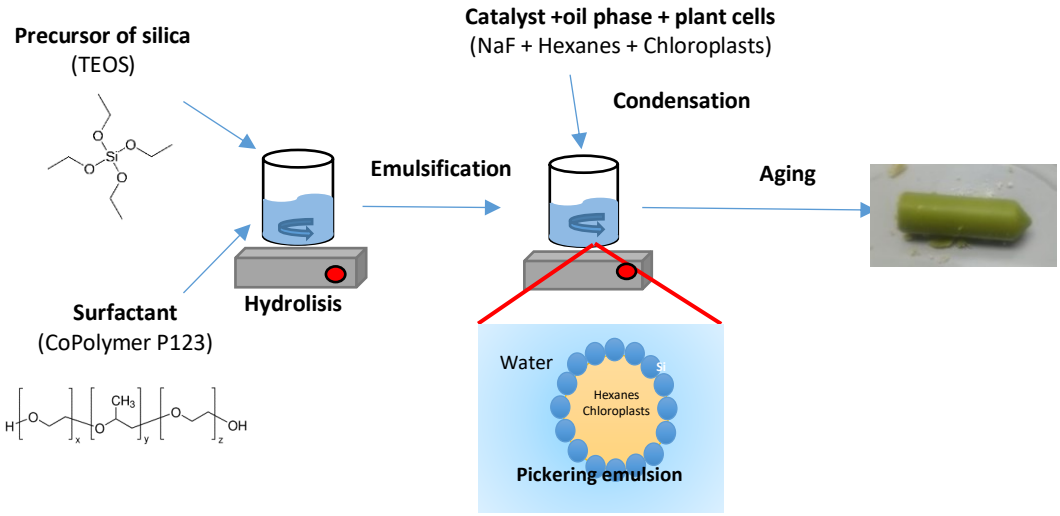


**Figure 5.2.** Scheme of the procedure to synthesize Chl/Si hybrid monolith.

Five samples were selected from the whole pool of the synthesized ones to analyse principally 2 factors. The influence of 1) Cu-Zn mainly used as stabilizing agents (in this work were used with the purpose of increasing the bactericidal properties and the possibility of having micronutrients, Cu and Zn, respectively) and 2) the amount of silica used on the photochemical activity of the chloroplasts. Also, it was followed the photochemical activity of spinach and grass chloroplasts within the matrix over time. In **Table 5.1** are summarized the synthesis conditions of the selected samples.

**Table 5.1.** Synthesis conditions of the samples Si3-mono/ChlS, Si1-mono/ChlS, CuZn/Si1-mono/Chl, Si3-mono/ChlG and Si1-mono/ChlG.

| Sample | TEOS (mL) | Hexanes (mL) | Chloroplasts Source | Metals | $V_{TEOS}/V_{oil+Chl}$ |
|---|---|---|---|---|---|
| **Si3-mono/ChlS** | 3 | 5.1 | Spinach | | 0.42 |
| **Si1-mono/ChlS** | 1.075 | 5.1 | Spinach | | 0.15 |
| **CuZn/Si1-mono/Chl** | 1.075 | 5.1 | Spinach | Cu-Zn | 0.15 |
| **Si1-mono/ChlG** | 1.075 | 5.1 | Grass | | 0.15 |

# 6 CHARACTERIZATION TECHNIQUES

Characterization of materials provides comprehensive, up-to-date coverage of material's structure and properties, widely applicable in the diverse field of materials research[51]. Mainly, optical imaging and spectroscopy are commonly used in this kind of research, due to one can have information about the morphology and spectral properties of biomaterials. Thus, optical and confocal microscopy, fluorescence, UV-Vis, and reflectance diffuse UV-Vis spectroscopies were performed in this work to analyze the samples (extracts and hybrids).

## 6.1 OPTICAL MICROSCOPY

Optical microscopy is a non-destructive and real-time imaging technique usually employed for biological samples analysis and also used to access the morphology of gels[52]. This technique is employed in this research project to study the evolution of the isolated chloroplasts after the extraction process as well as to prove their preservation after the emulsion. This analysis was performed with a Microscope Axio Imager.M2p with LED for transmitted-light brightfield and simple polarization, using a phototube 30°/25°, with PC interface.

## 6.2 CONFOCAL MICROSCOPY

Confocal microscopy offers a real-time observation of the structure and dynamics of biological systems with extremely high resolution. This technique eliminates the out of by spatial filtering, utilizing a point source of light for excitation, and a pinhole confocal with the excitation pinhole in front of the detector. A combination of transverse resolution with non-invasive optical sectioning offers images of biological specimens with high resolution[53]. This study uses this technique to analyze the influence of stabilizing agents in the preservation of the activity of the chloroplasts. The autofluorescence of living cells was analyzed using a Confocal Microscope Zeiss LSM 510. The 488 nm excitation ray line of an Argon ion laser was used, and the emitted fluorescence between 600 nm and 700 nm was collected.

### 6.3 ULTRAVIOLET-VISIBLE SPECTROSCOPY

UV-VIS spectroscopy is one of the important characterization techniques to study optical properties, widely applied to measure spectra of microscopic specimens. When lights interact with matter, it can be absorbed, and therefore promotes electrons from their fundamental state into excited states that depend on the light energy and the absorbing material. The spectrum maximum (the peak) corresponds to electronic transition[54]. In this work, it was measured the characteristic spectrum of the chloroplasts in order to follow the activity of the photosynthetic pigments in the cell culture. Also, it was used to follow the photoreduction of the ferricyanide described before. This technique was performed with the values of the spectrum recorded from 400 nm to 800 nm with a PerkinElmer® Lambda 1050+UV/Vis/NIR Spectrophotometer.

### 6.4 FLUORESCENCE SPECTROSCOPY

Other photophysical effects can occur when light promotes electrons to excited states in a molecule, such as the emission of light, called luminescence. This effect can also happen due to chemical mechanisms other than physical (light absorption). The emission spectrum is very similar to the absorption spectrum of fluorescent molecules because there is no alteration of the molecule geometry after excitation; therefore, its vibrational modes are similar[52]. Thus, fluorescence spectroscopy can study the physico-chemical properties of organic molecules, such as photosynthetic pigments. It was applied to analyze the evolution of the isolated chloroplasts within the matrix, using a fluorescence spectrophotometer Cary Eclipse VARIAN. The spectra were recorded with excitation at 430 nm.

### 6.5 DIFFUSE REFLECTANCE ULTRAVIOLET-VISIBLE SPECTROSCOPY

DRUV-Vis Spectroscopy is a non-destructive technique widely applied to measure the absorption and dispersion of light inside a medium that provides information about the environment of the chromophores, e.g., organic species, present in the material. With high success rates, this technique has been used for the detection of optical properties in biological media. It allowed following the activity of the photosynthetic pigments in the

silica matrix through the time. The data was recollected with a PerkinElmer® Lambda 1050+UV/Vis/NIR Spectrophotometer from 400 nm to 800 nm

## 7.1 CHLOROPLASTS EXTRACTION

After chloroplasts extraction from spinach and grass leaves, it was necessary to check if their integrity is conserved in order to carry out the encapsulation. Therefore, optical and confocal microscopies and UV-Vis spectroscopy techniques were crucial to estimate and observe chloroplasts intactness as well as their photochemical activity**.**

### 7.1.1 Morphology of extracted chloroplast and intactness criteria

As mentioned before, one main feature of chloroplasts that can help to know if they are still alive is their capacity to fluoresce. By confocal microscopy analysis, one can clearly visualize the emitted fluorescence (in green) between 650 nm and 700 nm corresponding to the chloroplasts after excitation at 488 nm, shown in **Figure 7.1a.** The images were compared with the fluorescence emitted by isolated chloroplasts reported in the investigation of Schulz *et al*.[55], shown in **Figure 7.1b**, where the chloroplasts suspension concentration used was 0.0025% (w/v).

The analysis was performed just after the extraction, and the quantity used in this work was around 1 mL of chloroplast suspension homogeneously dispersed on the glass slide. The concentration of chloroplasts was approximately 16% (w/v). Also, this study allows to give a reference for the fluorescence of the chloroplasts with regard to their encapsulation within the silica monoliths.

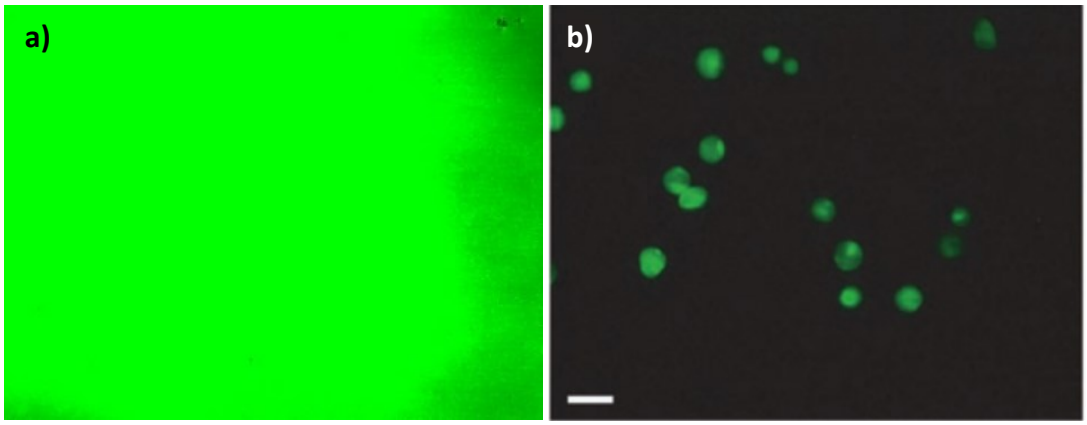


**Figure 7.1.** a) Isolated chloroplasts suspension and b) reference image of isolated chloroplast suspension reported by Schulz *et al.* [55]. Bar:10µm, viewed with a fluorescein-specific filter in confocal microscopy.

When the chloroplasts preserve their surrounded membrane, one can say that they remain intact, and by optical microscopy, this membrane is seen as a halo[55]. In **Figure 7.2a,** it is observed that the extraction procedure from the spinach leaves was successful because it clearly shows the intactness of the morphology of the chloroplasts. They have a spherical shape with an average size of about 5µm, which matches with the size reported in the investigation of Schulz *et al*. [55], shown in **Figure 7.2b**. In this manner, it is demonstrated that the encapsulation can be performed after the extraction taking the advantages that the unbroken chloroplasts can offer for photosynthesis purposes.

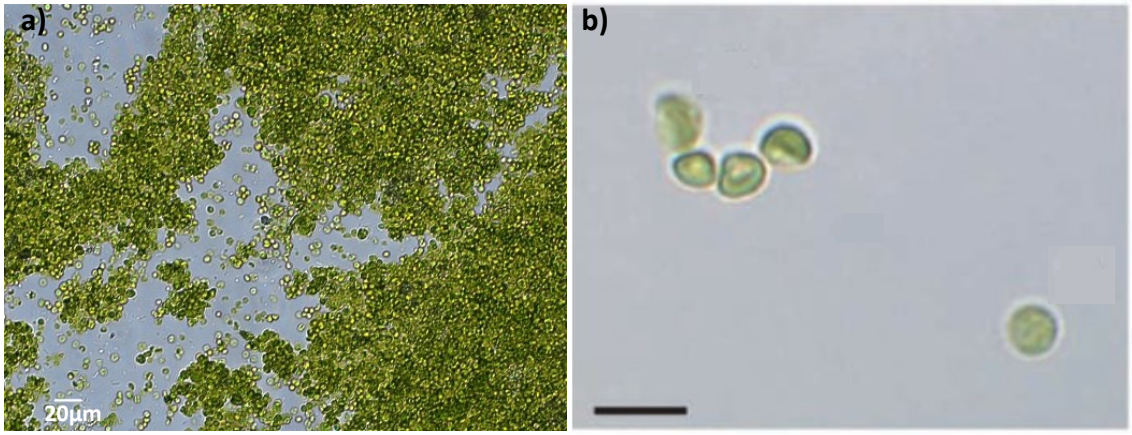


**Figure 7.2. a)** Isolated chloroplasts suspension. Bar:20 µm. and **b)** reference image of isolated chloroplast suspension reported by Schulz *et al.* [55]. Bar:10µm, viewed with brightfield objective in the optical microscope.

To estimate the percentage of the intact chloroplasts, the criteria exposed by Heber & Santarus[56] described before was applied. **Figure 7.3a** and **Figure 7.3b** shows the absorption spectra obtained after the ferricyanide reduction reaction with and without the osmotic shock, respectively. The upper-right image corresponds to the spectrum of the potassium ferricyanide in the extraction medium, which present a an absorption maximum at ~420 nm. In **Figure 7.3a**, it is shown that there is a more relevant decrease in the absorbance band at ~420 nm than in the **Figure 7.3b**. This decrement corresponds to the reduction of the potassium ferricyanide when it interacted with the proteins located inside the chloroplasts, which are exposed when the chloroplast membranes are broken, as was explained detailed in section 2. Therefore, the difference in those cases correspond to the percentage of the broken chloroplasts present in the chloroplast suspension.

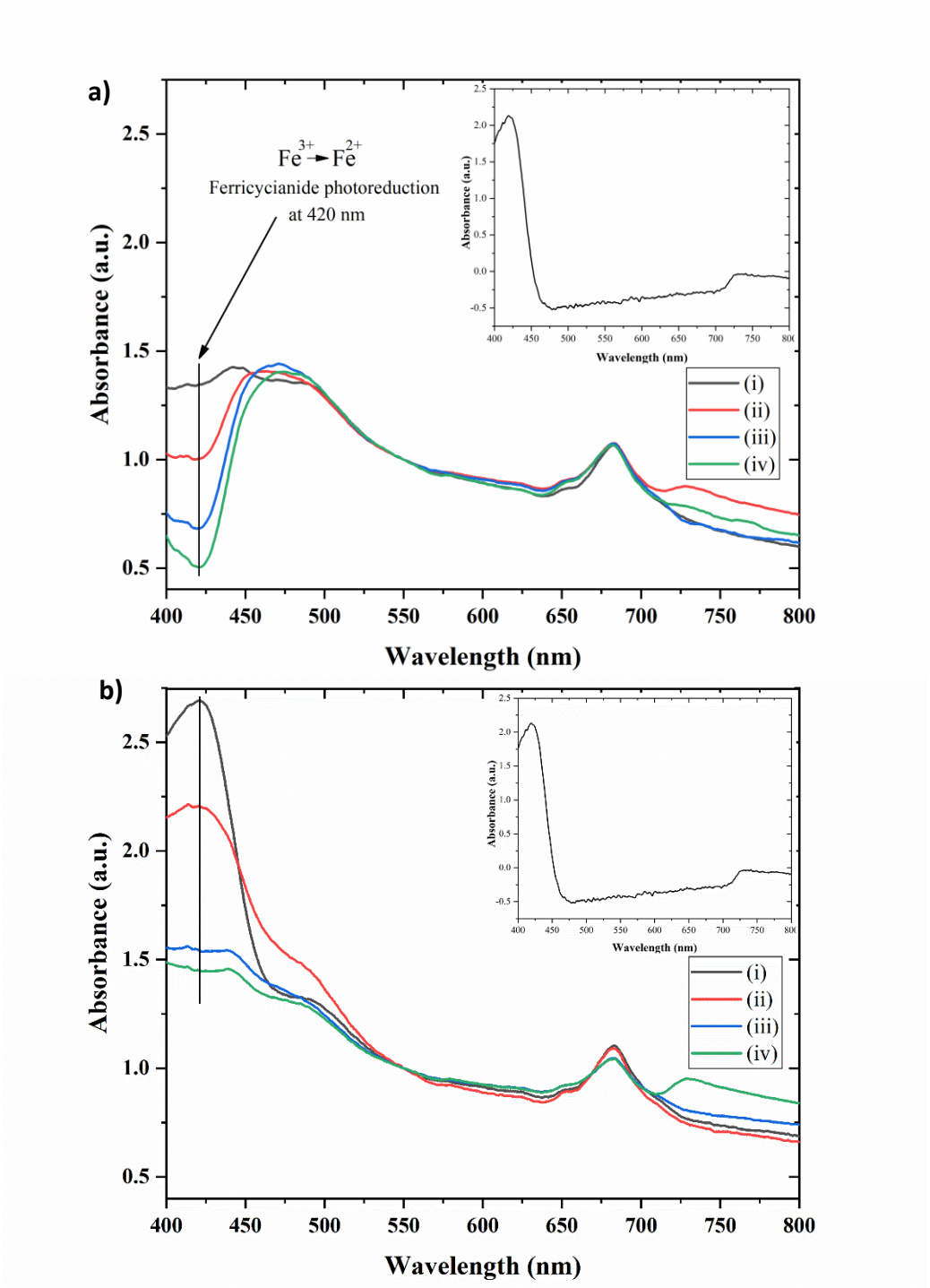


**Figure 7.3.** Absorption spectrum of ferricyanide photoreduction reaction a) with and b) without osmotic shock. Lines (i) before illumination, and after (ii) 2 min, (iii) 4min, (iv) 6 min of illumination. Upper-right image: potassium ferricyanide solution spectrum.

The estimation of the percentage of the intact chloroplasts is assessed by comparing the rate of ferricyanide reduction upon illumination before and after the osmotic shock of the chloroplasts. In a previous work published by Sigma-Aldrich company, it is reported a value of 88% of intact chloroplasts present in the final suspension using their chloroplasts isolation kit[49]. In this work, the obtained results showed in **Figure 7.4** indicate that 67.7% of intact chloroplasts were present after the extraction method, taking 32,3% of broken chloroplasts as a source of Chl *a* to enrich the monolith.

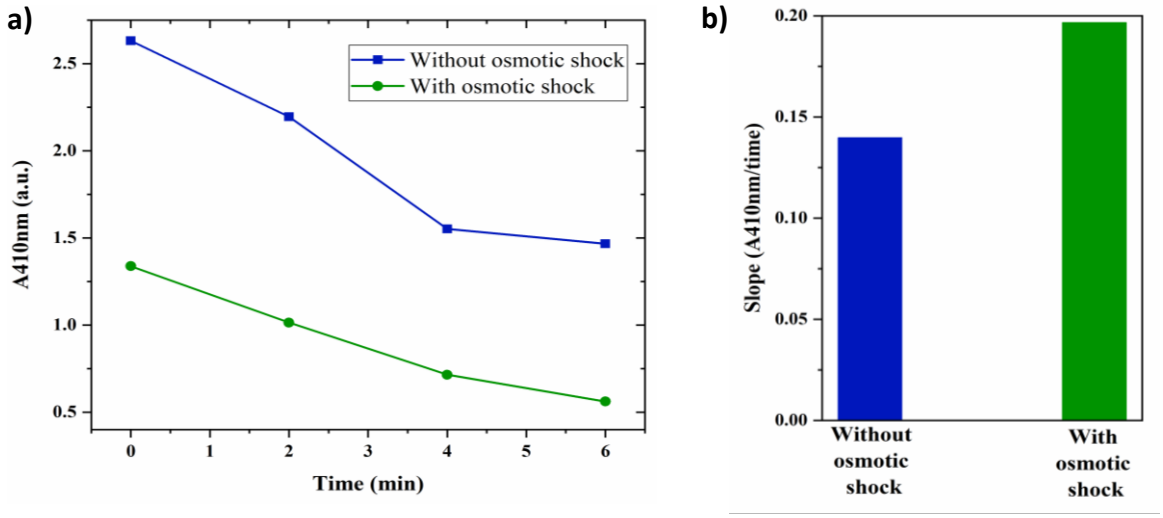


**Figure 7.4.** a) Absorbance values at 410 nm of the samples (with and without osmotic shock), for 6 minutes. b) Bar graph corresponding to the rates of ferricyanide reduction obtained from the slopes of the lines in graph a), taking the initial and final values.

### 7.1.2 Spectral properties of chloroplasts and chlorophyll content

The absorption UV-Vis spectrum allowed to follow the activity of the photosynthetic pigments contained in the chloroplasts. This was studied by their characteristic absorption peaks, which also permitted to have an estimation of the chlorophyll content. When acetone at 80% was used to perform the data collection from UV-Vis spectroscopy, Chlorophyll *a* (Chl *a*) presented an absorption maximum at ~664 nm ($Q_y$ band) and 430 nm (Soret band). The absorption maxima for each peak is very dependent upon solvent polarity, as shown in **Table 7.1**. Generally, a red-shift occurs to the absorption band when the polarity of the solvent increase.

**Table 7.1** Comparative UV-Vis absorption and fluorescence data of interest.

| Sample | Soret band | $Q_y$ band |
|---|---|---|
| Chla a (acetone 90%) | 430 | 662 |
| Chl a (acetonitile) | 431 | 662 |
| Chl a (alcohol)* | 430 | 665 |
| Chl a monolayer on smectite+ | 418 | 669 |
| Phaephetene (acetone 90%) | 409 | 665.5 |

Data collected from Blankenship[12], *Manna, *et al.* [57] and +Itoh, *et al*[58]

The spectrum of Chl *a* showed in **Figure 7.5** was measured in 80% acetone. Since the Chl *a* is the only primary pigment in the role of photosynthesis, the Soret and Q bands for this molecule are highlighted in the spectra.

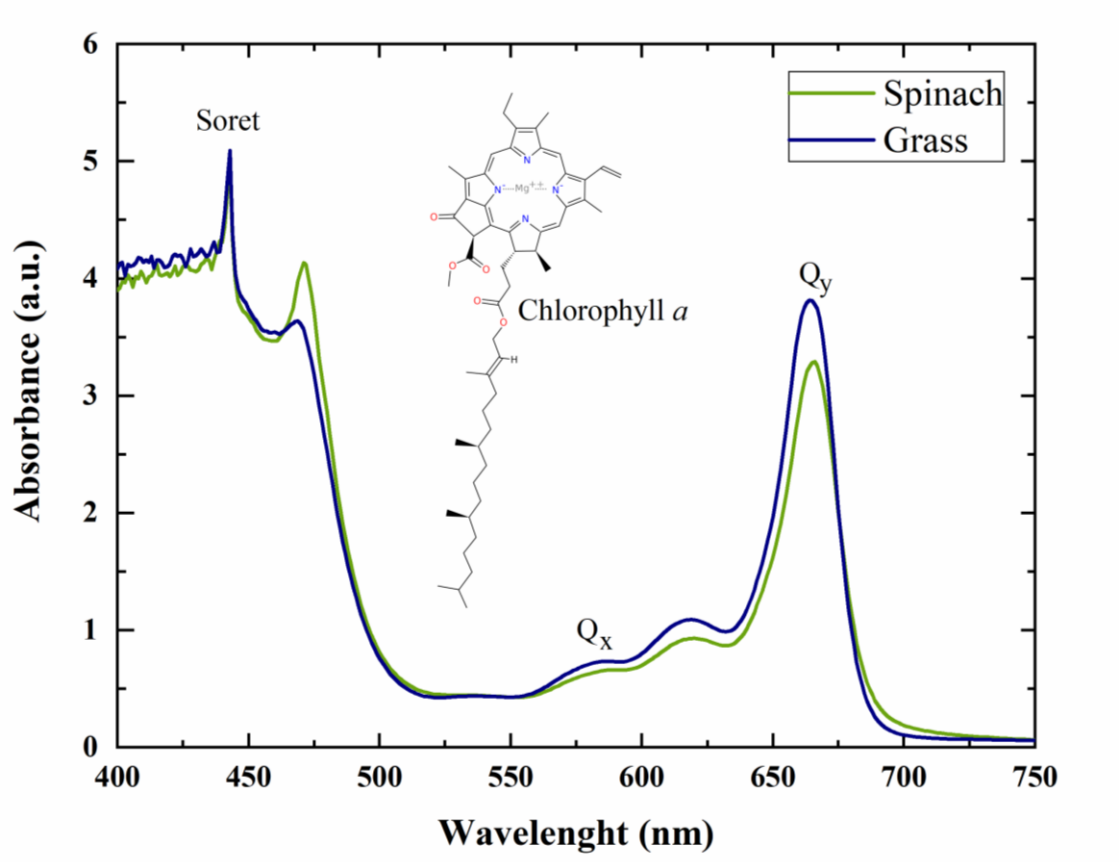


**Figure 7.5.** Absorption spectra of photosynthetic pigments from spinach chloroplasts (green line) and from grass chloroplasts (blue line) suspension in 80% acetone, highlighting the characteristic bands of Chl *a*.

Using the absorbance values deduced from UV-Vis measurements and applying the equations 10.1, 10.2, and 10.3 from **Appendix 2,** the quantity of Chl *a* and Total Chl were estimated. The calculated values are reported in **Table 7.2** as well.

**Table 7.2.** Q-bands and Soret band corresponding to Chl *a* as well as Chl content for spinach and grass chloroplast suspension.

| | $Q_y$ (nm) | $Q_x$ (nm) | Soret band (nm) | Chl *a* (μg/mL) | Total Chl* (μg/mL) |
|---|---|---|---|---|---|
| **Spinach** | 666 | ~580 | 443 | 35.94 | 52.41 |
| **Grass** | 664 | ~580 | 443 | 39.97 | 57.69 |

*Total Chl=Chl *a*+Chl *b*, only Chl *a* is reported in the table due to it is the primary pigment in the photosynthesis.

The slightly higher content of chlorophyll found in the chloroplasts extracted from grass can be attributed to environmental factors that affect plants growth, including dehydration, flooding, freezing, ozone, herbicides, competition, disease, insects, and deficiencies in N fertilization[59]. However, this difference between fresh spinach purchased at the market and grass harvested from the garden at the Campus University is not significant. Consequently, chloroplasts extracted from grass demonstrate to be a very attractive source for different applications, and especially for making the encapsulation of them.

## 7.2 CHLOROPLASTS ENCAPSULATION

Once it was successfully demonstrated that plant cells still have the ability to photosynthesize, the analysis for the encapsulated ones into the silica monoliths was carried out. The optical microscopy, confocal microscopy, UV-Vis spectroscopy, DRUV-Vis spectroscopy analysis offer evidence that silica shell confers the necessary conditions to extend the light-harvesting complex activity over time.

### 7.2.1 Influence of synthesis conditions on chloroplasts integrity

Optical microscopy was carried out to ensure the survival of chloroplasts upon synthesis conditions. The analysis was made just after the emulsion preparation. Micrographs shown

in **Figure 7.6** demonstrate the intactness of the chloroplasts, which are widely spread into the silica matrix. Indeed, it is found that the structural integrity of chloroplasts is kept during this process, so it can be concluded that the use of the oil phase offers protection against Na ions and alcohol groups during the synthesis. This process also occurs under a mild pH range (5-8) that helps to avoid the apoptosis of the cells. At this point, the chloroplasts of all samples appear to conserve their integrity, without matter the concentration of silica or the presence of metals.

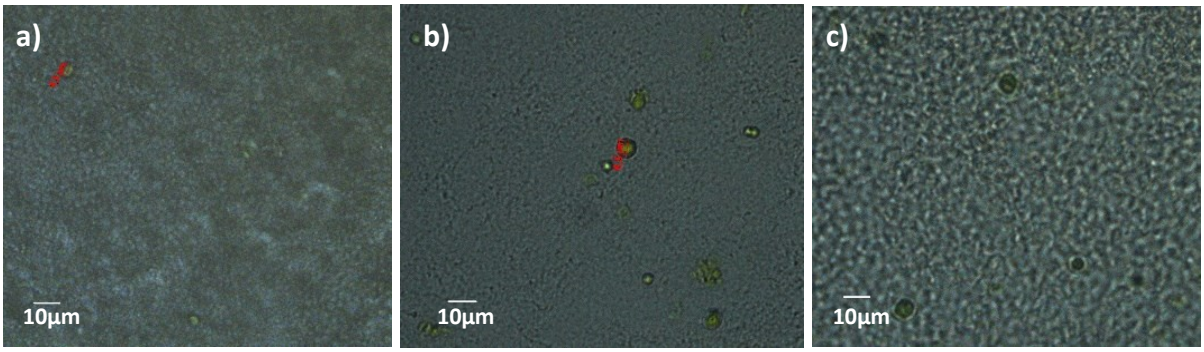


**Figure 7.6.** Optical micrograph of a) ***Si1 mono/ChlS***, b) ***Si3 mono/ChlS*** and c*)* ***CuZn/Si1-mono/ChlS*** just after the emulsion with 40x. Bar:10µm.

### 7.2.2 Influence of the presence of metals Cu-Zn on the chloroplasts activity inside the Silica-mono/Chlx hybrid monoliths

The chlorophyll content of cells is linked to the photosynthetic activity and the number of living cells. If the photoautotrophic cells died, then the hybrid monolith would show no longer fluorescence. Therefore, fluorescence analysis showed in **Figure 7.7** was performed after 21 days to prove the photochemical preservation of chloroplasts in samples ***Si1 mono/Chl*** and ***CuZn/Si1-mono/Chl.*** Many attempts trying to avoid the degradation of pigments have been carried out. One of the methods used has been the functionalization of the silica with some divalent metals, e.g., zinc or copper sulfate[60]. In this work, the use of these two metals was directed to the bactericidal effect of Cu[61,62] and the micronutrients necessaries for cell plants as for example, the Zn as an element of life [63] in order to prolong the chloroplasts activity. So, it has been evaluated the influence of both zinc and copper together, which were present in the matrix. The interesting fact to be highlighted in this analysis is that only the monolith without metals showed fluorescence. It means that the

presence of metals affects the chloroplasts integrity, negating the fact that the presence of metals (Zn-Cu) using the HIPE method can contribute to extending their photosynthetic ability. It can be assumed that Cu and Zn are forming complexes with the chlorophyll, resulting in a loss of activity as explain Petrovic *et al*[64]

The fluorescent signal shown in ***Si1 mono/Chl*** clearly shows the beneficial effect of the silica matrix. This result can be corroborated by the mechanism proposed by Sarkar *et al* [57]. This reaction mechanism involves the formation of a π complex between the π electron cloud of pyroll type ring of chlorophyll structure and the empty d orbital of silicon of silica matrix. The aromatic nitrogenous compounds tend to form π –d interactions (delta like bond), which provides a localized steric hindrance that prevents the diffusion of water and transportation of hydrogen ion. This leads to structural stabilization of chlorophyll molecule as well as resistance to degradation by water. Based on these results, further studies were made only for the monoliths ***Si1-mono/Chl*** and ***Si3-mono/Chl*** with a different silica precursor concentration to find the most suitable condition for the preservation of the light-harvesting complex.

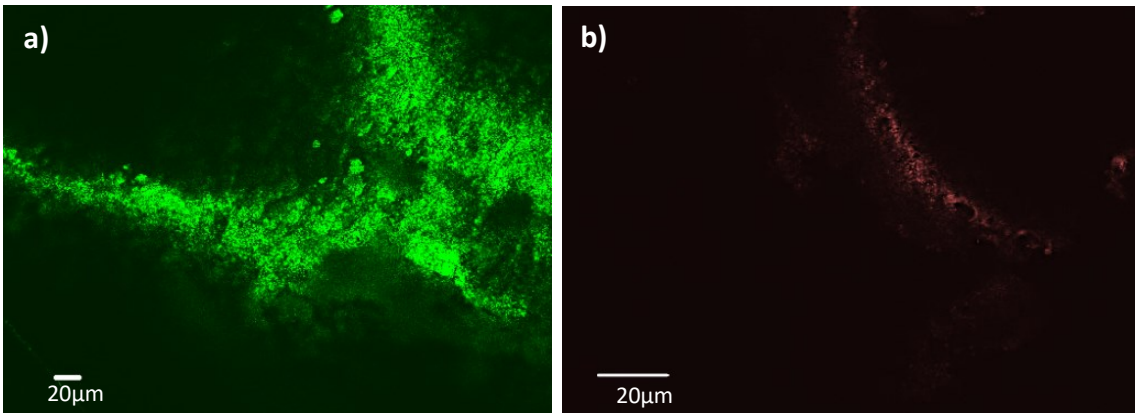


**Figure 7.7.** Confocal microscopy images for the monoliths a) ***Si1-mono/ChlS***. Bar: 20 µm, and b) ***CuZn/Si1-mono/ChlS*** Bar: 20 µm, obtained after 21 days. It is viewed with fluorescein-specific filter to chloroplasts shown in green and silica shown in red.

### 7.2.3 Intactness criteria of the chloroplasts inside the Silica-mono/Chlx hybrid monoliths

As was mentioned before the photoreduction reaction of the potassium ferricyanide can be used to know the presence of broken chloroplast. Therefore, the procedure exposed by Heber & Santarus[56] performed also for the chloroplast suspension was also applied for the

monolith ***Si1-mono/ChlS***. **Figure 7.8a** and **Figure 7.8b** show the absorption spectra obtained after the ferricyanide reduction reaction with and without the osmotic shock, respectively. In this case, it is not possible to estimate the amount of chloroplasts and chlorophyll is present in the sample because the silica, homogeneously distributed on the chloroplast envelop, confers protection to the chloroplasts. Thus, the chloroplasts remain intact, even with osmotic shock reaction, avoiding having a reference of the percentage of broken chloroplasts located in the monolith. However, the graphs clearly show that there is not a significant decrement of the absorbance at 420 nm, demonstrating the presence of a significant amount of intact chloroplasts inside the matrix.

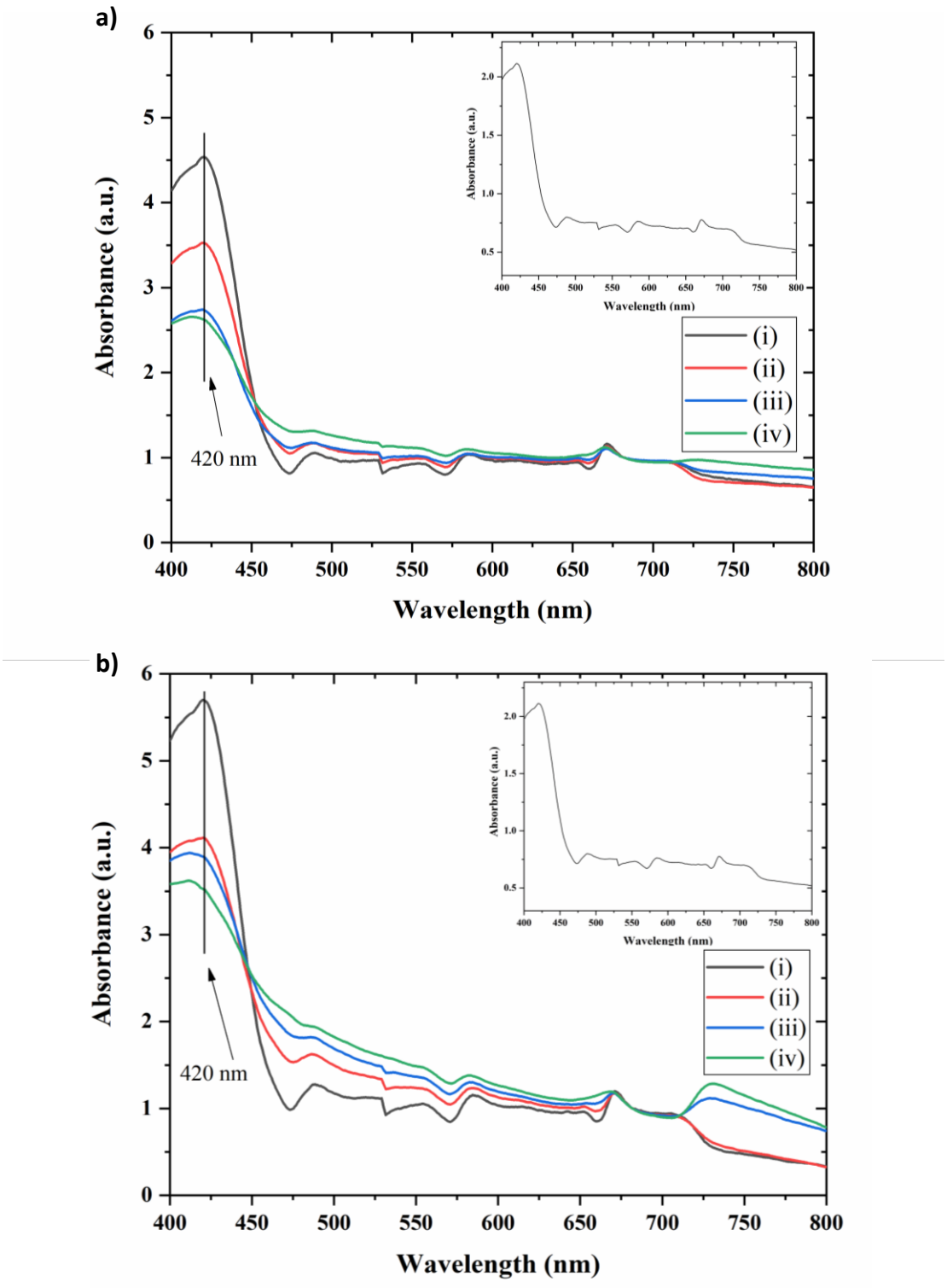


**Figure 7.8.** Absorption spectrum of ferricyanide photoreduction reaction of **Si1-mono/ChlS** a) with and b) without osmotic shock. Lines (i) before illumination, and after (ii) 2 min, (iii) 4min, (iv) 6 min of illumination. Upper-right image: Potassium ferricyanide spectrum.

### 7.2.4 Influence of $V_{TEOS}$ on the Silica-mono/Chlx hybrid monoliths

An additional aspect to be analyzed is the precursor-to-template ratio selected because the preservation of physiological function of chloroplasts is linked to the textural properties of the matrix. In **Figure 7.9** is shown the fluorescence spectra of ***Si1-mono/ChlS*** *and* ***Si3-mono/ChlS*** monoliths after 45 days. There is a significant increment of the fluorescent peak using a volume ratio of 0.15 in contrast to 0.42, meaning that using a lower volume ratio $V_{TEOS}/V_{oil+Chl}$ can be preserved better the ability of the light-harvesting complex. This result is corroborated with the work of Shen *et al.* [10] about the influence of silica concentration on the chloroplasts. They found that it is helpful to use smaller volumes of silica to have a thin monodispersed deposition around the bio-entities in order to avoid a detrimental contraction and shearing strain in the biological structures.

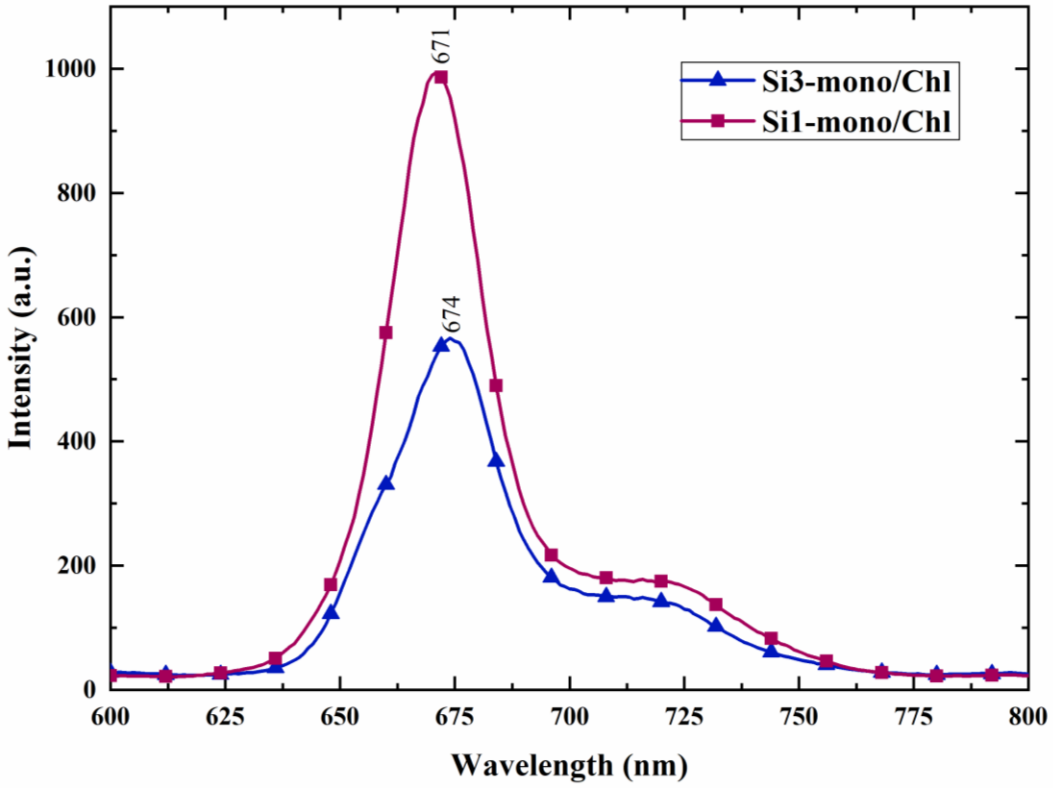


**Figure 7.9.** Fluorescence results of ***Si1-mono/ChlS*** (blue line) and ***Si3-mono/ChlS*** (red line) monoliths after 45 days, with an excitation at 430 nm.

The fact that a lower volume of silica is favorable to chloroplasts stability can be explained by the electrostatic attraction between the biomaterial and the silica matrix. Some previous

studies[1,30] demonstrate that in any solution with pH between 2 and 7.2, silica has associated a negative net surface charge, while chloroplasts have a positive net surface charge; thus, if the silica concentration is high enough, the chloroplasts are embedded in the bulk silica gel. Moreover, it can be assumed that by using a lower $V_{TEOS}$ ratio, one can avoid as much as possible the opacity of the monolith, allowing better the passage of light through the matrix.

Chlorophyll free molecules give an intense fluorescence peak at around 675 nm depending on the solvent used upon excitation at 430 nm[12].The group of Ishii and Itoh[5,58] demonstrated that the Chl *a* molecules when are adsorbed on smectite tend to cause a shift of their absorption maxima wavelengths to longer wavelengths because of the interaction with the matrix. Also, the authors observed that when more amount of chlorophyll is adsorbed the wavelength shift increase more and more. This behaviour was also observed by Sommer *et al.* [65] for both UV-Vis absorption and fluorescence emission of chlorophyll adsorbed into layered double hydroxides. This can be compared with the data obtained for intact leaves having an absorption maxima of chlorophylls at 678 nm[48], which indicates that the matrix also confers a different behaviour on light absorption.

The degradation product of chlorophyll called pheophytin has a maximum of fluorescence at a longer wavelength[12]. As well as for chlorophyll adsorbed into an inorganic matrix, pheophytin molecules suffer a red-shift on the wavelength of its maximum emission band [65]. In the red range, the strong absorption of Chl *a* in the samples ***Si1-mono/ChlS*** and ***Si3-mono/ChlS*** is observed as an intense fluorescence peak at 671 nm and 674 nm, respectively. It is confirmed that the behaviour, reported by Ishii-Itoh and Sommer, is also encountered in this work even for chloroplasts**.**

In **Figure 7.9,** no significant change of fluorescence characteristic is observed, demonstrating that the chlorophyll has not been degraded. This idea can be extended to prove the intactness of chloroplasts, otherwise the maximum in the fluorescence emission will suffer a more noticeable bathochromic shift.

### 7.2.5 The photochemical activity of chloroplasts within Silica-mono/Chlx hybrid monoliths over time

Once it was found that using a lower volume of silica without the addition of stabilizing agents preserve better the activity of the light-harvesting complex, two monoliths, ***Si1mono/ChlS*** and ***Si1-mono/ChlG*** using spinach and grass chloroplasts with that conditions were synthesized and analyzed over time. **Figure 7.10** shows the DRUV-Vis spectra of the two samples, in which the reflectance for both monoliths over time has an increase once the optimum aging time passed (7 days), and they were dried. Then, there was an increase of the reflectance signal for both samples until 16 days, which could be attributed to the the stabilization of the organisms into the matrix, as explained by Rooke, *et al.* [4]. After this time, the signal began to decline because of stressors factors such as pH changes, contraction effects related to the scaffold, among others, induce the apoptosis of the chloroplasts. This analysis indicates that the preservation of the photosynthetic apparatus; therefore, their capacity for doing photosynthesis is maintained.

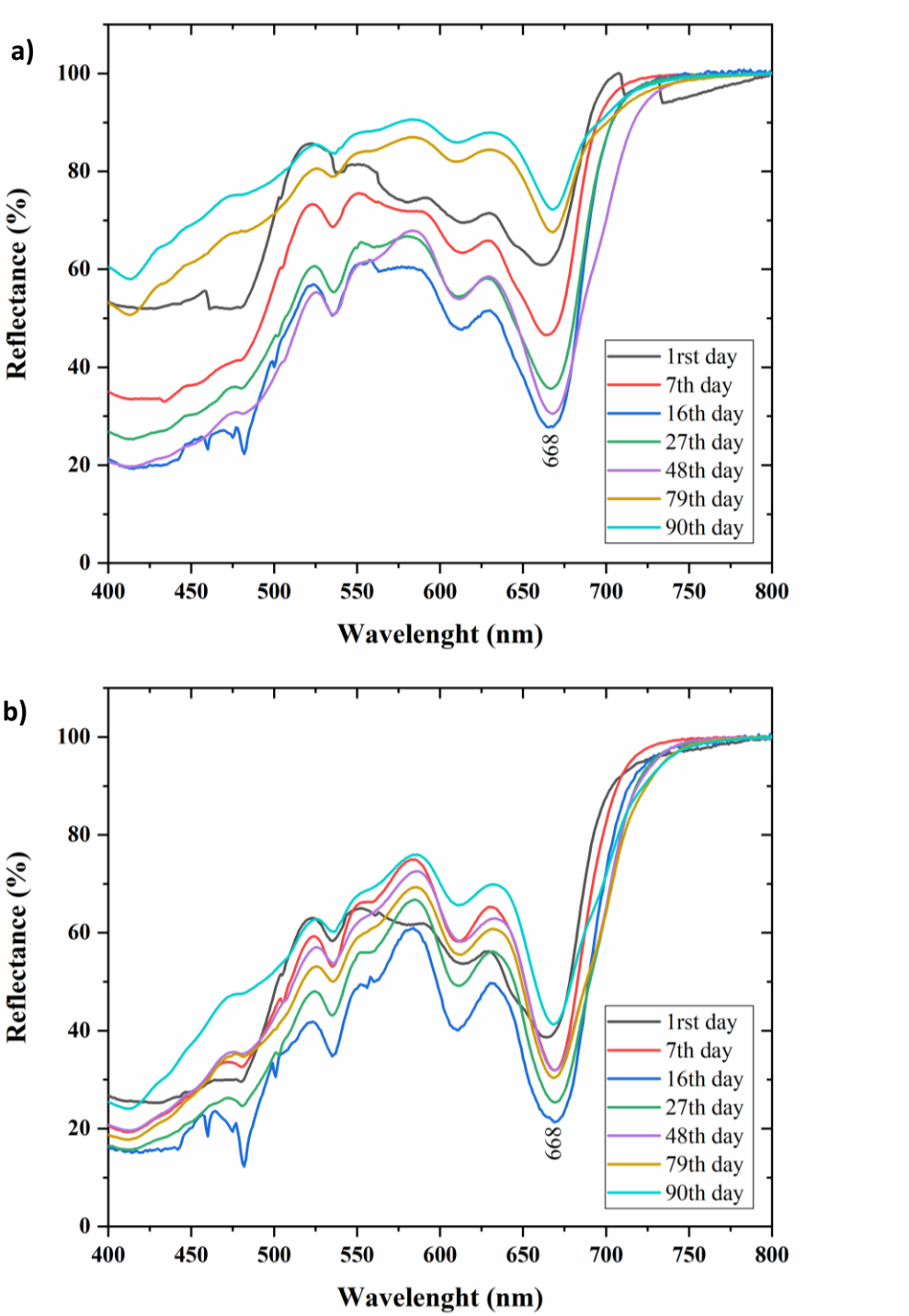


**Figure 7.10.** DRUV-VIS spectra of a) ***Si1mono/ChlS*** and b) ***Si1-mono/ChlG***, during 90 days.

To clearly show the reflectance dependence of the samples in respect with the time. The reflectance measurements corresponding to the wavelength at 668 nm (corresponding to the entrapped chloroplasts) versus time was analysed, and it is showed in **Figure 7.11.** For

the reasons previously explained, there is no gradual decrement of the reflectance intensity.

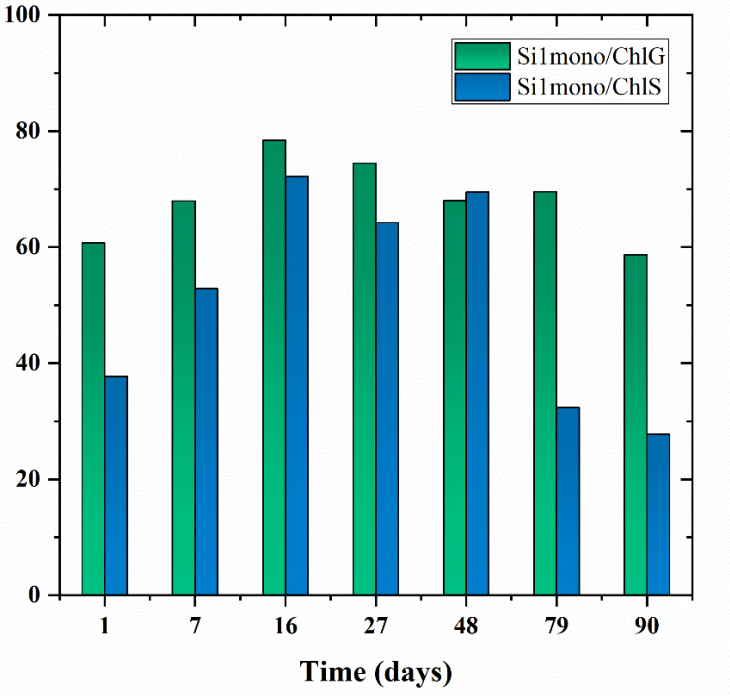


**Figure 7.11.** Comparison of the light-harvesting activity of encapsulated chloroplasts in ***Si1mono/ChlG*** and ***Si1-mono/ChlG***, during 90 days.

As was described before, the light-harvesting activity of chloroplast suspension decreases approximately 90% after three days. So, comparing with the present results, it is demonstrated for the first time that encapsulating chloroplasts by HIPE method extend their photosynthetic activity for a longer time (at least 90 days). Obtaining these functional bio-hybrids materials from grass waste residues is a very useful and timely development since they have an enormous potential to generate sustainable products and bioenergy. Besides, it is also important to highlight these monoliths also could be applied to remove heavy metals due to the capacity of the chlorophyll to form complexes, where the $Mg^{+2}$ can be replaced by some heavy metals such as $Hg^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$, among others[66] .Therefore, this approach considers the grass residues as potential raw material rather than disposing them, to avoid contamination and transmission of hazardous materials to the environment.

## 8 CONCLUSIONS AND FUTURE WORK

This work allowed us to investigate the development of leaf-like silica monoliths, using chloroplasts as photosynthetic material. The isolation of chloroplasts from spinach and grass leaves by the method reported was proved to be successful, giving a good percentage of intact chloroplasts. Also, it was found that it is possible to use pruning waste instead of the spinach due to their comparable good amount of chlorophyll.

It was found that in general, the use HIPE method was a suitable synthesis route to in situ encapsulate chloroplasts extracted from both spinach and grass leaves.

Once proved that HIPE is a successful method for chloroplasts encapsulation, two factors were studied in detail: the presence of metal and the quantity of silica. The presence of metals in the hybrid monolith prejudiced the bio-entities, showing no longer fluorescence. Moreover, by fluorescence spectroscopy was proved that using a lower volume ratio of $V_{TEOS}/V_{OIL}$ is better to preserve the ability of the light-harvesting complex. Besides, the typical spectral reflectance of chlorophyll pigments was maintained for more than 90 days for the stored samples.

Based on these results, it was demonstrated, for the first time, that HIPE method is suitable to prolong the photosynthetic activity of encapsulated chloroplasts within a silica matrix. Besides, in this sense, obtaining these bio-hybrids materials from the pruning residues is a potential development to generate useful products as well as to be applied in metal decontamination.

It is important to optimize the synthesis conditions as well as the interactions between the chloroplasts and the matrix host to find the softest conditions to develop photobioreactors capable of absorbing $CO_2$ to produce biogas, ‘‘green” fuels or other valuable chemicals. Moreover, this technique could be used to encapsulate other kind of organisms with applications in other fields, such as drug delivery, medicine, environmental monitoring, among others.

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# 10 ANNEXES

## 10.1 APPENDIX 1: ESTIMATION OF CHLOROPLASTS INTACTNESS

To estimate the percentage of intact chloroplast we used the ferricyanide photoreduction procedure reported by Heber & Santarus[56] was carried out.

The procedure followed is described below, using a volume equivalent to 100 µL of chlorophyll

- ***A**. Without osmotic shock:* Mix chloroplasts suspension or monolith sample with 4mL of isolation medium. Add 60 µL of 10 mM potassium ferricyanide $K_3Fe\ (CN)_6$.
- ***B.** With osmotic shock:* Mix chloroplasts suspension or monolith sample with 2 mL of distillate water. Leave it stirring for 1 min to allow the osmotic shock. Add 4 mL of isolation medium and 60 µL of 100 mM potassium ferricyanide $K_3Fe\ (CN)_6$.

Steps followed:

I) Place the tubes in ice-bath.

II) Then, illuminate with a 40 W bulb. Take 1 mL sample before the illumination, and one every 2 min after the illumination. Continue illumination for 6 min.

III) Measure the absorbance between a range of 400 to 800 nm after each lapse of time. Finally, the percentage of intactness is calculated with the slope (reaction rate) obtained from the absorbance at the spectrum maximum (the peak) before the illumination and after 6 min.

## 10.2 APPENDIX 2: ESTIMATION OF CHLOROPHYLL CONTENT

To estimate the amount of Chl *a* and total chlorophyll (Total Chl) of the grass and spinach chloroplast suspension the method reported by Sheikh *et al*. [17] was applied. This study was performed with the values of the spectrum recorded from 400 to 800 nm with PerkinElmer® Lambda 1050+UV/Vis/NIR Spectrophotometer. The amount of chlorophylls

was calculated using the absorbance values by the following equations and expressed as µg/mL:

$$\text{Chl } a \text{ [µg/mL]} = 11.63 \times A_{665} - 2.39 \times A_{649}) \quad (10.1)$$

$$\text{Chl } b \text{ [µg/mL]} = (20.11 \times A_{649} - 5.18x \times A_{665}) \quad (10.2)$$

$$\text{Total Chl [µg/mL]} = (6.45 \times A_{665} + 17.79 \times A_{649}) \quad (10.3)$$

## 10.3 APPENDIX 3: PAPER PUBLISHED IN MATERIALS RESEARCH SOCIETY ADVANCES

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# Immobilization of chloroplasts from grass within a silica matrix synthetized by HIPE method

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*ABSTRACT*

*The deficient disposition of the pruning waste, from grass (Poaceae), has been converted into a considerable environmental problem since it is discarded in common garbage dumps. As a result, gases and lixiviates are generated producing a negative impact on the environment. This project takes advantage of these residues to isolate their chloroplasts, with the aim of subsequently developing bioreactors that absorb $CO_2$. The encapsulation of grass chloroplasts into silica monolith with a hierarchical texture, using high internal phase emulsion (HIPE) method was carried out. The isolated chloroplasts were analysed by UV-Vis spectroscopy to estimate the amount of chlorophylls a and b present in the grass. Moreover, the synthesized samples were characterized by fluorescence spectroscopy for monitoring their photosynthetic activity, having an activity up to at least 90 days.*

## INTRODUCTION

The potential of photosynthetic organisms to convert $CO_2$ into useful products has been an attractive topic of investigation, especially in the biosynthesis and biosensors fields. So, researches have been widely interested in finding a suitable way to immobilize them into some different matrices creating a leaf-like photo-bioreactor [1-3]. Many attempts and efforts have been carried out in order to find a way to immobilize plants, algae and cyanobacterial cells into an artificial matrix. There have been numerous studies using the isolation of spinach chloroplasts for encapsulation [4-6] with promising results. However, it is still a challenge to obtain the proper conditions to avoid their chemical and

physical stress, because the chloroplasts are unfortunately fragile and unstable when they are isolated of their own medium. The most studied suitable matrixes include: alumina, alginates, calcium carbonate and silicates [2,7,8]. The latter offers one of the easiest ways of cell encapsulation, due to many factors such as: inexpensive synthetic procedure, hydrophilic nature, chemical inertness, porosity, transparency, stability and mechanical resistance [9]. In fact, the huge existence of silica hosts in nature like diatoms is a hint to develop "cell/silica" materials [10]. Additionally, silica monoliths can have hierarchical and ordered structure, which allows both the diffusion of the products released by themselves as well as $CO_2$ absorption [11]. One way to obtain a leaf-like material has been sol-gel method, but it presents some disadvantages like the high release of alcohol and sodium ions that induce an osmotic stress to the cells [7,12]. Another method is using emulsion-polymer double template, which offers both a controlled hierarchical morphology and a structure keeping also a high surface area [13-14]. Cam et al. [13] reported a new method to synthetize hierarchal inorganic porous monoliths with a high control degree over the size and shape of the porosity. Throughout this method, they obtained monoliths with organized vermicular-type mesoporosity that makes use of a double template, i.e. direct emulsion at the macroscale and micellar templates at the mesoscale. They used sol-gel process to create a system where the inorganic material is deposited around the oil droplets.

In the present work, it is shown for the first time a very innovative way of grass chloroplasts encapsulation by using a high internal phase emulsion method (HIPE) in one-pot process for obtaining a hybrid monolith/chloroplast. After chloroplasts extraction an estimation of total chlorophyll content was done by the method reported by Sheikh,et al [15]. Fluorescence spectroscopy was carried out in order to follow the photosynthetic activity after encapsulation as well as diffuse reflectance UV-Vis spectroscopy.

## EXPERIMENTAL DETAILS

### Chloroplast extraction

30 g of fresh natural grass leaves (*Pannisetum clandestinum*), taken from the Campus at the University Yachay in Ecuador, were washed with distilled water and dried with an absorbent tissue. All steps were made in the dark in order to avoid starch accumulation. The leaves were blended in a commercial blender with 120 mL of 0.1 M Tris-HCl (Sigma Aldrich) pH 7.6 at 25°C. The mixture was filtered through 4 layers of commercial miracloth and centrifuged (Centronic-BLT) for 3 min at 1200 RPM. This step has the objective to discard tissue debris, which appears as a white pellet at the bottom of the Eppendorf tubes used for centrifugation. The supernatant was transferred to new Eppendorf tubes and were centrifuged for 7 min at 3500 RPM. Then, the newly obtained supernatant was discarded, and the green pellet of each tube was resuspended with 1-2 mL of the isolation medium, by gently pipetting up and down.

### Hybrid silica monolith/chloroplast synthesis

Hydrolysis of the silica precursor (TEOS, Sigma Aldrich 98%) was carried out in an acidic aqueous solution, pH = 2 (where the rate of condensation exhibits a minimum), of a 2.0 mL of 20 %(w/w) tri-block copolymer (Pluronic® P123, Sigma Aldrich, $M_n$ ~5800). Silica precursor polymerization and pH regulation was enhanced by addition of 20 µL of 8 g/L NaF (Sigma Aldrich assay: ≥ 99%). Immediately after, the emulsion was performed by slow addition of 5.1 mL of cyclohexane (Fischer Chemical,

99.9%), previously mixed with ~2 mL of chloroplasts suspension. The final product was poured into plastic molds and let into a desiccator at room temperature for aging. The entire procedure is described schematically in Figure 1.

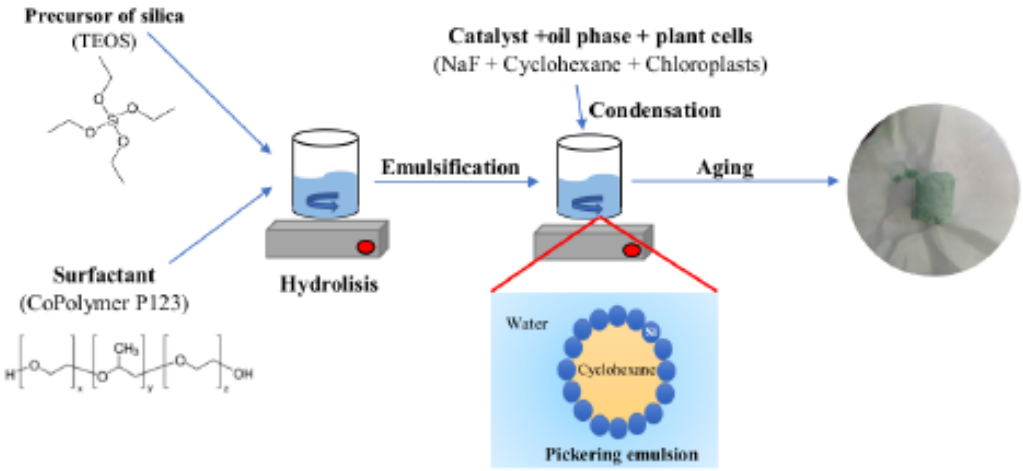


Figure 1. Scheme of synthesis procedure of the hybrid monolith.

## Characterization techniques

To estimate the amount of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Total Chl) of the grass chloroplasts extract, the method reported by Sheikh,et al [15] was applied and taken the amount of chlorophyll extracted from spinach chloroplasts as a reference. This study was performed with the values of the spectra recorded from 400 to 800 nm with PerkinElmer® Lambda 1050+UV/Vis/NIR Spectrophotometer. The amount of chlorophylls were calculated by the following equations and expressed as µg/mL:

$$\text{Chl a [µg/mL]} = 11.63 \times A665 - 2.39 \times A649) \quad (1)$$

$$\text{Chl b [µg/mL]} = (20.11 \times A645 - 5.18x\ A665) \quad (2)$$

$$\text{Total Chl [µg/mL]} = (6.45 \times A665 + 17.79 \times A649) \quad (3)$$

The evolution and the preservation of the isolated chloroplasts within the matrix were analyzed with a fluorescence spectrophotometer Cary Eclipse VARIAN with an excitation at 430 nm in a range of wavelength from 600 nm to 800 nm. Also, a diffuse reflectance UV-Vis analysis of the hybrid materials was carried out with a PerkinElmer® Lambda 1050+UV/Vis/NIR Spectrophotometer from 400 to 800 nm using a Praying Mantis module.

# DISCUSSION

## Comparison of chloroplasts extraction efficiency between spinach and grass

The estimated values of chlorophyll a, chlorophyll b and total chlorophyll were calculated using equations (1), (2) and (3) and are reported in Table I. These close related values reveal that the chloroplasts isolated from grass can be an attractive alternative

source to spinach, one of the most studied for the encapsulation. Additionally, it gives an added value to this pruning waste and a solution to the environmental problem produced, because they are normally discarded in common garbage dumps and harmful gases and lixiviates are released.

Table I. Chlorophyll a, Chlorophyll b and total content of chlorophyll values calculated by equation (1), (2) and (3).

| Type of leaves | Chl a [µg/mL] | Chl b [µg/mL] | Total Chl [µg/mL] |
|---|---|---|---|
| Grass | 39.97 | 17.72 | 57.69 |
| Spinach | 35.94 | 16.46 | 52.41 |

In the Figure 2 it is shown the spectra of the chloroplast extracts from grass (red line) and spinach (black line) demonstrating a slightly higher content of chlorophyll in the grass.

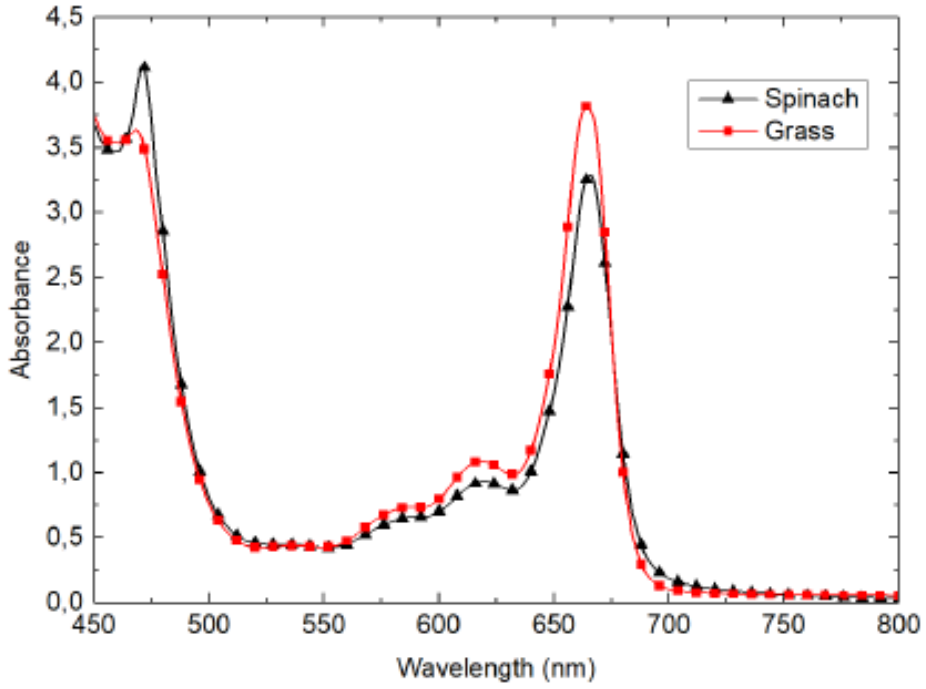


Figure 2. Absorption spectra of photosynthetic pigments present in plant leaves.

The higher content of chlorophyll found in the chloroplasts extracted from grass can be attributed to environmental conditions. According to Carter & Knapp [16], the quantity of chlorophyll depends on variety of stressors including dehydration, flooding, freezing, ozone, herbicides, competition, disease, insects, and deficiencies in ectomycorrhizal development and N fertilization. However, this difference between spinach and grass harvested from the garden at the Campus University of Yachay is not that significant so grass chloroplasts can be used as well for studying the encapsulation.

## Efficiency of chloroplasts immobilization inside silica monoliths

In the Figure 3, one can see the fluorescence spectra of the grass chloroplast immobilized into the hierarchical silica monolith after 57 and 93 days of encapsulation. It is observed a peak at 665nm corresponding to the chlorophyll a, matching the wavelength reported in the literature [17]. It is interesting to note that, throughout HIPE method, the oil phase seems to create a layer around the chloroplasts avoiding to have a direct interaction between cell/matrix. Thus, it provides a protection against the $Na^+$ ions and alcohol groups released during the synthesis [12].

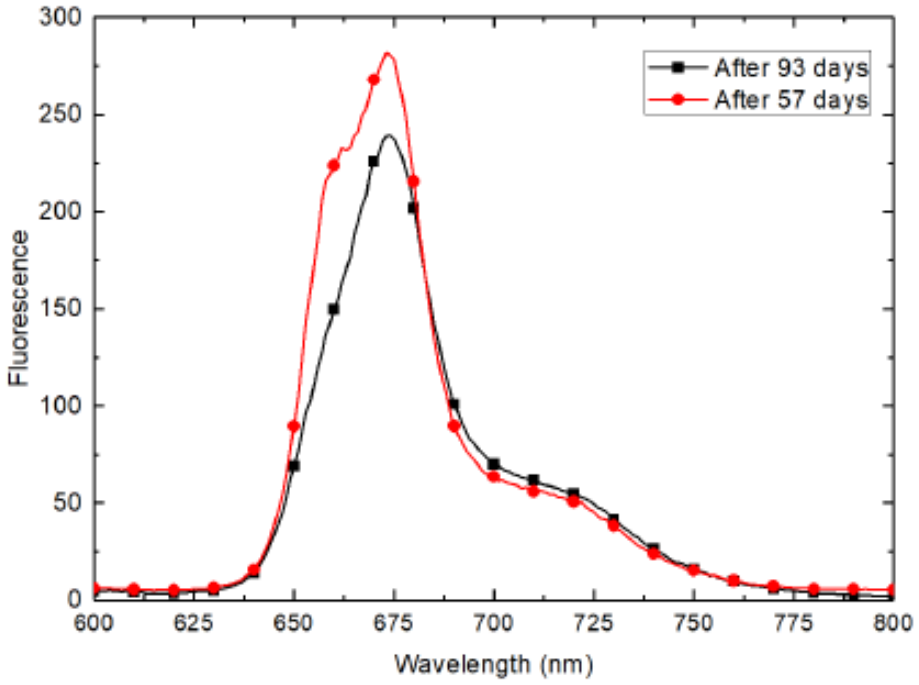


Figure 3: Fluorescence spectra of grass chloroplast immobilized into hierarchical silica monolith after 57 days (red) and 93 days (black) of encapsulation.

According to the results showed by diffuse reflectance UV Vis spectroscopy encapsulation enhance chloroplasts resistance to more than 100 days, Figure 4. The band at 671 nm highlights the presence of intact chlorophyll [12,18,19]. When chlorophyll decompose into its derivative pheophytine then this wavelength suffers a bathochromic shift due to the loss of the central magnesium in the chlorophyllin ring [17]. However, the same shift can also be observed upon immobilization [18,19]. To decide upon the cause of this shift, the presence of the band at around 610 nm (also called $Q_x$ band) ensures that chlorophyll remains intact even when encapsulated [18].

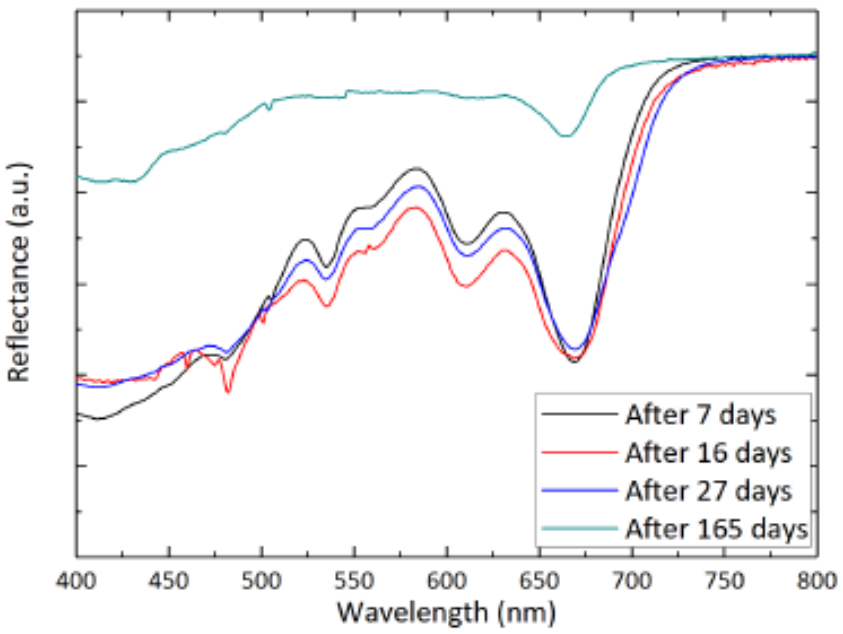


Figure 4: Diffuse Reflectance UV-Vis spectra of grass chloroplast immobilized into hierarchical silica monolith after 7 (black), 16 (red), 27 (blue) and 165 (green) days of encapsulation.

The well-known advantage of encapsulation is clearly demonstrated once more in this research taking into account the lifetime of isolated chloroplasts, which is approximately 48h [12]. When compared with other matrices used for encapsulation and even with the same matrix obtained by a different synthetic pathway [12], one can say that the employed method in this work really helps to maintain the photosynthetic activity of chloroplasts extracted from grass. For example, the encapsulation within a silica matrix synthesized via sol-gel only enhance the lifetime of chloroplasts to 30 days [12] whereas it is shown in this work a stability of at least 165 days. Additional studies are required to analyze the proper conditions as well as the interactions between the chloroplasts and the matrix host. Subsequently, the adsorption of $CO_2$ tests should be performed in order to develop 'green technology' devices.

## CONCLUSIONS

This investigation showed that it is possible to isolate chloroplasts from the grass with high yield. Moreover, this study demonstrates for the first time that the immobilization of chloroplasts within silica matrix by HIPE method is a suitable pathway to preserve the photochemical activity of the cells, as showed by fluorescence and diffuse reflectance UV-Vis spectroscopies analysis. Finally, this work should be extended in order to find the best conditions for developing sustainable devices capable of $CO_2$ adsorption as well as for producing fuel from the sugars liberated by the photosynthetic reaction.

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