Biological activity and/or electrochemistry of membrane proteins, bacteria and bacteriophages in a hybrid-based sol-gel material

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Activité biologique et électrochimie de protéines membranes, de bactéries et de bactériophages dans un matériau sol-gel hybride

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Abbreviations

Materials

TEOS: Tetraethoxysilane
TMOS: Tetramethoxysilane
PEI: Poly (ethylenimine)
PBS: Phosphate buffer solution
PEG: Poly (ethylene glycol)
SWCNT: Single-walled carbon nanotubes
SWCNT-$(EtO)_8$-Fc: Single-walled carbon nanotubes chemically-modified with poly (ethylene glycol) linker and ferrocene mediator
MWCNT: Multi-walled carbon nanotubes
MWCNT-Os: Multi-walled carbon nanotubes wrapped with osmium polymer
AuNP: Gold nanoparticles
GFP: Green fluorescent protein
PI: Propidium iodide
PVA: poly(vinyl alcohol)
(PVA-g-P(4-VP)): poly(vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)) complex
DCPIP: 2,6-dichlorophenol indophenol
ManDH: L-Mandelate dehydrogenase
FDM: 1,1-Ferrocene dimethanol
PDDA: Poly(dimethyldiallylammonium chloride)

Electrodes

CE: Counter electrode
WE: working electrode
GCE: Glassy carbon electrode
ITO: Indium-Tin-Oxide electrode
PGE: Pyrolytic graphite electrode

Techniques

CV: Cyclic voltammetry

*Bac*light: *Bac*light bacterial viability test

AFM: Atomic force microscopy

TEM: Transmission electron microscopy

Scientific terms

EC: Electrochemical communication

ET: Electron transfer

DET: Direct electron transfer

MET: Mediated electron transfer

EABE(1S): Electrochemically-assisted bacteria encapsulation in one-step

EABE(2S): Electrochemically-assisted bacteria encapsulation in two-step

EABE: Electrochemically-assisted bacteria encapsulation

Introduction générale

Le travail décrit dans cette thèse a été mené à l'interface entre trois disciplines: l'électrochimie, la science des matériaux et la microbiologie. L'objectif de cette recherche était tout d'abord d'étudier l'activité de bactéries immobilisées dans un film de silice déposé par le procédé sol-gel à la surface d'électrodes. Le film peut être préparé par électrochimie ou par simple dépôt d'une goutte de sol à la surface de l'électrode. Il est alors primordial de conserver la viabilité de la bactérie dans ce film sol-gel et de favoriser les réactions de transfert électronique entre la bactérie et l'électrode. L'immobilisation de protéines membranaires au sein de couches minces sol-gel a ensuite été considérée et appliquée à l'électrocatalyse enzymatique. Ces protéines sont associées à des fragments membranaires ou vésicules qui peuvent être stabilisés dans le film sol-gel en utilisant des stratégies similaires à celles impliquées pour l'immobilisation de cellules entières. Les études proposées ici sont de natures fondamentales, mais peuvent trouver des applications dans les domaines des biocapteurs ou bioréacteurs. Un dernier travail, périphérique par rapport aux travaux précédents, a concerné l'encapsulation de bactériophage pour étudier l'influence de mode d'immobilisation sur l'infectivité du virus.

L'électrochimie a tout d'abord été utilisée pour induire l'encapsulation de bactéries au sein de films hybrides organique-inorganique préparés par le procédé sol-gel (chapitre 2). En effet, il est possible d'utiliser l'électrolyse du sol pour produire localement de façon contrôlée à la surface de l'électrode les espèces OH^- qui catalysent alors la gélification du matériau. Cette approche a été initialement proposée par Schacham *et al*. à la fin des années 90 avant d'être appliqué par Walcarius *et al.* à la génération de films silicatés mésostructurés. Cette même approche a ensuite été appliquée en 2007 à l'immobilisation de protéines rédox par le groupe ELAN du LCPME. L'application du dépôt sol-gel par assistance électrochimique pour l'encapsulation de bactéries se situe ainsi dans la continuité des travaux précédents, l'enjeu étant ici de protéger la viabilité des bactéries dans cette couche mince, malgré le stress de l'électrolyse. Deux modes de dépôt ont été développés pour l'encapsulation des bactéries par assistance électrochimique, en une étape par introduction des bactéries dans le sol avant dépôt, et en deux étapes, avec une première étape d'immobilisation des bactéries à la surface de l'électrode avant application de l'électrolyse dans un sol qui cette fois ci ne contient pas de bactéries. Dans cette première série d'expériences, la viabilité des bactéries a été évaluée en

utilisant un kit de marqueurs fluorescents de l'ADN, Live/dead BacLight, permettant de caractériser l'intégrité membranaire. L'activité métabolique a également été testée en utilisant des bactéries génétiquement modifiées pour exprimer des protéines fluorescentes ou luminescentes en réponse à leur environnement.

L'électrochimie a ensuite été utilisée comme un moyen analytique pour caractériser les réactions de transfert électronique entre des bactéries et différents médiateurs rédox (chapitre 3). Dans une première approche, un médiateur soluble, $Fe(CN)_6^{3-}$, a été utilisé, en nous inspirant ainsi des travaux décrits par le groupe de Dong. L'immobilisation du médiateur a ensuite été considérée. Deux stratégies ont été mises en œuvre. La première fait appel à des nanotubes de carbone fonctionnalisés par des groupements ferrocène à l'aide d'un bras poly(oxyde d'éthylène). La seconde implique un médiateur naturel, le cytochrome c, qui est alors immobilisé dans la matrice de silice avec les bactéries, mimant de cette façon une stratégie développé par certains biofilms naturels pour augmenter les transferts électroniques vers l'accepteur final, minéral ou électrode. Cette dernière stratégie a d'abord été testée avec *Shewanella Putrefaciens* avant d'être approfondie avec *Pseudomonas Fluorescens*.

Le troisième sujet traité dans cette thèse est l'électrochimie des protéines membranaires immobilisées dans un film sol-gel (Chapter 4). Plusieurs travaux décrits dans la littérature ont montré que ces protéines, associées à des fragments membranaires, pouvaient être stabilisés au sein de matériaux sol-gel, mais l'utilisation de ce mode d'immobilisation pour l'électrochimie reste rare ou inexistant selon le type de protéine considéré. Deux systèmes ont été étudiés. Le premier était un cytochrome de type P450 (CYP1A2) qui peut être utilisé pour l'élaboration de biocapteurs. Une propriété de ce type de protéine rédox est de pouvoir accepter un transfert électronique direct entre l'électrode et le centre catalytique. Nous avons étudié ici l'intérêt de la chimie sol-gel pour protéger la protéine afin de favoriser la stabilité de ce transfert électronique. Le second système est la mandélate déshydrogénase (ManDH). L'électrochimie de cette protéine implique un transfert électronique médié. La stabilité de cette réponse électrochimique, notamment en milieu convectif, a été testée et comparée à la réponse du système impliquant une simple adsorption des vésicules contenant cette protéine à la surface de l'électrode de carbone vitreux.

La dernière section s'intéresse à l'influence de l'encapsulation du bactériophage ΦX174 dans une matrice sol-gel hybride sur son infectivité (chapitre 5). Ce type de virus, découvert au début du 20ème siècle présente une infectivité spécifique pour certaines souches bactériennes pouvant être utilisée pour le traitement antibactérien, en remplacement ou

complément des antibiotiques. L'encapsulation de virus dans un matériau sol-gel a été récemment considérée afin de permettre un relargage contrôlé dans l'organisme pour la thérapie génétique des cancers. Ce mode de relargage a également l'avantage de ralentir le développement d'anticorps en réponse à ce virus. L'apparition de souches bactériennes développant des résistances aux antibiotiques actuels a récemment remis en lumière les bactériophages comme moyen de lutte contre les infections bactériennes. Leur immobilisation contrôlée permettrait sans doute d'étendre le champ d'application de ces virus. Dans ce travail, l'effet de l'encapsulation des bactériophages dans une matrice sol-gel a été étudié, en tenant compte notamment de l'effet du séchage du matériau sur l'infectivité. Les matériaux ont été préparés sous la forme de monolithes et conservés en atmosphère humide ou sèche. Ces monolithes ont ensuite été réintroduits en solution pour relargage des bactériophages dont l'infectivité a été mesurée en présence d'Escherichia *Coli*. Différents additifs ont été introduits dans le gel de silice comme le glycérol ou de polymères chargés afin d'évaluer leur effet sur l'infectivité résiduelle.

Toutes ces études sont précédées par une introduction sur l'intérêt des matériaux biocomposites dans les domaines médicaux et biotechnologiques (chapitre 1). Des généralités sur le procédé sol-gel, certains additifs organiques pouvant être introduits dans les matrices inorganiques pour l'élaboration de matériaux hybrides sont présentées en discutant leur intérêt potentiel pour notre travail. L'intérêt de l'encapsulation de protéines ou de microorganismes est également présenté en considérant les applications possibles, notamment en bioélectrochimie. Le principe de l'électrochimie des protéines rédox et des microorganismes et notamment les stratégies pour favoriser les réactions de transfert d'électron sont décrites. Enfin, le positionnement du sujet par rapports à la littérature est donné.

Les méthodes et les techniques utilisées pour décrire les propriétés physico-chimiques des systèmes étudiés dans cette thèse, les différents protocoles sol-gel, notamment pour la modification des électrodes sont décrits dans la partie expérimentale. Enfin, une conclusion générale est proposée.

General introduction

The work reported in this thesis has been developed at the interface between three disciplines, i.e., electrochemistry, material science and microbiology. The purpose of this research was first to study the activity of bacteria immobilized in silica-based films prepared by the sol-gel process on electrode surfaces. The film can be prepared either by electrochemistry or drop-coating on the electrode surface. In such systems, the fundamental keys are to retain the cell viability in a sol-gel film [1] and to promote the electron transfer reactions between entrapped bacteria and electrodes materials [2]. Then, the immobilization of membrane associated redox proteins in sol-gel films have been considered and applied for electrocatalysis. These proteins are associated to membrane fragments or vesicles that can be stabilized in sol-gel films with similar strategies as reported for whole cells [3]. The approaches of the different works shown here with bacteria and membrane-associated proteins, are fundamental, but can find application for example as electrochemical biosensors or electrochemical bioreactors. A peripheral work of this PhD, will be also presented, i.e., the influence of encapsulation in sol-gel matrix on bacteriophage infectivity.

Electrochemistry was first used to induce the encapsulation of bacteria in hybrid sol-gel films (Chapter 2). Indeed, the controlled cathodic electrolysis of the sol can be used to produce locally at the electrode surface a basic pH, which catalyzes the rapid gelification of the sol. The approach has been initially proposed by Schacham *et al.* at the end of the nineties [4] before to be applied by Walcarius *et al.* to the generation of mesostructured films [5]. The same approach has been also applied in 2007 to the immobilization of redox proteins by the group ELAN of LCPME [6]. The application of electrochemically-assisted sol-gel deposition for the encapsulation of bacteria is a continuation of these previous investigations, the challenge being here to protect the viability of the bacteria in such thin film, despite the stress of electrolysis. Two approaches have been developed for electrochemically assisted bacteria encapsulation (EABE), within one step with the bacteria introduced into the sol before deposition or within two steps with the bacteria immobilized on the electrode surface (ITO) before to apply the electrolysis of the sol that do not contain, in this case, any bacteria. In this first set of experiments, viability of the microorganism was simply controlled with the commercial Live/Dead *Bac*Light test based on staining of the DNA material of bacteria with fluorescent dyes. Metabolic activity was also tested with using genetically modified bacteria that could express fluorescent proteins or luminescent light in response to the environment.

Electrochemistry was then considered as an analytical method. Bacteria have been encapsulated in silica-based films and the electron transfer reactions from bacteria to different redox mediators have been monitored (Chapter 3). First approach simply involved ferricyanide as soluble mediators as reported by the group of Dong [7]. Then, the immobilization of the mediator was considered. Two different strategies have been implemented. First one involved carbon nanotubes functionalized with ferrocene moieties through a long poly (ethylene oxide) chain. The second strategy involved a natural mediator, i.e. cytochrome c form bovine heart, which was immobilized inside the silica gel, mimicking in some respect some strategies involved in natural biofilm to increase the ability to transfer electron towards a final acceptor, electrode or mineral. This latter strategy was initially proposed for *Shewanella putrefaciens*, before to be studied systematically with *Pseudomonas fluorescens*.

The third topic of this thesis was the electrochemistry of membrane associated proteins immobilized in a sol-gel film (Chapter 4). Several reports in the literature have shown that such proteins, associated to membrane fragments could be stabilized in sol-gel materials, but application to bioelectrochemistry remains seldom [8,9] or does not exist depending on the considered proteins. Two systems have been studied. The first one was cytochrome P450 (CYP1A2) that is actually considered for biosensor development [10,11]. One major interest of this protein is the ability to operate direct electron transfer (DET) during the bioelectrochemical reaction. Here, it has been studied the potential interest of sol-gel chemistry to provide a protective environment to the protein in order to promote stable direct electron transfer reaction. The second system studied in this section was membrane associated mandelate dehydrogenase (ManDH). The electrochemical application of this protein involves mediated electron transfer (MET). The stability of the electrochemical response, notably in convective environment was tested and compared with the simple adsorption of proteins on glassy carbon electrodes.

A final section considered the influence of encapsulation in a hybrid sol-gel matrix on the infectivity of bacteriophage ΦX174 (chapter 5). Such virus, discovered at the beginning of the 20th century [12] can show infectivity for specific bacterial strains to be considered for antibacterial treatment. Encapsulation of virus in sol-gel materials has been recently considered in order to promote a controlled release in the organism for cancer gene therapy [13]. The silica gel-based delivery had moreover the advantage to slow down the development of anti-adenovirus antibodies. With the appearance of bacterial strain displaying resistances to

antibiotics, the interest of bacteriophage for development of antibacterial treatment recently reemerged [14]. Their controlled immobilization could help in extending their application. Here, the effect of their encapsulation in sol-gel matrix was studied taking into account the effect of drying on their infectivity. Material have been prepared in the form of monolith and stored in humid or dry atmosphere. After storage, the gels were introduced in solution for release of the bacteriophage, whose infectivity was measured in the presence of *E. coli*. The different organic additives such as glycerol or positively charged polyelectrolyte have been introduced in the gel and their effects on the infectivity of the released bacteriophage were studied.

All these studies have been preceded in the thesis by a brief introduction to the interest of biocomposite in biomedical and biotechnological fields (Chapter 1). Knowledge of sol-gel based materials, different organic additives and advantages of incorporation to fabricate sol-gel based hybrid materials are discussed. The advantages of proteins or microorganism encapsulation and their applications in bio-electrochemical and bio-technological fields as well as the principles of wiring redox proteins or microorganisms for bioelectrochemical devices have been illustrated as reported in literature. Finally, positioning of the subject compared to literature survey has been described.

Methods and techniques has been also proceeded to describe the physico-chemical properties of the studied compounds, various sol-gel preparation methods, electrode modifications and experimental techniques and analyses used in this work. A conclusion will close the manuscript.

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Chapter I. Knowledge and literature survey

This chapter is providing some general information from the literature to serve as a basement for the positioning of this PhD thesis. Interests of biocomposite materials in the biological, chemical and technological fields is discussed and illustrated. General information about sol-gel process and materials is given. Methods for film deposition have been summarized highlighting those based on solvent-evaporation and assisted electrochemically. Advantages of hybrid materials combining organic and inorganic components for preserving biological activity of proteins or microorganisms are discussed. Different organic additives are described in details. The interest of encapsulation in hybrid sol-gel materials for proteins, bacteria and viruses is discussed. The principle of electrochemical communication between proteins or bacteria and electrode materials and potential applications are described. Finally, the positioning of thesis is given.

1. Interest of biocomposite materials

Enzymes, bacteria and viruses nowadays are interesting systems for technological devices due to their simplicity of manipulation, high selectivity and sensitivity. Their bioanalytical features are increasingly attracting attention in the pharmaceutical, environmental, medical, and industrial fields. The real challenge faced by efficiency of biological devices is to fulfill the ever growing requirements of environmental legislation in terms of rapidity of response, selectivity, stable reactivity and cost [1,2]. To achieve that, enzymes, bacteria and viruses should be entrapped in biodegradable composite to maintain a high viability/activity rate during the storage process and to enhance the communication with the surrounding environment [1], *i.e*, the entrapped cells provide advantages over free cells such as: increased metabolic activity, protection from environmental stresses and toxicity, increased plasmid stability and they might act as cell reservoir systems prolonging reaction times [3,4].

Several chemical or physical methods for bio-entrapment have been proposed in the literature. In chemical methods, enzymes and bacteria can be attached to an inert and biocompatible matrix through cross-linking using a bi-functional reagent. Proteinic supports, *e.g*., bovine serum albumin [5,6] and gelatin [7,8] are typically used to constitute the network with glutaraldehyde (GA) as the cross-linking agent. This method is rather simple and rapid. Nevertheless, it has also some drawbacks especially when considering microorganisms. In particular, cross-linking involves the formation of covalent bonds between the functional groups located on the outer membrane of the microorganisms/enzymes and GA. This mode of immobilization is consequently not suited when cell viability is absolutely required or when enzymes involved in the detection are expressed at the cell surface [2]. Physical methods represented by physical adsorption, is the simplest and softest method for biomolecules and microganisms immobilization. Nevertheless, it results in a weak electrostatic bonding that may cause easy desorption of the proteins and microorganisms from the surface during storage or analysis [2,9]. Another way to avoid leakage is to entrap them in an adequate material. Different strategies have been developed in this context. In a first strategy, the cells are filtered through a porous membrane, *e.g.,* Teflon [10], polycarbonate [11], cellulose nitrate [12], silicon [13] or nylon [14] which is subsequently fixed to the electrode. In a second strategy, cells are retained near the transducer surface by a dialysis membrane [15]. These two modes of immobilization have been widely used for biosensors construction. In a third approach, proteins and microorganisms are entrapped in a chemical or biological

polymeric matrix. Sol-gel silica [16–18] or hydrogels, such as poly(vinyl alcohol) (PVA) [19] and alginate [20] are typically used for that purpose. These polymers can efficiently protect microorganisms from external aggression. On the other hand, they may form a diffusion barrier that restricts the accessibility of the cells to the substrate and/or decrease electron transfer reactions [2]. The swelling properties of hydrogels may also limit their practical application in some cases. The combination of sol-gel and hydrogel materials by using a sol-gel-derived composite based on silica sol and PVA–4-vinylpyridine copolymer prevents silica glass from cracking during the sol-gel transition, limits hydrogel swelling and protect the bacterial viability [21].

To date, natural or engineered proteins, antibodies, antigens, DNA, RNA, cell membrane fractions, organelles and whole cells, have been encapsulated in a diverse range of inorganic, organic, and hybrid materials [22]. The realm of sol-gel biocomposite offers significant advances in technologies associated with biology, *i.e,* for fabrication of biosensors, biocatalysts, and bioartificial organs to the fabrication of high-density bioarrays and bioelectronic devices. Indeed, it is expected that the coming years will witness the realization of a variety of research and industrial applications, especially those aimed at the catalysis, sensing/monitoring, diagnostics, biotechnology, and biocomputing sectors [22]. Next section will provide some general information about the sol-gel process.

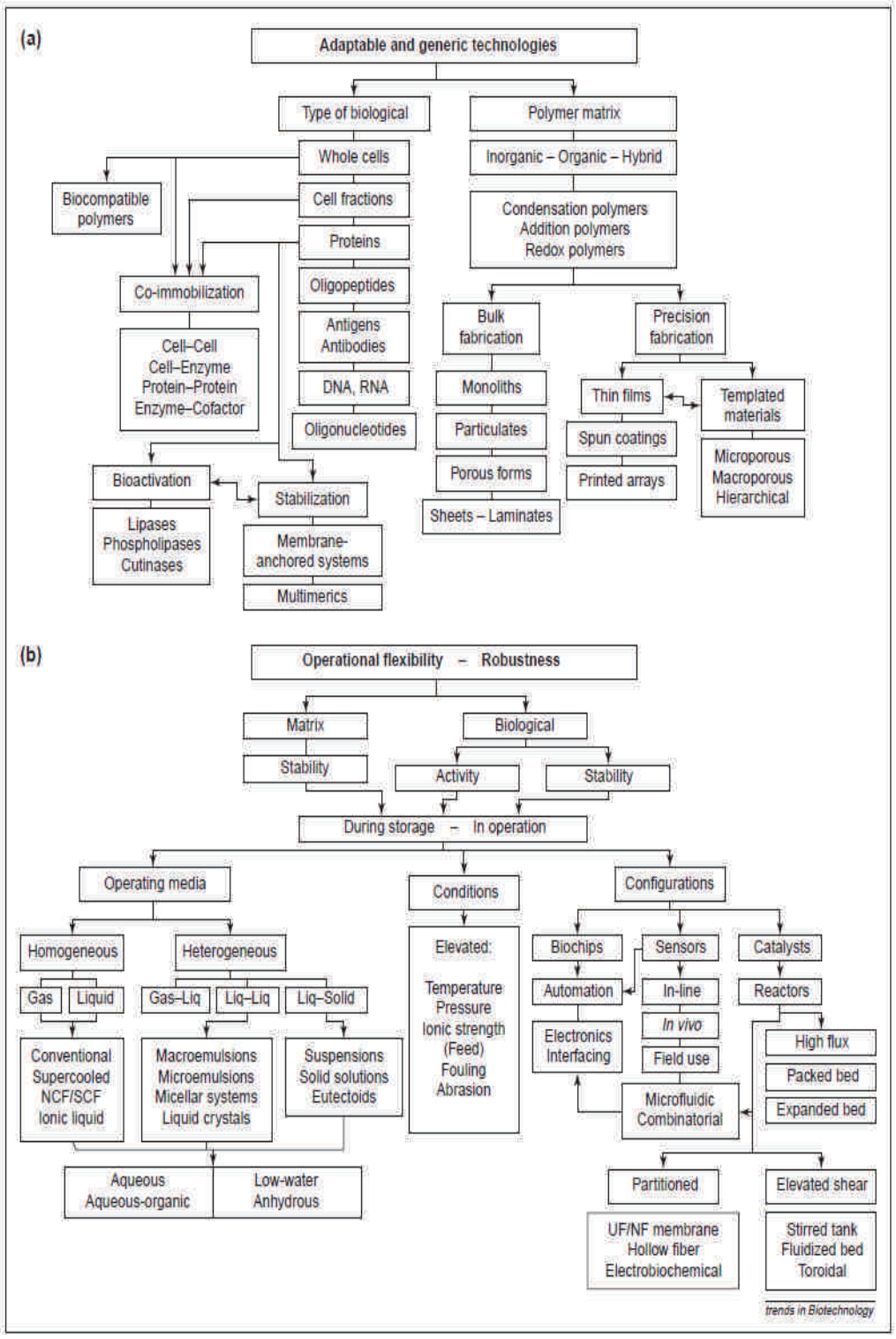

(a)
Adaptable and generic technologies
Type of biological
Polymer matrix
Whole cells
Inorganic – Organic – Hybrid
Biocompatible polymers
Cell fractions
Condensation polymers
Addition polymers
Redox polymers
Proteins
Co-immobilization
Oligopeptides
Bulk fabrication
Precision fabrication
Antigens
Antibodies
Cell–Cell
Cell–Enzyme
Protein–Protein
Enzyme–Cofactor
DNA, RNA
Monoliths
Thin films
Templated materials
Oligonucleotides
Particulates
Spun coatings
Microporous
Macroporous
Hierarchical
Porous forms
Printed arrays
Bioactivation
Stabilization
Sheets – Laminates
Lipases
Phospholipases
Cutinases
Membrane-anchored systems
Multimerics
(b)
Operational flexibility – Robustness
Matrix
Biological
Stability
Activity
Stability
During storage – In operation
Operating media
Conditions
Configurations
Homogeneous
Heterogeneous
Elevated:
Temperature
Pressure
Ionic strength
(Feed)
Fouling
Abrasion
Biochips
Sensors
Catalysts
Gas
Liquid
Gas–Liq
Liq–Liq
Liq–Solid
Automation
In-line
Reactors
In vivo
Electronics Interfacing
High flux
Field use
Conventional
Supercooled
NCF/SCF
Ionic liquid
Macroemulsions
Microemulsions
Micellar systems
Liquid crystals
Suspensions
Solid solutions
Eutectoids
Packed bed
Expanded bed
Microfluidic Combinatorial
Aqueous
Aqueous-organic
Low-water
Anhydrous
Partitioned
Elevated shear
UF/NF membrane
Hollow fiber
Electrobiochemical
Stirred tank
Fluidized bed
Toroidal
trends in Biotechnology

Fig I-1. *Applications and issues of bioencapsulation.* ***(a)*** *Summary of the major fabrication requirements of bio-immobilization technologies. Critical demands are broad applicability to a diverse range of biological materials, accommodation of specific co-immobilization, activation and/or stabilization prerequisites, availability of a variety of polymer chemistries, and amenability to macro- and micro-fabrication in different formats.* ***(b)*** *Overview of the performance characteristics of ideal bio-immobilizates. The major issues are the physico-chemical robustness of the polymer matrix and the stabilization of the immobilized biological. These are especially important in relation to storage stability, sustained performance in gaseous and liquid media and heterogeneous environments, efficient functioning in biologically, chemically and physically aggressive environments, sustained catalytic performance in large-scale bioreactors, and close-tolerance functioning of electronics-interfaced micro-fabricated immobilizates (adapted from reference* [23]).
Abbreviations: liq, liquid; UF, ultrafiltration; NF, nanofiltration; NCF, near-critical fluid; SCF, super-critical fluid.

2. Principles of sol-gel chemistry and processing

The sol-gel process is a wet-chemical technique widely used in the fields of material science and ceramic engineering. In this procedure, the initial precursor 'sol' gradually evolves towards the formation of a gel-like diphasic system containing both a liquid phase and solid phase whose morphologies range from discrete particles to continuous polymer networks. A sol is a stable dispersion of colloidal particles in a liquid. Colloids are solid particles with diameters of 1-1000 nm. A gel is an interconnected, rigid network with pores of submicrometer dimensions and polymeric chains whose average length is greater than a micrometer [24].

In the sol-gel process, the starting precursor could be either in an alkoxide or an aqueous form. Alkoxides are available as pure, single molecules whose reactivity toward hydrolysis can be efficiently controlled and thus the nature of the species that will effectively condense to form a gel can be selected [25]. Overall, the alkoxide-based sol–gel route is more flexible in terms of reaction conditions, chemical nature, functionality, and processing. However, when one considers biology-related applications of sol–gel chemistry, alkoxide precursors exhibit several limiting features. Alkoxide hydrolysis leads to the release of parent alcohol molecules that may be detrimental to biological systems. This is a major concern for material design itself, but also in terms of ecological impact when large-scale applications are to be developed [25,26]. Considering the actual knowledge in the field of sol-gel material, it can be proposed that “alkoxide” and “aqueous” routes may be suitable for different applications, related to their processing. Silicon alkoxides appear convenient precursors for the

encapsulation of proteins and enzymes which are not highly sensitive to alcoholic byproducts, While silicates and colloidal silica are more adapted to encapsulate sensitive microganisms such as bacteria and viruses [26].

The sol-gel process consists of hydrolysis, condensation, gelation, drying and aging steps in order to form gels or xerogels as summarized in Figure I-2.

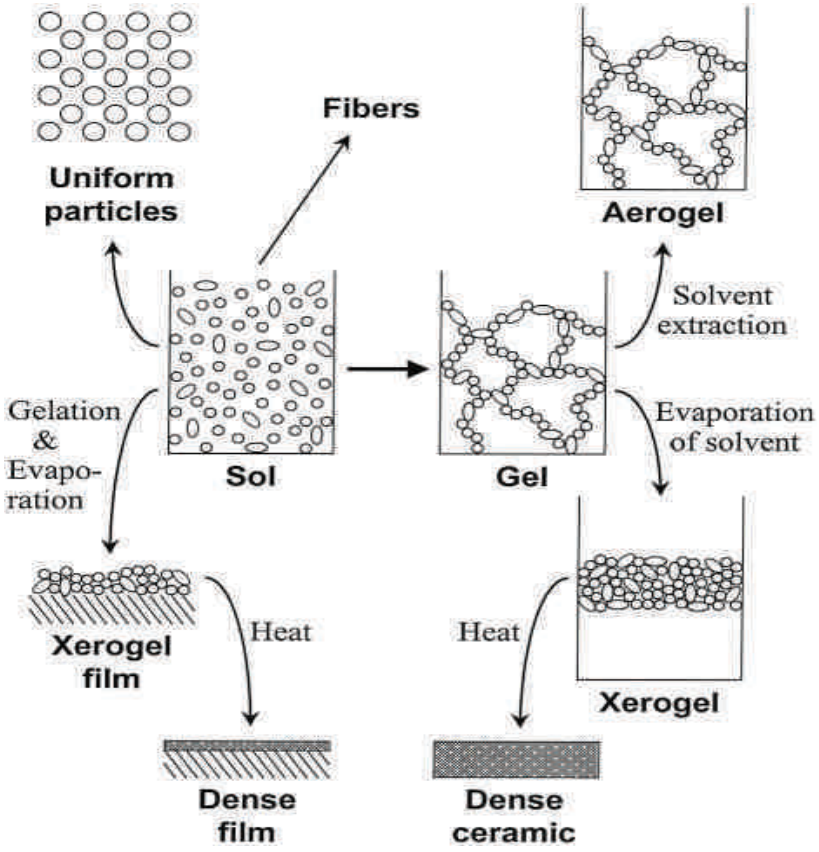


Fig I-2. *Overview of the sol-gel process (adapted from references* [25,27]*).*

2.1. Hydrolysis

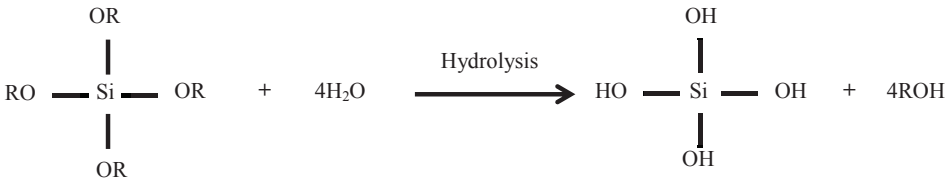
Sol-gel silica synthesis is based on the controlled condensation of $Si(OH)_4$ entities. These may be formed by hydrolysis of soluble alkali metal silicates or alkoxysilanes. The commonly used compounds are sodium silicates, tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS) [23].

Hydrolysis of aqueous silica

Silicate solution species are controlled by the pH medium and silica concentration [25]. Monomolecular $Si(OH)_4$ is the predominant solution species below pH 7and at higher pH, anionic and polynuclear species are formed. For that, sodium silicate solutions are acidified in order to generate $Si(OH)_4$ species that will then condense to form siloxane bonds. The sodium silicate solution characteristics are dependent on the SiO_2:Na_2O ratio.

Hydrolysis of alkoxysilanes

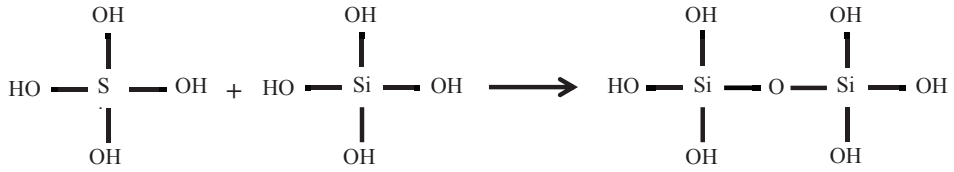
The preparation of a silica glass can begin with an appropriate alkoxide, such as $Si(OR)_4$, where R is mainly CH_3, C_2H_5, or C_3H_7, which is mixed with water and/or a mutual solvent to form a homogenous solution. Hydrolysis leads to the formation of silanol groups (SiOH) (Eq. I-1). It has been well established that the presence of H_3O^+ in the solution increases the rate of the hydrolysis reaction [24,25].



Eq I-1. *Hydrolysis of alkoxide silica precursor in acidic medium.*

2.2. Condensation

In a condensation reaction, two hydrolyzed or partially hydrolyzed molecules can link together through forming siloxane bonds (Si-O-Si) (Eq. I-2). This type of reaction can continue to build larger and larger silicon-containing molecules and eventually results in a SiO_2 network. The H_2O (or alcohol) expelled from the reaction remains in the pores of the network. When sufficient interconnected Si-O-Si bonds are formed in a region, they respond cooperatively as colloidal (submicrometer) particles or a sol-gel [25].



Eq I-2. *Condensation of silica precursors.*

The gel morphology is influenced by temperature, concentrations of each species (especially R ratio, R = [H2O]/[Si(OR)4], and mainly pH. Hydrolysis and condensation occur simultaneously. In acidic conditions, the rate of hydrolysis is faster than that of condensation [24,25]. Sol-gel are generated by polycondensation of inorganic in nature or both inorganic and organic as induced either by evaporation of a sol solvent or electrochemically-assisted deposition [28,29].

2.2.1. Solvent evaporation

- **Coating mode** (drop-, spin- and dip-coating): A process where the substrate (biomolecules or microorganisms) is mixed with sol to be dropped, spinned or dipped on the surface allowing solvent to be evaporated (Fig. I-3). The drawbacks of this protocol are the restriction to basically flat surfaces and simple-shaped substrate only and the unselectively of film deposition on both conducting and insulating materials [29].

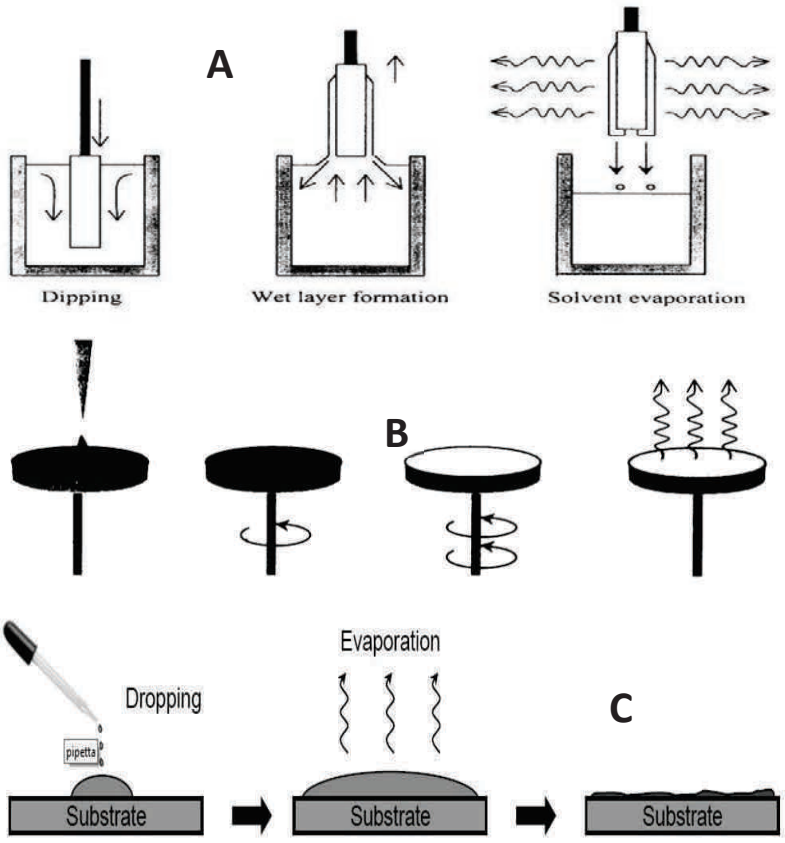


Fig I-3. *Schematic representation of coating processes via solvent evaporation such as A) dip coating; B) spin coating and C) drop-coating (adapted from reference* [28]*).*

- **Monolith:** are defined as a bulk gel (smallest dimension ≥ 1 mm). Monolithic gels are potentially of interest because they are stable at room and low temperature [25]. In addition, bulk gel could be more convenient for entrapment of microorganisms and viruses due to its higher protection which is contributed to its thickness [17].

2.2.2. Electrochemically assisted deposition

An alternative method for polycondensation of hydrolyzed silica precursors has been proposed by Shacham *et al.* involving the incorporation of electrochemistry with the sol-gel processing [30]. The basic idea of electrodeposition is to facilitate the polycondensation of sol precursors by an electrochemical control of the pH at the electrode/solution interface [31], thereby affecting thereby the kinetics associated to the sol-gel process. Starting from a sol solution where hydrolysis is optimal (*i.e.*, pH 3) and condensation very slow, electrodeposition by applying a negative potential are likely increasing pH at the electrode/solution interface (Fig. I-4), thereby catalyzing the polycondensation on the electrode surfaces [29]. The film thickness deposited on the electrode surface is likely to be affected by the applied potential, the electrodeposition time, and the nature of the electrode [30].

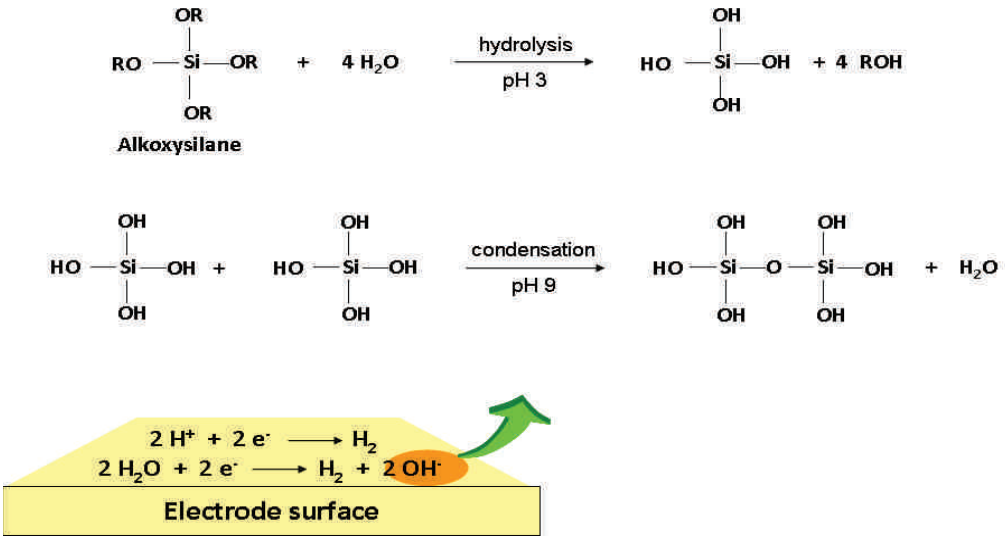


Fig I-4. *The principle of electrochemically-assisted generation of sol-gel on the electrode surface.*

The electrochemically assisted deposition could be also applied for non-silica precursors such as zirconia or titania [32,33]. In addition, electrochemically-assisted deposition can be advantageously combined with the surfactant templating process to generate highly ordered mesoporous sol-gel with unique mesopore orientation normal to the underlying support [34,35] and this was also exploited to prepare vertically aligned silica mesochannels bearing organo-functional groups [36,37].

2.3. Gelation

The hydrolysis and condensation reactions discussed in the proceeding sections lead to the growth of clusters that eventually collide and link together to form a network called gel. Gels are defined as "strong" or "weak" according to the stability of bonds formed which could be reversible or permanent. The difference between strong and weak is related to time of gelation [25]. The gelation rate also influences the pore structure. Fast gelation gives an open structure because the particles are quickly connected and cannot undergo further rearrangements [38].

2.4. Aging

Aging is taking place when a solution starts to lose its fluidity and takes on the appearance of an elastic solid. During aging, four processes affect the porous structure and the surface area of the silica gel [25,45]. These processes are:

- **Polycondensation** is the further reaction of silanols and alkoxy groups in structure to form siloxane bonds. The reaction results in densification and stiffening of the siloxane network.
- **Syneresis** is the shrinkage of the gel network. This shrinkage is caused by the condensation of surface groups inside pores, resulting in a pore narrowing. In aqueous gel systems, the pore size controlled by equilibrium between electrostatic repulsion and Van der Waals forces. In this case, shrinkage is produced by addition of an electrolyte.
- **Coarsening or Ostwald ripening** is the dissolution and re-deposition of small particles. Also necks between particles will grow and small pores may be filled in.

This results in an increase in the average pore size and decrease in the specific surface area.

- **Phase transformation** can occur in aging with several types such as separation of solid phase from liquid (microsyneresis), segregation of partially reacted alkoxide droplets (gel turns white and opaque), crystallization and precipitation.

Summarizing, the pore structure, surface area and stiffness of the network are changed and controlled by the following parameters: time, temperature, pH and pore fluid [9].

2.5. Drying

The gel drying process consists of removal of water from the interconnected pore network with simultaneous collapse of the gel structure, under conditions of constant temperature, pressure, and humidity. Large capillary stresses can develop during drying when the pores are small (＜20 nm). These stresses will cause the gels to crack catastrophically unless the drying process is controlled by decreasing the liquid surface energy by addition of surfactants or elimination of very small pores, by supercritical evaporation, which avoids the solid-liquid interface, or by obtaining monodisperse pore sizes by controlling the rates of hydrolysis and condensation [24,25].

3. Sol-gel based bio-hybrid materials

Sol-gel is a suitable matrix for immobilization of enzymes, viruses and bacteria to develop biotechnological applications. However, sol-gel precursors and conditions are sometimes not mild enough for designing a stable and safe biocomposite. For instance, the release of alcohol during the hydrolysis-condensation of silicon alkoxides has been considered an obstacle, due to its potential detrimental effects on the entrapped proteins and microorganisms [39,40]; the ionic strength of aqueous precursor has been also considered as harmful environment for microorganisms entrapment [39,41]. In addition, sol-gel constraints and shrinkage occurring during gelation and drying processes respectively lead to fracture of the material, pore collapse and loss of biological activity [42]. In order to overcome the limitations of inorganic matrices, sol-gel based hybrid materials have been proposed as a stable systems containing inorganic precursor and some organic and/or polymeric additives [22]. Hybrid materials can effectively eliminate the brittleness of pure inorganic sol–gel and the swelling property of some pure polymer or hydrogel.

3.1. Biocompatible silica precursors

Biocompatible silica precursors have to be used in order to avoid the denaturation of the entraped biological life. TMOS [$Si(OMe)_4$)] and TEOS [$Si(OEt)_4$)] are commonly used as biocompatible precursors behind the evaporation of methanol and ethanol, respectively, under vacuum. Aqueous sol–gel precursors are otherwise commonly used in order to avoid any trace of alcohol, for example a sodium silicate solution [43], or a mixture of sodium silicate and Ludox® suspension [18,39]. Another way to avoid denaturation by alcohol, is to use biocompatible alcohols such as polyol-based silanes as hydrolyzable groups that can be hydrolyzed under mild pH conditions [44].

3.2. Organic additives

3.2.1. Sugar

Sugars can be used as additives to stabilize biological life within sol-gel matrices. Chymotrypsin and ribonuclease T1 have been trapped in the presence of sorbitol and N-methylglycine. Glucose and amino acids significantly increase the thermal stability and biological activity of the proteins by altering their hydration state and increasing the pore size of the silica matrix [45]. D-glucolactone and D-maltolactone have also been covalently bonded to the silica network via a coupling reagent aminopropyltriethoxysilane (APTES), giving rise to non-hydrolyzable sugar moieties. Firefly Luciferase, trapped in such matrices, has been used for the ultra-sensitive detection of ATP via bioluminescent reactions [46]. Trehalose and sucrose are non-reducing disaccharides that may be accumulated at high concentrations by many organisms capable of surviving complete dehydration. They were shown to be excellent stabilizers of many biomolecules and living cells in the dry state, and appeared to be superior to other sugars [47,48]. The stabilization effects of trehalose were attributed to several mechanisms/interactions with critical biomolecules or cellular structures such as membranes and proteins. The stabilization mechanism of membranes often referred to the water replacement hypothesis. The formation of hydrogen bonds with membrane phospholipids and proteins suggests to replace water molecules which have been evaporated during drying [49]. This, in turn, inhibits Van der Waal's interactions between adjacent lipids thus preventing the elevation of membrane phase transition temperatures (Tm). Such a Tm change could cause membranes to shift from their native liquid-crystalline state to a gel state under environmentally relevant temperatures. As the membranes of biomolecules and

microorganisms pass through this phase transition, regions with packing defects make the membranes leaky. Also, trehalose may interact by hydrogen bonding of its -OH groups to polar residues in proteins, hence preserving the conformational state against dehydration processes [50]. Trehalose has also demonstrated to decrease oxidative damage caused by oxygen radicals [51] and to be accumulated in response to heat shock and cold exposure, allowing bacteria viability at low temperatures. It was shown in literature that trehalose considerably increases the tolerance of *E. coli* to drying processes [41].

3.2.2. Glycerol

Glycerol (or glycerin) is a simple polyol compound. It is water-soluble, sweet-tasting and non- toxic compound which is widely used in pharmaceutical formulations. As known in literature, glycerol is an example of compatible solute which can be accumulated in cells under hyperosmotic conditions to allow them to tolerate osmotic stresses [47]. Thus, glycerol is used as a powerful osmotic stabilizer, which lowers water activity, prevents contraction of the gel, and also protects cells against encapsulation and freezing stress [17,18,41,47]. Glycerol in aqueous sol-gel monolith has succeeded with its specific properties as water solubility, biocompatibility and maintenance of silica gel cohesion to preserve 60 % of bacterial viability from stresses of sol gelation and aging along one month [17].

3.3. Polymer additives

3.3.1. Poly(ethylene Glycol) (PEG)

PEG is flexible, water-soluble polyether polymer and highly applicable from industrial manufacturing to medicine. It can play several important roles in sol-gel applications, apart from structure modification: they decrease the non-specific binding of proteins and living cells to the surrounding matrix and fill the pores and spaces in the matrix, thereby eventually preventing matrix shrinkage and collapse [52]. In turn, this contributes to protect the entrapped biomolecules and microorganisms from denaturation and activity loss [42,47,53]. The introduction of PEG to sol-gel material has proven to be efficient for safe immobilization of bacteria in stable sol-gel film [54].

3.3.2. Poly(ethyleneimine) (PEI)

The cationic polymers such as PEI, poly(allylamine) and poly(dimethyldiallylammonium chloride) have shown critical effects on the stability of encapsulated enzymes [55]. PEI which

is a polymer with repeating unit structure composed of the amine group and two carbon aliphatic (CH_2CH_2) spacer, has been used for irreversible bacterial adhesion on the solid materials via electrostatic interactions between its amine groups and membranes of protein and bacteria [56]. Thus, PEI could be a safe agent for immobilization of biological life on the surface of conducting electrodes for construction of electrochemical biosensors.

3.3.3. Chitosan

Chitosan is a very abundant biopolymer obtained by the alkaline deacetylation of chitin. It is a heteropolymer containing both glucosamine and acetylglucosamine units. The introduction of chitosan polymer aims at counterbalancing some of the silica disadvantages especially cracking upon aging/drying. Moreover, it may provide additional properties such as ion binding capacity, photoluminescence or chirality [57]. On the basis of IR studies evidencing interactions between chitosan and silica materials, a model proposed H-bonds of silanol groups with amide- and oxy-groups of chitosan, ionic bonds between chitosan amino groups and silanolate as well as covalent links resulting from transesterification of chitosan hydroxy-groups by silanol (Fig. I-5) [57,58]. However, the fact that amino groups remain available for further binding suggests that these functions are only weakly bonded to the silica network [59]. Due to the strong compatibility and interactions between chitosan and sol-gel, these hybrid materials were also shown to be suitable for the encapsulation of enzymes [60–62].

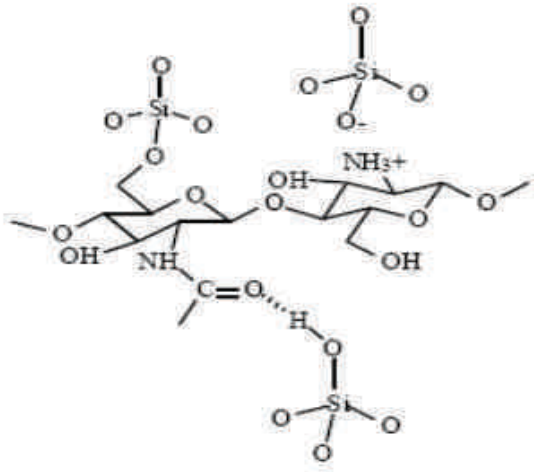


Fig I-5. *Possible interactions between chitosan and silicates (adapated from reference* [57]*).*

Moreover, chitosan has shown a considerable possibility to be electrodeposited with proteins for biosensor constructions [63,64]. The electrodeposition mechanism involves a cathodic electrolysis as for the electrodeposition of silica which could be interesting for co-deposition of both chitosan and silica to form electrodeposited hybrid materials. First, chitosan can be protonated and dissolved in acidic solutions. At low pH, protonated chitosan becomes a cationic polyelectrolyte (Equation 1) [63].

$$\mathrm{Chit}\cdots\mathrm{NH_2} + \mathrm{H_3O^+} \rightarrow \mathrm{Chit}\cdots NH_3^+ + H_2O$$

Eq I-3. *Protonation of chitosan polymer in acidic medium.*

However, the increase in solution pH results in a decreasing charge and at pH=6.5 amino groups of chitosan become deprotonated. High pH can be generated at the cathode surface using the cathodic reduction of water discussed before in section 2.2.2 (Fig. I-4) [63].

$$2\mathrm{H_2O} + 2e^- \rightarrow \mathrm{H_2} + 2\mathrm{OH^-}$$

Eq I-4. *Cathodic reduction of water molecule.*

Then electric field provides electrophoretic motion of charged chitosan macromolecules to the cathode, where chitosan forms an insoluble deposit (Equation 3) [63].

$$\mathrm{Chit}\cdots NH_3^+ + \mathrm{OH^-} \rightarrow \mathrm{Chit}\cdots\mathrm{NH_2} + H_2O$$

Eq I-5. *Cathodic electrodeposition of chitosan polymer by precipitation.*

The development of electrochemically-assisted codeposition between silica gel and chitosan polymer could provide a stable biodegradable and safe composite for biomolecules and cell entrapment towards biosensing applications [62].

Finally, the sol-gel based hybrid materials have gained the interest in combining the attractive properties of both organic and inorganic materials for biotechnologies and electrochemistry [65]. The hybrid materials exhibit chemical and physicochemical features that might be readily exploited when used to modify electrode surfaces due to the versatility of sol-gel chemistry in the design of electrochemical devices. Applications cover various fields such as sensors, reactors, batteries, and fuel cells [27]. Next section will discuss the interesting features of sol-gel based hybrid materials for entrapment of biological life and their potential applications in biotechnologies.

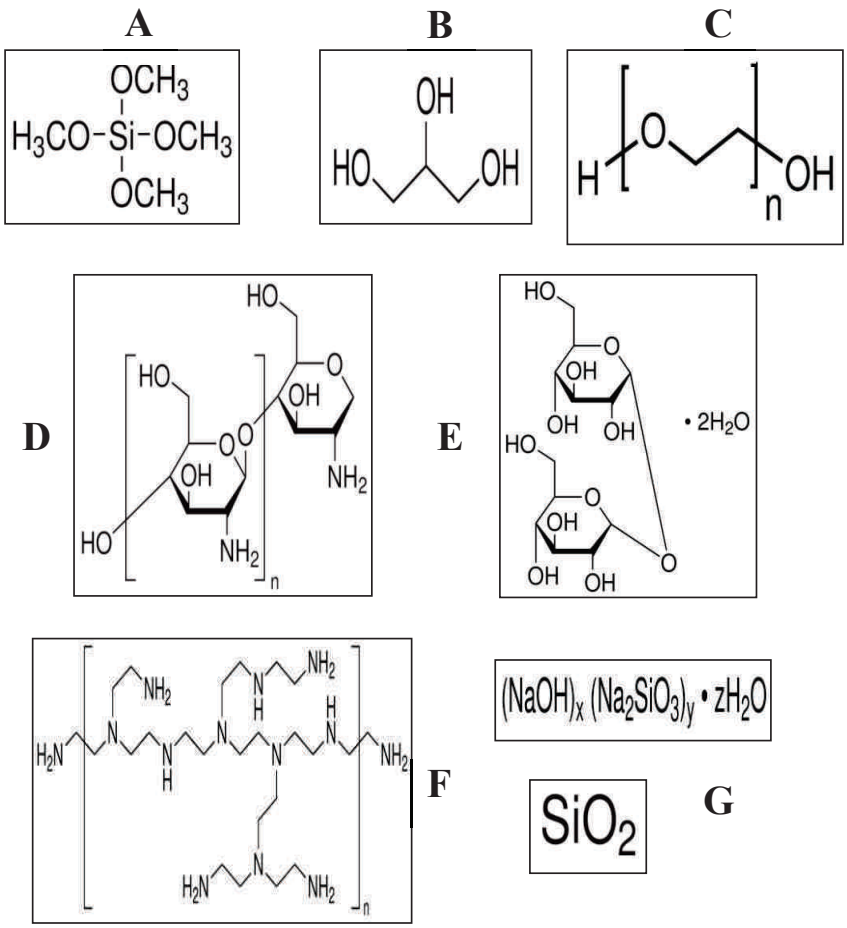


Fig I-6. *Chemical structures of Sol-gel based hybrid material i.e: A: Tetramethyl orthosilane; B: Glycerol; C: Poly(Ethylene Glycol); D: Chitosan; E: Trehalose dehydrate; F: Poly(Ethyleneimine); G: sodium silicate with colloidal silica.*

4. Sol-gel bioencapsulation and applications

The last decade has seen a revolution in the area of sol-gel-derived biocomposites since the demonstration that these materials can be used to encapsulate biological species in a safe functional state. The technological advancements in immobilization of biological species over several decades has also resulted in a revolution in the use of biological objects for the selective extraction, delivery, separation, conversion and detection of a wide range of chemical and biochemical reagents. The use of biological species such as proteins, peptides,

nucleic acids and even whole cells in these applications relies largely on the successful immobilization of the biological objects in a physiologically active form [26,66].

4.1. Encapsulation of proteins

Proteins can be broadly categorized as either soluble or membrane-associated. Soluble proteins, which include enzymes, antibodies, regulatory proteins and many others, reside in an aqueous environment, and thus are generally amenable to aqueous processing methods for immobilization. The solubility of these proteins arises owing to the presence of polar or charged amino acid residues on the exterior surface. Therefore, immobilization techniques for soluble proteins must provide a hydrated environment at a pH that does not alter the membrane of proteins and does not have significant polarity differences relative to water. The group of Avnir was one of the pioneers in developing the sol-gel encapsulation technique for soluble proteins as enzymes [67], whose field has become an important area of research and technology [68]. More recently, the group of Walcarius has succeeded to preserve and stabilize the activity of different enzymes in sol-gel film based on solvent evaporation protocol [69,70] and electrochemically-assisted protocol [71,72] for bio-electrochemical applications. In addition, the bioencapsulation field has also included the entrapment of the redox protein c-cytochrome in silica nanoparticles for designing electrochemical biosensors [73].

On the other hand, membrane-associated proteins, contain either completely (intrinsic membrane proteins) or partially (extrinsic membrane proteins) embedded within cellular lipid membranes [66] (Fig. I-7). The distinguishing characteristic of membrane proteins, with respect the soluble ones, is the presence of both hydrophobic residues, which associate with the lipophilic membrane, and hydrophilic amino acid residues that associate with the aqueous environment on either side of the membrane [66].

Therefore, successful immobilization of membrane proteins must address two important issues. Firstly, the method must allow retention of the tertiary folded structure of the protein as is the case for soluble proteins. Secondly, it must accommodate the phospholipid membrane structure, which is held together mainly through hydrophobic interactions [66]. These issues are necessary to retain intact the membrane, or at a minimum the protein-associated lipids, in order to accommodate both the hydrophilic and hydrophobic portions of

the protein. This latter requirement suggests that the use of organic solvent should be avoided and highlights the need for aqueous processing methods during immobilization [66].

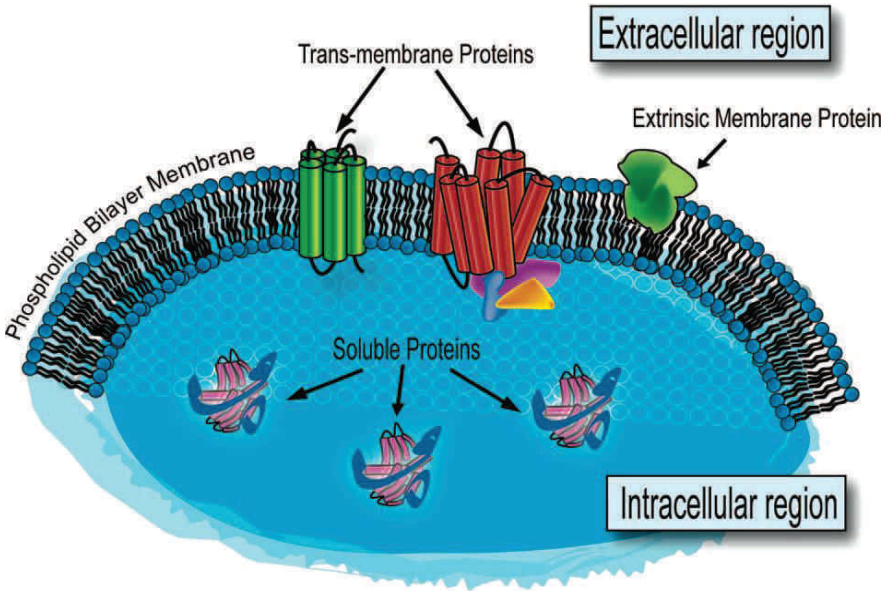


Fig I-7. *Simplified illustration showing the different types of proteins which are utilized for various immobilization formats. Soluble proteins are located within the hydrophilic intracellular compartment of the cell. Intrinsic trans-membrane proteins span the cellular phospholipid bilayer membrane, whereas extrinsic membrane proteins are partially embedded within the membrane and are exposed to either the intracellular or extracellular regions (adapted from reference* [74]*).*

There have been a wide number of strategies directed to the immobilization of bilayer lipid membrane [75], including physical adsorption of a bilayer through deposition, covalent attachment of a monolayer, or bilayer of phospholipids to a solid surface, and attachment via avidin-biotin linkages. Some of these typical strategies are illustrated in Fig. I-8.

The biocompatibility and stability of silica-based gel has gained an attention for immobilization of membranes and membrane proteins such as: pyrene-labeled liposomes and dye-encapsulating liposomes [76,77], photoactive proton pump bacteriorhodopsin (bR) [78], two ligand-binding receptors, the nicotinic acetylcholine receptor (nAChR) and the dopamine D2 receptor [79], hydrogenases [80] and cytochrome P450s [81]. In most of cases, the water soluble polyethylene glycol (PEG) or glycerol were required as an additive to maintain the receptors in an active state (ca. 40–80% activity relative to solution) [74,80]. The transition from traditional silica precursors and processing methods toward more biocompatible

processing methods has been critical for the successful entrapment of many membrane-bound proteins. The use of polyols such as glycerol or polyethylene glycol have also proven to be highly beneficial for stabilization of entrapped membrane proteins, yet the underlying mechanisms of stabilization by these additives being still not fully understood. Fundamental and technological advances would expedite the use of these membrane protein doped materials for applications including microarrays, bioaffinity chromatography, biosynthesis, biocatalysis, bioselective solid phase microextraction, and energy storage [66].

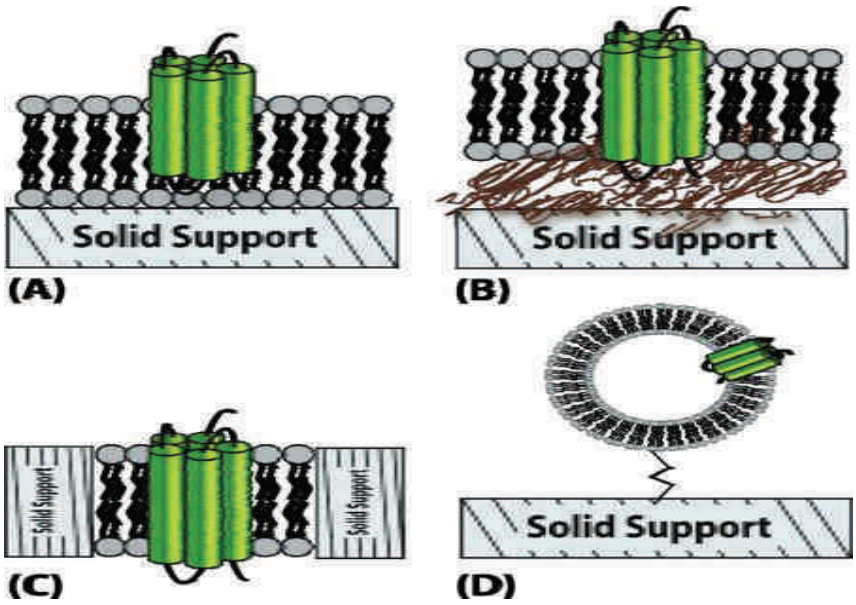


Fig I-8. *Illustration of some typical strategies used for immobilization of membrane proteins. (A) Direct physical adsorption of the lipid bilayer and membrane protein onto a solid support. (B) Adsorption or covalent attachment of the lipid bilayer onto a solid support, with an intermediate layer of hydrated polymer. (C) Immobilization of the lipid bilayer and membrane protein in the pores of a solid support. (D) Immobilization of a phospholipid vesicle through covalent or avidin—biotin bioaffinity attachment (adapated from reference* [66]).

4.2. Encapsulation of bacteria

An ideal immobilization matrix should be functional at ambient temperature and biocompatible, should enable complete retention of the cells, and allow the flow of nutrients, oxygen, and analytes through the matrix. In theory, encapsulated cells should remain viable but not growing [21]. This aspect is of paramount importance when immobilized cells have to

be stored for long periods of time without loss of viability. In this manner living cells can be regarded as a "ready-to-use reagent" that can be stored for prolonged time under carefully controlled conditions and, once activated, *e.g*., by a defined temperature change or addition of nutrients, a constant and reproducible number of viable cells can be obtained for selected applications (*e.g.,* biosensing, bioremediation, fermentation) [1]. Cells have been successfully encapsulated in organic polymers (*e.g.,* hydrogels such as agar) [82] and inorganic matrices (*e.g*., sol–gel) [41] and in sol-gel based organic-inorganic hybrid materials [17,18,21,41,47]. All of them performed well in the terms of solute diffusion and cell entrapment maintenance whereas encapsulation in organic polymers may hamper from cell leaking due to its swelling behavior. The real challenges in this field are to avoid the sol-gel constraints during gelation and aging processes and the impact of gel drying on long-term cell viability.

Most of the cell encapsulation described in the literature have applied the mode of solvent evaporation and monolithic form [18,21,39,83,84], in addition to the thick sol-gel layer [85,86] in order to keep as long as possible the water/solvent ratio for preserving cell viability. Only a limited number of works succeeded in keeping cell viability in thin films either by cell encapsulation in multi-layered silica thin films [87] where protecting encapsulated cells from direct contact with the surrounding media, avoiding leaching and rapid drying, or in cell-directed assembly protocol [88] where the phospholipids can self-organize around cells and confine water at their vicinity allowing long-term preservation under ambient conditions. To date a limited number of works was based on the mode of electrochemically-assisted cell encapsulation. One approach has succeeded to encapsulate protein and bacteria in thick electrodeposited hybrid sol-gel film but the authors have just confirmed the conserved membrane integrity of encapsulated bacteria without providing the accurate details, such as the ratio of PEG to silica material, the duration and % of conserved viability [89]. The other approaches have just performed the deposition with alginate polymer instead of sol-gel polymer [90,91].

Actually, there is a tremendous interest for immobilization of living cells (bacteria, yeast, or microalgae, …) in porous matrices for biotechnological applications such as bioreactors, bioreporters, biosensors and biofuel cells [1,2].

4.2.1. Bioreactor

Some cells like cyanobacteria are classified as photosynthetic prokaryotes, employing the same reaction as plants to synthesize bio-organic compounds such as sucrose, starch and cellulose from CO_2 and water in presence of light (Fig. I-9). Cyanobacteria-based bioreactor could minimize the CO_2 level in the environment and produce a novel, reusable carbon source to replace the rapidly depleting fossil fuel [92]. The encapsulation approach in sol-gel materials is likely to retain its photosynthetic activity for conversion of carbon dioxide into biofuels under light energy [83,84]. Thus, scientific impact of bioencapsulation in sol-gel relies on cell viability accompanied with cell integrity, metabolic activity and protein synthesis of the encapsulated bacteria for environmental biotechnologies [4,17]. All these works have exploited the sol-gel deposition protocol based on solvent evaporation and monolithic form.

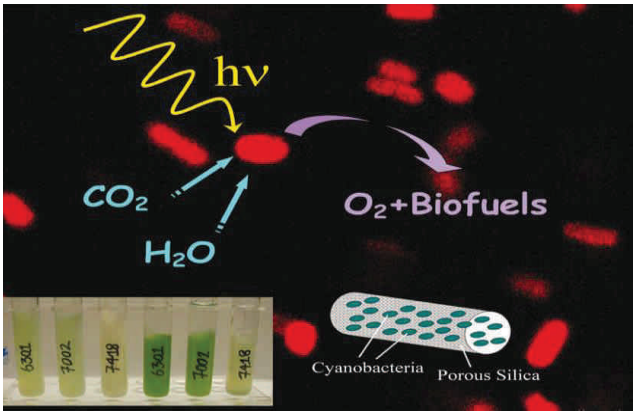


Fig I-9. *Photosynthesis of cyanobacteria encapsulated in porous silica (adapted from reference* [83]*).*

4.2.2. Bioreporter

Genetically-engineered reporter consists of a gene promoter as a sensing element for chemical or physical changes and a reporter gene(s) coding for measurable protein(s) relative to the environmental effects. The promoter senses the presence of target molecule (s) and activates transcription of the reporter gene and subsequent translation of the reporter mRNA produces a protein/light as a detectable signal (Fig. I-10) [93,94]. The genetically-engineering process has generated diversity of cells for designing bioreporters such as bacteria modified with zntA

gene for detecting toxic metals [95]. ZntA gene could be incorporated to luminescent or GFP reporter for early detection of toxic metals such as cadmium, mercury, *etc.*

The approach based on the encapsulation approach in sol-gel materials has opened a route for designing of long-term efficient fluorescent and luminescent reporters based on cells genetically modified with the green fluorescent protein (GFP) or luminescent light properties in response to general toxicity and genotoxicity [85,86,96]. All these works have used the sol-gel deposition protocol based on solvent evaporation and thick sol-gel film.

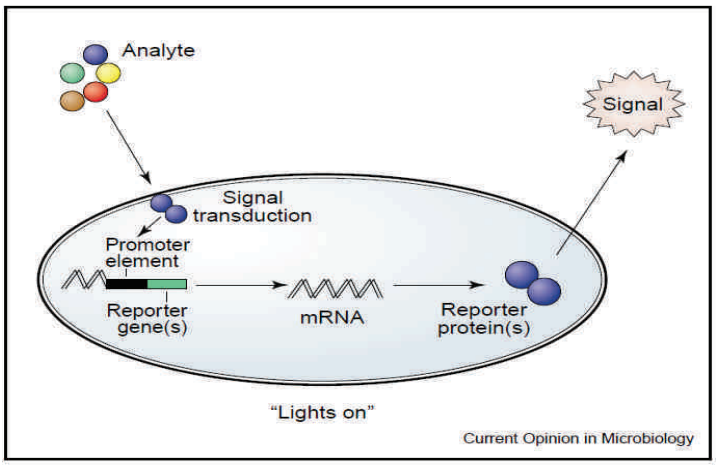


Fig I-10. *Bacterial toxicity assay principle. A quantifiable molecular reporter is fused to specific gene promoters, known to be activated by the target chemical(s) (adapted from reference* [93]*).*

4.2.3. Biosensor

A biosensor is a device that enables the identification and quantification of an analyte of interest from a sample matrix, for example, water, food, blood, or urine. As a key feature of the biosensor architecture, biological recognition elements that selectively react with the analyte of interest are employed (Fig I-11) [97,98].

The approach based on the encapsulation in sol-gel matrices has also opened a route for development of sol-gel based biosensors such as for biochemical oxygen demand (BOD) or detection of toxic organophosphates which result from the extensive use of pesticides and insecticides as well as potential chemical warfare agents [99]. BOD is used to characterize the organic pollution of water and wastewater, which is estimated by determining the amount of oxygen required by aerobic microorganisms for degrading organic matters in wastewater

[21,100,101]. The encapsulation approach has succeeded to preserve the cell viability and its metabolic activity in solvent evaporation based protocol [98]. Moreover, the electrochemically-assisted cell encapsulation in a sol-gel matrix could extend the biotechnology into the micro- and nano-scale level of environmental and diagnostic analysis for developing of bioelectronics and biosensors [90,91].

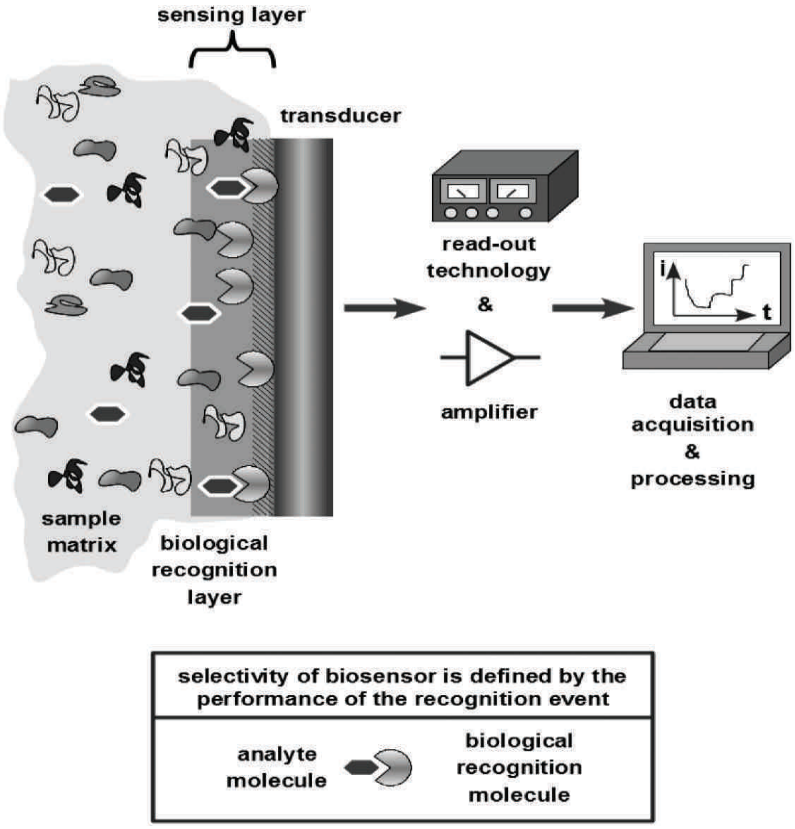


Fig I-11. *Typical biosensor setup (adapted from reference* [97]*).*

4.2.4. Biofuel cells

A new form of green energy based on the efficient conversion of organic matter into electricity is now feasible using biofuel cells. In these devices, microorganisms support their growth by oxidizing organic compounds and anodic electrode serves as the sole electron acceptor, so electricity can be harvested. These electrons then flow from the anode, through the device to be powered, or a resistor in experimental studies, and onto the cathode (Fig. I-12) [102,103]. Since the efficiency of biofuel cells depends on the bacterial density, most biofuel cells have been performed with microbial biofilms. The sol-gel encapsulation

approach has preserved the viability and activity of these biofilms for few months [104] but up to now, the encapsulated biofilm have a limited applications in applied for construction of microbial fuel cells [105].

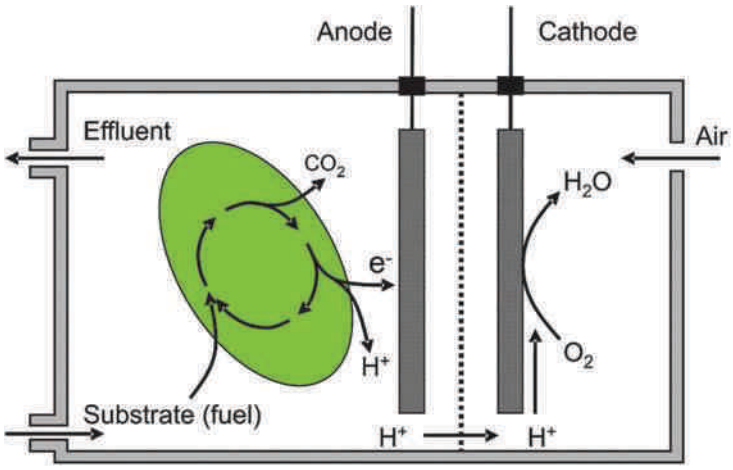


Fig I-12. *Schematic illustration of a microbial fuel cell (adapted from reference* [106]*).*

4.3. Encapsulation of virus

The possibility to immobilize biological active agents like virus and bacteriophage within silica gels has opened the route for development of biomedical applications [107–109]. These microorganisms are important agents for the medical field such as oncolytic viruses including replication-competent adenoviruses which are emerging as a promising tool for the treatment of cancer [110] and bacteriophages which reduce food-borne pathogens during the preharvest and postharvest stages of food production [111]. The key factors for these biomedical applications are the conservation of viral infectivity and sometimes the extended release of virus to reach the specific release rate and target. For that, the possibility of immobilizing viruses into sol-gel materials without the loss of biological activity has led to the development of vaccination and viral epitopes carriers for treatment of diseases [107,109]. The process is based on sol-gel polymerization in conditions compatible with biological life. Silica materials are biodegradable in vivo to control the release rate of encapsulated viruses then they are subsequently secreted into urine [112]. The sol-gel encapsulation of viruses is designed to contain substantial amounts of water to ensure large pores in an aqueous environment, which can be a hydrophilic environment for viruses. In principle, viruses might therefore remain

encapsulated and infective for months [113]. In addition, the encapsulation of viruses and bacteriophage in sol-gel materials has been developed for biotechnologies such as ordered mesoporous silica with designed pores according to the size and morphology of biomaterials (Fig I-13) [114,115].

Fig I-13. *Mesoporous template Synthesis of Hierarchically Structured Composites (adapted from reference* [114]*).*

5. Investigation of bioelectrochemical communication

As discussed above, redox proteins and bacteria have supplied the driving force for development of bio-electrochemical systems such as electrochemical bioreactors, biosensors and biofuel cells. The key criterion in bio-electrochemical devices is the electrochemical communication (EC) between these biomolecules/cells and an electrode surface [106,116]. In case of redox proteins (such as cytochromes and enzymes), the active site is the critical domain for electron transfer (ET) between the protein, its cofactor and the electrode surface [117]. In case of bacteria, the EC between its intracellular enzymes and electrode surface is expected to occur via extracellular electron transfer (EET) [118,119]. The EET is a catalytic mechanism of organic substrates with different intracellular enzymes (respiration or fermentation for aerobic and anaerobic species, respectively). Electrons produced from microbial respiration at microbial ET chain and transported through the periplasmic space and cell wall to the outer electron acceptor (*i.e.,* electrode). Electrodes can serve either as electron

donors or electron acceptors for microorganisms/redox protein, depending on whether the electrode is considered as a cathode or anode, respectively [120].

An electron transfer pathway is generally divided into 2 categories:

1) Direct electron transfer (DET).
2) Mediated electron transfer (MET).

The DET takes place via a physical contact of the bacterial cell membrane, or protein active site, with the electrode surface without requirements of any electron shuttle [106,117]. The exclusive drawbacks for DET are (i) limiting to electroactive bacteria since most of living cells are assumed to be electronically non-conducting, such a transfer mechanism has long been considered impossible and (ii) working electrode overpotential could be toxic to the microbial and enzymatic life [97,106]. The MET, as an alternative to direct-contact pathways, involves an additional molecule capable to ensure redox cycling process (*i.e.,* electron shuttle or mediator). Redox artificial mediator can enhance the ET transferring electrons between enzymatic active sites/microbial cell membranes and the electrode surface. Moreover, it can operate at low overpotentials in order to avoid drawbacks discussed in DET [97,106].

In case of bacteria, the MET mechanism of electron shuttling can be further divided into 2 categories:

a) Endogenous shuttling.
b) Exogenous shuttling.

Endogenous shuttle, which is microbially-produced, is secreted outside the bacteria to exchange electrons by redox mechanism with the electrode surface. This process is known as self-mediated ET [118]. Few bacteria have self-mediated ET property such as *S. oneidensis* strain MR-1 which is able to excrete a quinone/flavin molecule to serve this purpose [121–123].

Exogenous shuttle is an artificial mediator used to enter/contact the bacteria to redox shuttling electrons with the electrode surface such as ferricyanide as a chemical origin [124] The development of different mechanisms for facilitating the ET between enzymes/bacteria and an electrode surface has extended the electrochemical technologies such as sensors and fuel cells into biological life.

5.1. Electrical wiring of protein

The development of EC between redox proteins and an electrode surface has gained an interesting attention for biosensors and enzymatic fuel cells fabrication [71,75,125,126]. Some redox proteins such as laccase, bilirubin oxidase, hydrogenase, hemoglobin and c-cytochrome are likely to conduct electrons directly with the electrode surface [125,127–129]. Mediators such as ferrocene dimethanol (FDM) [72], osmium polymer [55] or vitamin K_3 [70] attached to carbon nanotubes (CNT) have been investigated for wiring of redox proteins. Furthermore, biological mediators such as cytochromes have been also utilized to wire enzymes efficiently and to avoid using toxic chemical substances [130–133]. In addition, the immobilization of cytochrome c into layer-by-layer systems has improved the EC between enzymes and the electrode surface than monolayer system [134,135]. Research works have shown that an enzyme can be durably immobilized by simple entrapment in porous gel networks without requiring any covalent bond between the support and the enzyme, thus maintaining its native properties and biological activity [68,70,136]. The immobilization of artificial mediator along with enzyme and its cofactor for reagentless biosensors fabrication, has gained an attention due to stability of enzymatic activity and electrical wiring in porous materials [16,70].

5.2. Electrical wiring of bacteria

Whole cells have also gained an increasing attention in EC with electrode surface for fabrication of biosensors and microbial fuel cells [20,103]. Whole cells can easily and rapidly communicate with their surrounding environment and their utilization can save the expensive and time-consuming enzyme purification step and preservation in natural environment [98]. In case of DET, only electroactive bacteria were able to directly-conduct electrons with the electrode surface [106]. The DET in electroactive species is facilitated by outer-membrane (OM) redox protein (c-cytochrome) that allows the ET from ET chain located in cell membrane to the electron acceptor (outside the cell) as the case of *Shewanella* species [102] or by conductive appendages such as nanowires in flagella and pilli as the case of *Geobacter* species [103]. Electroactive bacteria has been considered as building blocks for construction of microbial fuel cells for its efficiency in DET with electrode materials [137].

In case of MET, soluble mediator such as ferricyanide $K_3[Fe(CN)_6]$ has gained an attention especially for wiring gram-negative bacteria for BOD sensors because of its solubility, higher

than oxygen, and its ability to enter through the porins of cell walls to accept electrons directly from the bacterial electron transfer chains [124]. However, high ferricyanide concentration (≥ 50 mM) can also damage the membrane of these bacteria [138]. One approach tried to immobilize ferricyanide in an ion exchangeable polysiloxane to fabricate BOD sensor for detection of organic pollution in industrial wastewater, effluent or natural water. This approach has improved the BOD assay than the conventional one which requires a 5 day period and complicated procedures and skilled analysts to obtain reproducible results [100]. Polymeric mediators have also gained a significant attention in electrical wiring field especially with the flexible osmium polymer which has been stably-bound on the electrode surface [139,140]. they can wire efficiently different gram-negative bacteria due to their cationic charge and flexibility [124,139,140]. The electrode modification with osmium polymer for wiring bacteria has been described either by using organic polymers with/without CNT in presence of gluteraldehyde as cross-linker [124,139,140], or by conducting polymer for enhancing ET with electrode surface [141]. Up to now, c-cytochrome has not been utilized for wiring bacteria as an artificial mediator. A limited work has been done for investigation of the electrochemical communication of entrapped bacteria in sol-gel materials [100,142].

Following the development of EC between planktonic bacteria and electrode surfaces especially the current and electric responses in electrochemical biosensors and microbial fuel cells respectively, the building of multilayers of electro-active bacteria as biofilm has gained more and more interests especially for microbial and hybrid fuel cells [125,143,144]. A biofilm can facilitate bacterial ET efficiency in biofuel cells, primarily due to much higher biomass densities and higher bacterial viability, caused by anode respiration. However, natural anodic biofilms usually have a considerable thickness varying from several to tens of microns, which may cause diffusion limitation of nutrients and insufficient interaction of bacteria with anode materials [144]. For that, the mimicking of biofilm within silica matrix and/or conducting polymers eliminates the significant cultivation times and variability typically associated with natural biofilm formation and avoids the loss of biomass in addition to serving as efficiently as natural biofilm [105,141,143]. All the discussed mechanisms for ET between bacteria or biofilm with the electrode surface have been summarized in figure I-14.

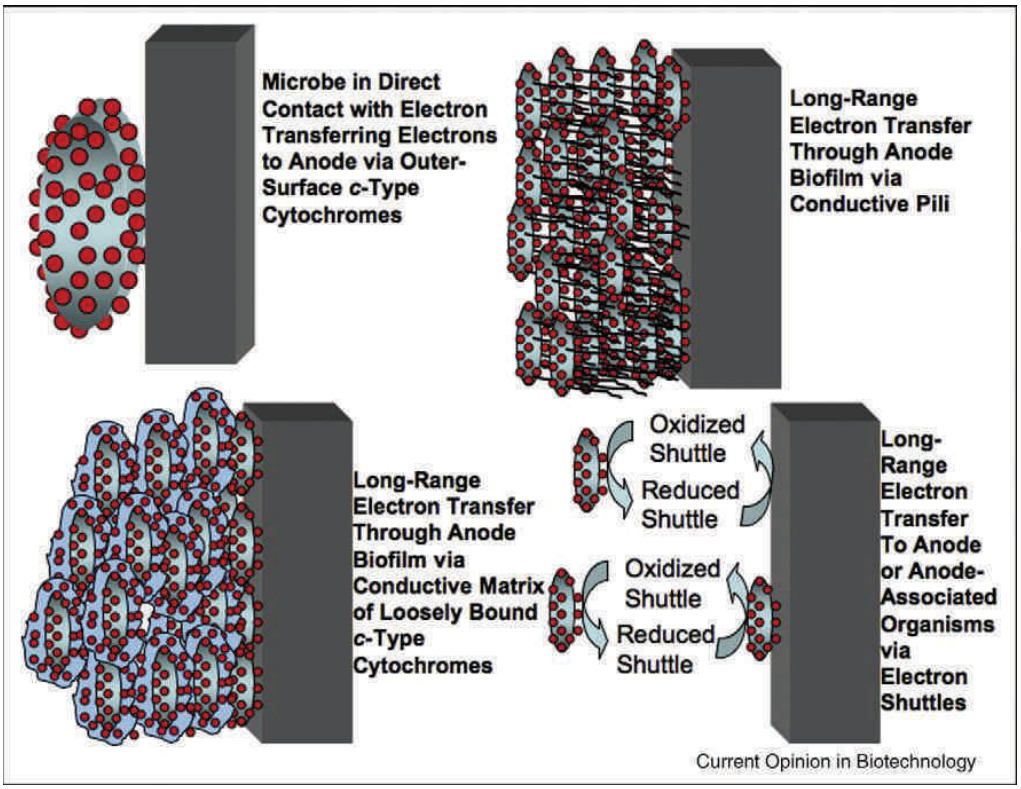


Fig I-14. *Summarizing the proposed mechanisms for ET to the anodic electrode. Red dots represent outer surface cytochromes, black lines represent nanowires, and the blue clouds represent the possible extracellular matrix which contains c-type cytochromes conferring conductivity (adapted from reference* [102]*).*

6. Positioning of the thesis

As it was discussed in the literature survey, encapsulation of bacteria, proteins [55,70] or even viruses [107,113] in silica-based materials prepared by the sol-gel process has been already described. Electrochemistry of bacteria or redox proteins is also a major field of research, providing opportunities for application in biosensors, bioreactor and biofuel cell technologies. In these active areas, the complementary expertise in electrochemistry, sol-gel chemistry and microbiology still provide opportunities for original research. Several directions will be considered in this thesis. First, we will investigate the application of electrochemistry to trigger the encapsulation of bacteria in thin hybrid sol-gel film (Chapter 2). Electrochemistry was then considered as an analytical method to study the reactions of electron transfer

between bacteria immobilized in a sol-gel layer and the electrode material. The immobilization of these bacteria could limit the electron transfer reactions to the electrode surface because of the insulating character of silica matrix, thus strategies to favor electron transfer reactions will be implemented, for the preparation of artificial biofilm (Chapter 3). The expertise developed for electrochemistry of whole cell will then be applied to the electrochemistry membrane-associated proteins, i.e. P450 and Mandelate dehydrogenase, with the goal to improve the stability of the electrocatalytic response (Chapter 4). Finally, a last topic has been investigated, which concerns the infectivity of bacteriophages after their encapsulation in a silica-based sol-gel monolith (Chapter 5).

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Chapter II. Electrochemically-assisted bacteria encapsulation in thin hybrid sol-gel film

In this chapter, a novel method, based on the electrochemical manipulation of the sol–gel process, was developed to immobilize bacteria in thin hybrid sol–gel films. This enabled the safe immobilization of *Escherichia coli* on electrode surfaces. *E. coli* strains C600, MG1655 pUCD607 and MG1655 pZNTA-GFP were incorporated and physically encapsulated in a hybrid sol–gel matrix and the metabolic activity and membrane integrity of the bacteria were assessed as a function of the aging time in the absence of nutrients at + 4 °C or + 80 °C. Live/Dead *Bac*Light bacterial viability analysis detected by epifluorescence microscopy indicated good preservation of *E. coli* C600 membrane integrity in the sol–gel film. The presence of chitosan, trehalose and polyethylene glycol additives was shown to strongly improve the viability of *E. coli* cells in the electrodeposited matrix for 1 month after encapsulation. Finally, the bioluminescent activity of *E. coli* MG1655 pUCD607 was preserved by approximately half of the cells present in such composite films.

1. Introduction

There is a tremendous interest for immobilization of living cells (bacteria, yeast, microalgae, …) in porous matrices for biotechnological applications [1–5]. Bacteria can be encapsulated in organic polymers (e.g., agar hydrogels) [6] or inorganic matrices (e.g., sol–gel-derived silica-based materials) [7,8]. Compared to organic polymers, silica gel offers several advantages such as improved mechanical strength, chemical inertness, biocompatibility, resistance to microbial attack and retention of the bacterial viability out of growing state [9,10]. In order to combine the advantage of both organic and inorganic components, sol-gel based hybrid materials have been also proposed (e.g., sol–gel including gelatin [9], glycerol [11], or copolymers of poly(vinyl alcohol) and poly(4-vinyl pyridine)) [10].

A challenge in this field is to avoid the impact of drying on cell viability so that bacteria were essentially encapsulated in monoliths [9,11] or thick films [7,8] while keeping enough water in the final materials. Both encapsulation in monolith or thick film have succeeded to preserve the viability and activity of encapsulated microorganisms for several months. While only a limited number of works succeeded in preserving cell viability in thin films (*i.e.*, a configuration often required for practical applications). This was notably achieved by multi-layered silica thin films [12] or by introducing phospholipids in cell directed assembly [13]. On the other hand, the incorporation of organic polymers, especially those bearing amine or amide groups (e.g., chitosan), in the sol-gel matrix allows the formation of organic-inorganic hybrids (often stabilized by strong hydrogen bonding) with improved protection against cracking upon aging/drying [14]. The incorporation of glycerol or poly(ethylene glycol) prevents excessive contraction of sol-gel matrices and protects the cells against osmotic stresses [15,16]. Finally, sugars (particularly trehalose) have been shown to stabilize bacteria during freezing and aging [17,18].

At the end of the nineties, besides the classical evaporation methods applied to generate thin sol-gel films [19], a novel approach was described to deposit such thin films on electrode surfaces, based on an electrochemically-driven pH increase likely to accelerate the gelification of a sol, and hence film deposition [20]. This has been rapidly extended to the generation of functionalized sol-gel layers [21] or ordered and oriented mesoporous silica films [22], as well as to the encapsulation of biological objects (e.g., haemoglobin [23] or dehydrogenases [24]) in thin sol-gel films. This approach offers some advantages compared to

other sol-gel deposition methods that are based on solvent evaporation (spin-, dip-, spray-coatings), especially in terms of enabling homogenous film deposition on small electrodes (e.g., ultra-microelectrode) [25,26] or non-flat supports [27] and on conducting supports, which can be useful for bioprocessing and electrochemical biosensing applications [28–31]. The electrochemical encapsulation has been recently developed for bacteria in alginate and studied for short-term analysis [30,31], but it has not been reported yet for bacteria in sol-gel films.

The aim of this chapter is to present the application of electro-assisted deposition technique for the encapsulation of bacteria in thin hybrid sol-gel films. This technique utilizes low voltages for base-catalyzed condensation of a sol doped with suitable additives on an indium-tin-oxide (ITO) electrode. The approach involves the immersion of the electrode into a stable hydrolyzed sol in moderately acidic medium, which is followed by applying a constant negative potential to the electrode surface in order to induce a local pH increase leading thereby to the polycondensation of the hydrolyzed monomers and thus film deposition along with entrapping bacteria in the final deposit. This led to thin films contrary to thick films described elsewhere [26]. Here, we also show how organic additives can improve the electrochemical encapsulation of bacteria in hydrophilic thin sol-gel films by protecting the bacteria against both electrochemical and aging stresses. In order to assess the state of encapsulated bacteria in the film, the Live/Dead *Bac*Light viability kit has been applied. It enables differentiation between bacteria with intact and damaged cytoplasmic membranes (membrane integrity) [32]. *E. coli* C600 has been used as a simple model for adjusting the optimum protocol of electrochemically-assisted encapsulation in sol-gel film. In addition, the metabolic activity of encapsulated bacteria was evaluated by measuring the luminescent activity of *E. coli* MG1655 pUCD607 [33] and the fluorescence activity of *E. coli* MG1655 pZNTA-GFP, as expressed in the presence of low concentration of Cd^{2+}.

2. Factors affecting the electrochemically assisted deposition of the hybrid sol–gel film

The electrochemically assisted bacteria encapsulation has been optimized with specific parameters for successful entrapment of bacteria and optimal viability. The sol–gel reaction is

catalysed by the local variation of pH that can be induced by the electrolysis of the sol. In this work, a potential of - 1.3 V versus Ag/AgCl (3 M) was applied to the working ITO electrode. This potential was found optimal in preliminary experiments as more negative potential was detrimental for the bacterial viability and less negative potential was inefficient to produce rapidly the films containing, or covering, the bacteria. Under these conditions, the thickness of the material could be controlled from less than 100 nm to more than 2 µm by varying the deposition time from 10 to 60 s. The film thickness was also significantly affected by the sol composition, especially the presence of the polymeric additives (chitosan, PEG) used to improve the cell viability in the final films. To assess the effect of these components, thickness measurements were made using AFM on films deposited under the same conditions, *i.e.*, at - 1.3 V for 30 s, for various sol compositions. Electrolysis from the starting sol containing only the silica precursor (0.25 M) did not produce a visible deposit under these conditions. Note that changing these conditions can allow faster film deposition for other applications [23]. The addition of PEG (12.5% m/v as a final concentration in the hybrid sol-gel film) increased the deposition rate but the thickness remained very small, *i.e.*, 30 ± 7 nm. In contrast, the introduction of chitosan (0.25% m/v as a final concentration in the hybrid sol-gel film) in the sol led to a dramatic increase in the film thickness, by a factor of 10, to reach 350 ± 70 nm. The thickness increased even more by mixing together the three components, *i.e.*, the silica precursor (TMOS), chitosan and PEG, to reach 1.9 ± 0.1 µm (Fig. II-1).

Such variations could be basically explained by different polycondensation rates, which are affected by the presence of organic additives, in agreement with previously reported observations for other related systems [22,24]. But this is not the only reason. Indeed, chitosan has the ability to be electrodeposited by applying negative potential on the conducting electrode [34,35]. The thickness of a film prepared with chitosan and PEG only (no silica precursor) was rather small (24 ± 4 nm), suggesting little contribution of this process. But in the presence of the silica precursor, chitosan can be co-deposited to form a hybrid material displaying improved properties compared to the individual components taken separately. Moreover, PEG strongly influences the film drying and limits shrinkage during the aging process as reported before [36], whose effects could also be beneficial for preventing a mechanical stress to the bacterial membrane encapsulated in the film.

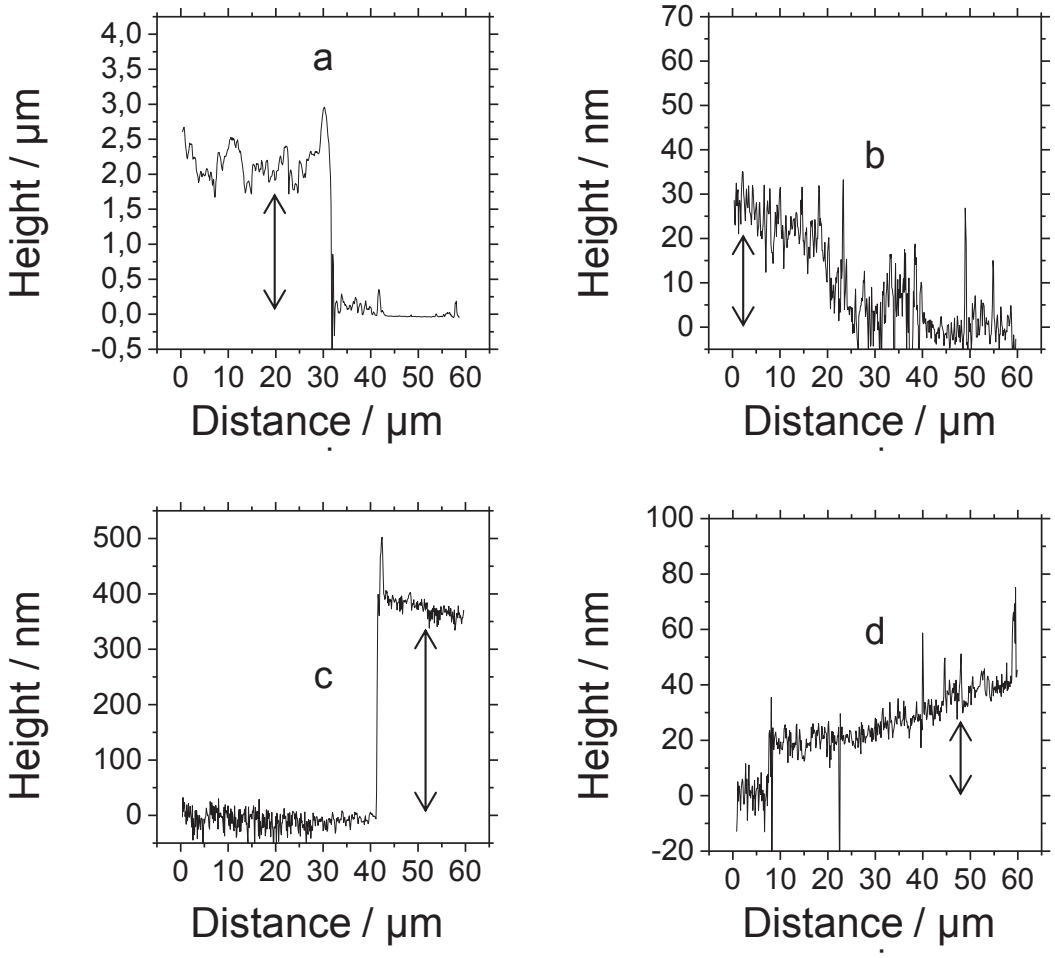

***Fig II-1**. AFM profiles measured on films composed with (a) TMOS, PEG, and chitosan, (b) TMOS and PEG, (c) TMOS and chitosan, (d) chitosan and PEG. N.B. E= -1.3 V, deposition time = 30s.*

3. Electrochemically assisted bacteria encapsulation in two steps (EABE(2S))

3.1. Principle and preliminary characterization

Immobilization of bacteria was first performed using a two-step procedure (EABE(2S)), implying first the adhesion of the bacteria on the ITO surface, as described elsewhere [37], and then the electrochemically assisted sol–gel film deposition and bacteria entrapment, as illustrated in Fig. II-2.

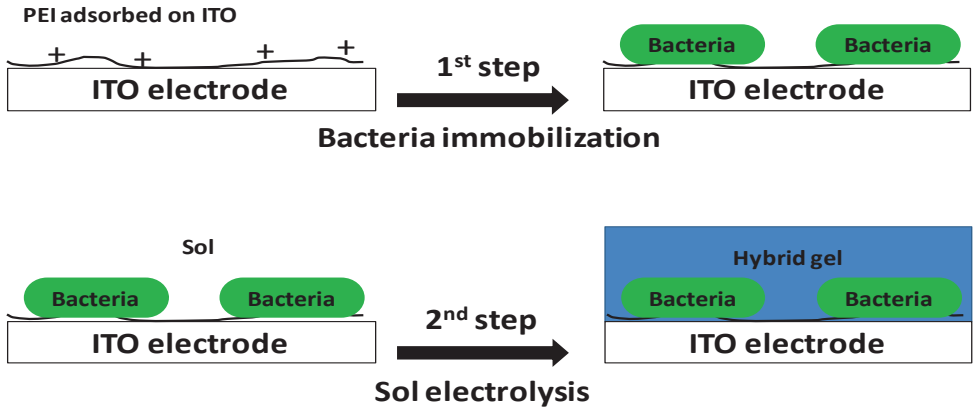

Fig II-2. *Mechanism of EABE(2S) of bacteria in a thin sol–gel film. PEI: poly(ethylene imine), ITO: indium tin oxide.*

Thicknesses of the films deposited with various electrolysis times were measured using AFM. Values of 82 ± 5 nm, 130 ± 20 nm and 1.9 ± 0.1 µm were obtained for deposition times of 10, 20, and 30 s, respectively (Fig. II-3). This variation was not linear with time as reported previously for other electrochemically assisted sol–gel deposition [21].

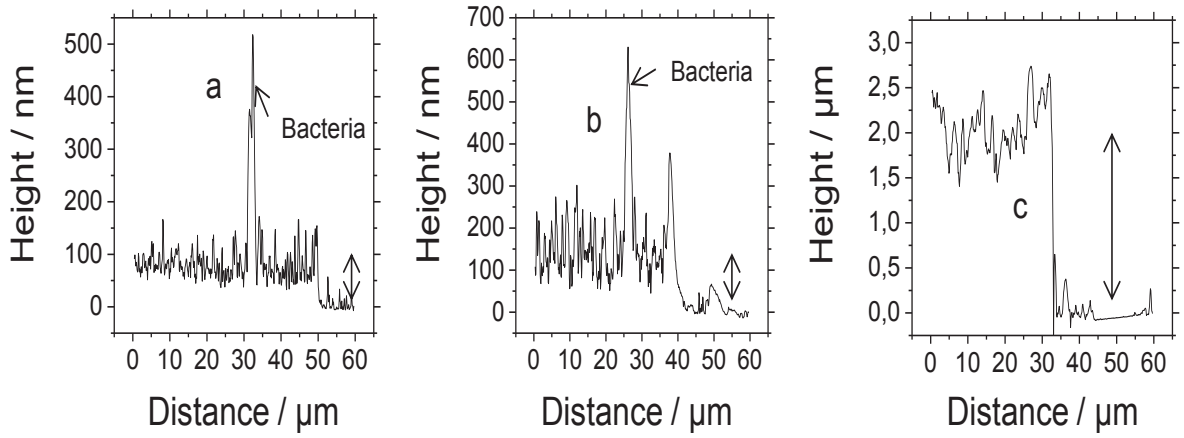

Fig II-3*. AFM profiles for electrodeposition of optimal hybrid sol-gel films varying with deposition times: (a) 10 s, (b) 20 s, (c) 30 s. N.B. E= -1.3 V.*

AFM characterization also provides additional information concerning the gradual encapsulation of *E. coli* C600 with cell density (4×10^6 cells mL^{-1}) (displaying about 500 nm diameter of bacteria) with electrodeposition time (Figures II-3 and II-4). Bacterial cells were still visible on the planar substrate after deposition of 80-nm thick film (Fig. II-3a&4a) but was gradually masked after 20 s (Fig. II-3b&4b) and 30s (Fig. II-3c) deposition. Note that the roughness of the film (measured after drying) also strongly increased when increasing the film

thickness. We will see in the next section that the time of deposition also strongly influences the bacterial viability.

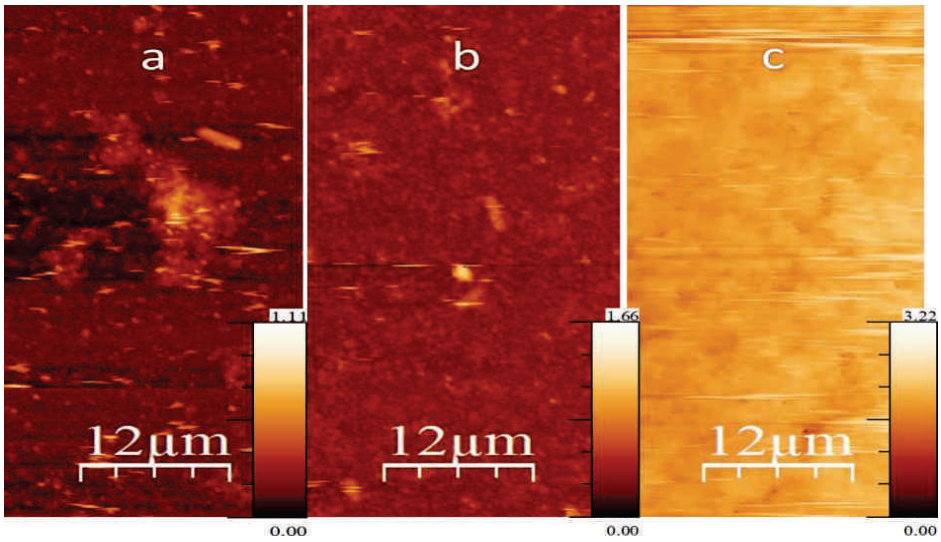

Fig II-4. *AFM images of EABE(2S) for E. coli C600 (4×10^6 cells mL-1) encapsulated in optimal sol-gel: a) 10 s, b) 20 s and c) 30 s. N.B. E= -1.3 V.*

3.2. Viability of bacteria immobilized by EABE(2S)

Live/Dead *Bac*Light viability analysis is a dual DNA fluorescent staining method used to determine simultaneously the total population of bacteria and the ratio of damaged bacteria. It was applied here on encapsulated bacteria in sol–gel films. *Bac*Light kit is composed of two fluorescent nucleic acid-binding stains: SYTO 9 and propidium iodide (PI). SYTO 9 penetrates all bacterial membranes and stains the cells green while PI only penetrates cells with damaged membranes. The combination of the two stains produces red fluorescing cells (damaged cells) whereas those with only SYTO 9 (green fluorescent cells) are considered viable [32].

At first, the short-term viability, *i.e.*, one hour after the encapsulation of *E. coli* C600 in the optimal sol-gel containing silica precursor, PEG and chitosan, was studied and high viability was observed (close to 100 %). One great advantage of EABE (2S) is that the bacterial density can be easily controlled by the first bacteria adsorption step, and the uniform orientation of the cells allows precise analysis of the viability (Fig. II-5). At the opposite, the encapsulation in one step (EABE(1S)) leads to randomly distributed bacteria in the all volume of the film (see next section).

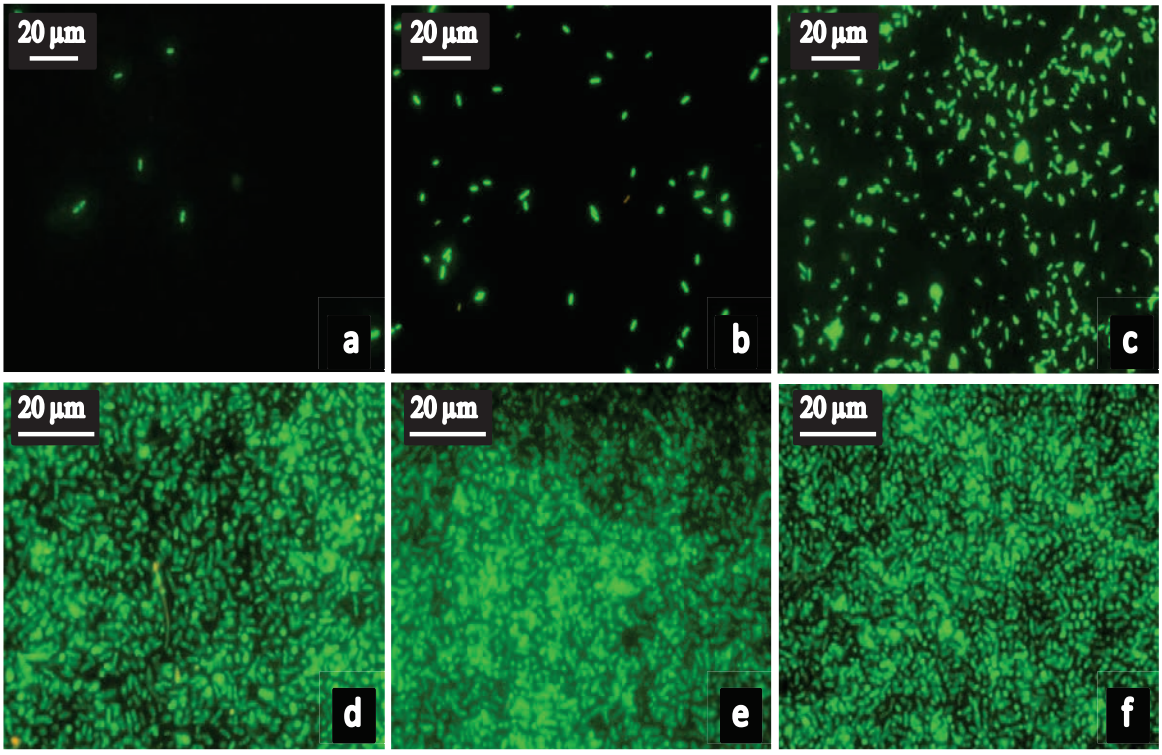

Fig II-5. *Short-term BacLight analysis of EABE(2S) for six E. coli C600 concentrations encapsulated in optimal sol-gel: a) [4 ×10^4 cells ml^{-1}], b) [4 ×10^5 cells ml^{-1}], c) [4 ×10^6 cells ml^{-1}], d) [4 ×10^7 cells ml^{-1}], e) [4 ×10^8 cells ml^{-1}] and f) [4 ×10^9 cells ml^{-1}]. Same magnification for the 3 upper figures a,b and c, same magnification for the 3 lower figures d, e and f. N.B. E= -1.3 V, deposition time = 30s.*

The effects of several parameters, *i.e*. the hybrid sol–gel components (Fig. II-6), the storage conditions (Fig. II-7) and the deposition time parameters of EABE(2S) (Fig. II-8) were then analyzed by Live/Dead *Bac*Light over several weeks.

3.2.1. Influence of film components on the long-term viability of EABE(2S)

As shown in Fig. II-6, the optimal film composition was achieved from a sol solution containing the silica precursor, PEG and chitosan (in the presence of trehalose) as it provided the best protection of membrane integrity for the encapsulated bacteria (96 % of encapsulated *E. coli* C600 exhibited non-damaged membrane after 2 weeks) (curve a). Silica gel, chitosan and PEG have here a complementary role to provide an efficient protection for the immobilized bacteria. Removal of one of the component of the hybrid material led to a dramatic drop in bacterial viability. After 15 days, only 50 % viability was kept with the film that did not contain silica (curve b), only 20 % in the absence of PEG (curve c) and only 10 % in the absence of chitosan (curve d).

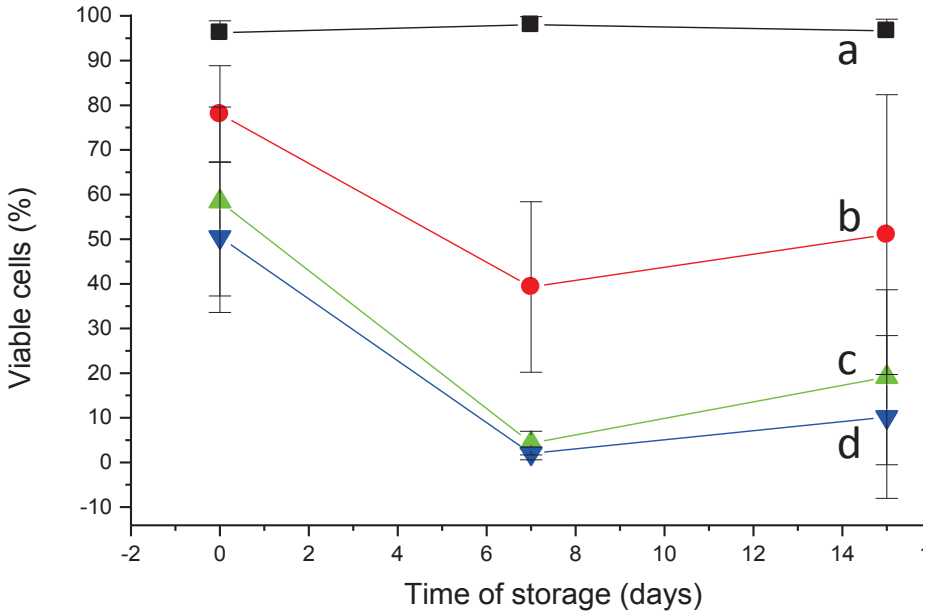

Fig II-6. *Long-term BacLight analysis for EABE(2S) of E. coli C600 (4×10^6 cells mL^{-1}) in films composed with (a) sol-gel silica, PEG and chitosan, (b) PEG and chitosan, (c) sol-gel silica and chitosan, (d) sol-gel silica and PEG. Trehalose was also introduced in all films. The first measurement was done 1 hour after electrochemically assisted bacteria encapsulation. All films have been prepared by electrolysis at -1.3 V for 30 s. All samples were stored at + 4 °C in moist air.*

The positive influence of silica gel on the cell viability was already discussed in the literature [9,10]. In addition; the presence of silica precursor, PEG and chitosan in the electrolysis bath allows here the rapid gelification of a hybrid film, thick enough to protect the bacteria as discussed in section (4.1) where the thickness reached 2 µm (approximately 4 times the diameter of bacterial cell ≈ 0.5 µm). In particular, chitosan, which is characterized by the presence of $-NH_3^+$ groups, can interact with silicate monomers to facilitate the deposition of a stable hybrid sol-gel film [38]. As a result, the samples prepared with chitosan exhibited larger film thicknesses (≥ 100 times) than those without chitosan (see the first section of Results and discussion).

In addition, PEG lowers the interaction of entrapped bacteria with the silicate matrix, minimizes the interaction with toxic solvent produced during the hydrolysis and prevents excessive contraction during sol-gel transition phase; it fills the pores and spaces in the matrix, thereby eventually preventing matrix shrinkage and collapse [36,39].

3.2.2. Influence of storage conditions on the long-term viability of EABE(2S)

Fig. II-7 reports the evolution with time of the bacterial viability of encapsulated *E. coli* C600 under different storage conditions, *i.e.*, 1 mM KCl solution (curve a) or moist air (curves b and c). All samples have been stored at +4 ºC which was given as an optimum temperature for storage of encapsulated *E. coli* inside inorganic matrices in the literature [40].

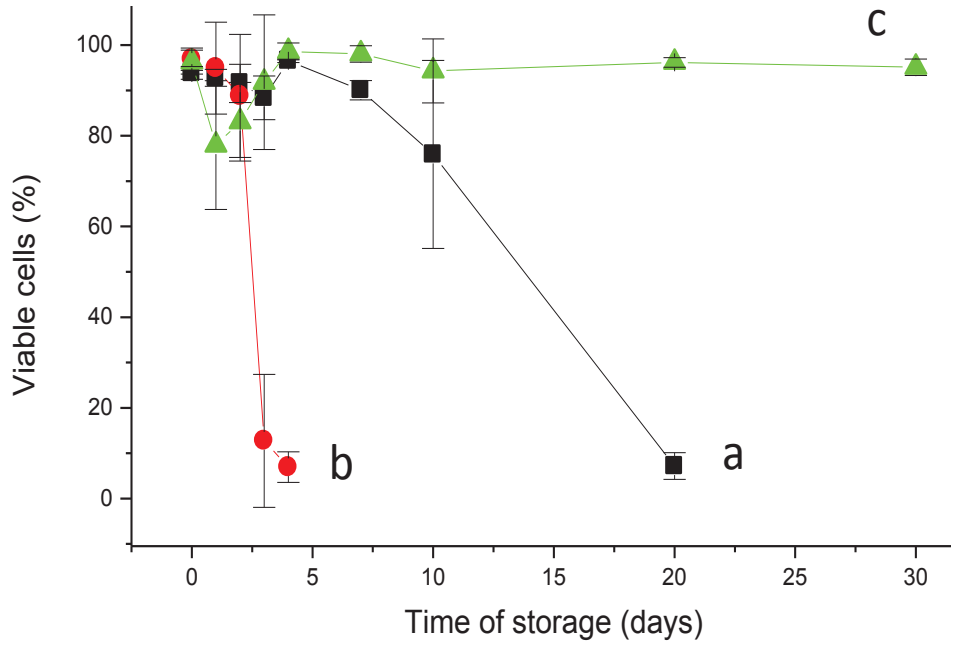

Fig II-7. *Long-term BacLight analysis for EABE(2S) of E. coli C600 (4×10^6 cells mL^{-1}) in KCl 1 mM (a), moist air only (b), and moist air + trehalose inside sol-gel film (c). All films have been prepared by electrolysis at -1.3 V for 30 s. N.B (zero: 1 hour after encapsulation).*

According to results of Fig. II-7, the storage in moist air without introducing trehalose into the film did not succeed in preserving bacterial viability for more than 3 days due to complete drying of the sol–gel film (curve b). The storage in KCl solution could solve the problem of bacterial damage due to sol–gel film drying (curve a). On the other hand, storage in wet medium has also some disadvantages in terms of limiting long-term viability due to aqueous dissolution of the sol–gel material for long storage conditions [12], and providing higher probability of contamination than dry storage. The storage in moist air with introducing trehalose inside the sol–gel film has solved these problems, showing the best preservation of the membrane integrity as 95% of viable cells after 30 days of encapsulation (curve c). Here, trehalose was the water-replacing molecule in dry storage. As described in the literature [17,18], it provides a hydrophilic humid medium by replacing water bonding during gel

drying to avoid protein denaturation and lipid phase transition of bacterial membrane. This, in turn, inhibits van der Waals interactions between adjacent lipids thus preventing the elevation of membrane phase transition and stabilizes the structural conformation of bacterial membrane proteins against denaturation.

The conditions used in this experiment are not favourable for cell division. We suppose that the observed increase in cell viability for short time, *i.e.* between 0 and 5 days (curve c), could be due to recovery of cell membrane integrity. The error affecting these measurements is higher for short time than for longer time. This variability seems to be related to the stress that affected some cells, long-term measurement allowing higher ratio of viable cells and lower variability in the measurement.

3.3.3. Influence of the electrodeposition time on the long-term viability of EABE(2S)

Finally, we have studied the effect of the electrolysis time, from 10 to 30 s, on the viability of encapsulated *E. coli* C600 (Fig. II-8). The films have been produced from the optimal sol composition defined before, in the presence of silica gel, PEG, chitosan and trehalose. All samples were stored at +4 ºC in humid air.

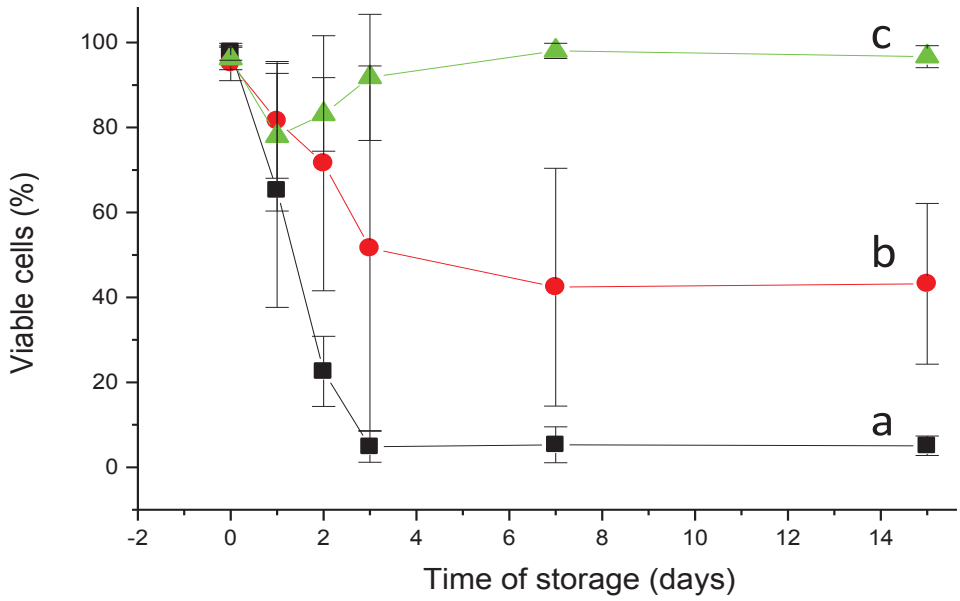

Fig II-8. *Long-term BacLight analysis for EABE(2S) of E. coli C600 (4×10^6 cells.mL^{-1}) with 10 s (a), 20 s (b) and 30 s (c) deposition times. All films have been prepared by electrolysis at -1.3 V. N.B (zero: 1 hour after encapsulation).*

For all samples, the short-term viability measured one hour after encapsulation was high, close to 100 %. However, this viability dramatically decreased for thinner films and almost no

viability could be found after 3 days when the film was produced with using 10 s electrolysis (curve a). The viability after 15 days was greatly improved by increasing the deposition time, as 40 % (curve b) and 96 % (curve c) viability were observed in the films prepared with 20 s and 30 s electrolysis, respectively. The effect of electrolysis time has to be correlated with the film thickness that increases dramatically from 82 ± 5 nm and 132 ± 17 nm for 10 and 20 s, respectively, to 1.9 ± 0.1 μm for 30 s. Here, the film deposited with applying 30 s electrolysis was thick enough for effective protection of the encapsulated bacteria with no significant cell membrane destruction by electrochemical stress. Thinner films did not ensure enough protection because the bacteria were not properly covered with the hybrid layer. Thicker films can be produced by using longer electrolysis times but it was observed that too long electrodeposition processes became detrimental for the cell integrity (data not shown).

Practical applications of immobilized bacteria for biosensing could involve a large number of bacteria. Also, a large amount of encapsulated bacteria is necessary for studying the metabolic activity, notably in the luminescent mode of analysis. As shown in Fig. II-5d–f, the cell density of bacteria can be controlled during the first step adhesion. However, one disadvantage of the EABE(2S) protocol is the limited viability preservation when such high bacterial density is encapsulated. We observed that a dense bacterial film strongly limits the electrodeposition process and/or alters the film stability. For this reason, we have investigated the electrochemically assisted bacterial encapsulation in one step (EABE(1S)) in order to achieve larger amounts of encapsulated bacteria and to be able to analyze their metabolic activity once entrapped in the hybrid sol–gel films. The main results are described hereafter.

4. Electrochemically assisted bacteria encapsulation in one step (EABE(1S))

Fig. II-9 illustrates the EABE(1S) protocol. Here, bacteria are introduced in the starting sol, in the presence of the silica precursor, PEG, chitosan and trehalose. When the electrolysis is performed, the sol–gel transition occurs and bacteria are immobilized in the course of the hybrid sol–gel layer formation. During the immobilization, the bacteria are expected to be homogeneously distributed in the material but are randomly oriented.

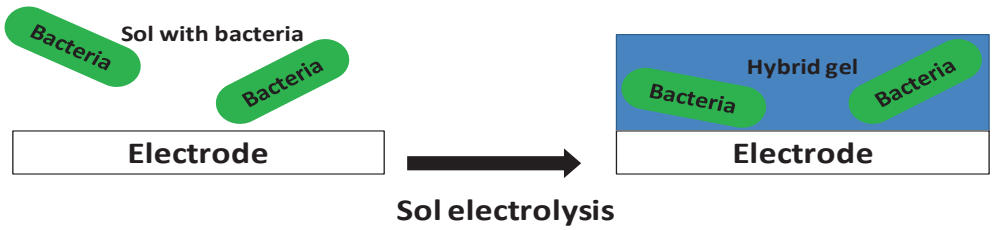

Fig II-9. *Scheme of electrochemically assisted bacteria encapsulation in one–step (EABE(1S)).*

4.1. Principle and preliminary characterization

As we explained before that EABE(1S) implying the electrodeposition of hybrid sol including bacteria suspension on the electrode surface. Thicknesses of the films deposited with various electrolysis times but optimal potential -1.3 V and same hybrid sol-gel were measured by AFM (Fig II-10). Values of 367 ± 55 nm, 2.1 ± 0.4 µm and ≥ 3.5 µm were obtained for deposition times of 10, 20, and 30 s, respectively.

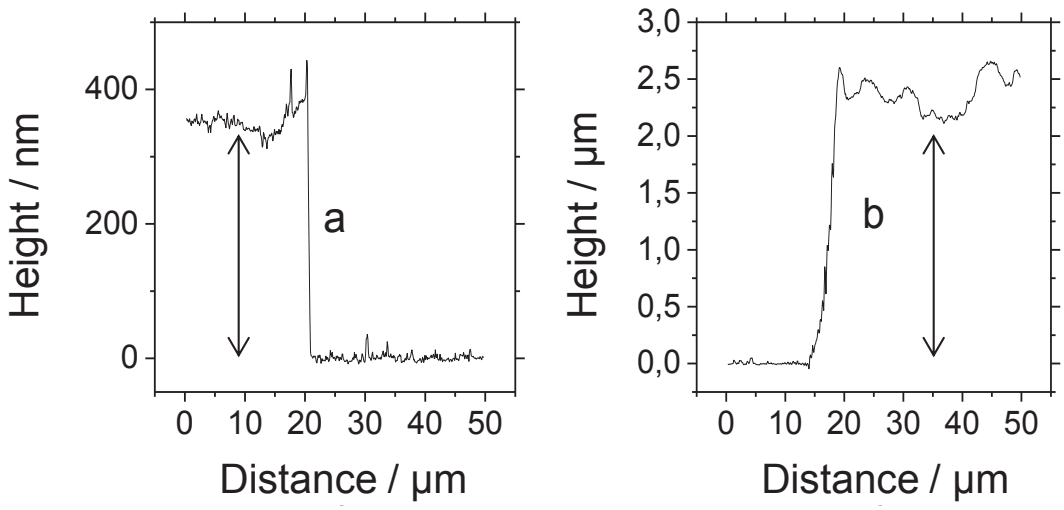

Fig II-10. *AFM profiles for electrodeposition of optimal hybrid sol-gel films obtained with various deposition times: (a) 10 s, (b) 20 s (30 s was not measurable). All films have been prepared by electrolysis at -1.3 V with final cell density 2 × 10^9 cells/mL.*

Using the same parameters (optimal potential, hybrid sol-gel components and optimization, time of electrodeposition) as EABE(2S), the AFM measurements for EABE(1S) have shown much higher film thicknesses than those of EABE(2S) probably due to the presence of bacteria that affected electrodeposition rate on the ITO surface.

AFM characterization also provides additional information concerning the gradual encapsulation of *E. coli* C600 with cell density (8×10^9 cells mL^{-1}). With the one-step protocol, bacteria can be observed on all samples, even when using long electrodeposition time, i.e. 30s, leading to relatively thick films (Fig. II-11). This confirms the random distribution of bacteria encapsulated by EABE(1S) inside the deposited gel. The roughness of the film (measured after drying) also strongly increased when increasing the film thickness.

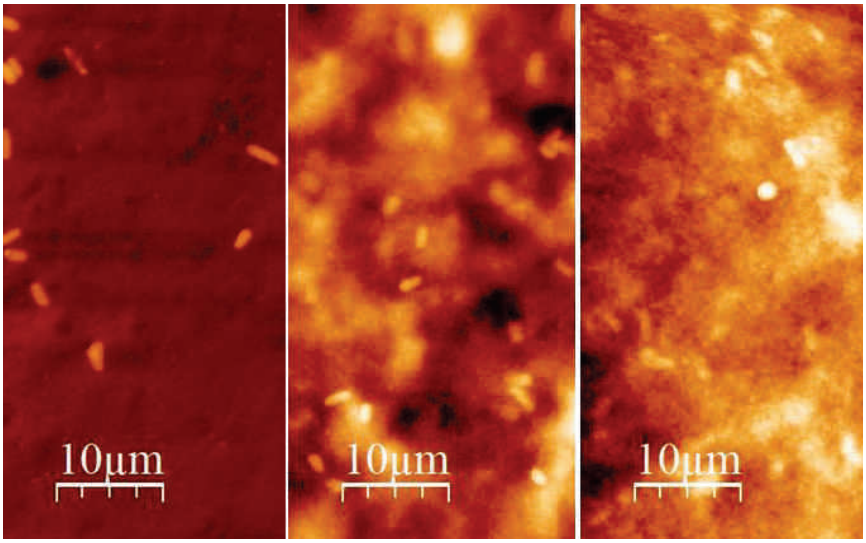

Fig II-11. *AFM images of EABE(1S) for E. coli C600 (8×10^9 cells mL^{-1}) encapsulated in optimal sol-gel: a) 10 s, b) 20 s and c) 30 s. All films have been prepared by electrolysis at -1.3 V.*

4.2. Viability analysis of bacteria immobilized by EABE(1S)

Fig. II-12 reports the short-term viability of *E. coli* C600 immobilized by EABE(1S) in films produced with electrolysis times varying from 10 s (Fig. II-12a) to 60 s (Fig. II-12d). The bacterial cells exhibited high viability, but precise counting of viable bacteria was difficult because of their random orientation, especially for the thickest films. The quantity of bacteria immobilized on the electrode surface was controlled by the bacterial density introduced in the sol and the time of electrodeposition. As observed previously for EABE(2S), too long electrolysis time in EABE(1S) can be detrimental for the bacteria as shown in Fig. II-12d (corresponding to the film prepared with 60 s electrolysis). In this experiment, a significant number of non-viable cells were observed (but it was not possible to quantify them accurately). Note that the cell density (4×10^8 cells per mL) used for the EABE(1S)

experiment reported (Fig. II-12) is 100 times lower than the cell density for EABE(2S) reported previously (4×10^6 cells per mL was used in Fig. II 6-8). In fact, in the two steps protocol, the adhesion of the bacteria during the first step (see Fig. II-2) allows concentrating the bacteria on the electrode surface before sol electrolysis and bacteria densities become comparable for the two approaches.

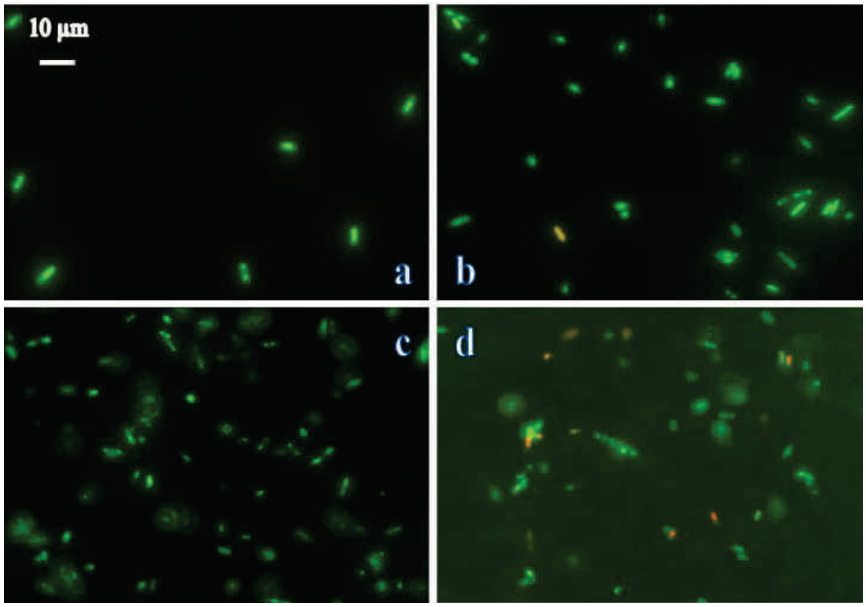

Fig II-12. *Short-term BacLight analysis of EABE(1S) for E. coli C600 (4×10^8 cells mL^{-1}) along deposition times of optimal sol-gel: a) 10 s; b) 20 s; c) 30 s and d) 60 s. Same magnification for all pictures. All films have been prepared by electrolysis at -1.3 V with final cell density 10^8 cells/mL.*

4.3. Analysis of bacterial metabolic activity

In order to confirm the viability of the encapsulated bacteria, the EABE(1S) protocol was applied on the bioluminescent bacterial biosensor *E. coli* MG1655 pUCD607. The pUCD607 plasmid contains the lux- CDABE operon under the control of the constitutive tetracycline resistant promoter [41], which is responsible for continuous light production. The bioluminescent reaction is catalyzed by luciferase (encoded by the luxA and luxB genes) which requires a long-chain aldehyde (provided by the luxCDE gene products) as a substrate, oxygen, and a source of reducing equivalents, usually reduced flavin mononucleotide ($FMNH_2$) [42]. Since the $FMNH_2$ production depends on a functional electron transport system, only metabolically active cells produce light. Here, the *E. coli* MG1655 pUCD607

system was used to assess the effect of encapsulation and subsequent storage conditions on the overall metabolic activity of bacteria.

E. coli MG1655 pUCD607 (8×10^9 cells per mL) was encapsulated in the sol gel matrix with the optimal composition (as described above) and compared to non-encapsulated *E. coli* (only deposited on PEI-modified ITO electrode). All samples have been stored at - 80 ºC prior to the reactivation step of bacteria in medium for long-term luminescent analysis. All results are presented in Table II-1. After four weeks storage, the electrochemical encapsulation of *E. coli* MG1655 pUCD 607 in a film with optimal composition has preserved 50% of the continuous luminescence production compared to approximately null metabolic activity of non-encapsulated bacteria, which is most probably due to the absence of cell stabilizers against freezing stresses [18].

Luminescence (CPS)		
Storage time (days)	Encapsulated bacteria	Non-encapsulated bacteria
1 week	51000 ± 6000	37000 ± 4000
2 weeks	50000 ± 4000	1000 ± 500
3 weeks	43000 ± 15000	2300 ± 200
4 weeks	26000 ± 5000	600 ± 150

Table II-1. *Long-term metabolic activity for EABE(1S) of E. coli MG1655 pUCD607 stored at - 80 ºC. All films have been prepared by electrolysis at -1.3 V for 30 s.*

On the other hand, the same encapsulation protocol has been applied to the biosensor strain *E. coli* MG1655 pZNTA-GFP, which expresses GFP in response to cadmium exposure (P. Billard, unpublished data). This bacterial strain could be used for designing of fluorescent microbial sensor based on assessment of heavy metal bioavailability [43]. After one week of storage at -80 °C, the latter strain still produced GFP fluorescence upon overnight incubation in TSB medium supplemented with 1 µM $CdCl_2$ (Fig. II-13). However, this long incubation time caused silica dissolution and release of bacteria that could grow as planktonic cells, thereby preventing the quantitative measurement of the cell response to Cadmium stress.

Nevertheless, this test confirmed that cells were viable after encapsulation and storage and even retained their ability to sense and respond to adverse environmental conditions.

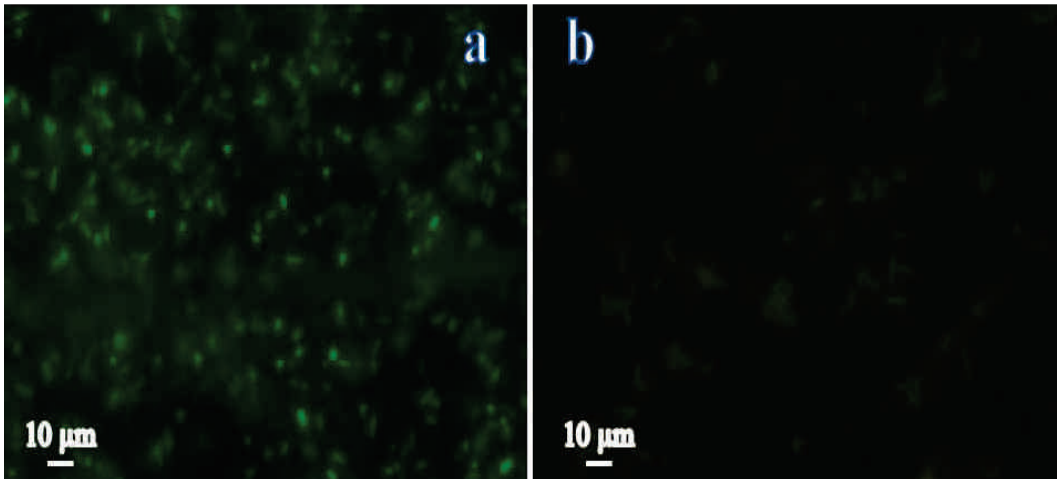

Fig II-13. *Fluorescence metabolic activity for EABE (1S) of* MG1655 pZNTA-GFP *(4×10^8 cells mL^{-1}) encapsulated in optimal sol-gel after 1 week: a) stressed by* $CdCl_2$ *and b) Unstressed (Control).*

5. Conclusions

Three *E. coli* strains (C600, MG1655 pUCD607 and MG1655 pZNTA-GFP) have been used as models of encapsulated microorganisms in thin sol-gel films generated in a controlled way by electrochemical protocols. The encapsulation can be performed with bacteria pre-immobilized on the electrode surface or from a suspension of the bacteria in the starting sol. The possibility of utilizing electrochemistry for effective bacteria encapsulation in sol-gel films with preserving a high percentage of viability and activity for the entrapped microorganisms was demonstrated here. The presence of organic additives was proved to be essential as they contributed to modify significantly both the reactivity and the stability of the biocomposite films, notably by providing high protection levels for the encapsulated microorganisms for a rather long period (1 month). Bacteria immobilized in such films can be reactivated for expression of luminescent or fluorescent proteins and they kept sensitivity to their environment, as demonstrated by the fluorescent signal in the presence of cadmium. The development of such electrochemical protocol provides new opportunities for bacteria immobilization on electrode surfaces, with possible applications in the field of electrochemical biosensors for environmental monitoring, or to optimize the electrochemical communication between bacteria and electrode collectors, which constitutes major challenges in the development of biotechnological devices.

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Chapter III. Investigations of the electrochemical communications between bacteria and the electrode surface

In this chapter, the work focuses on the electrochemical communication between *Shewanella putrefaciens* CIP 8040 *or Pseudomonas fluorescens* CIP 69.13 encapsulated in a silica-based sol-gel film and a glassy carbon electrode. As silica is an insulating material, strategies have found to improve the electron transfer from bacteria to electrode in this environment. Several configurations have been considered, i.e., direct electron transfer from the bacteria to the glassy carbon electrode, introduction of soluble $Fe(CN)_6^{3-}$ mediator in the solution, introduction of carbon nanotubes on the electrode surface or in the sol-gel layer, functionalization of the carbon nanotubes with ferrocene mediator and long ethylene glycol arm and finally introduction of a natural mediator , i.e., cytochrome c in the gel, in close interaction with the bacteria. The comparison of these different approaches allows a discussion on the interest and limitation of these different strategies. Sodium formate and glucose have been used as electron donors for *S. putrefaciens or P. fluorescens*, respectively.

1. Introduction

Development of living cell-based electrochemical devices holds great promise in biosensors, bioreactors and biofuel cells. It eliminates the need for isolation of individual enzymes, and allows the active biomaterials to work under conditions close to their natural environment, thus at a high efficiency and stability [1–4]. Living cells are able to metabolize a wide range of chemical compounds. Microbes are also amenable for genetic through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes [2]. However, the main issues are the preservation of cell viability and efficient electron transfer (ET) between bacteria and electrodes for high performance and feasible large-scale implementation for these electrochemical devices [1,4].

The preservation of cell viability could be materialized by encapsulation technology in biocompatible matrices resembling the natural environment in order to conserve the cells alive in open porous matrix for passage of oxygen, solvents and nutrients but avoiding cell leaching [3]. Whole cells have been successfully encapsulated either by physical or chemical methods. The latter includes covalent interactions between functional groups located on the outer membrane of the microorganisms and glutaraldehyde as cross-linking agent. This encapsulation mode is consequently not suited when cell viability is absolutely required whereas excessive degrees of cross-linking can be toxic to the cells [4,5]. On the other hand, the physical entrapment is a simple and soft method to encapsulate microorganisms onto a transducer. Microorganisms can be encapsulated in organic or inorganic polymeric matrix, e.g., sol-gel [6,7], or hydrogels such as poly(vinyl alcohol) (PVA) [8], alginate [9] or agarose [10] . In order to combine the advantages of both organic and inorganic matrices, microorganisms have been entrapped in hybrid materials based on silica sol and poly(vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)) [11,12]. The encapsulation can protect the cells from external aggression but at the same time might form a barrier that decreases the electrochemical interaction with the electrode surface [4].

Immobilization of the bacteria onto an electrode surface is also a key issue in the development of cell-based electrochemical devices by providing close proximity between the viable cells and the solid electrode surface to achieve fast and efficient electron transfer (ET) reactions [4]. The challenge is to enhance the link between the microbial catabolism and the communication with the electrode surface. Microorganisms can adopt different strategies for

ET, most common of which are: (1) direct electron transfer (DET) and mediated electron transfer (MET), through either exogenous or endogenous diffusive electron mediators [13]. DET has been developed between electrode and monolayer bacteria through c-type cytochromes (c-Cyts) associated with bacterial outer membrane (OM) such as *Shewanella oneidensis* [14] or putatively conductive nanowires such as *Geobacter sulfurreducens* strains [15] or with conductive biofilm [16]. Mediated ET has been performed with either bacterial self-mediated transfer such as *Pseudomonas aeruginosa* [17] or by an artificial mediator to enhance the ET between microbial cells and electrodes. Thus, the natural final electron acceptor (e.g., dioxygen in the case of aerobic bacteria, or other oxidant such as Fe(III) oxides in the case of anaerobic respirating organisms) can be replaced to prevent the problem of limiting concentrations of the electron acceptors [18]. Soluble artificial mediator such as ferricyanide, p-benzoquinone and 2,6-dichlorophenol indophenol (DCPIP) have been successfully used for applications of microbial fuel cells and electrochemical biosensors [19–21] due to their ability to circumvent the cell membrane, collect and transfer the maximum current to the electrode surface. Ferricyanide ($Fe(CN)_6^{3-}$) has greater attention especially for gram-negative bacteria because the high solubility of synthetic electron acceptor overcame the limitation from lower oxygen solubility and its ability to enter through the porins of cell walls. However, high ferricyanide concentration ($\geq$ 50 mM) can also damage the membrane for some bacteria like *E. coli* [22]. Polymeric mediators have also succeeded to facilitate the electrochemical communication (EC) of different bacterial species through flexible polymer stably binding on the electrode surface to avoid the problem of releasing potentially human-toxic compound in the environment [20,23–25]. The conductive properties of osmium polymer promote a good EC between the electron donating system and the electrode surface due to the permanent positive charge of the redox polymer which favors its electrostatic interactions with charged cell surface [25]. In addition to the mediators, carbon nanotubes (CNT) has attracted considerable attention in electrochemical biosensors to provide large surface areas for enhancing the conduction of ET between bacteria and the electrode surface alone [26,27] or in presence of osmium polymer [28]. Actually, it seems to be of interest to consider using covalently-linked chemical mediator or biological mediator for wiring bacteria with electrode surface, but this approach has not been described so far in the literature.

2. Investigation of bioelectrochemical communication

The goal of this study is to investigate an original strategy based on ET between *shewanella putrefaciens* and an electrode material using novel artificial mediators. Sodium formate was used as a substrate for this bacterium. For the first investigation, $Fe(CN)_6^{3-}$ has been used to optimize the EC of encapsulated bacteria or film modified with bacteria and bare CNT with the glassy carbon electrode (GCE). In addition, Bare CNT and CNT covalently-linked with flexible ferrocene mediator have been compared in different entrapment protocols in order to investigate and evaluate the EC of encapsulated bacteria with GCE.

The different configurations of electrode are schematically described in Figure III-1.

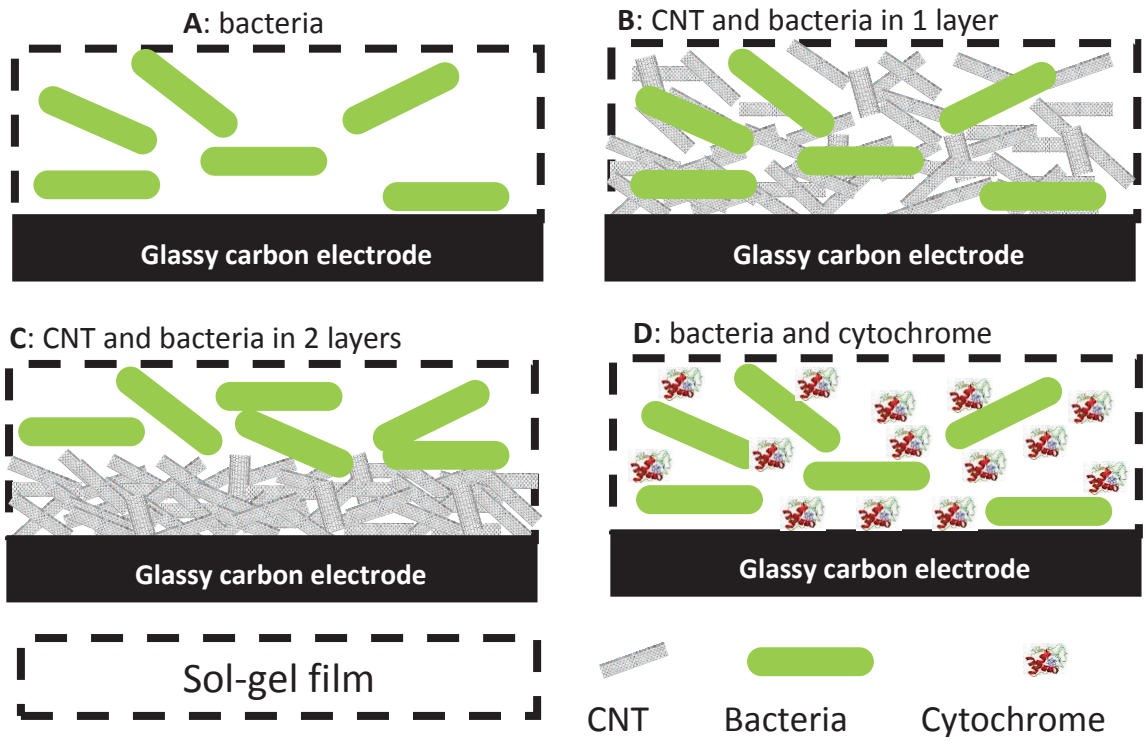

Fig III-1. *The various electrode configurations used in this study: in (A) only the bacteria are immobilized in the sol-gel layer deposited on GCE; in (B) SWCNT has been introduced in the sol-gel layer with bacteria; in (C) a first layer of SWCNT was deposited on GCE and then a second layer of silica gel containing bacteria was overcoated; and in (D) cytochrome c was introduced in the sol-gel layer with bacteria in order to serve as electron mediator.*

2.1. Electrochemical activity of bacteria in a silica gel layer

Figure III-2A shows a typical cyclic voltammetric response of *S. putrefaciens* encapsulated in a sol-gel layer deposited onto the GCE surface (i.e., configuration as in Fig. III-1A). In these conditions a complex cathodic response can be observed with a well-defined redox signal

around -0.22 V and a smaller signal at -0.32 V (peak potentials). On the reverse scan, the anodic response appears even more complex with three successive redox signals visible at -0.28 V, -0.15 V and +0.06 V. One can reasonably suppose that only the bacteria located close to the electrode can transfer efficiently some electrons to the GCE. These signals are compatible with redox proteins secreted by the bacteria or present in the outer membrane of the *S. putrefaciens* cells such as membrane-bound cytochrome [28,29] or secreted riboflavin molecules [30] which could participate to electron transfer reactions to the electrode surface. Curve "a" in Figure 2B (see inset for a closer view) reports the chronoamperometric response of this electrode upon two successive additions of 20 mM sodium formate. Despite the visible electrochemistry of *S. putrefaciens* in the sol-gel layer, the resulting current in the presence of electron donor remains very low, almost undetectable in the range of few nA. A so low response can certainly be explained by the limited number of bacteria that are able to transfer electron to GCE in the insulating environment formed by the silica gel [2,4].

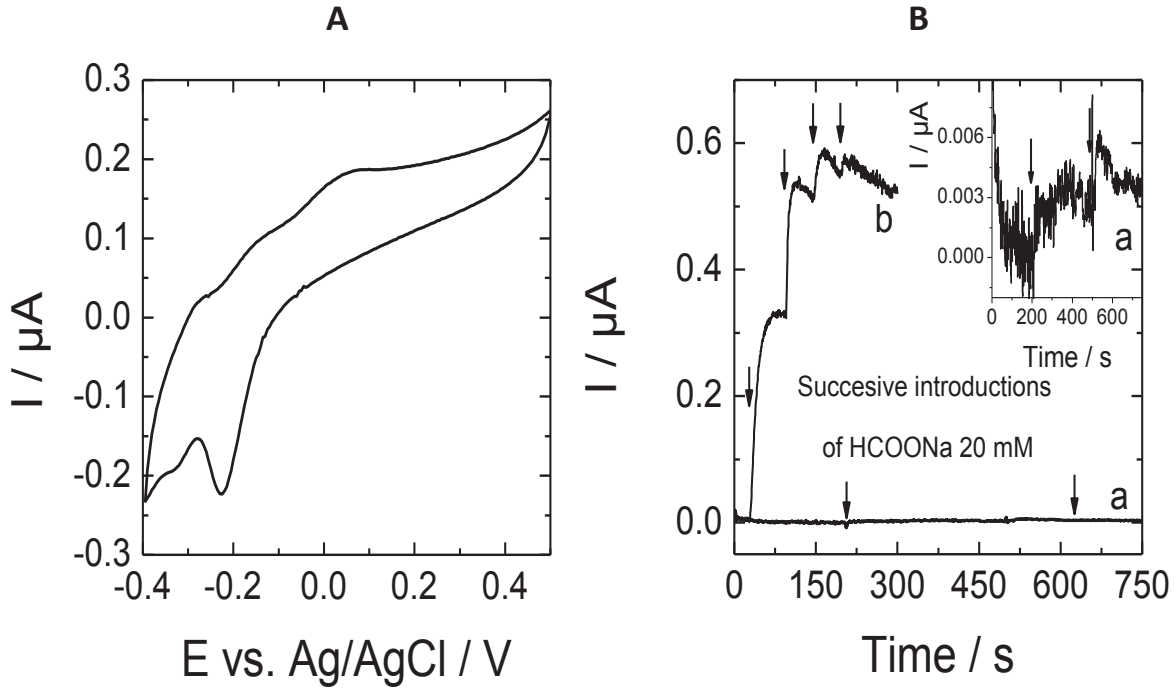

Fig III-2. *A) Cyclic voltammograms recorded with a GCE modified with a sol-gel layer containing S. putrefaciens. The measurement was performed in phosphate buffer at room temperature at scan rate of 20 mV s^{-1}. B) Amperometric response obtained upon successive additions of sodium formate 20 mM (arrows) with a similar electrode as (A). The measurements were performed at room temperature in PBS under stirring, by applying +0.35 V versus Ag/AgCl reference electrode (a) in the absence and (b) in the presence of 5 mM*

$Fe(CN)_6^{3-}$. The inset shows the measurement for (b) at a lower scale current in order to give a better resolution.

On the other hand, one already know that soluble mediators like ferricyanide have been used successfully to shuttle electrons between gram-negative bacteria physically encapsulated in a sol-gel layer and an electrode surface [21,31,32], notably for the fabrication of biochemical oxygen demand (BOD) sensor [21,32]. Ferricyanide can indeed diffuse through the porins of the outer cell membrane for collecting the electrons from the bacteria [20,31]. Curve "b" in Figure III-2B confirms such observation through the electrochemical response measured with the same system as reported in curve "a", but this time in the presence of 5 mM $Fe(CN)_6^{3-}$. In these conditions, a well-defined current increase, in the range of hundreds of nA, was measured after each addition of 20 mM formate in the solution, which is an indication of the good activity of the bacteria in this environment. The comparison between experiments performed in the absence (curve "a") and in the presence (curve "b") of ferricyanide in the solution clearly indicates that electron transfer from bacteria encapsulated in a sol-gel layer can be strongly hindered if no electron shuttle is added. The presence of a soluble mediator is indeed an effective approach to improve this reaction, but other strategies could be implemented in order to reach more efficient electrochemical communication. The next sections will discuss the possible interest of using SWCNT for enhancement of the electrochemical communication. This will be made first in the presence of soluble mediator and then by attaching the mediator, i.e. ferrocene, on the surface of SWCNT. Finally a last strategy based on the encapsulation of cytochrome c as natural mediator in the sol-gel layer along with bacteria will be investigated and compared with strategies involving SWCNT and chemical mediators.

2.2 Influence of SWCNT on the electrochemical communication with entrapped bacteria in a silica gel layer

The introduction of carbon nanotubes has been performed following two different protocols, i.e., cases B and C as reported in Figure III-1. In case B, SWCNT was introduced in the sol-gel layer with the idea to promote a network of bacteria and nanotubes. In case C, a layer of SWCNT was first deposited on glassy carbon electrode and covered by a second layer containing the bacteria, in order to increase the underlying electrode surface area in the

objective to improve the interactions between the bacteria and the SWCNT at the interface between these two layers. The electrochemical responses of these assemblies were first tested in the absence of mediator in solution. The electrochemical response observed upon additions of 20 mM formate with the one-layer configuration (curve "a" in Fig. III-3A, corresponding to case B in Fig. III-1) was slightly improved when compared to the response observed in the absence of SWCNT (curve "a" in Fig. III-2B) but remained very low. The current response was enhanced with the two-layer configuration (curve "b" in Fig. III-3A, corresponding to case C in Fig. III-1), however this response dropped dramatically and rapidly with time.

The same systems, i.e., cases B and C (Fig. III-1), were then tested in the presence of 5 mM ferricyanide. As expected the current increased dramatically upon addition of 50 mM formate, the current value in the one-layer configuration (curve "a" in Fig. III-3B, corresponding to case B in Fig. III-1) being two times lower than the two-layer configuration (curve "b" in Fig. III-3B, corresponding to case C in Fig. III-1). On the other hand, it is noteworthy that the two-layer configuration suffers from longer response time, probably because of some resistance to mass and charge transfer in the insulating silica layer to reach the conductive electrode surface (a phenomena which is expected to be less constraining in the composite layer made of CNTs distributed in the whole film). For both configurations, the electrochemical response recorded in the presence of carbon nanotubes was improved by comparison to that observed with the bacteria alone in the gel (i.e., without SWCNT, see curve "b" in Fig. III-2B).

SWCNT could be in principle appropriate for networking bacteria encapsulated in the sol-gel material. The good electrochemical responses measured in the presence of soluble mediators indicates that the bacteria kept good activity in the sol-gel layer and was not disturbed by the presence of SWCNT or additives used to disperse them. However, the small improvement observed in these experiments in the absence of soluble mediators indicates that optimal use of this nano-material cannot be reached here, possibly because of the limited dispersion of SWCNT in the sol that would lead to insufficient networking. Moreover it was observed that silica is likely to cover the nanotube surface and this effect could hinder the electron transfer reaction between them or here with the bacteria. One way to overcome this limitation is the surface functionalization of SWCNT with a suitable mediator that would allow efficient interaction between bacteria and neighboring carbon nanotubes.

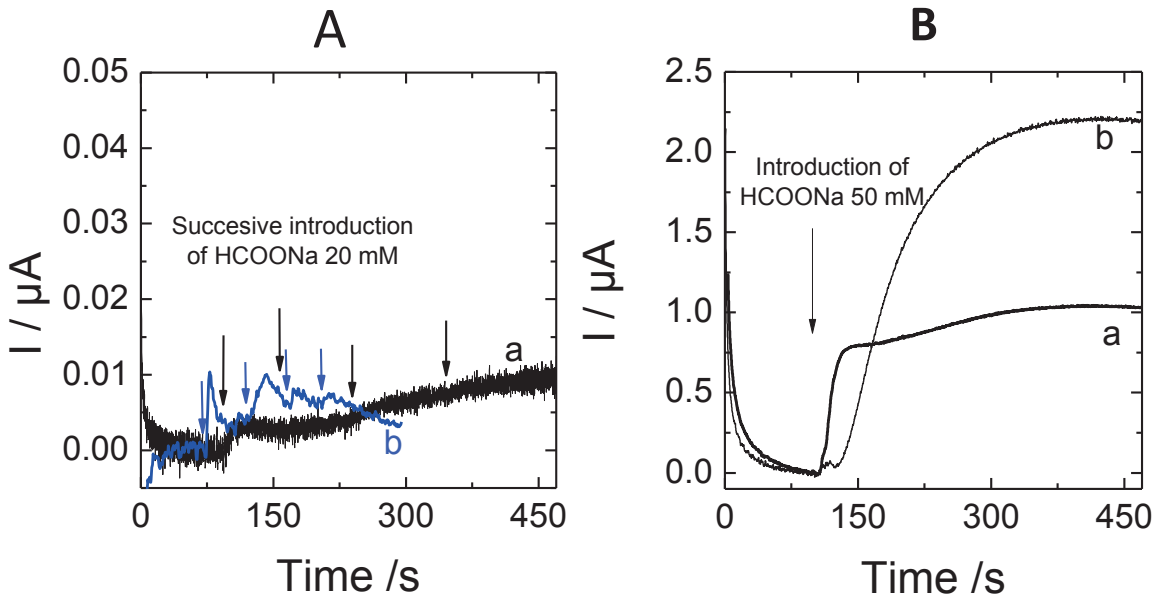

Figure III-3. *Amperometric current responses measured (A) in the absence and (B) in the presence of 5 mM $Fe(CN)_6^{3-}$ mediator in solution upon successive additions of sodium formate (arrows) using glassy carbon electrodes modified with (a) a sol-gel layer containing both SWCNT-COOH and S. putrefaciens (as reported in Fig. III-1B) and (b) sol-gel overlayer containing S. putrefaciens on chitosan/SWCNT-COOH underlayer film (as reported in Fig. III-1C). The measurements were performed in PBS at room temperature by applying +0.35 V versus Ag/AgCl reference electrode.*

2.3. Application of SWCNT-$(EtO)_8$-Fc for electrochemical communication with bacteria entrapped in a silica gel layer

Our group recently described the functionalization of SWCNT with ferrocene mediator with a flexible poly(ethylene glycol) linkers, and a spacer length of 8 ethylene glycol moieties was reported as the most effective one [33]. This functionalization provides both a good mobility to the ferrocene moieties in order to get well-defined cyclic voltammetric response (Fig. III-4A) and to achieve suitable electrocatalysis and an improved dispersion of the nanotubes in aqueous environment used here for bacterial immobilization. The polyethylene glycol chain could also provide a hydrophilic protection for preserving microbial viability [12]. Figure III-4B shows the electrochemical response measured with these SWCNT functionalized by ferrocene. Both anodic and cathodic signals, though not so intense, are well resolved, indicating the effective immobilization of Fc on the carbon nanotube surface. Additional

physico-chemical characterization has been carried out and is reported elsewhere [34]. The two electrode configurations described before, i.e. one-layer configuration (case B in Fig. III-1) and two-layer configuration (case C in Fig. 1) were tested here with the functionalized carbon nanotubes.

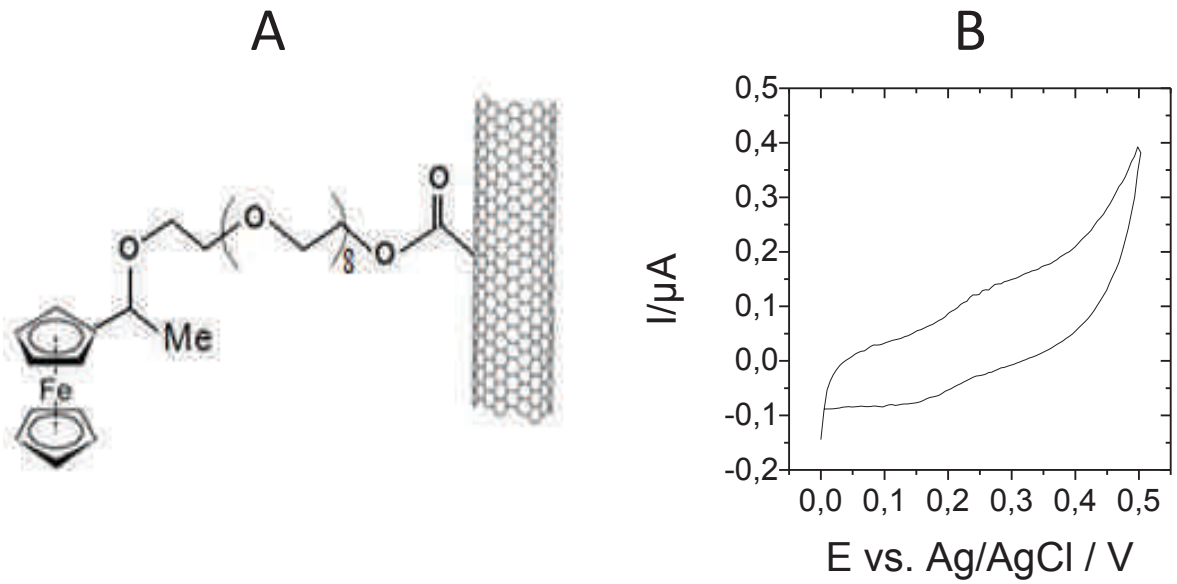

Fig III-4. *(A) Scheme of SWCNT functionalized with ferrocene linked by flexible Poly(ethylene glycol) linker. (B) Cyclic voltammogramm measured with GCE modified with SWCNT-$(EtO)_8$-Fc. The measurement was performed in PBS at room temperature and at scan rate of 20 mV s^{-1}.*

Curve "a" in Figure III-5 shows the electrochemical response of the bioelectrode prepared in the one-layer configuration (case B in Fig. III-1) with SWCNT-$(EtO)_8$-Fc and *S. putrefaciens*. No mediator was introduced in solution, only the mediators immobilized on the surface of SWCNT allowed to transfer electrons from the bacteria to the glassy carbon electrode. In these conditions, the successive additions of 20 mM formate in the solution led to gradual increase of current, however not proportionally to the total concentration of formate in solution. At the opposite, the electrode prepared in the two-layer configuration (Case C in Fig. III-1) with SWCNT-$(EtO)_8$-Fc and *S. putrefaciens* gave rise to a more linear response with a gradual increase in the electrochemical response upon successive additions of 20 mM formate. The response of the bioelectrode was linear up 80 mM with a sensitivity of 0.1 µA $M^{-1}cm^{-2}$. The very good behavior of the two-layer configuration indicates that bacteria and

SWCNT-$(EtO)_8$-Fc interacts in a very efficient manner at the interface between the two layers. Actually, the deposition of the second layer of silica gel most probably induced a slight redissolution of the firstly deposited network of SWCNT-$(EtO)_8$-Fc, allowing thus a very favorable arrangement of these different components, before gelification of the silica material.

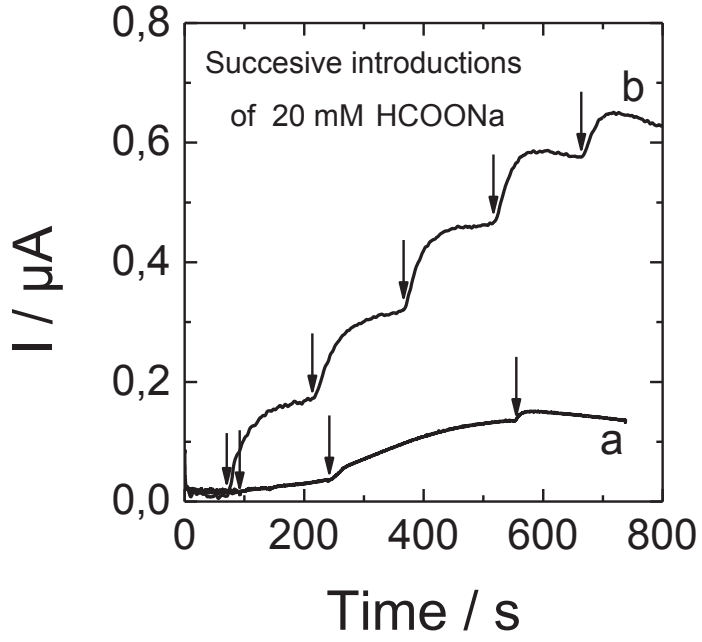

Fig III-5. *Amperometric current responses measured upon addition of sodium formate using glassy carbon electrodes modified with (a) sol-gel layer containing CNT-$(EtO)_8$-Fc and S. putrefaciens (as reported in Fig. III-1B); (b) sol-gel overlayer containing S. putrefaciens on chitosan/SWCNT-$(EtO)_8$-Fc underlayer film (as reported in Fig. III-1C). The measurement was performed under stirring in PBS at room temperature by applying +0.35 V versus Ag/AgCl reference electrode.*

2.4. Effect of biological mediator on cell communication

The electrochemical behavior of *S. putrefaciens* in sol-gel has been studied in the presence of cytochrome c (biological mediator) to be compared with results obtained from SWCNT-Fc described in the previous section. Fig. III-6A reports the electrochemical response of cytochrome c in the sol-gel layer. A pair of well-defined current peak is observed located at 0.07V. Contrarily to the response measured with *S. putrefaciens* that display several irreversible peaks (Fig. III-2A), the signal coming from cytochrome c is clearly reversible. Investigations have shown that the electrochemical response of cytochrome c was controlled

by diffusion, indicative of the good mobility of this small protein in the silica gel (See section 3 of this chapter).

This film displayed a significantly different behavior from previous system with chemical mediators (Ferrocene or $Fe(CN)_6^{3-}$). The amperometric response was indeed measured upon successive additions of 0.2 mM formate in the solution *i.e.,* 100 times lower concentration than those reported in the previous investigations reported in this this section. Under this conditions a maximum sensitivity of 1 mA M^{-1} cm^{-2} was observed, which is ten times higher than the best sensitivity observed with using SWCNT-$(EtO)_8$-Fc. Moreover, the operating potential was lower with cytochrome c (*i.e.,* +0.15 V) than with chemical mediators (*i.e.,* +0.35 V), which is clearly advantageous for several applications, including biosensing and biofuel cell. But the linear range of the response was much narrower when using cytochrome c as mediator (mM range) by comparison to other mediators (in the range of 100 mM). In the present state of our investigations, it is difficult to clearly explain the different behavior of the two classes of mediators, but their different nature could be at the origin of distinct interactions with bacteria. The interaction mechanism of chemical mediator such as $Fe(CN)_6^{3-}$ or flexible osmium polymer linked to CNT has been mentioned in literature as entrance of mediator through the porins of outer cell membrane in order to accept electrons from electron transport chain located at the cell membrane [20,31] or even from cytosolic enzymes [35] while biological mediator could collect electrons only from the outer cell membrane without entering through its porins.

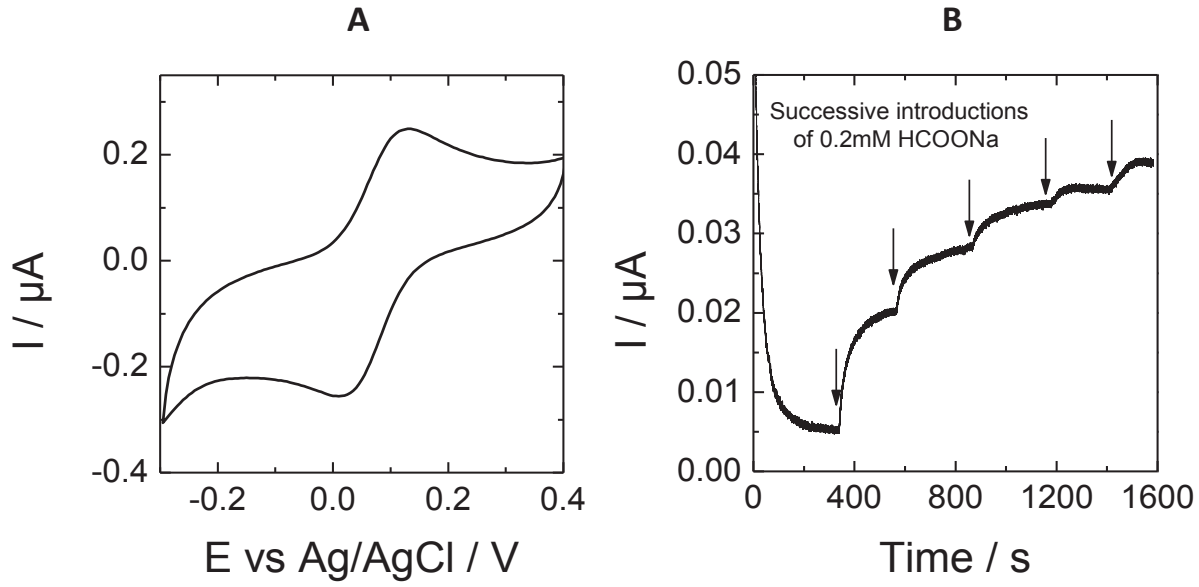

Fig III-6. *(A) Cyclic voltammogramm measured with GCE modified with a sol-gel layer containing bovine heart cytochrome c. The measurement was performed in PBS at room temperature and at a scan rate of 20 mV s^{-1}. (B) Amperometric current responses recorded upon successive additions of 0.2 mM sodium formate, as measured using GCE modified with a sol-gel layer containing bovine heart cytochrome c and S. putrefaciens. The measurement was performed under stirring in PBS at room temperature by applying +0.15 V versus Ag/AgCl reference electrode.*

As a matter of comparison, a recent report involving *S. putrefaciens* CIP 8040 , in connection with osmium redox polymer display a maximum current of 45 μA cm^{-2} in the presence of 18 mM sodium lactate [24], which would correspond to a sensitivity of 2.5 mA M^{-1} cm^{-2}, twice the sensitivity reported here with using cytochrome as electron mediator in a sol-gel film. Interestingly the artificial immobilization in the same osmium polymer led to a comparable sensitivity as reported in our work. Some limitations arise from the forced immobilization of the bacteria in sol-gel material in this kind of artificial biofilm. Further improvement in the strategies used for bacteria immobilization will be necessary to make sol-gel encapsulated bacteria competitive with more freely growing biofilms. But one also has to consider that freely growing biofilms take more than a day to express the maximum current, while the artificial biofilm reported here is rapidly operating, which is clearly an advantage when considering biosensing applications.

To resume this section, different ways of electrochemical communication between bacteria encapsulated in a sol-gel layer and a glassy carbon electrode has been investigated. In the absence mediator, the bacteria display poor ability to transfer electron to the electrode surface, because of the insulating nature of the matrix of encapsulation. The introduction of SWCNT on the glassy carbon surface or in the sol-gel layer does not improve significantly this transfer of electron, while the bacteria kept good activity as demonstrated in the presence of soluble mediator. SWCNT-$(EtO)_8$-Fc and cytochrome c have demonstrated their ability to facilitate the electron transfer reaction between *S. putrefaciens* encapsulated in the silica layer and the glassy carbon electrode. The covalent linkage of ferrocene on SWCNT with polyethyelene glycol linker has provided the suitable flexibility for efficient interaction with *S. putrefaciens*. The better sensitivity (1 mA $M^{-1}cm^{-2}$) was obtained with using cytochrome c as mediator while the widest linear response versus the concentration was observed with SWCNT-Fc. The different behavior observed in the presence of SWCNT-$(EtO)_8$-Fc and cytochrome c is indicative of the different mechanism involved by the bacteria to transfer the electrons to the

mediator, as soluble mediator or ferrocene are susceptible to enter the porins of the outer bacterial membrane, while cytochrome c do not, limiting with the latter electron from the outer membrane of the bacteria.

Further optimization has been done with using cytochrome c as natural mediator. Experiments have been performed with *Pseudomonas fluorescens* and are presented in the next section. The modification of the strain was only motivated by practical reason, this latter strain was simply found easier to cultivate than *Shewanella putrefaciens*.

3. Investigation of biological-mediated bioelectrochemical communication

The electrochemical investigation of cytochrome c as redox-active protein at electrodes [36] has gained an interesting area of research. Indeed, cytochrome c has succeeded to mediate the ET between an electrode surface and some enzymes [37,38] without chemical mediator, forming a stable protein-protein system for construction of biosensors [39,40]. Up to now, the biological mediator has not been tested for wiring bacteria, especially those having membrane-bound cytochromes. Cytochrome c displays many important features of such as harmless effect on bacterial viability, good electroactive properties and is of prime importance for the electrochemical communication into biofilms [16]. The encapsulation of both cytochrome c and bacteria in porous sol-gel matrices could mimic the natural biofilms and avoid their limitations such as insufficient interaction of bacteria and electrode materials or the poor control on the bacterial strains involved. The silica matrix mimics in these conditions the exopolysaccharide ‘glue’ that binds cells in a natural biofilm.

The goal of this study is to evaluate an original strategy based on ET between *Pseudomonas fluorescens* CIP 69.13 cells and an electrode using bovine heart cytochrome c as biological mediator. The encapsulation of this bacteria-protein couple in a sol-gel film produces an artificial biofilm likely to be considered in electrochemical biosensors, biofuel cells or bioreactors. Glucose was used as a model analytical probe to investigate such novel system.

3.1. Electrochemistry of bovine heart cytochrome c in sol-gel

The immobilization of cytochrome c was achieved in situ by physical entrapment in the silicate film in the course of the sol gelification. The redox-active protein cytochrome c is likely to mediate ET between other proteins and an electrode surface [36]. The first experiments were thus directed to evaluate the electrochemical characteristics of the entrapped cytochrome mediator in the sol-gel film. Figure III-7A reports the cyclic voltammetric responses measured with the bare glassy carbon electrode (curve a), and the electrodes modified with a thin sol-gel film containing the cytochrome c alone (curve b) or in the presence of *P. fluorescens* (curve c). As expected, no faradaic current was measured on the unmodified electrode. At the opposite, the introduction of cytochrome c in the film allows measuring a well-defined pair of current peaks characteristic for quasi-reversible redox system, located at 0.05 V versus Ag/AgCl and exhibiting 0.1 V potential peaks separation (as measured at 20 mV s^{-1}). These electrochemical characteristics are consistent with the signal of cytochrome c previously published [41].

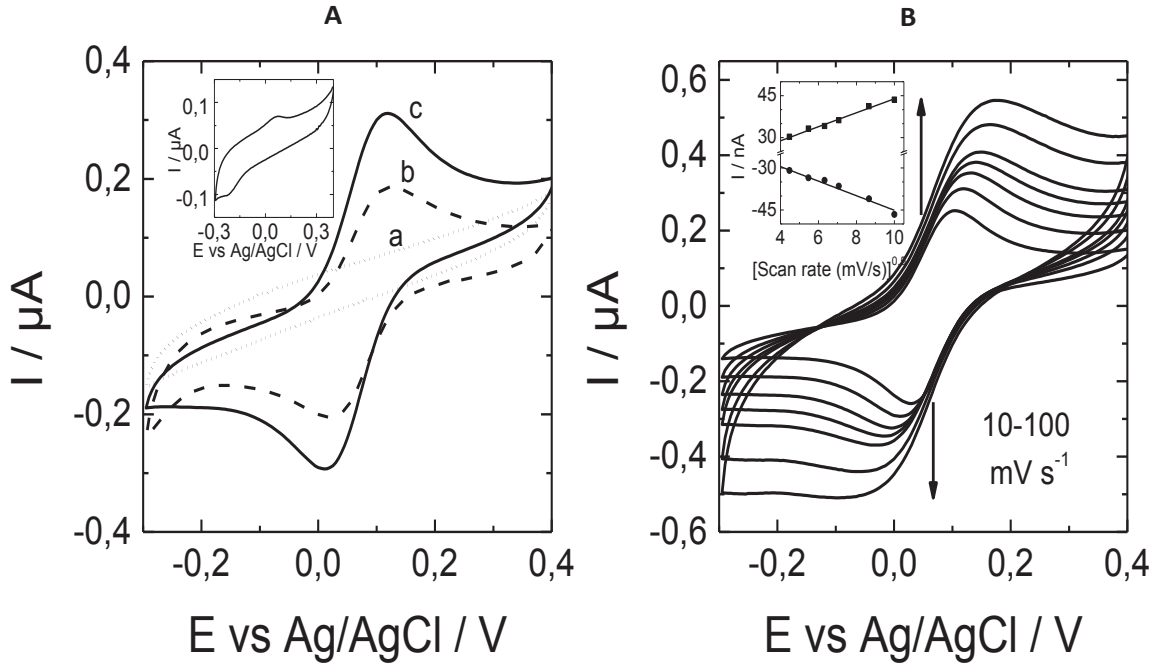

Fig III-7. *(A) Cyclic voltammograms for (a) bare GCE, (b) bovine heart cytochrome c in a solgel film on GCE, (c) cytochrome c and P. fluorescens in a sol-gel film on GCE. Inset: Cyclic voltammetric response of P. fluorescens alone in the sol-gel film. (B) Variation of the current response measured by cyclic voltammetry at different scan rates for a sol-gel film*

containing both cytochrome c and P. fluorescens. Inset: linear variation of the measured current versus the square root of scan rate.

The introduction of *P. fluorescens* in the film led to a slight increase in the redox current, without affecting significantly the electrochemical characteristics of cytochrome c signal. The electrochemical signal was measured by varying the potential scan rate as reported in Figure III-7B. The current intensity of both oxidation and reduction peaks varies linearly with the square root of scan rate in the range 10 to 100 mV s^{-1}, which is an indication of a diffusion limitation of the electron transfer reaction, as it could occur if electrons are hopping from cytochrome to cytochrome during the electrochemical reaction (Fig. III-7B). Using an electrode modified with *P. fluorescens* in absence of cytochrome c led to the observation of a small signal in the same potential region (anodic peak located at + 0.08 V versus Ag/AgCl) but more complex feature can also be observed at -0.20 V (see inset in Fig. III-7A). This signal could be assigned to redox proteins present in the bacterial membrane. The electrochemical comparison of *P. fluorescens* with *S. putrefaciens* species (anodic and cathodic peaks are located in the range of -0.1 to +0.1 V and -0.2 to -0.4 V respectively with respect to bacterial strains) [29,42] has demonstrated the compatibility between *P. fluorescens's* redox peaks and peaks related to membrane-bound cytochromes present in electroactive bacteria. These membrane-bound cytochromes are able to transfer electrons to the electrode surface and are probably involved in the electron transfer reactions to the cytochrome c introduced in the sol-gel film.

3.2. Feasibility of bacteria/protein communication in sol-gel

It is well known that viable cells in microbial biosensors and biofuel cells are likely to catalyze the oxidation of various substrates (such as glucose, lactose, methanol …etc.) producing electrons in a process called microbial respiration [2,43]. The role of artificial mediators is to enhance the ET between microbial cells and the electrode. In order to study the feasibility of bacteria / c-cytochrome ET the in sol-gel layer, amperometric measurements have been applied to detect the microbial communication with the electrode upon successive additions of glucose as a substrate for respiration (Fig. III-8). As shown in Fig. III-8A, cytochrome c alone was not able to generate any current response upon addition of glucose, as expected from the absence of bacterial respiration, while *P. fluorescens* CIP 69.13 alone have generated very small current response upon addition of glucose (Fig. III-8B). By contrast, *P.*

fluorescens CIP 69.13 encapsulated with bovine heart cytochrome c has shown much higher current responses upon successive additions of glucose (> 10 times that of bacteria alone, Fig. III-8C). This big difference of intensity between measurements reported in figures III-8B and III-8C can be explained by the critical role of cytochrome c mediator added into the film ensuring an electron relay between the microorganism and the electrode surface, while in the absence of this mediator, extracellular ET in sol-gel film only occurs for bacteria located very near to the electrode surface. The mediation with cytochrome c occurs probably via electron hopping between the heme centers of the proteins [44], which obviously improves the microbial electrochemical communication with the electrode. A direct relation was found to exist with respect to an increasing glucose concentration and current density, up to a maximal saturation value (see the inset in Fig. III-8C).

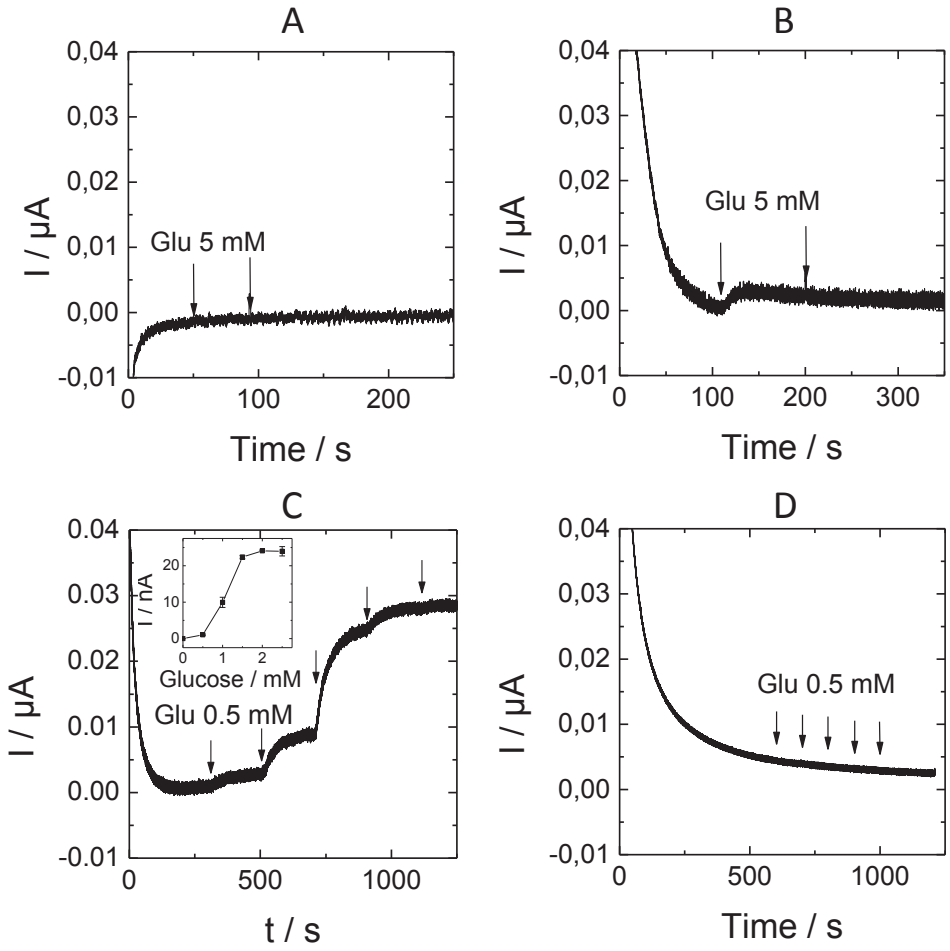

Fig III-8. *Amperometric current responses measured upon the addition of glucose using glassy carbon electrodes modified with sol-gel film containing (A) bovine heart cytochrome c, (B) P. fluorescens, (C) P. fluorescens with bovine heart cytochrome c and (D) E. coli with*

bovine heart cytochrome c. The measurements were performed under stirring in phosphate buffer (pH 7) at room temperature at an applied potential of + 0.15V versus Ag/AgCl reference electrode.

At the contrary, no electrochemical communication with *E. coli* c600 realized upon addition of glucose was observed (Fig. III-8D). The absence of electrochemical communication between bovine heart cytochrome c and *E. coli* c600 could be explained by the absence of extracellular ET between bacterial ET chain and cell wall for exporting the electrons outside the cell and shuttling them to the electrode surface. This control experiment confirms that the presence of bacterial cytochromes located between outer cell membrane and cell wall in the case of *P. fluorescens* [45] facilitated the extracellular ET and communication with the outer environment.

3.3. Communication disruption/competition

In order to further confirm the mechanism of microbial communication with the electrode, some toxic chemicals have been tested as inhibitors of ET between *P. fluorescens* and the electrode surface, either by killing bacteria [46] or inhibiting the cell respiration [45]. On the basis of amperometric measurement performed upon addition of NaClO (200 mg L^{-1}), it was shown that a rapid inhibition of the electrochemical communication occurred, with a decrease in the current response to approximately 80 % within 1 minute (Fig. III-9A).

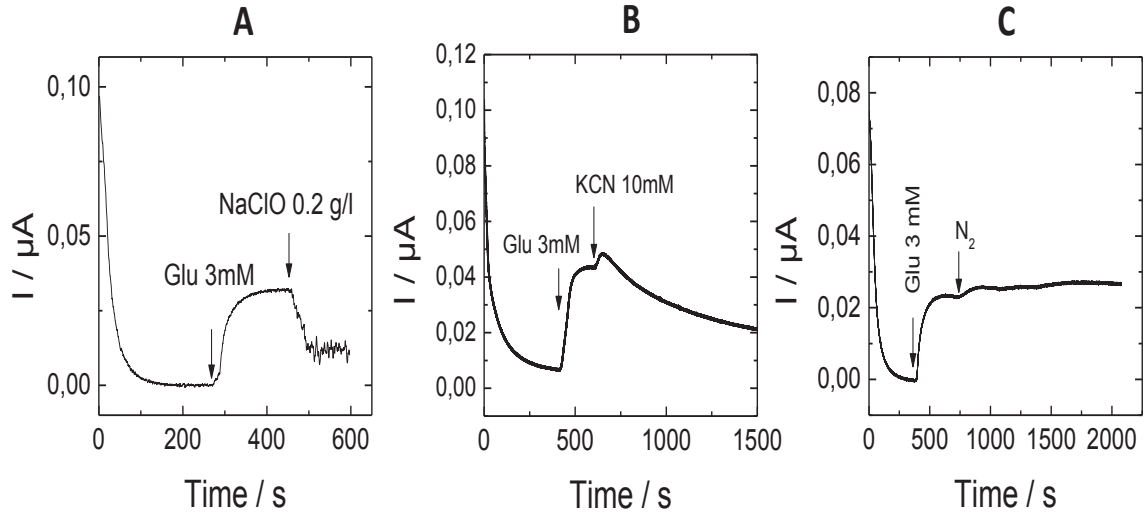

Fig III-9. *Amperometric current responses measured in the presence of 3 mM glucose with glassy carbon electrodes modified with sol-gel film containing P. fluorescens and bovine*

heart cytochrome c, upon the addition of (A) NaClO (200 mg L^{-1}), (B) KCN (10 mM) and (C) N_2. Other conditions are similar as in Figure III-8.

Potassium cyanide also caused rapid modification of the bacterial communication, but displaying a much more complex amperometric profile, characterized by a first small current increase and then followed by a continuous decrease in the measured currents (Fig. III-9B). In addition, investigating the effect of N_2 on the electrochemical communication of *P. fluorescens* and cytochrome c led to the observation of very small increase in the amperometric signal (by ca. less than 10 % of the total current response, see (Fig. III-9C) than in anaerobic medium. This experiment indicates that cytochrome c has good competition with O_2 as a final electron acceptor during electrochemical communication with the electrode surface.

3.4. Factors affecting the bioelectrochemical communication

For further optimization of the conditions to improve the electrochemical communication between *P. fluorescens* and bovine heart cytochrome c immobilized in sol-gel films, the effect of cytochrome c concentration, cell density, temperature, pH and applied potential have been investigated and studied via amperometric measurements (Fig. III-10).

Figure III-10A shows that increasing the cell density (from 4 to 9 x 10^9 cells mL^{-1}) encapsulated with constant concentration of cytochrome c generated higher current response. Fig. III-10B displays the electrochemical response of the biofilm as a function of the initial concentration of bovine heart cytochrome c (from 0.1 to 1.5 mM) in the sol used for electrode modification, as co-encapsulated with constant density of *P. fluorescens* [9 x 10^9 cells mL^{-1}]. The highest response was obtained with using 1 mM cytochrome c. The small decrease in the current response observed at higher concentration could be explained by some leaking of cytochrome c due to alteration of sol-gel film stability at this high protein concentration (indeed, too high contents of additives are expected to result in more open silica structures and, thereby, more easy leaching of entrapped species). Temperature variations from 20 to 35 °C resulted in significant variations in sensitivity (Fig. III-10C), with a maximum current response for the electrochemical communication obtained at 30 °C, which corresponds actually to the temperature used for growth of this bacterium. Finally, pH 7 was observed as optimal (Fig. III-10D) for this bioelectrode. The optimal pH and temperature are here in

coincidence with literature studying the electrochemical communication of *Pseudomonas* species in the presence of glucose as the substrate [5,27].

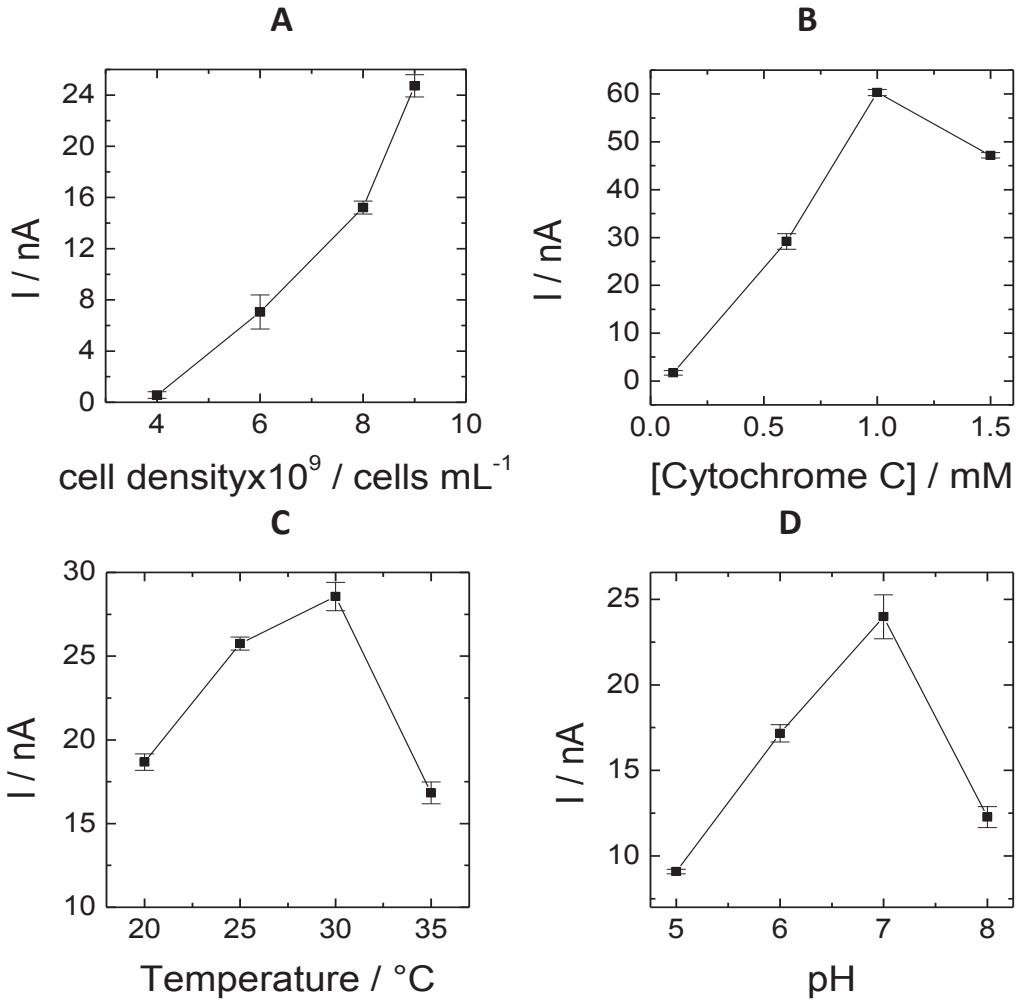

Fig III-10. *Amperometric current responses measured with glassy carbon electrodes modified with sol-gel film containing P. fluorescens and bovine heart cytochrome c with respect to (A) P. fluorescens cell density in the presence of 1mM c-based cytochrome, (B) cytochrome c concentration in the presence of P. fluorescens (9 x 10^9 cells mL-1 as initial cell density), (C) temperature and (D) pH of the medium. Other conditions are similar as in Figure III-8, with the exception of the parameters under study.*

The film thickness also influences the electrochemical response. Different aliquot volumes, *i.e.* 2, 4 or 6 µL of the sol (containing the silica precursors, the bacteria and the cytochrome c), have been drop-coated on the glassy carbon electrode. The electrodes have been then tested for the detection of 3 mM of glucose. In these conditions, the current varied linearly with the quantity of sol deposited on the glassy carbon electrode (Fig. III-11A). Films obtained after

gelification had thicknesses of several micrometers. The precise measurement of the film thickness was not possible because of the heterogeneity induced by the dryness of the drop on the electrode surface. However, it can be considered that the quantity of encapsulated bacteria (and cytochrome c) is directly proportional to the sol volume deposited onto the electrode surface. So, the proportional increase in the current response with increasing the quantity of sol-gel material strongly supports the argument for efficient ET at rather long distance through the sol-gel material due to the co-encapsulated cytochrome c. Not only the bacteria located at the sol-gel/electrode interface are transferring the electron to the glassy carbon electrode, but also those located inside the gel, far away from the electrode surface.

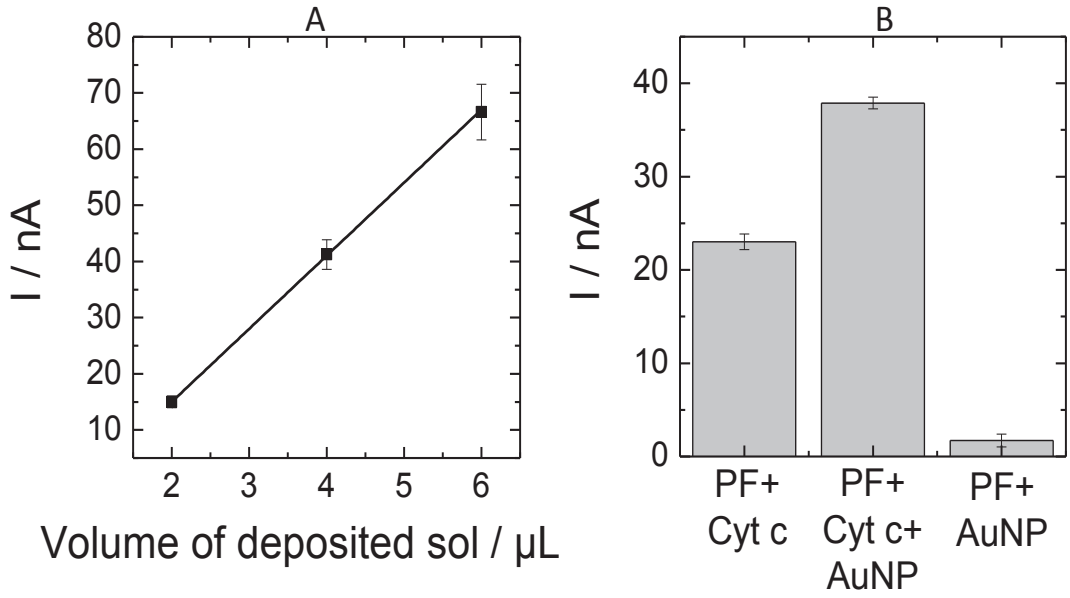

Fig III-11. *Influence of (A) the quantity of artificial biofilm and c-cytochrome (volume of deposited sol solution) and (B) the presence of gold nanoparticles in the biocomposite film on the electrochemical response of glassy carbon electrodes modified with sol-gel films containing P. fluorescens and bovine heart cytochrome c, upon the addition of 3 mM glucose as substrate. Other conditions are similar as in Figure III-8. (N.B: PF and AuNP are the abbreviations of P.fluorescens and gold nanoparticles respectively)*

In view of potential application in the biosensors field, efforts should be also made to increase the sensitivity of the bioelectrode response. In this context, it was checked if the electron transfer pathway between *P. fluorescens*, cytochrome c and glassy carbon electrode surface can be further improved by the introduction of gold nanoparticles in the hybrid sol-gel layer. In the presence of gold nanoparticles (*i.e.* 0.04 mg mL^{-1} in the starting sol), the electrochemical response was doubled (Fig. III-11B). However, the introduction of more

nanoparticles, i.e., 0.08 mg mL^{-1} in the sol, led to a significant decrease in the electrochemical responses. These limitations could be related to toxicity to biological life or to modification in the composite structure and hence charge transfer properties could lower the current values [28]. In attempting to circumvent this limitation, the incorporation of multi-walled carbon nanotubes (instead of gold nanoparticles) was also evaluated as an alternative way to improving electrochemical communication between the encapsulated bacteria and the electrode (using 0.4 and 0.8 mg mL^{-1} in the sol), but no significant enhancement of current responses was observed in these conditions. As a final control experiment, the introduction of gold nanoparticles in absence of added cytochrome c in the gel was showing only a small current response, which confirms the importance of cytochrome c as electron shuttle between *P. fluorescens* and the electrode surface in the construction of the artificial biofilm for electrochemical biosensing. The sensitivity of the bioelectrode was found to be 0.38×10^{-3} mA $mM^{-1}cm^{-2}$ glucose in the presence of the optimal concentration of gold nanoparticles. Note that the concept of artificial biofilm was reported only recently in the literature, with planktonic bacteria wrapped with graphite fiber and encapsulated in polypyrrole [16] and biofilm covered with sol-gel layer [47]. Both considered the facilitated EC between bacteria and the electrode surface by accumulation of flavin mediator in the polypyrrole or sol-gel layer, respectively. The strategy proposed in this thesis is original, providing a new approach for the elaboration of artificial biofilms to be applied in electrochemical studies or devices.

4. Conclusion

The poor electrochemical communication between bacteria encapsulated in a silica-based sol-gel film and a glassy carbon electrode can be increased with using soluble mediator $Fe(CN_6^{3-})$, or ferrocene mediator chemically linked to single walled carbon nanotubes. This “chemical” strategy, as tested with *Shewanella putrefaciens*, leads to rather comparable results, which would be indicative of similar mechanism involved with these two different mediators. The interpretation of these results, in agreement with other reports from the literature, can be that the mediators are able to enter the outer membrane of the bacteria, through the porins, to collect electrons [48]. Another strategy, of “biological” nature, involves cytochrome c that can be co-immobilized with the bacteria in the silica-based gel. However, cytochrome cannot enter the outer membrane of the bacteria and only collect the electron from the outer

membrane cytochrome, leading to a different behavior, by comparison with chemical mediators, both in term of sensitivity and range of response to the addition of electron donor.

The application of cytochrome c for electron transfer from bacteria to the electrode was then studied systematically with *Pseudomonas fluoresecens*. The resulting systems could be considered as an artificial biofilm, with the silica matrix mimicking the exopolymers involved in natural biofilms, but not only. Here the strategy involved to increase the electron transfer rate is also mimicking a strategy involved in natural biofilms, in which cytochrome can be expressed to relay the electron from the bacteria to a final acceptor, this latter being a mineral or the electrode of a microbial fuel cell. The system display high competition against O_2 as electron acceptor, and displayed clear response to the environment as demonstrated by the dependent relationship of current with glucose concentration. The introduction of gold nanoparticles has slightly enhanced the electron transfer reaction between the electrode and the bacteria/cytochrome c composite. This concept of artificial biofilm is probably an important topic to be developed in future works for application as biosensors or bioreactors.

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Chapter IV. Sol-gel encapsulation of membrane-associated proteins for bioelectrochemical applications

In this chapter, the work focuses on the immobilization of membrane-associated proteins in the hybrid sol-gel matrix for bioelectrochemical applications. According to the literature, the sol-gel material could provide a suitable environment for safe immobilization of these proteins, which would be beneficial for bioelectrocatalysis. The bioencapsulation process was achieved via drop-coating of the starting sol from aqueous silicate including enzymes. PEG has been introduced to design a protective environment for encapsulation of the membrane fragments. Two different proteins have been chosen for this study, *i.e.*, cytochrome P450 (CYP1A2) according to its ability to directly transfer electrons to the electrode surface and L-Mandelate dehydrogenase that can transfer electron only through electrochemical mediators. The biohybrid materials developed could be further used for construction of reagentless bioelectrochemical devices such as biosensors and bioreactors.

1. Introduction

Technological advancements in immobilization of biological molecules over several decades has resulted in a revolution of biodevices for the selective extraction, delivery, separation, conversion and detection of a wide range of chemical and biochemical reagents. The use of biological molecule such as proteins, peptides and nucleic acids in these applications relies largely on the successful immobilization of the biomolecule in a physiologically active form [1]. In aqueous solutions, biological molecules such as enzyme lose their catalytic activity rapidly [2]. For that, the utilization of immobilized enzymes has gained more considerable attentions as an active sensing element for fabrication of biosensors due to their stable inherent properties such as specific recognition and catalytic activity [1,2].

In general, the key challenge of immobilization techniques is the stabilization of enzymes without altering its catalytic activity. The limitation of adsorption techniques is the weakness of bonding, thus adsorbed enzymes lack the degree of stabilization and leak easily into the solution. On the other hand, the strengthening of the bonds between support and enzyme by chemical linkage could inactivate or reduce most of the enzymatic activity [2]. For these reasons, the physical entrapment of enzyme in a porous carrier such as sol–gel materials has drawn great interest in recent years. This is due to its simplicity of encapsulation process, chemical inertness and biocompatibility of silica, tunable porosity, mechanical stability, and negligible swelling behavior [2,3]. Sol-gel encapsulation approach has succeeded to maintain the enzymatic activity to fabricate reagent less biosensors and bioreactors [4–8]. A diversity of works has been briefly reported about sol-gel encapsulation of enzymes as soluble protein but a limited works on the membrane-associated proteins.

Membrane-associated proteins are either completely (intrinsic membrane proteins) or partially (extrinsic membrane proteins) embedded within an amphiphilic bilayer lipid membrane (BLM). Thus it is important to retain at least the essential bound lipids, and often the entire BLM, to keep the membrane-associated protein properly folded and functional. This makes such species particularly difficult to immobilize by conventional methods and highlights the need for protein-compatible immobilization methods [1,9]. Some of membrane-associated proteins have been successfully encapsulated in sol-gel materials, as reported for photoactive membrane proteins [10], membrane-bound receptors [11], two-ligand channel receptor [12] and cytochromes P450 [13,14]. The use of polyols such as glycerol or polyethylene glycol

with sol-gel materials have been proven to be highly beneficial for stabilization of entrapped membrane proteins [1]. The goal of this study is to evaluate the interest of such approaches for application of membrane-associated proteins in bioelectrochemical processes, with the goal to immobilize these proteins in an active form on the surface of electrodes. Two systems have been considered. First, cytochrome P450 was immobilized in a sol-gel layer on pyrolytic graphite electrodes, with the idea to favor and keep direct electron transfer reactions between the electrode and the protein (this work was done in collaboration with the Groupe of GM. Almeida, Université de Lisbonne). The second system was the Mandelate dehydrogenase, isolated as vesicle from *Pseudomonas putida*. This protein was kindly provided by Gert Kohring(Saarland University, Sarrebruck, Allemgane). Direct electron transfer reactions are not possible with this protein and had to be wired using mediators, either soluble or immobilized on the electrode surface.

2. Direct communication of membrane-associated cytochrome P450

The basis of third generation biosensors is the direct communication between electrodes and proteins especially those containing heme groups such as cytochromes [15]. For biosensors based on DET, the absence of mediator is the main advantage to provide a superior selectivity, operate in a potential window closer to the redox potential of the enzyme itself, and therefore, less prone to interfering reactions [16].

Cytochromes P450 belong to the group of external monooxygenases [17,18]. In nature, the iron heme cyt P450s utilize oxygen and electrons delivered from NADPH by a reductase enzyme to oxidize substrates stereo- and regioselectively. Significant research has been directed toward achieving these events electrochemically [19]. Improving the electrochemical performance of cytochrome P450 enzymes is highly desirable due to their versatility in the recognition of different biological and xenobiotic compounds for application as biosensors [20], and biocatalysis [21,22]. Interfacing these enzymes to electrode surfaces and electrochemically driving their catalytic cycle has proven to be very difficult [23]. The protein was immobilized directly on electrode surface with taking advantage of electrostatic interactions on graphite electrode or by covalent linkage on gold surfaces, in lipid membranes, or using additional additives, i.e., surfactants, polymers or nanoparticles [23].

The application of sol-gel chemistry for the immobilization of CYP has been rarely considered. One report by Iwuoha et al. described the immobilization of P450cam in a complex hybrid matrix prepared with methyltriethoxysilane in the presence of didodecyldimethyl ammonium bromide (DDAB) vesicles, bovine serum albumin (BSA) and glutaradhyde (GA) [13]. Direct electron transfer reactions between the electrode surface and the immobilized are described and applied for camphor or pyrene oxidation and an improved stability is discussed, considering the application in organic solvent. However DDAB is already known for stabilizing P450 proteins and the critical role of the inorganic precursor is not clearly demonstrated in this complex matrix including BSA and GA. Another report by Waibel et al. described screen-printed bienzymatic sensor based on sol-gel immobilized acetylcholinesterase and a cytochrome P450 BM-3 (CYP102-A1) mutant [14]. P450 was stabilized in the sol-gel matrix and applied for the catalytic oxidation of parathion in the presence of NADPH and O_2. The resulting molecule, i.e. paraoxon, could inhibit the enzymatic activity of acetylcholine esterase that was electrochemically monitored. Different protocols for sol-gel immobilization were described, but no tentative of direct electron transfer reactions were considered with the immobilized CYP.

This work was performed by Patrícia Rodrigues as cooperation with Maria Gabriela Almeida (*Chemistry Department. Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa)* under our co-supervisions. It will report the immobilization of cytochrome P450 1A2 (CYP1A2) in sol-gel thin films prepared from water-based precursor in the presence of polyethylene glycol (PEG) with a special focus on direct electron transfer reaction. The use of sodium silicate prevents the introduction of alcohol in the starting sol while PEG improves the stabilization of the membrane proteins. The direct electron transfer reaction was characterized electrochemically in the absence and in the presence of O_2.

2.1. Critical role of the sol-gel matrix for direct electron transfer electrochemistry

Figure IV-1A shows the electrochemical response of CYP simply adsorbed on pyrolytic graphite electrode (PGE) surface. A small signal could be observed for some experiments, corresponding to the DET from the electrode to the CYP protein. This signal was not systematically observed but sometimes observed as a very small intensity. This is probably

due to the small amount of protein presenting a good orientation and conformation DET reaction. PEG which is known as stabilizer for electrochemistry of P450 at high temperature [24] was tested here to immobilize CYP1A2 (Fig. IV-1B). However, no signal was observed in these conditions. The immobilization in silicate has pointed a slight improvement in the measured electrochemical signal (Fig. IV-1C).

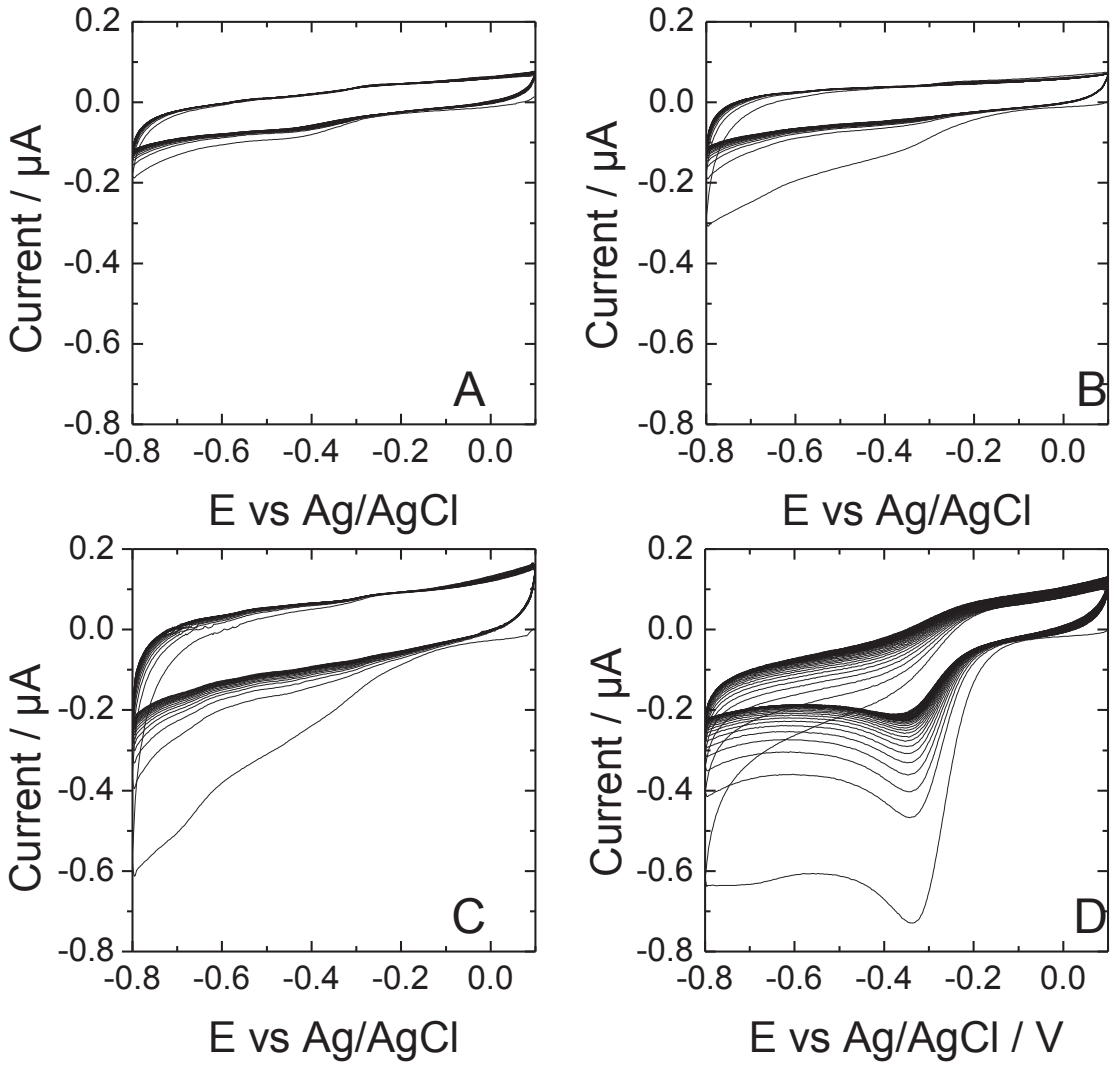

Fig IV-1. *Electrochemical responses of CYP1A2 (A) simply adsorbed on PGE surface, (B) immobilized in PEG400, (C) in sodium silicate and (D) in a hybrid sol-gel matrix made of sodium silicate and PEG400. All measurements have done in 50 mM phosphate buffer at pH7 after degassing the solution for 20 min with Argon. The 40 successive scans have been performed at 50mV/s.*

But, only the immobilization in hybrid gel containing both PEG and silicate led to a well-defined redox peak at -0.335 V versus Ag/AgCl (Fig. IV-1D). This redox signal decreased

rapidly by a factor of free during the first minutes of experiments before to be stabilized at a peak current of -0.2 µA. It is supposed that weakly immobilized proteins are first leaching out from the film and only the stably immobilized proteins are remaining entrapped in the hybrid sol-gel film for a long time of experiment. This set of experiment clearly show that neither PEG nor silicate are able to immobilize CYP1A2 in an electrochemically active form and only the synergetic effect from their combination allows to express the redox active of the membrane-bound protein.

The electrochemistry of sol-gel encapsulated CYP1A2 was studied by changing systematically the potential scan rate from 5 to 100 mV s^{-1} as reported in Figure IV-2. For all experiments, the redox peaks were irreversible as only the reduction peaks could be observed. The current intensity of this redox signal increased linearly with the square root of the scan rate which is indicative of a diffusion limitation. It indicates that electron transfer from cytochrome to cytochrome can be considered.

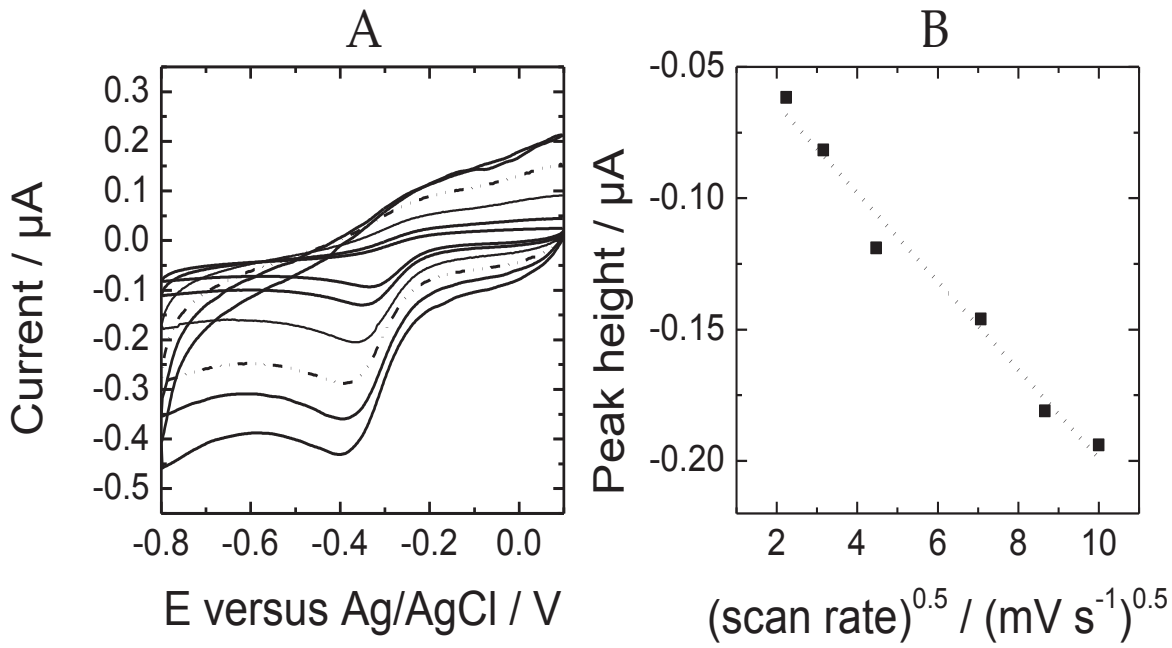

Fig IV-2. *(A) Cyclic voltammetric characterization of CYP1A2 electrochemistry in sodium silicate – PEG400 hybrid matrix at different potential scan rate: 5 mV s^{-1}, 10 mV s^{-1}, 20 mV s^{-1}, 50 mV s^{-1}, 75 mV s^{-1}, 100 mV s^{-1}. (B) Linear relationship between the peak current intensity and the square root of scan rate. All measurements have been done in 50 mM phosphate buffer at pH 7 after degassing the solution for 20 min with Argon.*

2.2. Electrocatalytic reduction of O_2

CYP are very sensitive to oxygen that can be used to evaluate the DET reactions [25]. Figure IV-3A reports the electrochemical response of CYP1A2 immobilized in the sol-gel-PEG hybrid film to increasing amounts of oxygen. The experiment was initiated in oxygen free solution, deareted with argon. Then, small volume of air saturated solution was introduced into the cell, leading to a gradual increase in the electrocatalytic response of the P450 protein due to oxygen reduction. The mechanism of oxygen reduction with CYP can involve the production of H_2O_2 species with detrimental effect on the protein activity [19]. This is verified here by increasing more the concentration of oxygen in the solution, by equilibration with the atmosphere (Fig. IV-3B). The electrochemical response was measured for the first cyclic voltammogram was well defined with peak current intensity higher than 8 µA and maximum peak potential at -0.355 V vs Ag/AgCl. However continuous cyclic at 50 mV s^{-1} leads to a continuous degradation both in peak current intensity and peak potential. The last CV measured here was indeed located at -700 mV vs Ag/AgCl and displayed only 3 µA intensity.

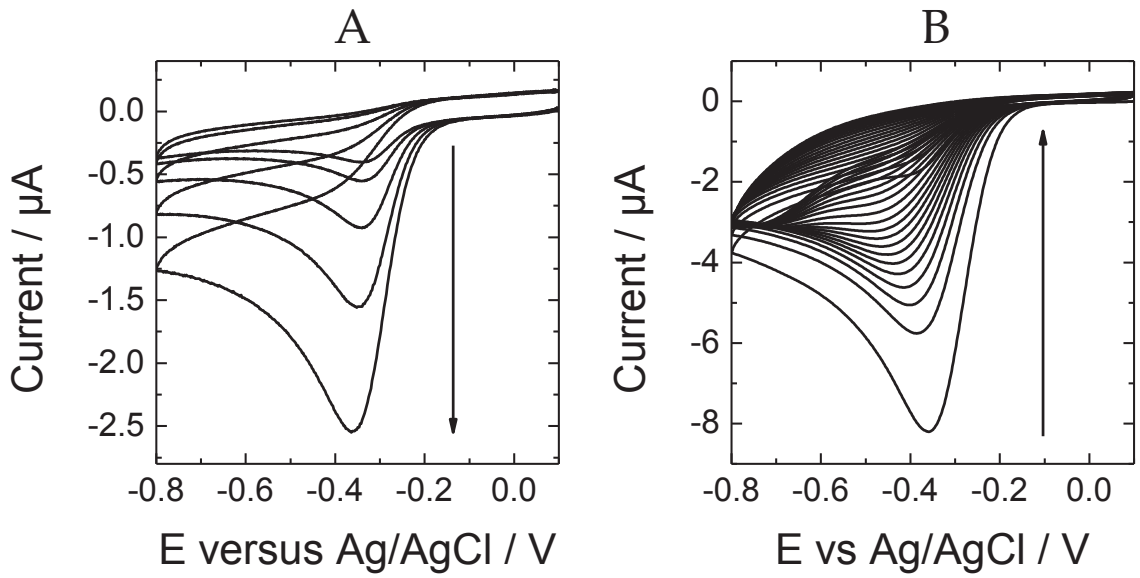

Fig IV-3. *(A) Influence of O_2 addition on the electrochemical response of CYP1A2 immobilized in sodium silicate – PEG400 hybrid sol-gel matrix. Detrimental influence of higher concentration of O_2 on the direct electron transfer reaction. All measurements have been done in 50 mM phosphate buffer at pH 7 after degassing the solution for 20 min with Argon. Potential scan rate was 50 mV s^{-1}.*

To resume, the direct electrochemistry of P450 proteins in water-based sol-gel thin film is described here for the first time. The electrochemistry of the heme center and the electrocatalytic reaction with O_2 is greatly improved in the presence of PEG. A careful comparison of the film composition shows that only the combination of the inorganic matrix and the PEG additive allows the observation of DET reaction and the electrocatalytic activity for immobilized P450.

A second system has been thus studied here, involving another membrane protein, i.e. L-Mandelate dehydrogenase. The possible immobilization of the vesicle containing the proteins in the silica-based layer developed in this section was first considered in order to evaluate the interest of this approach to stabilize the electrochemical response of this protein, whose response is based on mediated electron transfer.

3. Mediated communication of membrane-associated L-mandelate dehydrogenase

L-Mandelate dehydrogenase (ManDH) could be applied for the production of enantiopure α-hydroxy acids as educts for the preparation of semi synthetic antibiotics [26,27]. Their catalytic activity is expressed by several proteins, which can be soluble or membrane bound and use different co-factors like nicotine amide dinucleotide (NAD^+) or flavine mononucleotide (FMN). The Uniprot databank lists 63 ManDH related proteins from bacteria and fungi but there are many more α-hydroxy acid dehydrogenases expressing mandelate oxidizing or benzoylfomate reducing activity. Here we chose L-ManDH from *Pseudomonas putida* (EC 1.1.99.31) encoded in the mdlB gene as a membrane bound FMN-dependent protein [28]. The enzyme has been cloned and heterologously expressed in *Escherichia coli* as active catalysts and it was characterized in detail [29–31]. The enzymatic activity, determined by reduction of 2,6-dichlorophenol indophenols, could be demonstrated with S-ManDH bound to membrane vesicles.

In the case of mediated communication, redox mediators shuttle electrons between the active side of enzymes, *i.e.* containing the FMN cofactor, and the electrode. The main advantages of employing mediated electron transfer (MET) within a biosensor device are that the electron transfer process is independent of the presence of natural electron acceptors or donors [32].

Most of dehydrogenases are communicating directly with the external mediator instead of the electrode surface. As mentioned above, they are interesting enzymes for electrosynthesis applications, especially for the production of rare sugars as building blocks for pharmaceutical and food industry [33]. One of the main requirements for electrosynthesis is the stable immobilization of a large amount of active proteins on the electrode surface of the reactor. A huge diversity of soluble dehydrogenases have been encapsulated in sol-gel materials without altering their enzymatic activity and stability [2,34]. However, the sol-gel encapsulation of membrane-associated dehydrogenase has not been reported yet. All the membrane-associated dehydrogenases have been studied in literature depending on simple adsorption on the electrode surface for bioelectrochemical biosensors [35–37].

3.1. Feasibility of the L-mandelate dehydrogenase in a hybrid SiO_2/PEG film

The sol-gel used for the encapsulation of L-mandelate dehydrogenase have been prepared according the protocol optimized previously for encapsulation of cytochrome P450 studied in section A (SiO_2 55mM/ PEG 6wt % - pH 7). L-mandelate dehydrogenase activity in sol-gel film has been measured in presence of ferrocene dimethanol (FDM) as a soluble mediator in buffer medium using electrochemical measurements (Fig. IV-4).

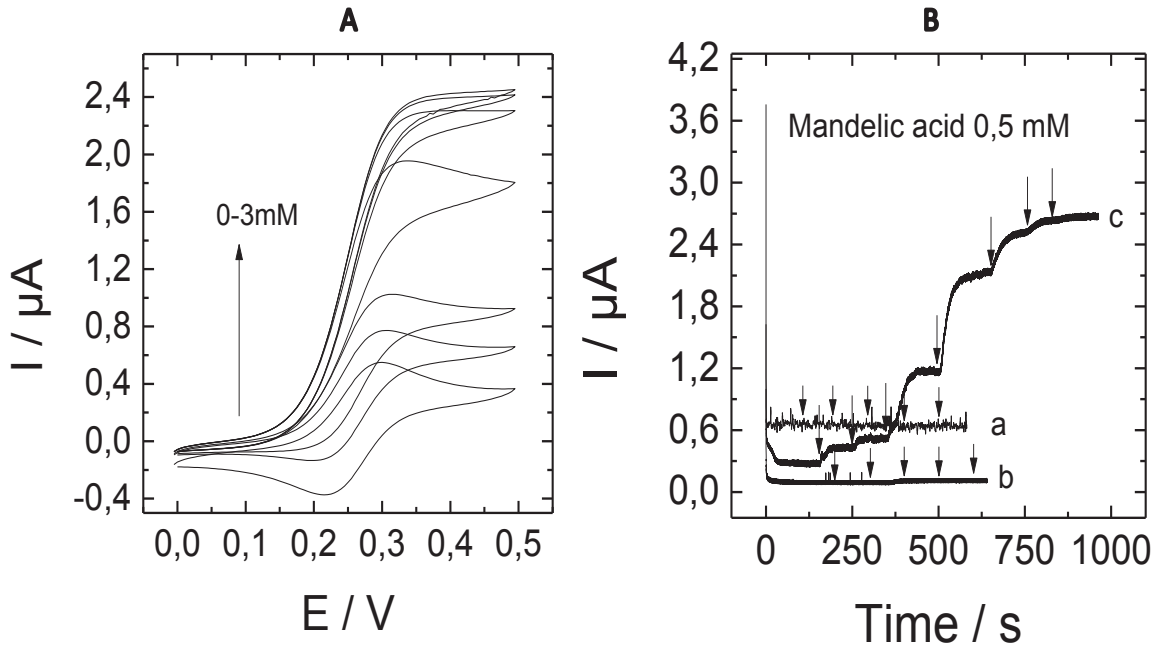

Fig IV-4. *(A) Cyclic voltammetry for a sol-gel film containing L-mandelate dehydrogenase. The measurement was performed in phosphate buffer (pH 7) and FDM (0.1 mM) at room temperature. Potential scan rate was 20mVs^{-1} (B) Amperometric measurements for (a) bare GCE, (b) L-mandelate dehydrogenase in a hybrid sol-gel film on GCE in absence of FDM, (c) L-mandelate dehydrogenase in a hybrid sol-gel film on GCE. The measurements were performed under stirring in 50 mM phosphate buffer (pH 7) and FDM (0.1 mM) at room temperature by applying a constant potential of + 0.3V versus Ag/AgCl reference electrode.*

The CV measured in the absence of mandelic acid only show the typical electrochemical response of the ferrocene mediator, whose signal is controlled by diffusion into the sol-gel film (Fig. IV-4A). The addition of mandelic acid, from 0.5 to 3 mM leads to a gradual increase in the anodic signal concomitant with a decrease of the cathodic signal, which is the signature of an electrocatalytic process. This electrocatalytic response was further tested under hydrodynamic conditions, using the chronoamperometric measurement by applying 0.3 V at the working electrode (Fig. IV-4B). Under these conditions, the bioelectrode modified with L-ManDH vesicles in sol-gel led to a gradual increase of the measured current upon additions of increasing amount of mandelic acid, in agreement with the CV experiments (curve "c" in Fig. IV-4B). Control experiments on bare glassy carbon electrode (curve "a" in Fig. IV-4B) and with GCE modified with ManDH vesicle in sol-gel, but in the absence of electrochemical mediator in solution (curve "b" in Fig. IV-4B), confirms that the electrochemical response was due to the enzymatic activity, and that it could be observed only in the presence of mediator.

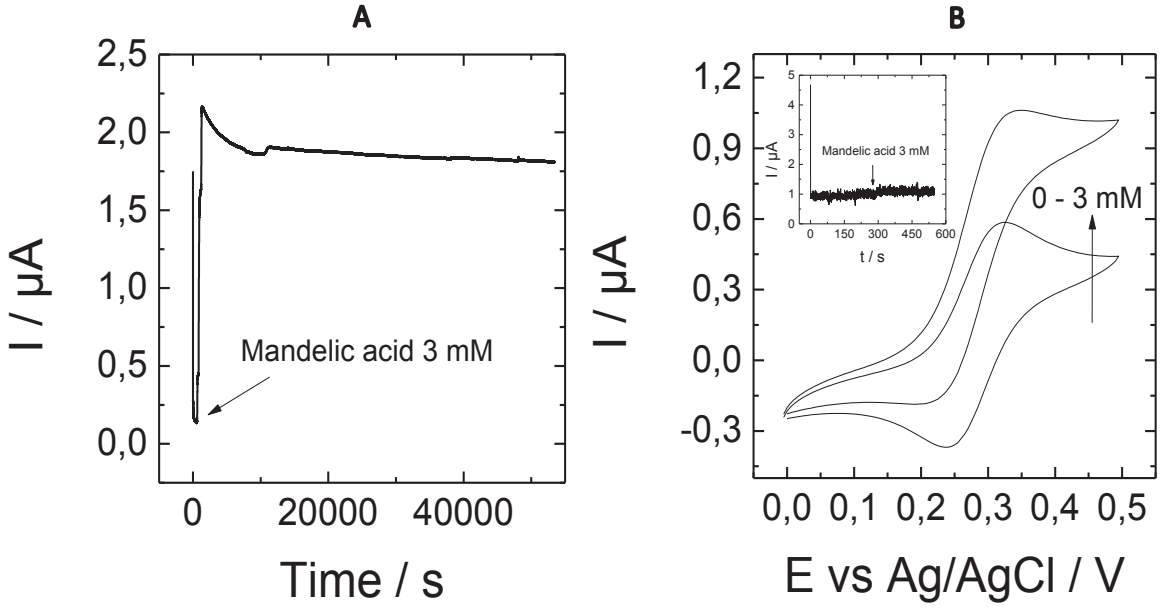

Fig IV-5. *A) Amperometric measurement for L-mandelate dehydrogenase in a hybrid sol-gel film on GCE. B) Cyclic voltammetry for a L-mandelate dehydrogenase simple adsorbed on GCE without gel. The measurement was performed in phosphate buffer (pH 7) and FDM (0.1 mM) at room temperature. Potential scan rate was 20mVs^{-1}. (Inset) Amperometric measurement for L-mandelate dehydrogenase simply adsorbed on GCE without gel. Amperometric measurements were performed under stirring in 50 mM phosphate buffer (pH 7) and FDM (0.1 mM) at room temperature by applying a constant potential of + 0.3V versus Ag/AgCl reference electrode.*

Additionally, few tests of stability in presence and absence of hybrid sol-gel were done under hydrodynamic conditions, showing the electrochemical response was rather stable for at least 15 hours in the presence of 3 mM mandelic acid (Fig. IV-5A). Only a slight decrease in the measured current was observed. For comparison, the simple adsorption of ManDH GCE without any sol-gel protection has shown a very small catalytic response on CV measurements (Fig. IV-5B). The chronoamperometric measurement made under convection by applying 0.3 V at the working electrode didnot show significant response, probably because of the leaching of ManDH from the electrode surface (see inset in Fig. IV-5B). These results confirm the importance of sol-gel materials to maintain the stability of catalytic reactions and maximize the catalytic response by stabilizing the enzymes on the electrode surface.

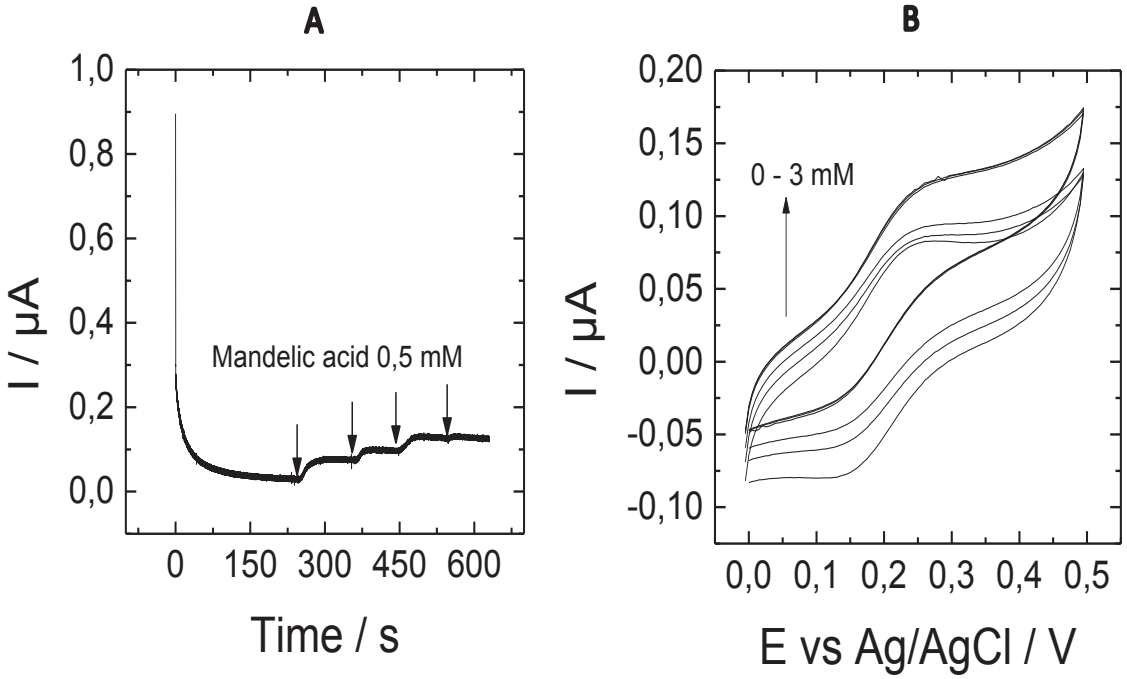

Fig IV-6. *(A) Amperometric measurement for a hybrid sol-gel film containing L-mandelate dehydrogenase and MWCNT-Os on GCE The measurements were performed under stirring*

in 50 mM phosphate buffer (pH 7) at room temperature by applying a constant potential of + 0.3V versus Ag/AgCl reference electrode. (B) Cyclic voltammetry for a sol-gel film containing L-mandelate dehydrogenase and MWCNT-Os on GCE. The measurement was performed in phosphate buffer (pH 7 at room temperature. Potential scan rate was 20mVs^{-1}.

A last set of experiment was done to evaluate the possibility to co-immobilize the ManDH vesicle and osmium polymer for reaching a reagentless configuration. Here a sol-gel layer containing both CNT wrapped with osmium and the ManDH has been deposited on GCE. The amperometric measurement and cyclic voltammetry measurement (Fig. IV-6) have shown good electrocatalytic responses of encapsulated L-ManDH with osmium polymer wrapped multi-walled carbon nanotube (MWCNT-Os) upon successive additions of 0.5 mM L-mandalic acid. The reactivity and current response were lower than soluble mediator as expected from higher facility of soluble mediators to reach enzymatic active sites than immobilized ones. These results could open an interesting window for fabrication of reagentless electrochemical devices based on membrane-associated proteins.

3.2. Encpasulation of L-ManDH using other sol-gel protocols

The experiment made in the presence of P45O have shown that some component like PEG could be necessary for the stabilization of the proteins or to favor a conformation suitable for direct electron transfer reaction. Two different conditions have been tested, 1) the same sol as in section 3.1, but in the absence of PEG and 2) a sol based on TEOS, prepared at slightly acidic pH (5) and in which a small amount of EtOH was produced during hydrolysis reactions (Fig. IV-7).

Surprisingly, all systems led to a quite comparable response, showing increasing current upon addition of increasing amount of mandelic acid, measured by CV or chronoamperometry under hydrodynamic conditions. The vesicle as prepared from *E. coli* is rather robust and was efficiently encapsulated using these different protocols. The better stability of ManDH response compared to P450 could be due to the lower requirement for MET by comparison with DET. Indeed, DET requires a suitable conformation to be achieved by the protein on the electrode surface in order to operate the final electron transfer reaction. At the opposite, in MET mechanism, the mediator can diffuse through the gel to the protein located in the vesicle. Here the only requirement is that the vesicle should not be dissolved in the sol-gel environment, which is obviously the case.

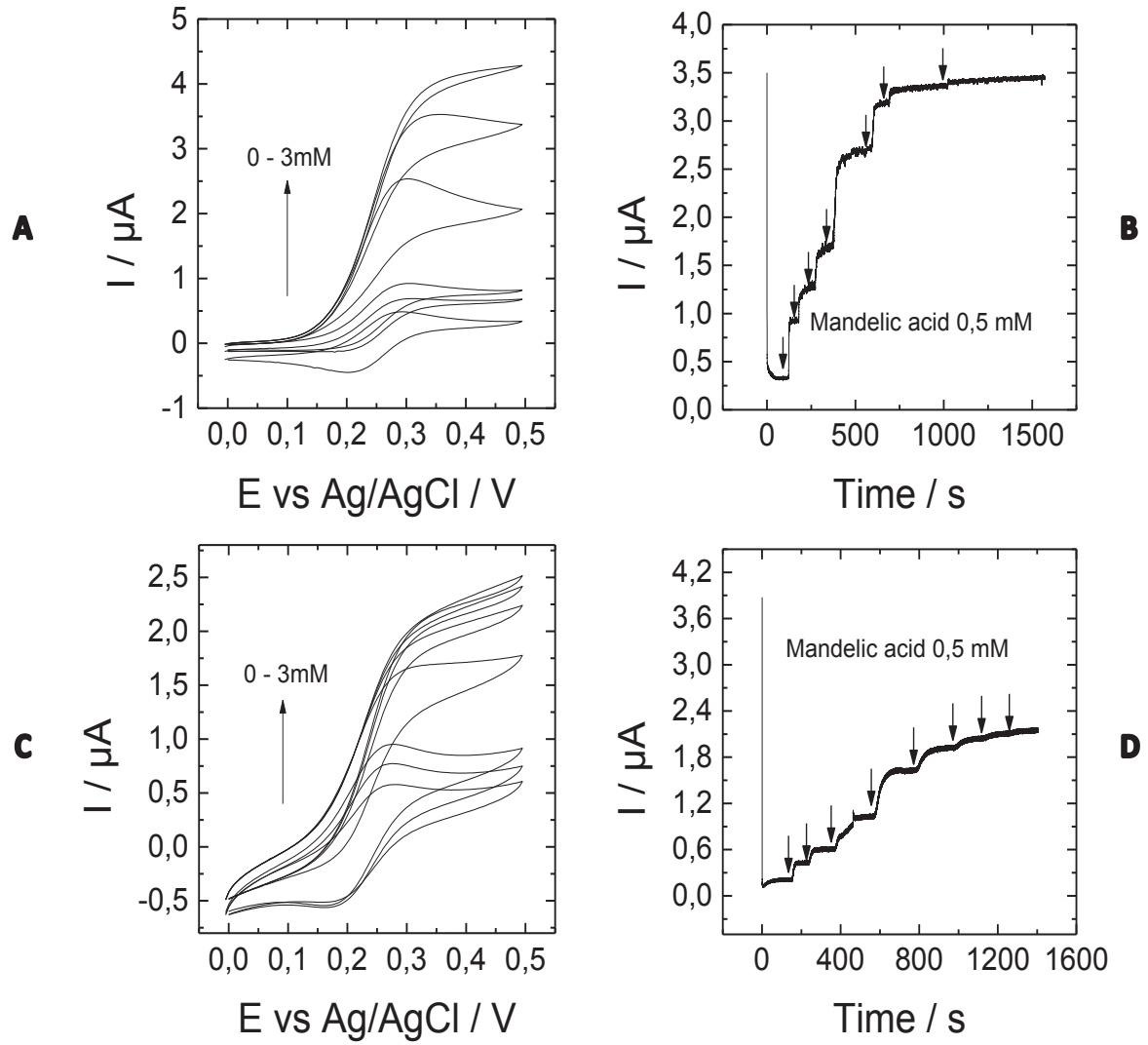

Fig IV-7. *Cyclic voltammetry for L-mandelate dehydrogenase in (A) SiO_2 (55mM – pH 7) and (C) TEOS (0.25 M - pH 5). The measurements were performed in phosphate buffer (pH 7) and FDM (0.1 mM) at room temperature. Potential scan rate was 20mVs^{-1}. Amperometric measurement for L-mandelate dehydrogenase in (B) SiO_2 (55mM – pH 7) and (D) TEOS (0.25 M - pH 5). The measurements were performed under stirring in 50 mM phosphate buffer (pH 7) and FDM (0.1 mM) at room temperature by applying a constant potential of + 0.3V versus Ag/AgCl reference electrode.*

4. Conclusion

The electrochemistry of membrane-associated proteins can be promoted in sol-gel environment. Direct electron transfer (DET) has been found more sensitive than mediated electron transfer (MET) reaction. DET requires the proteins to be located at close distance to

the electrode surface in a conformation that is suitable to operate this transfer of electron. We suppose that a fraction of the total P450 CYP1A2 proteins introduced in the sol-gel material are not able to react with the electrode and only those located at the film/electrode interface leads to a measured current. Nethertheless the sol-gel material and the presence of PEG clearly allowed the electrocatalytic signal for oxygen reduction. Further experiment should involve electrochemical detection of other substrate of P450 proteins, e.g. propanolol or paracetamol.

Mediated electron transfer does not display the same requirements. Proteins located far from the electrode surface can transfer their electrons to the electrode if the mediator can diffuse in the silica gel to the redox center of L-Mandelate Dehydrogenase to react with the FMN cofactor before to react again with the electrode. The immobilization of vesicle with ManDH was found rather insensitive to the sol-gel route used for immobilization on the electrode surface. The benefit of encapsulation has been shown for electrochemical measurement under convection. The electrochemical response kept stable for at least 15 hours, while no response was observed with the vesicle simply dropped on the glassy carbon electrode surface. These experiments were all developed with using a mediator in solution, i.e. ferrocenedimethanol. We could finally show that the mediator, in this case an osmium polymer wrapped on carbon nanotubes, could be co-immobilized with the protein in a reagentless configuration. Further studies could involve the implementation of such biohybrid materials in electrochemical reactors for electroenzymatic synthesis.

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Chapter V. Encapsulation of infectious bacteriophage in a hybrid sol-gel monolith

The effect of encapsulation of bacteriophage ΦX174 in silica-based sol-gel matrices on the infectivity has been studied. The purpose of this study is to investigate some strategies to protect bacteriophage against deactivation. The encapsulation was achieved via aqueous-based sol-gel, similar as those used in chapter 3 and 4, but the protocol here is applied for the preparation of small monoliths. The materials were aged, and then introduced into water for release of the virus and assessment of the infectivity. Organic and polymeric additives have been introduced to design a protective environment for encapsulation of sensitive bacteriophage. The efficiency of sol-gel encapsulation has been evaluated by studying the viral infectivity as a function of lysis plaques in the presence of *Escherichia coli*.

1. Introduction

Bacteriophages have been discovered by Felix d'Herelle in 1917. They have the ability to infect selectively target bacteria, allowing, e.g., the detection and control of foodborne diseases [1,2]. They can also find application in biosensing, as molecular biology tools or for drug delivery [3].

Upon infection of a host bacterium, a bacteriophage utilizes the cell machinery for amplification of new virions, thereby causing the death of the host bacterium. Bacteriophages can be produced in large quantities, they display a better stability than antibodies and they are able to recognize only living bacteria [2]. The research on bacteriophage as antibacterial agent was important before the discovery of antibiotics, but then decreased, being active only in few locations, notably in Georgia. The recent emergence of bacterial strain that developed resistances to existing antibiotics treatments has refocused the research on this biological antibacterial treatment.

To maintain the infectious character of virus is also crucial when considering their use in medical applications [4]. Bacteriophages can be produced, concentrated and stored in aqueous solutions at low temperature, showing in these conditions slow decay of the infectivity with the time. Immobilization in a protective matrix is another approach that could be used to maintain the infectivity of the virus in conditions that would be suitable for specific applications [3].

As discussed in previous chapters, sol-gel encapsulation technology has already been successfully used to preserve the viability and activity of many encapsulated biomolecules such as vegetal cells [5], enzyme [6,7], bacteria [8,9], algae [10], yeast [11]. Several example of encapsulation of viruses in sol-gel matrix can be found in the literature, with the goal to use this biological entity as a template during the gelification of the inorganic matrix [12,13], but it is important to consider that these studies did not consider the infectious character of the immobilized virus. Only recently, this approach was used to encapsulate adenovirus with the goal to extend the release time from implants [4]. One has to note that in this example the gel containing the virus was stored in aqueous solution before implantation in mice, whose conditions are favorable for keeping high ratio of infectious virus.

Actually, the influence of sol-gel encapsulation on the infectivity of bacteriophage has not been reported. Elaboration of hybrid materials allowing the preservation of this infectivity would be of great interest for development of antibacterial coating or to control the release of the bacteriophage for biomedical purposes, enhancing the persistence of the phage at the site to be treated.

The aim of this work is to study the influence of encapsulation in inorganic matrix on the biological activity of bacteriophage, considering both wet and dried gel. Bacteriophage ΦX174 was chosen as model system. It is a virus isometric about 25 nm in diameter, having single-stranded circular DNA and icosahedral shape of 20 triangular faces and 12 vertices (Fig. V-1) [14]. It is capable to infect a number of enterobacteria strains such as *Shigella sonnei*, *Salmonella typhimurium Ra*, or *E. coli* C by recognition of its target cells through lipopolysaccharides (LPS) present on the bacterial membrane [15,16].

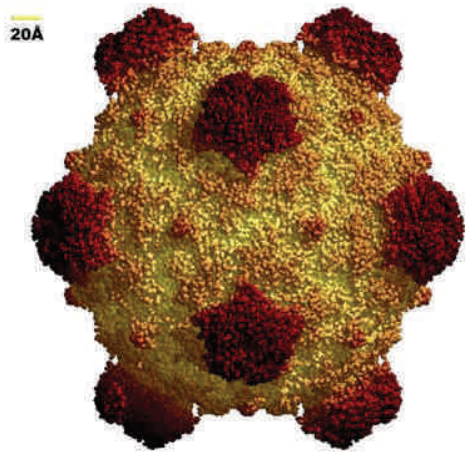

***Fig V-1**. Representation of bactériophage ΦX174 - Image de Jean-Yves Sgro ©2004 virology.wisc.edu/virus world*

The approach involves the polymerization of aqueous silicate monomers in the presence of silica nanoparticles for encapsulation of bacteriophage in stable bio-composite matrix. Various additives were introduced in the sol with the goal to improve the protection of the immobilized bacteriophage, especially after drying of the gel. After aging, the gel was reintroduced in solution for phage release and the infectivity of ΦX174 has been analyzed.

2. Assessment of viral infectivity

2.1. Principle of infectivity determination for encapsulated bacteriophage

The infectivity analysis of encapsulated ΦX174 has been tested from single-agar-layer plaque assay [17]. The protocol is reported in Figure V-2. First, ΦX174 was encapsulated in sol-gel monolith and aged at +4 °C. Then the monolith containing bacteriophage was dissolved in water to release free ΦX174 followed by entrapping together with *E. coli* CN in agar plate and incubation overnight at 37 °C to allow the interactions between ΦX174 and *E. coli* and lysis plaques formation in the agar plate.

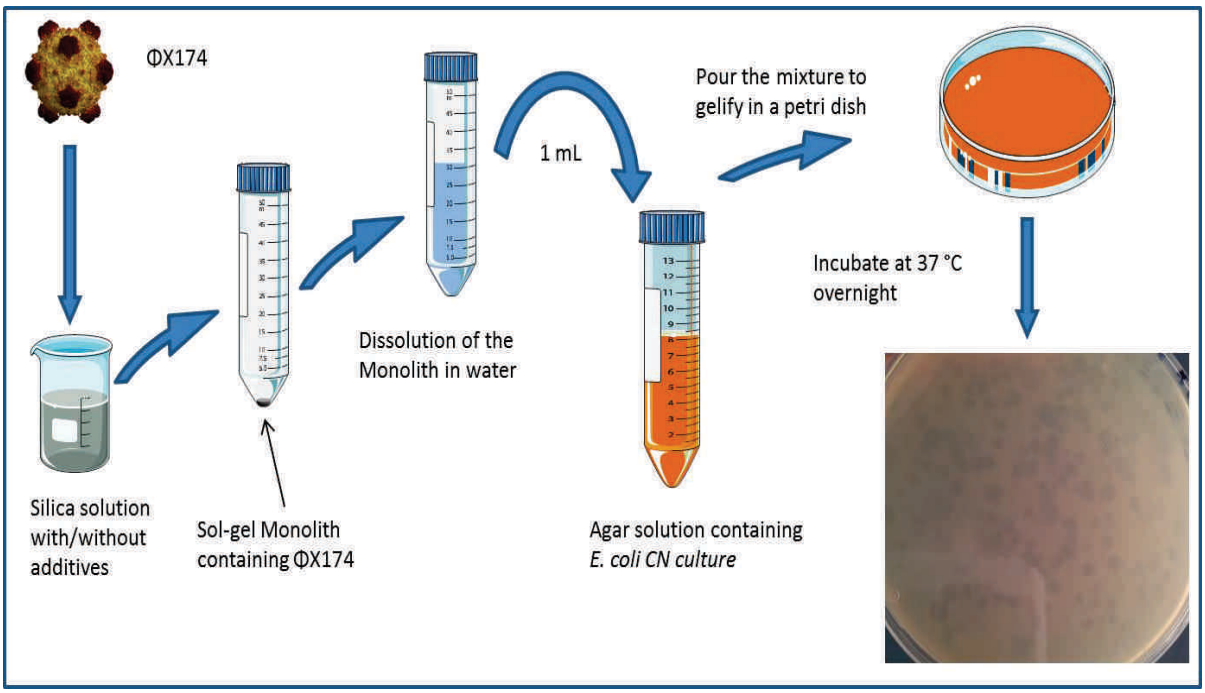

***Fig V-2**. Schematic representation of bactériophage ΦX174 encapsulation and determination of its infectivity against E. coli CN.*

As shown on the picture reported in Figure V-2, lysis plaques can be counted, this parameter being used to compare the influence of aging, and the impact of additives on viral infectivity after release in solution (units forming plaque per mL of solution, UFP mL^{-1}).

2.2. Encapsulated in semi-dry silica monolith

The compatibility of the aqueous sol-gel route (sodium silicate solution and Ludox nanoparticles), i.e. not involving alcohol [18], for bacteriophage encapsulation was first studied by dispersion of the bacteriophage in saturated solution of sodium silicate, pH 7. The

infectivity was then tested for 6 days (Fig. V-3). The infectivity of ΦX174 against bacteria remained approximately constant (250-300 UFP mL^{-1}) during this time, which confirm the sol was harmless on the viral infectivity and could be applied for encapsulation of bacteriophages.

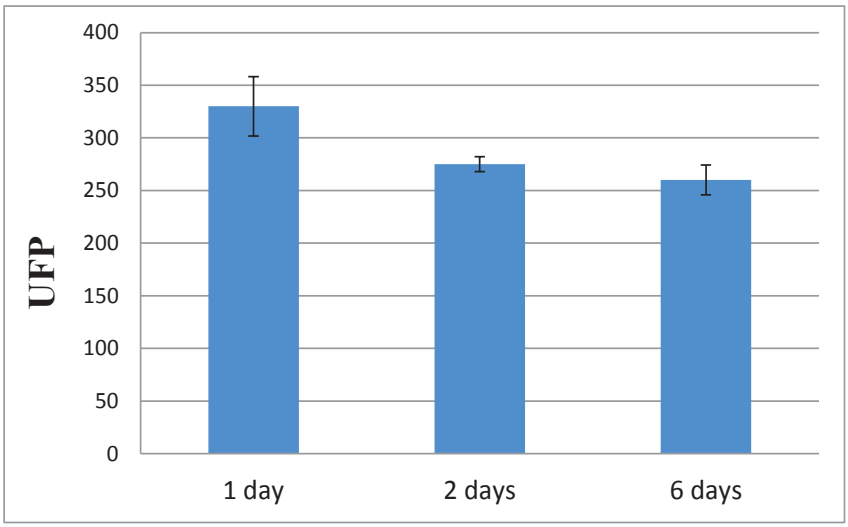

***Fig V-3**. Assessment of bacteriophage ΦX174 infectivity along 6 days of dispersion in saturated solution of sodium silicate.*

Bacteriophages ΦX174 have been encapsulated in sol-gel monoliths (thickness > 1mm) and then stored at 4 °C in petri-dish covered with a lid in order to avoid, in this first set of experiments, the complete drying of the gel. Some additives have been introduced so as to protect the bacteriophage against constrains during sol-gel transition and aging phases and to evaluate their influence on the viral infectivity after release in solution. Fig V-4 reports the effect of encapsulation in pure and hybrid sol-gel on preservation of infectivity against bacteria along 6 days. The infectivity of ΦX174 encapsulated in pure sol-gel (no additives) has decreased from 180 UFP mL^{-1} (initial value) to 13 UFP mL^{-1} and 1 UFP mL^{-1} after 1 and 6 days respectively; this rapid deactivation of viral infectivity during 6 days could be explained by capsid protein denaturation during sol-gel transition and aging phases or, this cannot be totally excluded, from a somehow limited release in solution. At first, we made the hypothesis that deactivation was mainly observed during the encapsulation in the gel. In order to improve the protection against the rapid viral deactivation from sol-gel matrix, trehalose and glycerol which are known in literature as microorganism's stabilizer against sol-gel constraints and aging stresses [19–21] have been introduced into the sol-gel material. The infectivity of ΦX174 encapsulated in hybrid sol-gel containing either glycerol or trehalose or

both additives has decreased from 180 UFP mL^{-1} into 4 UFP mL^{-1}, 3 UFP mL^{-1} and 4 UFP mL^{-1} respectively after 6 days at +4 °C. The introduction of these additives in the gel has thus only a limited impact on protection against rapid deactivation of viral infectivity during 1 week. Finally, the influence of polyelectrolytes added into the gel film has been tested. The infectivity of ΦX174 encapsulated in hybrid sol-gel containing either Poly(ethylene imine) (PEI) or Poly(diallyldimethylammonium chloride) (PDDA) has decreased from 180 UFP mL^{-1} into 10 UFP mL^{-1} and 1 UFP mL^{-1} respectively after 6 days at +4 °C. The introduction of PEI to the sol-gel has apparently improved the protection against rapid deactivation of viral infectivity during 1 week while PDDA did not. This improvement of PEI could be explained by the favorable electrostatic and H-bonds interactions between PEI and bacteriophage capsides, protecting the bacteriophage from surrounding sol-gel materials as reported before for some redox proteins [19].

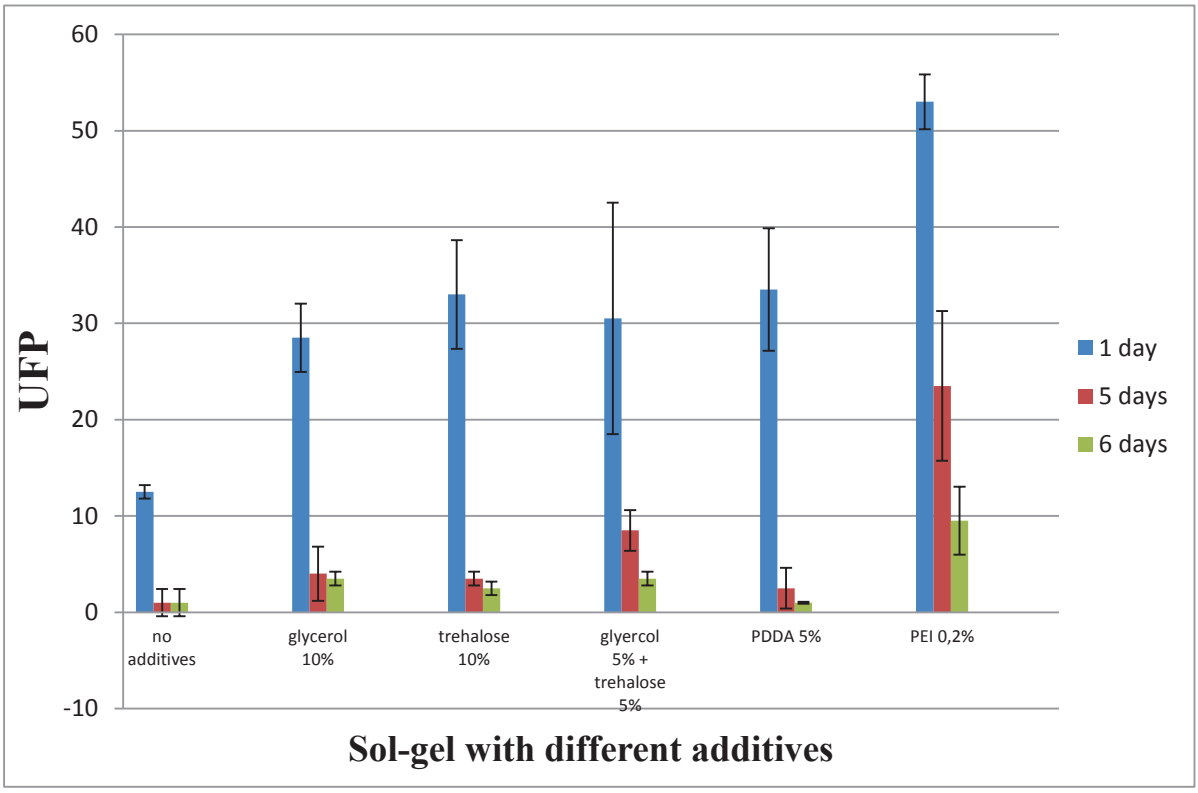

***Fig V-4**. Assessment of bacteriophage ΦX174 infectivity along 6 days of encapsulation in hybrid sol-gel monolith and storage in semi-dry environment.*

2.3. Encapsulated in dried silica monolith

In a second set of experiments, bacteriophage ΦX174 has been encapsulated in a monolith of hybrid sol-gel and stored at 4°C for 6 days in petri-dish without lid, allowing a complete

drying of the material. In this experiment, higher concentration of ΦX174 (1800 UFP mL^{-1}) has been encapsulated for better quantification of viral infectivity (Fig. V-5). As it can be observed, only the sol-gel materials prepared in the presence of PEI (359 UFP mL^{-1}), glycerol (55 UFP mL^{-1}) and mixture of glycerol and trehalose (23 UFP mL^{-1}) have maintained some viral infectivity in harsh dry state, the higher infectivity being observed in the presence of PEI.

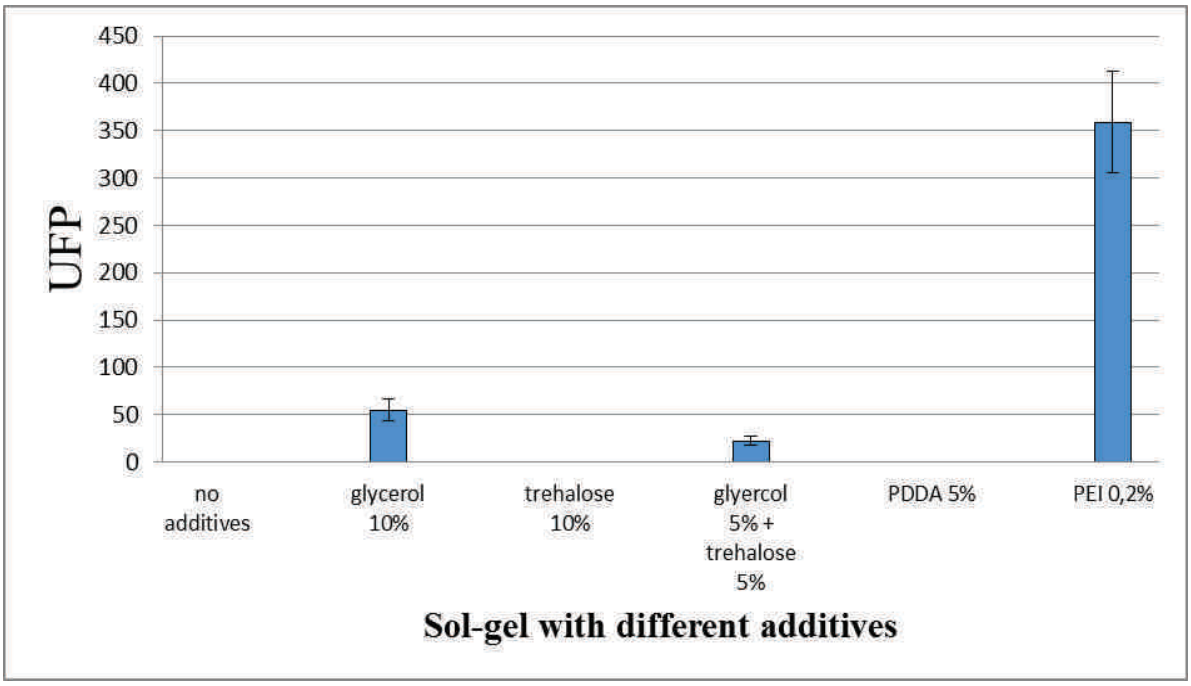

***Fig V-5**. Assessment of bacteriophage ΦX174 infectivity along 6 days of encapsulation in hybrid sol-gel monolith and storage in harsh-dry environment.*

TEM analysis of the resulting has been performed in order to evaluate the influence of the additive of the gel texture, but no obvious influence could be observed as the texture of the material was dominated by the silica nanoparticles of Ludox (Fig. V-6). No bacteriophage could be observed due to their relatively low concentration.

In the present state of our investigation, we first suppose that the beneficial influence of PEI and glycerol on the bacteriophage activity is due to interactions established between the additives and the viral capside, preventing complete deactivation in the harsh conditions of the study. Note that the composition of the gel could also affect the liberation of bacteriophages from entrapped into free active state. To the best of our knowledge, this work

represents the first systematic investigation on the chemical environment that could favor the better infectivity of bacteriophage encapsulated in inorganic sol-gel materials.

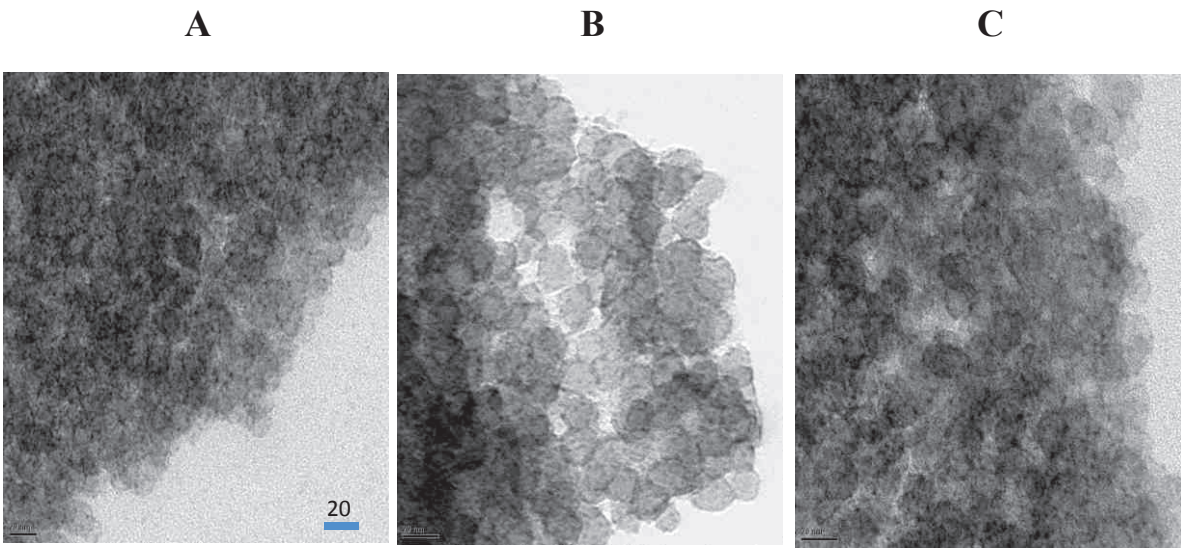

***Fig V-5**. Representative transmission electron micrographs (TEM) for A) gel made with sodium silicate and Ludox nanoparticles, B) with glycerol and C) with PEI as additives. Same scale for all figures is 20 nm.*

3. Conclusion

The bacteriophage ΦX174 has been used as a model for a systematic investigation on the conditions to favor infectivity after encapsulation in sol-gel inorganic material. The encapsulation process has been performed by mixing a suspension of the bacteriophage in the starting sol followed by rapid gelification. The presence of glycerol and polyethyleneimine was proved to be essential to modify significantly the viral infectivity against bacteria in dry environment for 6 days, the better protection being obtained with the polyelectrolyte. The favorable electrostatic and H-bonds interactions between bacteriophage and the polymer could explain this better resistance to inactivation.. This work could provide more opportunities for applications of immobilized bacteriophage in antibacterial protection and in medicine.

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Conclusion et perspectives

Le sujet principal de cette thèse concernait l'immobilisation et la caractérisation de l'activité de bactérie dans des films de silice préparés par le procédé sol-gel. L'électrochimie a été utilisée avec succès pour induire l'immobilisation de *Escherichia coli* dans un films sol-gel hybride en utilisant l'électrolyse contrôlée du sol initial contenant les précurseurs de silice, le poly(éthylène glycol) du chitosan et du tréhalose. Les bactéries étaient soit adsorbées à la surface de l'électrode avant le dépôt sol-gel soit codéposées pendant l'électrolyse. Nous avons montré que l'intégrité membranaire était conservée pendant plus d'un mois et que les bactéries immobilisées pouvaient exprimer de la luminescence ou de la fluorescence en réponse à leur environnement.

L'électrochimie a ensuite été utilisée pour caractériser la communication électronique avec *Shewanella Putrefaciens* et *Pseudomonas fluorescens* en présence de donneurs d'électrons, respectivement le formiate de sodium et le glucose. Ces deux souches bactériennes sont capables de transférer des électrons par leur membrane externe. Mais l'immobilisation de ces bactéries limite fortement les transferts électroniques de la bactérie à l'électrode en raison de la nature isolante de la matrice de silice. Deux stratégies ont été proposées afin d'augmenter ces processus de transfert électronique dans le gel, en utilisant des nanotubes de carbone fonctionnalisés par des groupements ferrocène ou par co-immobilisation dans le gel avec les bactéries d'un médiateur naturel, le cytochrome c. Ces deux approches ont conduit à des résultats sensiblement différents du point de vue de leur sensibilité à l'ajout d'un substrat en raison des mécanismes impliqués. En effet, le ferrocène est susceptible de traverser la membrane externe, par exemple au travers des porins, pour collecter les électrons de la respiration alors que le cytochrome ne peut collecter que les électrons transmis au travers de la membrane externe. L'utilisation du cytochrome c au sein de la matrice de silice qui permet d'immobiliser les bactéries mime dans une certaine mesure une stratégie développée dans certains biofilms naturels pour favoriser le transfert électronique vers un accepteur final, minéral ou électrode. Il est ainsi possible de considérer que notre approche conduit à la formation d'un biofilm artificiel.

L'expérience développée sur l'immobilisation de bactéries a ensuite été appliquée à l'immobilisation de protéines membranaires for la bioélectrochimie. Deux types de protéines rédox ont été étudiées, un cytochrome P450 (CYP1A2) et une mandélate déshydrogénase (ManDH). Pour ces deux systèmes, une activité électrocatalytique a pu être mesurée après

encapsulation dans un film sol-gel à la surface de l'électrode, par transfert direct d'électron (P450 - CYP1A2) ou transfert médié (ManDH). Nous avons pu observer que le cytochrome était plus sensible que la déshydrogénase, ce résultat pouvant s'expliquer par la nature directe du transfert d'électron entre l'électrode et la protéine. Cependant, pour ces deux systèmes, l'immobilisation dans un film sol-gel a permis d'augmenter l'intensité et la stabilité de la réponse électrocatalytique.

Finalement, les études préliminaires sur l'encapsulation du bacteriophage ΦX174 au sein d'une matrice sol-gel hybride a permis de montrer que l'infectivité du virus pouvait être augmentée par la modification judicieuse du matériaux. La présence de glycérol ou de polyéthyleneimine a ainsi permis de protéger cette infectivité dans une atmosphère sèche pendant plus de 6 jours, la meilleure infectivité étant observée en présence de polyélectrolyte. L'interaction favorable entre le bactériophage chargé négativement et le polymère chargé positivement pourrait expliquer, au moins en partie, cette meilleure résistance à l'inactivation. Ce travail pourrait procurer de nouvelles opportunités d'application pour les bactériophages immobilisés pour la lutte contre les souches bactériennes résistantes aux antibiotiques ou en médecine.

Dans une tentative de proposer des perspectives à cette thèse, nous pouvons tout d'abord dire que de nombreux systèmes différents ont été étudiés dans cette thèse, chacun conduisant à imaginer de nouvelles directions de recherche. Ces développements potentiels concernent l'application de l'encapsulation bactérienne par assistance électrochimique, l'exploitation des biofilms artificiels, l'application des protéines membranaires immobilisées pour l'électrocatalyse et de nouveaux développements avec les bactériophages.

Le lecteur aura certainement noté que le protocole d'encapsulation bactérienne par assistance électrochimique développé au chapitre 2 n'avait pas été appliqué aux études décrites au chapitre 3. Ces deux études ont en effet été menées en parallèle et le lien entre les deux travaux n'a pu être fait ici. Une difficulté est que la composition du film adaptée pour l'électrodépôt est différente de celle utilisée pour les études de communication électronique. Il sera alors nécessaire de faire de nouvelles optimisations pour appliquer cette approche originale à l'électrodépôt de biofilm artificiel applicable en électrochimie.

Les biofilms artificiel semblent être une voie de recherche intéressante pour l'élaboration contrôlée de bioélectrodes à base de bactérie. Cette approche a ses limites dans la mesure ou

l'encapsulation empêche la bactérie ou le biofilm de croître naturellement. Cependant elle a aussi ces qualités. Elle permet d'abord un très bon contrôle de la souche bactérienne présente dans le biofilm, ce qui permettrait de mener des études fondamentales dans lesquelles plusieurs souches bactériennes serait associées d'une façon bien contrôlée afin d'évaluer leur synergie dans une configuration biofilm. La croissance d'un biofilm peut demander beaucoup de temps. Le biofilm artificiel est une stratégie intéressante pour élaborer rapidement des dispositifs microbiologiques pour des applications électrochimiques telles que les biocapteurs ou les bioréacteurs.

L'immobilisation des protéines rédox membranaires dans un matériau sol-gel est une voie intéressante pour augmenter la stabilité de leur réponse électrochimique, celle-ci pouvant alors être testée en réacteur électrochimique pour l'électrosynthèse enzymatique. Le sol pourrait être optimisé pour augmenter autant que possible la stabilité de la réponse électrocatalytique sans perturber l'activité enzymatique. D'autres protéines membranaires présentent un intérêt pour la conversion d'énergie, telle que les hydrogénases. Il serait intéressant de considérer l'intérêt de la voie sol-gel pour l'immobilisation de cette dernière classe de protéines.

Enfin, les études sur l'encapsulation des bactériophages pour contrôler leur relargage sous une forme infectieuse n'en est qu'à ses débuts. Il conviendrait d'étudier plus en détails les paramètres qui influencent à la fois la libération des batériophages et le maintient de leur infectivité. Des études futures pourraient considérer l'application de ce type de matériau sous la forme de film pour le relargage contrôlé de cet agent antibactérien.

Conclusion and perspective

The main topic of this thesis was related to the immobilization and the characterization of activity of bacteria in silica-based sol-gel films. The electrochemistry was successfully used to induce the immobilization of *Escherichia coli strains* in a hybrid sol-gel layer by using the controlled electrolysis of the starting sol containing the silica precursors, polyethylene glycol, chitosan and trehalose. Bacteria were either adsorbed on the electrode surface before sol-gel electrodeposition or codeposited during the electrolysis. We could show that the membrane integrity of the bacteria was kept for more than one month and that bacteria were able to express luminescence or fluorescence in response to their environment.

Electrochemistry was then considered for the communication with *Shewanella putrefaciens* and *Pseudomonas fluorescens* strains in the presence of sodium formate and glucose as electron donor, respectively. Both strains are able to transfer electrons from their outer membrane. The immobilization of these bacteria strongly limits the electron transfer reactions from the bacteria to the electrode surface because of the insulating character of silica matrix. Strategies have proposed to increase this electron transfer pathway in the gel, using either immobilized ferrocene mediators on carbon nanotubes or by co-immobilization of a natural mediator, i.e. cytochrome c, in the silica gel including bacteria. Both strategies lead to very different behaviors from the point view of sensitivity to the addition of the substrate, as ferrocene could be able to cross the outer membrane of the bacteria, through the porins, and to collect electrons in the inter-membrane space. At the opposite cytochrome c only collects electrons from the outer membrane. Implication of cytochrome c within silica based gel entrapping bacteria mimics in some respects the strategies developed in natural biofilms to favor electron exchanges with a solid substrate, mineral or electrode and can this system can be defined for this reason as being an artificial biofilm.

The experience developed on immobilization of bacteria was further applied to the immobilization of membrane associated proteins for Bioelectrochemistry. Two different proteins have been evaluated, P450 (CYP1A2) and L-Mandelate dehydrogenase (ManDH). Both systems display electrochemical activity after immobilization, by direct electron transfer and mediated electron transfer, respectively. We observed that the electrochemistry P450 (CYP1A2) was more sensitive to the sol-gel environment than L-ManDH, whose result can be explained the nature of the electron transfer reaction between the protein and the electrode.

Nevertheless, in both systems, the immobilization in sol-gel matrix was improving the intensity and the stability of the electrocatalytic response.

Finally, the some preliminary studies on encapsulation of bacteriophage in hybrid sol-gel inorganic material have shown that the infectivity of this virus could be improved by careful control of the material composition. The presence of glycerol and polyethyleneimine was proved to be essential to modify significantly the viral infectivity against bacteria in dry environment for 6 days, the better protection and/or release being obtained with the polyelectrolyte. The favorable interaction between negatively charged bacteriophage and the positively charged polymer could partially explain this better efficiency (resistance to inactivation). This work could provide more opportunities for applications of immobilized bacteriophage in antibacterial protection and in medicine.

In a tentative to propose some perspectives to this thesis, we can first comment that many different systems have been studied in this thesis, each of them providing new opportunities of continuation. These futures developments concern the application of electrochemically assisted bacteria encapsulation, future development on artificial biofilm, application of immobilized membrane associated proteins and new development on bacteriophages.

The reader have certainly noticed that electrochemically assisted bacterial encapsulation developed in chapter II was not applied to electrochemical studied as reported in chapter III. These two studies have been developed in parallel and the link between these two different approaches was not done here. The difficulty is that the film composition suitable for electrodeposition was different to the one used in electrochemical communication studies, so optimization are necessary for application of electrodeposited biohybrid films to electrochemical devices.

Artificial biofilm appears as a promising route for controlled elaboration of bioelectrode. Such concept as some drawback, as the immobilization hinders the natural growing of bacteria. However it provides some qualities. It allows the careful control of the bacterial strain; it would allow fundamental studies in which several strains would be mixed together in a controlled manner in order to study their synergy for bioelectrochemical reactions. Biofilm growth needs time; artificial biofilm would be a valuable strategy for the rapid elaboration of microbial devices for electrochemical application such as biosensors or bioreactors.

Immobilization of membrane associated proteins in sol-gel materials could be a valuable approach for increasing the stability of the electrochemical response, which could be further tested in electrochemical reactor for electroenzymatic synthesis. The sol could be further optimized for improving as much as possible the stability of the electrocatalysis without affecting the enzymatic activity. Other membrane proteins are of interest for energy conversion, like hydrogenase. It could be of interest to consider the interest of sol-gel for immobilization of this later class of proteins.

Finally, studies on bacteriophage immobilization in sol-gel materials for controlled release are in their infancy. Additional studies should consider more deeply the effect of additives on the release of liberated infective bacteriophage from silica matrix and the effect of their concentrations on the viral infectivity. Further studies could consider the potential application of such biomaterial in the form of form for controlled released as antibacterial treatment.

Methods and Techniques

To conduct the work presented in this thesis, several chemicals and techniques have been used to prepare and characterize the active film. This chapter presents the description of sol-gel precursors, polymeric and organic additives, chemical and biological mediators, conducting nanomaterials used as materials for fabrication of biocomposite. The preparation protocols of biomaterials such as bacteria, bacteriophage and membrane-associated enzymes have been described in details. A series of protocols used to encapsulate biomolecules and microorganisms in pure inorganic and organic/inorganic hybrid sol-gel are also described. In addition, the analyses used to investigate the viability and bioactivity (electrochemical measurements, thickness measurements, membrane integrity and metabolic activity) have been described in this chapter. At last, the techniques and analytical methods used to confirm these studies have been described and illustrated.

1. Chemicals and biological species

1.1. Sol-gel reagents

This work focuses on developing safe bioencapsulation techniques based on pure silica or hybrid sol-gel film to construct effective whole cell biosensors. A series of precursor with different properties are used (Table 1). Tetramethoxysilane is the most common precursor used to electrodeposit sol-gel film on the conducting electrodes. Sodium silicate solution and Ludox® HS-40 colloidal have been used for bacteria and protein encapsulation by drop-coating in order to avoid any trace of alcohol (i.e. aqueous sol-gel route).

Chemicals	Formula	Grade	MW ($g.mol^{-1}$)	Suppliers
Tetramethoxysilane (TMOS)	$Si(OCH_3)4$	98 %	152.22	Aldrich
Sodium silicate solution	Na_2O SiO_2	14 % 27 %	-	Aldrich
Ludox® HS-40 colloidal (40 wt. % suspension in H2O)	SiO2	40 %	60.08	Aldrich
Tetramethoxysilane (TEOS)	$Si(OC_2H_5)_4$	98 %	208.33	Alfa Aesar

Table 1. *Silica precursors.*

1.2. Additives

A series of additives with different physical properties have been used in this work. These additives have been introduced either for adsorption of bacteria on electrode surface or for optimization of hybrid sol-gel materials. The effects of additives introduction into sol-gel for bioencapsulation are also investigated later. Table 2 provides some technical information on these organic additives.

Chemicals	Abbreviation	Grade	Suppliers
Poly(ethyleneimine) solution	PEI	50 % w/v	Aldrich
Poly(ethylene glycol) solution	PEG 400	pure	Prolabo
Chitosan (Medium MW)	-	75-85% deacetylated	Aldrich
D-(+)-trehalose dehydrate	trehalose	99%	Fluka
Glycerol	-	pure	Descharmes

Poly(dimethyldiallylammonium chloride)	PDDA	20 wt % in water	Aldrich

Table 2. *Additives for preparation of hybrid materials*

1.3. Bacteria and bacteriophage species

Bacteriophage ΦX174 and series of microbial species such as *Escherichia coli*, *Shewanella putrefaciens and Pseudomonas fluorescens* have been used in this work. These microorganisms have been encapsulated in different sol-gel protocols and materials. The physical properties of microorganisms encapsulated in sol-gel are also investigated. Table 3 provides some information on these microorganisms.

Strains	Physical properties
Escherichia coli K12 derivative C600	*E. coli* C600 is an aerobic Gram-negative, rod-shaped bacterium. Optimal growth occurs at 37 °C.
Pseudomonas fluorescens CIP 69.13	*P. fluorescens* CIP 69.13 is an aerobic Gram-negative, rod-shaped bacterium with multiple flagella. Optimal growth occurs at 28 °C.
Shewanella putrefaciens CIP 8040	*S. putrefaciens* CIP 8040 is a facultative anaerobic Gram-negative bacterium. Optimal growth occurs at 30 °C.
Escherichia coli MG1655 pUCD607 [1]	*E. coli* MG1655 pUCD607 is an aerobic Gram-negative, rod-shaped bacterium. It is genetically-modified with plasmid pUCD 607 containing constitutive luminescence promoter fused to lux CDABE gene. Optimal growth occurs at 37 °C in presence of ampicillin.
Escherichia coli MG1655 zntA-GFP [2]	*E. coli* MG1655 zntA-GFP is an aerobic Gram-negative, rod-shaped bacterium. It is genetically-modified with plasmid zntA-GFP containing toxic metal-inducing promoter fused to green fluorescent gene. Optimal growth occurs at 37 °C in presence of kanamycin.
Escherichia coli CN	*E. coli* CN is an aerobic Gram-negative, rod-shaped bacterium. Optimal growth occurs at 37 °C.
Bacteriophage ΦX174	ΦX174 is an aerobic virus isometric, having single-stranded circular DNA and icosahedral shape of 20 triangular faces and 12 vertices. Optimal growth occurs at 37 °C in presence of bacteria and nalidixic acid 25mg/mL.

Table 3. *Microorganism used in this thesis.*

Pseudomonas fluorescens CIP 69.13 was kindly provided by F. QUILES (Laboratory of chemistry, physics and microbiology for environment; LCPME – Lorraine University, France).

Escherichia coli MG1655 pUCD607 and *Escherichia coli* MG1655 zntA-GFP were kindly provided by P. Billard (Laboratory of interactions microorganisms, minerals and organic materials in the sol; LIMOS – Lorraine University, France).

Escherichia coli CN and Bacteriophage ΦX174 were kindly provided by Prof. C. Gantzer (Laboratory of chemistry, physics and microbiology for environment; LCPME – Lorraine University, France).

1.4. Membrane-associated enzymes

The membrane-associated L-Mandelate dehydrogenase (L-ManDH) solution (30 units/mg) isolated from *Pseudomonas putida* has been provided by Prof. G. W. Kohring (Laboratory of microbiology - Saarland University, Germany).

The membrane-associated cytochrome P450-CYP1A2 has been provided by G. Almeida (Faculty of science and technology - Nova de Lisboa University, Portugal).

1.5. Culture medium for microorganisms

All the culture media used for bacteria and bacteriophage growth has been sterilized prior each experiment using autoclave system for 15 min at 121 °C to avoid contamination with other species. Table 4. shows the chemical composition of the culture media.

Culture medium	Chemical components
Trypticase soy broth (TSB)	TSB medium contains 17 g/L casein peptone (bovine), 3 g/L soy peptone, 5 g/L sodium chloride, 2.5 g/L dipotassium hydrogen phosphate, and 2.5 g/L dextrose (= glucose) dissolved in 1 L of deionized water. (pH= 7.3 ± 0.2)
Trypticase soy agar (TSA)	TSA medium contains 17 g/L casein peptone (bovine), 3 g/L soy peptone, 5 g/L sodium chloride, 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L dextrose (= glucose) and 10 g/L agar dissolved in

	1 L of deionized water. (pH= 7.3 ± 0.2)
Minimal salt broth (MSB)	MSB medium contains 10 g/L peptone, 3 g/L yeast extract, 12 g/L beef extract, 3 g/L NaCl, 5 ml of Na_2CO_3 (150g/mL), 0.3ml of [$MgCL_2$, $6H_2O$] (2g/mL) and 10mL of nalidixic acid (25mg/mL) dissolved in 1 L of deionized water. (pH= 7.3 ± 0.2)
Minimal salt agar (MSA)	MSA medium contains 10 g/L ^peptone, 3 g/L yeast extract, 12 g/L beef extract, 3 g/L NaCl, 5 ml of Na_2CO_3 (150g/mL), 0.3ml of [$MgCL_2$, $6H_2O$] (2g/mL), 10mL of Nalidixic acid (25mg/mL), and 10 g/L agar dissolved in 1 L of deionized water. (pH= 7.3 ± 0.2)

Table II-4. *Chemical components of culture media.*

1.6. Redox mediators

Redox mediators	Formula	MW ($g.mol^{-1}$)	Suppliers
1,1'ferrocenedimethanol	$C_{12}H_{14}FeO_2$, 98 %	246.08	Sigma
Potassium hexacyanoferrate (III)	$K_3[Fe(CN)_6]$, ≥ 99 %	329.24	Fluka
Bovine heart cytochrome c	Purity 95 %	13.327	Sigma

Os-containing redox polymer was kindly provided by the group of Prof. Wolfgang Schuhmann, and synthesised by Sascha Pöller (Analytische Chemie – Elektroanalytik & Sensorik & Center for Electrochemical Sciences – CES; Ruhr-Universität Bochum, Bochum, Germany).

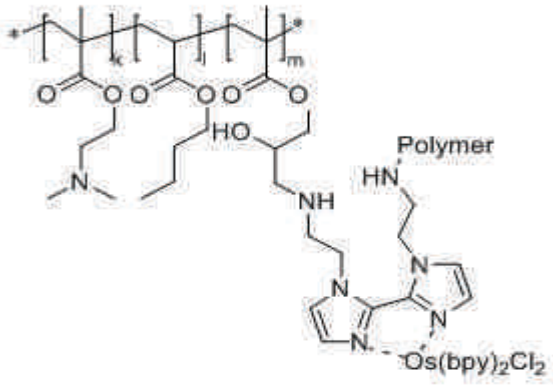

Figure I. *Molecular structures of the synthesized Os-containing redox polymer P0072.*

1.7. Other chemicals and solvents

All other reagents are of analytical grade. Hydrochloric acid HCl solution (1M; Riedel-de Haën), sodium hydroxide solution NaOH (1 M, Riedel-de Haën), acetic acid solution CH_3COOH (99.8 %, SDS) and nitric acid solution HNO_3 (65%, Aldrich). The buffers are KCl solution (1 mM, Aldrich) and phosphate buffer prepared from $NaH_2PO_4.2H_2O$ (≥ 99.6 %; Prolabo) and $Na_2HPO_4.2H_2O$ (≥ 99 %; Fluka). The fluorescent DNA dyes are propidium iodide (PI) and SYTO9 from the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen). The conducting nanoparticles are single-walled carbon nanotubes functionalized with poly (ethylene glycol) or multi-walled carbon nanotubes functionalized carboxylic acid (MWCNT-COOH and SWCNT-EtO, Nanolab). The other chemicals are cadmium chloride $CdCl_2$ (99 %, Prolabo), D-(+)-glucose anhydrous (HPLC ≥ 99 %; ACROS), L-(+)-Mandelic acid (98 %, Sigma), sodium formate NaCOOH (GC for analysis ≥ 99 %; MERCK), potassium cyanide KCN (99 %, Prolabo), sodium hypochlorite solution NaClO (15 % of active chlorine; Prolabo), trisodium citrate dihydrate (Riedel-de Haën) and gold (III) chloride hydrate (99.99% trace metals basis, Aldrich).

2. Preparation of nano-material suspensions

1) MWCNT-COOH: 2 mg of MWCNT-COOH were dispersed in 1 mL of deionized water. The solution was sonicated for 10 min followed by stirring overnight at room temperature.
2) SWCNT-EtO: 2 mg of SWCNT were dispersed in 1 mL of chitosan. The solution was sonicated for 10 min followed by stirring overnight at room temperature.
3) SWCNT-$(EtO)_8$-Fc: the chemical modification of SWCNT with poly (ethylene glycol) linker and ferrocene has been prepared by SRSMC laboratory [3,4]. Its dispersion in chitosan has been applied using the same protocol as SWCNT-EtO.
4) MWCNT-Os: first, 2 mg of MWNCT-COOH were dispersed in 1mL of deionized water by sonication. Then, a mixture of 150 µL of osmium polymer P0072 and 250 µL of dispersed MWCNT-COOH were sonicated for 1 h followed by stirring overnight at room temperature. Finally, MWCNT wrapped with osmium P0072 was dispersed in chitosan 0.2 wt % (ratio 1:1) [5].
3) Gold nanoparticles: HAuCl4 was dissolved in 20 mL of deionized water to get a final concentration of 1mM and heated. When the solution started boiling, trisodium citrate dihydrate (1 wt%, 2 mL) was added. The final solution was stirred and boiled until changing

color into brick-red color. The final concentration of gold nanoparticles was estimated at 0.2 $mg.mL^{-1}$ [6].

3. Electrodes

Glassy carbon electrode (GCE, 3mm in diameter), indium-tin-oxide glass slides (ITO, Delta Technologies) or pyrolytic graphite electrode (PGE) served as working electrode. Prior each measurement, GCE was first polished on wet emery paper 4000, using Al_2O_3 powder (0.05 mm, Buehler), then rinsed thoroughly with water to remove the embedded alumina particles. ITO electrodes were cleaned only with acetone to remove any chemical or biological contamination. Basal plane pyrolytic graphite (3 mm diameter disks). The surfaces were treated by abrasion with sandpaper and polished with alumina slurry (0.3 μm), followed by brief sonication in deionized water. Finally, the electrodes were washed with deionized water and dried with compressed air. The counter electrode was a platinum wire and the reference electrode was Ag/AgCl 3M KCl.

4. Preparation of sol-gels for bioencapsulation

We have explored various starting sol compositions for bioencapsulation (Table 5). Aqueous sol-gel (**Sol A, B and D**) has been applied in order to avoid any trace of alcohol in drop-coating and monolith protocols of bioencapsulation. Alkoxide-based sol-gel (**Sol C**) has been applied for electrochemically-assisted bioencapsulation and (**Sol E**) has been applied for bioencapsulation of membrane-associated enzyme. The release of alcohol after the hydrolysis of TMOS has been considered an obstacle, due to its potential denaturing activity on the entrapped bacteria. Methanol has been removed from the TMOS sol by evaporation. A natural polymer (chitosan) and PEG were used as additives which were advantageous in providing a biological protection and deposition improvement for electrochemically-assisted bioencapsulation. In addition, trehalose and glycerol has been used for better bacterial protection against sol-gel constraints and aging. PEI has been used for either better protection of viral infectivity in sol-gel monolith or bacterial adsorption in electrochemically-assisted bioencapsulation. Note that control experiments have been applied in absence of one of hybrid sol-gel components to check its effective role on sol-gel bioencapsulation.

Materials	Procedures used to prepare the starting sols for films formation
Sol A	Sodium silicate solution (0.22 M, 10 mL) was mixed with LUDOX HS-40 (40 wt %, 10 mL). HCl (1 M, 2.4 mL) was added to adjust pH at 7.
Sol B [7]	Sodium silicate solution (0.27 M, 13 mL) was mixed with LUDOX HS-40 (40 wt %, 7 mL) and 5 wt % of pure glycerol. HCl (1 M, 2.4 mL) was added to adjust pH at 7. Finally, this sol mixture has been diluted 10 times with deionized water for further use.
Sol C [8]	The starting sol was prepared by mixing 4 ml TMOS with 0.5 mL of 0.1 M HCl and 2 mL of distilled water. The mixture was sonicated (Transsonic T 080) for 10 min. Then the acidified sol has been diluted to concentration 1 M with deionized water and evaporated for a weight loss of 3.52 g of methanol. Then, alcohol-free sol has been stored at 4 °C for further use.
Sol D	Sodium silicate solution (55 mM, 20 mL) was mixed with PEG 6% wt. HCl (1 M, 0.7 mL) was added to adjust pH at 7.
Sol E	The starting sol was prepared by mixing 2.25 ml TEOS with 2.5 mL of 0.01 M HCl and 2 mL of distilled water. The mixture was mixed overnight at room temperature. NaOH (1 M, 0.7 mL) was added to adjust pH at 5. Finally, this sol mixture has been diluted 3 times with deionized water for further use.

Table 5. *Information of different methods used to prepare the starting sols*

5. Microorganism cultures

5.1. Bacteria

All the strains used in encapsulation experiments are from our laboratory collection. All the bacterial strains (except *E. coli* CN) were streaked on TSA plates from cultures stored at -80 ºC on beads supplemented with glycerol. When needed, a 0.1 mL overnight culture from single colony was inoculated in 200 mL TSB and incubated at the optimal growth temperature for each strain under stirring (160 rpm) for 24 h (stationary growth phase). Except *S.*

putrefaciens strain needs stirring for 48 h (stationary growth phase) in order to have depletion of oxygen inside growth medium and strain starts to produce cytochromes to adapt the anaerobic medium. Ampicillin sodium salt [100 µg/mL] and kanamycin (25 µg/mL) were added only to growth media for cultivation of *E. coli* MG1655 pUCD607 *and E. coli* MG1655 zntA-GFP strains, respectively in order to control the growth of genetically-modified bacteria only. Bacterial growth (turbidity) was measured by monitoring the optical density at 600 nm. The culture was harvested by centrifugation at 5000 g for 10 min at room temperature. The pellet was washed twice with 1 mM KCl, and then suspended in 1 mM KCl. This washing procedure eliminated nutrients to avoid any bacterial growth in the sol-gel. Finally, bacterial suspension in KCl [1mM] could be used directly for sol-gel bioencapsulation or stored at +4 °C for few days.

The strain *E. coli* CN has been cultured following a different protocol in order to adapt its physiological properties at exponential growth phase. Bacteria were stored at -80 ºC on beads supplemented with glycerol. When needed, a 0.1 mL overnight culture from single colony was inoculated in 200 mL MSB Nal medium and incubated at 37 ºC under stirring (160 rpm) for 3 h (exponential phase growth). Bacterial growth was measured by monitoring the optical density at 600 nm.

5.2. Bacteriophage

The bacteriophage ΦX174 was produced according to ISO10705-2:2000(F). A suspension of ΦX174 (107 PFU/ml as a final concentration) was incubated in MSB Nal medium containing E. coli CN cells (adapted at exponential growth phase) for 5 h at 37 °C. The culture was harvested by centrifugation at 3000 g for 20 min at room temperature. Then, supernatant was filtered by filter membrane (pores of 0.22 µm), divided into aliquots and stored at 4°C in dark until further use.

6. Sol-gel bioencapsulation protocols

6.1. Bacteria encapsulation by electrodeposition (Chapter II)

The preparation of sol used for EAD protocols has been described in the previous section (preparation of **sol C**) without any additives. The introduction of additives allowed enhancing deposition and stability of sol-gel film in addition to its protective role for bacteria.

6.1.1. EABE(1S)

It is electrodeposition of a film from a sol containing the bacteria in suspension in one-step protocol (scheme is illustrated later. The preparation of hybrid sol for one-step protocol is: 5 mL of **sol C** [1 M] was mixed to 5 mL of 50 % PEG (w/v), 5ml of chitosan (1% w/v), 1 % (w/v) of trehalose. Then, NaOH solution (0.2 M, 1.6 mL) was added to raise the pH to 5.3 and 5mL of *E. coli* MG1655 pUCD607 or *E. coli* MG1655 zntA-GFP were introduced into the hybrid sol. Finally, the final mixture was poured into the electrochemical cell where electrochemically-assisted deposition was performed at -1.3 V at room temperature for several tens of s (deposition time). Then, ITO plates were rinsed immediately with deionized water carefully in order to remove non-deposited sol and stored at -80 °C for further analysis. N.B. the final concentrations for the hybrid sol-gel components are: TMOS (0.25 M), PEG (12.5 % w/v), chitosan (0.25 % w/v), trehalose (0.25 % w/v) and *E. coli* MG1655 pUCD607 or *E. coli* MG1655 zntA-GFP (2×10^9 cells/mL).

6.1.2. EABE(2S)

It is divided into 2 steps: adsorption of the bacteria on the ITO plate surface through the aid of polycationic PEI polymer followed by the electrodeposition of the hybrid sol-gel film through/over the bacteria layer (scheme is illustrated later).

1- Adsorption protocol: ITO plate was successively treated in nitric acid (65 %) for 1 h, sodium hydroxide solution (1 M) for 20 min, and PEI (0.2 % w/v) for 3 h. Between each step the ITO plate was washed with ultrapure water and dried in sterilized air at room temperature. The treated ITO plate was then introduced in *E. coli* C600 suspension (4×10^6 cells.mL-1) and incubated at room temperature in sterile environment for 2 h. Finally, the ITO plate was washed with and stored in a KCl solution (1 mM) for two-step EAB [9].

The preparation of hybrid sol for two-step protocol is: 5 mL of **sol C** (1 M) was mixed to 5 mL of 50 % PEG (w/v), 5ml of chitosan (1% w/v), 1 % (w/v) of trehalose. Then, NaOH solution (0.2 M, 1.6 mL) was added to raise the pH to 5.3. Note that some control experiments were done by replacing PEG, chitosan or sol C with deionized water. N.B. the final concentrations for the hybrid sol-gel components are: TMOS (0.25 M), PEG (12.5 % w/v), chitosan (0.25 % w/v) and trehalose (0.25 % w/v).

2- Electrodeposition step: ITO plate modified with bacteria has been introduced into hybrid sol-gel mixture (described above) and electrochemically-assisted deposition has been performed at -1.3 V for several tens of s (deposition times). The, ITO plates were rinsed immediately with deionized water carefully in order to remove non-deposited sol and stored at 4 °C for in humid air further analysis. Note that some control samples have been stored either in KCl solution [1mM] or humid air without introduction of trehalose into sol C.

6.2. Bacteria encapsulation by drop-coating (Chapter III)

The preparation of sol used for drop-coating protocol has been described in the previous section (preparation of **sol B**). The protocol has been divided into 2 branches:

6.2.1. One-layer encapsulation

In section A, 10 µL of **sol B** was mixed with 10 µL of *S. putrefaciens* CIP 8040 (9×10^9 cells/mL) and/or 10 µL of SWCNT-EtO/ SWCNT-$(EtO)_8$-Fc (2 mg/mL) dispersed in chitosan (0.5 wt%). Finally, 5µL of obtained mixture has been drop-coated on the glassy electrode and kept in humid air at 4 °C for further analysis. N.B. the final concentrations for the hybrid components are: *S. putrefaciens* (3×10^9 cells/mL) and SWCNT-EtO/ SWCNT-$(EtO)_8$-Fc (0.67 mg/mL).

In section B, 30 µL of **sol B** was mixed with 10 µL of bovine-heart cytochrome c [1 mM], 10 µL of one of these strains (*E. coli* C600, *P. fluorescens* CIP 69.13 or *S. putrefaciens* CIP 8040 (9×10^9 cells/mL) and/or 10 µL of MWCNT-COOH dispersed in water (2 mg/mL) or 20 µL of gold nanoparticles. Finally, 5µL of obtained mixture has been drop-coated on the glassy electrode and kept in humid air at 4 °C for further analysis. N.B. the final concentrations for the hybrid components are: bacteria (1.8×10^9 cells/mL), SWCNT-EtO/ SWCNT-$(EtO)_8$-Fc (0.4 mg/mL) and bovine-heart cytochrome c [0.2 mM].

6.2.2. Two-layer encapsulation

First, 5µL of SWCNT-EtO/ SWCNT-$(EtO)_8$-Fc (2 mg/mL) dispersed in chitosan has been drop-coated on the glassy electrode and kept for 2 h to be fully dried. Then, 5 µl of suspension (containing 10 µL of **sol B** and 10 µL of *S. putrefaciens* CIP 8040 (9×10^9 cells/mL) has been drop-coated on the glassy electrode and kept in humid air at 4 °C for further analysis. N.B. the

final concentrations for the hybrid components are: *S. putrefaciens* (4.5×10^9 cells/mL) and SWCNT-EtO/ SWCNT-$(EtO)_8$-Fc (2 mg/mL).

6.3. Membrane-associated protein encapsulation (Chapter IV)

Cytochrome P450: 10 µL of **sol D** was mixed with 10 µL of Cytochrome P450 (CYP1A2) membrane protein. 5µL of obtained mixture has been drop-coated on the pyrolytic graphite electrode and kept in humid air at 4 °C for further analysis.

L-Mandelate dehydrogenase: 10 µL of **sol D or E** was mixed with 10 µL of L- ManDH membrane protein. 5µL of obtained mixture has been drop-coated on the glassy electrode and kept in humid air at 4 °C for further analysis.

6.4. Bacteriophage encapsulation (Chapter V)

The preparation of sol used for monolith bioencapsulation has been described in the previous section (preparation of sol A. A collection of organic additives have been introduced uniquely into sol A in each experiment such as PEI (0.2 wt %), glycerol (10 wt %), trehalose (10 wt %), PDDA (5 wt %) and a mixture of trehalose-glycerol (5wt % - 5 wt %) as a final concentrations in the sol-gel monolith. 2 mL of HCl solution (1 M) was added to raise the pH up to 7.4. Then, ΦX174 (1 mL, 6×10^7 UFP/mL) was added to the sol and/or hybrid sol. Finally, aliquots samples (15 µL) of mixture containing sol and bacteriophage were aged at 4°C for 6 days in humid air or harsh dry air for further analysis. Note that control experiment has been performed in absence of any additive.

7. Methods of analysis

7.1. Electrochemical measurements

7.1.1. Cyclic voltammetry (CV) [10]

Cyclic voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions. It is often the first experiment performed in an electroanalytical study. In particular, it offers a rapid location of redox potential of the electroactive species, and convenient evaluation of the effect of various parameters on the redox process. This technique is based on varying the applied potential at a working electrode

in both forward and reverse directions (at selected scan rates) while monitoring the resulting current. The corresponding plot of current versus potential is termed a cyclic voltammogram. Figure II-1 shows the response of a reversible redox couple during a single potential cycle. It is assumed that only the oxidized form O is present initially. Thus, a negative-going potential scan is chosen for the first half-cycle, starting from a value where no reduction occurs. As the applied potential approaches the characteristic E0 for the redox process, a cathodic current begins to increase, until a peak is reached. After traversing the potential region in which the reduction process takes place, the direction of the potential sweep is reversed. During the reverse scan, R molecules (generated in the forward half cycle, and accumulated near the surface) are reoxidized back to O and an anodic peak results. The important parameters in a cyclic voltammogram are the two peak potentials (Epc, Epa) and two peak currents (ipc, ipa) of the cathodic and anodic peaks, respectively. Cyclic voltammetry can be used for the study of reaction mechanisms and adsorption processes, which can also be useful for quantitative purposes, based on measurements of the peak current.

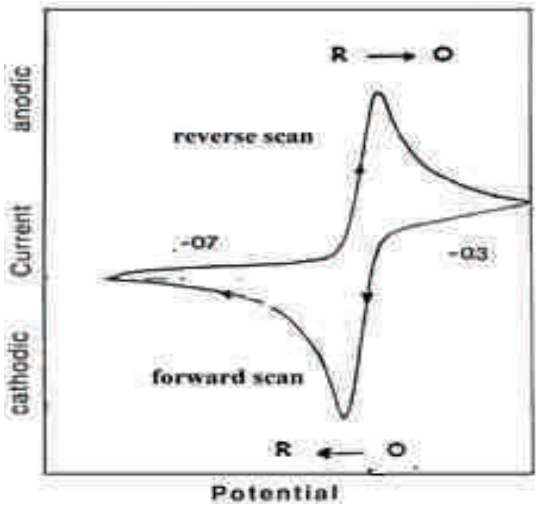

Figure 1. *Typical cyclic voltammogram for a reversible redox reaction (O + ne-⟶ R and R ⟶ O + ne-).*

7.1.2 Amperometric measurement [10]

The basis of amperometry technique is the measurement of the current response to an applied potential. A stationary working electrode and stirred solution are used. The resulting current-time dependence is monitored. As mass transport under these conditions is solely by convection (steady state diffusion), the current-time curve reflects the change in the concentration gradient in the vicinity of the surface, which is directly related to concentration in solution (Fig. 2).

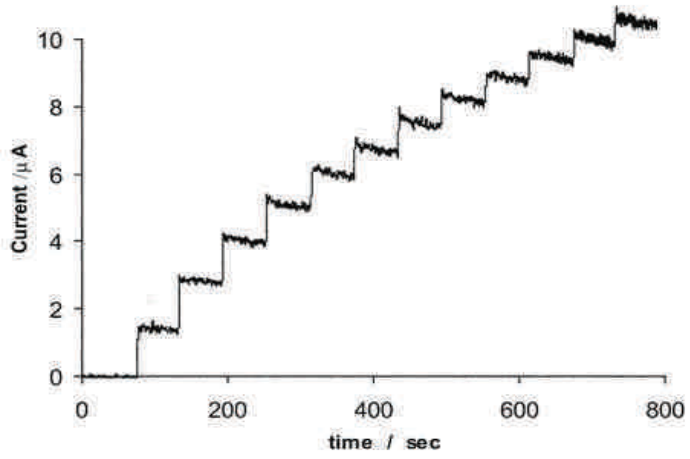

Figure 2. *Typical amperometric detection for a chemical reaction upon successive additions of substrate.*

All the electrochemical measurements (amperometric or voltammetric) were carried out the potentiostat Palm.Sens and monitored by the software (PS Trace) using a conventional three-electrode cell. Amperometric and cyclic voltammetry measurements (Chapters III and IV) were performed using glassy carbon electrodes (GCE) as working electrode in presence of platinum counter and Ag/AgCl reference electrodes. Amperometric measurement (Chapter II) was performed using ITO plates as working electrode in presence of platinum counter and Ag/AgCl reference electrodes for electrodeposition.

7.2. Atomic force microscopy (AFM)

Atomic force microscopy experiments were carried out using MFP3D-BIO instrument (Asylum Research Technology, Atomic Force F&E GmbH, and Mannheim, Germany). Triangular cantilevers with square pyramidal tips (radius of curvature 15 nm) were purchased from Atomic Force (OMCL-TR400PSA-3, Olympus, Japan). The spring constant of the cantilevers measured using thermal noise method was found to be 20 pN/nm. The films have been imaged in AFM contact mode and were collected at a fixed scan rate of 1 Hz with a resolution of 512 x 512 pixels. Experiments were performed in air at room temperature. The Film was scratched and its thickness was measured from the cross section profiles of the AFM topographical images. Values were calculated by statistical analysis, as averages of 5 measurements obtained from several regions of the film.

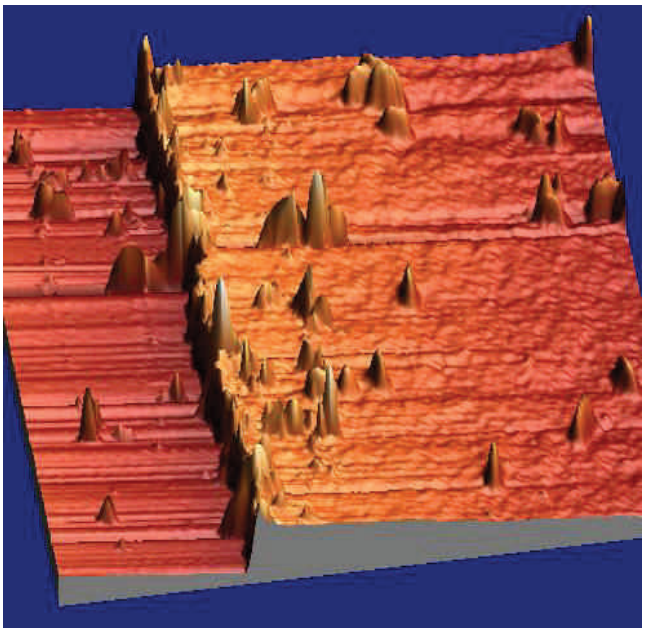

Figure 3. *Typical AFM 3D image for a sol-gel film scratched on the left side*

7.3. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy technique whereby a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera. TEM could be used for morphological studies of silica materials in addition to the biological species encapsulated in these materials.

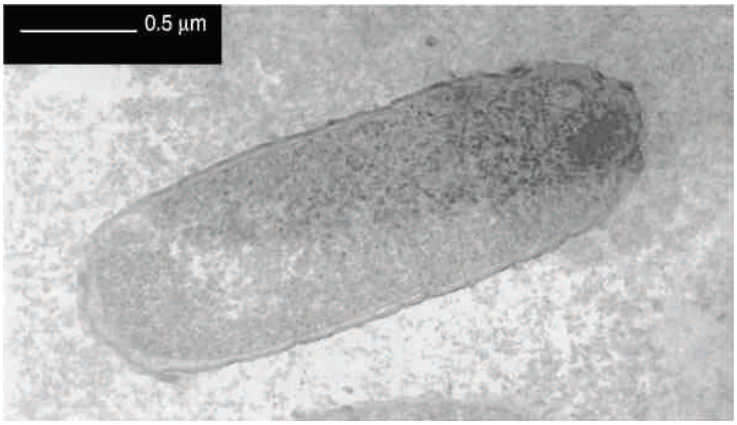

Figure 4. *Transmission electron microscopy of an E. coli cell trapped within a silica gel (adapted from reference* [11]*).*

7.4. *Bac*Light™ bacterial viability analysis

LIVE/DEAD BacLight viability analysis is a dual DNA fluorescent staining method used to determine simultaneously the total population of bacteria and the ratio of damaged bacteria. It was applied here on encapsulated bacteria in sol-gel films. BacLight is composed of two fluorescent nucleic acid-binding stains: SYTO 9 and propidium iodide (PI). SYTO 9 penetrates all bacterial membranes and stains the cells green while PI only penetrates cells with damaged membranes. The combination of the two stains produces red fluorescing cells (damaged cells) whereas those with only SYTO 9 (green fluorescent cells) are considered viable [12].

The two dye solutions, SYTO 9 (1.67 µM) and PI (1.67 µM), were mixed together with the same volume equivalent. Then, 3 µL of this solution was diluted in 1 mL of non-pyrogenic water. The sample of sol-gel film encapsulating bacteria was covered with 0.2 mL of diluted dye solution and incubated for 15 min in dark condition. Finally, the sample was washed in KCl solution (1 mM) and the counting was performed with epi-fluorescence microscope (OLYMPUS BX51) with emersion oil at 100X magnification. Viability values were calculated as averages of ten fields obtained from two samples.

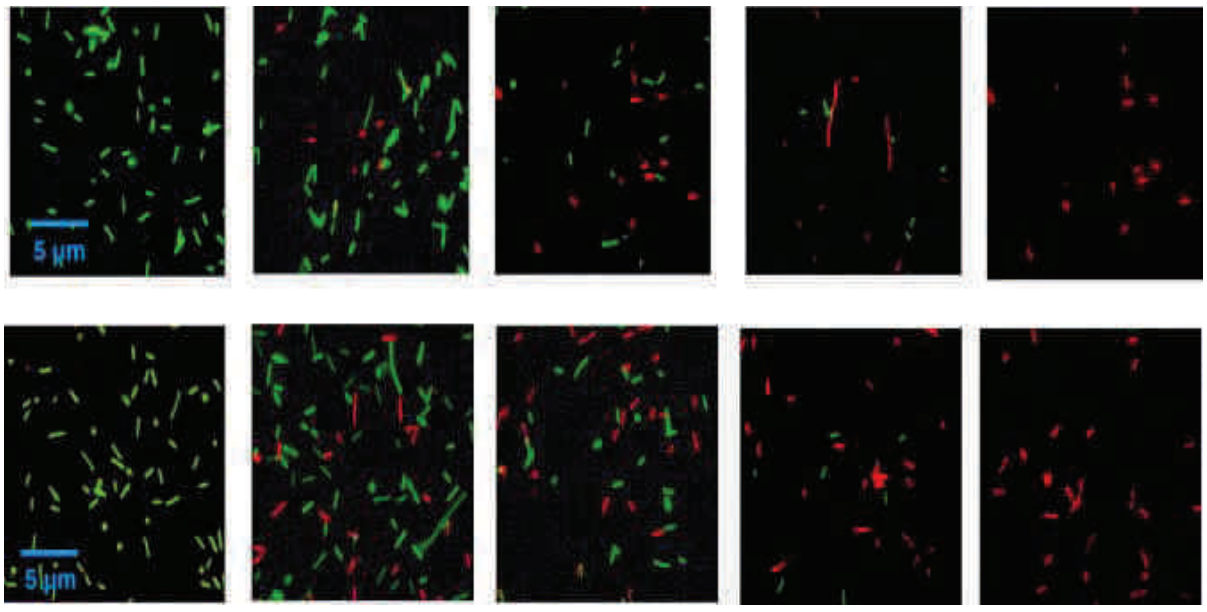

Figure 5. *Image series for comparison of dye combinations in mixed E.coli suspensions (adapted from reference* [13]*)*

7.5. Bioluminescence analysis

Bioluminescence is simply light produced by a metabolic reaction which originates in a microorganism. The luminescent bacteria modified with plamisd PUCD607 contain the luxCDABE operon which is responsible for continuous light production. The bioluminescent reaction is catalyzed by luciferase (encoded by the luxA and luxB genes) which requires a long-chain aldehyde (provided by the luxCDE gene products) as a substrate, oxygen, and a source of reducing equivalents, usually reduced flavin mononucleotide ($FMNH_2$) [14,15]. Encapsulation and storage stresses applied on the encapsulated bacteria could block the light production by altering the bacterial metabolic activity.

The sample sol-gel film encapsulating bacteria was incubated at 30 °C in diluted TSB (1/10) in presence/absence of cadmium chloride (0.1 mM) for 4 h. Then, the luminescence was measured with a FLX Xenius microplate reader (SAFAS, Monaco). Activity values were calculated as averages of three fields obtained from three samples.

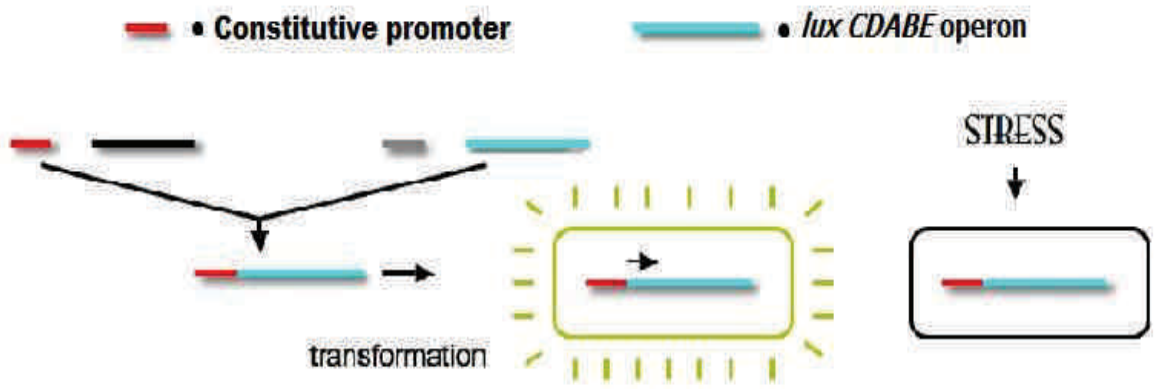

Figure 6. *Typical luminescent scheme for a bacteria modified with plasmid PUCD607*

7.6. Biofluorescence analysis

Biofluorescent is simply light produced by a metabolic reaction which originates in a microorganism. The fluorescent bacteria modified with plamisd zntA-GFP contain the green fluorescent protein (GFP) operon which is responsible for green fluorescence production. The GFP operon is connected to zntA promoter which is inducible upon exposure to toxic metals.

The sample sol-gel film encapsulating bacteria was incubated at 37 °C in diluted TSB (1/10) in presence/absence of cadmium chloride (10 µM) for 12 h. Then, the sample was washed in

KCl solution (1 mM) and the counting was performed with epi-fluorescence microscope (OLYMPUS BX51) with emersion oil at 100X magnification.

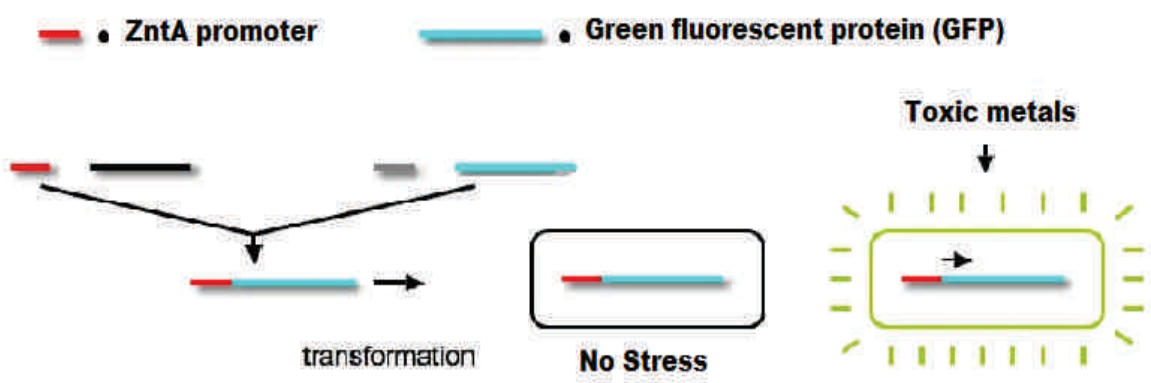

Figure 7. *Typical fluorescent scheme for a bacteria modified with plasmid ZntA-GFP*

7.7. Infectivity analysis

The infectivity analysis of encapsulated ΦX174 has been tested from single-agar-layer plaque assay [16]. The aliquots of encapsulated ΦX174 samples were dissolved in sterile water (50 mL) for at room temperature for testing the preserved infectivity to bacteria. 10mL of MSA Nal soft medium (MSB containing 10g/L Agar) are steamed to liquefy and adjusted at 55°C in a water bath. 1mL of E.coli CN (adapted at exponential phase) and 1mL of dissolved aliquots samples were introduced into the melted MSA. The final mixture has been gelified in petri dish. Finally, the latter was incubated overnight at 37°C and lysis plaques were counted by naked eye. Values were calculated as average of 2-3 samples. Note that some control test has been perfomed using same protocol for ΦX174 suspension dispersed in saturated solution of silica.

Figure 8. *Typical plaque assay for encapsulated bacteriophage upon exposure to bacteria*.

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Résume

Le travail décrit dans cette thèse a été mené à l'interface entre trois disciplines: l'électrochimie, la science des matériaux et la microbiologie. L'objectif de cette recherche était tout d'abord d'étudier l'activité de bactéries immobilisées dans un film de silice déposé par le procédé sol-gel à la surface d'électrodes. Les applications potentielles de ce travail fondamental sont les biocapteurs, les bioréacteurs ou biopiles. L'encapsulation bactérienne assistée par électrochimie a été développée en utilisant l'électrolyse du sol de départ pour immobiliser la bactérie *Escherichia Coli* dans une couche mince sol-gel hybride. La combinaison de précurseurs de silice, de chitosan, de poly(ehtylène glycol) et de tréhalose permet de préserver l'intégrité membranaire et l'activité métabolique. L'électrochimie a ensuite été utilisée comme moyen analytique. *Shewanella putrefaciens* et *Pseudomonas fluorescens* ont été encapsulées dans un film à base de silice et les réactions de transfert d'électron de la bactérie à différent médiateurs rédox ont été analysées. Des nanotubes de carbone fonctionnalisés par des espèces ferrocène et la protéine rédox cytochrome c ont été utilisés pour faciliter ce transfert électronique au sein de cette matrice de silice isolante, permettant l'obtention d'un biofilm artificiel. Ces deux types de médiateurs, chimique ou biologique, ont conduit à des sensibilités différentes de la bioélectrode à l'ajout du substrat pourvoyeur d'électron en raison des mécanismes différents impliqués pour transférer ces électrons. L'immobilisation de protéines rédox membranaires a également été considérée dans ces couches minces inorganiques pour favoriser la stabilité de la réponse électrocatalytique. Les protéines considérées impliquent des mécanismes de transfert électronique différents, soit direct pour le cytochrome P450 (CYP1A2), soit médié pour la mandélate déshydrogénase. Finalement, l'influence de l'encapsulation dans une matrice sol-gel hybride sur l'infectivité du bactériophage ΦX174 a été étudiée, montrant l'effet protecteur de la polyéthylènenimine ou du glycérol.

Mots clefs: sol-gel, matériaux hybrides, bioencapsulation, électrodépôt, bactérie, biofilm artificiel, protéine rédox membranaire, bactériophage, transfert électronique, cytochrome c.

Abstract

The work reported in this thesis has been developed at the interface between three disciplines, i.e., electrochemistry, material science and microbiology. The purpose of this research was first to study the activity of bacteria immobilized in silica-based films prepared by the sol-gel process on electrode surfaces. Potential applications concern biosensors, bioreactors and biofuel cells. Electrochemically assisted bacterial encapsulation has been developed, using sol electrolysis to immobilize *Escherichia coli* in a hybrid sol-gel layer. The combination of silica precursors, chitosan, poly(ethylene glycol) and trehalose allowed preservation of cell membrane integrity and metabolic activity. Electrochemistry was then considered as an analytical method. *Shewanella putrefaciens* and *Pseudomonas fluorescens* have been encapsulated in silica-based films and the electron transfer reactions from bacteria to different redox mediators have been monitored. Single-walled carbon nanotubes functionalized with ferrocene moieties and bovine heart cytochrome c have been considered as redox shuttles to facilitate the electron transfer in the non-conducting silica matrix, leading to the elaboration of artificial biofilms. Interestingly, these two classes of mediator, i.e. chemical and biological, led to different substrate sensitivity because of their different mechanism of interaction with the bacteria. Immobilization of membrane associated redox proteins in sol-gel films have been then considered and applied for electrocatalysis. Direct and mediated electrochemical communication has been investigated between the electrode surface and cytochrome P450 (CYP1A2) or mandelate dehydrogenase, respectively, showing the interest of sol-gel to stabilize the bioelectrocatalytic reaction. Finally, the influence of encapsulation in a hybrid sol-gel matrix on the infectivity of bacteriophage ΦX174 has been studied and the protective effect of polyethyleneimine or glycerol was shown.

Keywords: sol-gel, hybrid materials, bioencapsulation, electrodeposition, bacteria, artificial biofilm, membrane-associated enzyme, bacteriophage, electron transfer, cytochrome c.