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Delneuville, Cyrille

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Dissertation présentée par
Cyrille DELNEUVILLE

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Composition du jury :

Prof. Marc Boutry (UCLouvain)
Prof. Fabrice Franck (ULiège)
Prof. Pierre Van Cutsem (UNamur)
Prof. Alain Krief (UNamur)
Prof. Stéphane Vincent (UNamur, président du jury)
Prof. Bao-Lian Su (UNamur, promoteur)

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Université de Namur
FACULTÉ DES SCIENCES
rue de Bruxelles 61 - 5000 Namur
Tél. : +32 (0)81 72 41 11

Conception de matériaux hybrides pour la création de systèmes cyanobactéries@silice

Cyrille DELNEUVILLE

Résumé

Face aux défis environnementaux et sociétaux actuels, il est important de développer des technologies propres et durables. Les progrès de la microbiologie et l'essor des biotechnologies permettent aujourd'hui de proposer des solutions efficaces à certains de ces défis liés entre autres à la diminution des ressources fossiles ou à la pollution produite par l'activité industrielle. Les microorganismes ont en effet de multiples qualités. Ils sont capables de répondre à certains stimuli, de produire des molécules complexes, certains sont également capables de transformer l'énergie lumineuse du soleil. Tous ces procédés sont réalisés avec toujours une importante efficacité énergétique et une sélectivité remarquable. Néanmoins, leur exploitation intensive et le besoin de viabilité à long terme dans des environnements artificiels conduisent à des conditions d'utilisation de ces microorganismes de plus en plus drastiques. Il est donc fondamental de concevoir des stratégies pour les protéger et faciliter leurs manipulations.

En s'inspirant des processus de biominéralisation fréquents dans la Nature, il est possible de modifier l'environnement proche de ces microorganismes en les entourant d'un matériau abiotique protecteur, à l'instar des diatomées et des œufs de poules où des coquilles à différentes échelles protègent le matériel biologique.

L'objectif de ce travail est de concevoir une méthode d'encapsulation de cyanobactéries de type *Synechoccocus* PCC 7002 au sein d'une nanocoquille à base de silice pour les confiner et les protéger dans le but d'améliorer l'utilisation de ces procaryotes photosynthétiques. La synthèse de matériaux hybrides organiques-inorganiques est un point clé de ce travail. Ceux-ci seront obtenus en utilisant la méthode de couche par couche et différents additifs qui dirigeront la synthèse par voie sol-gel du matériau de silice.

Dans un premier temps, six matériaux sont obtenus suite à la déposition de différents polycations (poly(hydrochlorure d'allylamine, poly(chlorure de diallyldiméthylammonium), poly(éthylèneimine), poly(hydrochlorure de l-arginine), poly(hydrochlorure de l-lysine) et hydrochlorure de diéthylaminoéhtyle dextran) autour des cellules. Cette couche préliminaire fait office de support pour le dépôt ultérieur de silice suite à l'addition d'acide silicique. En comparant ces matériaux, il a été mis en évidence que la composante organique de la nanocoquille hybride a une réelle influence sur les propriétés de celle-ci. En modifiant la nature et la taille des polyélectrolytes, il est possible donc d'induire certaines propriétés à la matrice abiotique.

Continuant dans cette direction, l'ajout de groupements aromatiques, grâce à l'addition de poly(styrène sulfonate) de sodium, absorbant les rayonnements ultraviolets a permis de protéger les cellules de ses effets délétères. Malgré la modification de l'environnement direct de la paroi cellulaire, les cyanobactéries ont conservé leurs propriétés utiles pour la bioremédiation. Les capacités de biosorption de métaux lourds (Cd^{2+}, Cu^{2+} et Pb^{2+}) par les cyanobactéries sont ainsi très similaires avant et après le procédé d'encapsulation. Leur recyclage s'est également avéré possible sans perte de fonctionnalité après cinq cycles.

Ensuite, l'utilisation de silanes organiquement modifiés a été envisagée afin de contrôler les interfaces du matériau en insérant des groupements organiques. Il est ainsi possible de contrôler l'interface entre les cellules et le matériau de silice par l'intermédiaire d'organosilanes aminés. La modification du matériau de silice a également permis l'addition de groupements sulfhydryles en surface, créant ainsi des points d'ancrage pour d'éventuels greffages. Ces groupements ont ainsi permis l'insertion de particules magnétiques en surface de la nanocoquille et de conférer ainsi une certaine mobilité aux cyanobactéries. Le greffage réussi des cyanobactéries sur lames de verre préalablement traitées ouvre la voie à de nouveaux types de systèmes hybrides multifonctionnels et innovants.

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University of Namur
FACULTY OF SCIENCE
rue de Bruxelles 61 - 5000 Namur
Phone: +32 (0)81 72 41 11

Design of hybrid materials towards single cyanobacteria@silica

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Abstract

Faced with current environmental and societal challenges, it is important to develop clean and sustainable technologies. Advances in microbiology and the rise of biotechnology now make it possible to propose effective solutions to some of these challenges, related among others to the decrease of fossil resources or pollution produced by industrial activity. Microorganisms have many qualities. They are able to respond to certain stimuli, to produce complex molecules, some are also able to transform the sun's light energy with always high energy efficiency and remarkable selectivity. Nevertheless, their intensive exploitation and the need for long-term viability in artificial environments lead to increasingly drastic conditions for the use of these microorganisms. It is therefore essential to design strategies to protect them and facilitate their handling.

By drawing inspiration from the biomineralisation processes that are common in Nature, it is possible to modify the environment around these microorganisms by surrounding them with a protective abiotic material, such as diatoms and chicken eggs, where shells at different scales protect biological material.

The objective of this work is to design a method to encapsulate cyanobacteria *Synechococcus sp.* PCC 7002 within a silica-based nanoshell within the purpose to contain and protect them improving the use of these photosynthetic prokaryotes. The synthesis of hybrid organic-inorganic material is a key point of this work. This can be obtained by using the layer-by-layer method and various additives that will direct the sol-gel synthesis of the silica material.

First, six materials are obtained following the deposition of six different polycations (poly(allylamine) hydrochloride, poly(diallyldimethylammonium chloride), poly(ethyleneimine), poly-l-arginine hydrochloride, poly-l-lysine hydrochloride, diethylaminoethyl dextran hydrochloride) around the cells. This preliminary layer serves as a support for the subsequent deposition of silica obtained after the addition of silicic acid. By comparing these materials, it has been shown that the organic component of the hybrid nanoshell has a real influence on its properties. By modifying the nature and size of polyelectrolytes, it is therefore possible to induce certain properties to the abiotic matrix.

In this context, the addition of aromatic groups, by adding poly(styrene sulfonate), which absorbs ultraviolet radiation, has protected the cells from its harmful effects. Despite the modification of the direct environment of the cell wall, cyanobacteria have retained their properties useful for bioremediation. The biosorption capacities of some heavy metals ions (Cd^{2+}, Cu^{2+} et Pb^{2+}) by cyanobacteria are thus very similar before and after the encapsulation process. Their recycling has also been shown to be possible without loss of functionality after five cycles.

Then, the use of organically modified silanes was considered in order to control the interfaces of the material by inserting organic groups. This allows the interface between the cells and the silica material to be controlled using amino organosilanes. The use of these silica materials has also allowed the addition of sulfhydryl groups on the surface, thus creating anchor points for possible grafting. These groups have thus made it possible to insert magnetic particles on the surface of the nanoshell and thus confer a certain mobility on cyanobacteria. The successful grafting of cyanobacteria onto previously treated glass slides opens the way for new types of multifunctional and innovative hybrid systems.

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List of main abbreviations used

5-FM	N-(5-fluoresceinyl)maleimide
AA	Atomic absorption
AEAEAPTMS	3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane
AEAPTMS	[3-(2-aminoethylamino)propyl]trimethoxysilane
APTMS	(3-aminopropyl)trimethoxysilane
BET	Brunauer-Emmet-Teller
BJH	Barret-Joyner-Halenda
DAD	Diethylaminoethyl dextran hydrochloride
DE	Direct excitation
DHA	Dihydrolipoic acid
DTT	DL-dithiothreitol
EDX	Energy-dispersive X-ray spectroscopy
FDA	Fluorescein diacetate
GelH	Cyanobacteria entrapped in silica gel obtained from H_2SiO_3
GelNa	Cyanobacteria entrapped in silica gel obtained from Na_2SiO_3
GSH	Glutathione reduced
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IUPAC	International Union of Pure and Applied Chemistry
LbL	Layer-by-layer
LPS	Lipopolysaccharide
MAS	Magic angle spinning
MPTMS	(3-mercaptopropyl)trimethoxysilane
MTT	2-mercaptoethanol
M_w	Molecular weight
NMR	Nuclear magnetic resonance
OD730	Optical density measured at 730 nm
Ormosil	Organically modified silica
PAH	Poly(allylamine) hydrochloride
PBS	Phosphate buffered saline
PCC7002	Synechococcus sp. PCC7002
PDA	Poly(diallyldimethylammonium chloride)

PDA_l	Poly(diallyldimethylammonium) large size: Molecular weight from 400.000 to 500.000
PDA_m	Poly(diallyldimethylammonium) medium size: Molecular weight from 200.000 to 300.000
PDA_s	Poly(diallyldimethylammonium) small size: Molecular weight <100.000
PEI	Poly(ethylenimine)
PLA	Poly-l-arginine hydrochloride
PLL	Poly-l-lysine hydrochloride
PSS	Polystyrene sulfonate (sodium)
PUT	Putrescine
SEM	Scanning electron microscopy
SPD	Spermidine
SPM	Spermine
TCP	Tris(2-carboxyethyl)phosphine hydrochloride
TEM	Transmission electron microscopy
TEOS	Tetraethyl orthosilicate
TMOS	Tetramethyl orthosilicate
UV	Ultraviolet
XPS	X-ray photoelectron spectroscopy

Part I
General introduction and objectives

Chapter 1 – Introduction

1.1. Clean technologies for a sustainable future

Nowadays, the world is facing environmental and societal challenges. In September 2015, the member states of the United Nations, including Belgium, adopted a list of 17 Sustainable Development Goals (SDGs)[1], several of which refer to energy, health, food, water issues calling to the ingenuity of scientists and researchers for the development of clean and sustainable technologies.

1.1.1. Inspiration from Nature

During evolution, Nature has developed ingenious adaptation mechanisms towards more complex, more efficient, more resilient and less energy-consuming systems. From tiny entities to complex systems, Nature enables to create coherent beings composed of billions of differentiated parts with different functions working together in harmony.

Replicating these effective systems to solve complex problems that our society is dealing with is a promising approach called bio-inspiration that thereby reducing environmental and energy costs compared to other types of engineering. Careful observation and deep understanding are the starting point for designing powerful bio-inspired systems. Technological advances have contributed to more and more accurate observations and understanding of the mechanisms that become in that way more and more detailed. That information has facilitated the design of these new systems. The recent Nobel Prize in Chemistry (2017), rewarding researchers for their advances in electron microscopy, attests to the significance of the deep observations on the structure of biomolecules [2]. This gigantic source of information in which living systems consist of could inspire new technologies. The advantages of these bio-inspired products, such as their reliability, low cost, low energy demand, intelligence, adaptation, miniaturisation, etc., cater to the current demands for sustainability [3]. However, innovation does not only come from observation, interpretation and duplication of the intelligence of the living world, but also from the ingenuity of researchers who design new systems with harmonious combination between Nature and human creation [4].

The advanced material field has been rapidly expanding in recent years. The beauty and the perfect functionality of natural structures have always impressed the scientific and

engineering fields. First of all, macroscopic observations have already left their mark on bio-inspired technologies. The most famous classic example is the invention of the Velcro™. In the 1940s, when a Swiss engineer removed the burrs from his dog's fur, he discovered the hook shape of the plants which can help to disperse their seeds (Figure I-1a). Nowadays, bio-inspired inventions exist in every corner of our life. For example, concerning the design of Japanese high-speed trains, scientific minds were focused on the bio-inspired innovation mimicking the aerodynamics of birds (Figure I-1b) [5].

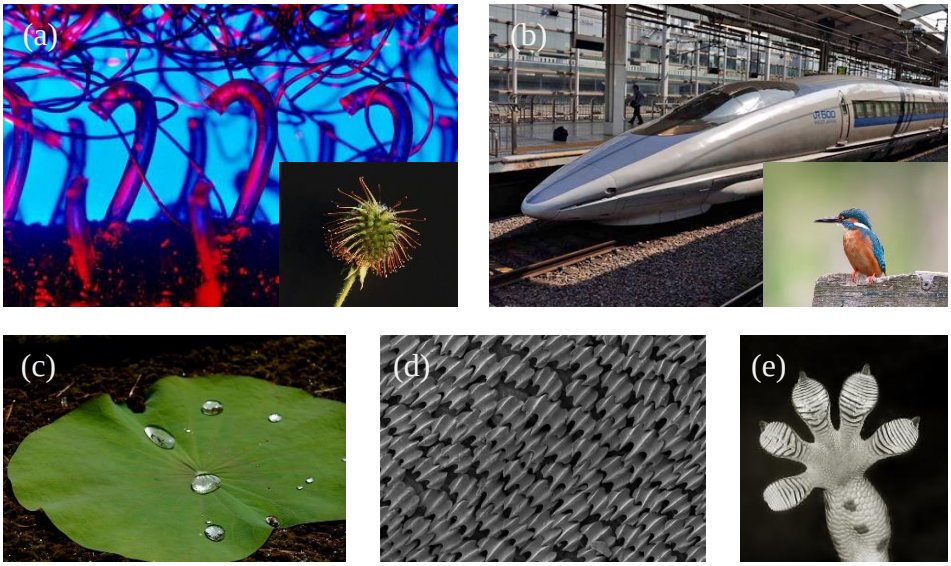


Figure I-1: Some examples of bio-inspiration: (a) Micrography of Velcro™ and photography of burrs seeds; (b) Japanese bullet train and fisher bird; (c) lotus leaf; (d) micrography of shark skin and (e) gecko foot (images from Wikimedia Commons[1])

Micro- and nanoscale observations, developing with the advancing of technique, contribute to the design of materials with high efficiency. Observation of the microstructure of lotus leaves (Figure I-1c) has highlighted its microscopic roughness and thus explained their hydrophobic behaviour towards water drops. This structure has inspired the design of syperhydrophobic coatings for the fabrication of self-cleaning glass. The self-cleaning enables the degradation of organic soiling due to photocatalytic properties related to its chemical composition and then their elimination because of its microstructure properties. Inspired by the microstructure of the skin of certain fish some brands of sports equipment have designed clothing for professional swimmers by mimicking the microstructure of shark skin (Figure I-1d) in order to improve the hydrodynamic properties of these garments for better performance of the swimmer. Similar studies and progress have been made on aircraft structure for fuel

[1] Creative Commons License (CC BY-SA), https://commons.wikimedia.org/

saving by reducing air resistance [6, 7]. A new example is the development of new adhesives inspired by geckos. These reptiles are able to adhere to all types of surfaces because of the presence of the hierarchical microstructures of the fingers of their feet (Figure I-1e). The structure and organisation of the keratin hairs on their fingers allow an incredible adherence to these lizards due to a large number of low interactions (type Van der Waals)[8].

Nature processes interesting structural models on several scales. Recently, the understanding of phenomena related to hierarchical structures (leaf veins, insect spirals, blood networks, etc.) has been revisited to predict certain behaviours of porous materials. The new materials designed following Murray's law show optimised mass transfer, thus making them a promising candidate to be new generation battery materials where Li-ion transfer process is maximised [9].

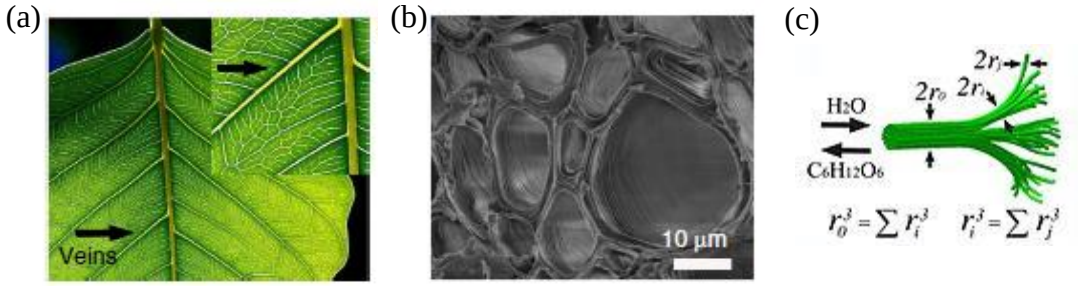


Figure I-2: Murray's law illustration: (a) photography of leaf veins; (b) micrograph of leaf veins and (c) hierarchically porous network models for the veins (adapted from reference [9])

However, the performance of synthetic materials still cannot reach the ones of Nature even though a series of natural phenomena have been explored deeper and deeper. For example, the highly selective reactions conducted by certain enzymes, the energy-saving and complexity biosynthesis conducted by living cells have not yet been duplicated artificially. Nowadays, more and more researches in material chemistry are focusing on carbon dioxide mitigation. This molecule, produced with large quantities by the industrial activities, poses problems related to climate change. Scientists have therefore tried to deal with this situation by recycling CO_2, which is the most abundant C1 compound. But this molecule is very stable with a Gibbs free energy of about -395 $kJ.mol^{-1}$, so its transformation remains a challenge and presents a large energy demand. Photosynthesis is a promising way to deal with the CO_2 issue, nevertheless, it is difficult to reproduce photosynthesis of plants, micro-algae or photosynthetic bacteria despite significant advances in catalysis [10] or research into the creation of materials for artificial photosynthesis [11].

This is the reason why, at the present time, the use of whole or partial living systems is a compromised way towards artificial photosynthesis design. This is generally referred to the biocatalysis where natural catalysts such as enzymes perform chemical transformation. It is also possible to exploit these biocatalysis in the entire living cell. The use of the entire cell has several advantages such as high stability and reaction versatility. In addition, the use of whole-cell can reduce the costs associated with enzyme purification [12, 13].

1.1.2. Microorganisms: "tiny factories"

Cells are considered the smallest fundamental structural and functional biological entities of all living beings. In this way, they are the smallest living entity capable of reproducing, thus are considered as a small factory [14]. The use of living cells for production is an ancestral practice. It is assumed that Palaeolithic humans had already noticed that fruits under certain conditions fermented to produce alcoholic mixtures. The first archaeological evidence of the production of fermented beverages dates back to 7000 BC and was discovered in China [15]. In the 1990s, a study performed a comparison between *Saccharomyces cerevisiae* yeast collected in nature and the one collected in vineyards. This showed that the species had not evolved in the same way, thus evidencing the human domestication of these microorganisms [16]. Currently, living organisms are widely used in biotechnology. The Organisation for Economic Co-operation and Development (OECD) also ranks biotechnology in emerging technologies at the beginning of the millennium. It is defined as follows by the OECD [17]:

"Biotechnology is the application of science and technology to living organisms, as well as parts, products, and models thereof, to alter living or non-living materials for the production of knowledge, goods, and services."

Advances in genetic engineering have made it possible to further expand the range of the applications of these microorganisms. Since 1978, when synthetic human insulin was produced successfully from the bacterium *Escherichia coli* [18], discoveries and advances have continued to develop. As evidenced by a more recent and powerful example that is the exploitation of plant cells as a platform for the production of human antibodies [19].

Among all these living organisms of interest, photoautotrophic organisms have attracted a lot of attention in energy and environmental researches. They are able to use their energy from solar light to synthesise the organic matter for their metabolism via the bioenergetic process of photosynthesis. It is therefore possible to imagine that a large number

of complex molecules can be produced essentially from CO_2 and water, which are very inexpensive or even free. The development of photobioreactors is a promising way to address many energy-related challenges.

Nevertheless, the exploitation of these living organisms has some limitations. They are often difficult to be manipulated because of their sensitivity to the changes of environment. In addition, there are several requirements related to their intensive use, such as long-term viability, harsh environment adaptation, new functionalisation possibilities, etc. The development of new strategies to protect them, facilitate their handling or improve their productivity is a current challenge for the scientific world [20].

1.2. Living materials

Observing Nature, understanding and replicating its phenomena could be a promising way to find solutions and research methods to face these challenges.

1.2.1. Combining living world and materials: a powerful association

Over the course of evolution, many organisms have developed methods for mineral synthesis. They have several functions and their synthesis is referred as biomineralisation. Some molluscs have calcite exoskeletons. The shells of birds' eggs are composed of the same basic mineral and have a protective function. The endoskeletons of vertebrates are composed of hydroxyapatite such as human teeth. Bacteria as well as certain fish (tuna and salmon) are capable of synthesising magnetite for magnetic navigation [21-24]. After secreting some polysaccharides, some cyanobacteria colonies are able to precipitate the carbonates present in their aqueous environment and create solid protection against harmful ultraviolet rays [25, 26].

One of the most beautiful examples of biomineralisation remains the exoskeletons of diatoms. These unicellular micro-algae, which are found in most aquatic environments, are capable of creating microorganised and hierarchical structures from silica dissolved in their environment (mainly in the form of orthosilicic acid). These structures are already used in natural form (diatomite) in industry. However, the exploration of synthesis mechanisms and duplication of synthesis processes remain a great challenge [27-29].

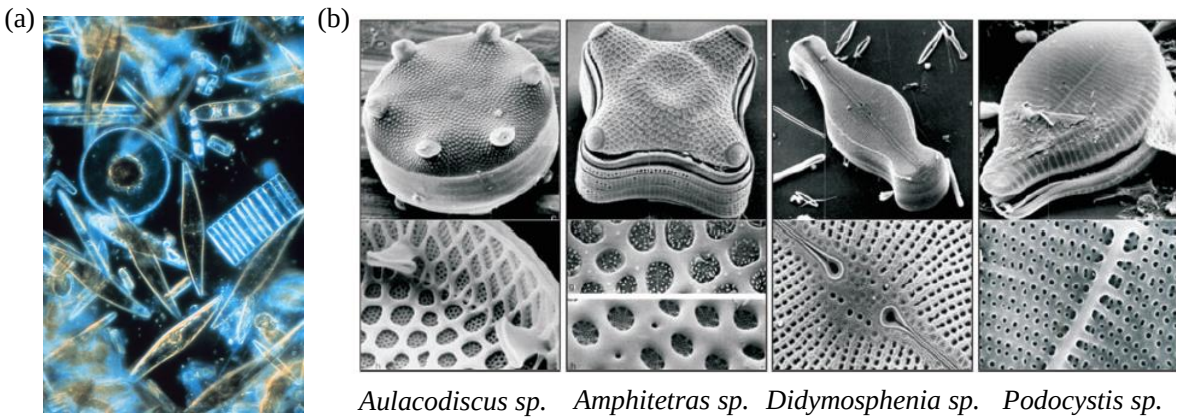


Figure I-3: Diatoms illustrations: (a) micrographs obtain by optical microscopy (adapted from Wikimedia Commons) and (b) by scanning electron microscopy (adapted from reference [28])

Inspired by the cell protection by biomineralisation, it is possible to imagine strategies for protecting and stabilising living entities that are incapable of biomineralisation by combining them with strong materials. The creation of these "living hybrid systems" requires both biocompatible materials and biocompatible immobilisation method to maintain the viability and activity of immobilised biological entities.

1.2.2. Cellular immobilisation

1.2.2.1. An overview

It seems that the oldest written records of cell immobilisation were described in the book of Qimin Yaoshu (544 C.E). The ancient Chinese agricultural texts mention "jiuqu". That was starch pellets contained bacteria and yeasts and was used for the fermentation of wine [30]. The oldest scientific article concerning the immobilisation of biological entities dates from the 1910s and describes the immobilisation by adsorption of enzymes on solid supports to improve their stability [31]. Two decades later, it appears the idea of immobilisation of whole cells. In 1933, Vincenzo Bisceglie demonstrated that tumour cells encapsulated in polymer membranes remained viable for a long time after transplantation into the abdominal cavity of pig [32].

Since then, research has continued to evolve. Today, in order to present processes and abiotic supports with high biocompatibility, high stability and lower costs, several materials have been considered. The first choices of supports were synthetic polymers, natural organic polymers or inorganic (clays, glass beads) type material. Natural polymer matrices (agarose, alginate, chitosan, etc.) have been widely used in the field of biotechnology because of their very low toxicity, low cost and facile implementation [33, 34]. However, these biopolymers do not have sufficient mechanical stability for immobilisation. They suffer from the chemical and

biological degradation and often have high release rates of biological material over time. Synthetic organic polymers (polyvinyl alcohol, polyacrylamide, polyurethane, etc.) have also been widely used [35, 36]. These polymers generally have better stability than natural polymers but require synthesis conditions that are often too drastic for the immobilisation of biological material. The range of applied materials then was extended to inorganic materials. Metal oxides have also been used as a matrix for the immobilisation of biomolecules and microorganisms [37-39]. These supports have good mechanical stability, good chemical resistance and good biocompatibility.

Another inorganic compound has very interesting characteristics that make it a material of choice in the case of cellular immobilisation. Silicon dioxide is a material with great mechanical strength, very low chemical reactivity and good resistance to microbial attacks. This material is also very easy to be synthesised under mild (and so adapted to living cells) conditions through a sol-gel process [40]. Last but not least, this mineral is present in all biological kingdoms, from primitive animals (sponges) to complex plants, including unicellular micro-algae, so it is expected to be highly biocompatible [41, 42]. Indeed, it is this material that makes up the exoskeletons of the diatoms described above. By studying the natural models provided by these organisms and their biosynthesis mechanisms of a highly hierarchical mineral [27-29, 43, 44], it could be possible to design adapted materials for cell immobilisation.

1.2.2.2. Sol-gel: a safe-way for safe encapsulation

Synthesising a material via the sol-gel process involves the use of alkoxy precursors as an inorganic source. For example, in the case of silica, $Si(OMe)_4$, $Si(OEt)_4$, $Si(OPr)_4$, etc. are most often used as precursors. An oxide network is formed via hydrolysis and condensation reactions. First, small clusters are formed in solution (sol). Subsequently polymerisation of the species continued to form a silicate network being rich in water (gel) (Figure I-4). The formed network can be treated with an ageing and drying stage to obtain powders with different textural properties under various preparation conditions [45-47].

The use of alkoxides for cell encapsulation suffers from a disadvantage which is the release of cytotoxic alcohol (methanol, ethanol, isopropanol, etc. depending on the starting precursor) during hydrolysis and condensation reactions. A first solution to overcome this concern is to eliminate the alcohol of prehydrolysed sol by heating before the addition of biological material [4]. Another solution is using biocompatible precursors. The use of polyolsilanes for immobilisation of biological entities has also been considered. Their synthesis

releases biocompatible products, e.g. glycerol [48]. Other approaches involve the use of stabilised colloidal particles (Ludox®), sodium silicate (Na_2SiO_3) or polysilicic acid (H_2SiO_3), which lead to the formation of a silicate network by the "aqueous" route and no longer by the "alcoholic" route [49, 50].

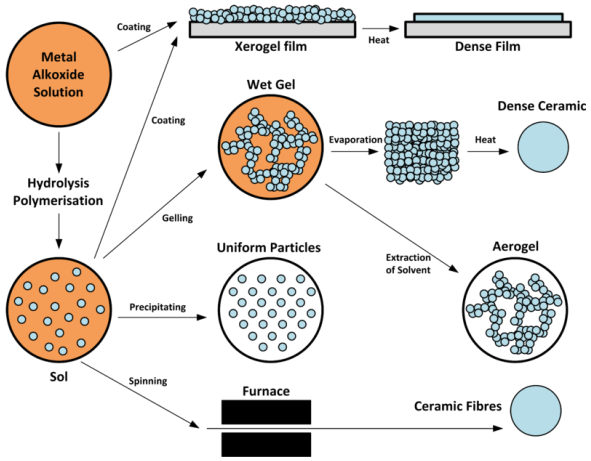


Figure I-4: Illustration of the sol-gel process (adapted from Wikimedia Commons)

1.2.2.3. Organic-inorganic hybrid with modulated properties

To further improve the performance of inorganic materials, it is interesting to add organic additives. This type of study focuses on the creation of organic-inorganic hybrid materials. The versatility of the sol-gel process makes it easy to incorporate functional groups into the network of the obtained material. The properties of the latter are then modulated. Thus, by using additives, it is possible to modify material properties such as the thermal behaviour, the hydrophobic/hydrophilic balance, the chemical stability, the optical properties, the biocompatibility as well as additional functionalities [51-54].

Professor Clément Sanchez from the "Collège de France" and his collaborators classified hybrid materials into two categories according to the link bond between the organic part and the inorganic part. The first type corresponds to the material where the two components are linked together via interactions type Van der Waals forces, ionic bonds, hydrogen bonds or hydrophilic-hydrophobic balance. The second class of hybrid materials possess covalent bonds that link organic and inorganic components. So that the two components of the structure are "grafted" together [51].

The sol-gel process eases the design of hybrid material. By adding organic compounds during the polymerisation process, the material resulting is a composite with mixed properties. Concerning the second category of hybrid material, covalent bonds are easy to be formed using

organically modified silanes as precursors. These molecules are the starting point for the insertion of reactive organic groups within the inert silica material creating organically modified silica (ormosil).

Several examples of cellular immobilisation involving the first category of hybrid material have been reported. For example, the addition of protective additives (glycerol) to silica matrices enable to prolong the life of the encapsulated cells [37]. The layer-by-layer (LbL) modification before the introduction of an inorganic layer is also widely used [55-57]. These methods combine the advantages of each part involved in the processes and contribute to the design of improved materials.

The second category of hybrid material is also well developed. The use of glycidoxypropyltrimethoxysilane (GPTMS) for the creation of organically modified silica network has shown good results for the immobilisation of bacteria [58]. While using gluconamidylsilane as an additive to form the silicate networks have been investigated for the encapsulation of plant cells. This molecule is obtained by reaction between amino silanes and a lactone [4]. The functionalisation of artificial shells of cells by the use of silanes organically modified by thiol groups is also interesting [59, 60]. Furthermore, it is possible to obtain a reversible system using the behaviour of the organic component under various conditions [61].

1.2.2.4. Immobilisation methods

The immobilisation of biological materials involves two categories of methods: post-synthesis methods and *in-situ* methods.

1.2.2.4.1. Post-synthesis immobilisation

Post-synthesis immobilisation refers to immobilise biological entities (enzymes, cells, etc.) on a material, which has been already synthesised. The immobilising materials include, among others, cellulosic materials (modified cellulose, sawdust, etc.) or inorganic materials (porous glass, montmorillonite, etc.). These materials can further be treated with polyelectrolytes to improve their adsorption capacity. These strategies are based on the interactions between the material and the biological material, which often refers to weak bonds and surface adsorption (Figure I-5) [62, 63]. In addition, the difficulty of creating contact between the biological entities and the preformed abiotic matrix under optimal conditions leads to the release of the biological part over time termed the instability of immobilisation.

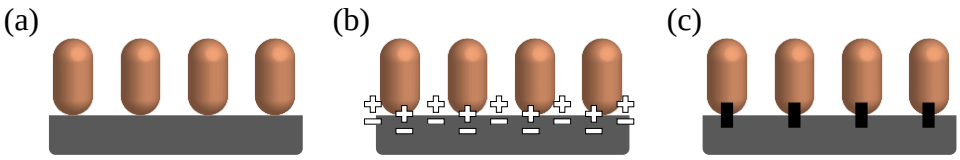


Figure I-5 : Post-synthesis immobilisation based on (a) adsorption on the surface ; (b) electrostatic binding on the surface and (c) covalent binding on the surface

1.2.2.4.2. In-situ immobilisation

During *in-situ* immobilisation strategies, the matrix is synthesised directly around the biological material. In these cases, the synthesis process, secondary products, reaction conditions, etc. must be in accordance with the stability conditions of the biological material in order to maintain the viability of the immobilised cells. A matrix completely entrapping cells within it is the general process of encapsulation. The Figure I-6 shows the different *in-situ* approaches. The first one is to disperse and immobilise biological entities within a monolith [48, 64-66]. The second one allows the creation of small cavities in which the cells have the opportunity to proliferate [67, 68]. A similar process is used to create beads that trap dispersed biological material [69, 70]. Finally, the fourth strategy is creating artificial shells around each of the cells individually [30].

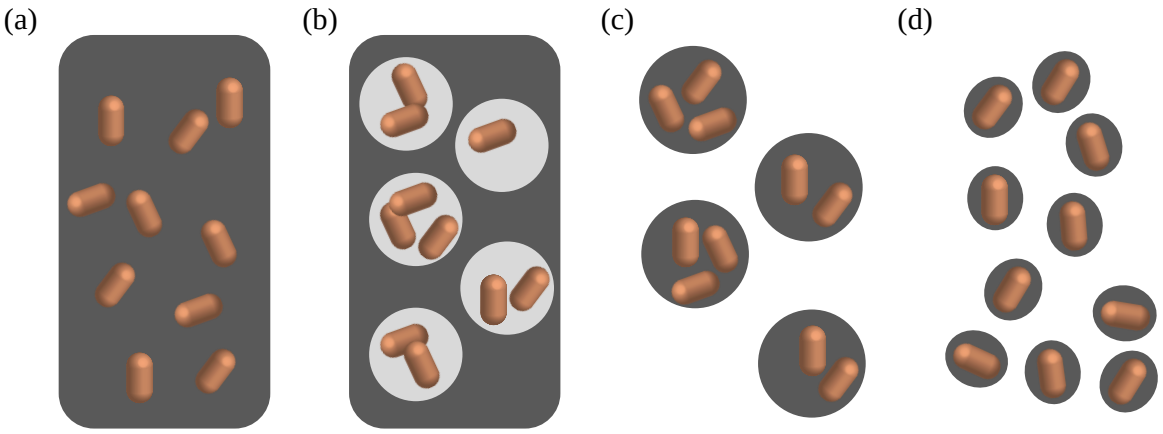


Figure I-6: Diagrams of *in-situ* encapsulation: macroencapsulation (a-b) and microencapsulation (c-d)

The creation of monoliths (method a and b) is generally referred to macroencapsulation. Materials obtained by macroencapsulation have good properties for the storage and conservation of biological entities. It also allows the creation of bioreactors that are easy to create and manipulate. But because of the size of the materials (several centimetres), problems appear associated with the diffusion of nutrients, light, etc. through the material. The synthesis of small independent entities (method c and d) is categorised as microencapsulation. Beads

present very good hydrodynamic and less diffusion-related problems, making it a good candidate for the creation of continuous flow bioreactors. However, in the first three strategies, although the encapsulation of biological entities could be done and facilitate the handling of the cells, it is difficult to control the environment around the cells as well as to understand the behaviour of the cells within the material. This is why the fourth strategy, individual encapsulation, could bring certain innovations.

1.3. Cellular level: individual encapsulation

1.3.1. Generalities

The individual encapsulation of cells, by keeping the system at the micrometres level, presents some advantages presented below [71]. It is easier to understand the behaviours of the living system and the involvement of its components since all of them are in close interactions. This working level also allows use of innovative microfluidic systems for material synthesis [72]. Microfluidics and associated technologies improve the homogeneity of the system by providing the same amount of light or nutrients throughout the system or by creating variable conditions. In addition, the individual encapsulation also can make the system homogeneous. Diffusion is more facile through shell than through bulk materials. Moreover, the savings of chemicals in individual cell encapsulation can reduce the costs associated with immobilisation handling and thus meet the demand for low-cost technologies.

This type of encapsulation also provides controls at different levels. It allows the confirmation of the composition of the cell environment with nanometre accuracy. This control further leads to regulate the behaviour of the cells such as their population (by blocking cell division), or the equipment with new properties.

Strategies for the design of these materials are inspired by Nature, there are several examples of individual encapsulation by biomineralisation (Figure I-7). The most famous macroscopic example is the egg of chickens (or other birds). The fragile embryo is confined by a mineral layer of calcite. This egg shell allows the exchanges between eggs and the outside surroundings while provides important protection. By the closest inspection of this shell, several components can be distinguished from it: a layer that induces mineralisation is covered by the solid outer layer of calcite [73]. The second example, which is now at the microscopic level, is diatoms. The biogenesis of the silicate exoskeletons of these unicellular micro-algae is extraordinary and is full of lessons. They set up very interesting mechanisms for harvesting dissolved silica in their environment and then creating these hierarchical structures. In this

biomineralisation process, silica deposition vesicles (SDV) and organic biomolecules such as sillafins or long-chain polyamines play an important role which have been attempted to be replicated in artificial biomineralisation [43]. Drawing inspiration from such those mechanisms is an extraordinary way to design powerful living materials [28, 29].

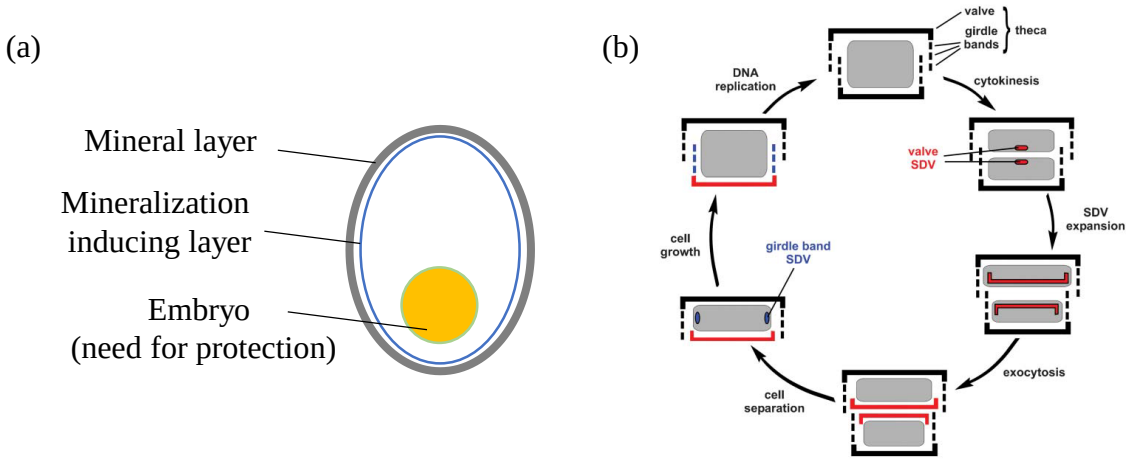


Figure I-7: Bio-inspiration for single cell encapsulation: (a) chicken egg and (b) the diatom cell cycle (adapted from reference [43])

The first referenced design of "cell by cell" living system dates from the early 2000s and concerns the individual encapsulation of yeast in multilayer polymers [74]. Since this research, several strategies have been put into practice for the design of bio-hybrid systems by individually encapsulating whole cells.

1.3.2. Strategies

Hybrid material synthesis strategies around cells are based on different parameters [30, 71]. The chemical composition of cell walls and especially the presence of functional groups on their surface are the key point for designing "shellization" strategies. Indeed, the surface of the cells is composed of several thousand biomolecules (lipids, proteins and carbohydrates) arranged in the three dimensions of space with different levels of organisation [75]. Groups such as the hydroxyl, carboxylic acids, amines, amides, esters or thiols can be found on the outmost surface. These groups are responsible for the surface properties of the cells. They lead to the appearance of charge, the presence of hydrophobic/hydrophilic sites, regulate the chemical reactivity, etc. Surface modification strategies can thus be designed based on these properties. These groups are the basic scaffold for the creation of the coating by playing on the possible interactions between the abiotic material and the cell surface.

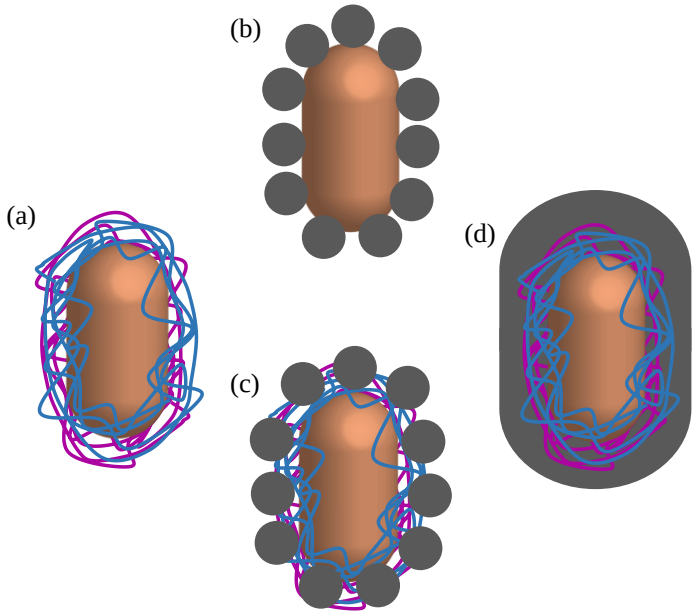


Figure I-8: Strategies for single cell encapsulation: (a) Layer-by-layer (LbL) deposition of polyelectrolyte; (b) nanomaterials auto-assembling; (c) LbL combined with nanomaterials depositions and (d) LbL induced mineralisation

On Figure I-8, the different strategies for single cell encapsulation are presented. They are described briefly in the next sections. For more meaning, some of the examples from the literature are shown in Figure I-9.

1.3.2.1. Layer-by-layer (LbL) process

The layer-by-layer method is a widely used method for the construction of functional materials in the shape of films [76, 77] or microcapsules [78]. This process is particularly interesting in the case of cell encapsulation. It is the first to be described in the literature as mentioned above [74]. The layer-by-layer method is built on the creation and reinforcement of interaction between each component one after the other. Used for individual encapsulation, it consists of adding compounds step by step that will together form a stable multilayer. This makes it possible to control the direct environment around the cells (first layers) and to modify the properties of the shell using additives.

1.3.2.2. Nanomaterials self-assembling

The self-assembly of preformed abiotic material around living cells is another method for coating. Geng, Wang *et al.* classified those deposition methods depending on the dimensions of the nanomaterials (nanoparticles=0D, nanotubes=1D or nanosheet=2D). Simply by contact interactions can appear between the cells and nanoparticles, it is then possible to cover microorganisms up. This strategy has been developed for the deposition of iron particles around bacterial cells to bring them magnetic properties [79]. This deposition is possible thanks

to the layer of polysaccharides present around the bacteria that can interact with the nanomaterial. However, it is often necessary to cover the cells previously with additional layers to create strong links. The reproduction of a layer-by-layer scheme is then widely used for the deposition of presynthesised material. The polyelectrolytes can promote the deposition of nanomaterials for construction of the bio-hybrid system and stabilise the formed layer.

For the first aspect, some example of the 0D nanoparticles deposition showed interesting perspectives. Electrical conductivity can be provided to individual bacteria by the deposition of gold nanoparticles which is initiated by first layers of lysine [80] or poly-(l-lysine) [81]. Concerning the second category and the deposition of building blocks with one dimension (1D), some cases have highlighted the deposition of carbon nanotubes on the surface of yeast cells. This encapsulation is mediated by the use of a LbL method and the use of different polyelectrolytes. The researchers were able to confirm that this deposition modified the transfer of electrons between living yeast and an artificial acceptor, thus opening the door to many applications [82]. Examples related to the last classification and coating by nanosheet (2D building blocks) on living cells have been addressed for the first time by a group of Korean researchers. Their researches have demonstrated the appearance of new magnetic properties and the new possibility of yeast functionalisation following the deposition of graphene oxide sheet (assisted by a prior deposition of polyelectrolytes) [83].

1.3.2.3. In-situ material synthesis

In-situ methods consist of the construction of nanostructures around whole cells. These are generally formed via a surface-induced polymerisation of a precursor. These methods are most often described as bioinspired methods, as its mechanism comes from examples as biosilification of diatom exoskeletons [71]. Among this type of encapsulation is the *in-situ* construction of a calcium phosphate shell. These provide the living cells with improved resistance to external aggression [84]. The design of silica-based shells around yeast was also considered. A previous deposition of poly(ethyleneimine)/poly(sodium 4-styrenesulfonate) bilayer allows the polymerisation of a hydrolysed solution of tetramethyl orthosilicate around the cells [85]. The same group has also developed a bioinspired method for creating titanium shells around micro-algae cells. In this case, the polymerisation of the precursor (titanium bis(ammonium lactato)dihydroxide) is initiated by a first layer of engineered peptides [39]. A similar process is designed to protect micro-algae cells. The polymerisation of a silica precursor is scaffolded by peptide layer. The final material provided thermotolerance to these cells when compared to native ones [86].

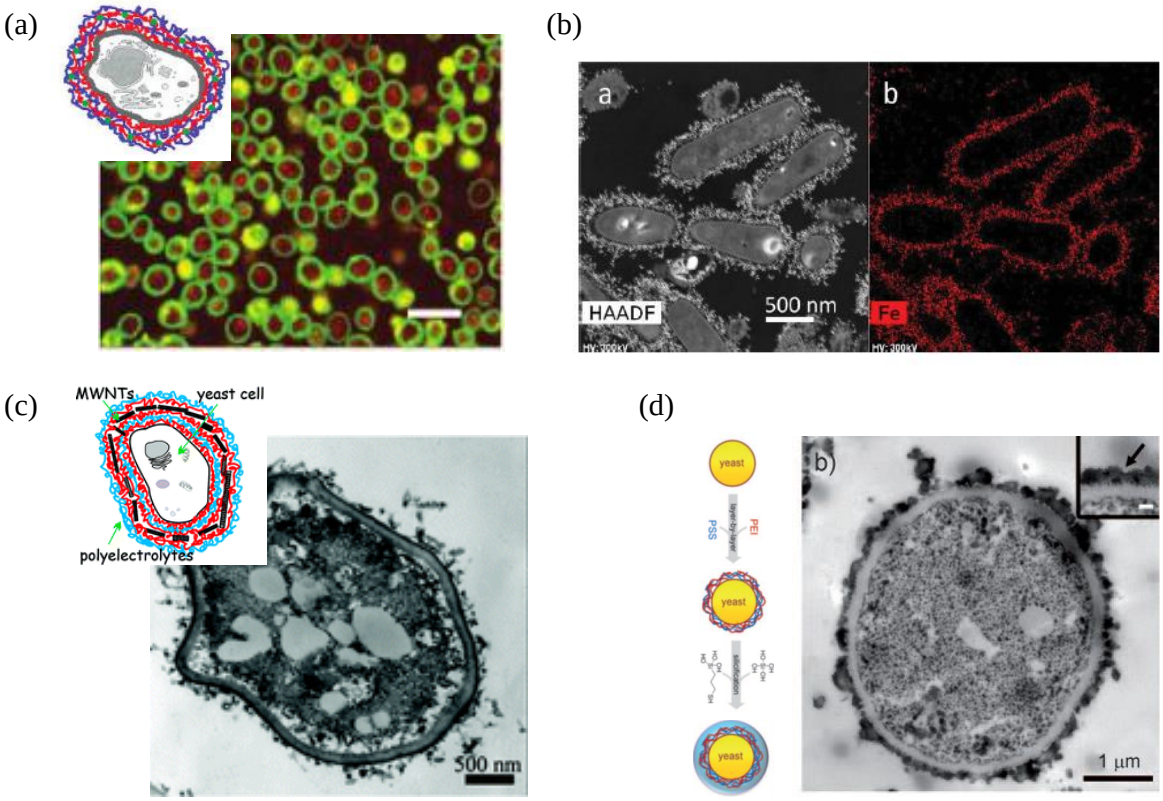


Figure I-9: Strategies for single-cell encapsulation: (a) Yeast encapsulated via consecutive adsorption of poly(allylamine hydrochloride) and poly(styrene sulfonate sodium salt) marked with fluorescein isothiocyanate (merged confocal micrographs) (adapted from reference [74]); (b) electron microscopy micrograph and EDX mapping of artificial magnetic bacteria obtained by direct deposition of iron nanoparticles on the cell surface (adapted from reference [79]); (c) polyelectrolyte-mediated assembly of multiwalled carbon nanotubes on living yeast cells (scheme and electron microscopy micrograph) (adapted from reference [82]) and (d) individual yeast cells in the thiol-functionalised silica (scheme and electron microscopy micrograph) (adapted from reference [85])

1.3.3. Surface engineering

Individual encapsulation belongs to cell surface engineering [73]. By controlling the chemical components of the outmost surface, it is possible to control the behaviour of the cells. The examples described above have already shown that individual cell encapsulation is a promising approach to bring new properties to living entities. An overview of these properties is proposed thereafter and is illustrated by the Figure I-10.

1.3.3.1. Resistance to external stress

The first function of artificial shells is to protect against certain stresses and external aggression. When microorganisms are used intensively, they are confronted with artificial environments, which often threaten their survival. For example, in order to produce metabolites, cell cultures are placed in bioreactors or photobioreactors. For a high yield, the requirements are high cell density, agitation, artificial lighting and a mixed supply of nutrients in the reactor. The cells are therefore subjected to shear forces, pathogens, high light flows, various pH, etc. Although this problem can be solved technically, the chemical pathway of cell

encapsulation can provide undeniable support to deal with these problems. On the other hand, the advent of bioelectronic devices also brings many constraints for the use of biological materials. Strategies must therefore be developed to help these cells to survive on solid supports, under certain electrical flows or oxidative stress, etc.

1.3.3.1.1. Protection to shear forces

One of the common stresses to which cells are exposed in some bioreactors is shear forces due to the agitation in the containers. An example of protection against these stresses is the creation of a film of polyelectrolytes via a layer by layer approach around HepG2 cancer cells that has increased their resistance to centrifugation: "free" cells lose more than 90% viability after 6 cycles, whereas encapsulated cells show a survival rate greater than 90% after 18 centrifugation cycles [87].

1.3.3.1.2. Protection to osmotic stress

When microorganisms are subjected to changes in aqueous media during intensive use, it appears that they often lose stability because of differences in composition due to osmotic shocks. For example, a team of researchers improved yeast stability by chemically encompassing cells with reduced graphene oxide. These have a much greater tolerance after successive baths in pure water (hypo-osmotic shock) [88].

1.3.3.1.3. Photo-protection

The optical properties of abiotic materials are very important. Indeed, visible light is essential to photoautotrophic organisms, while ultraviolet light is harmful to most living organisms. A challenge is therefore to obtain a coating that is transparent to visible light and has a protective effect against harmful lights (absorbing ultraviolet rays). This modification of photochemical properties was considered using several materials. Certain optical properties are assigned to lanthanide phosphate ($LnPO_4$), making it a matrix of choice for photoprotection by individual encapsulation. A coating based on $LnPO_4$ has then protected zebrafish embryos against ultraviolet type B radiation. As these materials respond to this radiation, the cells are less affected by ultraviolet degeneration [89]. The combination of polyelectrolyte layer and artificial biosilification protected cyanobacteria from high photoflux [90]. Cyanobacteria are photosynthetic prokaryotes, their metabolic activity is highly dependent on light. The addition of gold nanoparticles has also used in protecting cyanobacteria again against harmful ultraviolet-c light [57]. The construction of bio-hybrid shells based on a thiolated amino acid (l-cysteine) and Au nanoparticles has also shown its photoprotection. Yeast covered by this

coating showed significant survival under harmful UV-c radiation. After 5 hours under the irradiation, 98% of the initial activity of the treated cells was maintained whereas 6% of the initial activity maintained in the "free" cells [91].

1.3.3.1.4. Thermotolerance

Thermal stability cannot always be guaranteed in artificial systems. It is therefore interesting to provide a certain thermotolerance to cells. As mentioned above, silica-titanium shells have provided an interesting thermotolerance to micro-algae cells. After two hours at 45°C, the native cells have lost more than 80% of their activity while the algae@SiO_2-TiO_2 has a survival rate of almost 60% [86]. Yeast (*Saccharomyces cerevisiae*) was also encapsulated in a silica shell, which provides protection and a better tolerance to heat compared to native cells [92].

1.3.3.1.5. pH tolerance

Cell encapsulation is effective strategy to protect cells from several external stresses. A silica shell, formed with the assistance of amino acids (l-cysteine) and the incorporation of Au nanoparticles, provided photoprotection to photosynthetic prokaryotes. These amino acids could also provide a buffered microenvironment and thus allow cells to survive harsh conditions with various pH. The example presented show encapsulated cells were able to survive over a wider pH range than native cells (4 to 10 versus 6 to 8) [57].

1.3.3.1.6. Resistance to biotic stresses: effect of lyticases

Pathogen attacks are also important problems when using living organisms. For example, yeast cells are sensitive to lyticases that can break their cell walls. The synthesis of an organic/inorganic double-layered shell around the cells can increase the resistance of yeast cells against those harmful enzymes. More than 50% of the activity is maintained after 4 hours of incubation with enzymes, while only 10% survival is observed for native wild-type yeast [93].

1.3.3.2. New electrical properties

1.3.3.2.1. Conductivity

With the advent of bioelectronics, microorganisms are in the forefront. They are able to respond to certain changes in the environment with great precision, and their cellular metabolism involves oxidation-reduction reactions and therefore electron exchange. However, the organic material that makes up all the biological entities show very low conductivity. Introducing conductive materials on the cell wall would facilitate the development of

bioelectronics. The material would act as a link between the cells and the outside to control exchanges. Examples already mentioned have already shown the possibility to equip yeast cells with electrical conductivity properties by covering them with gold nanoparticles [82]. Coating cells with reduced graphene nanosheets offer in addition to conductivity, an electron exchange to the outside of the hybrid material opening the way to electricity production [83].

1.3.3.2.2. Magnetism

Controlling cell mobility is always an essential part of some biotechnology, especially in cell culture recycling and target cell therapy. The cell surface modification strategies are particularly interesting in this case because the experimenter has control over the chemical composition of this artificial shell. It is then easy to introduce magnetic particles that will drag the cells into their movements under certain effects. By binding Fe_3O_4 nanoparticles to the reduced graphene-covering yeast cells, it not only can provide electrical conductivity but also can provide them with movement under the effect of a magnet [83]. Another example was to incorporate Fe^{3+} ions with the tannic acid coating around the cells. The results proved the possibility of arranging the cells with different patterns using magnetic fields [94].

1.3.3.3. Control of cell behaviour via stimuli-sensitive material

The cells are already sensitive to their environments and show good adaptability following some stimuli. By playing on the properties of the encapsulating material, it is possible to create a shell that responds to stimuli as well. This “intelligent” coating can multiply their potentialities.

During biomineralisation processes, the conditions of the reactions are important and the pH is one of those parameters. The form of the reactive species and the equilibria occurring during the polymerisation processes are regulated by the various pH. So, a thin layer of crystallised octacalcium phosphate built through the mineralisation induced by LbL deposited polymers, prevents the division of yeast cells and thus provides control over the cell population. The addition of a small amount of hydrochloric acid dissolves this shell and then allows the cells to proliferate again [84]. This approach provides important control and an easy way to control cell behaviour using a common compound (HCl). Yeast coated by a silica coating built by the LbL of poly(methacrylic acid) based copolymers show behaviour dependent on the pH of the solution. Studies have shown that the growth of the encapsulated yeast population highly depended on the pH [95]. Surface engineering provides control over the cell population. pH variations are not the only parameter on which it is possible to work to create a sensitive shell.

Researchers have worked on the properties of disulphide bridges, these covalent bonds are quite easy to cut by reduction reaction. Cell surface engineered yeast with cross-linked LbL multilayers of poly(2-(dimethylamino)ethyl methacrylate-co-2-(pyridyl disulphide)ethyl methacrylate) (PDMAEM-co-PPDEM) and poly(methacrylate-co-2-mercaptoethyl methacrylate) (PMA-co-PMEM) shows a response to l-glutathione reduced (GSH). GSH addition leads to a breakage of the disulphide bonds, decomposition of the artificial wall and then the appearance of a cellular activity [61].

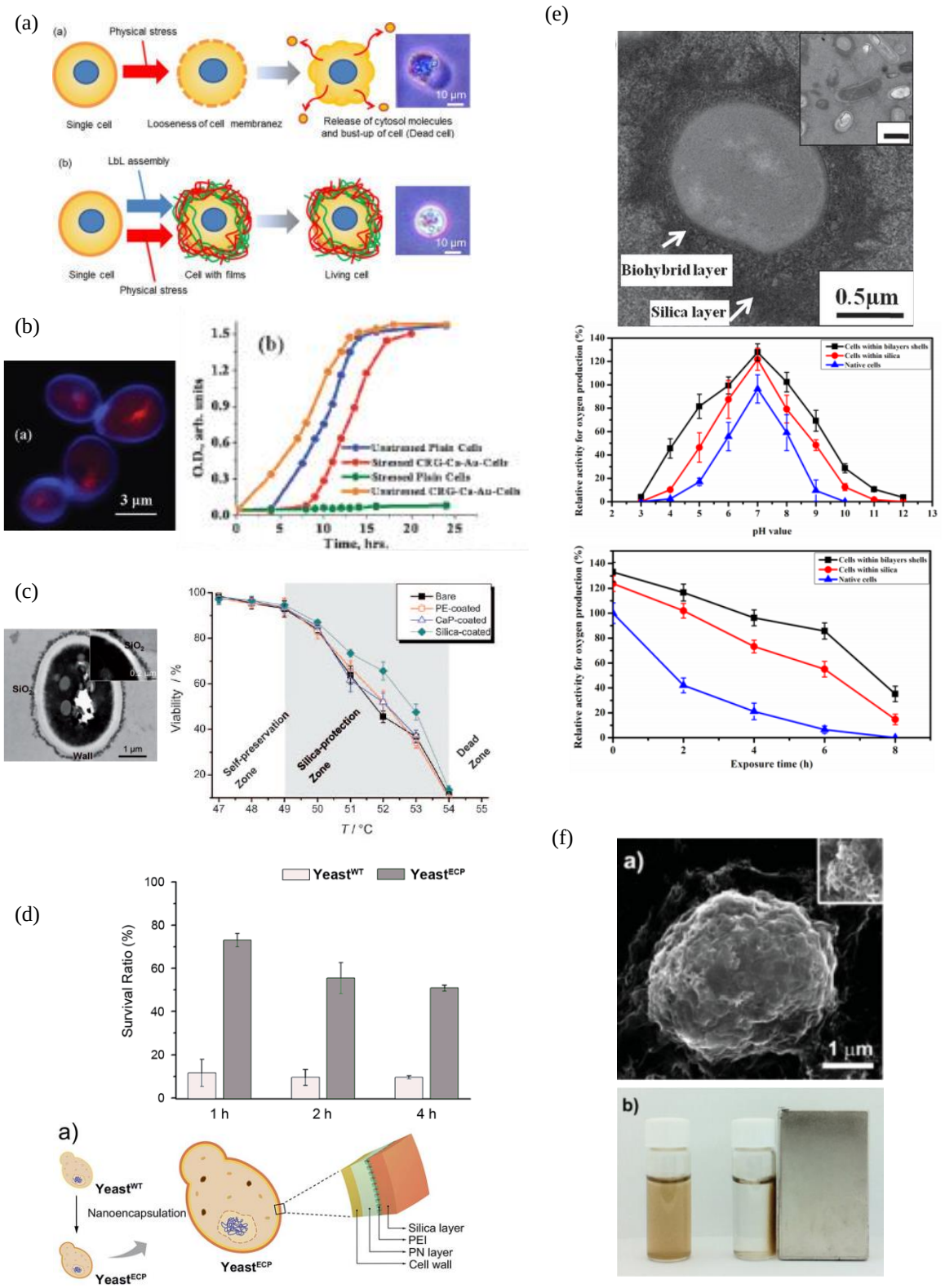


Figure I-10: (a) Resistance against centrifugation brings to yeast from LbL assembling of polyelectrolytes (adapted from reference [87]); (b) resistance to osmotic chocks brings to yeast from a graphene reduced nanosheet deposition (adapted from reference [88]); (c) thermotolerance bring by silica-coated yeast (adapted from reference [92]); (d) resistance to biotic aggression (lyticase) brings to yeast by an organic/inorganic double-layered shell (adapted from reference [93]); (e) photoprotection and pH variation resistance bring to cyanobacteria from a bilayered shell (adapted from reference [57]) and (f) new magnetic properties of yeast from Fe_3O_4 nanoparticles inserted in the graphene oxide nanosheet coating (adapted from reference [83])

1.3.4. Applications

The applications of living materials are as diverse as the microorganisms are numerous and powerful. However, individual encapsulation is quite recent (less than 20 years for the first individual immobilisation and less than 10 years as a full part research topic). In the following section we will attempt to present some relevant examples to demonstrate the power of cell surface engineering from labs to future larger scale applications.

1.3.4.1. Electricity production

Yeast coated with poly(l-lysine)/nanoparticles bilayer had already demonstrated their electrical conductivity. In this case, placing the yeast cells with a monolayer coating of Mg-Au nanoparticles between two electrodes as a bridge resulted in a non-linear current [96]. Even though, there is no actual current generation, this study presented the use of biological entities for the construction of nanoelectronic devices. However, the current generation of single cells is observed when they are immobilised on solid graphene aerogel electrodes decorated with platinum nanoparticles [97] or deposited on an organised microstructure [98] for an improvement of these microbial fuel cells (MFCs). Combining the progress made in individual encapsulation through nanoshell engineering and the improvement of MFCs seems therefore extremely promising.

1.3.4.2. Biocatalysis

Microorganisms have been used as biocatalytists for a long time. They have good stereoselectivity and conversion rate properties. By playing on the physico-chemical properties of artificial coatings, it would then be possible to improve the performance of the cells by providing external assistance (better affinity for the substrate, mass transfer improved, electron donors, etc.). Recent research has shown that the conversion rate of a reaction biocatalysed by polydopamine-coated yeast is significantly higher than one catalysed by native yeast. These microorganisms, protected by this shell, show a longer lifetime and therefore have a better recycling capacity [99].

1.3.4.3. Bioremediation

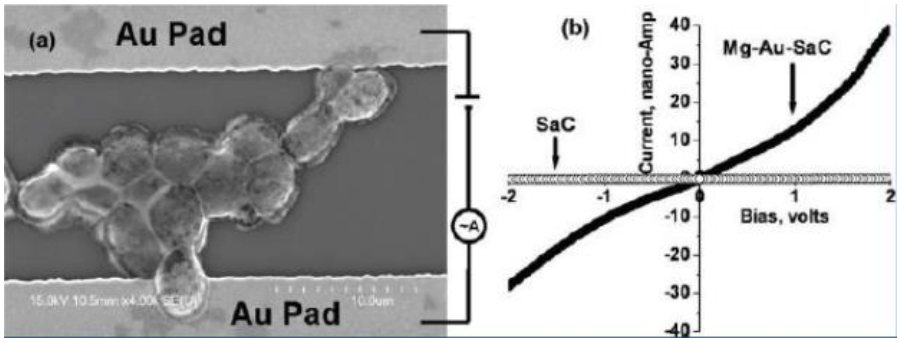
Microorganisms and biomass are starting points that are already widely used in wastewater treatment, such as working as biosorbents to remove toxic substances [100]. The construction of nanoshells around cells has also proved its worth in this area on a laboratory scale. The degradation of dibenzothiophene (DBT) by desulfurizing bacteria was tested and

compared to the cells encapsulated in titanium-based shells. The study shows that the hybrid shell does not disrupt cell metabolic activity and DBT degradation while protecting cells [101].

1.3.4.4. Medical applications

Living cells have been expected to be widely used in the medical field. Cellular therapy is an emerging and promising biotechnology thanks to advances in genetic engineering. However, many challenges remain. In this context, the creation of artificial surfaces around the cells makes it possible to play on their behaviour and function. For example, by making them invisible to the immune system in the case of transplantation. The first example selected is the research on cancer therapy. First, the researchers created liposome-like "nanocarriers". These synthetic particles can be stably attached to haematopoietic stem cells (HSC) without being harmful to their functions. HSCs decorated with the nanocarriers (loaded with glycogen synthase kinase-3 inhibitors) allow receiver animals to be reconstituted rapidly following bone marrow transplants without affecting the differentiation potential of these cells [102]. Delivering drugs is a particularly powerful point. Another therapy is to transplant cells capable of producing the necessary molecules themselves for the therapy. An example is the transplantation of insulin-producing cells for the cellular treatment of type 1 diabetes. However, these therapies are often limited by the immune system responses of patients. At present, this type of case is managed by the creation of microcapsules acting as a shield and encompassing several cells [103]. The use of individually coated cells could also lead to some progress. A recent article describes the upcoming trends in biotechnology and highlights the relevance of miniaturising these microgels to single cells [104].

(a)



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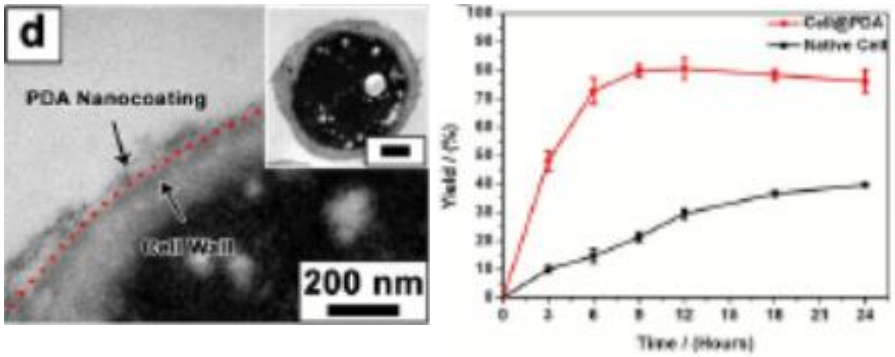

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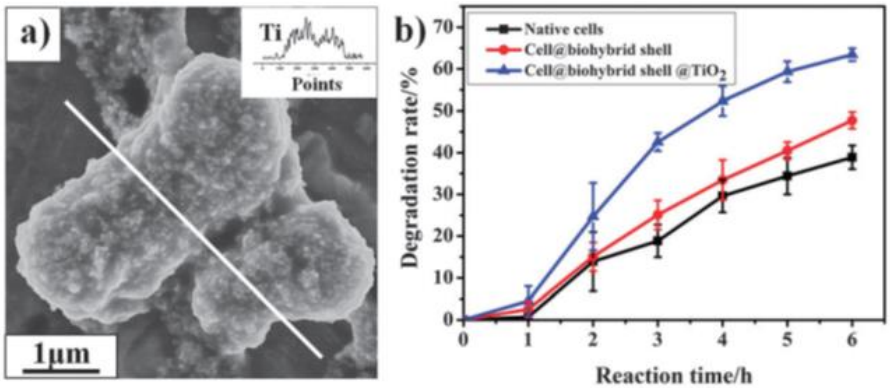

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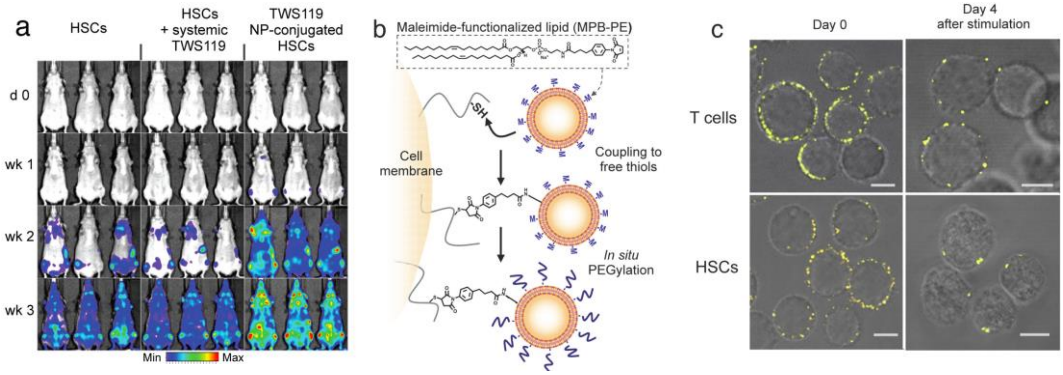

Figure I-11: Single-cell applications: (a) non-linear current response from the bridge between two pads made by yeast coated by Mg-Au nanoparticles (adapted from reference [96]); (b) biocatalysis performance of polydopamine nanocoated yeast (adapted from reference [99]); (c) degradation rate of desulfurizing bacteria encapsulated in a TiO_2-based nanoshell (adapted from reference [101]) and (d) illustration of HSCs carrying drugs reconstitute recipient animals with rapid kinetics (adapted from reference [102])

1.4. Challenges and future

Although good progresses have been made in surface engineering and therefore individual encapsulation, this field of research still needs to be further developed. The demands of performance and stability of new applications as well as the expectations of multifunctional purposes induce new strategies to be approached.

1.4.1. Deeper understanding of the bio-hybrid system

The behaviour of confined cells is not always easy to be predicted. They are subject to certain stresses when they are covered with an artificial coating. Understanding and studying these systems allows us to optimise the shells for cell encapsulation. Despite the numerous researches, it is still not easy to define the limitations related to the encapsulation of living organisms. Understanding and responding to these limitations is a challenge for researchers. This is how, in the case of macroencapsulation, protective additives were added to the silica gel that reducing then the stresses on the encapsulated cells and allow extension of the lifetime of the cells [105-107]. Same strategies have to be developed for individual cell encapsulation.

Studying the different interfaces found in living materials is also essential. First of all, there is the interfaces not only between the cell and the nanoshell, but also between the artificial material and the exterior. Defining favourable and unfavourable interactions is of great help in intervening and promoting those that help the survival of the cells while reducing harmful interactions. Each interface must be carefully controlled. For example, studies on plant cells immobilised in silica macrogels have shown that cell-matrix interactions must be controlled to avoid mineralisation of the cell wall. This mineralisation reduces nutrient bioavailability and reduces cell life. The addition of organic function has reduced direct interactions and has prolonged the biological activity of plant cells [108]. On the other hand, a case previously mentioned concerning yeast biocatalysis showed that the material is favouring contact between the cells and the substrate [99]. In this case, it is the interface between the exterior and the living material which is the key point of interest and that must be understood.

1.4.2. New strategies for the synthesis and improvement of nanomaterials

Although the sol-gel process is well known and allows the synthesis of a material under mild conditions, it is not a feasible option for all types of structures. Developing innovative synthesis methods is essential. These must be biocompatible and allow the maintenance of the vital functions of encapsulated microorganisms. Despite their synthesis conditions, materials

must not have a deleterious effect on the cells and their properties. The design of new nanostructures appeal to the inventiveness of scientists.

The creation of increasingly sophisticated materials (following the design of hybrid materials) is the future of nanoshell design. As described above, hybrid materials are numerous and classified into several categories, depending on their bonding between different components [51]. In the creation of nanostructures, it is possible to focus on various strategies. The ingenuity of adding and mixing additives in a basic structure around the cells will allow new advances and the creation of multifunctional materials. While the creation of a matrix with covalent bonds can be achieved by copolymerisation. The case of silica is particularly interesting since the appearance of organically modified silanes that makes possible to bring new properties to the material and its interfaces.

Finally, material engineering can also increase their diffusion properties. As the material is at the interface between the cells and the outside, it is directly involved in the exchanges (of energy, mass and information). Building complex, porous and hierarchised structures with defined properties (hydrophobicity/hydrophilicity, charge, affinity, electron availability, etc.) is a key point for the design of materials with high-performance.

1.4.3. Multifunctional technologies

One of the principles of obtaining a high-performance and resilient technology is its multifunctionalisation and then principles used for sustainable macrosystems [109] could be applied to the bio-hybrid microsystems. On the one hand, each compound of the hybrid material must have several functions (protection, functionalisation, performance improvement, electron donors, etc.). On the other hand, each function of our system must be fulfilled by several components to improve their effect. For example, this is how UV resistance is provided both components of the hybrid nanoshells *i.e.* polyelectrolyte layers and the silica who are UV absorbant for an improved protection but also the combination of the biocatalytic effect of the cells with the physicochemical properties of the material for enhanced catalysis. To address the challenges related to the need for long-term sustainability and new functions, the combination and positive interaction of all components of the bio-hybrid system need to be carefully designed.

1.4.4. Scale-up

Last but not least, the challenge is to exploit these technologies on a large scale. Building efficient production systems for individual cell encapsulation will be a challenge in

the coming years. It is therefore necessary to develop simple and efficient synthesis methods and to link them to innovative devices. In this way, emerging technology as microfluidics and its flexibility will probably have a predominant role in single-cell encapsulation. This technique allows, under very homogeneous conditions, to add components to the system step by step. This would allow the cells to be encapsulated under the flow path.

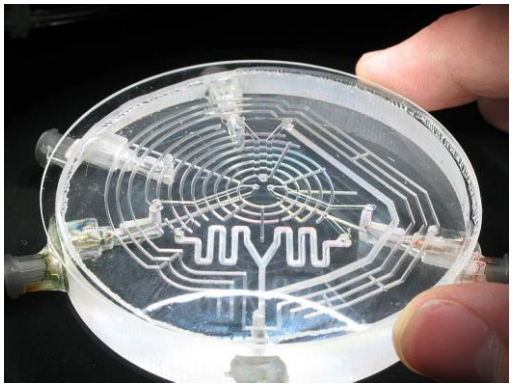

Figure I-12: Microfluidics system (adapted from Wikimedia Commons)

1.5. Afterword

The design of living materials by individual cell encapsulation is a multidisciplinary field. It is located at the interface between biology, chemistry, material science and engineering. It is a promising sector that proves the need for fundamental research and the close connections that have to be maintained with applied research to industries. Although this research has already made its way, its history has yet to be written.

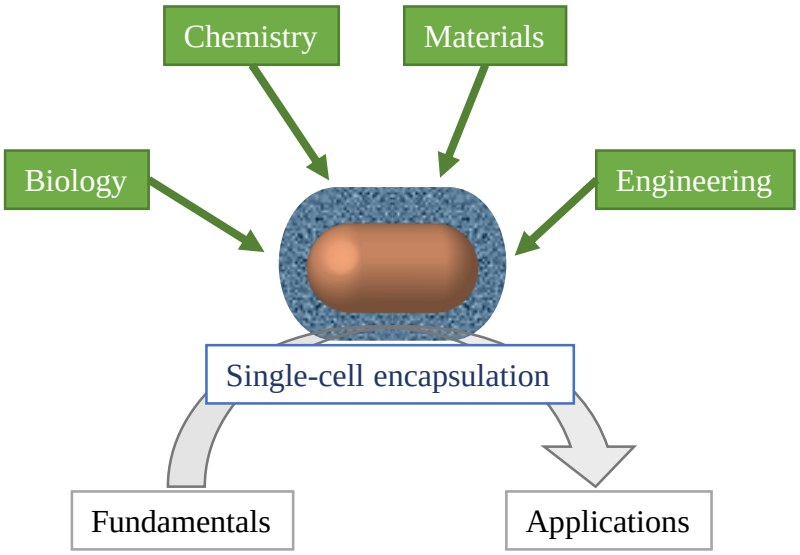

Figure I-13: Single-cell encapsulation challenges

Chapter 2 - Cyanobacteria and cell encapsulation

2.1. Cyanobacteria and their potential for the development of clean technologies

This research focuses on the use of cyanobacteria (Figure I-14) for the development of clean technologies. These photosynthetic prokaryotes are the basis for the design of biotechnology. It is also proposed that the first phototrophic organisms were anaerobic ancestors of cyanobacteria ("procyanobacteria") [110] that modified the Earth's atmosphere billions of years ago [111]. They are the smallest living entity capable of photosynthesis and their rapid growth rate, ease of use at different scales, high photosynthetic efficiency and easy and inexpensive culture make them ideal candidates for various uses. Cyanobacteria are then microorganisms with characteristics required for the design of high-performance technologies and have been used as powerful platforms for clean technologies [112]. Moreover, their cultivation does not compete with the use of arable land and uses many water resources (fresh water, seawater, waste water, etc.) [113].

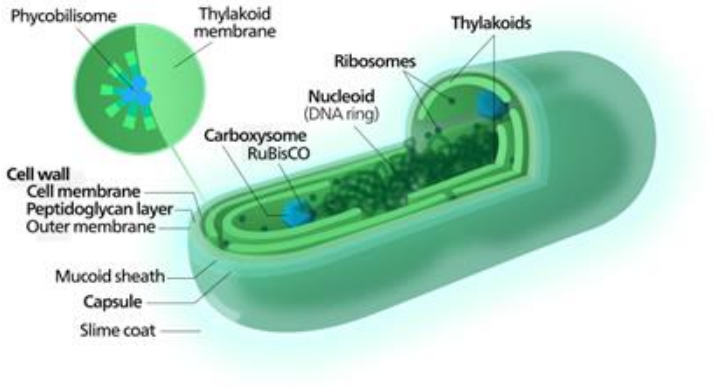

Figure I-14: Scheme of a cyanobacterium (adapted from Wikimedia Commons)

2.1.1. Chemicals production

The use of living photosynthetic organisms for the production of chemicals is a promising approach. Their photosynthetic abilities make it possible to convert light energy into chemical energy. It is thus possible to produce a wide range of molecules of interest for industry. Recent scientific research increase the knowledge about those powerful organism.

The development of genetic engineering tools that further develop the range of their potentialities [114]. Cyanobacteria are, in this way, considered as an industrial platform for the production of high value chemicals and biofuels through photosynthesis [115, 116].

2.1.1.1. Biofuels production

The use of cyanobacteria for the production of chemicals such as biofuels is the most exploited application of these photosynthetic prokaryotes. Several strains of cyanobacteria have even shown ability to produce dihydrogen [117]. This gas is considered as a green energy carrier. The production of ethanol by cyanobacteria is also well referenced [118]. For example, one of Algenol Biofuel Company's key products is ethanol produced in photobioreactors [119]. The exploitation of cyanobacteria for the production of various alkanes is also recognised [120]. Different processes implementing the use of different strains for the production of n-alkanes were performed by Joule Unlimited for their commercialisation [121]. The production of other chemicals such as fatty acids, other alcohols or ethylene is also investigated [112, 115, 116, 122].

2.1.1.2. Production of metabolites with high added values

A study conducted in the early 21st century identified marine cyanobacteria as a prolific source of useful natural products. By realising the effects of many natural chemical compounds present in cyanobacteria, the researchers highlighted the presence of anti-cancer drugs, antifungals, antibiotics, but also antivirals [123, 124]. Cyanobacteria therefore seem to be organisms of important interest for the production of metabolites with high added value .

The generation of these metabolites of highly added value can in addition be improved by genetic engineering, making cyanobacteria even more attractive for the production of such compounds [125].

2.1.2. Bioremediation

Wastewater treatment with the assistance of cyanobacteria targets several ways. The first is the degradation of organic pollutants. Thus, the degradation of various molecules such as phenols, aromatic polycyclic hydrocarbons or polychlorinated bisphenyls are attributed to different strains of cyanobacteria. Other examples include the species *Anabaena* that is capable of degrading 2,4,6-trinitrotoluene [126].

The second type of use is the recovery of toxic heavy metals from industrial wastewater. The treatment of this type of pollutant involves chemical or mechanical processes that are often

energy-intensive, expensive and time-consuming. The use of cyanobacterial bioadsorbent seems to be an effective and inexpensive method. The removal of these metal ions involves different mechanisms: adsorption on the surface of the cells, electrostatic interactions, ionic exchange with cations present on the surface, precipitation on the surface as well, complexation with available functional groups or even bioaccumulation [100]. The surface reactivity of cyanobacteria makes them the microbes of choice for this type of industrial wastewater remediation [127]. Various methods have also been considered to improve the recovery capacities of these ions by cyanobacteria, such as pre-treatment [128] or encapsulation of nonliving biomass [129].

2.1.3. Biosensing

The photosynthetic activity of cyanobacteria coupled with their ability to respond to certain stimuli makes them relevant candidates for biosensor design. It is reported that genetically modified cyanobacteria are able to respond to the presence of different herbicides with detectable changes in luminescence [130]. A change in luminescence is also observed in a modified strain of cyanobacteria following the addition of various toxic metals. It is thus possible to design biosensors for the determination of metal ions concentration of nickel, zinc or cobalt [131].

2.1.4. Electricity generation

Recently, the generation of electricity by devices involving the use of cyanobacteria has been highlighted. By coupling the electron generation of these organisms following photosynthesis with an electrode designed by materials engineering, it is possible to supply small electrical devices such as LED or a digital clock [132]. A schematic illustration of this biophotovoltaic hybrid cell is presented by Figure I-15.

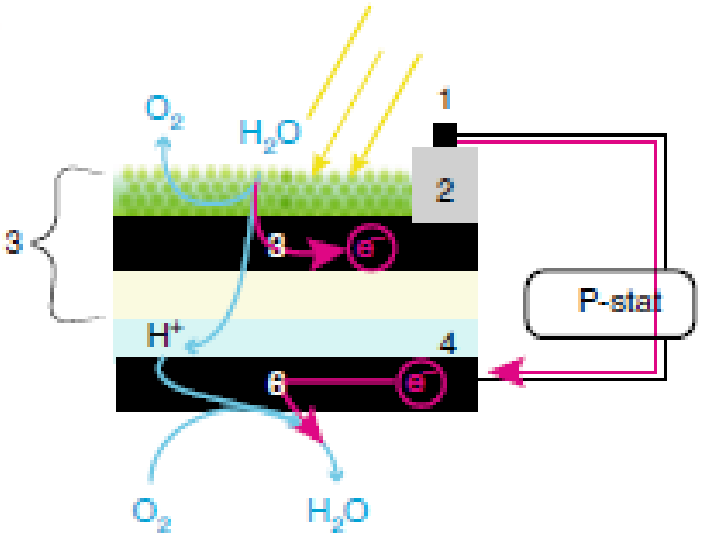

Figure I-15: Schematic illustration of the cross section of the biophotovoltaic cell where electrons, protons and oxygen flow are represented (adapted from reference [132])

2.2. Encapsulation of cyanobacteria

Despite their many qualities, the use of cyanobacteria suffers from several limitations. These living organisms are difficult to handle and they are sensitive to changes in their environments and external stresses, for example the influence of ultraviolet light limits their activity [133]. Some technical limitations related to their use in photobioreactors can decrease their productivity.

Encapsulation, as presented in the previous chapter (Chapter 1 – Introduction), makes it possible to overcome many constraints and improve the use of these living organisms. Entrapping cyanobacteria in abiotic material can facilitate their exploitation. Encapsulation provides containment and protection that can improve the efficiency of biotechnology based on these prokaryotes. It is recognised that bacteria are able to communicate via quorum sensing mechanisms. Their confinement within an artificial entity limits these communications and allows them to be used to direct the production through those encapsulated cyanobacteria [134, 135]. Limiting cell growth can direct cell activity towards the production of secondary metabolites as presented in the case of dihydrogen production using cyanobacteria [136]. Afterwards, cell density, a limiting factor in bioreactors, can be increased by encapsulating entities within abiotic matrices [40]. Finally, confining genetically modified organisms can also lead to greater safety and a reduced risk of release into the environment.

The first case of immobilisation of cyanobacteria dates back to 1983 and highlights a post-synthesis strategy of these organisms in polyurethane foams for dihydrogen photoproduction [137]. However, since this type of encapsulation suffers from some of the problems mentioned in the previous chapter, *in-situ* encapsulation has been preferred in more recent research.

2.2.1. Macroencapsulation

The example of encapsulation of cyanobacteria within monolithic gel is the most referenced. Taking as an example the methods developed at the end of the 20th century for the encapsulation of bacteria in silica gel [138], this research has shown promising results. It was about ten years later that researchers confirmed the preservation of cyanobacterial activity in this type of material for more than three months [139]. The pioneering research in our group is quickly followed by other researchers. Thus, the case presented in the previous section, concerning the production of dihydrogen by cyanobacteria encapsulated in silica matrices, has been published the following year (2009) [136]. A third research group is also interested in the design of this type of hybrid system where cyanobacteria are entrapped in protective abiotic matrices in the form of monoliths. This time, the material selected is magnesium phosphate cements instead of silica [140].

2.2.2. Microencapsulation

The microencapsulation of cyanobacteria is much more recent and poorly referenced. In addition, it only concerns individual encapsulation (cell by cell). In 2013, a Chinese group published a short communication highlighting the protection of cyanobacteria against significant light exposures inhibiting photosynthesis. The individual encapsulation follows a classic scheme presented in the previous chapter: an artificial biosilification induced by a polyelectrolyte layer deposition [90]. A complete protocol is then put in place by another Chinese research team whose research is published two years later. The construction of a similar system based on the modification of the interface between cells and silica material from cysteine amino acids is developed. The insertion of gold particles into the material provides additional protection for cyanobacteria against various stresses [57].

2.3. Strategies

For the entrapment of cyanobacteria within silica material, the strategies are relatively similar. Initially, macroencapsulation used the sol-gel pathway to obtain a silica matrix. Various precursors were studied, starting with silicon alkoxides, which were then replaced by the use of aqueous precursors whose synthesis is harmful to cyanobacteria. Supplementation with different additives has shown interesting effects on cell behaviour within materials.

This sol-gel strategy is modified for microencapsulation. Indeed, a layer-by-layer method allows the addition of organic molecules inducing the polymerisation of silica around the cells specifically. Among all the strategies presented in the previous chapter, it is an in-situ synthesis constructed following a LbL deposition that is highlighted in the works mentioned in the previous section.

2.4. Challenges

The use of cyanobacteria and the improvement of biotechnology are subject to many challenges. Cellular encapsulation is a promising way to address these concerns. The advent of bioelectronics and new types of biotechnology requires miniaturisation of systems. Strategies for the design of hybrid systems are subject to increasing constraints, homogeneity within the entire material is also required. Understanding changes in cell behaviour within abiotic matrices would allow some modulability, for example in their production capacity or selectivity. Understanding and modifying the interfaces within these hybrid systems are also a major challenge related to cyanobacteria encapsulation. The complexity of the cell wall and its extreme surface area are the starting point for much research. Modifying this exterior also provides additional properties to the cells as discussed in the previous chapter. Research related to the encapsulation of cyanobacteria has shown that it is possible to improve the capacity of these prokaryotes, but none yet presents the addition of new properties.

Based on these facts, it is clear that research has focused more recently on the individual encapsulation that manages many of these challenges. This type of research is at the centre of the investigation presented in this manuscript with innovative approaches to the design of this system.

2.5. The natural exterior of cyanobacteria, a starting point for individual encapsulation

To build our system, it is necessary to know the exterior surface composition of cyanobacteria. It is documented that the envelope of these cells is composed of three distinct parts: the cytoplasmic membrane, a layer of peptidoglycan and the outer membrane [141]. Cyanobacteria are therefore considered to be gram-negative bacteria. Nevertheless, despite the required characteristics of gram-negative bacteria, cyanobacteria have a thicker peptidoglycans layer than other bacteria: around 10 nm for thicknesses usually measured at 5 nm [142].

The outer membrane is the part of interest since its chemical composition will be the starting point for surface engineering via a cellular encapsulation process. It is composed of lipopolysaccharides (LPS). Studies on different strains of cyanobacteria have highlighted the chemical composition of these LPS (Figure I-16) [143, 144].

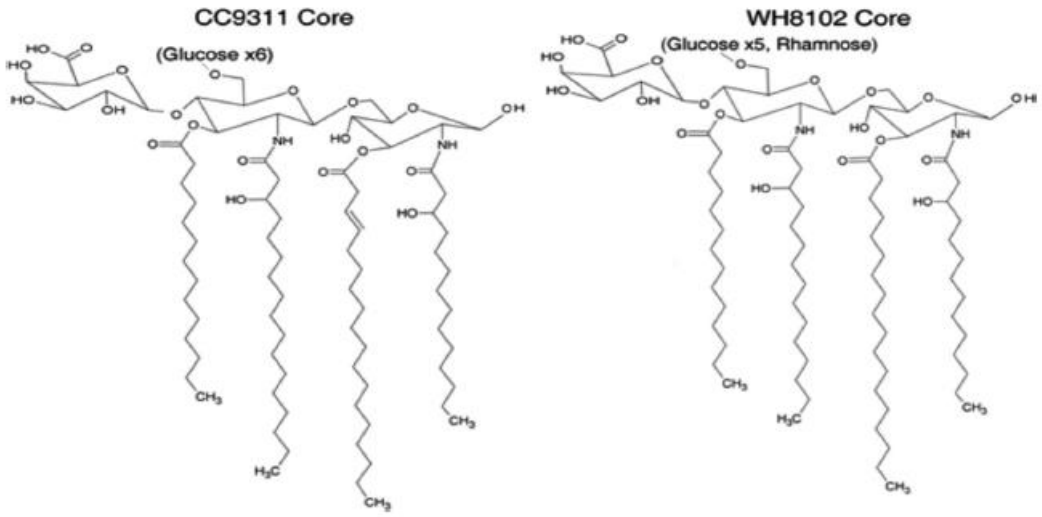

Figure I-16: Putative structure of *Synechococcus* minimal core LPS (adapted from reference [143])

Nevertheless, other studies highlight the presence of an additional outer layer called the s-layer (surface layer), whose chemical composition seems, even at the present time, not to be precisely known [145]. It is composed of a relatively crystalline structure of proteins and glycoproteins [146].

Chapter 3 – Objectives and strategies

3.1. Objectives

Facing to the current environmental and societal challenges, this work aims to synthesise bio-hybrid system composed of individual cells in an abiotic shell towards the development of sustainable technologies.

The main objective of this work is to design hybrid materials for individual encapsulation of whole active cyanobacteria. These bio-hybrid systems could show good performance in terms of long-term viability of encapsulated cells, enhanced cell resistance to certain stresses, improved performances as well as multifunction. The synthesis route needs to be approached carefully to avoid harmful effects on the cell survival, while the introduced nanoshell should have defined properties for the functionalisation of the encapsulated cells.

In order to achieve this objective, it is necessary to take into account several additional points which will be detailed below.

3.1.1. Design of encapsulation methods

The design and operation of encapsulation methods compatible with the viability and activity of cells is an important part of this work. In addition to being non-harmful to living cells, the design approach should direct the creation of nanoshells around individual cells.

3.1.2. Tuneable material properties

Synthesising the materials with controlled and various properties is an essential point in the design of nanoshells. For this reason, hybrid materials are appropriated candidates to be nanoshells for cell encapsulation. Combining inorganic and organic materials is a promising approach to be used in this research area.

3.1.3. Studies and control of interfaces

The construction of the shell is based on the interaction appearing in each of the interfaces containing cell-material interface and material-material interface. The formation of cell-material interface starts with the direct contact of the outer membrane and the material via chemicals or physical links. Care must be taken not to change the permeability of this membrane by generating those links. In addition to building these artificial interfaces, understanding the generated interactions is highly necessary.

3.1.4. Modification and functionalisation of the outer cell surface

In order to facilitate the cell manipulation, cell surface needs to be modified and engineered with proper properties or structures. Cell surface engineering makes it possible to create multifunctional system by bringing new properties to cells and allows additional manipulation of cells through material synthesis.

3.2. Scientific strategy

Several choices play an important role to achieve the objectives of this work. It is therefore necessary to be cautious to choose of living cells on the basis of the bio-hybrid system, the encapsulation methods and the components of the material.

3.2.1. Choice of the biological component

During this work, cyanobacteria of the species *Synechococcus sp.* PCC[2] 7002 (PCC7002) were selected. This is a well-known model for studies about photosynthesis or cyanobacterial metabolism and has great potential for biotechnological applications. They have all the characteristics required for laboratory use and carry all the necessary attributions for industrial purposes: rapid growth rates (with a duration of division around 3.7 h and a growth rate around 0.34 d^{-1} during the phase of growth [147]), high tolerances (salts, temperature and light) and no toxicity [148, 149]. Genetic engineering tools are known and used to make this cell strain even more versatile and efficient [149, 150]. The production of soluble sugar [151], aromatic carotenoids [152] or extracellular type-I cellulose [153] are attributed to wild or genetically modified PCC7002. While it is capable of degrading organic pollutants [154] or biosorbing certain heavy metals [127].

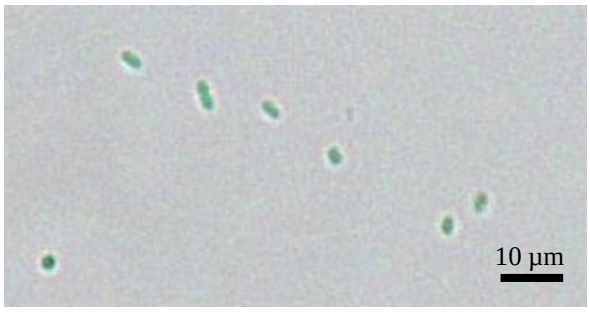

Figure I-17: Micrographs of native *Synechoccocus sp.* PCC 7002

[2] Pasteur Culture collection of Cyanobacteria

These bacteria are gram-negative types, with a size of about 0.8 µm and 1.6 µm, their cell enveloppe is complex and composed of several layers (cytoplasmic membrane, peptidoglycan layer and outer membrane). The outer wall is composed mainly of lipopolysaccharide with the decorations of proteins, lipids and carotenoids [141]. Its surface has been studied and shown to display a net negative charge (zeta potential measurements). In this study, its surface reactivity is attributed to the presence on the surface of carboxyl groups, phosphoryl groups, amine groups and hydroxyl groups [127]. This could be the starting point for the design of an efficient encapsulation method.

3.2.2. Choice of method

The encapsulation will be carried out *in-situ*. Layer-by-layer method is mainly used in the individual cell encapsulation processes. It provides the necessary control of the interfaces between the biological entity, the nanoshell and the external environment. This LbL method was proposed in the second middle of the 20th century. It is referenced in the Iler's work firstly [155]. Recently it has been used widely for the design of films [77], microcapsules [78] and single-cell encapsulation [74].

This technique will make it possible to deposit charged layers around cyanobacteria PCC7002 using their negative charged surface as substrate. It is then possible to introduce alternate charged components to build a stable coating. The layer-by-layer technique also allows full control, the composition of the shell offering additional functions to the PCC7002 through the introduction of the well-defined components.

3.2.3. Choice of components

The shell will be made of a hybrid material: an organic component will provide the shell with malleability and multifunctionality while an inorganic component offers chemical stability and mechanical resistance. Both types of hybridity will be used. First, an organic component will be entrapped in an inorganic matrix (Parts II and III of this work), while covalent bonds will be created between the two components, which will be discussed in the last part of the results of this work (Part IV). The strategies are described in the Figure I-18.

3.2.3.1. Inorganic component

The inorganic matrix will be made of silica. As mentioned above (Section 1.2.2.1. An overview), silica is a very good candidate for cell encapsulation. This material shows very good mechanical resistance and chemical stability. It is transparent to visible light (essential for photosynthetic organisms) and easy to be synthesised under mild conditions. In addition, it is

found in all kingdoms of the living world and has a protective action (improvement of physical properties, thermotolerance, resistance to microbial attacks, etc.).

3.2.3.2. Organic component

3.2.3.2.1. Polyelectrolytes

The first method to insert an organic part into the silica-based material will be layer deposition. By playing on the defined properties (net charge of working pH and diverse function) of the polymers, it will be possible to induce and direct the deposition of silica around the cells. This bioinspired mechanism mimics the biosilification process occurring in diatoms and the role of sillafins and long-chain polyamines.

3.2.3.2.2. Organically modified silanes

The second way to introduce an organic component into the silica matrix will be to use trimethoxysilanes with an organic group grafted in the fourth position. By inserting these silica monomers into the bulk network, a hybrid material will be obtained. In this case, a covalent bond will be created between the two components of the hybrid material.

3.2.4. Synthetic pathway

The silica material will be obtained from precursors that polymerise according to the sol-gel pathway (see Section 1.2.2.2. Sol-gel: a safe-way for safe encapsulation). A network containing all the other components of bio-hybrid materials (whole-cell and organic part) will be obtained first using the method. Subsequently, additional organic groups will be introduced by copolymerisation implying the design of ormosils.

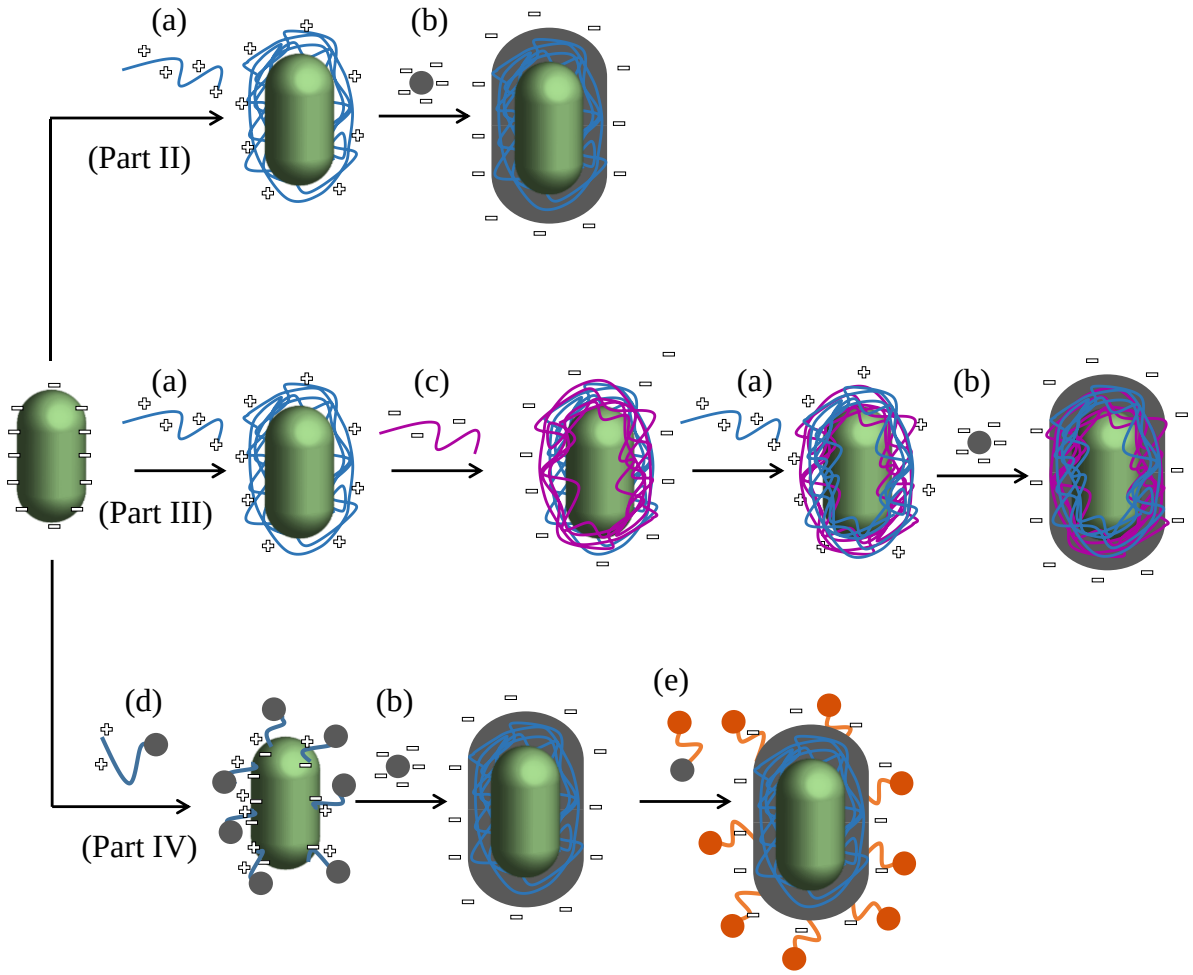

Figure I-18: Strategies for the design of bio-hybrid systems: step by step (LbL process) deposition on native cyanobacterium following the addition of (a) polycation; (b) silica precursor; (c) polyanion; (d) cationic organosilane or (e) functional organosilane

Chapter 4 – Cell culture conditions

Cyanobacteria are photoautotrophic prokaryotes. For their cultivation, it is necessary to place them in optimal conditions for their development. These conditions mimic their living natural environment. The selected species (*Synechococcus sp.* PCC 7002) was isolated in the 1960s by Professor Van Baalen's team [156]. It was collected around Magueyes Island in Puerto Rico (U.S.) and is now part of the Pasteur Institute's collection (Pasteur Culture collection of Cyanobacteria (PCC)). The culture conditions are those provided by the institute [157]. The cultures were provided by the Pasteur Institute (Paris, France)[3].

4.1. Culture method

Liquid strain cultures are maintained at room temperature under visible light (cool white: Omsram lamps type L18W-840) and slow agitation (70 rpm). The cells are transferred according to a monthly routine. The mother cultures are diluted six times in fresh medium (mix of BG-11:ASN-III with a volume ratio of 1:1) as presented in Figure I-19.

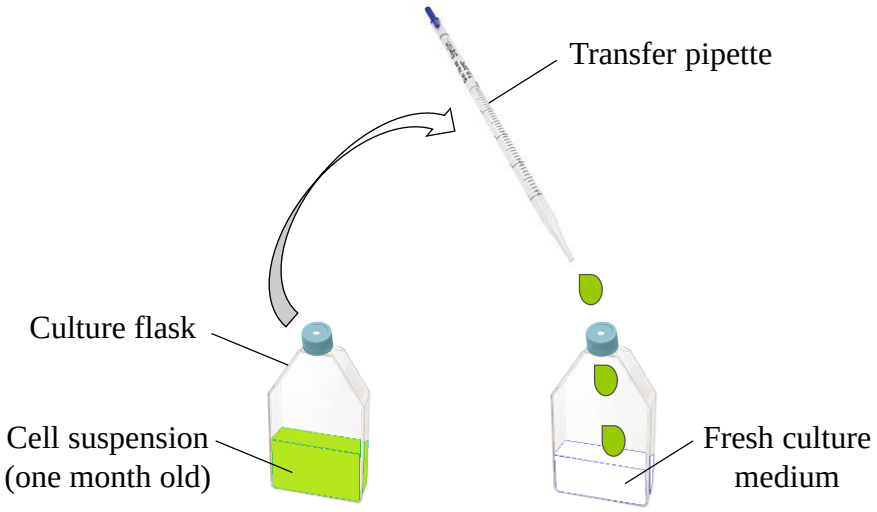

Figure I-19: Diagram related to the cell transfer protocol

4.2. Composition of culture media

Culture media are saline aqueous solution with the specific compositions which are listed below. After preparation, these media are adjusted to pH 7.4 using a 0.1 M HCl solution and sterilised by autoclaving. The ASN-III medium is then supplemented with vitamin B12 (10 $\mu g.l^{-1}$). For the preparation of the media, see the following tables.

[3] Institut Pasteur, 25-28 Rue du Dr Roux, 75015 Paris, France

Table I-1: BG-11 composition

Ingredient	Concentration	
	g. L^{-1}	mM
$NaNO_3$	1.5	17.67
K_2HPO_4. $3H_2O$	0.04	0.18
$MgSO_4$. $7H_2O$	0.075	0.30
$CaCl_2$. $2H_2O$	0.036	0.25
Citric acid	0.006	0.029
Ferric ammonium citrate	0.006	0.030
EDTA K_2 Mg. $2H_2O$	0.001	0.0024
Na_2CO_3	0.04	0.38
Trace Metal (see A5+Co)	Add 1 mL	
Deionised water	To 1 L	

Table I-2: ASN-III composition

Ingredient	Concentration	
	g. L^{-1}	mM
NaCl	25.0	428
$MgSO_4$. $7H_2O$	3.5	14.2
$MgCl_2$. $6H_2O$	2.0	9.8
KCl	0.5	6.7
$CaCl_2$. $2H_2O$	0.5	3.4
$NaNO_3$	0.75	8.8
K_2HPO_4. $3H_2O$	0.02	0.09
Citric acid	0.003	0.014
Ferric ammonium citrate	0.003	0.015
EDTA K_2 Mg. $2H_2O$	0.0005	0.0012
Na_2CO_3	0.04	0.38
Trace Metal (see A5+Co)	Add 1 mL	
Deionised water	To 1 L	

Table I-3: Composition of Trace metal A5+Co

Ingredient	Concentration
	g. L^{-1}
H_3BO_3	2.86
$MnCl_2$. $4H_2O$	1.81
$ZnSO_4$. $7H_2O$	0.222
Na_2MoO_4. $2H_2O$	0.390
$CuSO_4$. $5H_2O$	0.079
$Co(NO_3)_2$. $6H_2O$	0.0494
Deionised water	To 1 L

Chapter 5 – Characterisation techniques

Many analytical techniques are required to confirm the synthesis of the material, to provide the information of the properties of the materials and finally to monitor the activity of cells encapsulated within these nanoshells.

5.1. Material analysis

5.1.1. Electron microscopies

Electron microscopies allow the observation resolutions to reach in the nanometre range, which is essential for the studies of materials. Both scanning and transmission electron microscopies are used for their complementary information. Electron microscopy is based on the interaction between electrons and matter as described by the Figure I-20 [158].

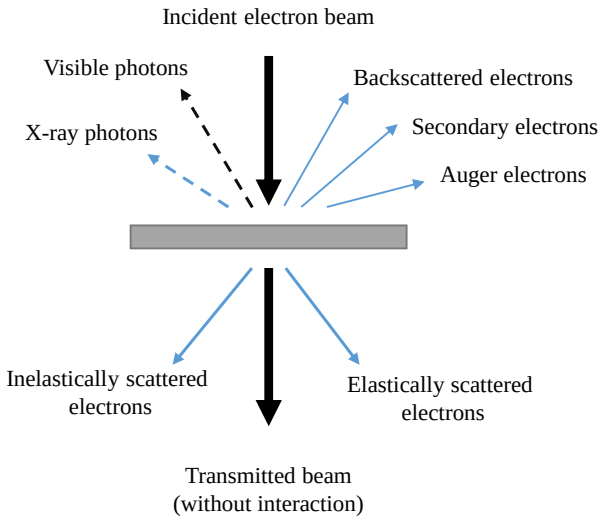

Figure I-20: The types of signal generated by the electron beam-sample interaction

5.1.1.1. Scanning electron microscopy (SEM)

Scanning microscopy provides information on the textural properties of materials. The principle of this analysis is to scan the surface of the sample point by point via an electron beam, and the electrons ensuing the interaction between the electron beam and the matter are collected by a detector. After computer manipulation, an enlarged image of the analysed surface is obtained.

Samples preparation. The synthesised samples are not compatible with the ultra-high vacuum required for electron microscopy analysis. Drying is necessary and carried out as follows: preliminary dehydration is carried out following successive baths with a concentration gradient

(25-100% ethanol) before undergoing a supercritical drying after ethanol-carbon dioxide exchange using a Leica EM CPD030. These materials show low conductivity, so it is necessary to cover them with a thin conductive layer which can lead to a higher secondary electron yield and the removal of problems related to charge accumulations for a better analysis. A thin layer of gold (10 nm) is sputtered onto the samples previously stuck to an aluminium disc using double-sided carbon tape.

Observations. The micrographs produced and presented in this work are obtained using two microscopes from JEOL: 7500-F and 6010LV microscopes with an acceleration potential of 15 kV.

5.1.1.2. Transmission electron microscopy (TEM)

In addition to the surface observations made using the SEM, the TEM provides transverse images of the samples. Based on the absorption of the incident ray by a material, ultra-fine cuts of the samples are observed. In this case, the images are reconstructed from the electrons transmitted through the samples. Once enlarged and focused, the images allow to study the thickness of the coating, the integrity of the cells as well as the structure of the cell-material interface. In this case, the samples also need to be pre-prepared.

Samples preparation. Biological samples are fixed with a 2.5% glutaraldehyde solution in a sodium cacodylate buffer (0.1 M; pH 7.4) and incubated overnight at 4°C. The samples are then rinsed with the buffer (0.2 M cacodylate, pH 7.4) and post-fixed in a 1% OsO_4 solution for another night. The samples are then washed with the cacodylate solution and dehydrated in successive baths of ethanol solution (at a concentration gradient of 25-100% ethanol). Dehydration is followed by washing with pure propylene oxide before the samples are coated with LX112 epoxy resin. The ultrathin cuts are made with an ultra-microtome III (from LKB) and are finally contrasted with uranyl acetate and lead citrate.

Nanoparticle material is dried at 40°C under the ambient atmosphere. They are then resuspended in ethanol before being deposited on copper grid covered with polymer film.

Observations. The observations are made with an acceleration voltage of 80 kV on Philips Tecnai 10 microscope.

5.1.2. Energy-dispersive X-ray spectroscopy (EDX)

The general chemical composition of the material can be studied by EDX. Following the illumination of the sample by an electron beam, several signals are generated, including X-

rays. This radiation is then collected by a Si(Li) semiconductor. The signal is amplified and transformed to obtain an X-ray spectrum. The elements found in the sample are identified by their energy peak and the quantitative analysis by the number of peaks.

Observations/analysis. A mapping of the chemical elements present in the sample is obtained using an EDX device coupled to the SEM JEOL 7500-F.

5.1.3. X-ray photoelectron spectroscopy (XPS)

Analysing the chemical composition at the extreme surface of the samples is essential in the context of individual encapsulation. XPS provides information on the bio-hybrid material/external environment interface. This technique is based on a measurement of the kinetic energy of electrons ejected from the material under the effect of monochromatic electromagnetic radiation X (photoelectric effect). This analysis only concerns the surface (maximum of 10 nm depth) of the sample because of the limited kinetic energies of the ejected electrons.

Analysis. X-ray photoelectron spectroscopy (XPS) is performed to determine the external chemical composition of bio-hybrid systems. Spectroscope Escalab 250 Xi (Thermo Scientific) is used for the analysis. X-rays are produced from a magnesium anode (Kα ray; hν = 1253.6 eV). Measurements are made at room temperature and under reduced pressure.

5.1.4. Nitrogen physisorption

The textural properties of hybrid materials are studied using gas adsorption/desorption. This technique provides a lot of important information: the specific surface area, the pore volume as well as the pore size. In this work, the properties of materials without biological material are studied. The reasons for this are that the handling of biological materials leads to several constraints, including very small amounts of samples to be analysed. Nevertheless, knowing their textural properties is essential to understand how to analyse the exchanges that occur through the nanoshell once synthesised.

The strategy put in place to obtain the desired information is to measure the amount of nitrogen adsorbed by the material at the boiling temperature of liquid nitrogen (-196°C) as a function of the pressure applied, measured at equilibrium. The results are first obtained in the form of an isotherm, the appearance of which is characteristic of the textural properties of the material. IUPAC classifications [159] gather them into 6 categories according to their shapes, which make it possible to assess the porosity of the materials discussed below (Figure I-21).

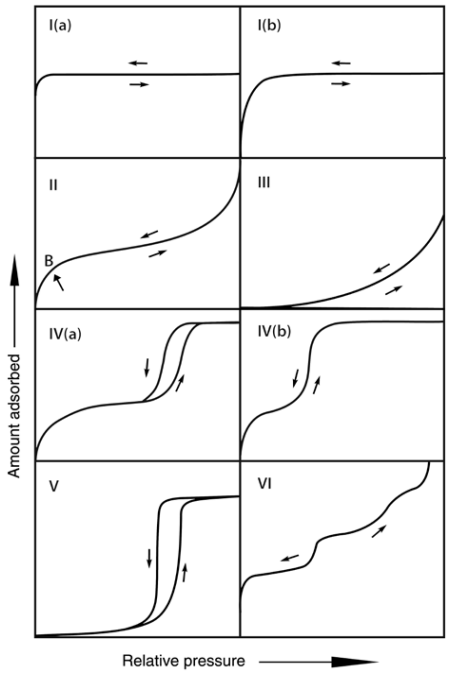

Figure I-21: Classification of isotherms according to IUPAC (adapted from reference [159])

Reversible Type I isotherms are the typical isotherm of microporous solids which have relatively small external surfaces. Reversible Type II isotherms are given by the physisorption of most gases on nonporous or macroporous adsorbents. Type III isotherm corresponds to macroporous adsorbents. Type IV isotherms are related to mesoporous adsorbents. Type V isotherms are observed for adsorption microporous and mesoporous adsorbents (during adsorption of water on hydrophobic adsorbents). The reversible isotherm of type VI is representative of the layer-by-layer adsorption on a uniform and non-porous surface.

Solid porous adsorbents are thus classified according to the diameter of their pore (d_{pores}): microporous (d_{pores}<2 nm); mesoporous (2<d_{pores}<50 nm) and macroporous (d_{pores}>50 nm).

The specific surface area can then be calculated from the isotherms using the Brunauer-Emmet-Teller equation (BET) [160], while the BJH method [161] (named after its authors: Barret, Joyner and Halenda) provides the mesopore size distribution.

Samples preparation. Nanoparticles are dried under the ambient atmosphere and at 40°C before analysis.

Analysis. The measurements are made using a TriStar 3000 analyser (Micromeretics). Analysis is performed after drying under vacuum (70 millitors) and low heating (90°C) overnight. The surface area is calculated within the BET method (0.05-0.30 p/p_0 range) and the pore size distribution is obtained from the desorption isotherm using the BJH model.

5.1.5. Silicon-29 solid-state nuclear magnetic resonance (^{29}Si NMR)

The properties of synthesised hybrid materials are also studied by nuclear magnetic resonance. It reveals the chemical composition of silica-based and functionalised materials.

NMR spectroscopy is based on the degeneration of the energy levels of nuclei under the effect of a magnetic field (B_0), called Zeeman interactions. These transitions can then be induced between these levels when another magnetic field perpendicular to the first one is added at a controlled frequency (radio frequency). This external magnetic field (B) is locally modulated by the electronic environment of the nuclei and induces a resonance frequency (ν) specific to each isotope:

$$\nu=(\gamma/2\pi).B \quad (1)$$

Where γ represents the gyromagnetic ratio specific to each isotope, the technique can therefore be regulated on a particular element.

^{29}Si NMR must be adapted because of dipole interactions between nuclear spins, so the spectra are acquired while the sample is rotated at the magic angle (54.74°). This magical angle spinning (MAS) thus cancels out these interactions and provides the clearest signal.

Samples preparation. Nanoparticles are dried under the ambient atmosphere and at 40°C and crushed with a pestle before analysis.

Analysis. Direct Excitation (DE) Magic Angle Spinning (MAS) NMR spectra are recorded on a Bruker Avance-500 spectrometer running at 11.7 T at 8.000 Hz spinning with a 4 mm Bruker probe.

5.2. Viability of cyanobacteria

It is essential to monitor the biological activity of the materials and thus to obtain information on the viability and the behaviour of the cells encapsulated within the nanoshell.

5.2.1. Oximetry

The first way to confirm the viability of photosynthetic organisms is to monitor their oxygen production which can be correlated to their bioenergetic process. This information can be provided using oximetry (or Clark's oxygen sensor). This device (scheme on Figure I-22) is a hydrodynamic voltammetry where a constant potential difference (700 mV) is maintained between two electrodes [162].

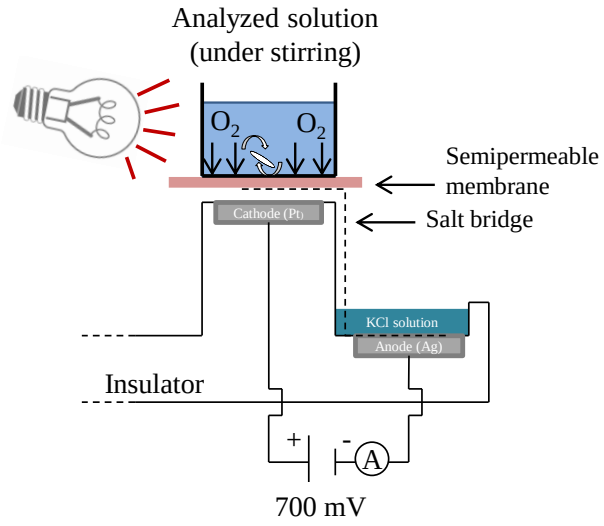

Figure I-22: Representation of the oximeter used during this work

The cathode is a platinum disc and the working electrode is an Ag-AgCl anode. A thin oxygen-permeable membrane (polytetrafluoroethylene) separates the electrochemical cell from the analysed solution. The oxygen present in solution then is reduced on the surface of the cathode, resulting in the generation of an electric current. The reactions are the followed:

On the cathode: $O_2 + H_2O + 4\ e^- \rightarrow 4\ OH^-$

On the anode: $4\ Cl^- + 4\ Ag \rightarrow 4\ AgCl + 4\ e^-$

$$4\ Ag + O_2 + 2\ H_2O + 4\ Cl^- \rightarrow 4\ AgCl + 4\ OH^-$$

After calibration (constant stirring, given temperature, etc.), the measured current is directly proportional to the dissolved oxygen concentration. It is thus possible to monitor its evolution over a given period of time.

Analysis. In a thermostatised reaction chamber (20°C), 1 mL of the solution is added. 10 µL of $NaHCO_3$ (0.6M) is supplemented to prevent the limitation of photosynthesis by insufficient dissolved CO_2. After degassing with gaseous nitrogen (up to an O_2 concentration of 0.100 µmol/ml), the chamber is put in the dark to exclude fluctuations related to natural light. The measurements were acquired under slow agitation (80 rpm) and under an artificial light (λ = 650 nm, 1200 $\mu mol.m^{-2}.s^{-1}$) to stimulate photosynthesis. The device used is an Oxy-Lab manufactured by Hansatech Instruments.

5.2.2. Fluorescence optical microscopy

Direct observation of the samples can be performed by optical microscopy. In bright field, this technique provides therefore information on its colour, shape, size, etc. Unfortunately, in our case, cyanobacterium is too small species for precise observation and only appears as a point. Therefore, the fluorescence properties will mainly be exploited to confirm the viability of the cells.

The fluorochromes observed are, on the one hand, chlorophyll which is naturally present in cyanobacteria and has an emission in red under excitation light and, on the other hand, fluorescein diacetate (FDA). The cell wall is permeable to this second molecule that is a substrate for the enzymatic activity of the cells. Thus, it provides dual information about first the enzymatic activity and, second, the integrity of the cell wall. After hydrolysis by intracellular esterases, FDA will be oxidised to be fluorescent and confined within the cytoplasm. The fluorescence can be observed as the green light under light excitation.

5.2.2.1. Bright field and cell counting

A cell count is carried out in the bright field using a Neubauer counting chamber. Thus the concentration of the cell suspension can be determined. With the precise volume of the chamber, mathematical manipulation makes it possible to obtain the cell concentration.

Analysis. Observations are made on a Nikon Multizoom Microscope AZ100. 500 µL of culture samples are added to the chamber. Ten measurements (ten different squares) are made per sample and the cell concentration is obtained using the following equation:

$$\text{Cell concentration (cells/mL)} = (\text{total number of cells/number of squares}).4.10^6 \quad (2)$$

5.2.2.2. FDA assays

Analysis. FDA is added to the cell suspension with the final concentration of 5 mM and incubated with the samples for 30 minutes. After three rinsing with distilled water, 500 µL of the culture sample is dropped on a microscope slide and spread out under cover glass.

Nikon Multizoom Microscope AZ100 was used to obtain fluorescent images. Observations are performed at 536/40 nm with a colour camera (DSRi, Nikon) by illuminating the samples with excitation lights of 482/35 nm.

5.2.3. Spectroscopy UV-visible

Cyanobacteria are very small microorganisms, it is quite difficult to monitor the evolution of biomass. It is therefore necessary to develop other alternatives to follow cell

culture activity. Optical density is a fast and accurate method of obtaining this type of information. It is generally measured at 730 nm for cyanobacteria culture [163]. This wavelength makes it possible to avoid the absorption of photosynthetic pigments and to base these studies on the evolution of turbidity in solution due to the cellular population.

This technique is based on Beer-Lambert's law, which allows the absorbance of a sample to be linked to a given wavelength and its concentration. The absorbance (A) is thus directly proportional to the concentration (C, unit = $mol.L^{-1}$) moderate by the effect of the molar extinction coefficient ($\boldsymbol{\varepsilon}$, unit = $L.mol^{-1}.cm^{-1}$) and the distance travelled by the light beam (l, unit = cm)

$$A = \boldsymbol{\varepsilon}.C.l \quad (3)$$

UV-visible spectroscopy provides then a light absorption profile for each sample.

It is important to note that, when operating at the limit of the visible, which is the case of 730 nm, another phenomenon appears. This is light scattering. In this case, it is the diffusion coefficient (*D*) that makes the transmittance dependent on the amount of species in presence.

Analysis. 700 µL of the solution is analysed after being placed in suitable cuvettes (l = 1 cm). Spectra are obtained for wavelengths from 400 nm to 900 nm on Lambda 35 UV/VIS Spectrometer from Perkin Elmer Instruments.

5.2.4. Relationship between cell density and optical density

Optical density is an easier measurement to implement, so it is necessary to establish the linear relationship between optical density and cell concentration. These measurements are dependent on the optical properties of the cells (related to their size, among other things) which vary according to certain parameters such as the age of the culture. A linear relationship is obtained in each case, but varies for each of the experience sets. Measurements of optical density at 730 nm (OD730) of native PCC7002 cultures are related to the cell concentration measured using the Neubauer measuring chamber (Figure I-23).

The mathematical analysis of this relationship provides a line with a good correlation coefficient (R=0.94). Following this specific set of analysis, a mathematical relationship can thus be applied to switch from one measurement to another valuable in a wide range of absorbance:

$$\text{Cell concentration } (10^6 \text{ cells/mL}) = (OD730 + 0.21)/0.047 \quad (4)$$

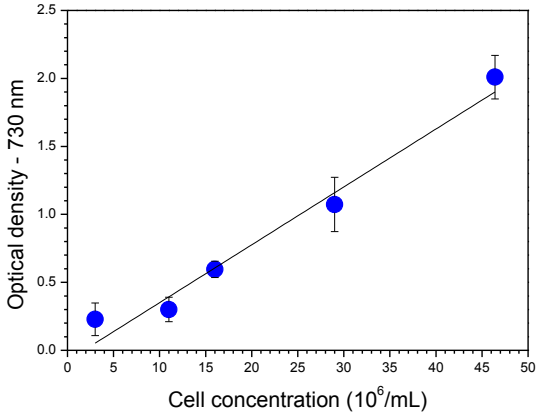

Figure I-23: Relation between the cell concentration and the OD730

5.3. Functions of the bio-hybrid systems

The functionalities of the cells are multiple and studied in this work. The bioremediation activity of cells is monitored by atomic absorption while the functionalisation of some bio-hybrid system is observed using confocal microscopy.

5.3.1. Atomic absorption (AA)

The metal ion concentration is determined by atomic absorption.

The analysis is based on the absorption of photons by atoms in the fundamental state. The solutions to be analysed are atomised in a flame and a lamp specific to the element to be analysed sends an incident ray through this flame. The difference in intensity between the incident and the resulting radiation is directly proportional to the quantity of the element under study. The relationship involved is very similar to that used for UV-visible spectroscopy where the absorbance (A) depends on the ratio of the incident light to the resulting light (I_0/I), but is also proportional to the absorption coefficient of the element at a given frequency (K_ν) and the length of the flame (l) (Equation 4).

$$A = \log (I_0/I) = K_\nu.l \qquad (5)$$

Analysis. A spectrometer AA7000 manufactured by the Shimadzu Company is used for the measurement. Calibration curve (at least four points) relies on the absorbance with the atomic concentration. The measurement is realised in triplicate.

5.3.2. Confocal microscopy

For a more detailed observation of our samples, confocal microscopy is used. This technique basically consists of the optimisation of conventional optical microscopy. It allows the acquisition of images with a very shallow depth of field (~600 nm). Confocal microscopy uses the fluorescence emitted by the sample as the acquisition mode.

It will be used to observe the nanoshell of synthesised bio-hybrid materials functionalised by thiol groups using a fluorescent marker N-(5-fluoresceinyl)maleimide dye (5-FM).

Analysis. The samples are observed in liquid in suitable cuvettes. The marking shall take place as follows: fluorescent marker (final concentration: 5 mM) in HEPES buffer (0.01 M, pH 7.4) is added to the sample and left for 30 minutes of incubation. After three rinses, observations are made using a confocal laser scanning microscopy Leica TCS SP5 device.

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Part II
Design of bio-hybrid system by encapsulation of single cyanobacteria

Chapter 1 - Two components for an efficient hybrid material

1.1. Foreword

In this part of the thesis, *in-situ* encapsulation of single cyanobacteria with silica shells will be discussed in terms of their synthesis component, synthesis strategies, material properties, functionalities and applications. The silica-based nanoshell have several components, generally including inorganic part and organic part. The organic part serves as the initial scaffolding to induce the synthesis of silica.

1.2. Influences of the components

The sol-gel pathway will be used for the synthesis of silica material. Silica networks could be easily obtained from several precursors under different physico-chemical reaction conditions [1, 2]. In our case, we will adapt these conditions to cyanobacteria (pH 7.4, saline environment, atmosphere and ambient temperature). To modify the properties of the material, it will be necessary to develop several approaches.

The function of scaffolds in the synthesis of organised silica material is already widely studied. A typical example of the use of scaffolds for the silica synthesis is the formation of mesoporous materials with the assistance of surfactants [3]. In the living world, silica is always associated with organic molecules in both assimilation and synthesis [4]. For instance, it is through defined biomolecules that diatoms build their well-constructed exoskeleton characteristic of hierarchical materials [5, 6].

These organic additives thus have an influence on the morphology and textural properties of the synthesised materials [7]. Some of these processes have been already studied. The reactivity of precursors as well as the size of molecules have been considered as two most important factors to influence on the properties of the materials [8-11].

In this part, we would like to make a quick overview of different bases for the construction of single cyanobacteria@silica and their corresponding influences. In a short first step, the use of different silica precursors will be discussed, before studying the influence of organic scaffolding.

1.3. Strategy

Silica monoliths will first be synthesised from different sources. Initially, organic-inorganic hybrid materials will be synthesised without the cells in order to free themselves from certain constraints. The designed strategy is an *in-situ* encapsulation based on the layer-by-layer method and the sol-gel pathway in order to obtain the bio-hybrid systems type cyanobacteria@silica. The organic layer will be composed of cations that will bind with the cell wall via electrostatic interactions as presented by the Figure II-1.

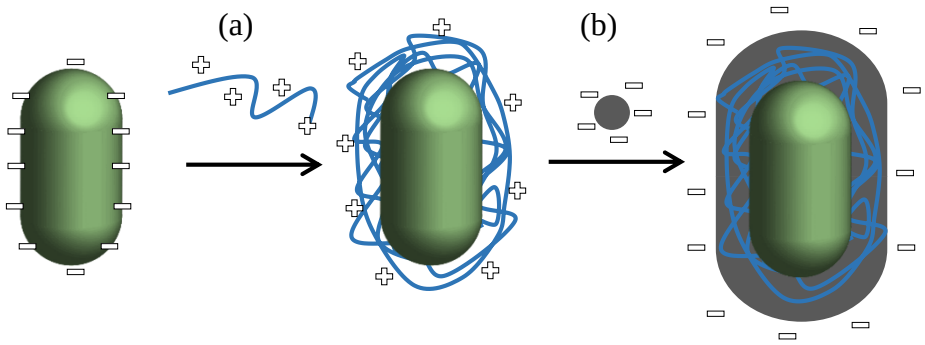


Figure II-1: Scheme representing the designed strategy of a single cyanobacteria@silica system synthesis from native PCC7002: (a) addition of a polycation layer and (b) addition of silica precursor towards the silica-based shell

Chapter 2 - Silica synthesis

2.1. Choice of the precursors

Nowadays, synthesis of inorganic materials are well-studied processes. From the second half of the 20th century, increasing knowledge about the sol-gel process allows chemists to obtain well-organised material in soft conditions in terms of temperature, pressure and pH [1].

This way of synthesis involves the hydrolysis and condensation of precursor monomers with the release of by-products, which would be cytotoxic. Therefore, the choice of the silica precursor is crucial for a living hybrid system synthesis.

2.1.1. Alkoxides

The use of alkoxides as silica precursor presents several drawbacks. First, release of alcohol appears during the polymerisation reactions of silicon alkoxides (most use are tetramethyl orthosilicate (TMOS, $Si(OCH_3)_4$) and tetraethyl orthosilicate (TEOS, $Si(OCH_2CH_3)_4$)). The different steps of the polymerisation steps are presented by the following reactions: hydrolysis (1) and alcoholic (2a) and aqueous condensation (2b) [12, 13].

$$Si(OR)_4 + n\ H_2O \rightarrow Si(OR)_{4-n}(OH)_n + n\ ROH \quad (1)$$

$$\equiv SiOR + HOSi\equiv \rightarrow \equiv Si\text{-}O\text{-}Si\equiv + ROH \quad (2a)$$

$$\equiv SiOH + HOSi\equiv \rightarrow \equiv Si\text{-}O\text{-}Si\equiv + H_2O \quad (2b)$$

In these reactions, the symbol "≡" represents the link between an outer group and the silica network (tetracoordinated silicon). The hydrolysis reaction involves the substitution within the silica precursor molecule of an alkoxy group by a hydroxyl group. During condensation reactions, siloxane bonds are formed (Si-O-Si) with the release of alcohol or water. Those alcoholic by-products have a negative effect on the activity of biomolecules as enzymes and thus can potentially be cytotoxic to cells.

Secondly, a problem of miscibility is observed in water, as reported by Wright and Sommerdick [12]. Following the ternary phase diagram of the system TEOS-alcohol-water

(Figure II-2) at standard temperature (25°C), the adding of alcohol is necessary for a homogeneous reaction as expected for the material synthesis.

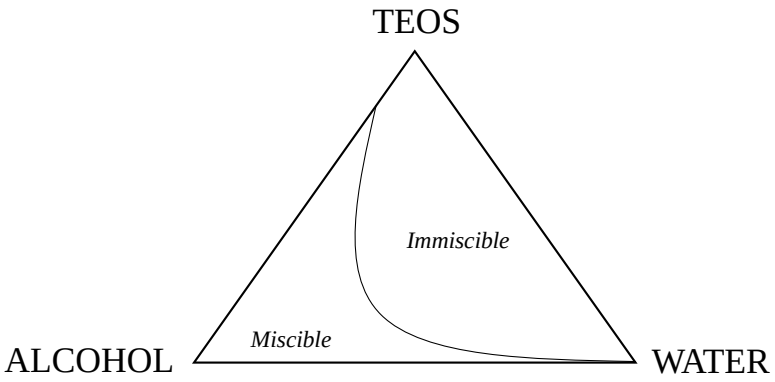


Figure II-2: Ternary phase diagram of the system TEOS-alcohol-water

Facing those two issues, the alkoxide way is to override. Another choice must be applied to the silica precursor. More recently, it appears the use of aqueous precursor obtained basically from sand (silica) dissolve in alkali solution.

2.1.2. Aqueous precursor

At the beginning of the 2000s, scientists involved in biomolecules entrapment have solved the problems related the sol-gel alkoxide route using aqueous precursor for silica synthesis [14]. They are quickly followed by the French group of Jacques Livage, leaders in the field of cell immobilisation, who used aqueous way for the entrapment of bacteria and prove that this pathway is less deleterious for the cells [15].

2.1.2.1. Sodium silicate

Sodium silicate is the generic name for small chain made from sodium silicate monomers with the general formula: Na_2SiO_3.

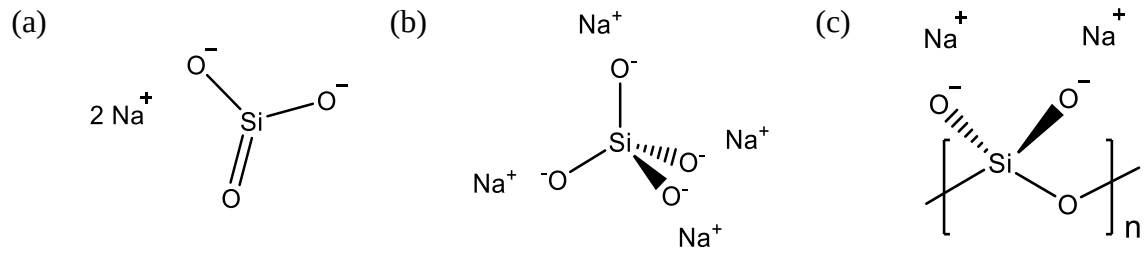


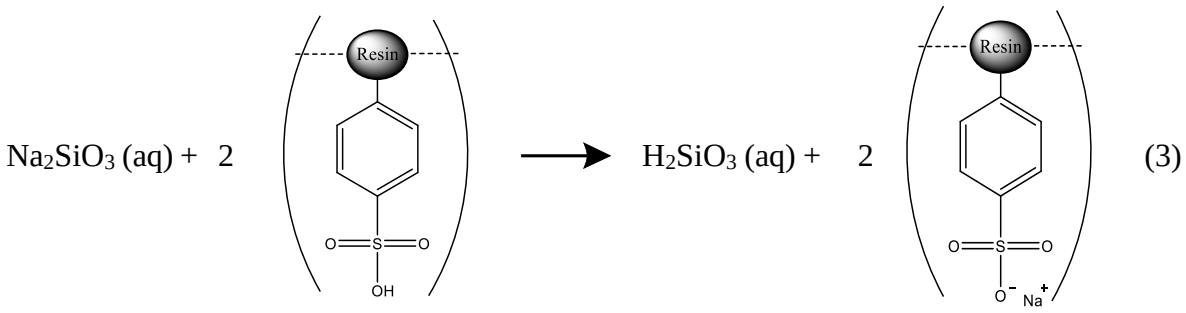
Figure II-3: (a) Sodium metasilicate; (b) sodium orthosilicate and (c) sodium silicate

A silica material can easily be obtained from sodium silicate via some reactions. The original solution presents a very high pH. Once pH decreases to neutrality, the polycondensation of the precursor will occur leading to the formation of the silica material. The by-products in this case are water and sodium chloride (NaCl). Cyanobacteria are very tolerant of these compounds since both are present in large amount in the culture medium for cyanobacteria PCC7002 cultivation.

2.1.2.2. Silicic acid

In order to have a fully hydrolysed precursor and so make the reaction as clean as feasible with only the release of water as a by-product, the use of silicic acid (more precisely known as polysilicic acid) can be considered [15, 16].

It is obtained via an ionic exchange reaction involving a resin (3):



The silicic acid solution is very reactive and presents a low pH. Here also, changing the pH of the precursor solution led to the polymerisation of the precursor, resulting in the formation of silica (SiO_2) and the release of water (H_2O).

2.1.3. Comparison between both aqueous route

In order to see the difference between two aqueous precursors, attempts of hybrid material synthesis have been performed following the entrapment of cyanobacteria in silica hydrogel.

2.1.3.1. Experimental methods

All those experiment are carried out under sterile condition (laminar flow hood).

2.1.3.1.1. Cyanobacteria

Cyanobacteria PCC7002, one month after the transfer, are diluted thrice four days before the manipulation. Cells are harvested and concentrated by centrifugation (2,500 rpm, 25

min) from 30 mL of the previous culture solution. The supernatant is removed and the cell pellet is resuspended in 1.5 mL of fresh culture medium.

2.1.3.1.2. Silica precursors

A sodium silicate solution (100 mL, 1.5 M) is obtained by dilution of sodium silicate purchased from Merck (assay 25.5-28.5%) and kept at 4°C. This solution is sterilised on filters (Rotilabo®, 0.22 µm) and used as it is.

The silicic acid solution is obtained from another sodium silicate solution. The $Na^+ \rightarrow H^+$-ion exchange is performed using Amberlite® IR120, H resin (Acros Organics). It is firstly washed (150 g resin) with 1.0 L HCl solution (pH 2) and cooled in the freezer (4°C). Then, a sodium silicate solution (100 mL, 1.5 M, 4°C) was mixed with the resin and the mix is vigorously shaked. The resulting silicic acid solution is recovered after filtration. The measured pH is around 2.0 (±0.3) and this final solution is kept at 4°C, sterilised on filters (Rotilabo®, 0.22 µm) and used in the following 4 hours.

2.1.3.1.3. Attempts of gel formation

The embedding of cyanobacteria in silica gel is performed in a two-step procedure as described in literature [15, 17]. In a first step, the pH of the sol made of silica precursor solution and concentrate culture medium is adjusted. The second step consists of the addition of cells in this mix and waiting gelation time.

The precursor solutions are obtained mixing 18.0 mL of the silica precursor (Na_2SiO_3 or H_2SiO_3, 1.5 M) with 2.0 mL of culture medium concentrated 10 times. The pH of the solution is adjusted to 7.4 (± 0.2) by adding a small amount of HCl 0.2 M solution or NaOH 0.2 M solution. From this time, all the manipulation must be performed quickly: 0.5 mL of concentrated cell suspension is added to precursor solution (Na_2SiO_3 or H_2SiO_3). In a multiwell plate (24 wells), 2 mL is poured ten times for each precursor. In this way, two kinds of living systems are obtained (Table II-1).

Table II-1: Samples characteristics

Abbreviation name	Aqueous silica precursor
GelNa	Na_2SiO_3
GelH	H_2SiO_3

Fresh culture medium (0.5 mL) is subsequently added over the synthesised materials. The multiwells plate is kept on orbital shaker (60 rpm), at room temperature and under light (cool white: Omsram lamps L18W/840).

2.1.3.1. Results: behaviour of the cells

The first quick observation can be achieved during the material synthesis. From silicic acid, solid hydrogels entrap cells after less than 10 minutes. In the case of sodium silicate, nanoparticles precipitation is observed in the wells after 20 minutes.

After one hour, sodium silicate and its polymerisation seem to have a negative effect on the cyanobacteria activity and confirmed in time as described in the Figure II-4.

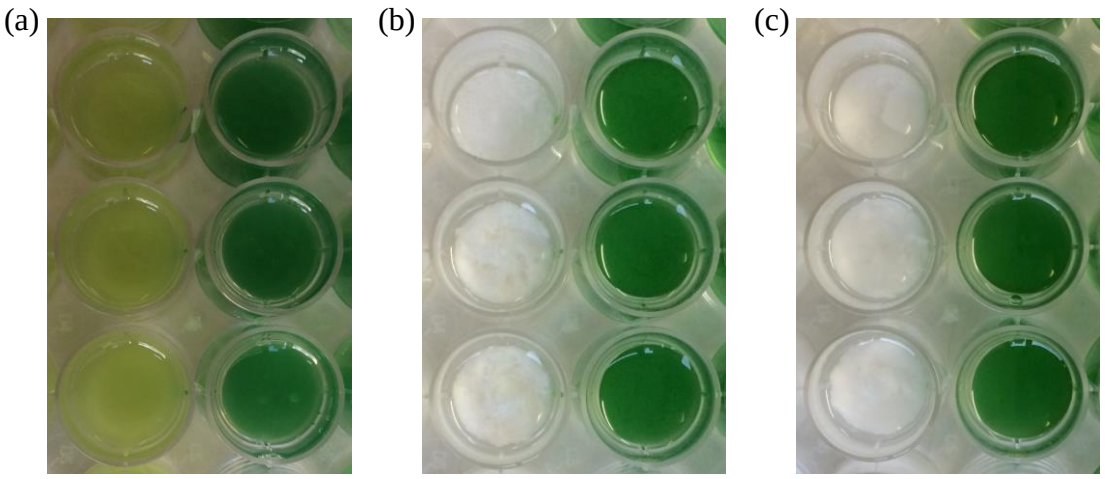
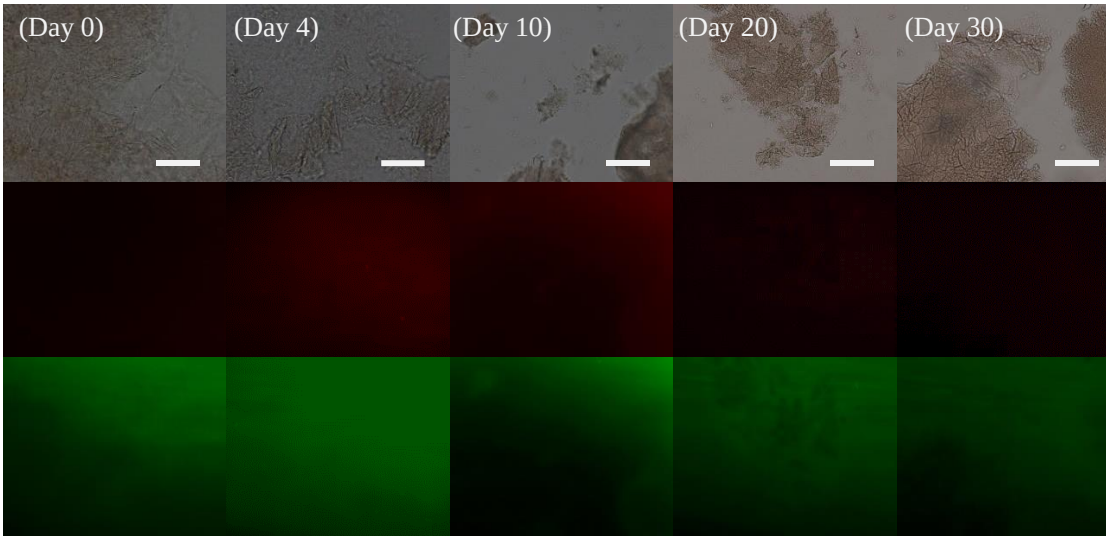


Figure II-4: Photographs of a part of the multiwell plate with a different time after experiments (3 wells left corresponding to GelNa, 3 wells right corresponding to GelH): (a) one hour; (b) 4 days and (c) 10 days

A direct difference in the colour of the samples can be seen. The cells incubated with sodium silicate show a yellow colour while the samples with silicic acid keep their strong green characteristics. Those observations led to think that Na_2SiO_3 precursor has a deleterious effect on cells type PCC7002. Viability tests were performed to confirm this conclusion. Firstly, a fluorescent dye (FDA) is used to label the living cells (as described in Part I – Chapter 5). The encapsulated cells with different acquisition conditions are observed using fluorescence microscopy. The results are presented in Figure II-5.

(a)



(b)

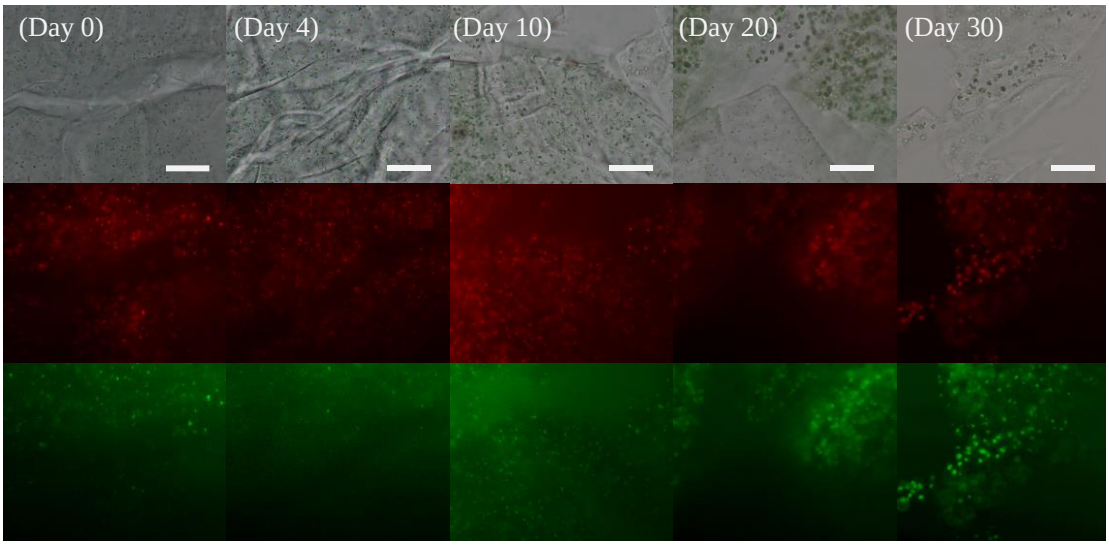


Figure II-5: Micrographs of PCC7002 entrapped in (a) GelNa (precursor = Na_2SiO_3) and (b) GelH (precursor = H_2SiO_3) different days after immobilisation treatment in bright field, red fluorescence (chlorophyll) and green fluorescence (related to FDA fluorescence) (bars = 200 µm)

Just after the immobilisation procedure, difference is already visible between the two experimental groups. The macroscopic observations are confirmed in the micro-scale. With nanoparticles (reflected as brown by the microscope light) are present in the samples synthesised using the sodium species. While well-defined transparent gels are obtained following the polymerisation of polysilicic acid. The stability of those gels are confirmed by time: after one month, gels are still in form. In terms of viability, in the case of the first set, no green dots can be seen on the micrographs, but only a diffuse fluorescence. The same

observations can be made for the autofluorescence of the chlorophyll (red fluorescence). In comparison with the set obtain from silicic acid, the viability is different. The bright field images reveal that the cells were well entrapped in the gel. The silica material seems not to have a too negative effect on the cells regarded that after ten days, small "settlements" are visible due to the cells division. The photosynthetic pigments are visible thanks to the red fluorescent dots corresponding to the cells visible in the bright field. That fluorescence can be detected in the green field, which confirms the cell viability. Those observations reveal the appearance of starting bacterial colonies where the enzymatic activity is more effective regarding the isolated cells also present in the gel. The hypothesis advanced to explain this difference in behaviour (dividing cells versus latent cells) in the silica gels is that the light and the nutrient diffusion are different depending on the depth of the cells in the material. Further investigations could provide precise explanation. But, in this case, it is clear that the cells keep their activities in the gel obtain with silicic acid compared with their behaviour in contact with the sodium silicate and the corresponding precipitated particles.

For the purpose of confirming these observations, the photosynthetic activity of the different samples as a function of time is followed by measuring their molecular oxygen (O_2) production. A control is set up using native cells with the aim of proving that no external issues interfere with the measurement procedure. For GelH samples, 500 mg of the hybrid system (gel containing cyanobacteria) is diluted in 1 ml of fresh medium in the analysis chamber. In the case of GelNa, 500 µl of the solution (mixed particles and cyanobacteria dispersed in the culture medium) is also diluted in the chamber with 1 ml of fresh medium. These samples are compared to the behaviour of a native cell culture (500 µl in the chamber, added to 1 ml of fresh medium). This activity is presented by the following graph (Figure II-6).

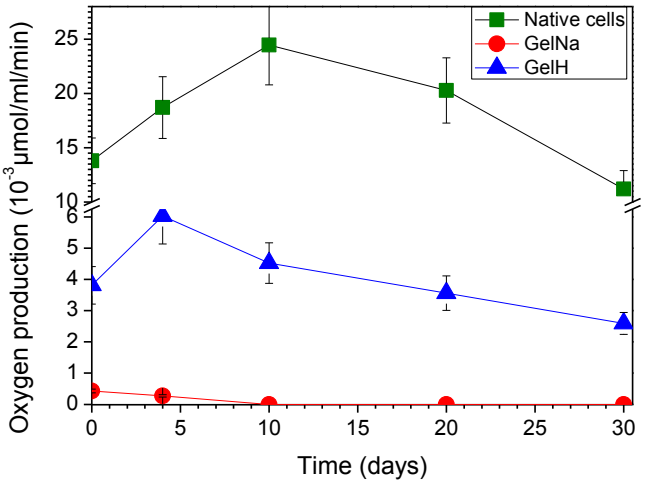


Figure II-6: Oxygen production of native cells and after immobilisation via the use of two aqueous precursors as a function of time (one set of measurement: inaccuracy is depending on technical and experimental manipulations)

Comparing red (GelNa) and blue (GelH) curves, the procedure involving the use of sodium chloride releasing precursor have a deleterious effect on the photosynthetic activity of the cyanobacteria compared to a procedure which implements a preliminary ionic exchange. A small activity can be detected in the first days before a total loss of oxygen production. On the contrary, the GelH samples produce oxygen. Even an increase is detected after four days probably due to the adaptation of the cells in the gels after the stress of the immobilisation procedure. Those results confirm the negative effect of the procedure involving the sodium silicate opposite to the silicic acid precursor. The native cells present quite a higher oxygen production due to two main reasons. The first is the different cell concentration between the initial cell culture and the "gel" samples because of the dilution of the cell pellets in the gel precursor mixtures. The second is related to the procedure itself that brings a significant stress to the cells and related to the material that constrains division. However, it allows advancing that none external factors interfere with the testing protocol.

2.1.4. Discussion and conclusions

The cyanobacteria strain PCC7002 seems to be sensitive to the change of ionic strength in the solution by the release of sodium chloride (or other unknown products) during the Na_2SiO_3 polymerisation, even though they have euryhaline properties. In the case of the gel synthesis, the change in ionic strength and osmotic pressure are too important to preserve the cell viability. Nevertheless, in the case of individual encapsulation, the amount of precursor

and the release of NaCl ensuing is very small. Further experiments are required to conclude that the loss of viability is due to the variation of NaCl concentration.

However, the long-term viability of the cyanobacteria in the silica gel is an indication that a silica material is not deleterious for the studied strain.

According to the results presented above, the use of silicic acid and the ionic exchange procedure to synthesise silica materials is preferable. This precursor avoids the release of deleterious alcohol and shows a less negative impact on the cyanobacterial activity when compared with the second aqueous precursor.

Chapter 3 - Cationic polymers

In this chapter, we present the synthesis of some bio-hybrid systems obtain via the layer-by-layer process: cyanobacteria are covered by a prior cationic layer and an outer silica layer (see Section 1.3. Strategy).

3.1. Organics as link between cells and material

The outer part of the *Synechoccocus sp.* PCC7002 presents an outer negative net charge as described in the literature [18]. This charge is mainly to outer chemical groups type carboxyl, phosphate and hydroxyl that induce the previous mentioned negative charge [19, 20]. On another hand, silica materials are also negatively charged due to the silanol groups at the external part of the silica particles [21]. The acidic behaviour of those groups was well studied [22] and from those previous observations, deprotonated hydroxyl groups are present at the working pH of 7.4 and contribute to this external charge. The appearance of electrostatic repulsion between those two during individual encapsulation process is expected as described previously. In order to counterbalance this unfavourable interaction and make a point of control of the shell properties, the addition of cations during the layer-by-layer procedure is planned.

The use of polycations for cell encapsulation is well depicted in literature even though more frequently, it is about polyelectrolytes [23-26]. In biological field, adhesive properties of cells on polymer surface is widely used and can be modulated owing to physical and chemical properties of the polymers [27]. While, on the other hand, interaction between polyelectrolytes and silica surface were studied and computational works highlight bonds type ion pairs, hydrogen bonds or other polar interactions between charged groups and surface silanol/siloxide groups [21].

3.2. Selected cations

The selection of the cationic organics is made following some criteria. Firstly, the use of polymer leading to polycharged molecule is fixed rather than monomers. The chemical groups relating to their pKa must present a cationic form at the working pH of 7.4. The price and the availability of the polymer are also taken into account as well as its solubility in water and its colour (no visible light absorbance).

Over a first phase, six polycations have been selected. Three polymers, two polypeptides and one polysaccharide have been tested for cell encapsulation. These polycations are presented in the following table and presented by the Figure II-7.

Table II-2: Polycations characteristics

Abbreviation	Usual name	Characteristics
PAH	Poly(allylamine) hydrochloride	M_w ~ 17,000
PDA	Poly(diallyldimethylammonium chloride)	N/A
PEI	Poly(ethyleneimine)	Branched, M_w 12,000-13,000
PLA	Poly-l-arginine hydrochloride	M_w 5,000-15,000
PLL	Poly-l-lysine hydrochloride	M_w 15,000-30,000
DAD	Diethylaminoethyl dextran hydrochloride	N/A

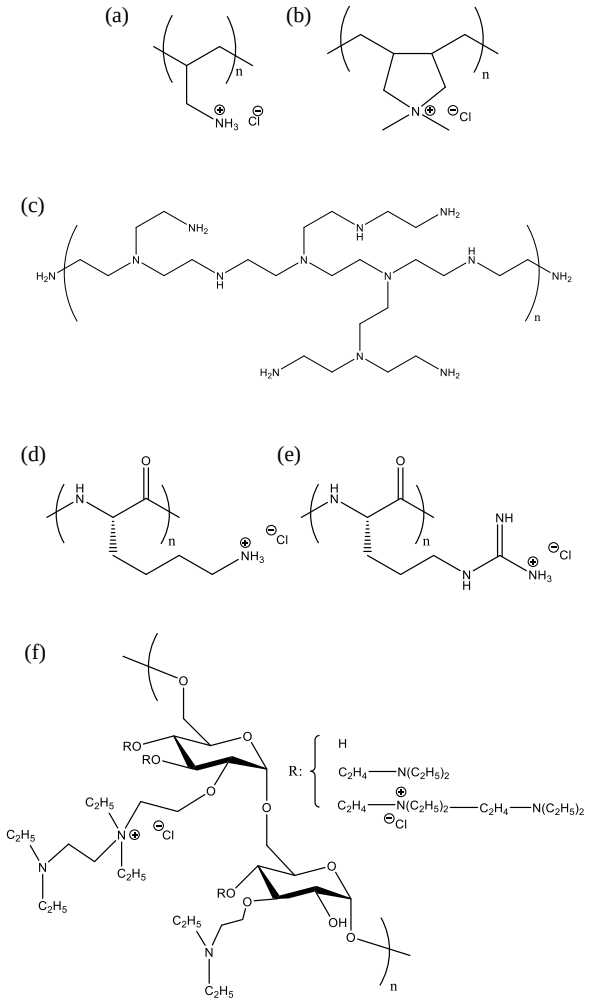


Figure II-7: Polycations : (a) PAH, (b) PDA, (c) PEI, (d) PLL, (e) PLA and (f) DAD

3.3. Bio-hybrid system synthesis and properties

3.3.1. Experimental procedure

The layer-by-layer deposition results from a step-by-step procedure. Cells (4 days after the transfer) are harvested by centrifugation (2,500 rpm; 20 min) from 15 mL of cell suspension for each of the six polycations. Previously rinsed after removal of the supernatant by fresh culture medium, they are treated by another centrifugation (2,500 rpm, 20 min) and are subsequently resuspended in 5 mL of polyelectrolyte solution (2% w/w, pH 7.4). After 30 minutes of incubation under slow stirring (orbital shaker, 60 rpm) cell pellets are gathered by centrifugation (2,500 rpm; 10 min) and rinsed by fresh culture medium in order to remove the amount of unbound polycations. They are then suspended in culture medium (4.83 mL) before the addition of 0.167 mL of H_2SiO_3 1.5 M (freshly prepared and sterilised, see section 2.1.3.1.2. Silica precursors) for a final concentration around 50 mM. The samples follow another incubation of 30 minutes and rinsed by fresh culture medium before being resuspended in 15 mL fresh medium. This procedure brings to a two-layered hybrid system in which silica is the outmost covering layer.

For porosimetry studies, silica-based nanoparticles were synthesised by preparing 2% polycation solutions in the aqueous culture medium and adjusted to the physiological pH of the cells (7.4). H_2SiO_3 is added with a final concentration of 50 mM. The reaction mixture remains under stirring for 24 hours before rinsing with distilled water. They are then dried at 40°C and ambient atmosphere.

3.3.2. Morphological studies

Individual encapsulation is related to the design of bio-hybrid systems at cellular level *i.e.* at a micro-nanoscale. The use of electron microscopy is then required for in-depth observations. After preparation of the sample (see Part I – Chapter 5), micrographs of the hybrid systems are taken using SEM and TEM.

Firstly, SEM micrographs could provide information about the general morphology of the cells and the changes caused by the encapsulation process. Indeed, the outer membrane of the cells is mainly composed of organic molecules, the addition of silica outer layer can be observed. The hybrid systems are compared with firstly native cells and described by Figure II-8. In this figure, in order to confirm the usefulness of the polycationic layer, another control is introduced. It consists of an attempt where the prior coating with polycations is prevented during the experimental procedure.

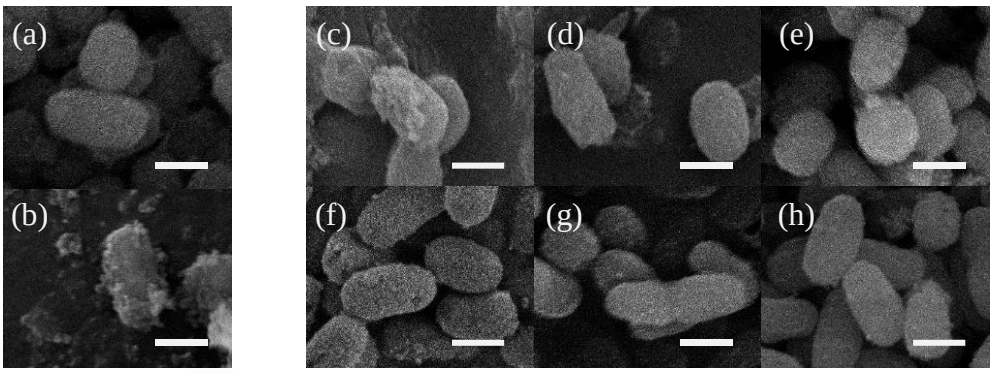


Figure II-8: SEM micrographs of native cells (a) and encapsulated cyanobacteria: (b) PCC7002@H_2SiO_3, (c) PCC7002@PAH/H_2SiO_3; (d) PCC7002@PDA/H_2SiO_3; (e) PCC7002@PEI/H_2SiO_3; (f) PCC7002@PLA/H_2SiO_3; (g) PCC7002@PLL/H_2SiO_3; (h) PCC7002@DAD/H_2SiO_3 (bars = 1 µm)

Despite the quality of resolution, it is possible to distinguish some differences between the various samples. The smooth appearance of the outer of the native cyanobacteria can be observed on the Figure II-8a. Particles can be observed on material PCC7002@H_2SiO_3 (Figure II-8b) which is prepared without cationic polymer even though the particles cannot form a homogeneous layer around the cells. While in another hand, particles are still visible in the other samples, but a more regular surface can be distinguished. For example, in Figure II-8f, in the case of the use poly-l-arginine, the smooth appearance of the cyanobacteria changes into cotton-like form. To go deeper in those observations, the imaging of slides is performed using transmission electron microscopy. The same samples as previously described were prepared; the micrographs are presented in the Figure II-9.

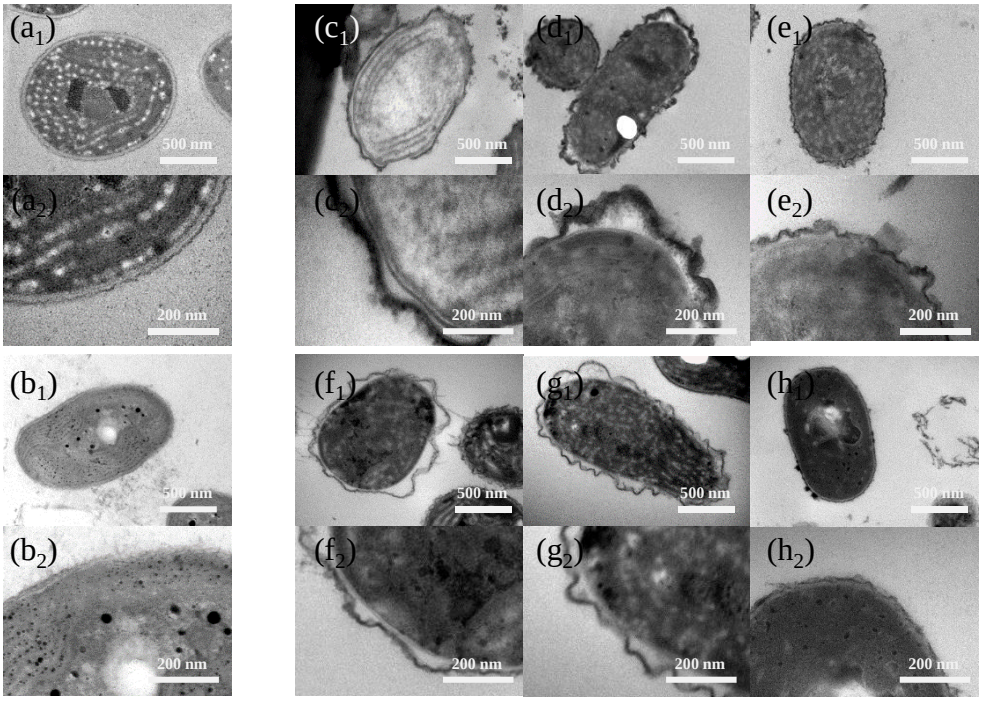


Figure II-9: TEM micrographs of native cells (a) and encapsulated cyanobacteria: (b) PCC7002@H_2SiO_3, (c) PCC7002@PAH/H_2SiO_3; (d) PCC7002@PDA/H_2SiO_3; (e) PCC7002@PEI/H_2SiO_3; (f) PCC7002@PLA/H_2SiO_3; (g) PCC7002@PLL/H_2SiO_3; (h) PCC7002@DAD/H_2SiO_3

In the micrographs (Figure II-9a_{1-2}), we can clearly see the cyanobacterium cell envelope. Two darker parts are surrounding a grey layer attributed to the peptidoglycan layer enclose between the two membranes. With further observation, it reveals additional outer line confirming the presence of the three parts of a gram-negative bacterium [19, 28]. Concerning the inorganic species presence, in both controls: native cells and PCC7002@H_2SiO_3 (Figure II-9b_{1-2}), the presence of silica seems very weak or non-existent.

Whereas, artificial shells comparable to an additional envelope can be synthesised using the two-layered procedure and cationic polymers. Indeed, the presence of a darker line that encircles the cells are attributed to the shell formed via the layer-by-layer method. The thickness of the layer approaches 10 nm to 25 nm as seen on the images Figure II-9c_2, d_2, e_2, f_2 and g_2. For those five samples, a space of a few dozen nanometres between the outer membrane and the shell is found. The efficiency of the encapsulation process is important in the case of the three polymers (PAH, PEI and PDA) where the entire cell population that appears to be surrounded by this artificial envelope. However, in the case of both polypeptides (PLA and PLL), this effectiveness must be nuanced. Cells still seem free (absence of this artificial layer) are visible among the encapsulated cells at lower magnification. Nevertheless,

it is difficult to calculate the efficiency rate of the encapsulation process because the images obtained are not sufficiently representative of the entire population.

Finally, the use of the polysaccharide (DAD) as cationic layer leads to a different morphology (Figure II-9h$_{1-2}$): the shell does not show a curving way to stick the cell and seems thinner than the one in the other cases. Despite the use of polysaccharides by cyanobacteria for biomineralisation (calcification) [29] and the use of polysaccharides for the construction of silica material with organised morphology [30], it seems that DAD is not a good candidate. The hypothesis is that this polysaccharide does not form enough bonds with the cell wall and could be ingested by these microorganisms. Without this link, deposition around the cells seems ineffective.

3.3.3. Physicochemical properties of the shells

The outmost layer observed around the cyanobacteria can be speculated to be silica, which shows darker (composed of a "heaviest" element) under the observation of TEM in comparison with the cytoplasm of the cyanobacterium (mainly carbon, hydrogen, oxygen and nitrogen) [31]. The chemical composition of this outer surface (depth of about 10 nm) of material is studied using X-ray photoelectron spectroscopy.

Firstly, a general comparison is made between the eight samples (after drying process): the two controls and the six bio-hybrid systems are presented in the Figure II-10.

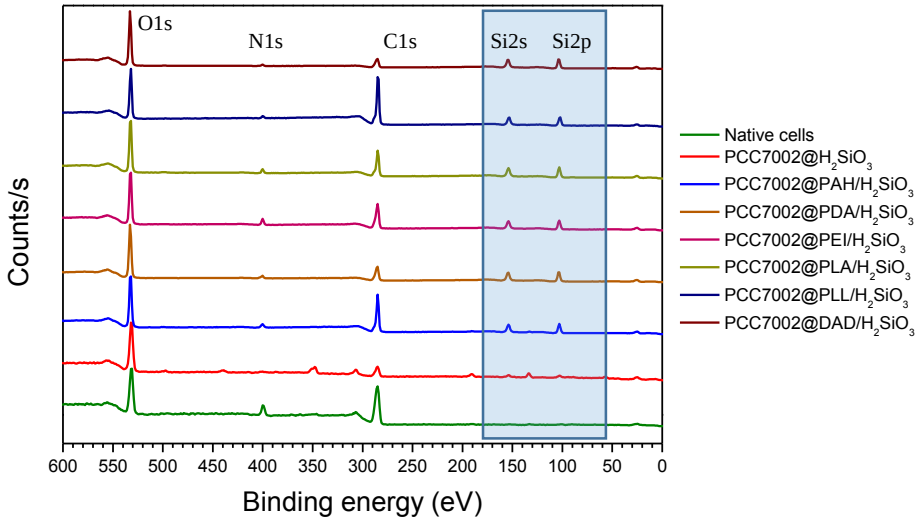


Figure II-10: Chemical composition of the outer part of the samples (measured by XPS)

The green curve is corresponding to the native cells profile. Three main peaks stand out this measure. As expected, they can be related to the main elements presented in the living world (often described as CHNO [32]): oxygen (binding energy around 530 eV), nitrogen (± 400 eV) and carbon (± 285 eV)[1].

For the samples PCC7002@H_2SiO_3 (second curve from the bottom), the same three peaks appear, verifying the presence of oxygen, nitrogen and carbon. The addition of other peaks, e.g. around 350 eV (calcium), corresponds to the nutrients present in the culture medium and other satellites (related to trace elements). Their presence is probably due to a non-efficient washing process before the drying of the cells or some contamination. However, for those two samples, the silicon peaks (around 150 eV corresponding to Si2s and 100 eV for Si2p), highlighted by a blue frame, seems absent which coincides with their morphology presented in the micrographs above (Section 3.3.2. Morphological studies).

In the cases of all the two-layered materials, those silica peaks appear. It is now possible to conclude that the hybrid shell is partly made of silica material as expected from the hybrid material synthesis. The ratio C/Si of the shells of the six samples is calculated and presented below.

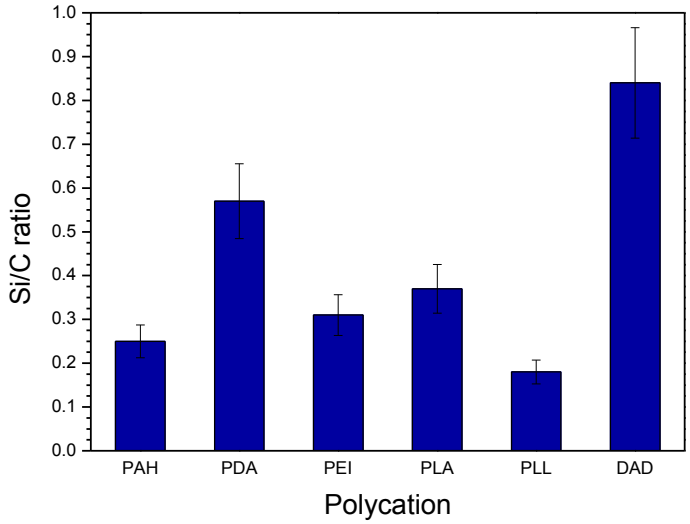


Figure II-11: Si/C ratio calculated for the two-layered materials depending on the polycations used in the process (inaccuracy dependent on technical and experimental manipulations)

[1] Hydrogen element can not be detected using XPS.

Although the reliability and reproducibility of these measurements are relatively low, they still provide a general idea of the efficiency of the encapsulation process. These data are a starting point for the comparison between the various synthesised systems and shows important difference confirming the influence of the organic layer.

The first information that demonstrated in the Figure II-11 is that the presence of carbon is higher than silicon in each sample. Significant difference is observed between the samples with a range of 0.18 for the use of poly-l-lysine hydrochloride and 0.84 in the case of the modified dextran. The important ratio in the last case can be attributed to the presence of silica nanoparticles which could disturb the measure and influence the result. Unfortunately, no trend can be extrapolated and the conclusion of those results is that the various polymer induce different material properties. It could be a great starting point for the design of control of the shells properties.

A zoom on the silicon peak (± 103 eV – Si2p) can also provide some information about the chemical environment of this atom and the silica form in the hybrid material. When the peak is deconvoluted (the peak fitting is presented only for PCC7002@PAH/H_2SiO_3 on Figure II-12), three components can be detected: around 103.5 eV, 103 eV and 102 eV. Respectively, the first two correspond to one overlapping $Si2p_{3/2}$ and $Si2p_{1/2}$ with the value of 103.5 eV corresponding to the silica form (SiO_2), the less energetic peak (102 eV) corresponds to silicon species surrounded or even linked with organic groups.

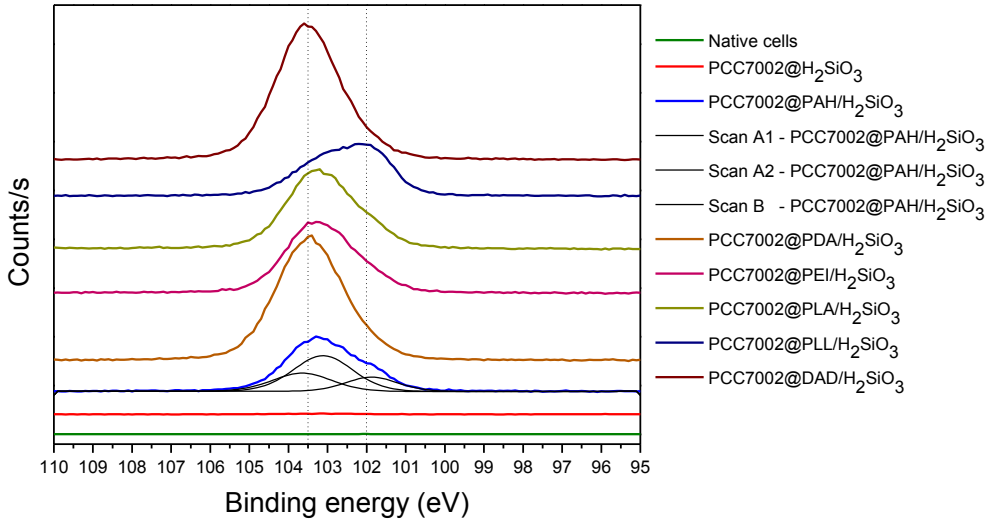


Figure II-12: Si2p peak (obtained by XPS**)**

For some material, the last contribution (102 eV) is very low or even inexistent. The main difference between the polyelectrolyte besides their aliphatic groups is the amino-groups. A summary of the amino-group of polymers and the presence of the "organic silicon" contribution is presented in the next table.

Table II-3: Characteristics of the polycations related to the physico-chemical properties of the silica material[2]

Polycation	Primary amine	Secondary amine	Tertiary amine	SiO_2 contribution	"Organic silicon" contribution
PAH	+++	/	/	++	+
PDA	/	/	+++	+++	/
PEI	+	+	+	+	+
PLA	+	+	/	+	+
PLL	+++	/	/	+	++
DAD	/	+	+	+++	/

The presence of the organic part is directly related to the presence of primary amines as seen in Table II-3. The effect of amino-group on the polymerisation reactions of silica is well known. It is considered as a catalyst for the hydrolysis reaction of silicon alkoxide where a hexacoordinate reaction intermediate occurs [8, 9]. The presence of long-chain amines in biological processes of biomineralisation in sponge or diatoms is also widely described in the literature [4-6, 22, 33]. From the observed properties of amino-group and silicon species, some hypothesis made to explain the "organic silicon" contribution in XPS spectra. A strong interaction between a silicon atom and a nitrogen atom can occur only if the doublet is "available". This is the case for primary amine unlike in the case of tertiary amine. It could be those interactions appearing in the silica network that provide this new observed species in some samples. Those hypotheses seem to be consistent with the previous results.

From this point, it would be interesting to know the influence of these organic parts and these interactions on the textural properties of hybrid materials.

[2] +: presence ; /: absence

3.3.4. Materials porosity

The properties of hybrid materials without biological material need to be investigated first because the small quantities of synthesised materials and some constraints on cells make the system more complicated to be studied.

A first direct observation is that in an environment without polycations, there is no precipitation visible observed using the naked eye. This roughly proves the effect of these organic molecules in the precipitation of silica.

The isotherms are obtained through the adsorption/desorption of nitrogen. All synthesised materials are demonstrated mesoporous except for the material synthesised with the assistance of PAH. According to the isotherm and using the mathematical models mentioned in the previous part, information concerning their specific surface area and pore size distribution is calculated and included in the Table II-4.

Table II-4: Hybrid material properties (porosity studied by N_2 ads/des)

Sample	**BET surface area (m^2/g)**	**BJH pore size (nm)[3]**
PAH/H_2SiO_3	0.05	N/A
PDA/H_2SiO_3	78	3.4
PEI/H_2SiO_3	68	5.2
PLA/H_2SiO_3	529	4.1
PLL/H_2SiO_3	526	3.8
DAD/H_2SiO_3	11	3.1

The material PAH/H_2SiO_3 has very low porosity. The other materials have various properties but with a rather small pore width range (between 3 nm and 5 nm). Specific surfaces are not very large compared to nanostructured mesoporous materials. However polypeptides show most significant surfaces. Even if this surface will have an impact on the exchanges between the cells and their environment, it is, however, more the pore size that presents a constraint in our case.

From these data, it is visible that the nature of the polycation has an influence on the synthetic silica-based material. Except for PAH, which has only a small porosity, and DAD,

[3] The maximum contribution is taken into account.

which does not allow the synthesis of a homogeneous shell around the cells, the other candidates are valid for single encapsulation.

3.3.5. Viability of the encapsulated cyanobacteria

A final point to be studied is the impact of these encapsulations on cell activity. The impact of the cationic layer will be studied as well as that of the silica-based shell. The oxygen production of the cells will be monitored and their cell development will be measured by their optical density.

The samples of cells with modified exteriors are compared to two controls, native cells and cells in the presence of polysilicic acid alone. The 35-day measurements are presented in the following figure.

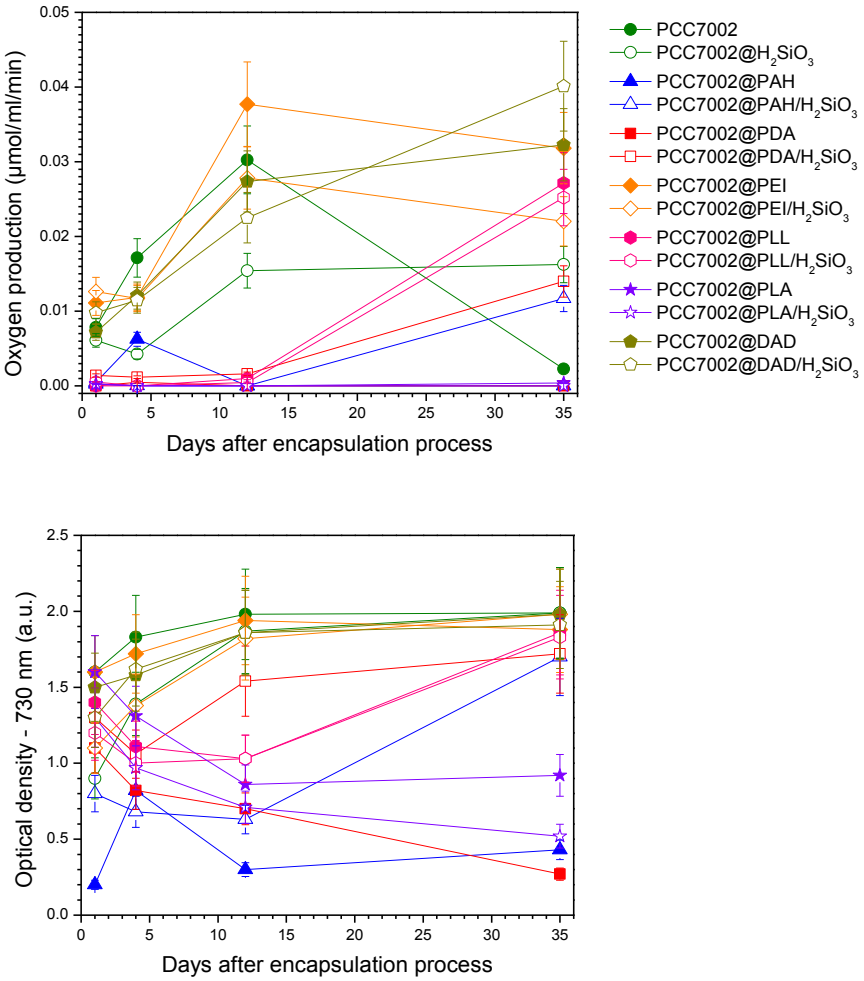


Figure II-13: Viability assay for native PCC7002, PCC7002@polycation and PCC7002@polycation/H_2SiO_3: evolution of oxygen production and optical density (one set of measurement: inaccuracy is depending on technical and experimental manipulations)

The effect on the first graph and therefore the photosynthetic activity followed by the production of oxygen is very different according to the different polycations. PLA (purple curves) has a marked negative effect with and without silica. For others (blue-PAH curves and red-PDA curves), the addition of silica reduces the negative effect. PLL has a short-term effect while PEI and DAD have no significant effect on the photosynthetic activity of the cells.

Looking at the evolution of optical density (OD730), we also see that the effects are very varied depending on the samples. By linking the measurements of photosynthetic activity with the optical density of native cells, the sudden drop in oxygen production can be explained by the lack of nutrients. Indeed a plateau related to cell population stabilisation is observed in the OD730 measurements. PLA (purple curves) shows a continuous harmful effect, while samples PCC7002@PAH and PCC7002@PDA also show that PAH and PDA are toxic without silica. However, after more than a month, all samples arrive at the same point (OD730 of value around 2) which is close to the detectable limitation of this technique.

3.4. Conclusions

In this chapter, several samples were obtained following different attempts to individually encapsulate cyanobacteria in silica-based nanoshells from a preliminary polycation layer. Since one of the objectives is to study the influence of the organic part of the hybrid material on the properties, several characteristics have been followed.

It appears that this organic component does have a marked influence, however, it is difficult to discover a clear trend. Other tests were therefore carried out using several approaches.

Chapter 4 - Influence of the number of amino-group

4.1. Polyamines: starting point and biomimicry

On the basis of the conclusion of the previous chapter, highlight a general trend related to the organic layer and its influence on the material properties could be a key information in order to be able to predict some material properties before their synthesis. In this chapter, the influence of the number of amino-groups present in the cationic molecules on the bio-hybrid systems will be discussed.

Compared to the previous chapter, the molecules mentioned below are quite small. The previous criteria are kept: presenting cationic charges at working pH (7.4), transparent, with the less negative effect on cell activity, etc. Keeping this in mind, the simplest polycations that can be easily found are a diamine, a triamine and tetraamine.

Polyamines are widely represented in the chemistry of life. Some of the smallest that can be found are putrescin (PUT): $H_2N(CH_2)_4NH_2$, spermidine (SPD): $H_2N(CH_2)_4NH(CH_2)_3NH_2$ and spermine (SPM): $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. Referenced for many years to be present in the animal [34] or plant [35] kingdom, but also in microorganisms [36]. These molecules have regulatory effects at several levels [37] and they contribute to the stabilisation of the structure and activity of tRNA and DNA [36]. However, a key point for this selection is that they are also implicated in the biosilification process in the diatoms or sponges [5, 22].

4.2. Bio-hybrid system synthesis and properties

4.2.1. Experimental procedure

A similar experimental procedure is established for the design of the nanoshell on the cyanobacteria. In this part, the LbL process will be implemented with the requirement of poly-amino species. Cyanobacteria (4 days after the transfer) are harvested by centrifugation (2,500 rpm; 20 min) from 15 mL of culture cells. Subsequently, they are rinsed by fresh culture medium and resuspended in 5 mL of polyamine solution (2% w/w, pH 7.4, hydrochloride form purchased from Sigma Aldrich). Next, they are placed under slow stirring (orbital shaker, 60 rpm) for 30 minutes of incubation. Cell pellets are then recovered by another centrifugation (2,500 rpm; 10 min) before being rinsed by fresh culture medium. They are then suspended in culture medium (4.83 mL) with the addition of 0.167 mL of silicic acid of 1.5 M (freshly

prepared and sterilised). The samples are treated with the incubation time of 30 minutes and then are rinsed by fresh culture medium and diluted in a final 15 mL fresh medium.

4.2.2. Morphological properties

In this new case too, electron microscopies techniques are helpful for in-depth observation of the bio-hybrid systems synthesised. These studies bring information on their outer morphology that is directly related to their surface modification via the LbL process.

Micrographs obtained using scanning electron microscopy are presented in the Figure II-14. The smooth texture of the cells outer wall is still visible, but it is supplemented by a grainy layer. Those observations shown in the micrographs prove that a surface modification took place following the two-layering process.

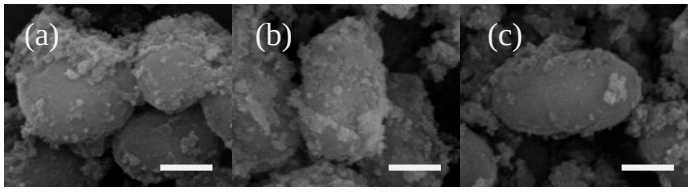


Figure II-14: SEM micrographs: (a) PCC7002@PUT/H_2SiO_3, (b) PCC7002@SPD/H_2SiO_3; (c) PCC7002@SPM/H_2SiO_3 (bars = 500 nm)

The coating visible seems inhomogeneous, it is necessary to correlate these observations with other studies. To go deeper in the interpretations, the samples are prepared for transmission electron microscopy studies.

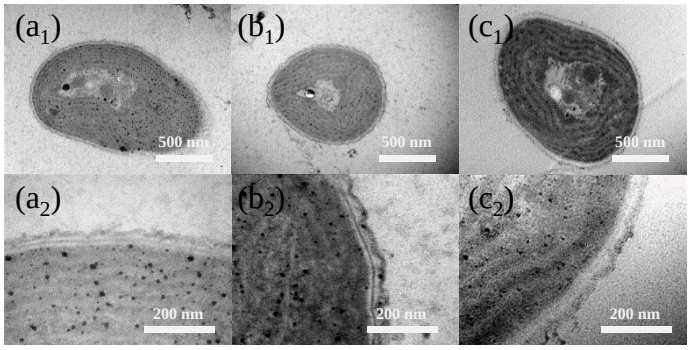


Figure II-15: TEM micrographs: (a) PCC7002@PUT/H_2SiO_3, (b) PCC7002@SPD/H_2SiO_3; (c) PCC7002@SPM/H_2SiO_3

The micrographs of the three samples (Figure II-15a-b-c) reveal a darker foggy-like layer around the cells. The boundary with the outer membrane is confused. It is clear that for each of the three samples, the silica layer is thinner compared to the shells described in the previous chapter. Indeed, once precise measurements are made, the thickness of the shells fluctuates between 10 nm and 20 nm for three samples with no marked difference.

It seems that a slight silica layer is present around the cells but the outer chemical composition and modification of the cells have to be further studied to confirm the presence of silica around the cyanobacteria.

4.2.3. Physicochemical properties of the shells

The outer chemical composition of the bio-hybrid systems is determined using XPS. The general surveys are included in the Figure II-16.

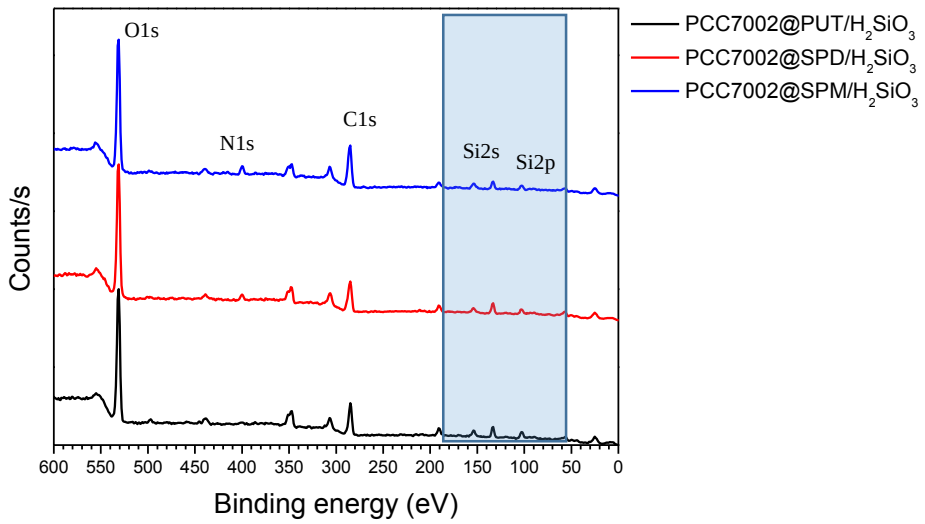


Figure II-16: Chemical composition of the outer part of the samples (measured by XPS)

Similar profiles than in the previous chapter are determined. Classic peaks are detected: oxygen (O1s ~530 eV), nitrogen (N1s ~400 eV), carbon (C1s ~285 eV) and the appearance of silicon peaks (Si2s ~150 eV; Si2p ~102 eV) highlighted by a blue frame. Two additional peaks also stand out: calcium (Ca2p ~350 eV) and phosphorus (P2p ~130 eV) that arise from salts composing the culture medium.

Expecting to determine the efficiency of the silica deposition around the cyanobacteria, the atomic ratio of Si/C is calculated for the three samples (see Table II-5). Interestingly, this

ratio decreases when the number of amines increases, which should be linked to a loss of efficiency in the silica polymerisation process.

Table II-5: Atomic ratio of the main elements present in the external part of the bio-hybrid materials (Error = ±2.5%)

Sample	Si/C ratio	O	C	N	Si
PCC7002@PUT/H_2SiO_3	0.23	56.18	35.56	Not detected	8.26
PCC7002@SPD/H_2SiO_3	0.13	54.58	35.83	4.79	4.80
PCC7002@SPM/H_2SiO_3	0.10	47.55	42.73	5.39	4.33

However, going deeper in the observations and considering more parameters allow to relativise those facts conclude with an initial approach. First, looking at the silicon molar ratio, a decreasing is observed as well as the oxygen molar ratio. They are the two components of a silica material. Subsequently, the ratio of carbon and nitrogen, increase when the molecule size increase. Those atoms compose the three amino-chemicals used in the two-layer process. When correlating those two observations, it seems that increasing the polyamine size increases its incorporation into the silica material even on the surface of the system.

A zoom on the binding energy between 110 eV and 95 eV (Si2p domain) prove the presence of silica in the materials (Figure II-17).

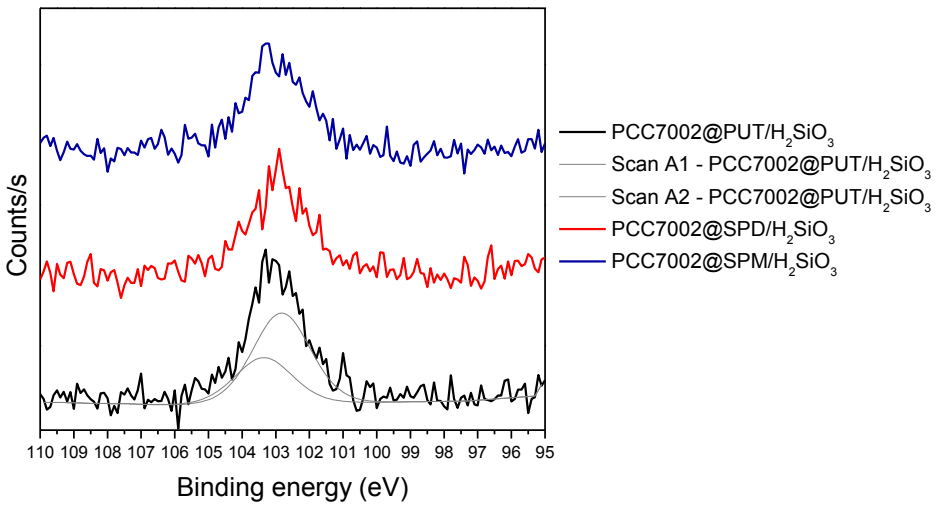


Figure II-17: Si2p domain (measured by XPS)

The deconvolution of the Si2p peak reveals that the major contribution result from SiO_2 species. The same observation is made for the three samples. In this case, no contribution from

"organic silicon" is detected even though the presence of free doublets on the amino species. The presence of primary or secondary amine does not seem sufficient to create the strong interaction detected previously between a silicon atom and the free doublet of the amino-groups.

4.2.4. Material properties

In order to understand the influence of the increase of the number of amines of the organic layer on the material properties, further studies are considered. Unfortunately once the bio-hybrid systems undergo the inevitable drying process, a huge loss of matter (loss of weight due to the removal of the main component: water) is observed and the experimental measurement procedure becomes impossible. The established strategy is to work without the biological part.

4.2.4.1. Materials synthesis

As mentioned before, studies on the properties of hybrid materials will be carried out partly on "white" materials (*i.e.* without cells). Identical molar concentrations of polyamines and silica precursor are mixed in culture medium (50 mL, 50 mM) and adjusted to pH=7.4. Left in reaction for 24 hours, the particles are removed from the reaction mixture by centrifugation (9000 rpm; 10 minutes) and washed in distilled water before being dried (40°C, ambient atmosphere).

4.2.4.2. Condensation degree of silica material

The condensation degree of the three silica materials are studied by solid-state NMR of ^{29}Si (Figure II-18).

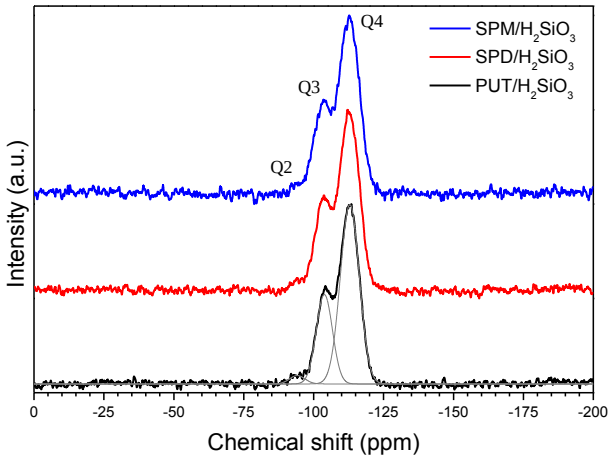


Figure II-18: ^{29}Si NRM spectra of the silica materials without cross polarisation

To analyse the previous spectra and determine the ratio of each silicon type, a deconvolution is realised using Gaussian. From the area of each contribution, the percentage of each species is calculated and presented in the Table II-6.

Table II-6: Silicon species taking part in the silica material (error = ±2.5%)

Samples	**Q_4 (%)** $Si(OSi)_4$	**Q_3 (%)** $Si(OSi)_3(OH)$	**Q_2 (%)** $Si(OSi)_2(OH)_2$
PUT	68.3	29.1	2.6
SPD	69.5	28.2	2.3
SPM	68.9	29.6	1.5

The silica particles synthesised are all characterised by silicon species from the intra-network (Q_4) and by silanols (Q_3 and Q_2). The three samples are mainly composed of $Si(OSi)_4$ structure that is the characteristics of a well-condensed material. In this case, the information confirms the form and the position of the silicon peak observed by XPS. The material is made of well-condensed silica materials. The only slight trend that can be deduced is the decrease of the Q_2 contribution that may cause a condensation degree increase with the number of amino-group. However, the measurement precision does not allow to take this observation for granted.

4.2.4.3. Porosity of the silica material

The properties of the silica materials synthesised are studied by nitrogen adsorption-desorption monitoring and the isotherms acquired are shown in Figure II-19a. In addition, the pore width distribution (Figure II-19b) is obtained from the desorption data using the BJH method.

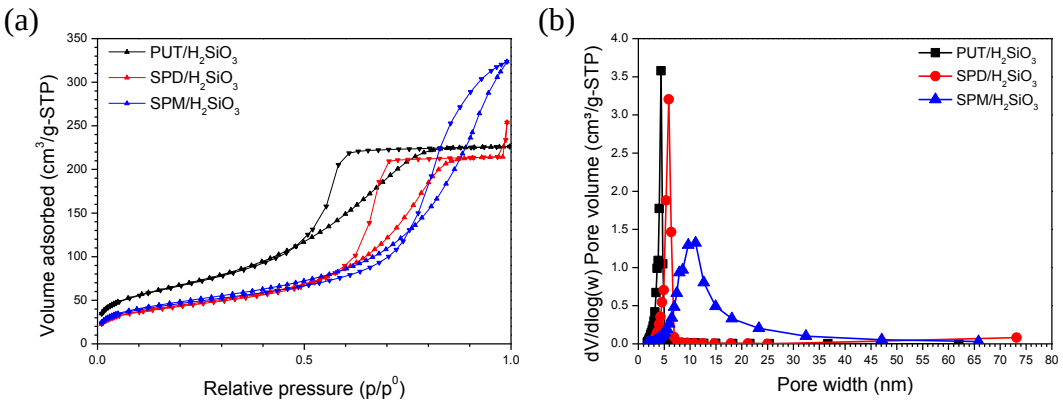


Figure II-19: (a) N_2 adsorption-desorption isotherms and (b) pore size distribution

The isotherms corresponding to the materials PUT/H_2SiO_3 and SPD/H_2SiO_3 are type IV. A small proportion of micropores is detected at the beginning of the isotherm, while the major contribution corresponds to the mesopores. This mesoporous property is confirmed by the adsorption plate. For these two samples, capillary condensation is accompanied by hysteresis. For the third sample (blue curves), the isotherm is closer to a macroporous material (type II isotherm), even though it still has some mesoporous characteristics (appearance of a light plateau for high pressures). The evolution of pore size distribution is also very specific to the characteristics of these materials. This evolves with the length of the molecules and the number of amines.

Table II-7: Hybrid material properties (porosity studied by N_2 ads/des)

Sample	**BET surface area (m^2/g)**	**BJH pore size (nm)[4]**
PUT/H_2SiO_3	251	4.4
SPD/H_2SiO_3	161	5.9
SPM/H_2SiO_3	175	10.3

Considering the evolution of the specific surface, we see that it decreases between the diamine and the triamine (Table II-7). The specific surface of the third sample (four amines) is a little higher than the second.

[4] The maximum contribution is taken into account.

Associated with the data of the previous studies, the results here show that the specific surface area decreases with the increase of the amine number. This is due to the aggregation of nanoparticles. Pore size increases as a result of the aggregation of uniformly sized silica nanoparticles. These studies have shown that natural polyamines influence aggregation and condensation. This is closely related to the size of their chain and the distance between amino groups [10, 11].

4.2.5. Viability of the encapsulated cyanobacteria

In order to monitor the effect of the encapsulation process of cell activity, two tests are performed. The first consists of monitoring photosynthetic activity over time (O_2 production), the second in studying the evolution of the cell population by measuring the optical density at 730 nm. These data are shown in Figure II-20.

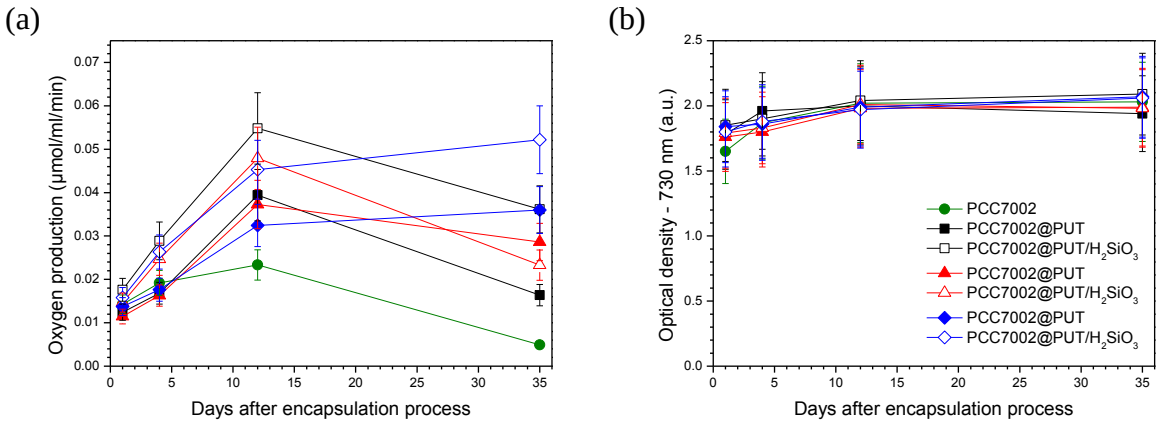


Figure II-20: Viability assay for native PCC7002, PCC7002@polyamine and PCC7002@polyamine/H_2SiO_3: (a) evolution of oxygen production and (b) optical density (one set of measurement: inaccuracy is depending on technical and experimental manipulations)

Focusing first on monitoring oxygen production, it is very clear that the addition of polyamines of all kinds boosts photosynthetic activity of cells. From day 12, native cells (green curves) have a lower production than all other samples. It is also interesting to highlight that the combination of polyamine/silica seems particularly favourable to the photosynthetic activity of cells. In this case, from the first day, the three samples show a higher oxygen production. If we refer to the evolution of the optical density, despite the cellular control which shows a slightly lower OD on day 1, all samples show the same tendency to reach a plateau after day 12. However, it is the material PCC7002@PUT/H_2SiO_3 that has the best activity after

35 days while oxygen production is higher for the system PCC7002@SPM/H_2SiO_3. The size of the amino molecule may influence cyanobacterial activity.

These data show that polyamines, deposited on the cell wall, do not have an adverse effect on cell activity. On the contrary, they seem to improve the photosynthetic activities of the cells. This effect is even more marked when polyamines is combined with silica. As described above, it is known that polyamines play certain roles in regulating biological processes. At this stage, the specific role of these molecules is uncertain. They increase perhaps the rate of chemical reactions involved in photosynthesis, or they can be a new carbon source for the cyanobacteria. At this stage, it is difficult to provide relevant hypothesis. Further investigations and studies in cell biology are needed.

4.3. Conclusions

This chapter has presented the use of polyamines of different sizes. The results showed that these molecules, which were smaller than the polymers used in the previous part, only induced a very low silica deposition.

The interesting point is that the size and amine number of these molecules led to different properties of the silica-based material in terms of porosity and degree of condensation. This seems to be a good initial point for controlling the properties of the silica nanoshell to build engineered materials.

Chapter 5 - Influence of polymer sizes

5.1. Polymer and microorganisms towards single cell encapsulation

The world of polymer chemistry and the world of microorganisms engineering meet in many ways. Thus, microorganisms are sometimes used to produce polymers [38], sometimes to degrade them [39]. Polymers are also widely used in cell encapsulation [40].

In this chapter, we will investigate the influence of the size of a polymer on the properties of bio-hybrid materials. Poly(diallyldimethyl ammonium chloride) (PDA) is selected for this study. This very cheap cationic polymer is already used for the engineering of silica materials [41] as well as cell encapsulation [42] and even for individual encapsulation [43, 44] but no studies on its effect on the bio-hybrid system (material properties or cell activity) are referenced.

5.2. Bio-hybrid system synthesis

5.2.1. Experimental procedure

The encapsulation process is the same as mentioned before. The same conditions are used: 2% w/w in polymer and 50 mM in H_2SiO_3. The layer-by-layer deposition is carried out after incubation, centrifugation and rinsing cycles.

The polymers with three different lengths are used, we will call them later: PDA_s (“s” for small), PDA_m (“m” for medium) and PDA_l (“l” for large). Their characteristics are shown in the table below.

Table II-8: PDAx properties

Abbr.	Molecular weight	Packaging	Supplier
PDA_s	< 100,000	Solution in water (35% w/w)	Sigma Aldrich
PDA_m	200,000-350,000	Solution in water (20% w/w)	Sigma Aldrich
PDA_l	400,000-500,000	Solution in water (20% w/w)	Sigma Aldrich

5.2.2. Morphological properties

Electron microscopy micrographs confirm the deposition of a silica-based nanolayer (thicknesses between 15 nm and 25 nm). The Figure II-21 shows that for each of the three

samples, the surface of cells is no longer as smooth as the surface of native cells, but presents folds. These are attributed to the silica coating.

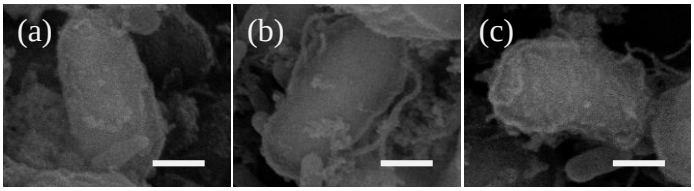


Figure II-21: SEM micrographs of (a) PCC7002@PDA$_s$: (b) PCC7002@PDA$_m$ and (c) PCC7002@PDA$_l$ (bars = 500 nm)

The observation of the slices by TEM confirms the presence of these silicate shells around the cyanobacteria in all three samples. The morphologies of this hybrid layer are similar in all three cases.

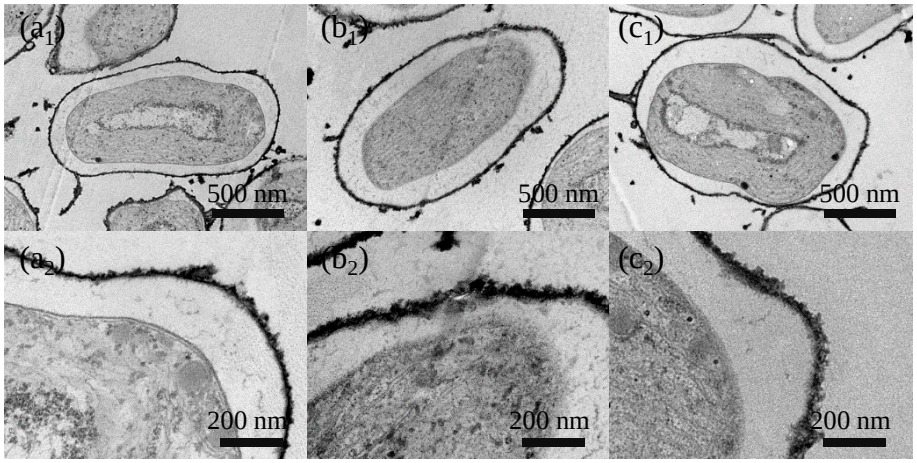


Figure II-22: TEM micrographies of (a) PCC7002@PDA$_s$: (b) PCC7002@PDA$_m$ and (c) PCC7002@PDA$_l$

5.2.3. Physicochemical properties of the shells

The chemical composition outside the materials is characterised using XPS. The results presented in Figure II-23 show the presence of silica on the extreme surface of the materials, confirming the composition of the nanoshell is indeed partly composed of silica.

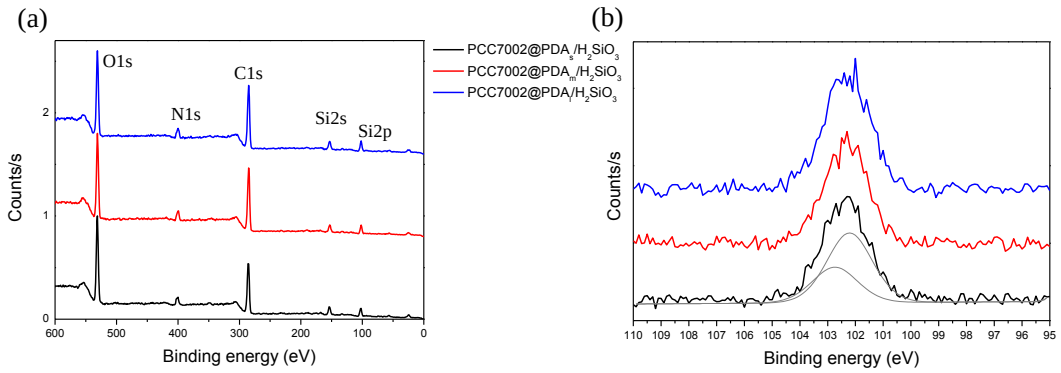


Figure II-23: (a) Outer chemical composition (XPS) and (b) Si2p peak for the three PCC7002@PDAx/H_2SiO_3 materials

The atomic ratios can be deduced from these spectra and are included in the Table II-9.

Table II-9: Atomic ratio of the main elements present in the external part of the bio-hybrid materials (error = ±2.5%)

Sample	Si/C ratio	O	C	N	Si
PCC7002@PDA$_s$/H_2SiO_3	0.14	33.67	51.80	7.46	7.07
PCC7002@PDA$_m$/H_2SiO_3	0.12	29.15	56.75	7.01	7.09
PCC7002@PDA$_l$/H_2SiO_3	0.15	29.24	55.79	6.72	8.25

The Si/C ratios do not follow any trend proportional to the size of the molecules. Nevertheless, the quantity of silicon seems to increase with the size of the polymers, the deposition would be more important according to this parameter.

5.2.4. Materials properties

For the following investigations, materials without the biological part has been synthesised again.

5.2.4.1. Material synthesis

Nanoparticles are obtained by precipitation of the silica precursor in the presence of our polycations of interest. The following conditions are used, the reaction mixture based on a culture medium is composed of 2% w/w of polymers, adjusted to pH 7.4 to which of H_2SiO_3 is added with a final concentration of 50 mM.

A 24-hour incubation, followed by three rinses in distilled water and drying at 40°C will allow the recovery of organic-inorganic hybrid materials.

5.2.4.2 Condensation degree of silica material

NMR spectra in direct excitation are obtained to monitor the influence of molecules size on the degree of silica condensation. From these spectra (Figure II-24), the ratios of each of the contributions of the condensed species are calculated and compiled in the following table.

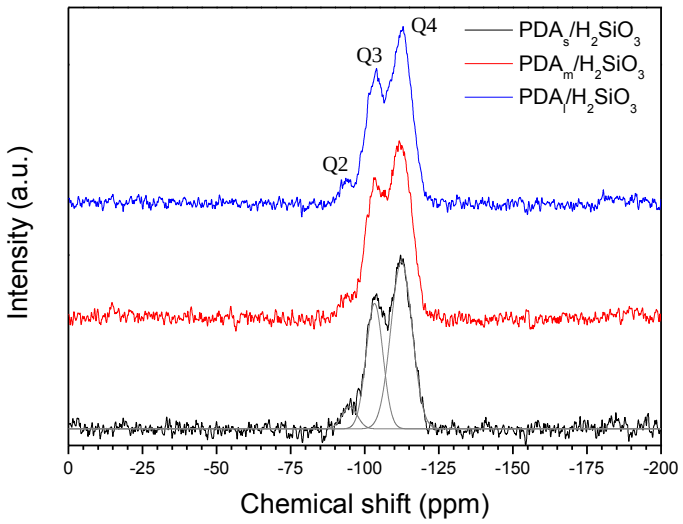


Figure II-24: ^{29}Si NRM spectra of the silica materials without cross polarisation

Table II-10: Silicon species taking part in the silica material. (Error = ±2.5%)

	Q_4 (%)	**Q_3 (%)**	**Q_2 (%)**
Samples	$Si(OSi)_4$	$Si(OSi)_3(OH)$	$Si(OSi)_2(OH)_2$
PDA_s/H_2SiO_3	59.2	35.0	5.8
PDA_m/H_2SiO_3	58.5	37.6	3.9
PDA_l/H_2SiO_3	57.1	38.1	4.0

Regarding the degree of condensation, the contributions of each species appear to be quite similar. Looking more closely at the contribution Q4 (completely condensed material), a slight decrease is observed with the increase in the molecular weight of the polymer. In the same way, Q3 contributions are increasing. Therefore, the size of the polymer seems to have a slight influence on the condensation of silica during the material formation process.

Despite the presumed absence of an electron pair free to interact with silicon atoms, we see here that the effect on the degree of condensation of the material is very similar to that in

the previous chapter and the use of polyamines. Unfortunately, it was not possible to highlight possible interactions between amino molecules and the silica network by nuclear magnetic resonance.

5.4.2.3. Porosity of the silica material

The next parameter concerns the textural properties of hybrid materials especially their porosity. The isotherms and BJH transformations are shown in Figure II-25.

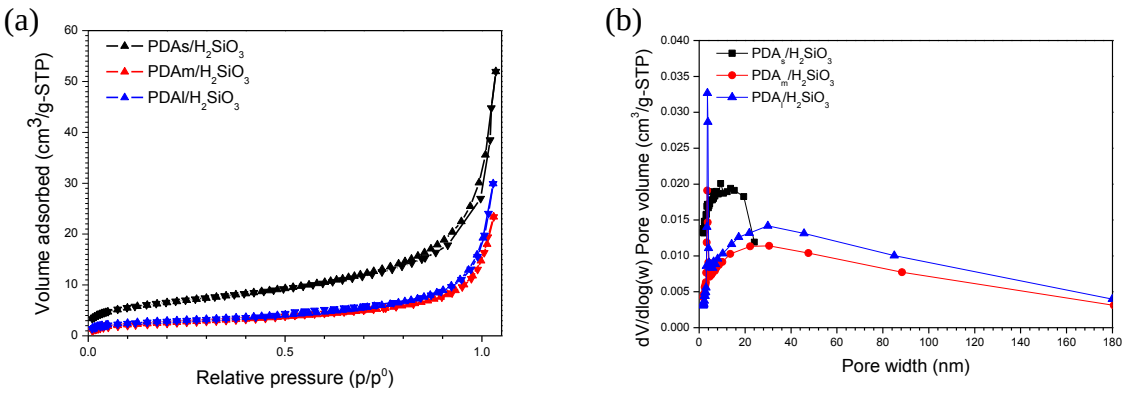


Figure II-25: (a) N_2 adsorption-desorption isotherms and (b) pore size distribution

These isotherms are type II and are connected to macroporous or even non-porous materials. However, as they are studied in-depth (see properties shown in Table II-11), additional contributions appear.

Table II-11: Hybrid material properties (porosity studied by N_2 ads/des)

Sample	BET surface area (m^2/g)	BJH pore size (nm)[5]
PDA_s/H_2SiO_3	24	Range from 1.8 to 24
PDA_m/H_2SiO_3	9	3.5
PDA_l/H_2SiO_3	11	3.6

The trend that the specific surface area decreases inversely with the size of the molecules seems to be confirmed. For medium to large polymers, the properties are very similar and indicate properties detected for the small PDA polymer. The folding or

[5] The maximum contribution is taken into account.

contributions of polymers therefore seem more coordinated and organised when the chain size reaches a certain limit.

5.2.5. Viability of the encapsulated cyanobacteria

The same approach as used in previous studies is applied here. The behaviour of the different synthesised bio-hybrid systems is compared and investigated. The evolution of photosynthetic activity and the cellular population are monitored by carrying out the same experiments as in the previous chapters.

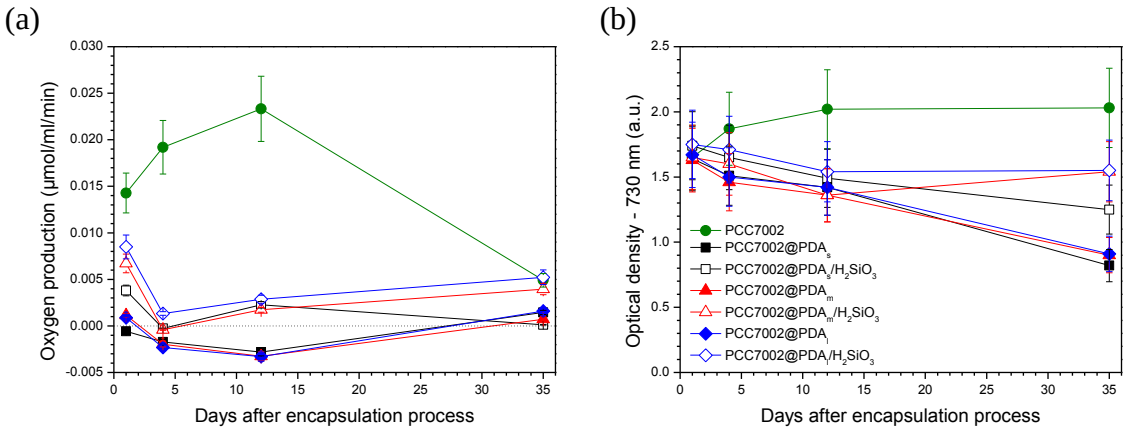


Figure II-26: Viability assay for native PCC7002, PCC7002@polymer and PCC7002@polymer/H_2SiO_3: (a) evolution of oxygen production and (b) optical density (one set of measurement: inaccuracy is depending on technical and experimental manipulations)

The data collected are presented in Figure II-26. The first visible thing is that the polymers used, regardless of their size, influence cell viability. On the first graph (evolution of oxygen production), three different behaviours are visible. First, the native cells show a rapid increase in activity from the first day, however, their activity decreased after day 12 probably due to a lack of nutrients. The second group includes cells covered only with polymers. Their oxygen production drops drastically from the very first day to even reverse the production trend. The cells then seem to breathe and no longer use photosynthesis to meet their energy needs. Finally, the third group concerns bio-hybrid materials that have been synthesised via the complete encapsulation process including silica. Despite a drop observed on the first day, stabilisation followed until a slow increase in photosynthetic activity. In this group, only sample PCC7002@PDA_s/H_2SiO_3 (black curve, hollow point), appears inactive after 35 days.

These groups are also found in the evolution of optical density. Stabilisation for native cells, a rapid decrease in materials without silica and finally a slower decrease or even stabilisation once silica precursor is added.

Several useful information can be extracted from this data. The first is that the silica layer reduces the harmful effect of these polymers. It is likely that the modification of the charge induced by the polycation layer modifies the exchanges with the outside thus modifying the behaviour of the cells. The addition of the silica layer (theoretically leading to a negative net charge) should stabilise these exchanges. The second information is that, in the case of polyamines, it seems that the larger molecules have either the most beneficial or the least harmful effect.

5.3. Conclusions

This part highlighted several points. The study consisted of using a linear polymer of different sizes to monitor the effect that this organic layer has on the hybrid material used for individual encapsulation of cyanobacteria.

The textural properties of the hybrid material depend on the organic part and can be modulated by changing the size of the polymers used. An important information is that this polycationic layer disrupts cell activity. Nevertheless, the effect can be modulated by adding the silica layer. We have highlighted here the usefulness of the two parts of the hybrid material that make up the nanoshell.

Chapter 6 – General conclusions

The purpose of this first part of the research work was to browse the synthesis of a range of bio-hybrid systems. A few manipulations have highlighted the relevance of using polysilicic acid as an aqueous precursor for the synthesis of our materials.

Then, the creation of a silica-based nanoshell was achieved using the layer-by-layer method and the sol-gel process for the polymerisation of silica. In this way, the first cationic layer could be put in place to counterbalance the negative net charge carried outside the cell wall by cyanobacteria. Following the addition of an aqueous silica precursor, these interesting prokaryotes were encapsulated in nanoshells.

Several polycations are then selected to be involved in the synthesis of the bio-hybrid materials. It appears from this study that the organic cationic part has a marked influence on the properties of the materials that make up the shell, but also on the material living in its entirety.

These studies showed that the nature of the cation seemed to have an influence on the silica network. Nevertheless, additional studies had to be carried out to go further. First, "small" polyamines were used to monitor the influence of the number of groups and size of molecules on the materials created. Studies have shown that these molecules have an influence on the properties of the material. First observations show that the porosity changes with the size of the polyamine and, further studies highlight that the photosynthetic activity seems to be boosted by the combination of the hybrid material (polyamine-silica). Secondly, it is the size of the polymer that has been studied. A linear polyammonium is used to study the influence of size, on the one hand, and the nature of cationic groups on the other.

The results highlighted confirm that the size of the polymer has a direct influence on the final properties of the material, both in terms of viability and textural properties. Nevertheless, no convincing results concerning the influence of the nature of the group on the silica network could be obtained despite the NMR spectra obtained.

This part has provided the basis for a method for the individual encapsulation of cyanobacteria and increases our knowledge of the mechanisms and influences involved in the individual encapsulation of our photosynthetic prokaryotes.

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Part III
Single cyanobacteria@silica through cell protection and use

Chapter 1 – Introduction

The design of new hybrid materials for cell protection and the use of the bio-hybrid systems for wastewater treatment will be described in this part of the manuscript. It should be useful to preface this part in order to clarify some choices. This part of the results were the first to be obtained. The synthesis and the studies of the following synthesised bio-hybrid systems have raised several questions regarding their properties, the cyanobacterial behaviour inside the hybrid shell, the modularity of the material properties that can be related to the process, the efficiency of this process, among others. This has led to explore further paths to discover trends and to improve our knowledge. The results obtained are described in the first part of the results (Part II of this manuscript) and present a fundamental approach. This second part of the results is focused on an approach towards the application of the bio-hybrid systems.

This part of the work therefore presents the genesis of the design of a method for constructing a silica-based nanoshell. It is designed in such a way as to provide protection for the cells. The additional point related to research on hybrid materials is their use. The shell must therefore not disturb the properties of cyanobacteria.

The following article presents the construction of a bio-hybrid system for the use of cyanobacteria in bioremediation and more specifically for the recovery of heavy metals.

An additional chapter is added to this work. It is about recycling the bio-hybrid systems for cyclic use related to heavy metals removal.

Chapter 2 - Article

Single cyanobacteria@silica porous microcapsules via a sol-gel layer-by-layer for heavy metals remediation

Cyrille Delneuville[a], Emeric P. Danloy[a], Li Wang[a*] and Bao-Lian Su[a,b*1].

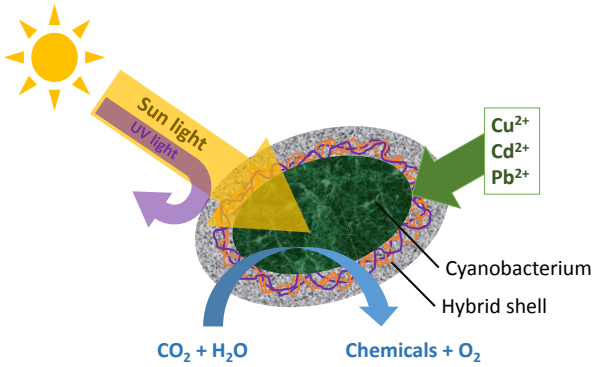
[a] Laboratory of Inorganic Materials Chemistry (CMI), University of Namur, 61 Rue de Bruxelles, B-5000 Namur, Belgium

[b] State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, 122 Luoshi Road, Wuhan, 430070, China

Highlights

- Individual cyanobacterium encapsulation within hybrid porous layers via a sol-gel method
- Bio-hybrid materials with UV-C irradiation resistance
- Bio-hybrid materials with a long-term viability
- Bio-hybrid materials for wastewater treatment

Key words Individual Cell Encapsulation, Silica, Cyanobacteria, Heavy Metals Remediation



Graphical abstract: Basic scheme representing the construction of a bio-hybrid system for cell protection and its use for heavy metal removal and potential photosynthetic production [2]

[1] * Emails : li.wang@unamur.be; bao-lian.su@unamur.be

[2] Note: This diagram highlights the potentialities of use of the encapsulated cyanobacteria. The photosynthetic activity represented in this diagram only present the production of metabolites excreted by the cells. The primary objective of this process (provide the cells with organic matter necessary for their activities) is omitted.

Abstract Heavy metal removal from industrial wastewater is a major issue. The use of microbial biosorbent is a low cost, sustainable and efficient way for water cleaning but the living organisms are limited by their sensitivity and fragility and subjected to water contamination by other relevant bacteria. In order to improve the use of the powerful photosynthetic prokaryote this work focused on the development of a new protection method of cyanobacterial strains of the genus *Synechococcus sp.* PCC7002 by a multiple coating including an external layer made of silica. This surfacing was performed using the layer-by-layer method and different polyelectrolytes. Polydiallyldimethylammonium chloride, a cationic polymer is used as a link between cell wall and silica shell while polystyrene sulfonate is inserted to play a protective role against ultraviolet light irradiation. This study discloses the synthesis of a highly efficient hybrid material and the excellent protection brought by the silica material. First of all, the layers, in particular four layers, deposited around single cells offer a strong protection against very harmful UV-C light irradiation while maintaining high viability and same bioadsorption capacity for heavy metal ions in solution. The long-term viability of this living material and its successful use for copper and lead sequestration, confirms the possibility of exploiting encapsulated cyanobacteria to improve current biotechnologies for wastewater purification.

INTRODUCTION

The last century has witnessed the rapid industrialisation. However, many problems have emerged following the progress. Especially pollution generated by certain activities that poses serious environmental problems. It is difficult to remedy heavy metals pollution generated by industries such as battery producers, mining activities, chemical industries (producers of fertiliser, catalysts, pesticides, etc.) and metallurgy. The large quantities of non-biodegradable scrap metals produced, some of them being toxic or even carcinogenic, are then directly or indirectly released in the environment [1]. For example, copper after passing through the blood, accumulates in the liver of human leading to cirrhosis and damages of the renal tubules or damaging brains and other essential organs [2]. It is well known that too much exposure to lead affects the nervous system and the brain function damages kidneys and liver and could result in loss of fertility [3]. According to the U.S. Agency for Toxic Substance and Disease Registry (ATSDR) cadmium accumulates in the liver, causing serious kidney problems while the exposure to cadmium fumes increases the risk of lung cancer.

Various techniques are currently used to treat wastewater in which these dangerous and toxic metals are increasingly found. The most widely method used is the chemical precipitation in which metal ions react with appropriate chemicals leading to insoluble residues (sludge) that can be recovered by simple sedimentation and from which the organic binders can be isolated and reused [4]. Ion exchange techniques are also used in this field: with strongly acidic synthetic resins (presenting sulfonic acid or carboxylic acid group) able to capture metal ions that can be released upon acidic treatment [5]. Filtration, electrodialysis, flotation, coagulation or electrochemical treatments have been also successfully used [6]. However, these more or less efficient methods still have certain limitations. Increasingly stringent regulations restrict the use of chemicals for metal ions removal. The effectiveness of most of these methods depends on different variables: contact time, temperature or pH are part of them. Other methods require a very important investment in expensive infrastructure or require very energy-intensive to recover polluting metals.

It is essential to develop clean, sustainable, low-cost and efficient strategies for wastewater treatment. The use of biological adsorbent is a very promising solution and attracts much attention. This fairly new process has undeniable advantages: the low cost of substrates, their efficiency in the removal of heavy metal ions and the rapidity of the adsorption process [7]. There are several sources of biosorbents that are already being used or in exploitation [8]. The first concerns all types of non-living biomass. Among these are peels, shells of all kinds (e.g. eggshell), sawdust, etc. which are potential biosorbents. Another source is biomass from algae. There is already much research on the adsorption of metal ions with algae. They have the advantage to be widely available with varied strains that have very good metal ions adsorption capacity. Bacteria also have very good characteristics for bioremediation. Widely known, these organisms can be easily genetically modified leading to the control of their abilities. In all bacterial kinds, cyanobacteria attract much attention. These photosynthetic prokaryotic organisms are found in all types of aqueous environment over sunlight exposure. Their cultivation is very simple with rapid growth and at very low cost. Compared to other microorganisms, cyanobacteria are considered to be very active. They have a very large contact surface and are known for their versatile properties [9, 10].

Despite the many advantages of using living organisms for wastewater treatment, it remains certain limitations. They are fragile and sensitive to contamination during their intensive exploitation [11]. Their use can be difficult because of poor control over the cell population as well as some difficulties in handling the cells and separating them from the

contaminated media or substrates. In the case of genetically modified organisms, serious precautions must be taken to avoid their release in outdoor water. Genetic drifts sometimes occurred and a loss of the characteristic of interest is observed after several generations. It is therefore essential to develop strategies to facilitate the use of these living organisms. They must be protected against physical and biological external aggression while confining them in a stable environment [12].

Since last decade a lot of work, with the ambition to protect living organisms, has been conducted [13]. One of the research axes developed taking inspiration from Nature has provided an abiotic protection to numerous living organisms against environment (mussels, sea shell, etc.) [14]. In nature, a range of unicellular organisms are able to synthesise protective shell (sponges, diatoms, etc.) [15, 16] which shows a highly hierarchical structure and allows to enhance the mechanical and biological properties of the cells. In this field, the idea of the synthesis of artificial protective shell for useful cells that are unable to do it by themselves appeared [17, 18]. The method used is generally called cell encapsulation. Like living organisms able of biomineralisation to build a physical barrier against aggressions, cellular encapsulation proposes to combine living organisms of interest with artificial protective materials. From beads [19, 20] to gels [21, 11], living materials made by the encapsulation have come a long way and open a new avenue for a large exploitation of biological cells with high potential in different fields. They can be used for the design of artificial organs, biosensors, for the creation of continuous flow bioreactors, etc. [22]. Immobilised biomass has already been used to adsorb heavy metals. First, dried bacterial cultures are immobilised in polysulfone-based structures and used for removal of various metal ions [23]. Phormidium laminosum nonliving biomass encapsulated in alginate beads has also been considered for Cu(II), Fe(II), Ni(II) and Zn(II) biosorption in a reusable material [24].

Focused on the idea of living material, it is possible to imagine the modification of cells in a nanometre scale and open a broad range of improved living biotechnologies. The design of specific shells around the cell wall can lead to the selection of the properties of the cell [25-28]. It becomes easy to imagine smart shells for smart cells. A control on the environment of the cells can enhance their abilities, improving their resistance against biotic and abiotic aggression but also allow a control on their activities. In the case of bacteria, the confinement in an individual shell removes the molecular interactions between them and allows to create artificial molecular messages [29, 16].

Following this pathway, cell surface engineering is defined at the beginning of the 21th century as a chemical approach in order to modify the surface chemistry of the cell walls [30]. One of the most used ways for the design of artificial cellular shells is the layer by layer method. By different steps of depositions, it is possible to create a controlled environment around the cell [31].

The present work focuses on the construction of a protective shell around cyanobacterium cell by the design of a silica layer. The presence of an artificial shell around cells is also a very important non-genetic way to modify the biological properties of cells [27]. Biomimicking diatoms, the use of sillafins for the synthesis of a very well-organised shell has been reported [15]. Here, the use of a cationic polymer and the layer by layer method for the deposition of silica around the cells have been investigated on cyanobacteria strain *Synechococcus sp.* PCC7002. This unicellular cyanobacterium is euryhaline and shows interesting properties [32] for use as depollutant owing to its great metal biosorption capacities [33]. Indeed, it is able to biosorb different heavy metals in an efficient manner. The protection created using the artificial silica shell is followed by comparison with free cells.

EXPERIMENTAL

Cyanobacteria cultivation

An axenic euryhaline strain of *Synechococcus sp*. PCC7002 is obtained from the Institut Pasteur (Paris, France). The culture medium used is that proposed by the Institut Pasteur, whose composition is available on their website: 1:1 BG11:ASN III with 1 µg dm^{-3} vitamin B12.

The liquid stock cultures were maintained at room temperature under visible light (cool white: Omsram lamps type L18W-840) with slow stirring (70 rpm) then were transferred into fresh culture medium on a monthly basis.

Innovative cyanobacteria encapsulation method

The method consists of the deposition of different layers by a so-called layer-by-layer process. This process deposits a cationic layer on the cell wall counterbalancing its negative charge and inducing silica deposition. Polydiallyldimethylammonium chloride (PDA) is chosen to be this essential link. Well-known as a biocompatible polymer, PDA is widely used in bioencapsulation [26]. In this work, the use of a second polyelectrolyte has also been investigated. Polystyrene sulfonate (PSS) is a polyanion that absorbs UV-light [34]. Inserting this molecule in the shell can bring a protection and act as a suncream against UV-light.

Sol-gel process allows to synthesise silica material in mild condition: room temperature, atmospheric pressure, in water at a neutral pH. The choice of the silica precursor is crucial. Silica can be produced in soft conditions using an alkoxide such as tetraethyl orthosilicate or trimethoxyl silane but the polycondensation of those monomers leads to the formation of toxic alcohol molecules (ethanol or methanol respectively) [13]. The use of polysilicic acid is the optimal way to follow to perform the synthesis of the silica shell [35]. The polycondensation of this precursor releases only water as a by-product. The orthosilicic acid was also the block used by diatoms to construct their wonderful exoskeleton [15].

Amberlite IR 120, H resin (Acros Organics) was used to obtain a polysilicic acid precursor via a $Na^+ \rightarrow H^+$-ion exchange of sodium silicate. 150 g of the resin was washed with 1L HCl solution (pH 2) and cooled in the freezer (4°C). A sodium silicate solution (0.1 L, 1.5 M, Assay 25.5-28.5%, Merck, 4°C) was mixed with the resin and vigorously shook. The resulting silicic acid solution, recovered after filtration and possessing a pH of 2.3 is kept at 4°C and used in the following 4 hours.

Solution (2% w/w) of polyelectrolytes is obtained from dilution in culture medium containing PDA (20 % w/w in water from Sigma Aldrich) and PSS (18 % w/w in water from Sigma Aldrich). The silica precursor solution is made of 50 mM polysilic acid solution. After pH adjustment to pH=7.4 using a solution of HCl 0.2 M (Sigma Aldrich) or of NaOH 0.2 M (Duchefa Biochemie), the solutions are sterilised on syringe (Minisart 0.22 µm).

The layer-by-layer deposition is realised by a cyclic method. Cells (4 days after the transfer) are harvested by centrifugation (2,500 rpm; 20 min) and suspended in PDA solution. After 30 min of incubation cell pellets are obtained by centrifugation (2,500 rpm; 10 min) and rinsed by fresh culture medium. At that stage and depending upon the case those cell pellets are suspended (i) in silicic acid solution followed by similar treatment as above leading to a two-layered hybrid material in which silica is the covering layer (PCC7002@PDA/SiO_2) or (ii) sequentially dipped in PSS, PDA and silicic acid solutions following the same protocol as above to provide a four-layered hybrid material with a silica layer as out-layer (PCC7002@PDA/PSS/PDA/SiO_2). Figure 1 represents a schematic view of the synthetic process. Those hybrid materials containing living cells are kept in fresh culture medium for a final cell concentration of around 25.10^6 cells/mL.

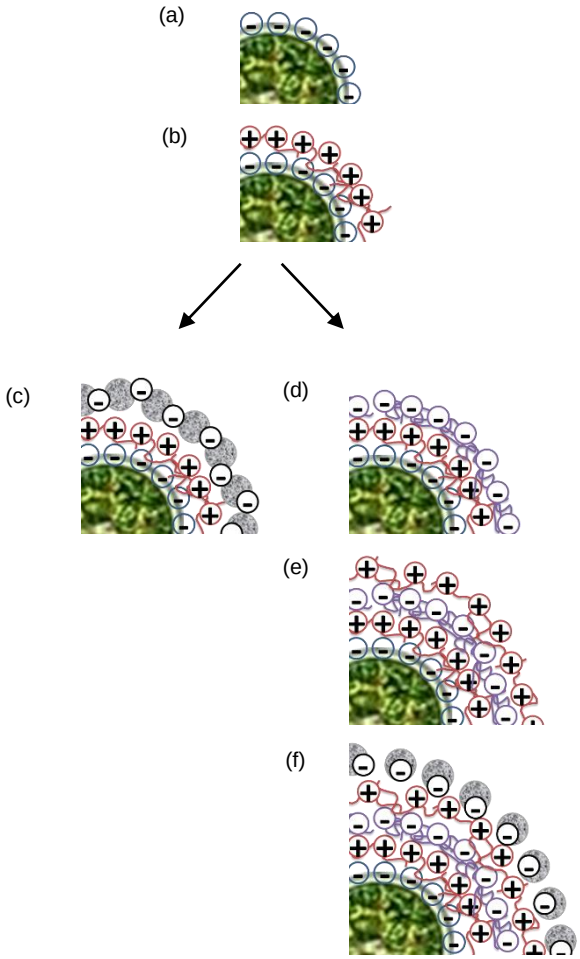


Figure 1: Schematic view of the layer by layer process: (a) free cyanobacteria; (b) addition of PDA; (c) addition of H_2SiO_3 : first hybrid material synthesised (2 layers) : cyanobacteria@PDA/SiO_2; (d) addition of PSS; (e) second addition of PDA; (f) addition of H_2SiO_3 : second hybrid material (4 layers) : cyanobacteria@PDA/PSS/PDA/SiO_2

Characterisation techniques

Material properties

The layer by layer process is followed by the evaluation zeta potential using a Zetasizer Nano ZS from Malver Instrument. The charge carried by the cell indicates the modification of the outside of the cyanobacteria. This procedure was performed in phosphate buffer saline solution (PBS) with a concentration of 0.1 M and a constant pH of 7.4 ± 0.1 after rinsing cells with 10 mL of the previous mentioned buffer.

The morphology and the shape of the deposition are observed using scanning electron microscopy (SEM) with a JEOL (JSM-7500F) microscope. Before microscopic observation the samples were prepared as follows. The samples were dehydrated using ethanol baths following a concentration gradient (25 - 100% ethanol) before undergoing a supercritical

drying. This preparation is essential in order to remove water from cells and pores without damaging the structure.

Transmission electron microscopy (TEM) was also used to investigate the deposition of silica on the cell wall using a TECNAI 10 microscope. Sample preparation in this case consisted of dehydration, fixation of biological material prior to embedding the sample in a resin. After centrifugation the cell pellets were suspended in distilled water and fixed with 2.5% glutaraldehyde in a sodium cacodylate buffer water solution (0.1 M, pH 7.4) and left to incubate overnight in the fridge (4 °C). The samples were then rinsed with the buffer solution (cacodylate 0.2 M, pH 7.4) and postfixed in a 1% OsO_4 solution overnight. The samples were washed with the cacodylate solution and dehydrated in ethanol baths with a concentration gradient (0 – 100 % ethanol). The dehydration is followed by washing in propylene oxide before being embedded into an epoxy resin LX112. Ultrathin sections were made with an ultramicrotome and were finally contrasted with uranyl acetate and lead citrate.

X-ray photoelectron spectroscopy (XPS) was performed to determine the outside chemical composition of the hybrid materials. Spectra are obtained from analysis performed on a spectroscope from Escalab (250Xi).

Viability and protection

Cells viability was studied by monitoring their photosynthetic activity. A Clark electrode (Pt/Ag - Oxy-lab manufactured by Hansatech) was used to monitor the oxygen concentration in a closed system. A milliliter of cell suspension of free or encapsulated cells was used for the measurements in a pre-calibrated system. After the addition of a $NaHCO_3$ solution (10 µL, 0.6 M), the system was flushed with nitrogen to remove any atmospheric oxygen and thus measured only O_2 production by the cells and the chamber subsequently sealed to exclude natural light fluctuations. Measurements were acquired under stirring (80 rpm) with an applied external light source (λ = 650 nm, 1200 µmol m^{-2} s^{-1}) to stimulate photosynthesis.

Viability of the cells is also followed using fluorescent labelling with fluorescein diacetate (FDA; 1 ppm) after 30 minutes of incubation with the samples and two rinses with bidistilled water. Micrographies are obtained using a microscope Multizoom AZ100 (Nikon) coupled with a camera (DSRi, Nikon). The fluorescent images (536/40 nm) are acquired under and excitation light of 482/35 nm.

The behaviour of the encapsulated cells placed in non-optimal mild and aggressive conditions was studied. A photoreactor was used to put the cells under UV-C light (centred

around 253.7 nm from 4 Philips lamps type TUV 8W G8T5) and under slow stirring (70 rpm). By comparing the photosynthetic activity of free cells with encapsulated ones, it was possible to determine the efficiency of protection conferred by the silica coating. The photosynthetic activity was investigated using the same protocol as described above for the Clark electrode.

Heavy metals removal

In order to study the removal capacity of the synthesised hybrid materials, they were exposed to contaminated water. The encapsulated and free cyanobacteria were harvested by centrifugation the day after the encapsulation process. Their supernatant were replaced with medium supplemented by 10 ppm of heavy metals. After a set period, the amount of heavy metals in the supernatant was determined by atomic absorption spectroscopy (AA7000 manufactured by the Shimadzu Company) and the value was compared to the original amount in order to track the biosorption of heavy metals by the as-synthesised hybrid materials.

We have also carried out control experiments using the same protocol on non-encapsulated cells in suspension.

RESULTS AND DISCUSSION

We have measured the zeta potential of the coated cells after each step (Figure 2) and found a significant change of the cell surface charge with a coating of different layers. The strain of cyanobacteria used in our study presents a negative zeta potential (-0.67 ± 0.1 mV) as disclosed in the literature [33].

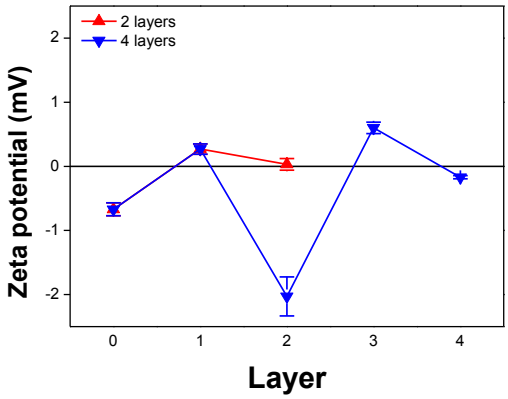


Figure 2: Zeta potential by step: 2 layers : Cyanobacteria@PDA/SiO_2; 4 layers : Cyanobacteria@PDA/PSS/PDA/SiO_2

The coating of the layer of PDA leads to a charge reversal from -0.67 ± 0.1 mV to 0.28 ± 0.08 mV. After the deposition of the silica layer, the charge of the outer layer of material

varies from positive to zero. This means that for the first material coated by two layers (PCC7002@PDA/SiO_2), the silica shell stabilises the charge with a potential close to zero.

For the second material (PCC7002@PDA/PSS/PDA/SiO2) coated with four layers, coating with the second layer (PSS) leads to a reversal of charge (from 0.28 ± 0.08 mV to -2.03 ± 0.03 mV) and coating with the polycation PDA contributes to the change of the charge to the potential of 0.6 ± 0.09 mV. This change of charge sign facilitates negatively charged silica deposition from the silicic acid solution that induces the last charge inversion: -0.17 ± 0.03 mV. The results confirm that by using different electrolytes in both materials the modification of the external properties of cyanobacteria cell walls occurs.

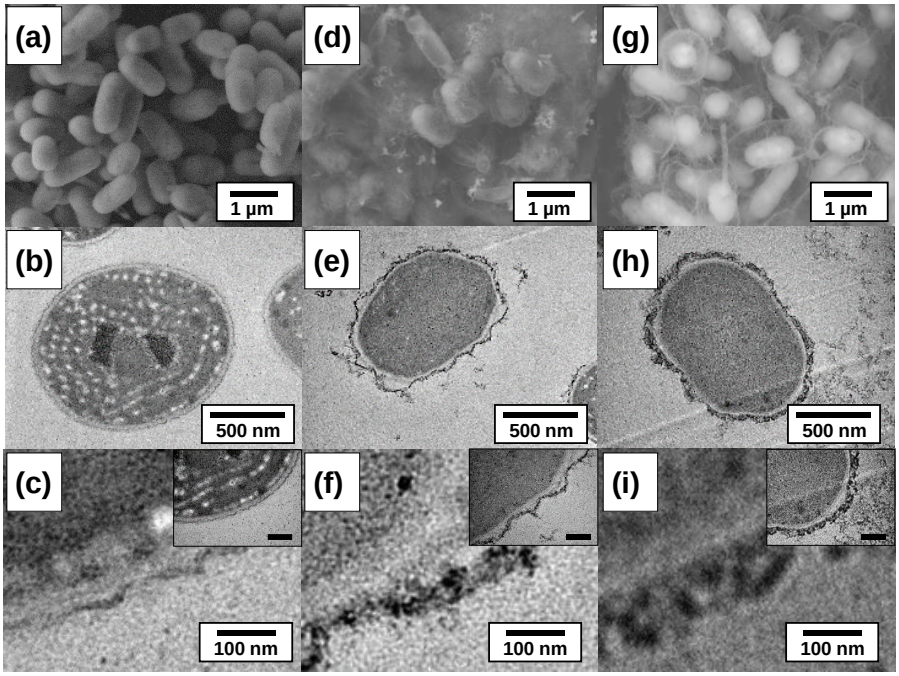


Figure 3: Micrographs of (a), (b) and (c): Free cells; (d), (e), (f) : 2 layers; (g), (h), (i): 4 layers

The textural properties of the shell are studied using electron microscopies (Figure 3). Compared with the native cells, the micrographs of the two materials confirm the presence of a shell around cells after individual encapsulation. According to the SEM micrographs (Figure 3a, b, c), the native cells show smooth surfaces (Figure 3a) and an intact cell wall (Figure 3b, c). Micrographs (Figure 3d, e, f) obtained from the material composed of two layer shells (PCC7002@PDA/SiO_2) reveal an extracellular morphology change of the cells. Thin shells are observed by SEM (Figure 3d) and TEM (Figure 3e), confirming the deposition of a very uniform shell around the cyanobacteria. The enlarged micrograph discloses the thickness of the

uniform layer is around 15 nm (Figure 3f). Micrographs (Figures 3g, h, i) obtained from the material composed by four layer shells (PCC7002@PDA/PSS/PDA/SiO2) show the smooth cell walls covered by a cloud-like layer (Figure 3g) and TEM images (Figures 3h, i) evidence the presence of the shells whose thickness range from 20 to 30 nm. Both the external coating of two layers and four layers could be formed by the aggregation of silica nanoparticles (Figure 3f, i and the inserted images), giving an interparticles porosity to guarantee the diffusion of the substances from outside of the shells to inside or the diffusion of metabolites from inside to outside of the shells.

These observations confirm the successful synthesis of bio-hybrid materials composed of a native cell as a core surrounded by porous layers in both materials. By correlating these results, it appears that a link between the charge and the deposition of silica exists. The polymeric multilayer of the second material induces a more positive charge than in the case of a single polycation layer before adding the silica precursor. The micrographs (TEM) reveal a difference in the thickness of the silica layer. A cationic charge therefore improves the polymerisation of the polysilicic acid around the walls of the cells, leading to a thicker layer of silica.

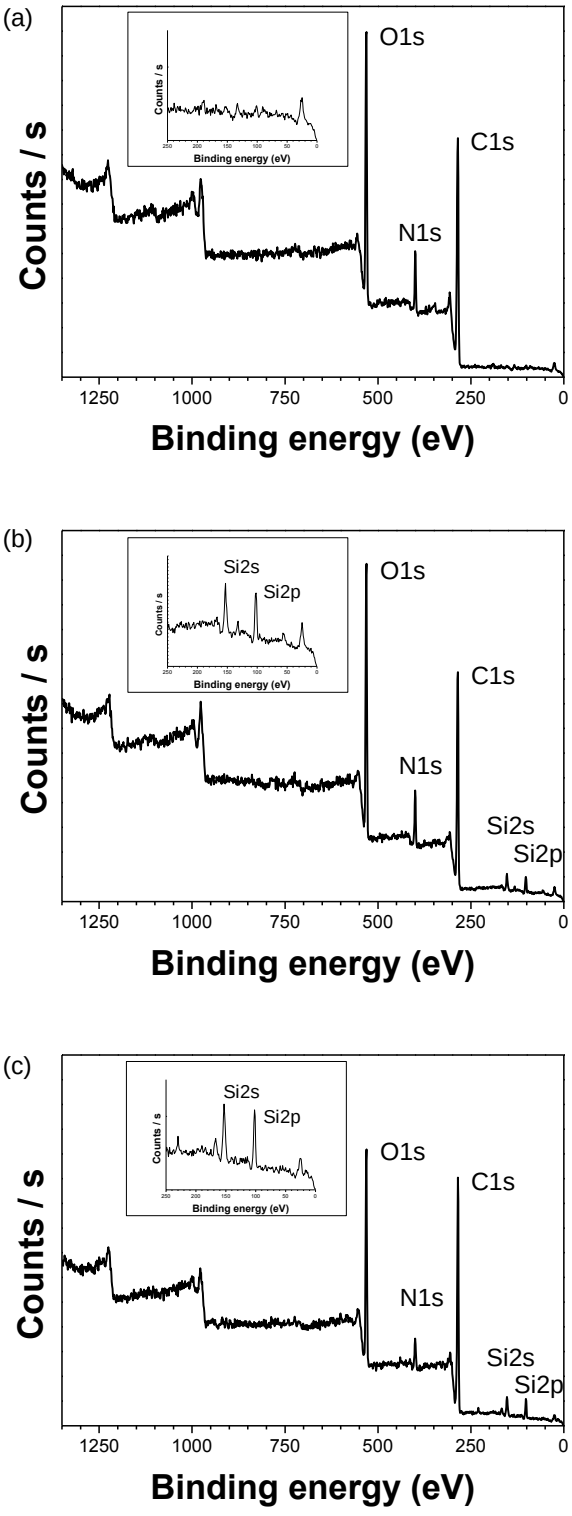


Figure 4: XPS spectra of (a) free cells; (b) 2 layers material; (c) 4 layers materials

The surface chemical composition of the materials was subsequently studied using XPS (Figure 4). In these experiments native cells are set as a control experimental group. As expected, the spectrum obtained after measurements on dried free cells reveals no detectable

trace of silicon and consequently confirm that before the encapsulation treatment there is no silica around or inside the cell wall. After cyanobacteria encapsulation with two and four layers, a peak related to silicium (Si2p) can be detected at around 103.5 eV (Figure 4a, b, c and the inserted images), meaning the presence of silica. In order to make a comparison with the three samples, the ratio between silicium and carbon is calculated. The absence of silica around native cells led to a ratio of zero. The first material presents Si/C ratio around 0.08 as well as the material obtained following four layers-deposition synthesis. These XPS measurement allows to conclude that the shell is composed of silica and confirms the observations by SEM (Figure 3a, d and g) and TEM (Figure 3).

The analysis above confirms the efficiency of the layer-by-layer process. Zeta potential proved the electronic interaction of each layer, while electron microscopies and XPS confirm the presence of a silica-based shell around the cyanobacteria. The second process (four steps) allows the insertion of aromatic groups and more silica in the shell.

It was essential to illustrate that the process of individual cell encapsulation is not deleterious to cells. The viability of cells is studied using oximetry to measure the production of oxygen by cells. The production of oxygen under a red light influence will be a direct indication of the photosynthetic activity of encapsulated materials and we compare the encapsulated cells to that of the native cells.

The curve (Figure 5) which shows the activity (oxygen production) of native cells is quite similar to a conventional growth curve [36]. A rapid increase in oxygen production is observed during the first 10 days. This could correspond to the proliferation of cyanobacterial population diluted in fresh medium. After 30 days, a plateau begins to appear which indicates the cell population is starting to stabilise.

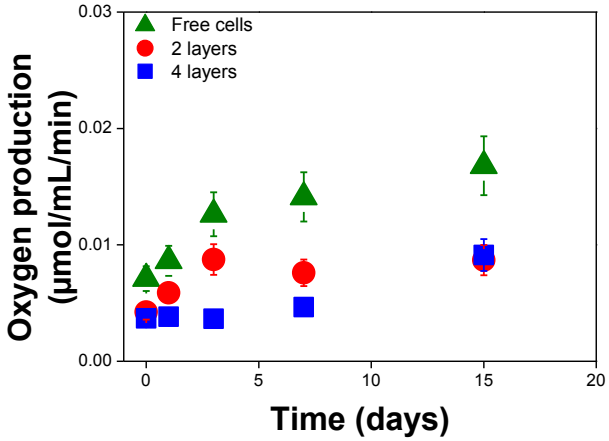


Figure 5: Evolution of the oxygen production of native cells and both hybrid materials

For the bio-hybrid materials, the photosynthetic activity is stable at the beginning of the cultivation, especially in the case of the materials obtained following the deposition of four successive layers. The synthesised shell plays a role in the stabilisation of the cell population. However, after the first week, rapid growth is observed. The process of cell encapsulation does not completely confine cells and only partially controls cell activity. The detection of photosynthetic activity in the three cases confirms that the encapsulation process carried out in the cells do not lead to any damage on the viability.

This part study shows that less than 20 days, cells are well confined in the electrolyte-silica layers. A longer time study reveals that after one month, the photosynthetic activity of both bio-hybrid materials is the same as the native cell showing a cell division in the three samples. It seems that a break of the coated layer is happening due to the growth of cells. The layer of 15 nm and 20-30 nm seems not to be thick enough to limit the growth of cells. A thicker layer should be needed to keep intact external shells around one single cell.

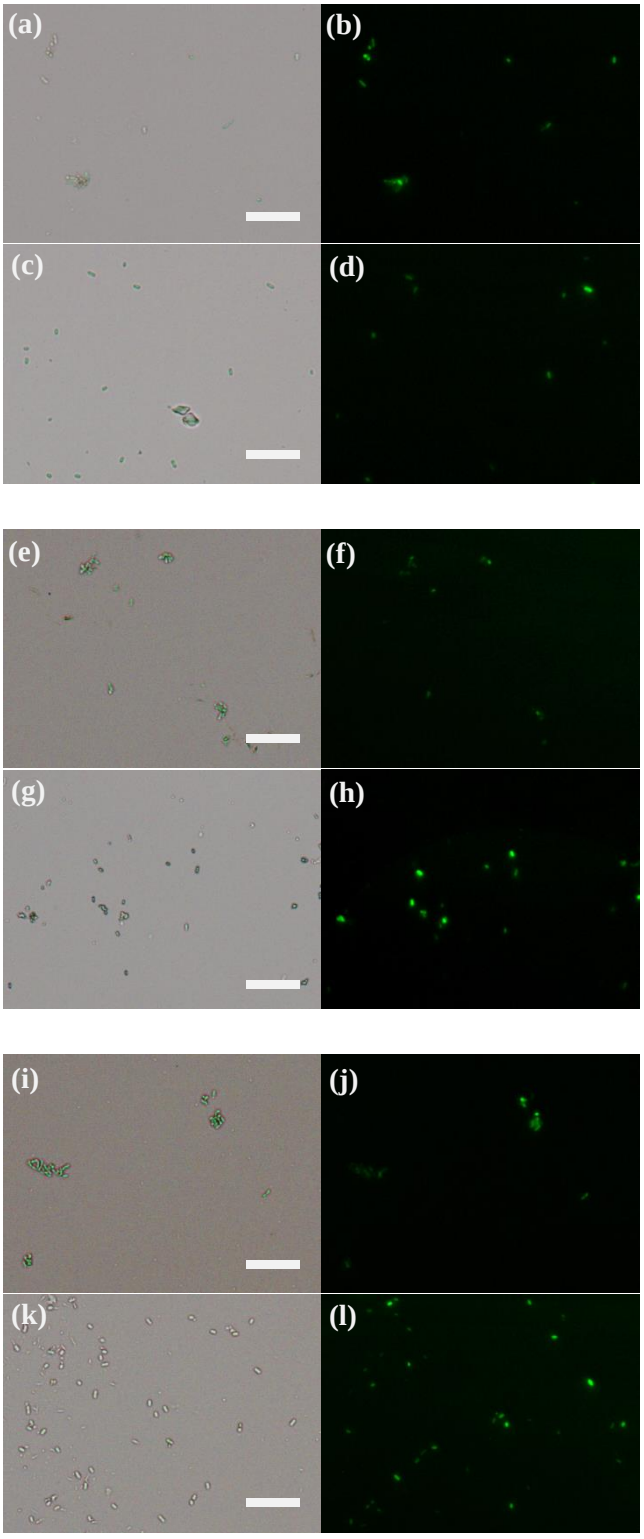


Figure 6: Micrographs of cells in bright field (BF) and fluorescence label (FDA) after incubation with fluorescein diacetate (FDA) dye : (a) BF of native cells (day 1); (b) Native cells labelled (day 1); (c) BF of native cells (day 50); (d) Native cells labelled (day 50); (e) BF of two-layered encapsulated cells (day 1); (f) Two-layered encapsulated cells labelled (day 1); (g) BF of two-layered encapsulated cells (day 50); (h) Two-layered encapsulated cells labelled (day 50); (i) BF of four-layered encapsulated cells (day 1); (j) Two-layered encapsulated cells labelled (day 1); (k) BF of four-layered encapsulated cells (day 50); (l) Four-layered encapsulated cells labelled (day 50)
(Bars = 250 µm)

The effect of the process on the viability is followed using a fluorescent dye (FDA). The Figure 6 presents optical micrographs of the three samples. It demonstrates the viability of native (Figure 6a-d) and encapsulated cyanobacteria (two-layered material: Figure 6e-h and four-layered material Figure 6i-l) is similar no matter one day or fifty days after encapsulation. The viability of cyanobacteria is not negatively influenced by the encapsulated procedures that almost all cyanobacteria can survive after the coating processes.

In addition to confining cells, the shell serves the purpose of protecting cyanobacteria from external aggression. In nature, some bacterial strains are able to synthesise sugars to precipitate dissolved carbonates to protect themselves from deleterious ultraviolet radiation from the sun [37]. These bacterial colonies thus form so-called stromatolites. However, the selected strain is sensitive to the effect of UV-C which inhibits the photosynthesis by breaking their photosystems. The multilayered shell can then protect the cells creating new interfaces that are able to absorb a part of the deleterious light. This protection can be reinforced by the presence of PSS in the four-layered hybrid material, due to the aromatic groups that play the role of absorbents of the UV-C light. The effect of C-type ultraviolet on PCC7002 is measured by following the photosynthetic activity (oxygen production) of those hybrid materials before and under UV-C radiation. The percentage of this activity is a direct reflection on the protection related to the materials synthesised around the cells.

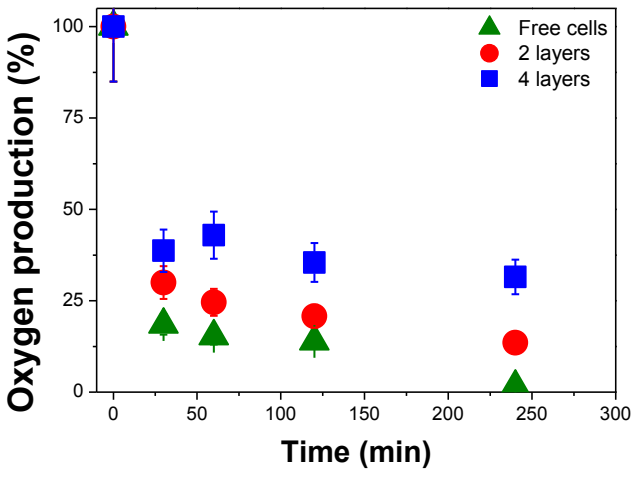


Figure 7: Evolution of the oxygen production of native cells and both hybrid materials under UV-C light[3]

[3] Note: As described above, the oxygen production of the samples at the starting point (before UV lamps are switched on) varies despite a similar optical density. The followed values were measured: 0.0112 µmol/ml/min for the free cells, 0.0065 µmol/ml/min for the two-layered material and 0.0044 µmol/ml/min for the four-layered material. In order to have a point of comparison, these values are considered as a maximum of activity (100%) of each of the cyanobacterial populations with or without encapsulation. To study the behaviour of the samples under ultraviolet light, it was chosen to compare these results in percentage terms.

The destructive effect of radiation on the native cells of cyanobacteria is visible from the first minutes (Figure 7) and after 30 minutes, only 20% of the photosynthetic activity of the native cells is conserved, meaning an important loss of more than 80% of the photosynthetic activity. The activity decreases then gradually with increasing irradiation time to zero after 240 minutes irradiation. However, under the same conditions, around 30% of the activity is maintained in the case of the two-layers hybrid material (PCC7002@PDA/SiO_2). The activity also decreases gradually with irradiation time. After 240 minutes irradiation, only around 15% of initial activity is found. However, over 40% is still maintained after 240 minutes irradiation, showing a strong protection effect of four layers coated on cells. For the four-layers hybrid material (PCC7002@PDA/PSS/PDA/SiO_2), the first minutes of irradiation are also destructive. However after the first minutes decrease in activity, the activity remains practically constant.

This study demonstrates very clearly that firstly the strong destructive effect of UV-C light irradiation at first minutes irradiation and secondly the strong protective effect of the layers coated on cells and finally more layers and layers containing aromatic groups give significant protection effect against UV-C light irradiation.

The various observations made after the synthesis of two living hybrid materials confirm that the layer-by-layer method and the exploitation of the sol-gel process make possible to obtain a shell around cyanobacteria type *Synechococcus sp.* PCC7002. The synthetic route not only does not interfere with the photosynthetic activity of cells but also induces a strong protection of cells against the harmful effects of ultraviolet light of the type C. This strain is also known for its surface reactivity [33].

The native and encapsulated PCC7002 are then placed in contact with different metals during the process: Cd^{2+}, Cu^{2+} and Pb^{2+} (from metal chloride purchased from Sigma Aldrich). The evolution of the concentration of these ions over time is followed by atomic absorption from a starting concentration of 10 ppm and presented on Figure 8.

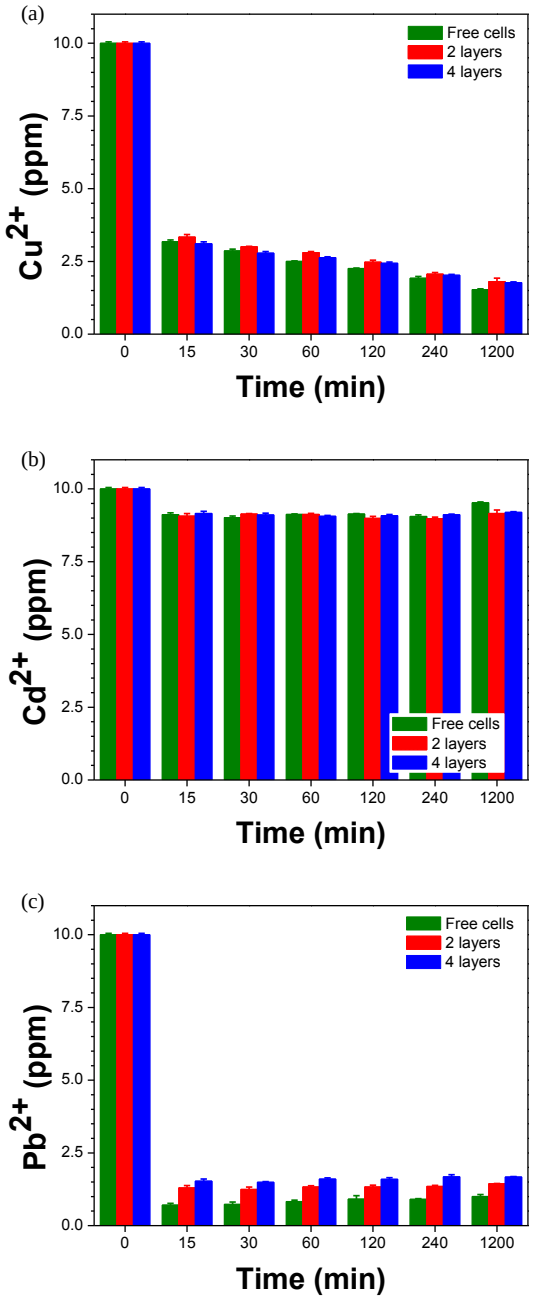


Figure 8: Metal removal abilities for native cells and both hybrid materials (a) Cu^{2+}; (b) Cd^{2+} and (c) Pb^{2+}

In the case of copper ions (Figure 8a), the biosorption is relatively similar to the three samples studied. A sharp adsorption of metal ions is observed during the first 15 minutes (7 ppm consumption by the cells) followed by a slow sorption during the following hours, almost similar to the three cases. Therefore, the ability of biosorption by the cyanobacteria is not affected by the encapsulation process.

In the case of cadmium ions (Figure 8b), the biosorption is not significant (less than 1 ppm) for all the three samples studied. The small difference observed after 20 hours in the

experiment with the native cell that seems to release a part (0.35 ppm) of cadmium bioadsorbed does not appear to be significant to be discussed.

The case of lead ions (Figure 8c) resemble that of copper ions (Figure 8a), the biosorption is relatively similar to the three samples studied. A sharp adsorption of lead ions is observed after the first 15 minutes (around 9 ppm of removal).

The results are promising. They confirm that the strategy developed allow the protection of living cells from very harmful ultraviolet radiation without affecting their capacity to capture heavy metals. This is an important step towards the exploitation of this type of living material. The layers coated on these cells give another strong advantage about the protection of cells from any pathogen attack. The layers coated on cells offer additional protection of cells from cell-cell adhesion and their aggregation which will affect the growth behaviour of cells and can lead to specific death of cells [38].

CONCLUSION

Bio-hybrid materials have been designed with protection from ultraviolet radiation, while the synthesis chemical route of a silica shell around individual cells has been discussed. It has been shown that the strategy used to build these shells has been possible to use the layer-by-layer method and the sol-gel route to obtain the hybrid materials under mild conditions (pH = 7.4, ambient temperature, atmospheric pressure). Textural studies confirmed that a coating was effective around the cells, allowing to modify the external composition of PCC7002.

Their successful use for wastewater treatment has been confirmed by comparative experiments that clearly show that the original properties of cyanobacteria are not affected by their encapsulation. These results make it possible to envisage the use of bio-hybrid system for the treatment of polluted water and to use the strategy disclose to further modulate and even improve their properties .

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COMPLIANCE WITH ETHICAL STANDARD

The authors declare that they have no conflict of interest. This article does not contain any studies with animals or human participants performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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Chapter 3 – Recycling process

The last part of the previous chapter demonstrates the possibility of using synthesised bio-hybrid materials for the removal of certain metal ions. At this point, their potential recyclability must be proven to show their ability to release metals. From this point a recycling system will be set up.

3.1. Recycling by time

The first set of tests of recyclability are related to a recycling process in the function of the time. The aim is to study the retention of metals by the bio-hybrid systems and their reuse after several days.

3.1.1. Experimental

The manipulation and studies follow the scheme previously carried out. The encapsulation procedure is executed according to the same experimental procedure as in the previous chapter. Three different samples considered: native cells and bio-hybrid systems composed of 2 and 4 layers. The samples to be studied are diluted to a final optical density at 730 nm with a value of 1.

The cells are then transferred to media (BG11:ASNIII 1:1 vol) containing 10 ppm of each of the metals to be analysed. After 30 minutes of incubation, the cellular pellets are isolated by centrifugation (2,500 rpm; 5 minutes) and the amount of metal ion is measured in the supernatant. In order to recover the metal ions biosorbed, the pellets are resuspend in the same volume of distilled water and left under agitation for 24 hours of incubation. Cells are separated from the supernatant by centrifugation (2,500 rpm; 5 minutes). The metal ion concentration of this second supernatant is analysed. The cellular pellets are then resuspended in medium supplemented by the heavy metals (10 ppm). This process is repeated for five cycles. All the solutions harvested (washing water and supernatant initially containing 10 ppm of metals) are analysed to quantify the amount of metal ions. This experimental procedure allows to follow the recycling abilities of the bio-hybrid systems synthesised as well as the native cells (free cells).

In total, each of the three metals, are analysed at nine different moments: cycles 1 to 4 (supernatant and wash water) and cycle 5 (supernatant only). These moments are spread over 5 days.

The amount of metal ions is analysed by atomic absorption as described in the first part of this manuscript.

3.1.2. Results

The results are shown in the following figure (Figure III-1). The cycles (1 to 5) parts are described as follows: "S" for starting supernatant solution and "W" for washing water.

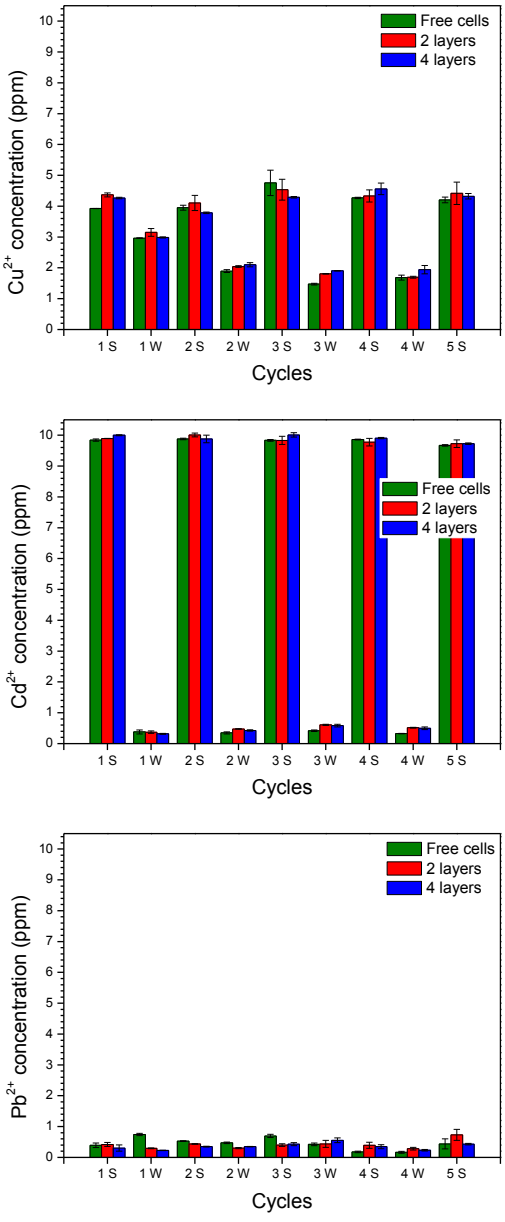

Figure III-1: Recycling by time: metal ion concentration after 5 cycles (S represent the supernatant (initially 10 ppm of metal) and W represent the "washing water")

Several information can be obtained from these data. The behaviour of each of the samples seems quite similar, however, the behaviour towards metal ions varies according to their nature.

The results of the concentration of copper ions show that the release of a certain amount occurs over time. Indeed, around 6 ppm can be removed after 30 minutes and can be partially recovered as shown by the detection of more than 3 ppm in the washing water. As the cycles progress, the samples always accumulate a similar amount of metals, but produce slightly less over time. We can therefore imagine that an additional process than surface adsorption occurs in the case of copper. However, these results show the feasibility of the reuse process for Cu^{2+} ions.

The results obtained in the case of cadmium were quite predictable because of the low biosorption capacity of this ion by cyanobacteria. The small quantity biosorbed is returned directly to the washing water. This shows the low interactions between Cd^{2+} and our samples.

Finally, when focusing on the lead ions removal abilities of the cyanobacteria, the high biosorption capacity of cyanobacteria appears again. Despite rinsing, only very small quantities are returned in the solution. After 5 cycles, it seems that cyanobacteria are able to biosorb more than 50 ppm of this metal ion. Based on those results, it seems that a simple washing is not sufficient to recover the biosorbed metal amount. Another strategy must be implemented.

3.2. Recycling after washing

The reactivity of the surface of microorganisms and their biosorption property depends on several parameters such as surface groups and their shape. Thus, a chemical modification of biomass makes it possible to improve the recovery capacities of metal ions from certain bacteria [1]. One way to recover some biosorbing metals from the surface of cells is to modify the shape of the chemical groups on the surface by adjusting the pH of the solution [2-4]. The surface reactivity being indeed dependent on this parameter, the equilibrium will be modified. This is the case for our cyanobacteria PCC7002 [5].

Previous studies have focused on the immobilisation of cyanobacterial biomass for the biosorption of heavy metals. This biomass is immobilised in polymer beads. The strategy for recycling material is an acid wash [6]. This strategy will be adapted in our case of study.

3.2.1. Experimental

Three samples are being studied for the removal tests of the three metal ions.

Following the same strategies of the previous recovery test, several cycles (a total of 5) will be applied to the samples. The steps of each of the cycles are: (i) the addition of 10 ppm of metal ions; (ii) a washing with a hydrochloric acid solution (0.01 M) and finally (iii) a rinsing with distilled water. The same volumes are used for each of the three steps, thus not altering the cell concentration. These three-step cycles are repeated 5 times. The separation of cell pellets and supernatants is carried out by centrifugation (2,500 rpm; 5 minutes).

3.2.2. Results

The results are shown in Figure III-2. As before, for clarity reasons, the cycle steps are represented by "S" and "W". Between this is inserted “acid washing” symbolised by the letter "A".

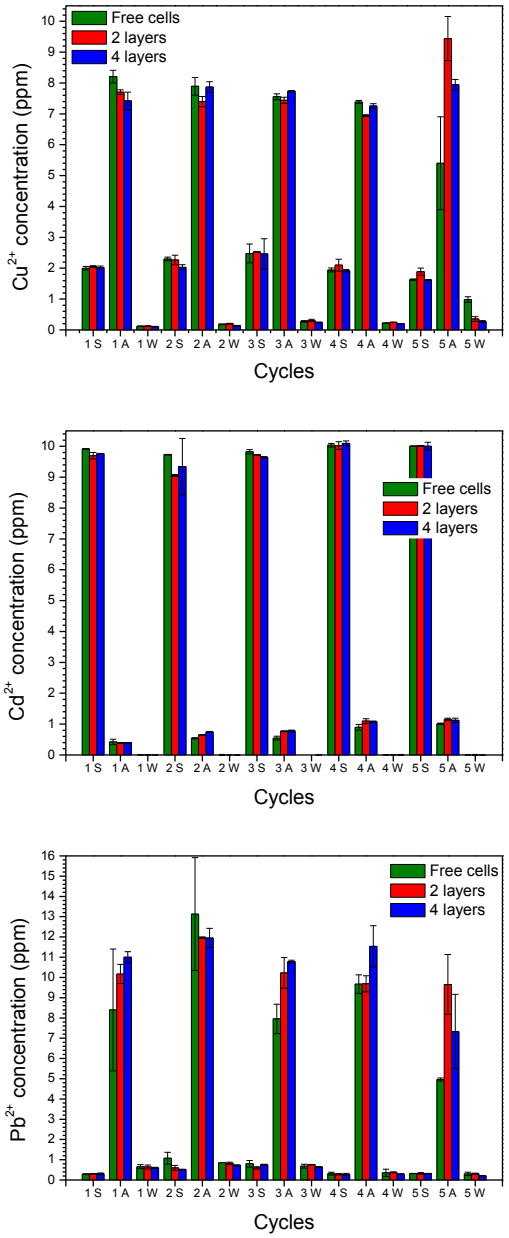

Figure III-2: Recycling by slow acidic solution: metal ion concentration after 5 cycles (S represent the supernatant (initially 10 ppm of metal), A represent the acidic washing solution and W represent the "rinsing water")

Following this acid washing treatment, it is possible to recover almost all copper ions (see first graph). These results are consistent with what had previously been shown where washing with water was sufficient. In this case, however, bio-hybrid systems retain their properties until the 5th cycle, when the free cells "return" only part of what they have biosorbed, while the bio-hybrid systems continue to have interesting capacities to be washed.

The behaviour of cyanobacteria towards cadmium ions (Figure III-2 - second graph) is identical: very little removal and very low cleaning abilities. Acid cleaning does not make cyanobacteria more suitable for cadmium ions removal.

Finally, in the case of Pb^{2+}, the usefulness of acid washing is demonstrated by the results shown in the third graph. From the first cycle, most lead ions are biosorbed (1 S). The 10 ppm (and more) are recovered by acid treatment (1 A) while a very small residual amount is recovered after washing with water (1 W). It should be noted that the observed concentration difference (above 10 ppm) is probably due to the device precision and/or residual lead between cycles. In this case, for most cycles (except the second one), recovery is more important during acid washing for bio-hybrid materials than for free cells. Cellular integrity seems to be better preserved when a nanoshell is present. Nevertheless, the size of the cells makes it difficult to observe them in optical microscopy. This hypothesis could not be confirmed.

Chapter 4 – Conclusions

The purpose of this part of the research work was to complete an entire process: from encapsulation method design, through the studies of the protection brought by the hybrid shell and finally the use of those systems for heavy metal removal. The nanoshell constructed through the layer-by-layer process provides protection for cyanobacteria under certain conditions. Meanwhile, this surface modification did not interfere with the properties of the cells and always allowed their exploitation.

The article in Chapter 2 highlights some interesting points. In this case, the cyanobacteria were protected from harmful ultraviolet radiation thanks to the engineering of this silica-based shell. The addition of organic groups within the material extends the survival of cells even longer under these dangerous rays. Then, it was proven that these materials could still be used in the case of wastewater treatment. This is the case for heavy metals that have been chosen in this section. Nevertheless, during this work, some points remain uncertain. The properties of the materials, as well as the efficiency of the process and its impact on the cells, must be studied and tested.

Additional experiments have shown that it is possible to reuse these materials. In the case of cadmium ions, it is possible to recover the small biosorbed amount by simply washing with distilled water. Copper ions can be partially recovered from a distilled water bath, but this process is not completely effective. Acid treatment improves recovery. Finally, in the case of lead, a washing with a slightly acidic solution is necessary. It is indeed recognised that the reaction properties of the cell surface can be modified by adjusting the pH of the solution.

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Part IV
Individual encapsulation of cyanobacteria towards cell functionalisation

Chapter 1 – Foreword

Surface engineering aims to provide cells with a new exterior. The purpose of this modification is bringing the cells with new properties in order to improve their activity or to equip them with additional functions. This section will present a novel encapsulation method for cell surface engineering towards the design of new hybrid materials. These new bio-hybrid systems will be synthesised through the design of organically modified silica (ormosils) network and the use of organosilanes. The interfaces between the cell surfaces and silica will therefore be controlled via the chemical groups added on the periphery of the silica material as well as the modification of the exterior of the bio-hybrid system.

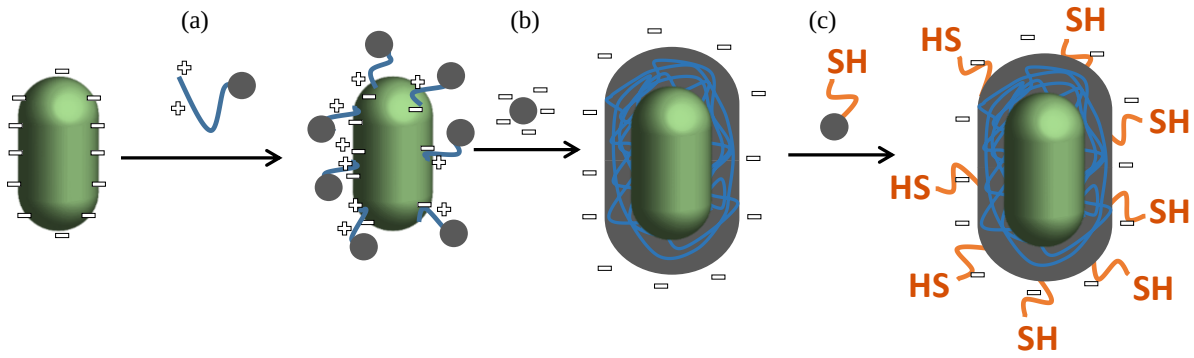

Figure IV-1: Scheme representing the synthesis strategy of a single cyanobacteria@ormosil system from native PCC7002: (a) addition of cationic organosilane, (b) addition of the silica precursor and (c) addition of (3-mercaptopropyl)trimethoxysilane

Chapter 2 – Article
Organically modified silanes for single cyanobacterium encapsulation and functionalisation

Cyrille Delneuville[a], Cassandra Toni[a], Emeric P. Danloy[a], Li Wang[a*] and Bao-Lian Su[a,b1*].

[a] Laboratory of Inorganic Materials Chemistry (CMI), University of Namur, 61 Rue de Bruxelles, B-5000 Namur, Belgium

[b] State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, 122 Luoshi Road, Wuhan, 430070, China

Highlights

- Organically modified silanes for single cell encapsulation
- Encapsulated cyanobacteria within porous shells
- Encapsulated cyanobacteria with long-term viability
- Encapsulated cyanobacteria with multiple functionalisation

Key words Single Cell Encapsulation, Cyanobacteria, Silica, Organically Modified Silanes, Multiple Surface Functionalisation

Abstract The use of microorganisms has increased in recent years for the development of highly selective biotechnologies. Cyanobacteria, one of prokaryote microorganisms, show good performance in this field because of their excellent photosynthetic abilities. These prokaryotes have been used as versatile factories for the synthesis of metabolites of highly added value. However, limitations due to their fragility still remain when they are used intensively (sensitivity to changes in their environment, demand for viability in artificial surroundings, etc.). It is therefore essential to improve their properties to facilitate their use. A chemical pathway to provide cells with new properties is the surface modification by artificial mineralisation of these cells.

[1] * Emails : li.wang@unamur.be; bao-lian.su@unamur.be

In this work, the cell surface has been engineered by organically modified silanes. At first, amino silanes, including (3-aminopropyl)trimethoxysilane, [3-(2-aminoethylamino)propyl]trimethoxysilane and 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane have been anchored to link the cell exterior to induce the formation of a silica matrix around the individual cyanobacterium. Furthermore, thiol-grafted silane ((3-mercaptopropyl)trimethoxysilane) has been exploited to introduce a sulfhydryl functional group outside the silica shells. The properties of encapsulating materials and encapsulated cells were studied in detail. By disulphide bonds formations, it is possible to link the cells with functional materials. The magnetisation of the cells has been made possible through the decoration of the encapsulating shells by magnetic nanoparticles. The modification of the cell surface also availed the graft of cyanobacteria on thiol-decorated glass slides for improved use.

INTRODUCTION

The current environmental, economic and social challenges call on the development of clean and sustainable technology [1]. The use of microorganisms meets these demands and attracted increasing attention. The high performance, low energy-consuming, low costs, flexibility, efficiency and specificity of microorganisms [2] make them suitable candidates for the design of biosensors [3, 4], biocatalysts [5], bioreactors [6] or for cell therapy [7, 8]. However, the use of these living microorganisms presents certain limitations because of their fragility and sensitivity to environmental variations. The improvement on the viability, resistance against harsh conditions and multiple functionalities of microorganisms is highly demanded for their efficient applications.

Single cell encapsulation is an emerging bioinspired technique of cell surface engineering. Like the protective shell structure of diatoms [9, 10], a coating can be constructed around an individual cell, leading to single cell encapsulation, which not only protects the cell from harsh environment, but also offers them with advanced functionalities without compromising their original viability and activity [11-13].

Nevertheless, the design of this shell is also subject to many constraints. The synthesis procedure as well as the chemical composition of shells of the latter must not be harmful to the life of the cells and its composition must not be cytotoxic. In addition, the shell must have controlled permeability and be transparent, to maintain the photosynthetic activity of the entrapped cell.

Currently, the materials for single cell encapsulation involve organic and inorganic components. Pure organic layers which are mostly composed of polymers can be formed around the cells by covalent bonding, hydrogen bonding or electrostatic force. This first layer induces the deposition of inorganic precursor to form a stable multilayered and multicomponent shell.

The advent of inorganic-organic hybrid materials has led to the revolution of material science [14-16]. These materials possess both advantages of inorganic and organic components. Hybrid materials present a good mechanical resistance and chemical stability due to the presence of the inorganic network, while they are malleable and functionalised because of their organic part.

As a versatile scaffold for hybrid material, silica has been considered as a promising choice for the design of living hybrid materials [17, 18]. This inorganic oxide can be synthesised using the sol-gel pathway [19]. Silica presents these great characteristics for combination with the living world: resistance, transparency and versatility. With the advent of organically modified silanes, inorganic-organic hybrid materials can be easily obtained. Since the beginning of research in 1985 [20, 21], many works proved the potential of those hybrid molecules. The rising of sol-gel science and technology led to the ormosils (organically modified silica) appearing as powerful precursors for material design [22-24].

In the context of single cell encapsulation, organosilanes can serve as a precursor of shells. They are composed of two parts and can link the whole cell and the abiotic shell material [25]. First, the organic part can be anchored on the surface of living cells. The inorganic precursor (methoxysilane group) is then involved in the construction of a silica scaffold. The controlled design of these interfaces is easy to implement via a layer-by-layer (LbL) process [26-28]. It is finally possible to manage the interface of the living bio-hybrid with its environment to artificially provide it with new functional exterior [13].

Cyanobacteria type *Synechoccocus sp.* PCC 7002 type (PCC7002) were selected for this work. As photosynthetic prokaryotes, cyanobacteria show promising perspectives in a wide range of fields such as energy, environment and genetic engineering. PCC7002 have been already used as a cellular model for various studies [29-31]. These cells show significant potentialities with their versatile structuration and functionalisation. Their encapsulation present various applications including cell storage, cell protection, cell imaging, etc. The encapsulation procedure consists of an optimised LbL process. First, amino silanes were added

into the cell suspension. Interaction including ionic bonding, hydrogen bonds, or low interaction type Van der Waals occurs between the chemical groups outside of the cells and the charged amino groups of the organically modified silanes [32, 33]. Subsequently, the amino group-containing silica induces the condensation of silica precursor around the cells to form a thick layer. Finally, the silica surface at the encapsulated cells could serve as a substrate to graft organically modified silanes for the post-functionalisation [28, 34].

This work demonstrates that ormosils are advantageous basic pieces in the design of living system such as single cell@silica. Three materials of type PCC7002@amino-silica were obtained. A fourth bio-hybrid system was synthesised through the post-functionalisation on the surface of the hybrid system by thiol group.

MATERIALS AND METHODS

Cell culture

The cyanobacterial strain *Synechococcus sp.* PCC 7002 was obtained from the Pasteur Culture Collection of Cyanobacteria (Institut Pasteur, Paris, France). Cyanobacteria were cultured in aqueous medium (BG11:ASN III 1:1 supplemented with 1 µg.dm^{-3} of vitamin B12) under visible light (cool white: Omsram lamps type L18W-840) with gentle shaking (70 rpm) at room temperature. Transfers are made on a monthly basis.

Experimental

Organically modified silanes for single cell encapsulation

Three amino silanes are selected: (3-aminopropyl)trimethoxysilane (APTMS); [3-(2-aminoethylamino)propyl]trimethoxysilane (AEAPTMS) and 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane (AEAEAPTMS), with a mono-, di-, and triamine respectively (Figure 1a,b,c).

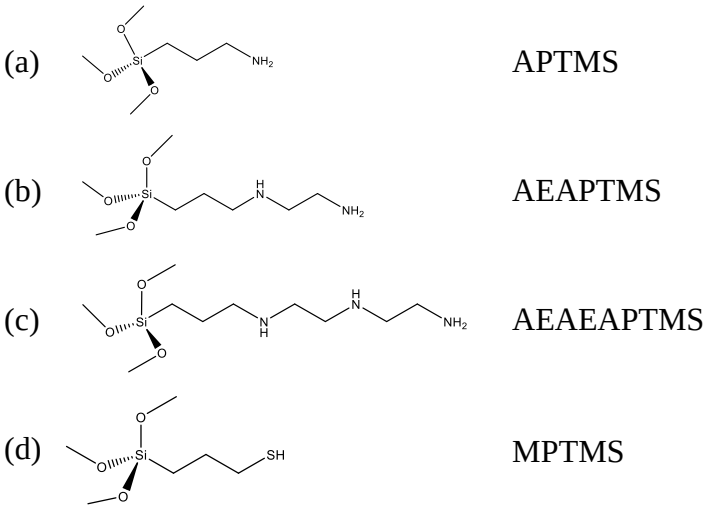

Figure 1: Selected organically modified silanes for single cell encapsulation

Aqueous silica precursor processing

Silica precursor was obtained via an ionic exchange reaction. First, 150 g of Amberlite IR 120, H resin (Acros Organics) were washed with 1 L HCl solution (pH 2) and cooled in the freezer (4°C). Then, a sodium silicate solution (0.1 L, 1.5 M, Assay 25.5-28.5%, Merck, 4°C) was mixed with the resin and subjected to vigorous shaking for 5 minutes. The Na^+ ions of the sodium silicate were exchanged by the H^+ ions of the resin, thus aqueous silica precursor solution with a pH value of around 2.0 ±0.3 was obtained. This silica precursor solution was further filtrated, then stored at 4°C and used in the following 4 hours.

Synthesis of ormosils

Organically modified silica networks were synthesised in the culture medium of cyanobacteria. Small amounts of amino silane (50mM, from 97% solution purchased from Sigma Aldrich) and the above obtained aqueous silica precursor solution (50 mM) was added in the aqueous solution to synthesise organically modified silica (ormosils). In order to control the hydrolysis and condensation of the precursors of modified silica, the pH of the reaction mixture was adjusted by adding HCl and NaOH (0.2 M). The reaction mixture was subjected to a gentle stirring for 24 hours. The synthesised ormosils were harvested by centrifugation (9000 rpm; 10 min), washed three times with bidistilled water and dried.

Single cell encapsulation procedure

Encapsulated cyanobacteria with hybrid shells were obtained via the layer-by-layer method. Amino silanes and aqueous silica precursor were used alternatively three times to synthesise the modified silica-based shells around the cyanobacteria.

Cyanobacteria were cultured for four days, harvested by centrifugation (2,500 rpm; 20 min) and washed with fresh medium. Then, cells in culture medium were mixed with amino silanes (1% w/w) and then incubated for 15 minutes under gentle stirring (stirring plate; 60 rpm) for the deposition of the first "scaffold" layer. After harvesting by centrifugation (2,500 rpm; 10 min) and washing with fresh culture medium, the cyanobacteria modified with silanes in the culture medium were mixed with aqueous silica precursor (50 mM) and then incubated for 15 minutes for the deposition of an additional silica layer. All the cell surface modification steps mentioned above were repeated three times, resulting in the encapsulation of cyanobacteria with hybrid ormosil shells. Finally, the encapsulated cyanobacteria were resuspended in fresh culture medium and placed in similar culture conditions to native cyanobacteria.

Post-grafting of the shell with thiol group

A thiol-grafted silane (3-mercaptopropyl)trimethoxysilane (MPTMS) (Figure 1d) was selected for grafting a sulfhydryl group outside the amino modified silica shell of the encapsulated cells. Cyanobacteria encapsulated cyanobacteria in hybrid shells formed by three double layers in the culture medium were mixed with MPTMS (1% w/w) and incubated for 15 minutes for thiol-grafting. The obtained thiol-grafted encapsulated cells were harvested, washed and placed in the fresh culture medium for the following analysis and functionalisation.

Functionalisation of the encapsulated cyanobacteria

Functionalisation of the encapsulated cyanobacteria with magnetic nanoparticles

Magnetic nanoparticles (Fe_3O_4, NPs) were synthesised following the protocol published in literature [35].

1.08 g $FeCl_3.6H_2O$ (Sigma Aldrich) and 0.39 g $FeCl_2.4H_2O$ (Sigma Aldrich) were added into 50 mL of ammonia solution (0.70M, Normapur). After 5 minutes of agitation, the solution was placed in falcons and left for 10 minutes. The black/brown sediment was harvested, washed with a solution of $HClO_4$ (60 %, Sigma Aldrich) and bidistilled water for three times, and placed in 50 mL of water.

For functionalisation, in a clogged Erlenmeyer, the suspension of Fe_3O_4 nanoparticles was diluted 20 times in denatured ethanol (99%, TechniSolv® from VWR). The pH was increased to 8-9 using an NH_3 solution (25%, purchased from Normapur). The reaction mixture was supplemented with 100 µL of MPTMS and then placed under mechanical stirring for 3

hours. The nanoparticles were harvested by centrifugation (9000 rpm; 10 min), rinsed three times with bidistilled water, and stored in bidistilled water for further use.

For the magnetisation of encapsulated cyanobacteria, 15 mL of encapsulated cells culture was mixed with 1 mL of magnetic nanoparticles suspension. After 30 minutes of incubation, magnetised encapsulated cyanobacteria were harvested by centrifugation (10 min; 2,500 rpm).

Functionalisation of the encapsulated cyanobacteria with selective adhesion

The protocol used is from the literature [36]. Microscope glass slides (purchased from VWR) were cleaned by a flame (4 times on each side) and then placed in a solution of HCl (4.7 M, Sigma Aldrich) and H_2O_2 (4.2 M, Acros Organics) for 24 hours for additional cleaning and the activation of surface silanols. Then, these glass slides were washed with bidistilled water twice and dried under nitrogen flow.

The activated slides were treated with MPTMS (97% w/w) for grafting thiol groups. The typical detailed process is described as follows: firstly, the side of the glass slides to be functionalised was covered with drops (50 µL) of MPTMS. Then, the glass slides were placed in a vacuum oven at 100°C for 30 minutes, finally placed in toluene and sonicated for 15 minutes. The recovered glass slides were further rinsed and stored in the refrigerator (4°C) in methanol (99%, Acros Organics) for the following use.

The immobilisation of thiol-grafted cyanobacteria was performed by the introduction of the modified glass slides in modified cells culture. After an incubation of one hour, the slide was washed with fresh culture medium and sonicated for 30 seconds in order to remove all the non-linked cyanobacteria.

Characterisation

Textural and structural investigation by N_2 adsorption/desorption, TEM and SEM

The ormosils materials were dried at 40°C before analysis. The porosity was characterised by nitrogen adsorption/desorption using a surface area and porosity analyser (TriStar by Micromeretics). The samples were degassed at 90°C overnight before analysis. The surface area was calculated by the Brunauer-Emmett-Teller (BET) method (0.05-0.30 p/p_0 range) and the pore size distribution was obtained by using the Barret-Joyner-Halenda (BJH) model from the desorption isotherm. The morphology and structure were studied by transmission electron microscopy (Philips Tecnai 10). Direct Excitation (DE) Magic Angle

Spinning (MAS) solid ^{29}Si Nuclear Magnetic Resonance (NMR) was applied to study the chemical environment of the silicon atom in the formed network. The NMR spectra were recorded by a Bruker Avance-500 spectrometer at 11.7 T and 8.000 Hz spinning with a 4 mm Bruker probe.

Transmission electron microscopy was also used to study the material around the encapsulated cyanobacteria. For the specimen preparation for TEM, cells were harvested by centrifugation, fixed with glutaraldehyde solution (2.5% in a sodium cacodylate buffer (0.1 M; pH 7.4, Sigma Aldrich)) overnight at 4 °C. Subsequently, the samples were rinsed with buffer (0.2 M cacodylate, pH 7.4) and post-fixed in 1% OsO_4 solution overnight. Then, the samples were washed with the cacodylate solution and dehydrated in baths of ethanol solution (with a gradient concentration from 25% to 100%). The dehydrated samples were washed with propylene oxide (>99%, Sigma Aldrich) and embedded into LX112 epoxy resin. The sample-containing resins were cut using an ultramicrotome and are contrasted using uranyl acetate and lead citrate, forming the specimen for TEM observations. The morphology of the silica layer was observed using scanning electron microscopy (SEM) microscope. The samples were also prepared before analysis as follows: the encapsulated cyanobacteria were dehydrated using ethanol baths with gradient concentrations (25-100% ethanol) and dried using a supercritical dryer for the exchange of ethanol and carbon dioxide. X-ray photoelectron spectroscopy (XPS, Spectroscope 250 Xi from Escalab) was performed to determine the chemical composition of the surface of the encapsulated cells. EDX measurement (coupled on the SEM) was also implemented to provide the general composition of the complete system synthesised.

Cell viability assessment by Clark electrode, fluorescent microscope and confocal microscope

The viability of cells was studied by monitoring their oxygen production. Clark electrode (Pt/Ag - Oxy-lab manufactured by Hansatech) was used to monitor O_2 concentration in a closed system. In the pre-calibrated system, 1 mL of cells was added into a reactor for measurements. After the addition of $NaHCO_3$ solution (10 µL, 0.6 M, from Sigma Aldrich), N_2 was bubbled into the reactor to remove most of the atmospheric oxygen (until 0.100 µmol/ml of O_2). Before the measurement, the reactor was put in the dark for 5 minutes to exclude fluctuations related to natural light. The oxygen production was measured under an artificial light (λ = 650 nm, 1200 µmol $m^{-2}s^{-1}$) with slow agitation (100 rpm).

Additional viability assessment was realised using fluorescent marker (fluorescein diacetate (FDA) purchased from Sigma Aldrich). FDA was added (final concentration: 5 mM) and incubated with the samples for 30 minutes. They were washed three times with bidistilled water before analysis. Micrographs were taken using an optical microscope (Nikon Multizoom Microscope AZ100), fluorescent images were obtained at 536/40 nm with a colour camera (DSRi, Nikon) by illuminating the samples with excitation lights of 482/35 nm.

N-(5-fluoresceinyl)maleimide dye (5-FM, purchased from Sigma Aldrich) was used to stain thiol groups. After 30 minutes of incubation with the fluorescent marker (final concentration: 5 mM) in HEPES buffer (0.01 M, pH 7.4), the samples were rinsed and observed using confocal laser scanning microscopy (Leica TCS SP5 device).

RESULTS AND DISCUSSION

Ormosil materials formed by amino silanes and silica

The pure materials (without any biological units) were synthesised as reference and characterised in detail. These experiments were performed in order to overcome the problems associated with the handling of biological material. It could lead to new knowledge which can be useful for cell encapsulation. This first part study taken place to study the effect of the nature of the amino silanes on the hybrid material.

TEM micrographs (Figure 2) have shown that all ormosils (APTMS/H_2SiO_3, AEAPTMS/H_2SiO_3 and AEAEAPTMS/H_2SiO_3) were formed by the aggregation of nanoparticles. From ten measurements on three different sites for each sample (30 final measurements), the average size of nanoparticles of the APTMS/H_2SiO_3, AEAPTMS/H_2SiO_3 and AEAEAPTMS/H_2SiO_3 was measured to be 22 ± 5 nm; 28 ± 8 nm and 35 ± 8 nm, respectively, which demonstrates a clear correlation between the size and the number of amines on amino silanes: the more amine groups, the larger size of nanoparticles is measured.

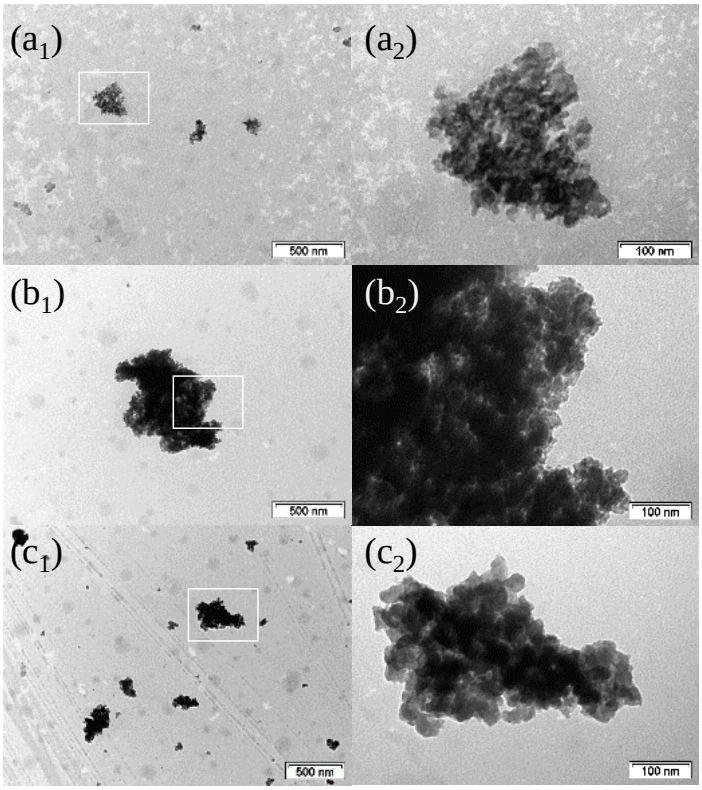

Figure 2: TEM micrographs of ormosils materials formed by: (a) APTMS/H_2SiO_3, (b) AEAPTMS/H_2SiO_3 and (c) AEAEAPTMS/H_2SiO_3

Similar studies have shown that, in the case of some polyamines [37-39], the increase of the length of the chain, thus more amine groups, could result in the increase of the size of nanoparticles. In addition, micrographs are also revealing the different aggregation behaviours of nanoparticles from these samples. Smaller aggregates (<200 nm) appear when APTMS and AEAEAPTMS are used for the synthesis of ormosils, while particles obtained from diamino silane (AEAPTMS) have larger aggregates (around 500 nm). It has been proposed that the chain length of the (poly)amine has a strong influence on the reaction rate and formed nanoparticle size. Surface properties are also influenced by the chain length of the (poly)amine. The advanced causes of this various properties are that the increasing number of amino group increase the reaction speed and aggregation rate due to charge matching leading to larger nanoparticles [37].

The surface area and porosity of the ormosils were characterised by N_2 physisorption method. According to the calculation from the isotherms, the ormosil synthesised by APTMS, AEAPTMS, AEAEAPTMS presented a surface area of 57 m^2/g, 6 m^2/g and 16 m^2/g respectively. The ormosil formed by AEAPTMS showed the lowest specific area. Increasing the chain size lead to an increase in the aggregation rate therefore, a decrease in specific area.

In this case, a change in the shape of the isotherm is observed beyond three amino groups leading to a slight specific surface gain. Further studies on molecules with longer chains should provide additional trends to explain these results. In this work, the specific surface area is an interesting characteristics criterion for the materials to be used in the single cell encapsulation. However, a larger specific surface area at this level should not have a significant influence on the mass transfer through the material.

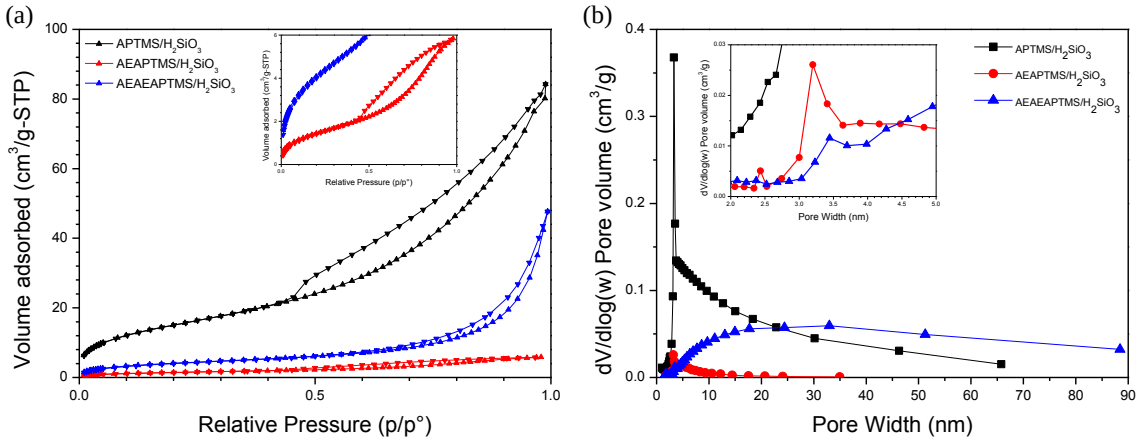

Figure 3: Porous properties of ormosils materials: (a) nitrogen physisorption isotherms and (b) BJH pore width distribution

As shown in Figure 3b, all three ormosils contain a very small amount of micropores. The hybrid material obtained from mono-and diamine (Figure 3a inset) have a high proportion of mesopore. For the third ormosil, the adsorption related to mesopores was lower, while the adsorption of this sample corresponding with macropores turned to be higher, confirming a large proportion of macropores present in this sample. According to IUPAC classification [40], the isotherms of these three samples belong to type II, characteristics of macroporous materials, but containing mesopores.

The pore size distributions of the ormosils determined by the BJH calculation are quite different (Figure 3b). Ormosils obtained from mono- and diamine silanes have pore size distributions centred at 3.3 nm and a broad shoulder from several nanometre to several tens nanometres. Focusing on the hybrid material obtained from triamine silane, their pore distribution gives a much broader range between 10 nm and 90 nm. Therefore, the number of amino silane influence the pore and particle size of the synthesised ormosils.

The reactivity of amino silanes with silica precursor was studied. The methoxy (present in the modified silanes) and hydroxyl (present in silica precursor) groups theoretically shows different reactivity in the polymerisation [41]. For the modified silanes, the polymerisation starts from the hydrolysis steps, while the aqueous silica precursor which is already completely hydroxylated, will form a network by polycondensation reactions. The question therefore remains concerning the incorporation of the amino groups in the silica matrix. The chemical environment in these three ormosils was analysed using nuclear magnetic resonance (NMR). The aqueous silica precursor and amino silanes were present with a molar ratio of 1:1. The peaks in the spectra, corresponding to the T-type contribution, come only from the incorporation of amino silanes. From the spectra (Figure 4), the percentages of each of the contributions can be obtained by deconvolution. The NMR data are presented in Table 1 where different silicon species are described.

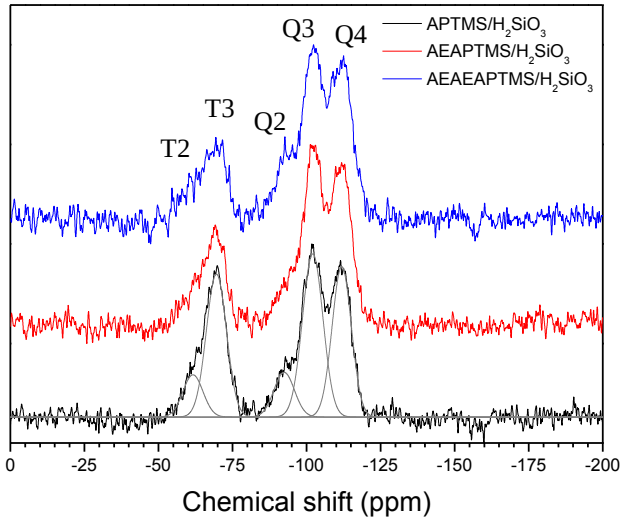

Figure 4: ^{29}Si DE-MAS-NMR spectra of the hybrid materials

Table 1: Condensed species from the hybrid materials (error = ±2.5%)

Hybrid material	**Q4 (%)** $Si(OSi)_4$	**Q3 (%)** $Si(OSi)_3(OH)$	**Q2 (%)** $Si(OSi)_2(OH)_2$	**T3 (%)** $Si(OSi)_3(R)$	**T2 (%)** $Si(OSi)_2(OH)(R)$	**Q/T ratio**
$APTMS/H_2SiO_3$	24	27	7	35	7	0.72
$AEAPTMS/H_2SiO_3$	34	28	13	8	17	0.33
$AEAEAPTMS/H_2SiO_3$	35	29	14	11	11	0.28

It is interesting to note that, firstly, the increase of the number of amines in the silanes increases the degree of condensation of silica species. While the size of the organic chain makes it more difficult to be incorporated into the matrix, since the Q/T ratio decreases as a function of the number of amines in amino silanes (respectively: 0.72, 0.33 to 0.28). Focusing on the different Q contributions, it is clear that it is the smallest molecule that most disrupts the degree of condensation. Indeed, the APTMS/H_2SiO_3, has the highest proportion of Q2. It seems consistent to propose that, given the greater incorporation of the organic group into the matrix, the frequency of amines that can interact with the silicate network (mainly hydrogen bridge between silanol and amino group) is higher. Focusing on the contributions of type T3 and T2, it is also shown that, the size of the amino chain influences the formation of the network. This time, it is a direct proximity to amino groups that disrupts the condensation of the silica network. This disturbance (less T3 type) is more pronounced when there is more than one amine group in the chain. It is diamine that seems to lead to greater disruption of the nearby network (greater proportion of T2).

Several points are particularly interesting. On the one hand, it is possible to obtain an organic-inorganic hybrid material with the incorporation of an amino chain into a silica-based network in an aqueous environment and under mild conditions (neutral pH, atmosphere and ambient temperature). On the other hand, this chain has a significant influence on the properties of the material. In terms of porosity, specific surface area or degree of condensation, the size of the organic chain determine the characteristics of these hybrid materials.

Encapsulation of cyanobacteria in ormosils-based shell

The cell encapsulation was carried out using the layer-by-layer method which can provide control over the chemical composition of the shell and the microenvironment around the cyanobacteria. The amino groups were used to link with the chemical groups outside the outer membrane, while the prepared silica precursor was used to construct a scaffold around the cyanobacteria. The addition of amino groups (cationic at pH 7.4) in direct proximity to the cell makes it possible the interaction of amino groups with the negative charged exterior of the cell via its outer chemical groups. This deposition can then initiate the polymerisation of the silica precursor around the cyanobacteria. With the deposition of different amino silanes and silica precursor, the encapsulated cyanobacteria with ormosil were obtained and named as PCC7002@APTMS/H_2SiO_3, PCC7002@AEAPTMS/H_2SiO_3, PCC7002@AEAEAPTMS/H_2SiO_3, respectively.

The formation of a silica layer around the cells was evidenced using transmission ad scanning electron microscopies. The results are presented in Figure 5.

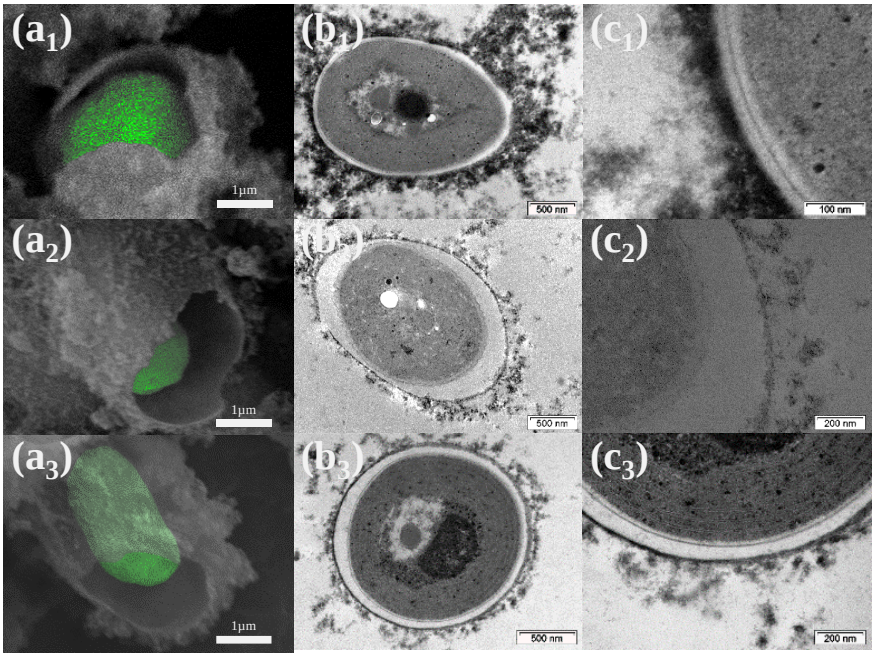

Figure 5: SEM and TEM micrographs of encapsulated cyanobacteria samples (a_1, b_1, c_1) PCC7002@APTMS/H_2SiO_3, (a_2, b_2, c_2) PCC7002@AEAPTMS/H_2SiO_3 and (a_3, b_3, c_3) PCC7002@AEAEAPTMS/H_2SiO_3

The SEM micrographs (Figures 5a_1, a_2 and a_3) reveal a cell-in-shell structure in all three samples. The cells in green colour were entrapped within a shell containing a smooth interior surface [42]. The samples were fixed in resin and cut by ultramicrotoming into thin slices. Transmission electron microscopy allows a more accurate study of the ormosil shell. The micrographs and their enlargements presented in Figures 5$b_{1\text{-}3}$ and $c_{1\text{-}3}$ evidence very clearly the presence of a layer around the cyanobacteria. The silica layer can directly be observed for each of the three samples as a darker circle around the cyanobacteria indicating that the efficiency of the process is quite good. All the cells are surrounded by this shell. In the case of PCC7002@APTMS/H_2SiO_3 samples, more silica aggregates are present and a thick and cloudy layer is clearly visible around the cyanobacteria (Figure 5b_1). For the other ormosils, this layer is thinner and more defined. By further comparing the two samples obtained from polyamines with the one from monoamine (Figure 5b_1), the silica layer appears to be in direct contact with the cell outer membrane, while a larger space between the cyanobacteria and the shell is visible for the other two samples (Figure 5$b_{2\text{-}3}$). The size of the carbon chain therefore has its influence on the shell formation as it is the link between the cell and the ormosil shell. The average ormosil thickness measured from 30 data (three different sites) is 121 ± 120 nm, 52 ± 21 nm

and 19 ± 8 nm for PCC7002@APTMS/H_2SiO_3, PCC7002@AEAPTMS/H_2SiO_3 and PCC7002@AEAEAPTMS/H_2SiO_3 respectively. The thickness decreases drastically with the increase of the chain length.

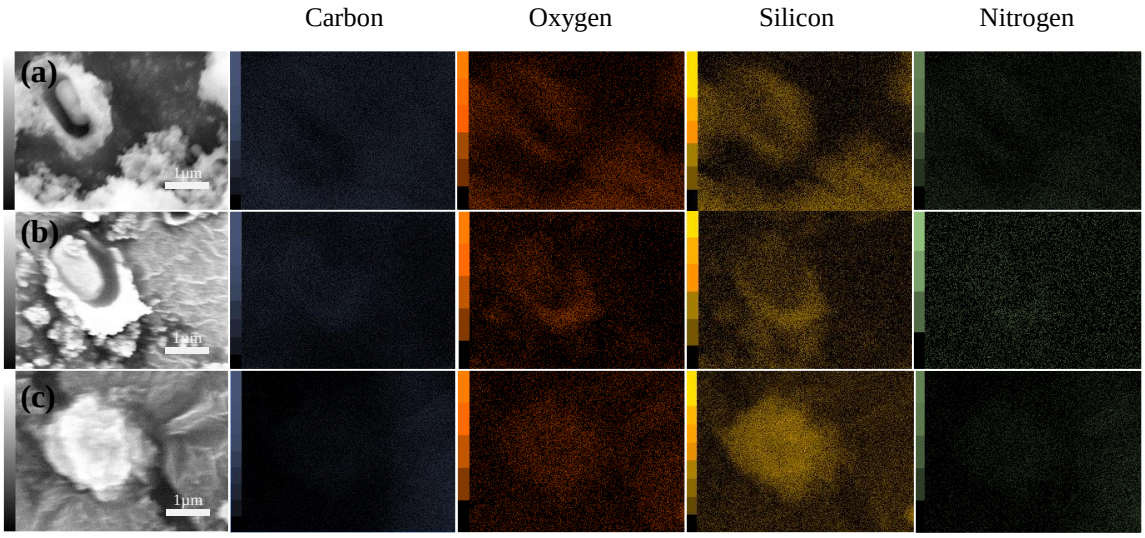

Figure 6: EDX mapping of (a) PCC7002@APTMS/H_2SiO_3, (b) PCC7002@AEAPTMS/H_2SiO_3 and (c) PCC7002@AEAEAPTMS/H_2SiO_3 (blue: carbon distribution; red: oxygen distribution; yellow: silicon distribution and green: nitrogen distribution)

The general chemical composition of samples was then analysed using energy-dispersive X-ray spectroscopy (EDX). The different mapping is presented in Figure 6. Four elements are selected and monitored using this technique (including carbon, oxygen, silicon and nitrogen). The images show a higher proportion of oxygen and silica in the corresponding areas of the silica shell for all three samples (Figure 6, red and yellow). The atomic percentages of the different elements can be obtained. The ratios of silicon to carbon percentages are as follows: $Si/C_{PCC7002@APTMS/H2SiO3}$ = 2.0/79.1 = 0.025, $Si/C_{PCC7002@AEAPTMS/H2SiO3}$ = 1.7/84.1 = 0.020 and $Si/C_{PCC7002@AEAEAPTMS/H2SiO3}$ = 2.1/75.8 = 0.028. These measurements confirm the presence of silica for all three samples. Nevertheless, there is no significant difference between the three ratios calculated for the various samples. The surface chemical composition of the shell is also studied using X-ray photoelectron spectroscopy (XPS). The results are listed in Table 2.

Table 2: Surface analysis of the three bio-hybrid systems (error = ±2.5%)

Sample	C1s (%)	Si2p (%)	O1s (%)	N1s (%)	C/Si ratio
PCC7002@APTMS/H_2SiO_3	12.5	25.0	59.2	3.3	0.5
PCC7002@AEAPTMS/H_2SiO_3	21.5	22.3	51.8	4.4	1.0
PCC7002@AEAEAPTMS/H_2SiO_3	18.5	23.3	53.0	5.2	0.8

The PCC7002@APTMS/H_2SiO_3 has the highest proportion of silicon. This is in line with previous TEM observations that the shell thickness is the most important for this sample. The amount of nitrogen is more important for the sample PCC7002@AEAEAPTMS/H_2SiO_3. AEAEAPTMS has the highest proportion of amino groups (triamine) and it seems consistent that a higher quantity (5.2% against 4.4% for the diamine and 3.3% for the monoamine) is found in this material.

The survival of the cells encapsulated within the material was monitored. Cyanobacteria are photosynthetic organisms and their oxygen production is an important parameter to indicate their living state. The oxygen production was performed by oximetry (Clark's oxygen sensor). In addition, cell viability is also evaluated by monitoring the evolution of their optical density at 730 nm over time. The measurement at this wavelength is related to the turbidity of the solution and is so linked to the cell population evolution. Dead cells would gradually be degraded and will no longer capture light in the same way as living cyanobacteria. The data obtained could reveal the potential effect of the encapsulation process on the cells. UV-visible spectroscopy was used for this measurement. Six different samples were monitored, including the three encapsulated cyanobacteria formed by the deposition of amino silane and silica precursor (PCC7002@organosilane/H_2SiO_3) and their corresponding ones without adding silica precursor (PCC7002@organosilane), in order to compare the behaviour of cyanobacteria in the presence of these different molecules.

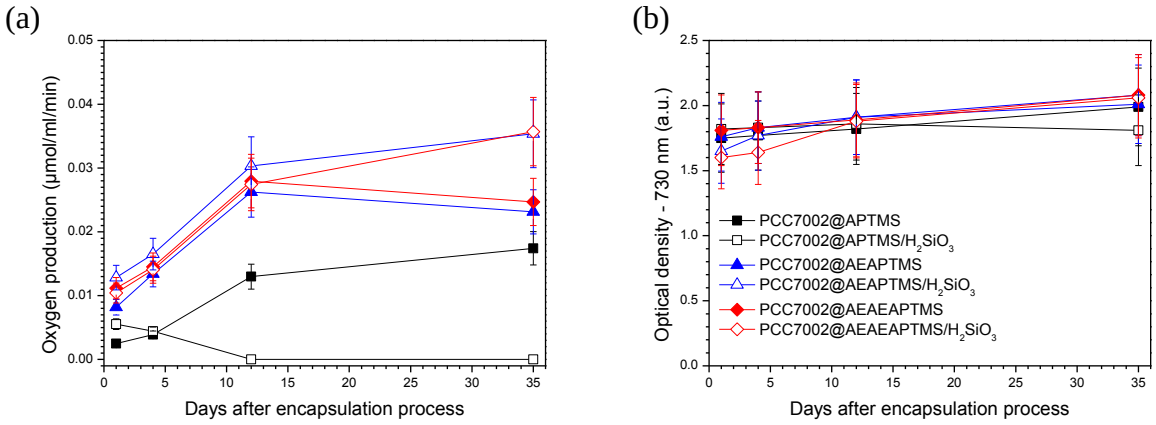

Figure 7: Viability assay for native PCC7002, PCC7002@organosilane and PCC7002@organosilane/H_2SiO_3: (a) evolution of oxygen production and (b) optical density
(one set of measurement: inaccuracy is depending on technical and experimental manipulations)

The evolution of oxygen production during one month is presented in Figure 7a for these 6 samples. The APTMS seems to have a negative impact on the oxygen production activity immediately after encapsulation process. Indeed, the oxygen production of the samples PCC7002@APTMS is the lowest (black curves) among all the samples. Despite its lowest initial oxygen production from the beginning, the oxygen production increases even after one month, showing good activity of cells with APTMS. However, the introduction of silica precursor after the addition of APTMS has a drastic effect on cell activity. In fact, on the twelfth day, the oxygen production activity of PCC7002@APTMS/H_2SiO_3 cannot be detectable anymore. This phenomenon can be correlated with the shell shape. The shell obtained following the encapsulation process using APTMS and silica precursor is thicker and less homogeneous than the other cases. This could prevent exchanges between the cyanobacteria and its outside, having a harmful effect on the cell life. Focusing on the use of the AEAPTMS, the presence of silica can reduce the impact of the organosilane on the oxygen production activity of cyanobacteria. In fact, the blue curve corresponding to the PCC7002@AEAPTMS/H_2SiO_3 (hollow blue triangles) is always above the one related to the PCC7002@AEAPTMS (blue filled triangles). One possible explanation for this phenomenon can be that the use of a diamine changes more deeply the charge carried by the cell wall: from a negative to a positive charge. This modification probably alters the exchange and transport of nutrients, leading to a loss of cellular activity. The addition of the silica precursor should offset this change in charge and allow better exchanges with the outside. Finally, for the third modified silane (AEAEAPTMS), the oxygen production seems significant, with a slow decrease after 35 days for the sample PCC7002@AEAEAPTMS (Figure 7a: red line, filled

diamonds). These results are in line with the previous observations obtained for diamine. The evolution of optical density over time (Figure 7b) seems quite similar to all the samples. The major difference is the appearance of a slow decrease in optical density on the day 35 for the sample PCC7002@APTMS/H_2SiO_3 (black hollow squares). Those graphs therefore highlight the deleterious effect of APTMS on cell viability. These data also show that the addition of the silica precursor balance the impact of the presence of polycations (AEAPTMS and AEAEAPTMS) around the cells.

All the above results demonstrated the feasibility of a single cell encapsulation of cyanobacteria in ormosil shells using the layer-by-layer method. The use of organically modified silanes was also shown as well as their effects on the properties of encapsulated cells.

Exterior surface functionalisation of the encapsulated cyanobacteria

Thiolated silane was used to functionalise the exterior shell surface of the encapsulated cyanobacteria. A first deposition of an amino silane has allowed to link the outer part of the cyanobacteria and the shell. The polycondensation of the silica precursor induced by this layer of amino silanes reinforces this ormosil shell. The addition of a thiol-grafted silane could bring new functionality to the exterior of the shells.

3-(mercaptopropyl)trimethoxysilane (MPTMS, Figure 1d), widely used for the functionalisation of silicon dioxide material [36, 43, 44], has been selected. The encapsulated cyanobacteria with the ormosil shell formed by AEAPTMS were used as a study model. This choice is based on its better control on the material pore size than using triamino silane. The material obtained using monoamino silane is excluded due to the inhomogeneous layer around cyanobacteria and low viability encapsulated using this organosilane.

Transmission electron microscopy (Figure 8) reveals that despite the addition of MPTMS, a thin shell around cyanobacteria is observed. It is interesting to note that the cell is in direct contact with the shell and no more space between the cell and the shell is visible. The average layer thickness is 32 ± 9 nm, which is slightly smaller than the average thickness for the material PCC7002@AEAPTMS/H_2SiO_3.

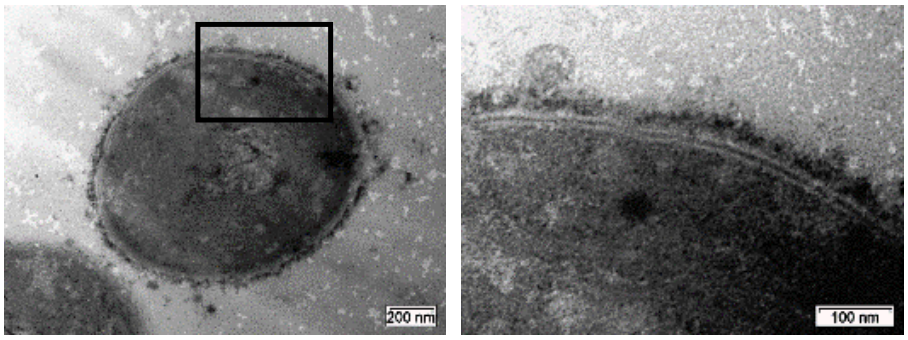

Figure 8: TEM micrographs of PCC7002@AEAPTMS/H_2SiO_3/MPTS

The chemical composition of outside of PCC7002@AEAPTMS/H_2SiO_3/MPTS is analysed using XPS. For comparison, the surface composition of PCC7002@AEAPTMS and PCC7002@AEAPTMS/H_2SiO_3 are also listed in the Table 3.

Table 3: Surface analysis of PCC7002@AEAPTMS/H_2SiO_3/MPTMS (error = ±2.5%)

Sample	Expected outer chemical group	C1s (%)	Si2p (%)	O1s (%)	N1s (%)	S2p (%)
PCC7002@AEAPTMS	« -NH_2 »	59.0	3.6	30.4	7.0	0.0
PCC7002@AEAPTMS/H_2SiO_3	« -OH »	72.6	3.3	20.8	3.3	0.0
PCC7002@AEAPTMS/H_2SiO_3/MPTMS	« -SH »	48.9	10.8	35.1	4.3	1.0

It reveals that it is during the last step that the silica proportion is the most important as shown by the higher proportion of oxygen and silicon. This is consistent with the fact that at each step, silica-based molecules are added around cyanobacteria. The proportion of nitrogen is the highest (with a percentage of 7.0%) after the addition of AEAPTMS around cyanobacteria and then decreases with the addition of silica precursor. After grafting MPTMS, a peak related to sulphur atom is observed. These measurements confirmed that the layer is composed of silica and the addition of sulfhydryl groups outside the ormosil shells is successful.

The evolution of oxygen production and optical density of the encapsulated cells were monitored and the results are shown in Figure 9. Native cells are set as a control for the comparison of the evolution of cell viability.

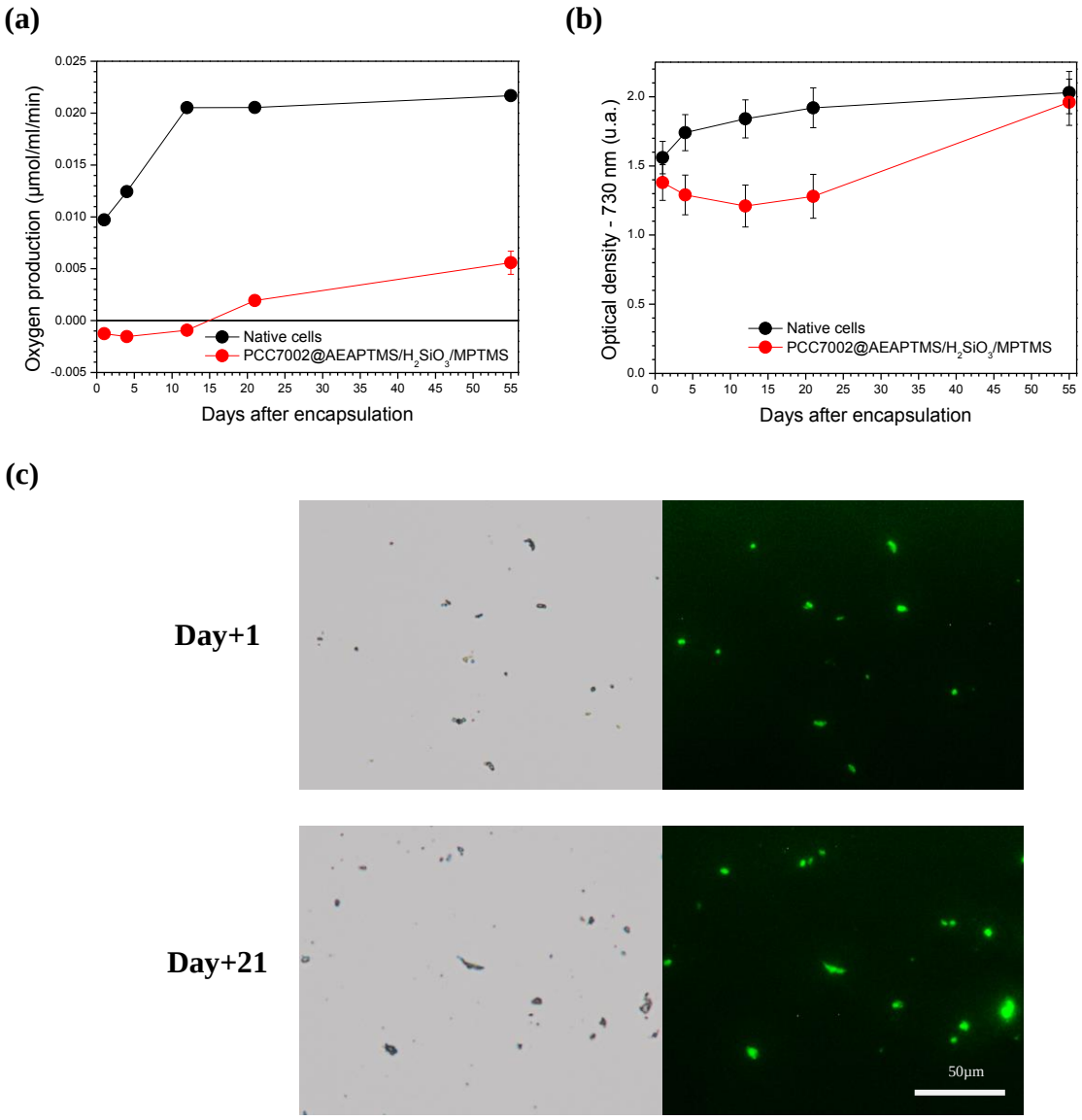

Figure 9: Viability assessments for the thiol-grafted bio-hybrid system: (a) oxygen production; (b) optical density and (c) micrographs in bright field and fluorescence (FDA marker)
(one set of measurement: inaccuracy is depending on technical and experimental manipulations)

The evolution of oxygen production over time of thiol-grafted encapsulated cyanobacteria reveals a harmful effect on the cell activity. In Figure 9a, native cells show classical evolution of oxygen production related to their activity [45], namely, a strong increase in the first few days before stabilisation which leads to a plateau indicates that the cell activity is constant. In the case of thiol-grafted encapsulated PCC7002, no oxygen production but O_2 consumption has been observed. Cyanobacteria respond to their modification by attempting to regulate their functions (homoeostasis). The evolution of optical density presents the same particular behaviour. A slight decrease of OD is observed during the first few days (Figure 9b) before stabilisation. After 21 days, the sample shows a positive oxygen production again. The

same trend is observed in optical density. The response of the cyanobacteria to the encapsulation process and more precisely to the addition of thiol groups is the oxygen consumption. PCC7002@AEAPTMS/H_2SiO_3 shows a very good oxygen production over time in the absence of a sulphur-based group. Post-grafting sulphur-based group could disrupt the permeability of the hybrid shell and block exchanges between the cells and their exterior.

A fluorescent dye marker, fluorescein diacetate (FDA), was used to stain living cells. The cell integrity can be evidenced by a marked green colour under light excitation. Thiol grafted encapsulated cells were observed by fluorescence optical microscopy. The micrographs were made 1 day and 21 days after encapsulation, as shown in Figure 9c. In both cases, fluorescent spots can be clearly observed, demonstrating that almost all the cells present a preserved structure.

This viability study of PCC7002@AEAPTMS/H_2SiO_3/MPTMS highlights that cell activity is affected by the encapsulation process and functionalisation by thiol-grafted organic component. The culture of these bio-hybrid systems shows a decrease in optical density related to the fact that the behaviour of the cells has been affected. Indeed, the first days of measurements show oxygen consumption and several days after, the oxygen production resulting from photosynthesis occurs again. The integrity of the cells does not seem to be too affected as observed by optical micrographs and the FDA staining. The cells are therefore undergoing significant stress.

It must be further demonstrated that sulfhydryl groups grafted on the shell are accessible for additional functionalisation of the external part. N-(5-fluoresceinyl)maleimide (5-FM) reacts with available thiol groups forming a covalent bond (Figure 10a) [46]. Using its fluorescence in the green spectrum allows marking the hybrid shell. The samples were then placed in the presence of this dye and were observed after rinsing using fluorescence confocal microscopy.

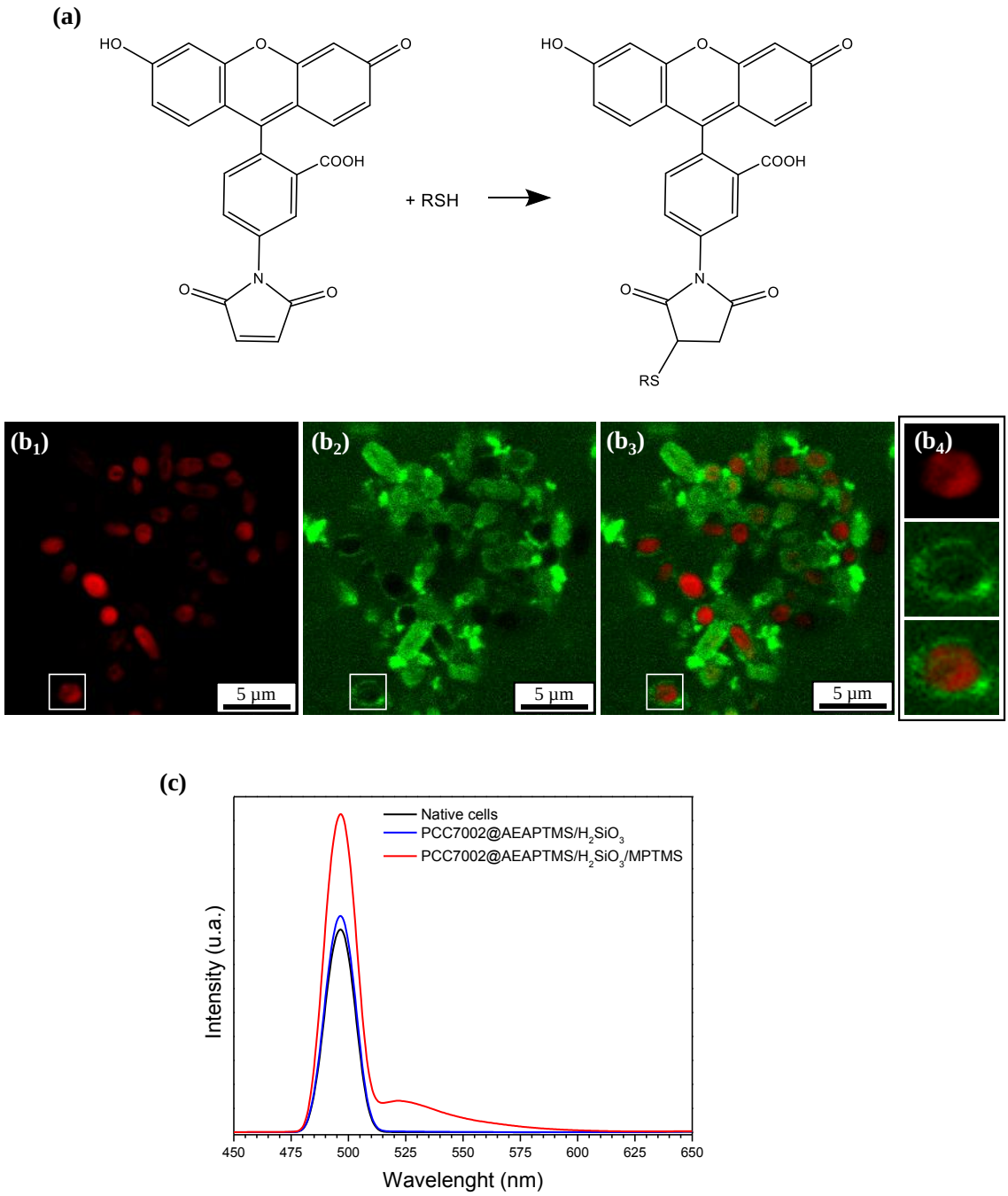

Figure 10: Thiol marking process: (a) reaction between 5-FM (open fluorescent form) and a sulfhydryl group; (b) micrographs of the thiol-grafted bio-hybrid system: (b_1) cholorophyll autofluorescence, (b_2) 5-FM fluorescence, (b_3) merged images of b_1 and b_2 and (b_4) magnification of the white squares (c) fluorescence spectra of samples after the addition of 5-FM

Figure 10b shows micrographs obtained by confocal microscopy. The first image was obtained by observing the fluorescence of the photosynthetic pigments of cyanobacteria (chlorophyll a) which emitted in red (Figure 10b_1). The form of cyanobacteria is distinctly observed. The second image was obtained following the fluorescence of the residual 5-FM after rinsing and thus grafted onto the hybrid shell (Figure 10b_2). The ormosil shells are visible

since inside some of them, the shadow of the "missing" cyanobacteria (because they do not emit into the green) can be observed. The encapsulated cells appear in their entirety in the merged images: cells entrapped in organic-inorganic hybrid shells grafted by external thiols (Figure 10b_3). The magnification presented by Figure 10b_4 present this process: the individual cell encapsulation and functionalisation. The spectra obtained (Figure 10c) also demonstrate the functionalisation of the material by thiols and their availability for subsequent grafting. In all three cases, a peak is present around 500 nm, it corresponds to the incident light (excitation: 494 nm). It is the only peak present for the first two samples (native cells and non-grafted bio-hybrid material). Finally, a peak appears in the last sample (around 524 nm, red curve) which corresponds to the fluorescence of 5-FM under the excitation of 494 nm. This proves that there are residual dye molecules linked around cyanobacteria. The grafting of thiols was successfully introduced on the shells and available for further functionalisation.

Functional materials

Equipping cyanobacteria with functional materials can provide them with new properties and functions thus widen the range of their applications. In this section, two functional materials were taken as examples to show the functionalisation of cyanobacteria. First, cyanobacteria were decorated with magnetic nanoparticles to facilitate their harvesting. Second, the thiol modified shells were constructed around cyanobacteria to control their attaching properties for a facile cell manipulation.

Magnetic particles grafting

Magnetic nanoparticles (NPs, composed of Fe_3O_4) were synthesised and decorated with thiols using MPTMS. The modified magnetic NPS are able to bind the thiol-grafted encapsulated cells, thus providing mobility for cyanobacteria under the impulse of a magnetic field. The functionalised cyanobacteria were then observed using transmission electron microscopy (Figure 11a). The shell can be observed around the cyanobacteria. Small square dots which are scattered in aggregates around the encapsulated cells are the magnetic nanoparticles.

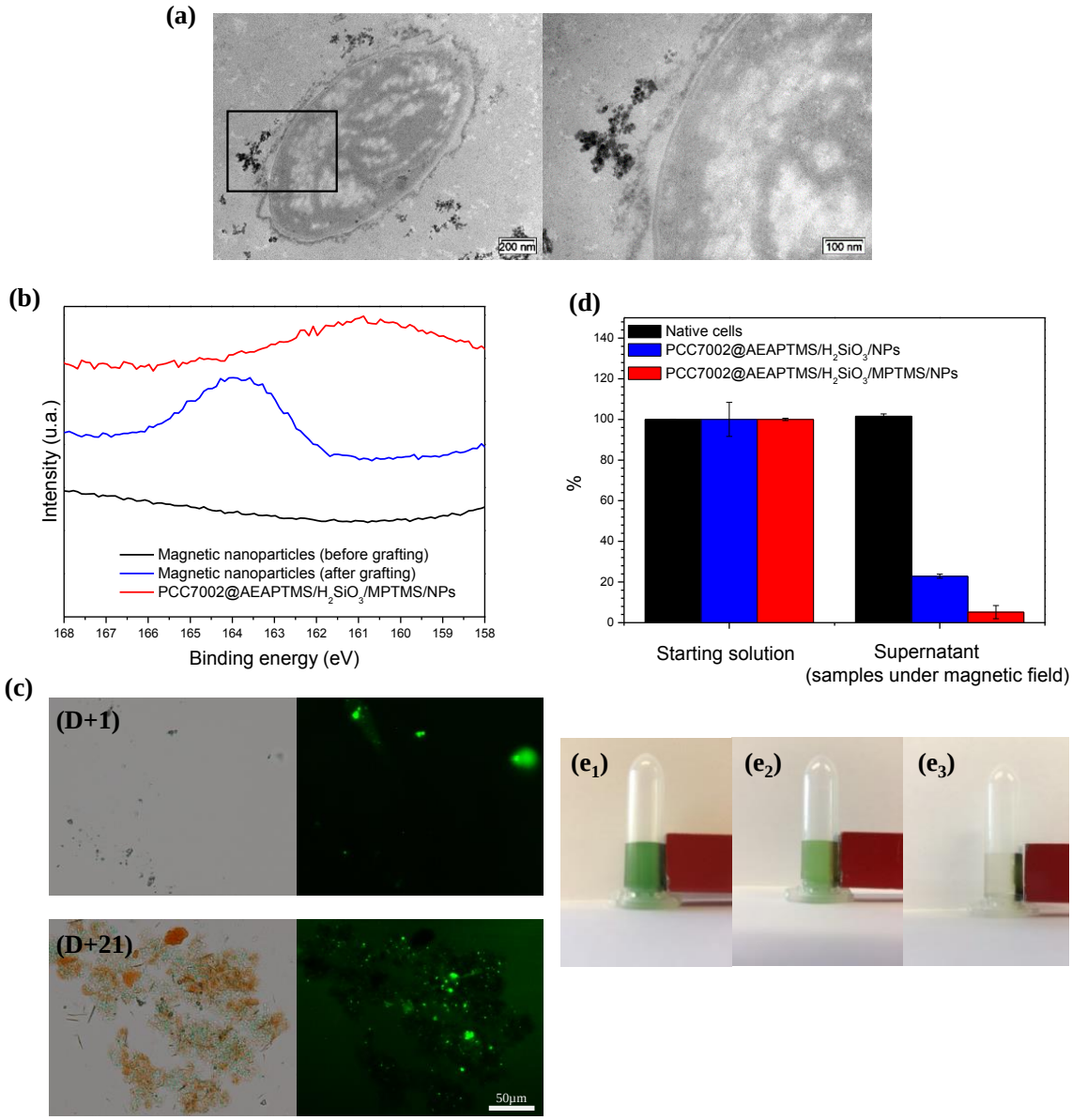

Figure 11: Magnetic nanoparticles grafting: (a) TEM micrographs; (b) surface chemical composition (detection area for sulphur peak); (c) optical micrographs (bright field and fluorescence) one day and 21 days after encapsulation and functionalisation; (d) efficiency of the functionalisation process: percentage of recovery of the cellular population (OD730) under magnetic field and photographs of (e_1) native cells, (e_2) PCC7002@AEAPTMS/H_2SiO_3 and (e_3) PCC7002@AEAPTMS/H_2SiO_3/MPTMS supplemented by modified Fe_3O_4 nanoparticles and exposed to magnetic field (red-coloured magnet, to the right of the images)

The surface chemical composition of the samples was also studied using XPS. The sulphur peak (~164 nm), characteristic of thiol groups, appears after the thiol addition on these NPs (blue curve, Figure 11b). A shift in the value of the chemical displacement from 164 nm to lower values (around 160 eV) can be observed in the sample containing cyanobacteria after the decoration by the modified NPs, which indicates the formation of disulphide bonds between magnetic particles and thiol-grafted encapsulated cyanobacteria.

An FDA fluorescent marker was used to monitor the preservation of cell integrity through enzymatic activity. The FDA-stained cells (PCC7002@AEAPTMS/H_2SiO_3/MPTMS/NPs) were observed using optical microscopy. Figure 11c shows the images that were taken 1 and 21 days after the encapsulation and functionalisation procedure. A few fluorescent dots confirm the survival of some cells, but the rate is low (less than 15 %)[2]. Aggregates appear to form over time as shown by the second set of micrographs (21-day post-encapsulation). Brown areas show the presence of magnetic nanoparticles in large quantities in these aggregates. Finally, these magnetic encapsulated cyanobacteria were put under the magnetic field in order to confirm the possibility of its recovery. Magnetic particles were added to a suspension of native cells and a suspension of encapsulated cells before the thiol-grafting step to confirm the function of thiol groups in the decoration. Photographs were taken 30 seconds after being exposed to a magnetic field. As shown in Figure 11d, the effectiveness of magnetisation is higher for the material that was submitted to the complete thiol-grafting process. By measuring the optical density at 730 nm of the starting solution and the supernatant under magnetic pulse, the efficiency of this process can be qualified. No link between magnetic particles and native cells was evidenced by the total recovery of cell culture in the supernatant. A bond was observed for the hybrid system PCC7002@AEAPTMS/H_2SiO_3 (*i.e.* not finally grafted by thiols) where only 23% can be recovered in the supernatant. Hydrogen bonds can be considered to explain this phenomenon. Finally, a high efficiency (95% of the cell population bound to the magnetic nanoparticles) was measured in the case of the encapsulated and thiol-grafted cells.

Grafting on glass slides

During this part of the work, a link between encapsulated cyanobacteria and substrate material will be constructed using the outer sulfhydryl groups of the encapsulated cyanobacteria. Microscope glass slides were functionalised using thiol-grafted silanes. XPS measurements confirm the appearance of thiol groups on the surface of the glass slides. As shown in Figure 12a, a peak appears around 164 eV for the red curve, which corresponds to the part of the slide that has been functionalised, unlike the black curve where the slide that has not been treated with MPTMS. The modified glass slides were then immersed in the cell culture and analysed.

[2] In this case, the quantification of the survival rate of cyanobacteria is made difficult by the limitations of the device.

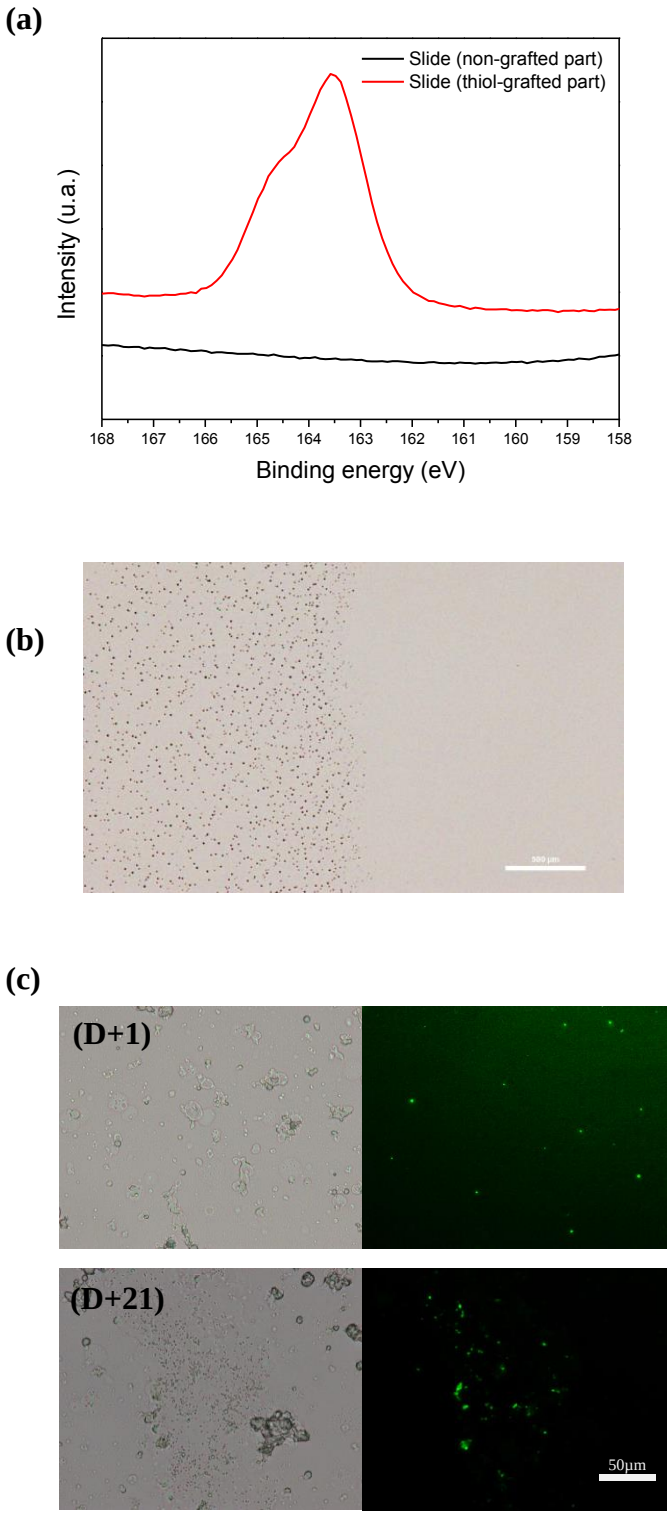

Figure 12: Grafting on glass slides: (a) chemical composition on the surface of slide (detection area for sulphur peak): comparison between a thiol-grafted side and a non-grafted side; (b) bright field micrograph of thiol-grafted cyanobacteria on modified slide (with the boundary between grafted and non-grafted part) and (c) bounded thiol-grafted cyanobacteria integrity assessment (slides are kept in culture medium) using FDA marker (bright field micrographs compared to fluorescence micrograph)

Visualisation of these slides by optical microscopy in the bright field clearly shows the deposition of the cells in the thiol-grafted part of the slides (Figure 12b). By adding the FDA dye and using fluorescence observation from the microscope, the cell integrity was studied. A few green fluorescent dots on the images in Figure 12c show that some cyanobacteria have retained their integrity. In this case, the cell preservation rate is quite low (less than 10%), but these results demonstrate the feasibility of this process.

Despite the requirement for improvement, the process of encapsulating and grafting living cells onto glass slides provides a wide range of uses. For example, these materials can be used as heterogeneous biocatalysts or as a basis for cleaning wastewater. In addition, attaching them to new materials would allow these cyanobacteria to be inserted into more complex systems such as “biobatteries” [47].

CONCLUSIONS AND PROSPECTS

This work demonstrates the use of ormosils for the single cell encapsulation of cyanobacteria. The encapsulation shells formed by organically modified silanes are quite versatile and therefore present a unique flexibility to control the microenvironment around living cells.

First, amino modified silica networks were synthesised by an aqueous silica precursor and amino silanes. The texture and intrinsic properties of the ormosil matrix were controlled by the organic chains of the organosilanes. This opens a path to adjust the properties of the abiotic material for single cell encapsulation. Then, the layer-by-layer method allowed the construction of ormosil shells around cyanobacteria. The sol-gel pathway facilitated this construction occurring under mild conditions (pH 7.4, temperature and ambient atmosphere), thus not having too much impact on cell survival. In all cases, a hybrid shell can be synthesised via the proposed synthesis route. However, the use of different organically modified for the design of ormosil materials have different impacts on cell viability over time.

The decoration of living systems with thiol groups was also investigated. A material of the type PCC7002@AEAPTMS/H_2SiO_3/MPTMS was successfully designed and synthesised. Although the cell integrity has been partially preserved, the final grafting influences cell activity negatively. It is necessary to design effective methods to monitor cell activity. Meanwhile, it would be useful to optimise the process to be less harmful to cyanobacteria. However, based on the previous results, it is considered to exploit these thiol groups available

on the surface to improve the performance of cyanobacteria. The possibility of linking those thiol-grafted cyanobacteria using disulphide bonds will be the initial step for their exploitation.

Magnetic nanoparticles and glass slides were also functionalised by MPTMS and thus present surface thiol available. This work showed that it was then possible to make magnetic cyanobacteria by bringing these living thiol grafted materials into contact with modified magnetic nanoparticles. The last part of the work involves grafting living materials onto modified glass slides. However, in these cases, the manipulations have an impact on the activity of the cells. It is therefore necessary to improve the processes to avoid these harmful effects for future uses.

The results presented above are the starting point for some improvements and uses of living organisms with single cell encapsulation. The design for further functionalisation starts from the modification of silica. As it is very versatile material, many methods for its functionalisation are available. The materials presented previously can be used in a wide range of applications. Having made cyanobacteria magnetic makes it easier to use them for biocatalysis, bioremediation, etc. While having obtained glass slides with an active biological part opens the way to the creation of biosensors, new heterogeneous biocatalysts, or even wastewater filters. Further research in this area could also make it possible to graft these living organisms onto electrodes. Their electron production, if canalised, should make it possible to feed a "biological" battery.

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COMPLIANCE WITH ETHICAL STANDARD

The authors declare that they have no conflict of interest. This article does not contain any studies with animals or human participants performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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Chapter 3 - Grafting/degrafting attempt

3.1. Purpose of the chapter

A reversible system was built based on the previous statements. The disulphide bonds can be opened easily by reducing agents, which contributes them to be used for the design of a reversible system.

Through the addition of such molecules to our system, we could build a switch for grafting our functionalised bio-hybrid system. The grafted cyanobacteria could be "ON", once functionalised with modified material. Then the reducing agent is added, they could be “OFF” when the degrafting occurs.

This type of strategy has already been used for the chemical modification of cell surface [1]. It allows to build shells around cells that are reversible [2,3]. This type of ON/OFF system can also be used in medicine for imaging [4] or especially the delivery of drugs *in vivo*. Based on these links and their reductions, it is possible to release active substances in targeted areas [5,6].

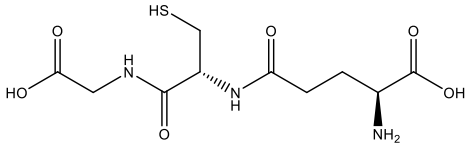
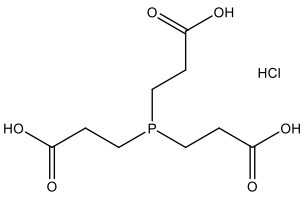
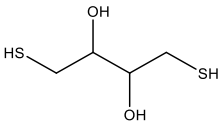
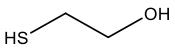
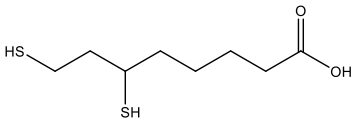
3.2. Experimental

Several reducing agents were selected to open the disulphide bonds between thiol-grafted encapsulated cyanobacteria and solid substrates (modified magnetic nanoparticles and modified glass slide). They are listed in the Table IV-1. Solutions of each reducing agent were prepared in phosphate buffer (PBS 1 M; pH 7.4) with a final concentration of 10 mM.

The first case study concerns the degrafting of nanoparticles. After the addition of nanoparticles (30 minutes of incubation) to the thiol-grafted encapsulated cells, the supernatant is removed (magnetic separation) and replaced by a reducing solution. The evolution of the release is monitored by dosing the OD730 of the supernatants under the effect of a magnet. Several measurements were made over time.

For experiments on glass slides grafted with cyanobacteria, the slide is rinsed, added in a reducing solution and sonicated for 30 seconds, thereafter, rinsed with distilled water and analysed using an optical microscope.

Table IV-1: Selected reducing agent

Molecule	Abbr.	Formula	Supplier
Glutathione reduced	GSH		Duchefa Biochemie
Tris(2-carboxyethyl)phosphine hydrochloride	TCP		TCI
DL-dithiothreitol	DTT		Sigma Aldrich
2-mercaptoethanol	MTT		Sigma Aldrich
Dihydrolipoic acid	DHA		Sigma Aldrich

3.3. Results

The results are shown in Figure IV-2. Photographs of each sample were taken. A control test was performed in the phosphate buffer alone. The photographs (Figure IV-2a) show the samples one hour after incubation. The yellow colour of the solution containing TCP (flask number 4) shows that the reducing agent also influences the cell structure of cyanobacteria. Protein structures are indeed highly dependent on disulphide bridges between sulphur containing amino acids. Therefore, the addition of a highly active reducing agent can be harmful.

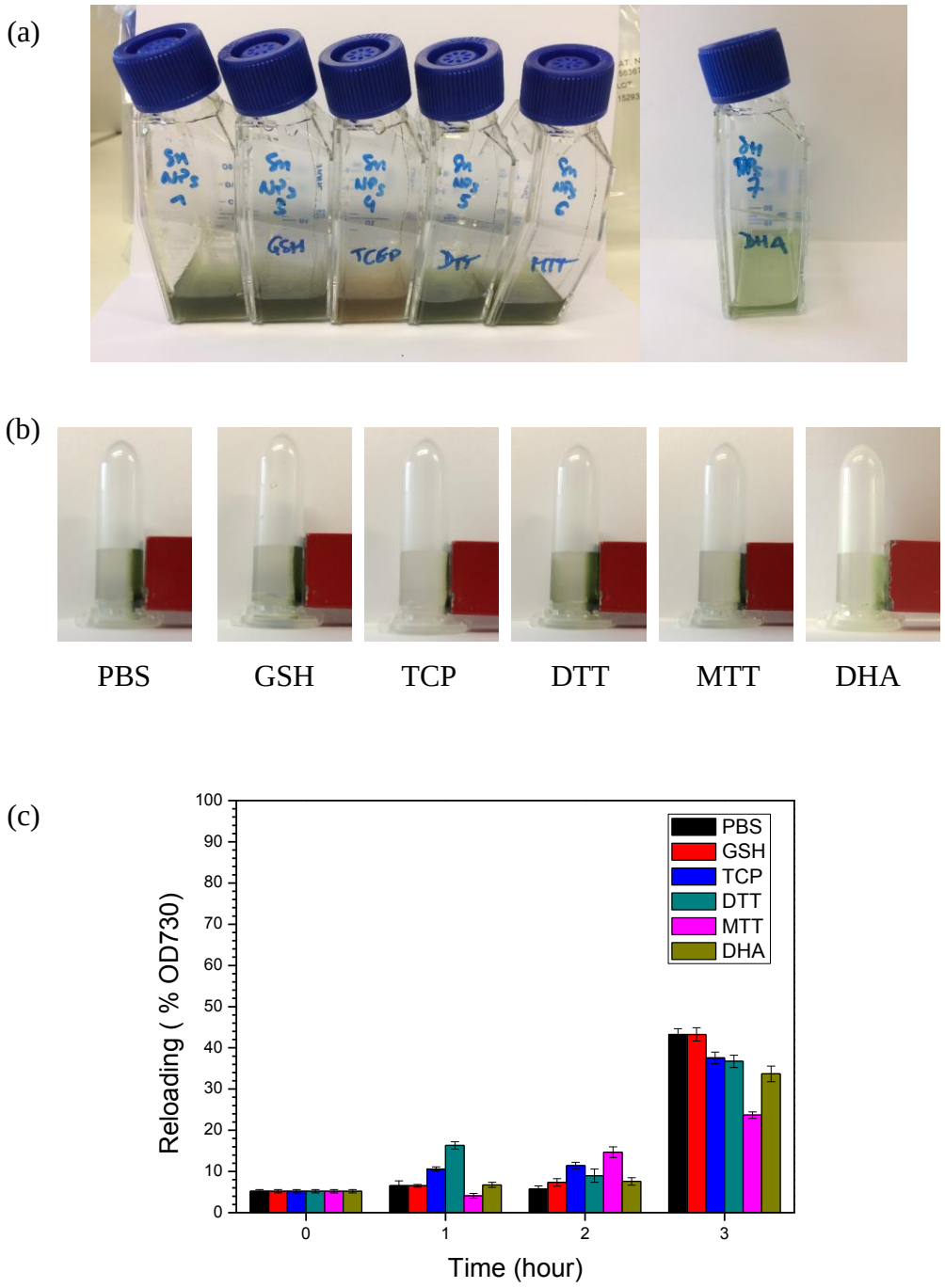


Figure IV-2: ON/OFF attempt on magnetic NPs grafted cyanobacteria: (a) photographs of the samples one hour after incubation; (b) photographs under the magnetic field and (c) reloading percentage by time measured by OD730

These same samples were exposed to a magnetic field in order to recover and analyse the supernatant. As shown in the images in Figure IV-2b, the effect of reducing agents is invisible for observation at the first approach. Optical density measurements were therefore carried out. Very low release is observed over time. After three-hour incubation, the optical

density rises. Nevertheless, it is difficult to differentiate between released cells and cellular fragments due to reducing agents.

However, after one hour, the DTT has shown the most encouraging results. It has been used for a test to remove cyanobacteria from the glass slides. The micrographs are made before and after adding the slide in the reducing solution and presented on the Figure IV-3.

(a) (b)

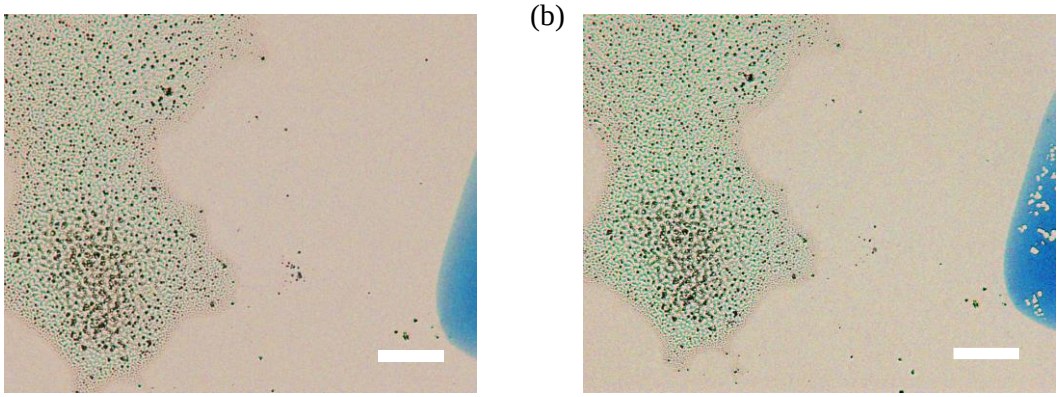


Figure IV-3: Optical micrographs of glass slide with modified cyanobacteria grafted: (a) before and (b) after the addition of DTT
(Bars = 50 µm)

This strategy seems not to be effective. The same location (located by a blue marker point) of the slide was observed at both times and no significant difference is visible. These observations lead us to believe that the cells remained attached on the modified part (by sulfhydryl groups) of the glass slide. In conclusion, this attempt was not successful. Reducing agents are not powerful enough and/or the conditions are not met to make them effective. Further testing is required in this two-system technology: “hooked/unhung”.

Chapter 4 – Conclusion

During this part of the project, new single cell hybrids were designed and synthesised. The innovative approach was to use organically modified silanes in the layer-by-layer process. This strategy has proven to be effective. In addition, by choosing among the different amino silane, it is possible to modulate the textural properties of the encapsulating ormosil shells.

The use of a thiol-grafted silane has made it possible to functionalise the cells with outer sulfhydryl groups. Our photosynthetic prokaryotes were thus decorated with magnetic nanoparticles and grafted onto a glass slide. This research is a starting point for the synthesis of new high-performance materials. Nevertheless, the synthesis processes still need to be improved and further studied.

An attempt was made to remove the magnetic nanoparticles grafting to cyanobacteria by using reducing agents. However, the strategy put in place does not seem adequate to achieve the purpose of those experiments. Additional studies will be needed in this innovative field of research.

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Part V
General conclusions

Chapter 1 – Conclusions

The main goal of this research work was the design of efficient processes for individually encapsulating cyanobacteria in silica-based nanoshells. The construction of these materials shows several constraints. A demand for duality leads to many challenges. Materials must have a certain rigidity to protect living organisms while being malleable to allow cellular activities. They must be porous and versatile for the possibilities of being functionalised or even multifunctionalised. To address these constraints, a general strategy has been established. It consists of the construction of hybrid materials. An organic part provides the shells with modulability and functionality while an inorganic part provides strength and stability.

Several materials have been built. They have in common the use of the layer-by-layer method which allows the synthesis of nanoshells by using sol-gel pathway.

While the layer-by-layer method and the use of polyelectrolyte to induce biomineralisation are widely used in cell encapsulation, many challenges remain. The properties of the materials obtained are poorly studied. No trends or predictions can be obtained from the literature. It is from this statement that the research bases for the first part of the results are defined. The Part II of this work aimed to go through the synthesis of several materials and study their properties in order to define trends allowing to predict their characteristics. Preliminary experiments have highlighted the relevance of the use of polysilicic acid as a precursor to form a silicate network. Then, several polycations were used to cover the cell wall and direct the synthesis of the silica-based nanoshell. The effects of these polycations on material properties have been demonstrated as well as on cell viability. Despite the potential harmful effects of these molecules on cellular activity and a mixed encapsulation efficiency, a demonstration of the method was carried out. To obtain trends afterwards, other molecules with certain characteristics have been selected. The use of small polyamines has made it possible to highlight the possibility of modulating the textural properties of the hybrid material by increasing the size of molecules. These have been shown to be beneficial to the cell's life. Then a cationic polymer (polyammonium) was used. Three sizes of molecules were studied, which again showed that the size of the molecule had an influence both on the properties of the materials and on the activity of the cells.

The second part of the results (Part III of this work) aimed to present a complete approach related to hybrid materials: design of a method, synthesis of a nanoshell, study of its

protection on living cells and exploitation of the bio-hybrid system synthesised. In this case, the choice was made to use the encapsulated cyanobacteria for wastewater remediation (removal of heavy metal ions). Indeed, cyanobacteria have a promising future in bioremediation. These nanoshells thus make it possible to protect our photosynthetic prokaryotes from harmful ultraviolet radiation while not preventing the cells from carrying out their biosorption activities of certain heavy metal ions problematic in wastewater (copper and lead). These materials have good stability against recycling over time. However, the results obtained identify some issues about the process efficiency, the control of the material properties, the effect on the activity of the cells, etc. leading to the general research work presented in the first part of results.

The construction of hybrid shells around cyanobacteria is generally constructed from numerous but weak interactions between each of the components of the hybrid artificial material. No method of individual encapsulation of cyanobacteria suggests the use of ormosil. Finally, in order to improve the functions of the cells and facilitate their handling, another method has been developed. The Part IV of this work has presented the synthesis of ormosil by covalent bonding between organosilanes and silica network. Those organically modified silanes have been used to manage the interfaces and build the silica shell using a modified layer-by-layer method. While individual encapsulation aims to modify the exterior of cells and provide them with new properties (mobility, specific recognition, etc.), no study has highlighted this type of modification for cyanobacteria. These organosilanes have, in this way, allowed the surface grafting of functional groups of the sulfhydryl type. However, post-grafting by thiol groups outside cyanobacteria has strongly affected cell activity. Nevertheless, those results are a starting point for linking those modified cyanobacteria through disulphide bridges. Thus, it was possible to decorate our modified cyanobacteria with magnetic particles, offering them mobility under the effect of a magnetic field and offering the experimenter additional ease of handling. The same method was used to attach bio-hybrid systems grafted with thiols to glass slides. Despite the marked effect on the cyanobacterial activity, the results obtained are a proof of the relevance of the concept leading to the potential design of new technologies. For example, controlled printing on a wider variety of substrates would be the basis for the design of new high-performance materials.

Chapter 2 – Prospects on the future

Several points still need to be addressed at the end of this work. We will try in this chapter to make an overview of those remarks.

2.1. Short term prospects

During this work, it has been shown that individual encapsulation of cyanobacteria can have adverse effects on cell activity. Moreover, the study of complete systems and their biological activity is not easy. It is therefore necessary to develop new strategies for their studies. This would then allow us to continue to optimise our synthesis processes to be as harmless as possible to the cyanobacterial activity.

It has been demonstrated that the organic part is essential to the properties of the material. It is now necessary to develop methods to modulate the diffusion properties of this shell. By controlling permeability, it is possible to protect the external aggressions (abiotic and biotic), on the one hand, but also to improve its production properties by allowing metabolites of interest to diffuse from the bio-hybrid system to its exterior.

The functionalisation of the material allows in addition to the sensitivity related to living cells to offer additional function to the cells. Developing "open/closed" or "on/off" systems are possible via surface engineering, it is necessary to continue research in this direction. Silica remains a material of choice for this type of research, as confirmed by its use and functionalisation for specific and precise tasks, such as nanocarriers [1, 2].

Functionalising the exterior of the material could also offer specific recognition and thus enlarge the field of exploitation of the cells, which in addition to being intelligent by their activity and sensitivity, would be intelligent in their direction and their choice controlled by the material. Strategies for grafting antibodies onto silica materials for specific recognition (affinity chromatography) exist [3]. It would therefore be wise to transpose them to cell encapsulation to offer new properties to the cells.

For intensive use, it is necessary to build systems that are resilient over time and efficient.

2.2. Long term

The long-term perspective of this project is, among other, an industrial use. The range of use is very varied for this type of cell. We have also shown its use (in research laboratories) for metals removal. By focusing again on bioremediation, it is possible to imagine the creation of efficient filters for the degradation of organic pollutants. Indeed, in the last part of the results, functional systems were built: easy recovery of cultures under the influence of a magnetic field or use of glass slides grafted with cyanobacteria. Cyanobacteria PCC 7002 are capable of degrading phenol to muconic acid [4]. This makes it possible to remove potential pollution by degrading a molecule that is toxic to humans into a much less problematic species. Combining the capacities of cyanobacteria and the functional materials obtained bring some potential innovations for the design of new bioremediation strategies through, e.g. new bio-filters.

Afterwards, the exploitation of this type of material with energy production objectives is a new vision. Photosynthesis is indeed rich in potentiality: production of metabolites with high added values [5], biofuels [6, 7] or even electricity [8]. First of all, it could be useful to focus on the production of beta-carotenes by those cyanobacteria. These high value-added molecules are widely used, among others, in the food and pharmaceutical industries. The cyanobacterial strain *Synechococcus sp.* PCC 7002 is studied for the production of an aromatic beta-carotene called synechoxanthin (Figure V-1) [9]. This production and the recovery and purification of the molecules could be facilitated by encapsulating cyanobacteria within hybrid nanoshells.

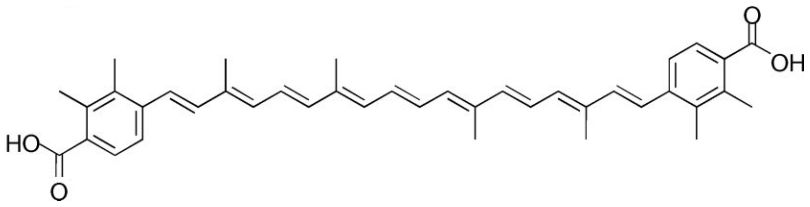

Figure V-1: Structure of the synechoxanthin produced by the *Synechococcus sp.* PCC 7002 (adapted from reference [9])

Nevertheless, the use of this type of bio-hybrid systems faces many challenges. The cost of chemical manipulation of living organisms and the lack of equipment for the synthesis of large quantities. Nevertheless, the development of new synthesis strategies, for example through microfluidics [10], makes it possible to imagine the commercialisation of this type of technology on a larger scale (Figure V-2).

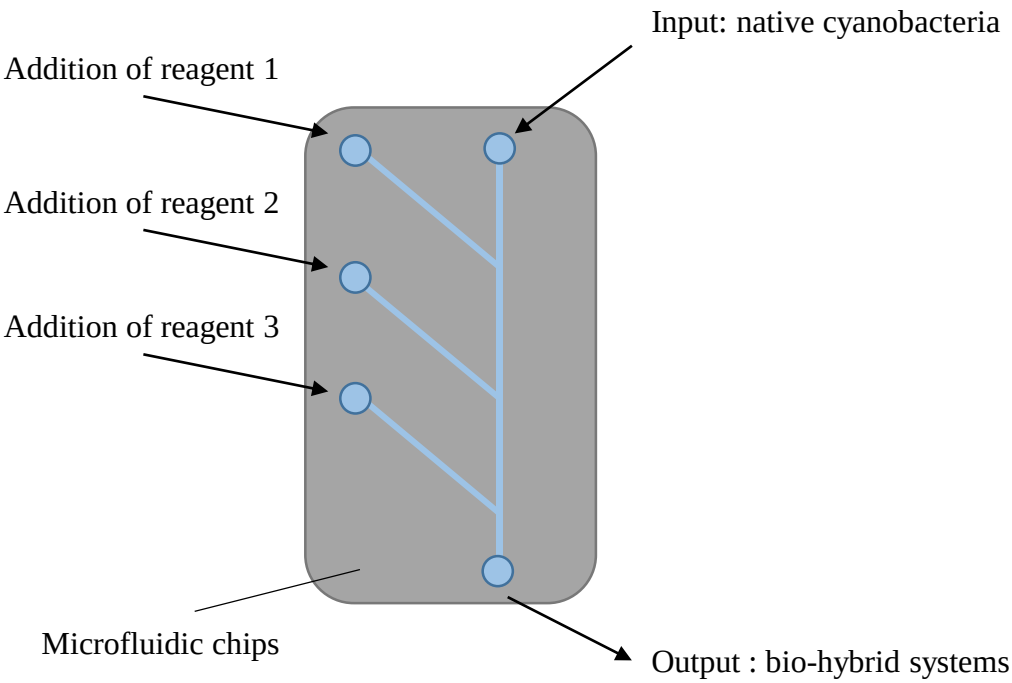

Figure V-2: Scheme of a hypothetical production of bio-hybrid system through microfluidic device where the input of various reagents step-by-step in a continuous flow allow a synthesis layer-by-layer of the designed hybrid nanoshell

This last remark highlights the need for dialogue and cross-research between biologists, chemists and engineers to achieve successful and sustainable technologies [11].

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Part VI
Annexes

Annex 1 – Single micro-algae@silica

1.1. Foreword

In order to study the feasibility of the method developed during this work and its replicability on other living organisms, another photosynthetic organism was selected. This appendix will highlight some of the results acquired during Jean Roussel's final internship in order to achieve his DUT degree, in Chemistry, from the Institut Universitaire de Technologie du Mans (France).

1.2. Introduction

The photosynthetic organisms are numerous and show good prospects for use. Micro-algae are also very popular for the design of photobioreactors and the production of high-value-added metabolites. These organisms are eukaryotic, their genetic machinery being more complex than cyanobacteria makes it possible to predict the production of more complex and specific molecules [1, 2]. These organisms have already been used for the design of living materials [3]. For example, they are the subject of research for the creation of continuous flow reactors following the creation of alginate beads containing micro-algae [4, 5]. Micro-algae have also been studied for the design of single cell materials and their protection by mineral-based nanoshells [6, 7].

The species selected in this case study is *Chlamydomonas reinhardtii* strain CC125 (CC125). This unicellular green alga has been used as a model since the 1960s [8] to study photosynthesis and some of the genomic features it shares with animals. It is in this organism that the presence of DNA in chloroplasts has been discovered. Genetic manipulation methods of this species have been well mastered for several years, making it a versatile species of choice for the design of new technologies [9]. It has already been studied in the context of macroencapsulation in silica gels [10].

In order to prove the transferability of the method developed during this research work, it will be adapted to the conditions of micro-algae culture, but will remain very similar to the approach used previously (Part II). Polycations will be selected for a layer-by-layer construction of a silica-based nanoshell.

1.3. Results and discussion

1.3.1. Experimental

1.3.1.1. Cell cultivation

Chlamydomonas reinhardtii type CC 125 (CC125) are cultivated. The growing conditions are adapted from Gorman *et al.* [11], *i.e.* every 14 days the photosynthetic cells are transferred (by dilution) in fresh culture medium (TAP aqueous buffered medium adjusted to pH 7.4). The culture cell is stored at room temperature, under light (cool white: Omsram lamps type L18W-840) and slight agitation (70 rpm).

1.3.1.2. Bio-hybrid materials synthesis

The nanoshell synthesis process is adapted from previous syntheses. Three deposition cycles are repeated. After removal of the supernatant (recovery of the cells by centrifugation: 2500 rpm; 10 min), the cells are (i) resuspended in a 1% w/w polycation solution (preparation in the TAP medium); (ii) rinsed with fresh medium (after centrifugation) and (iii) resuspended in a silica precursor solution (H_2SiO_3, 50 mM). After a new rinsing, this cycle is repeated twice for the final theoretical deposition of 6 layers around the cells.

1.3.2. Results

The use of three polycations leads to the synthesis of a hybrid silica-based coating around the cells. These are polymers previously used (presented in Part II of this manuscript): DAD, PDA and PEI.

The analysis of hybrid materials reveals mesoporous characteristics that vary according to the polymer. Under the appropriate culture and synthesis conditions, it is also possible to obtain a complex system type single cell@silica. The samples are prepared for analysis by electron microscopy and compared to native micro-algae. The micrographs of the four samples are collected in the Figure VI-1.

Native cells have a wrinkled surface following SEM observation (Figure VI-1a_1). The structure has been slightly affected by drying with supercritical CO_2. By TEM, we can clearly see the cell wall and the different organelles of these cells (Figure VI-1a_{2-3}).

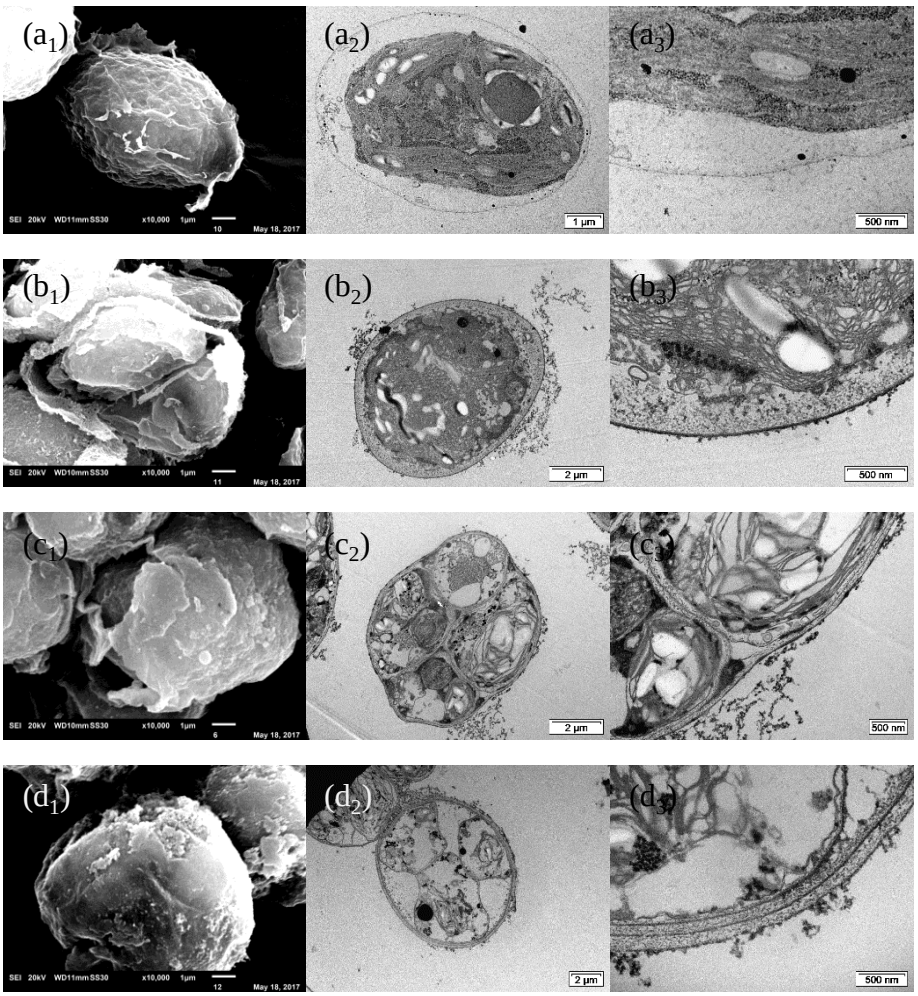

Figure VI-1: SEM and TEM micrographs of (a) native CC125; (b) CC125@DAD/H_2SiO_3; (c) CC125@PDA/H_2SiO_3 and (d) CC125@PEI/H_2SiO_3

By closely observing the other micrographs, it is possible to see as an envelope a few tens of nanometres thick around the micro-algae and this in all three cases (Figure VI-1b_1, c_1 and d_1). The crumpled surface of the native cells is replaced by a smoother surface with openings that suggest the foresee covered cells. Observations by transmission electron microscopy confirm these depositions. A thin darker layer is indeed visible, especially on the magnifications (Figure VI-1b_3, c_3 and d_3), for each observation. This is even clearer when compared to native cells (Figure VI-1a_3).

Nevertheless, viability measurement made by oximetry over time reveals the harmful effect of these shells on cell survival. The silica-based coating appears to block cell activity, either by preventing exchanges or its components have cytotoxic effects on cells.

1.4. Conclusions

The main objective of this work was to demonstrate the transferability of the synthesis pathway developed during work on other single-cell photosynthetic living organisms. The choice was therefore made to move away from cyanobacteria (prokaryotes) to micro-algae (eukaryotes).

The layer-by-layer method, the sol-gel method and the use of polycations were therefore adapted to the growing conditions of the CC125. Three polyelectrolytes were used to obtain bio-hybrid system type single cell@silica. Unfortunately, the synthetic route or synthesised nanoshells have a detrimental effect on micro-algae, which no longer exhibit any photosynthetic activity the day after the encapsulation manipulation.

However, these results are encouraging and show that it is possible to obtain a nanoshell on other living organisms. Although this method must be adapted to be less harmful to CC125 cells, we see that this technology is transferable.

Annex 2 – Bioenergetic processes in cyanobacteria

2.1. Photosynthesis

Photosynthesis is the bioenergetic process that directly or indirectly nutrients almost all living entities [12]. Indeed, in the biosphere, we find autotrophic and heterotrophic. The former can be considered self-sufficient as they develop their organic molecules from carbon dioxide and other inorganic raw materials from their environments, they are the producers. Heterotrophs are consumers since they feed on compounds synthesised by other organisms. A large part of the producers is photoautotrophs. They use light as an energy source to synthesise organic matter.

Reduced to its simplest expression, photosynthesis can be summarised by the following chemical equation (1).

$$n\ CO_2 + n\ H_2O \rightarrow [CH_2O]_n + n\ O_2 \quad (1)$$

This equation, which appears quite simple, represents a set of very complex processes. This has two phases: (i) a set of photochemical reactions (so-called light phase) and (ii) the Calvin cycle (dark phase). These steps are summarised in the diagram of the Figure VI-2.

The photochemical reactions include the steps that lead to the conversion of solar energy into chemical energy. The water molecule, then split, becomes the source of electrons and protons. The oxygen is then released. The photosynthetic pigments absorb visible light, these triggers the transfer of electrons and protons from water to temporarily store them within the nicotinamide adenine acceptor dinucleotide phosphate ($NADP^+$). This is reduced to $NADPH+H^+$, an energy-rich electron source. These photochemical reactions also induce the production of adenosine triphosphate (ATP) by phosphorylation of adenosine diphosphate (ADP), which is a chemical energy reserve.

Calvin's cycle follows photochemical reactions for the production of organic matter from the reserves of electrons and energy produced. This begins with carbon fixation: incorporation of atmospheric carbon dioxide. This is reduced to carbohydrates by the addition of electrons, the reducing potential comes from $NADPH+H^+$. The chemical energy required for this transformation comes from ATP.

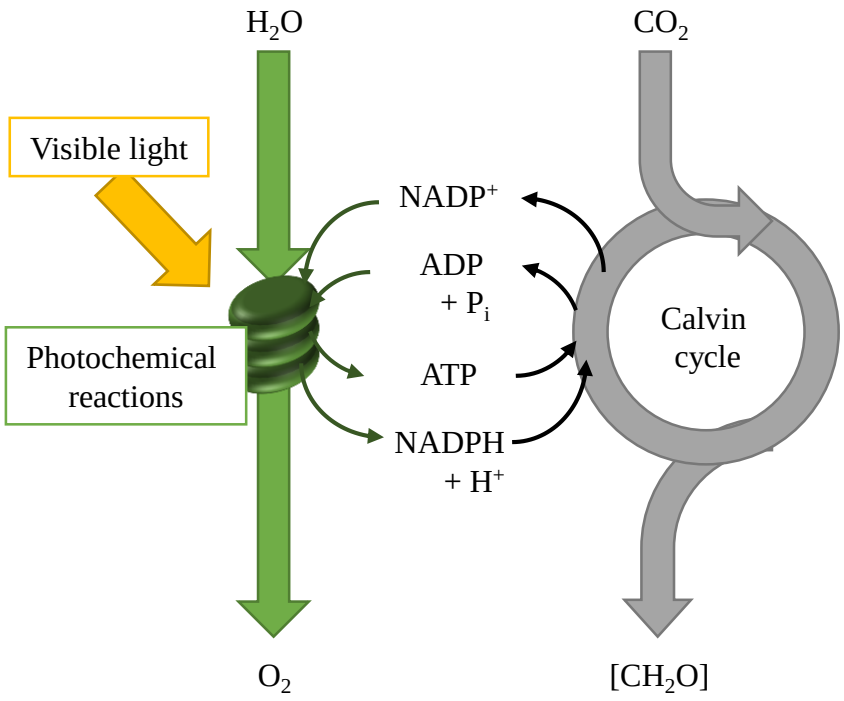

Figure VI-2: Diagram presenting the phases of the photosynthesis

2.2. Discussion: respiration

Despite their photoautotrophic properties, most photosynthetic organisms are also able to breathe. The function of this process is to generate a minimum amount of energy necessary for survival in the event of certain deficiencies (light or nutrients) [13]. During respiration, an oxidising species is at the origin of the creation of energy and organic matter. Most organisms use oxygen for this purpose.

Although breathing in its primary sense does not involve light energy, some metabolic processes exploit and consume oxygen via other chemical pathways. It is then common to talk about respiration [14]. Cyanobacteria are able to concentrate CO_2 internally and could thus inhibit respiration, nevertheless, it has been proven that this process is also essential in these photosynthetic prokaryotes [15].

During our work, the oxygen consumption of samples obtained under certain conditions were observed under the effect of light. The facts mentioned above lead us to imagine the possibility of a temporary blockage of photosynthesis following the addition of nanoshell compounds to each other. This then leads to the implementation of a survival process by PCC7002. Process that then results in oxygen consumption.

Annex 3 – Supercritical drying

3.1. Purpose

Biological samples are not compatible with the ultra-high voids of numerous characterisation techniques. These are in fact composed mainly of water, which will disturb this vacuum. Unfortunately, open-air drying leads to many deformations and cracking of the structures. Indeed, since water has a high surface tension in the air, the crossroads between the interfaces of the liquid to gaseous state during evaporation to the area brings forces that deform the structures. It is therefore necessary to use methods that allow the samples to be dried while preserving their morphology.

3.2. Critical point drying method

Critical point drying uses the properties of this interesting point where the characteristics of the liquid and gas are no longer distinguishable so as not to be damaging to the samples to be dried. At this point, compounds can move from one state to another without crossing an interface and thus avoid damage.

This strategy faces a technical challenge. The critical point of water is 374°C and 229 bar, conditions that destroy any biological material. It is therefore necessary to work with another compound. Carbon dioxide is an excellent candidate since its critical point is at 31°C and 74 bar, compatible with biological structures.

The only problem with CO_2 is that it is not miscible in water. It is therefore necessary to replace this with another solvent, in our case ethanol. Unfortunately, this one has critical point characteristics (241°C and 60 bars) unsuitable for the cells. An exchange of this exchange fluid by the CO_2 transition fluid is necessary.

After the H_2O/CH_3CH_2OH and CH_3CH_2OH/CO_2 exchange process, the drying process by removing CO_2 is started. The chamber, initially thermostatised at around 4°C (to obtain CO_2 in a liquid state during the process), is heated.

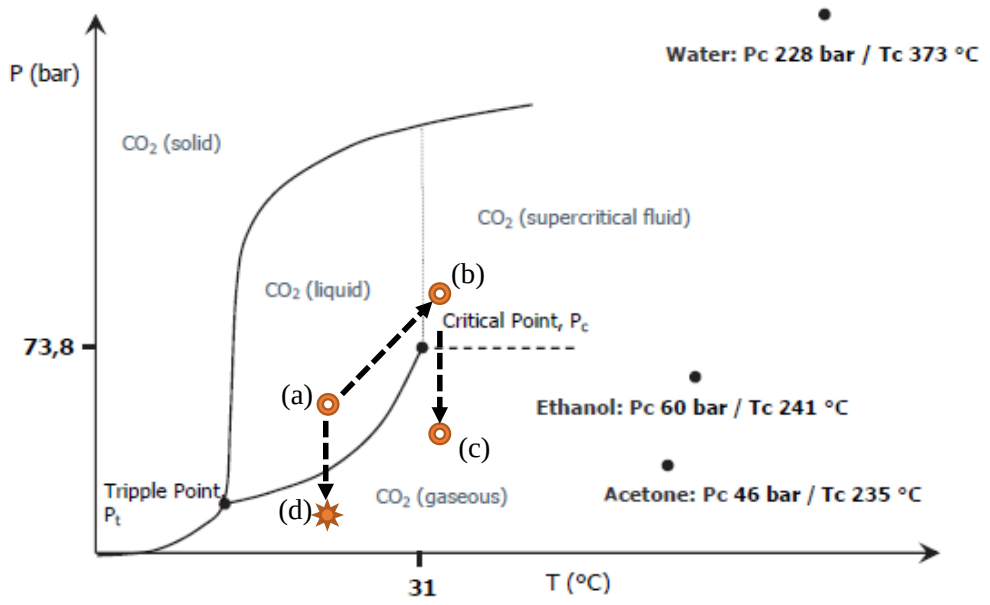

Figure VI-3: Supercritical drying process where P_c = critical point CO_2 and P_t = triple point CO_2 (adapted from reference [16])

Thus, the transition from the point (a) to point (b) on the Figure VI-3 shows the generation of supercritical CO_2. The pressure is then released by maintaining a constant temperature and opening the gas vent valve. The transition that occurs is represented by the arrow from the point (b) to point (c). This is how carbon dioxide becomes gaseous without crossing a boundary between two phases, unlike open air drying (path from the point (a) to point (d)).

3.3. Discussion

Drying biological samples is essential for the experimenter, but brings its share of challenges. Open air-drying leads to a collapse of the macro and microstructure, so it is necessary to go through additional technical manipulations. Another technique to preserve the structures of biological entities is freeze-drying. This technique is based on the sublimation of the solvent (usually water) from a sample previously frozen very quickly (use of liquid nitrogen).

This technique is widely used, for example, as the last step in protein purification processes. In our case, it is supercritical drying that is preferred. In the short term, this technique makes it possible to obtain samples of better quality according to certain criteria (even if, in the long term, the quality of the samples is similar due to the precarious stability of the samples) [17].

Annex 4 – PDA/PSS complex

4.1. Foreword

In this work, the use of polyelectrolytes is omnipresent. First the use of polycations is studied (Part II) before creating complex polycations/polyanions (Part III). This appendix will briefly discuss the complex formed: poly(diallyldimethylammonium chloride) and poly(styrene sulfonic acid) (PDA/PSS).

4.2. Discussion

The use of films [18] or capsules [19, 20] made from polymers had been already widely used. Their use quickly extended to the individual encapsulation of living cells [21]. The case of PDA/PSS complexes has undeniable advantages for the creation of protective coatings. The creation of capsules based on these two polymers has already been considered, studies show interesting properties.

The studies also confirm the presence of uncompensated charge in complexes of these two polyelectrolytes [22]. The layer-by-layer strategy and the use of loads for the construction of our bio-hybrid materials is therefore relevant. Capsules constructed in successive layers of PDA and PSS (Figure VI-4) show a fairly high modulus of elasticity (136 MPa [23]), especially compared to those of some cyanobacteria (between 1600 and 2000 kPa [24]). Although the measures were not taken under the same conditions, they differ by a factor of 100. Compared to other polyelectrolytes, the presence of cyclopentane rings in the PDA would bring a certain rigidity to the material [23]. It is therefore possible to have a potential protective effect under certain conditions: osmotic shocks and shear forces, among others. These capsules also have a certain heat resistance and therefore, despite the appearance of deformations, new protection could be provided to cyanobacteria against temperature variations [22, 25]. These complexes are also resistant to acid treatment. One study presents the formation of a PDA/PSS capsule formed by templating. The template is removed with acid, while the empty capsule is maintained [23]. This type of coating could also provide some tolerance to pH variations in the cells. Finally, PSS absorbs ultraviolet rays, so it is used as a protection against these dangerous rays.

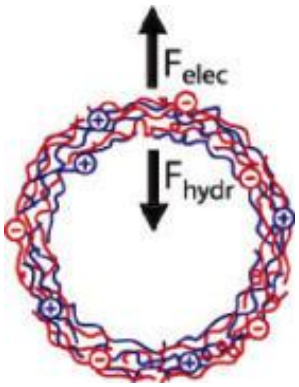

Figure VI-4: Polyelectrolytes capsules and competing forces determining their behaviour (adapted from reference [22])

Annex 5 – Removal of heavy metal from bacteria

5.1. Foreword

The recovery of toxic metal ions from contaminated water, or soil is a current challenge. Industrial activity leads, among other things, to this type of pollution, which brings new concerns related to the stability of those harmful components. The use of bacterial biomass and thus also cyanobacteria is a promising and inexpensive way to recover and isolate these heavy metals. Most of the biosorption mechanisms of these ions involve surface interactions of the cells. A modification by encapsulation from outside the cyanobacteria can thus potentially affect the biosorption capacities of metals.

In the following section, we will briefly review the general mechanisms operating for removal of heavy metals by microbacterial biomass. More specific parts will attempt to highlight the specific mechanisms of the ions selected during this work (Cu^{2+}, Cd^{2+} and Pb^{2+}).

5.2. Generalities [26-28]

Of all the mechanisms and interactions between metal ions and cyanobacteria, most depend only on surface properties of the prokaryotes. Thus, simple **physical adsorption** can bind the metal ions to the surface, allowing them to be recovered. Stronger bonds of **ionic interactions** types can also appear and fix these ions to the surface of the cells. Chemical reactions and exchanges also take place. **Exchanges** between metal ions and surface ions that then solubilise can occur to recover these ions. **Complexes** can also be formed between metal ions and functional groups on the surface (carboxyl, hydroxyl, sulfhydryl, etc.) leading to the retention of these ions by cells.

Other mechanisms depend on the biological functions of cyanobacteria. Surface **precipitation** may occur as a result of nucleation reactions between organic molecules produced by cyanobacteria with metal ions. Finally, an active transport allows the cells to assimilate (**absorption**) these metal ions and integrate them into their metabolism. For example, metals are necessary for the synthesis of metalloproteins [29].

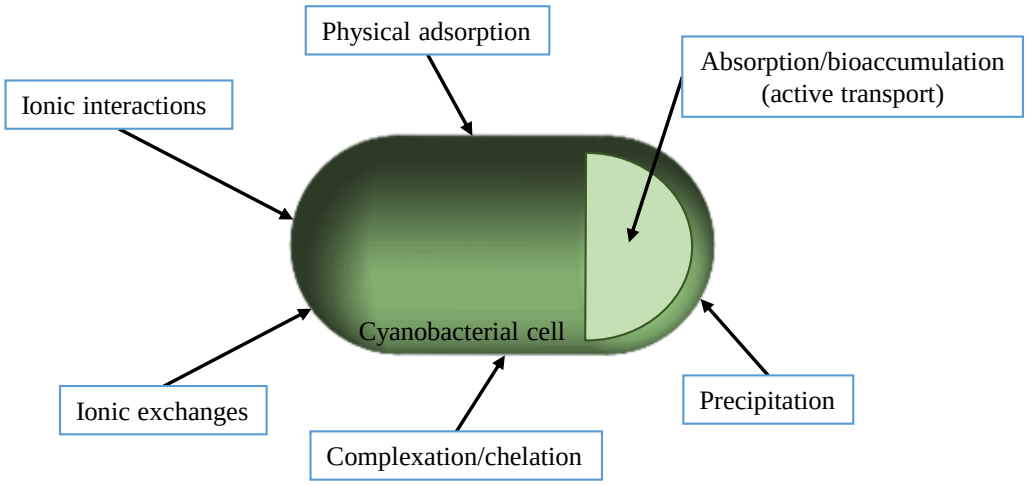

Figure VI-5: Diagram representing the basic mechanisms and interactions between metal ions and cyanobacteria allowing their removal from polluted water

5.3. Selected metal ions

5.3.1. Copper (II)

Copper has special properties and present abilities to form complexes (and special way of interacting with the ligands e.g. due to the Jahn-Teller effect [30]). The supposed mechanism of copper biosorption on the surface of cyanobacteria would therefore be the formation of complexes with functional groups [31, 32]. The possibility of complexing with outside carboxyl groups is recognised in the literature [33].

5.3.2. Cadmium (II)

The second metal also forms interactions with the surface of cyanobacteria. A link with functional groups on the surface (mainly carboxyl groups) is mentioned [34]. Nevertheless, a mechanism seems to be more frequent. Indeed, since the ionic radius of Cd(II) is near (a bit smaller) than the Ca(II) radius, an exchange with these ions present on the surface can occur, the intercalation being facilitated [35].

5.3.3. Lead (II)

The case of lead (II) is still different. The solubility of this metal ions is very low at working pH (around 7.4) in different forms. At this pH, the formation of poorly soluble species such as $Pb_2(OH)_3Cl$ and lead hydroxide ($Pb(OH)_2$) is expected [36]. A precipitation of these ions on the surface of the cyanobacteria is waited, allowing their removal. FTIR studies on bacterial species show the possible interaction between lead ions and functional groups on the surface [37] also adding a potential effect to the successful removal of these ions from the selected cyanobacterial species.

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Part VII
Scientific communication

Publications

From this work

1. Single cyanobacteria@silica porous microcapsules via a sol-gel layer-by-layer for heavy metals remediation
   Journal of Sol-Gel Science and Technology, **2018**, https://doi.org/10.1007/s10971-018-4687-x
   Cyrille Delneuville, Emeric P. Danloy, Li Wang and Bao-Lian Su
   (presented in this work: Part III – Chapter 2)

2. Organically modified silanes for single cyanobacterium encapsulation and functionalisation
   In preparation
   Cyrille Delneuville, Cassandra Toni, Emeric P. Danloy, Li Wang and Bao-Lian Su
   (presented in this work: Part IV – Chapter 2)

Related to this work

3. Single-cell yolk-shell encapsulation for long-term viability with size-dependent permeability and molecular recognition
   In submission
   Li Wang, Yu Li, Xiao-Yu Yang, Bo-Bo Zhang, Nöelle Ninane, Henk J. Busscher, Zhi-Yi Hu, Cyrille Delneuville, Emeric P. Danloy, Nan Jiang, Hao Xie, Gustaaf Van Tendeloo and Bao-Lian Su

Scientific events

1. Third International Conference on Advanced Complex Inorganic Nanomaterials (ACIN 2015), Namur (Belgium), **2015**

   *Poster presentation: "*Designing of an immobilisation method of individual cyanobacteria by a silica coating using the layer by layer method for a protection of the cells"

   Cyrille Delneuville, Emeric P. Danloy and Bao-Lian Su

2. Neuvième Journée-rencontres des jeunes chimistes de la Société Royale de Chimie, Bruxelles (Belgium), **2016**

   *Poster presentation: "*Silica and photosynthesis : cell protection technology through the immobilization of individual cyanobacteria within a silica shell via a layer by layer method"

   Cyrille Delneuville, Emeric P. Danloy, Li Wang and Bao-Lian Su

3. Fifth International Conference on Multifunctional, Hybrid and Nanomaterials (HYMA 2017), Lisbon (Spain), **2017**

   Oral presentation (speaker: Bao-Lian Su): "Design of bio-hybrid materials by the individual encapsulation of cyanobacteria in a silica porous matrix"

   Cyrille Delneuville, Emeric P. Danloy and Bao-Lian Su

4. Dixième Journée-rencontres des jeunes chimistes de la Société Royale de Chimie, Louvain-la-Neuve (Belgium), **2017**

   Oral presentation: "Bioencapsulation towards living cell improvement : design of silica shell around cyanobacteria"

   Cyrille Delneuville, Emeric P. Danloy and Bao-Lian Su

   Poster presentation: "Design of photosynthetically active bioceramics by encapsulation of cyanobacteria within porous silica matrices"

   Emeric P. Danloy, Cyrille Delneuville and Bao-Lian Su

5. Nineteenth International Sol-Gel Conference (Sol-Gel 2017), Liège (Belgium), **2017**
Oral presentation: "Design of bio-hybrid material: encapsulation of cyanobacteria via the sol-gel synthesis of silica shell around individual cells"
Cyrille Delneuville, Emeric P. Danloy and Bao-Lian Su

6. Fourth International Conference on Advanced Complex Inorganic Nanomaterials (ACIN 2018), Namur (Belgium), **2018**
Oral presentation: "Design of bio-hybrid materials: individual encapsulation of cyanobacteria towards cell protection"
Cyrille Delneuville, Emeric P. Danloy, Li Wang and Bao-Lian Su
Poster presentation: "Individual encapsulation of cyanobacteria trough cell surface modification"
Cyrille Delneuville, Emeric P. Danloy, Li Wang and Bao-Lian Su
(awarded a Best Poster Prize by Molecules (an Open Access Journal by MDPI))

Notes