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Applications nanobiothechnologies et thérapeutiques des membranes en bicouches lipidiques

Gwenael Scolan

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UNIVERSITE JOSEPH FOURIER

FACULTE DE PHARMACIE DE GRENOBLE

Année : 2012 N°

APPLICATIONS NANOBIOTECHNOLOGIES ET THERAPEUTIQUES DES MEMBRANES EN BICOUCHES LIPIDIQUES

THESE

PRESENTEE POUR L'OBTENTION DU TITRE DE DOCTEUR EN PHARMACIE

DIPLÔME D'ETAT

Gwenaël SCOLAN

Né(e) le : 20 Juillet 1987 A : Les Lilas (93)

THESE SOUTENUE PUBLIQUEMENT A LA FACULTE DE PHARMACIE DE GRENOBLE
Le 2 Avril 2012

DEVANT LE JURY COMPOSE DE

Président du jury : M. **Michel Sève**, Docteur en pharmacie, Praticien hospitalier et Professeur de l'UFR de pharmacie de Grenoble

Membres

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ABBREVIATIONS

AIDS: Autoimmune Deficiency Syndrome

AmB: Amphotericin B

AMPs: Antimicrobial Peptides

ATP: Adenosine Triphosphate

AuNP: Gold Nanoparticle

BLM: Black Lipid Membrane

BPM: Black Polymer Membrane

CpG: Cytosine-Guanidine

CRP : C-reactive Protein

cTnI: Cardiac Troponin I

CVD: Cardiovascular Disease

DNA: Deoxyribonucleic Acid

DVT: Deep Veinous Thrombosis

ECG: Electrocardiogram

ECL: Electrochemiluminescence

ELISA: Enzyme-Linked Immunosorbent Assay

EPR: Enhanced Permeability and Retention

FDA: Food and Drug Administration

FET: Field Effect Transistor

HBM: Hybrid Bilayer Membrane

HbV: Hemoglobin Vesicles

hCG: Human Cardiac Gonadotropin

HIV: Human Immunodeficiency Virus

ICS: Ion Channel Switch

ISE: Ion selective Electrode

LAPS: Light Addressable Potentiometric Sensor

LB: Langmuir-Blodgett

LbL: Layer-by-Layer

LUV: Large Unilamellar Vesicle

MC: microcantilever

MI: Myocardial Infarction

MIP: Molecular Imprinting

MIPs: Major Intrinsic Proteins

MLV: Multilamellar Vesicle

mRNA: messenger RNA

MVV: Multivesicular Vesicle

ODN: Oligonucleotide

PCa: Prostate Cancer

PCR: Polymerase Chain Reaction

pDNA: plasmid DNA

PEG: Polyethyl Glycol

PMP: Paramagnetic Particle

POC: Point-of-Care

PSA: Prostate Specific Antigen

QCM: Quartz Crystal Microbalance

RNA: Ribonucleic Acid

RNAi: RNA interference

ROS: Reactive Oxygen Species

SAM: Self-Assembled Monolayer

SAW : Surface Acoustic Wave

SDC: Serum Digoxin Concentrations

SERS: Surface Enhanced Raman Spectroscopy

siRNA: silencing RNA

SLB: Supported Lipid Bilayer

SOD: Superoxide Dismutase

SPR: Surface Plasmon Resonance

SUV: Small Unilamellar Vesicle

tBLM: tethered Bilayer Lipid Membrane

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TRH: Thyrotroponin-Releasing Hormone

TSH: Thyroid Stimulating Hormone

WHO: World Health Organization

INTRODUCTION

La membrane plasmatique des cellules est une matrice lipidique nanostructurée et sophistiquée, composée principalement d'un assemblage de phospholipides qui fourni une barrière à la diffusion entre le cytoplasme intracellulaire et l'environnement externe des cellules (1). La matrice lipidique est également capable de maintenir la morphologie cellulaire et constitue un échafaud pour l'intégration de protéines et glycoprotéines spécifiques, ce qui facilite le rôle clé des membranes cellulaires dans le processus d'adhésion cellulaire et les voies de communication entre les cellules. Le transport d'électrolytes et d'autres molécules à travers la membrane, nommé transport membranaire, est fondamental à la vie d'une cellule. La viabilité de la cellule est hautement dépendante des flux d'électrolytes et eau, générant et modifiant les gradients ioniques, et des échanges sélectifs de molécules à travers la membrane (2). La membrane plasmatique des cellules forme un modèle et une base très attractive pour le développement d'outils biotechnologiques, en particulier car les protéines membranaires sont la cible de plus de 50% des médicaments (3) (4) (5) (6) (7).

Les phospholipides sont constitués de deux parties distinctes avec des têtes hydrophiles polaires et des queues hydrophobes. Ce réarrangement spécifique leur donne la propriété d'être capables de s'assembler spontanément en une monocouche continue en milieu aqueux. La membrane cellulaire comprend deux de ces monocouches formant une bicouche lipidique, avec les faces hydrophobes de chacune des monocouches se faisant face. Par conséquent, les têtes hydrophiles sont orientées en direction du milieu aqueux et forment ainsi l'extérieur de la bicouche. Les protéines membranaires s'incorporent dans cette bicouche lipidique et préservent leur structure et leur fonctionnalité (8).

La bicouche lipidique a été largement acceptée comme étant l'élément structural des membranes lipidiques. Les biomembranes réelles sont extrêmement complexes avec de nombreux types de lipides, une asymétrie entre les deux feuillets et un enchevêtrement compliqué des composants, domaines et éléments du cytosquelette. Mais, même si elles représentent un modèle très simplifié, les membranes lipidiques peuvent imiter les membranes naturelles et sont donc appelées membranes biomimétiques.

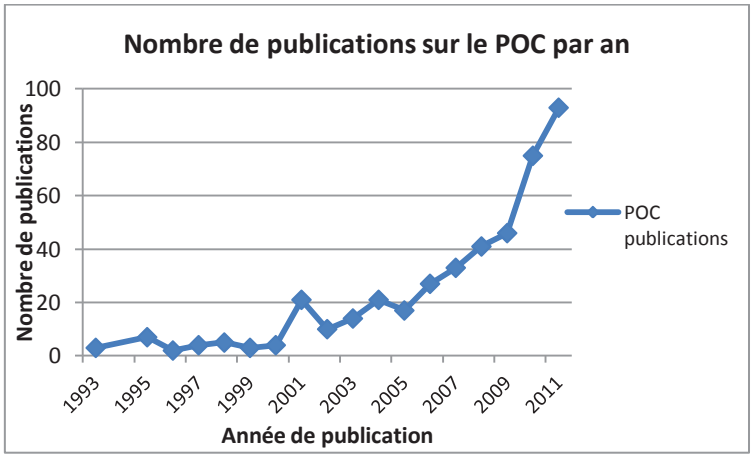
Ainsi peut-on s'interroger : **existe-t-il un moyen de tirer profit de la capacité des membranes lipidiques à imiter les membranes cellulaires pour des applications thérapeutiques ?**

Au long de cette thèse, nous allons essayer de répondre à cette question par des explications techniques et des exemples concrets.

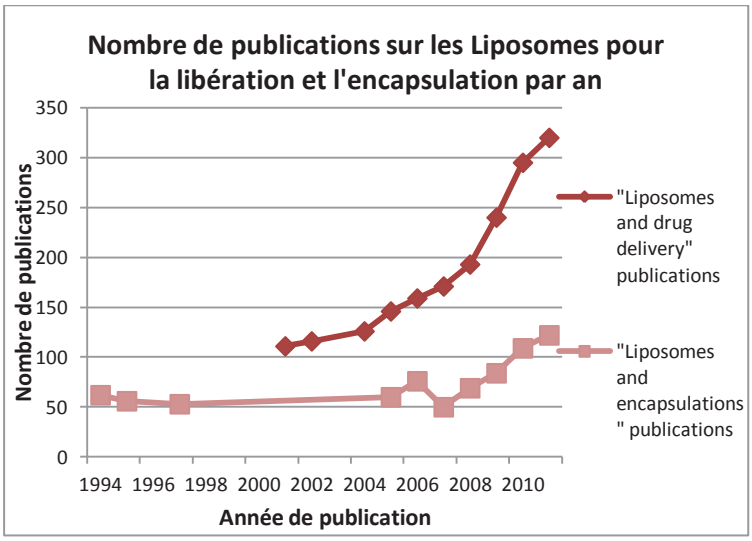
Dans une première partie, nous reverrons les applications *in vitro* des membranes lipidiques, avec une description du POC, des transducteurs et enfin des détails techniques sur l'incorporation des membranes lipidiques au sein de biocapteurs, en nous basant sur la littérature. Dans une seconde partie, nous nous concentrerons sur les applications *in vivo* des membranes lipidiques, toujours à partir de la littérature, comprenant les liposomes pour l'encapsulation et la libération de médicaments et les membranes lipidiques pour implantations.

Enfin, nous discuterons des différents éléments collectés dans les deux premières parties.

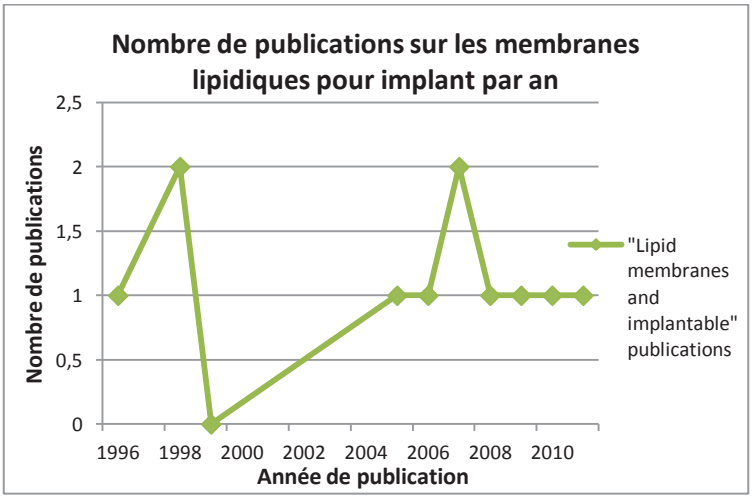
De manière générale, la littérature dans le domaine de la recherche sur les membranes lipidiques est en croissance avec des tendances et taux de publication différents selon le type d'application. Le taux de croissance des publications pour chacune des principales catégories d'applications est illustré en Figure 1.



a)



b)



c)

Figure 1 Graphique montrant l'évolution du nombre de publications par an pour chacune des principales applications des membranes lipidiques selon ISI Web of Science. a) Nombre de publications sur le POC. b) Nombre de publications sur les "liposomes and encapsulation" et "liposomes and drug delivery". c) Nombre de publications sur "Lipid membranes and implantable".

Pour les outils POC, la croissance est exponentielle, alors qu'elle est stable pour les publications relatives aux liposomes (à la fois les publications sur « encapsulation » et « drug delivery »). Le nombre de publications relatives aux membranes lipidiques à des fins d'implantations est assez stable, aucune réelle croissance n'est notable. De plus, le nombre d'articles pour chaque catégorie d'application est très disparate. Ceci est mieux illustré sur la figure suivante.

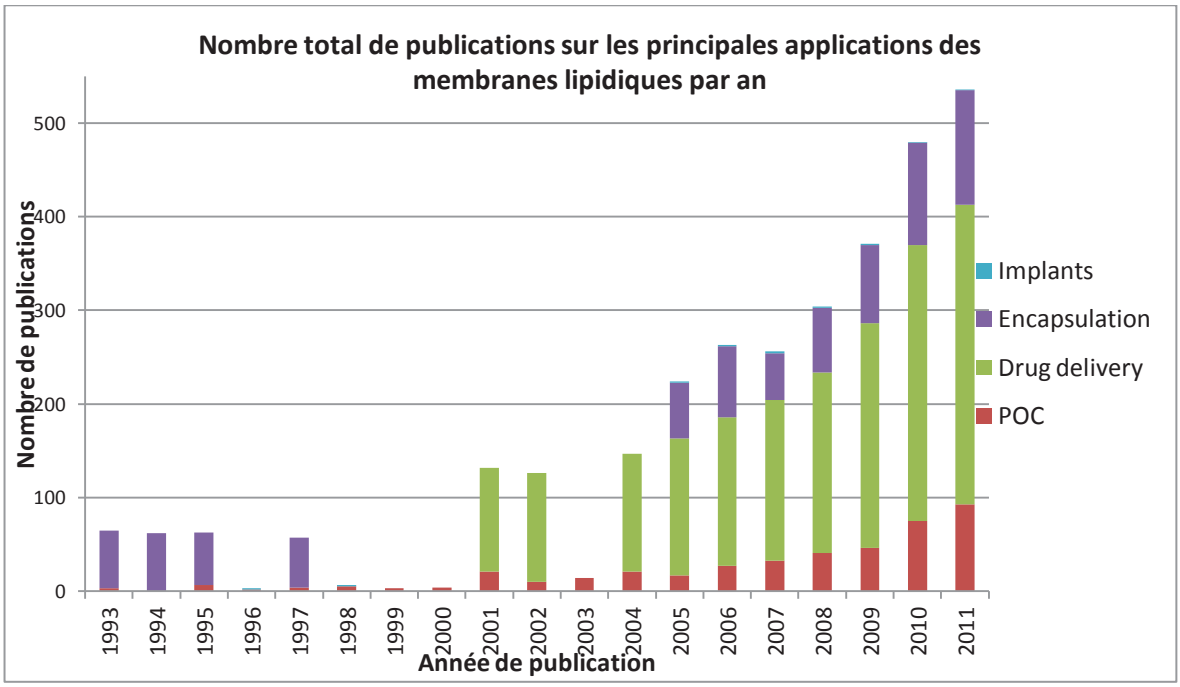


Figure 2 Graphique illustrant l'évolution du nombre de publications par an pour chaque catégorie d'application des membranes lipidiques et pour l'ensemble de ces catégories.

La recherche dans le domaine des applications implantables des membranes lipidiques est relativement insignifiante, alors que l'utilisation des liposomes pour l'encapsulation et la libération de médicaments est un champ très investigué. De manière générale, le nombre de publications sur les membranes lipidiques et leurs applications et donc la recherche dans ce domaine sont croissants, en particulier depuis les 10 dernières années.

The cell plasma membrane is a sophisticated nanostructured lipid matrix composed mainly of a phospholipid assembly that provides a diffusion barrier between the intracellular cytoplasm and external environment of the cells (1). This lipid matrix is also able to maintain cell morphology and provides a scaffold for integration of specific proteins and glycoproteins which facilitates the key-role of the cells plasma membrane in the cell adhesion process and in communication pathways between cells. The transport of electrolytes and other molecules across the plasma membrane, termed membrane transport, is fundamental to the life of a cell. The viability of the cell is highly dependent on the flows of electrolytes and water to generate and change ion gradients and the selective exchanges of molecules across the plasma membrane (2). The cell plasma membrane provides a very attractive model and basis for the development of biotechnological

devices, especially considering that the integrated membrane proteins are the target of more than 50% of drugs (3) (4) (5) (6) (7).

Phospholipids have the two distinct parts of hydrophilic head-groups (which are polar) and hydrophobic tails (alkyl chains). This specific arrangement provides them the property of being able to spontaneously assemble into a continuous monolayer when in the presence of an aqueous medium. The plasma membrane comprises 2 such monolayers to from a lipid bilayer with the hydrophobic sides of each monolayer facing each other. This results in the orientation of the hydrophilic head-groups being towards the aqueous medium and hence forms the outsides of the bilayer. Membrane proteins incorporate into that lipid bilayer membrane and maintain their structure and functionality. (8)

The lipid bilayer has been widely accepted as being the structural element of biological membranes. Real biomembranes are extremely complex with many types of lipids, an asymmetry between the two leaflets, and a highly complicated tangle between its components, domains and cytoskeletal elements. But even if they represent a very simplified model, lipid membranes can mimic the natural membranes and are thus named biomimetic membranes.

Also, one can wonder: **is there a way to take advantage of the capacity of lipid membranes to mimic cell membranes for therapeutic applications?**

Throughout this thesis, we will try to answer this question with technical explanations and concrete examples.

In a first part, we will see the *in vitro* applications of lipid membranes, with a description of POC, then transducers and then technical details of how lipid membranes are incorporated into biosensors, based on the literature background. In a second part, we will focus on the *in vivo* applications of lipid membranes, still on the basis of the literature background, including liposomes for drug encapsulation and delivery, and lipid membranes for implantable purposes.

As a final point, we will discuss the different elements collected along the two first parts.

Generally, the literature in this field of research is growing with different trends and publication rates according to the type of application. The publication growth rates for each main category of application are illustrated on Figure 1.

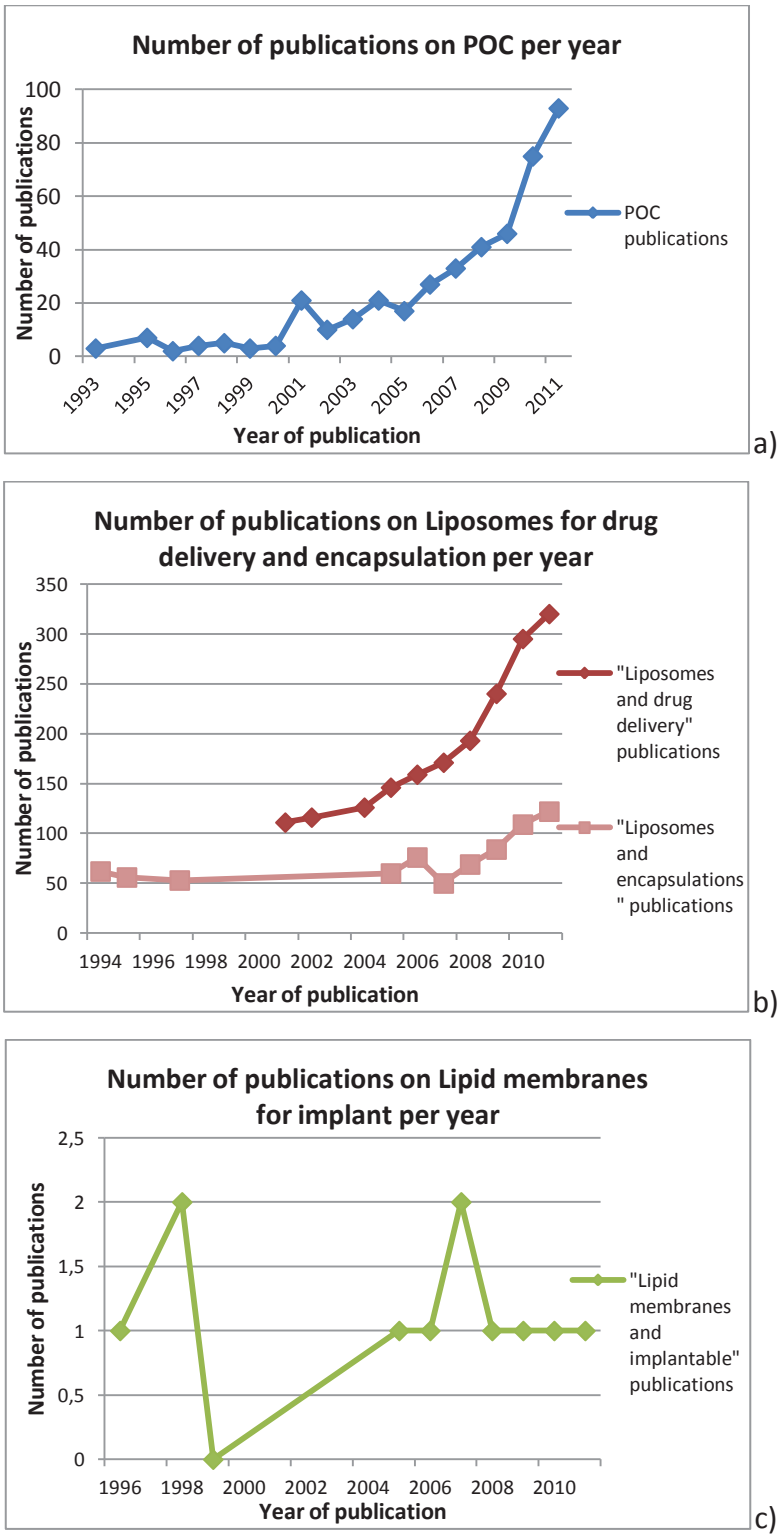


Figure 1 Graphs showing the evolution of the number of publications per year for each main application of lipid membranes according to ISI Web of Science. a) Number of publications dealing with POC. b) Number of publications dealing with "liposomes and encapsulation" and "liposomes and drug delivery". c) Number of publications dealing with "Lipid membranes and implantable".

For POC devices the growth is exponential, whereas it is steady for liposomes-related publications (both "encapsulation" and "drug delivery" targeted publications). The number

of publications dealing with lipid membranes for implantable purposes is quite stable, no real growth is noteworthy. Moreover, the numbers of papers for each category of applications is very disparate. This is better illustrated on Figure 2.

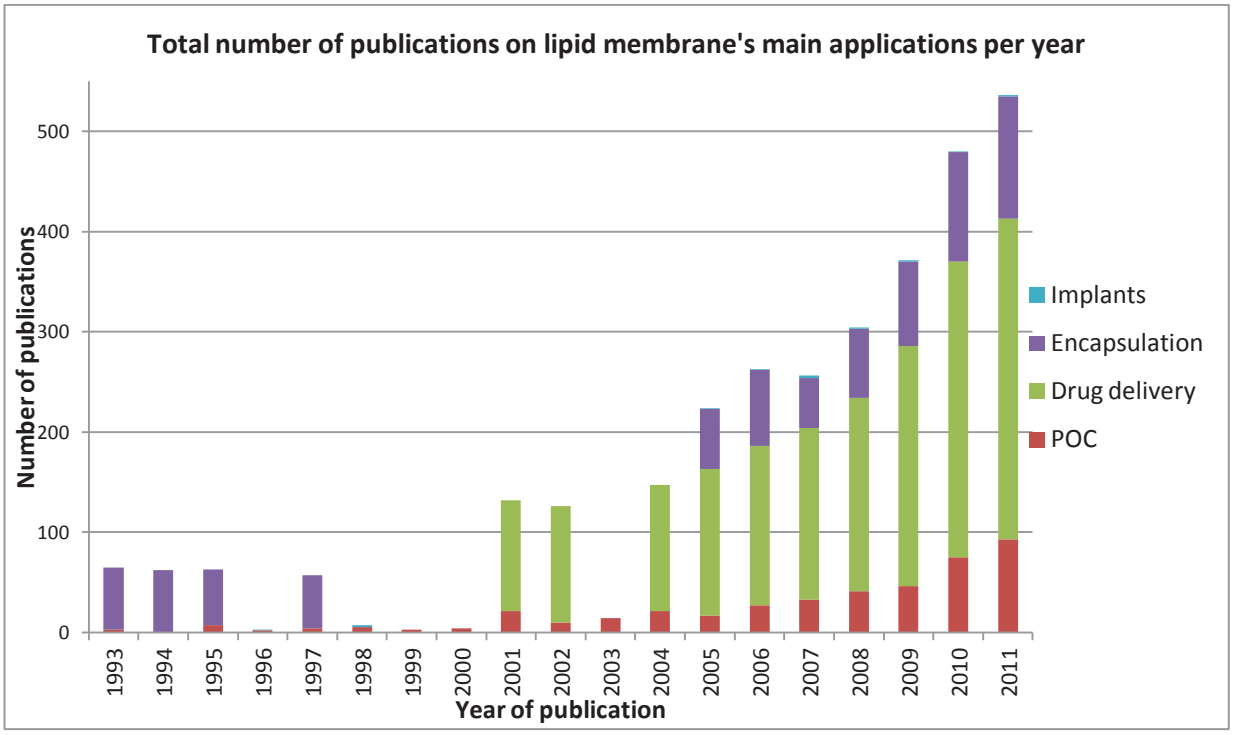


Figure 2 Graph showing the evolution of the number of publications per year for each category of lipid membrane application, and all categories together.

The research in the field of implantable applications of lipid membrane is quite insignificant, whereas the use of liposomes for encapsulation and drug delivery is a well-investigated area. In a general manner, the number of publications on lipid membrane applications and thus the research in this field is booming, especially for the last 10 years.

1. IN VITRO THERAPEUTIC USES OF LIPID MEMBRANES

The current trend in medicine is to move from curative intentions to a predictive and personalized support of the patient. The feasibility of this transition depends mainly on the existence of multianalyte point-of-care (POC) diagnostic tools in order to bring the analysis of clinical samples from centralized laboratories to the consulting room. This transition is often called "bench-to-bedside" analysis. Although, clinical laboratories provide a valuable service for diagnostic purposes, the priority is now to bring the diagnostics and the care closer to the first point of patient contact, in both temporal and physical dimensions. The development of new technological tools like POC testing devices enables the achievement of patient-proximity diagnostics. (10)

POC testing can be defined as an analytical integrated testing outside the normal central laboratory using devices that can easily be transported and employed wherever the patient is (11) (12). All POC diagnostic devices should share the following characteristics: (13) (14) (15) (16)

- *Cheap*: low manufacturing cost of manufacturing and low selling price
- *Obvious*: ease of use for anyone, including non-professional
- *Miniaturized*: to ensure their portability
- *Real-time measurement*: at the point of care, no transport of the samples
- *Automated*: built-in sample preparation, no handling except the addition of the sample to the device, ease of result interpretation
- *Robust:* resistant to difficult conditions, no "cold-chain" required
- *Quick*: short delay in obtaining results
- *Specific*: certainty of the results required to permit the deduction of a diagnostic
- *Multiplexed*: for a multi-analyte high-throughput testing
- *Unobstructive*: noninvasive collection procedure to reduce the fear of analytical tests
- *Sensitive*: requirement for only small volume of samples
- *Self-contained*: ease of disposal, reduced risk of cross-contamination of the sample.

The rapid and accurate measurement of cells, pathogens and any biomarker in biological samples has always been one of the major challenges in medicine. The expected long-term outcomes of POC testing are a better support of the patient, the implementation of the best adapted treatment, and thus shortening the duration of the hospital stay owing to earlier diagnostic and more effective therapies. The compliance of the patients should also be improved and the healthcare cost should be decreased. In summary, the principal aims of POC devices are to improve: (17)

- Quality of care
- Health outcomes
- Financial outcomes

From a technical point of view, most of the POC devices use blood samples, but diagnostics that use oral samples, such as saliva, are under investigation. For example, a saliva sample would permit the use of noninvasive assays, which is one of the main criteria for an ideal POC test. (18)

Although if the vast majority of the medical analyses are still conducted in classical laboratory facilities using a large equipment (19), POC testing devices constitute a growing portion of the cellular and molecular in vitro analysis market. For example, the European in-vitro-diagnostic (IVD) market was evaluated at $ 34 billions in 2007 with an annual growth-rate of 4-5%, and the portion held by POC was estimated at $ 1.3 billion in 2009 with a growing trend (20) (21). The graphic below illustrates (Figure 3) the growth of the proportion of POC testing usage versus laboratory testing.

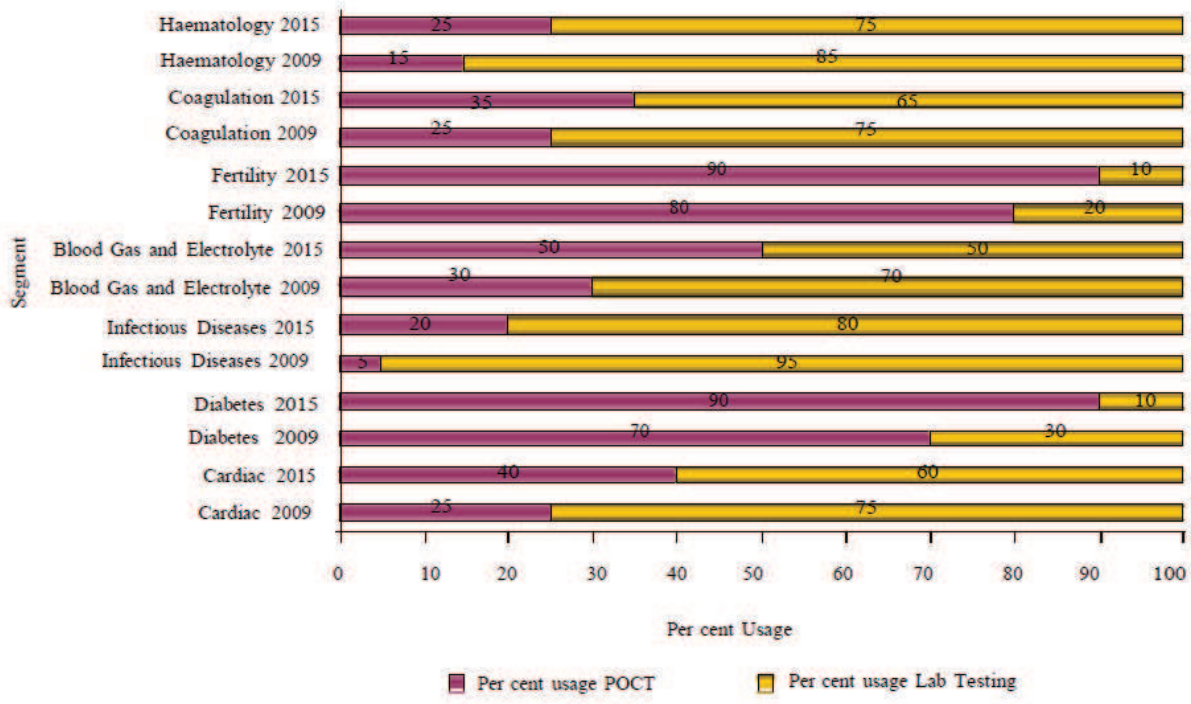


Figure 3 Illustration of Pera Innovation 2011 (21) showing the total European POC testing market in percentage of use versus the european Lab Testing market in percentage of use, for different segments in 2009 and predicted for 2015

As we can see on this bar chart, on current trends POC testing is predicted to take a larger share of the medical diagnostic analysis. This is best illustrated currently with diagnostics for diabetes and pregnancy where POC is already widely used in the form of respectively "finger pricker devices" to measure blood glucose levels and pregnancy test based on urine samples.

Biosensors are the key elements that enable POC devices to detect specific biomarker(s). These particular types of tools have an increasing impact in modern medical care (22). They will be described in more technical details later, but it is important to note that they have permitted the consideration of worldwide diagnostic. Their design is such that they are useful for both developed and underdeveloped countries, since the aim of biosensor development is to have a miniaturized portable molecular *in vitro* diagnostic device able to carry out the tasks of a large laboratory.

In fact, POC is the main aspect of biosensors applications. Considering that home diagnostics is in essence a type of POC, Figure 4 indicates that POC is a considerable proportion of the biosensor market, representing about 68% of the revenues.

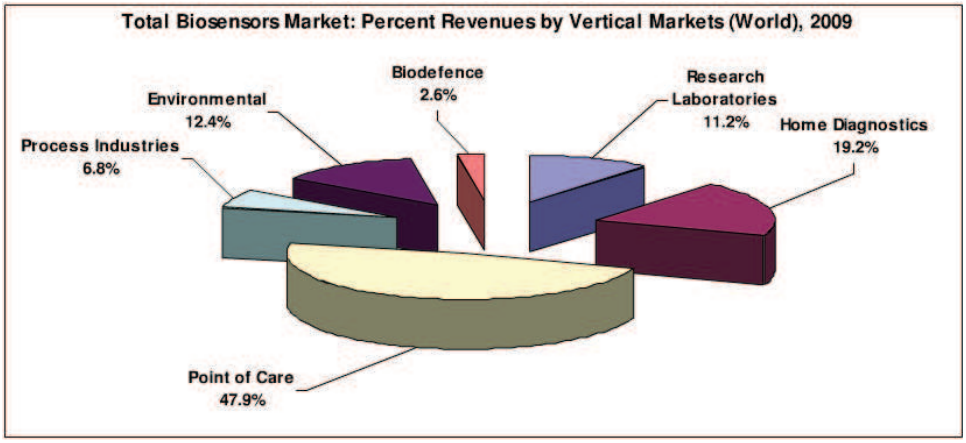


Figure 4 Illustration of Rajender Thusu for *Frost and Sullivan* (129) showing the total world biosensors market in percent of revenues by vertical markets for 2009

Different types of biosensors exist to detect different types of target biomolecules. Figure 5 shows the portion of the biosensor market held by each category of biomarkers, i.e. target biomolecules.

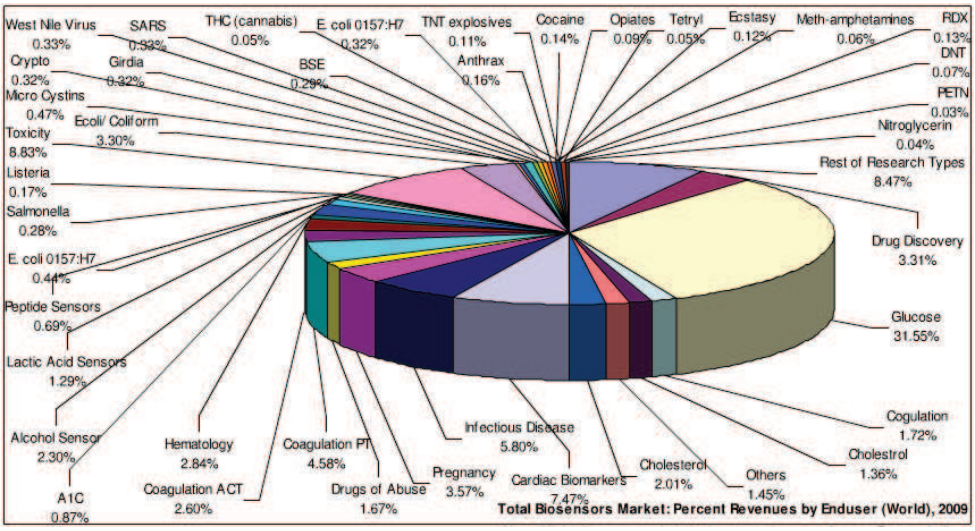


Figure 5 Illustration of Rajender Thusu for *Frost and Sullivan* (129) showing the total world biosensors market with the percent revenues by end-user application (detection target) for 2009.

Corresponding to majority of diabetes testing being conducted by POC (Figure 3), biosensors for the detection of glucose represents a major proportion of the revenues of the total biosensor market (31.55%). Biosensors for pregnancy testing also occupies a significant place, but the revenues generated are not as significant as one could expect by looking at Figure 3, where they represent around 80% of the POC fertility tests, they correspond to only 3.57% of biosensor market revenues. This is probably is due to the definition of biosensor used to compile the information in Figure 3, since pregnancy tests are mainly based on “strip-based” color changes from blood or urine samples rather than electronic biosensors.

One can also notice that cardiac biomarkers are an important and lucrative target for biosensors (7.47%).

Due to the growth in the need for POC testing devices, there is a continually expanding market for biosensors. Biomimetic lipid bilayer membranes provide an attractive system for enhancing the sensitivity and manufacture of biosensors for POC devices. Numerous biosensor designs rely on the use of biomimetic lipid membranes, which we will discuss in the following sections.

1.1 TYPE OF BIOSENSORS

1.1.1 DEFINITION

A biosensor can be defined as a miniaturized compact device incorporating a transducer within which a biological sensing element is integrated. The device consists of three main components: transducer, sensing component and readout (23) (14) (11). These devices are meant to allow highly sensitive and specific, simple, fast, accurate and response proportional, measurements. Different types of samples can be analyzed, including whole cells, bacteria, antigens, antibodies, enzymes, various proteins and other biomarkers (24). A major advantage of a biosensor is that it enables the diagnostic test to be undertaken anywhere: at the hospital, at the physician's office, at home, as well as in rural or underdeveloped countries. Thus biosensors can be classified as point-of-care devices (POC) (25) (26).

They are designed to be portable and cheap. In fact for a biosensor to be a viable POC device, it has to provide a combination of rapidity, reliability, simplicity, cost-effectivity, and the ability to detect low concentrations of the target analyte (27). They have many applications in various fields such as clinical diagnostics, drug discovery, research, environment and food monitoring. Here we choose to focus on the clinical diagnostic. For this purpose, it is preferable for a microfluidic flow system to be added as part of the sensor. Indeed, it enables the samples (e.g. blood) to be directed onto the sensor surface, with the flow of sample increasing the sensitivity of the biosensor.

Different types of transducers are used: (14) (11) (28)

- Electrochemical

- Piezoelectric
- Optical
- Thermometric
- Magnetic

The number of publications on biosensors is growing as is the interest and the popularity of these tools. However their marketing and commercialization haven't grown in the same proportion possibly due to the high production costs. However cheaper mass-production can introduce the problem of lower quality, accuracy and sensitivity of the measurements from the biosensor. Therefore one has to strike a balance between the different parameters required for manufacturing a biosensor to detect a biomarker (29).

As we have seen previously on Figure 3 and Figure 5, currently blood glucose monitoring dominates the market for biosensors (11).

1.1.2 Types

1.1.2.1 Types of transducer

The transducer converts the signal event such as biological and chemical binding into an electrical signal which can then be read and interpreted by the user or a health-care professional (e.g. physician, pharmacist, nurse).

It is important to note that label-free transduction means that the transducer does not require a reporter molecule to detect the presence of the target analyte. Thus, the samples do not need to be modified prior to testing and real-time measurement is possible (23).

Different types of transducers are used, (14) (11) (28), including electrochemical, piezoelectric, optical, thermometric and magnetic transducers and their subtypes as illustrated in Figure 6 below.

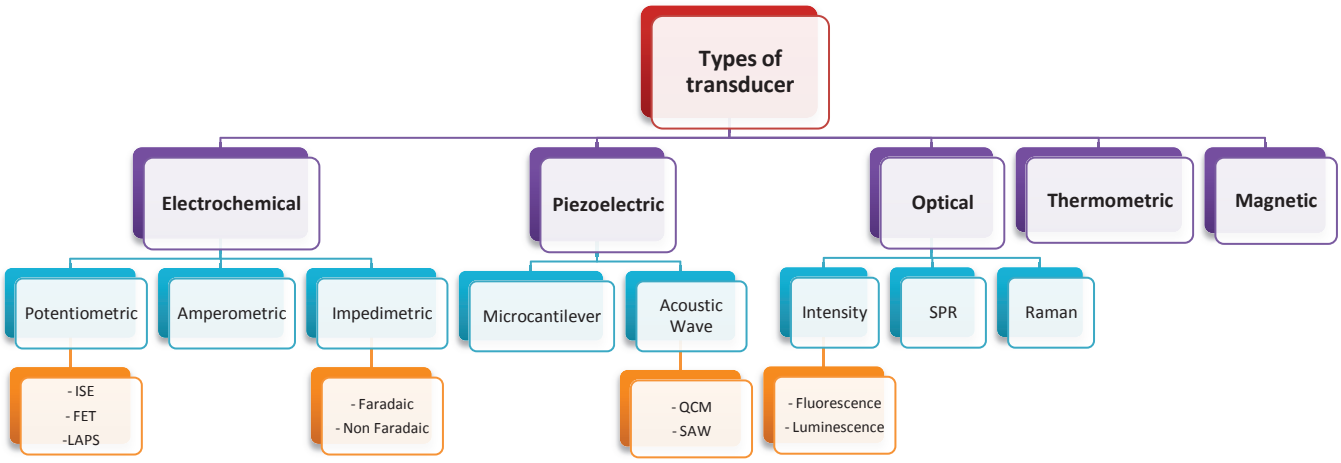


Figure 6 Summary of the different types of transducers which can be used for the design of biosensors.

ELECTROCHEMICAL TRANSDUCERS

These are the most widely investigated transducers. The functionality for a high variety of biomolecules has been proven and reported in many publications (30) (13). Electrochemical biosensors can easily be designed for POC diagnostics, and allow the design of a device to be portable, simple to use and disposable with a low cost of production. Diversity is provided for the selection of the size, shape, components, detection methods and surface (31). Most of the electrochemical biosensors would be suitable for real-time analysis. These types of transducers are of great value in situations that require low cost, portable equipment for a quick analyse, but accuracy and detection limits are not crucial (25) (32).

Nonetheless, most of electrochemical transducers also still require the use of a reporter molecule, unlike the label-free transducers, which is a reason why this kind of system has been slightly overlooked recently in preference for other systems. Also electrochemical transducers have other deficiencies that include problems with sensor fabrication and sensitivity. Indeed, the need for high-throughput manufacture for a widespread application is hard to attain. As seen previously, the combination of both a cheap manufacture in bulky quantities and a high selectivity and sensitivity of the device is not simple to achieve. Furthermore the sensitivity of electrochemical sensors is altered likewise by the nonspecific binding of proteins to the sensing platform.

Despite all of the known problems, the best example of a successful electrochemical biosensor is the glucose biosensor created to monitor the glucose blood level of diabetic patients (33).

There are two main categories of electrochemical transducers, which are potentiometric, amperometric and impedimetric (14). With potentiometric being the most popular electrochemical sensor (30).

- *Potentiometric*: measures the difference of potential (voltage) between either two reference electrodes separated by a membrane or a working and a reference electrode when there is no flow of current between both electrodes. The voltage is due to a specific reaction occurring at the sensor's surface which is generated by a change in the pH or in the redox or ionic state. They are the reference for immunosensing measurements where an enzyme-labelled antibody is bound to the electrode surface. The different categories of potentiometric biosensors:
    - Ionselective electrodes (ISE)
    - Field effect transistor (FET)
    - Light addressable potentiometric sensors (LAPS)
- *Amperometric*: measures the current generated at the working electrode, maintained at a specific potential relative to a reference electrode. This current is due to a redox reaction on the surface of the electrode. For example, when used as an immunosensor the current is proportional to the number of bound and labelled secondary antibodies (25) (30). The glucose biosensor, which is the most widely employed of all the biosensor, as mentioned above, is an amperometric biosensor (33).
- *Impedimetric:* measures the ratio of voltage to current between two electrodes. This ratio is modulated with the resistance and capacitance which are two components generating a resistance to current flow. Impedimetric transducers can be divided into two subcategories:
    - Faradaic detection
    - Non-faradaic detection

  Studies have shown that the binding of proteins or bacteria to the electrodes modify the value of the impedance. As a result, impedimetric biosensors also known as impedance biosensors would be appropriate as POC diagnostic devices. For

example, in 1988 an impedance biosensor was designed in order to detect acetylcholine. (25) (30) (13) (33)

PIEZOELECTRIC TRANSDUCERS

These are also called mass-sensitive transducers. The property of piezoelectricity was discovered in 1880 by Pierre and Jacques Curie (33). The first use of the piezoeletric effect was postulated by Raleigh in 1885 and then in 1959 Sauerbrey created the first piezoelectric biosensor. (24) (27) Currently, a number of sensors using the piezoelectric method are available on the market and used for various medical applications. For example, they can be used for immunosensing in order to detect cancer markers (34).

The piezoelectricity was first observed as an electrical potential generated by a pressure exerted on quartz plates in a particular direction. Piezoelectric sensors can be categorized as label-free transduction systems since the measurement is direct physical change and no label or tag is needed to detect the interaction between the biomarker and the receptor on the sensor surface (25).

Widely used as on-the-bench testing tools in a stable laboratory environment, piezoelectric biosensors are not suitable for field testing applications since their performance and sensitivity is strongly influenced by small external fluctuations. (28)

Piezoelectric transducers are divided into different subgroups:

- *Microcantilevers (MC):* only a part of these sensors is based on the piezoelectric effect. In this case, the MC transducer has a piezoelectric material integrated close to the upper surface of the cantilever. In a general manner, a change in the mass upon the sensor surface results in an observable and measurable change in the resonant frequency of the cantilever. The detection system does not need to be as powerful as for other types of transducers and it can be partly included within a chip. However piezoelectric MC sensors are limited in application by a complex manufacturing process. (25) (30)
- *Acoustic wave sensors*
    - *Quartz crystal microbalance (QCM)*: primarily used as gas sensing devices, this type of piezoelectric sensor includes a quartz crystal coated with a gold

electrode and two electrodes. That whole sensor constitutes a microbalance which is sensitive to changes in the mass on its surface. The QCM is driven at its resonant frequency and the sensing electrode is grounded so that the electrochemical interactions at the electrode are minimized. A variety of pathogens and biomarkers can be detected using QCM biosensors, including the CRP, a cardiac specific biomarker. (30) (27)

- *Surface acoustic wave (SAW)*: similar to the QCM, SAW are primarily used as gas sensing devices. SAW are mechanical vibrations propagating confined to the surface of piezoelectric crystals when excited by an electrical signal driven at the resonant frequency. This type of sensor is sensitive to changes in the mass on the surface of the sensor, but also to the viscosity of the material applied to the surface and the temperature of this surface. (30) (13)

Both the QCM and SAW, techniques share similarities regarding use and sensitivity, but the QCM is more popular at the moment. This might be explained by the complexity in the design of SAW in comparison with QCM design.

OPTICAL TRANDUCERS

The binding event on the sensor surface between receptor and biomarker is directly detectable as an emission of some form of light signal (25). This type of transducer is the "gold standard" in immunoassay (30).

Otherwise, optical sensors are commonly used for the detection of drugs, alcohols and pesticides (31). There are several test kits commercialised, with the home pregnancy test as the most well-known example. Several subgroups of optical sensors can be described:

- *Intensity methods*: the signals are easily produced; acquired and analysed by the means of simple and inexpensive apparatus, which make them attractive to be used in biosensors. There are two main sub categories: (30)
    - *Fluorescence*: the signal is produced by the excitation of a label molecule on the sensor's surface. The fluorophore label's excitation generates a fluorescence emission. Qualitative and quantitative measurements of

biomarkers can be achieved. Fluorescence is also the most common type of labelling technique (13).

- *Luminescence*: the signal is produced using a luminogenic substrate solution which undergoes an enzymatic reaction (chemiluminescence) or using an electrode on the surface of which a potential is applied leading to a highly energetic electron transfer of a luminescent compound set near the electrode surface (electrochemiluminescence or ECL). Immunoassays with a luminescent transducer were one of the first and most accurate means of biomarker detection (29). Although the efficiency and efficacy of chemiluminescent biosensors as POC devices has been proven, unlike fluorescent techniques, the measurements remain mostly qualitative. As far as the ECL biosensors are concerned, a quantitative measurement is possible but the material is cumbersome, not portable and so not suitable for POC applications. Nonetheless, they have been proven to be effective to detect cardio biomarkers.

- *Surface Plasmon Resonance methods (SPR)*: SPR rely on an excitation light and a metallic surface and is the most well-known example of an optical transducer for a biosensor (34). The signal is modulated by the thickness of the metal and the angle and wavelength of the light. The significant advantage is that the transduction is label-free and the SPR transducers allow quantitative, rapid, real-time measurements requiring only a small amount of sample. The main disadvantage is that no distinction can be made between a target biomarker specific and non-specific binding onto the sensor surface. Nonetheless, SPR is used to achieve a wide range of protein-membrane interactions studies due to recent advances in the modification of surfaces to mimic membranes. SPR even provides one of the most important techniques for studying the biochemical aspects and binding kinetics of protein-protein molecular interactions and protein-small molecule. Emerging SPR sensors are being developed to provide a mean to study specific membrane proteins of interest for the development of drugs or biomarkers. Their design is extremely studied and documented, based on patterned nanostructures. (30) (35) (5)
- *Raman methods*: these are label-free techniques using light spectroscopy. A specific spectrum of the target molecule is produced by the inelastic scattering of the incident photons on the surface of the molecule. Following the immobilisation of the target molecule on a rough surface of metal, the Raman signal can be

enhanced in the technique known as Surface Enhanced Raman Spectroscopy (SERS). The exact mechanism which generates this enhancement remains unknown, but the spectral signal transmitted is linearly proportional to the number of target particles. The detection limits of such techniques are several orders of magnitude lower than most of the fluorescence intensity transducers. SERS can be used for the quantification of multi-analytes. It was originally developed as an alternative to fluorescence labelling in biosensor development. (23) (30)

THERMOMETRIC TRANSDUCERS

Although rarely used in biosensors, the thermometric transducers are able to measure a change in the temperature. Enzymes are combined with sensors that are sensitive to temperature. When the target is exposed to these enzymes on the surface of the sensor, the enzymatic reaction generates heat that can be measured by the sensor. (11) (36)

MAGNETIC TRANSDUCERS

It is a method of transduction that is rarely used and relies on the magnetic properties of paramagnetic particles (PMPs), which are applicable as both a reaction label and a transportable sensor surface. In essence PMPs consist of an iron oxide core coated with a polymer layer that is biocompatible. The motion and polarization of the nanoparticles can be driven by an imposed magnetic field. The signals are produced by a sensor detecting these magnetic particles. Microfluidics could help reducing the time of PMPs assays which would be an interesting development for a use as POC devices. (30) (13)

1.1.2.2 TYPES OF SENSOR

In addition to the type of transducer used at the heart of the biosensor, there are major differences between the various overall designs of the biosensor platform. In this section we will review the main categories.

ANTIBODY-BASED BIOSENSORS OR IMMUNOSENSORS

The proof of concept of immunosensors as diagnostics tools was first demonstrated in 1987 through a fibre optical sensor used for the detection of a chemical carcinogen. Since then, numerous assays have been performed that established that immunosensors are able to reach the required sensitivity and selectivity to ensure they remain useful for diagnostic applications. Many devices have been developed using different transduction techniques for a broad range of targets. Electrochemical, piezoelectric and optical transducers are the most frequently employed and the literature is full of articles on sensing platforms used in immunosensor technologies. (14) For instance, immunosensors with QCM as transduction systems are reported in many publications and represent number of products in the biosensor market. Immunosensors are principally made with monoclonal and polyclonal antibodies. However, only a few diagnostic kits based on recombinant antibody technology are commercially available. Few publications deal with recombinant immunosensors, but the interest of researchers for such techniques is growing.

In a general manner, antibody-based biosensors always work in the same way. An immunoreaction between the antibodies of the sensor and the target antigen occurs which is processed into an analyzable signal by a transducer. The signal is then correlated to the concentration of the biomarker with a standard curve. (30) For these reasons, a "good" immunosensor has to be able to efficiently capture the target and to release a perceptible signal. This signal is most often produced by a label on a second antibody used for a second immunoreaction forming a sandwich with the target in between two antibodies. The type of label is depending on the transduction system chosen.

Immunosensors remain the "gold standard" as biomolecular event detection system for single-analyte (11). Devices for the detection of multiple analytes and analysis of complex samples often require more advanced technologies. Indeed, the antibody-based method has the significant limitation of cross-reactivity, which is a common phenomenon with immunochemical interactions (28). The technique of immobilization used also plays a great role in the specificity and sensitivity because it affects the orientation of the molecules on the solid support. The orientation directly influences the specific binding constants. (27)

A particular sort of immunosensors is designed with peptide aptamers, or "synthetic antibodies", which constitute a substitute to antibodies or other proteins. These immunosensors perform label-free detection tests. Aptamers are easy to produce and prevent cross-reaction. These two main advantages make them greatly attractive for the production of sensors. (33)

NUCLEIC ACID-BASED BIOSENSORS OR GENOSENSORS

They are mostly tailored for the detection of nucleic acids. One can define them as analytical devices incorporating an oligonucleotide whose sequence of bases is known or a complex sequence of nucleic acids combined with a transducer.

Nucleic acid-based biosensors might not be used to detect DNA or RNA fragments but rather to detect biological or chemical species. In this case, they are not called genosensor. But these types of sensor are uncommon.

A probe made of nucleic acids is tethered onto the surface of the sensor and used as the recognition element of the target molecule. The phenomenon of recognition consists of a hybridation between the probe's bases and the target DNA. The detection of this event can be performed using almost any sort of transduction technology requiring a label or not. Qualitative and quantitative results on the DNA analyte can be acquired (33).

If no previous polymerase chain reaction (PCR) is realized on the analytes, the target's concentration might be below the detection limit, so might be undetectable. In fact, genosensors' targets, namely microorganism genomes, are packaged inside the cell membrane *in vivo*. Thus, their concentration is very low, possibly too low to be diagnosed without an amplification by PCR.

Nucleic acids are easy to synthesize and functionalize. Artificial strands of oligonucleotides are called aptamers. Unlike the peptide aptamers seen previously for the immunosensors, these aptamers are single-strands of DNA or RNA, usually short, and therefore are DNA or RNA ligands which can be used as probes. DNA/RNA aptamers can also be generated against different kinds of molecules such as drugs or proteins.

With immunosensors, genosensors are the most popular sensors. They are studied by many research groups and the subject of many publications.

ON-CHIP PCR BASED BIOSENSORS

Previously, we have seen PCR as a preliminary step to the use of the biosensor, but PCR can also be used directly as a technology for a biosensor. PCR aims to amplify short DNA regions *in vitro* by the means of an enzyme-driven process. Millions of DNA copies can thus be created. The miniaturization of PCR onto a single-use chip could optimise the technique to reach its maximum potential. Indeed, through the use of microfluidics, rapid thermal cycling could be achieved by rapid heat transfer. Besides, due to the small size of the device a more homogeneous distribution of the temperature and an improved yield of the PCR would be possible. The cross-contamination risk would be reduced. Moreover, this type of tool would be useful for POC testing, for example in the diagnostic and follow-up of an infectious disease (12).

VIRAL BIOSENSORS

Viral particles such as Herpes simplex virus and Adenovirus have the ability to trigger the assembly of magnetic nanobeads which can be used as nanosensors for the detection of clinically relevant viruses. This type of biosensor is of great interest since it is more sensitive than ELISA methods and cheaper and faster than PCR methods, with fewer artefacts.

GOLD NANOPARTICLES (AUNPS) BIOSENSORS

These particular particles have the property to assemble onto the surface of the sensor only in the presence of a complementary target. Short fragments of DNA can easily be attached onto the surface of the AuNPs. If multiple strands are attached onto the surface of the sensor, millions of DNA sequences can be detected simultaneously. Their synthesis and functionalization with DNA or other biomolecules is easy to achieve. One of the main advantages of the gold nanoparticles is that they can be detected by various techniques. Consequently they represent a label of choice and the interest in these sensors has increased within the last few years. Recent developments of AuNPs-based tools for

molecular diagnostic are promising in terms of specificity and sensitivity, multiplexing capacity and quickness. Gold nanoparticles sensors show a great potential for the development of new POC tests. (37) (38)

CANTILEVER BIOSENSORS

A cantilever has the capability of transducing a chemical reaction into a mechanical motion at the nanoscale level. This motion can then be measured directly by a light reflected from the cantilever surface, in-effect to monitor surface stress changes. Cantilever technology is a label-free technology since the measure of the target molecules is direct. It constitutes a new method of DNA and protein microarrays and a valuable alternative to PCR. Cantilever biosensors offer many advantages besides the fact that they allow a label-free measurement, including fast screening of DNA sequences for the recognition of single-nucleotide polymorphisms, oncogenes or genotyping but also real-time monitoring of clinical parameters for POC utilizations. (25) (37)

AFFINITY SENSORS

The target binds by affinity to a probe immobilized on the surface of the sensor. This probe gives selectivity to the sensor. The transduction system used is an impedimetric transducer. The sensing surface is localized in the gap between electrodes or directly onto the electrode surface. The space between the electrodes is modified for affinity binding. When a target molecule links to the probe, changes in the resistance or capacitance occur which results in analyzable changes in the impedance. (33)

MOLECULAR IMPRINTING SENSORS (MIP)

The sensor comprises artificial receptors which have the ability to follow the shape of the target molecule. This can be performed, for instance, by the polymerization of the receptor around its target. After the target molecule is removed following the polymerisation, the receptor remains with a space matching very accurately with the size and shape of the molecule. The sensor then specifically recognizes this mold of the target. (33)

NANOWIRE SENSORS

This technology, based on metallic or semiconducting particles, as recently been reviewed due to their interest for biomolecular detection. These particles have a small size and electronic, optical and magnetic properties which make them suitable for almost any kind of transducer. (39) Two types of nanowire sensors can be distinguished:

- Optically encoded nanowire: also named barcoded nanowires, they provide a tool for highly sensitive multiplexing assays using readily available instrumentation.
- Semiconductor nanowire field-effect transistors (FETs): this sort of nanowire sensors allows the detection of a maximum of three target molecules at once (multiplexing) with a high sensitivity and no need for a label.

LIPID MEMBRANE-BASED BIOSENSORS

The interest in such tools is permanently on the rise. One entire section later in this thesis is devoted to them.

The detection of several targets at once is sometimes possible, which is called simultaneous multi-marker detection or multiplexing. For this purpose, more than one transducer might need to be incorporated into the sensor (27). Many are based on the immunosensor principle with several ELISA assays grouped on one chip in order to detect several target molecules, achieved usually with different clones of antibodies specific to a particular biomarker (25). The most commonly used category of transducer for multiplexed assay is the fluorescence intensity system coupled with immunoassay. This kind of multi-marker detection as well as alternative methods have been demonstrated in the literature (30).

Multiplexing is also called multi-analyte testing. Besides the detection of several biomarkers at once, it can also refer to devices able to make several parallel measurements

of the same analyte at once or devices which can detect and measure a range of biosamples simultaneously (40).

The design of multiplexed devices is one of the challenges of POC biosensor. They would be very interesting for the diagnostic of cancer: test of different cancer types. Infectious diseases also ideally necessitate a test capable to detect different pathogens, viruses or bacteria in order to establish an accurate diagnostic. Then, the question remains as to distinguish between the specific strain or type of virus or bacterium (20).

Nonetheless, one should not forget that the design of multiplexed biosensor currently still results in a very large piece of equipment and a complex set up which does not fit with POC diagnostic requirements. The development of multiplexed biosensor for POC diagnostic is thus still at an early stage.

1.2 Therapeutic applications of biosensors

Biosensors are already used for many therapeutic applications and should be developed more and more in the near future since many research groups focus their work on this type of technology in order to develop POC testing devices.

In this section, we will review the most well-studied therapeutic utilizations of biosensors. Obviously this list is not exhaustive and many other fields of application could be or are currently being considered.

Biosensors for the management of cancer

It is evident that cancers are one of the main health concerns worldwide and their management constitutes a major area of unmet needs. It is also important to consider that the earlier the detection happens, the highest are the chances of a positive outcome for the patient irrespective of the type of cancer. In other words, an early detection leads to early and more adapted support and follow up of the patient. In effect, it is almost the only way to improve patient survival and disease prognosis. Detection of cancer biomarkers by a biosensor is also a method of prevention of the disease. (11) (37)

One of the difficulties to design a functional biosensor is to find an appropriate target molecule. Since most of the biomarkers are not specific to a particular type of cancer, a combination of multiple markers detection can thus increase the diagnostic value. Simultaneous multi-marker detection systems are consequently interesting for cancer diagnostic and patient follow-up. It seems crucial to take into account all the knowledge acquired thus far on tumor molecular mechanisms and resistance to treatments in order to develop suitable POC diagnostic devices (41).

The most popular type of biosensor used in cancer management is a nucleic acid-based biosensor with an electrochemical transduction method for the detection of gene mutations of biomarkers and the detection of specific protein biomarkers. On the other hand, this technology is still at an early stage of development. (25)

Many articles that describe examples of biosensor for the management of cancer can be found. Here we chose to focus on one particular type of cancer which is prostate cancer (PCa) because it is one of the most commonly discussed in the literature (23). PCa is responsible for about 10% of the deaths from cancer. The marker of choice for this type of cancer is the level of serum prostate-specific antigen (PSA). Almost by default, the importance of this protein as an oncological marker is partially due to the absence of alternative. PSA is a serine protease produced by the prostate epithelium. It has a key-role in maintaining the seminal fluid in a liquid state. Currently no POC testing device exists. But a biosensor allowing home testing, would provide a major advance by eliminating the need for transportation of the sample, decreasing the waiting times, decreasing the cost for both the patient and the healthcare system, increasing quality-of-life for the patient and probably improve the clinical outcomes. Furthemore, recent developments in the field of biosensors technology keep bringing closer the creation of a POC testing device for PSA.

BIONSENSORS FOR INFECTIOUS DISEASES

In the context of an escalating threat of accelerated epidemic-to-pandemic-transition of worldwide spread outbreaks, decentralized diagnosis on the front-line would be helpful for the establishment of early public health responses. The morbidity and mortality attributable to infections remain noteworthy particularly in newborn babies (42).

An early, rapid and sensitive diagnostic is primordial for the management of an infectious disease. The main weaknesses of conventional diagnostic methods are the lack of high sensitivity and the time for getting results (37). POC tests allow near-patient real-time measurements which is a characteristic of major importance for routine infectious disease diagnosis. Indeed it is well known that the promptness of test results directly impacts patient outcomes since an adapted treatment can immediately be implemented. The utility of POC testing devices would be even greater in developing countries where diagnostic challenges are higher due to poor laboratory infrastructures and cost constraints. (12)

If an ideal diagnostic method for infectious agents had to be defined, the disposability would be one of the criteria required to avoid the risk of contamination. This criterion by itself ensures the commercial viability of cheap POC testing devices like biosensors. (29)

Similar clinical symptoms can be common to several infections due to different etiologic agents. For this reason the ideal biosensor would detect and accurately identify multiple pathogens and perform a subtyping. Multiplexing biosensors are thus of great interest to fulfil that ideal.

Moreover, to allow a successful decentralized diagnosis in hostile environment or developing countries, the ideal biosensor will need to require simplicity, economy, rapidity and accuracy all together. It has to be usable anywhere and by anyone.

The use of such tools will conversely lead to a limitation of the overuse of antibiotics. Indeed, the rapid diagnosis of viral agents would avoid the prescription a random antibacterial therapy and allow the most adapted empirical treatment can be utilised. Therefore, it would contribute to preventing the emergence of resistant strains and decrease the cost of the support associated with diagnostic procedures and hospitalization. The limitation of unnecessary treatments might also improve the compliance of the patients and limit side effects. (43)

Dengue fever is a good example of the requirement of POC testing devices for the diagnostic of infectious diseases. Potentially leading to a fatal hemorrhagic fever, four different forms of antigenically related viral serotypes can be responsible for the occurrence of the disease. The symptoms are non-specific and appear usually after around two weeks of incubation. Meanwhile viral particles arise in the blood, constituting an efficient biomarker. Clinical diagnosis is thus usually insufficient but can be

complemented by the use of an antibody-based technology based on dengue-specific antibodies. The use of such immunoassays is considered as a "gold standard". But this method still requires bulky material. The development of a corresponding sensor would be really useful.

Another example of deadly infectious disease, probably even more worrying, is AIDS. According to a quote from the World Health Organization (WHO) in 2008, HIV is responsible for the most serious infection challenge. A worldwide average of about 5700 people dies from AIDS each day. The use of POC testing devices could help for the diagnosis and monitoring. Such tools would provide ease of use and low cost, both of which are required particularly in underdeveloped countries. Biosensors have contributed to improve the understanding of HIV mechanisms. Now one could imagine the development of biosensors with anti-CD4-antibodies (for example) tethered to the surface of the sensor to detect CD4+ T-lymphocytes for HIV diagnosis. (44) (45) (46)

BIOSENSORS FOR CARDIOVASCULAR DISEASES

Cardiovascular disease (CVD) is at present one of the most prevalent medical conditions and is the cause of half of the deaths in the Western world. Each year, the conditions related to the disease and the health-care support cost billions of Euros to the European Union. Several biomarkers are needed because the disease is complex and thus one biomarker would not be enough for a definitive diagnosis. (30)

A representative example of CVD and the need of biosensors would be myocardial infarction (MI). The importance of laboratory tests in the diagnosis of myocardial infarction has been acknowledged for years. Since 1979, the WHO was already elucidating the important need for such fast and cost-effective tests (11). From a technical point of view, MI can be defined as the necrosis of cardiomyocytes resulting from a prolonged ischemia and causing irreversible damage to the heart. That damage can be limited by a quick, efficient and comprehensible diagnosis. Those criteria can be met by the combination of the measurement of specific cardiac biomarkers with the electrocardiograms (ECG). Indeed, the ECG reading by itself is not satisfactory due to the prevalence of false negatives. For this reason, in order to assist the physician in his decision-making, the assessment of specific cardiac biomarkers levels is required. POC

tests with measurements at the first contact with the patient would enhance the diagnosis, treatment and patient care and considerably reduce the expenses for the public health system. Troponin I (cTnI) is seen as the "gold standard" biomarker of MI specifically owing its presence during myocardial damage. Biosensors that detect cardiac biomarkers, including the troponin, are already commercially available and mostly based on an immunoassay technology. The result can be obtained within 5 to 20 minutes.

BIOSENSORS FOR ALLERGIC DISEASES

An allergic disease can be detected by *in vitro* blood tests to determine the fact that an IgE antibody sensitization occurred. The serologic assay method, which is based on immunoassay technology, to detect IgE was established about 40 years ago and remains almost unchanged since. Immunossays for allergic diseases have been developed subsequently to the discovery and identification of the "skin sensitizing antibody" as being IgE in the 1960s. Nanotechnology permitted the development of miniaturized IgE antibody assays on-chip. The main improvement of such a technique is that only a small amount of serum is required to perform a broad-spectrum allergy test which is an improvement of high interest especially for paediatric patients. Moreover, a biosensor-based test leads to automation of the process and thus a reduction of assay variability, a higher accuracy and a shorter time to achieve the result. The plausible issue which could be faced with antibody-based biosensors for the diagnostic allergic diseases is the antigenic competition among cross-reactive allergens. This would affect the quantitative accuracy and the sensitivity of the results. Another competition could be interference between IgG and IgE to bind to the allergens tethered to the surface of the sensor. (47) (48)

BIOSENSORS FOR NEUROLOGICAL DISORDERS

In order to diagnose such disease, several parameters have to be considered, including patient symptomatology, patient medical history and clinical laboratory testing. Multiple biomarkers have been identified and several biosensors designed for the diagnosis of neurological disorders are already commercially available. Because this type of disease and

cancer share some biomarkers and genetic mutations, some of the sensors used to detect cancer can be used for the detection of neurological diseases as well. (37) (49)

BIOSENSORS FOR THE DETECTION OF HORMONES

The most well-know of these biosensors and maybe of all the biosensors all application combined is designed to detect human chorionic gonadotropin (hCG) which is an important marker of pregnancy but also a carbohydrate tumor marker.

Immunosensors are the most commonly used combined either to an optical or an electrochemical transducer. (11) (32)

BIOSENSORS FOR AUTOIMMUNE DISEASES

The basic mechanism of autoimmune diseases is the production by the immune system of antibodies turned against oneself or anti-DNA antibodies targeting various organs which then generate various tissue damages. The use of biosensors for the detection of these detrimental antibodies is a powerful diagnostic tool (13) (50) (51) (52).

BIOSENSORS FOR *IN VIVO* DIAGNOSIS

They will be reviewed in the second part of this report. In a general manner, they are used for the continuous real-time monitoring of biomarkers *in situ*.

Table I below summarizes the state of the global biosensor market as it was in 2008. As explained previously, this list of biosensors for therapeutic use established above is not exhaustive. Many other therapeutic applications can be and will be developed in the next few years since the interest for biosensors as POC devices, among other utilizations, has kept growing within the last decades.

Company	Target/sector
Abbott sector Molecular Diagnostics and Diabetes Care	Glucose
Affymetrix	Pharmaceutical and general bioscience research
Applied Biosystems and HTS Biosystems	Affinity chip
ARKRAY, Inc.	Glucose
Bayer Diagnostics/Kyoto Daiichi/Menarini	Glucose
Becton Dickinson	Glucose
Biacore	Affinity sensors for pharmaceutical and general bioscience research
Biosite	Medical
Eppendorf	Glucose and lactate
LifeScan	Glucose
Molecular Devices	Diagnostics, pharmaceutical and defence
Nanogen	Diagnostics
Nova Biomedical	Glucose, urea, lactate and creatinine
Roche Diagnostics	Glucose
YSI	Glucose and lactate

Table I Table of M.Mascini and al. (11) of 2008 illustrating the market-leading biosensor companies in alphabetical order and target/sector involved.

1.3 Focussing on lipid membrane based biosensors

As seen previously, it has been widely acknowledged that the lipid bilayer constitutes the key component of biomembranes. Therefore a legitimate question arises as to whether biomimetic membranes can be used for biosensors applications. Indeed one could imagine using them to study membrane proteins function or for diagnostic purposes. (1)

It is important to note that the lipid bilayer can regulate protein function and conversely the protein can regulate the bilayer. By combining only some components of a biomembrane (lipid bilayer and a few membrane proteins) successful biosensors have been developed. The choice of the lipid species is mainly based on the requirements for stability. It also important to take into account that the lipid composition used to build the membrane might influence the protein folding and activity. The lipids have thus to be chosen also according to the protein requirements for an optimal functionality.

The first artificial lipid membranes are the Black Lipid Membranes (BLM) consisting of lipid films made of a single or a few lipid components. Their name is relative to their black appearance. BLM were initially observed by Muller and his research group in the 1960s from extracted brain lipids (53) (54). Since then, they have been well studied and used in the development of multiple devices. The utilization of BLM for protein and other biomolecule studies has been reviewed in a large number of publications. Black Lipid Membranes are also used for other applications in various fields such as immunochemistry,

drug development, drug testing, and biosensors. Black Polymer Membranes (BPM) have then been developed from BLM using polymerizable lipids. (6) (55)

Besides the BLM, lipid vesicles are another common form of lipid mono or bilayer that have been known and used for more than 30 years. Indeed, this type of lipid membrane in the shape of a sphere was first artificially synthesized in the 1960s by Bengham and his coworkers. (56)

Phospholipid vesicles are part of the three common systems used to mimic the cell membrane with self-assembled monolayers (SAM) and supported bilayers. SAM are molecular structures that spontaneously form from the interaction between a surface-active head group that is put in contact with a substrate in solution (3) (57) (58). For more details, the design of such membranes will be discussed below. Vesicles and supported membranes share major characteristics: they are made of the same lipid species found in plasma membranes, show the same fluidity as biomembranes, and are able to mimic the lateral rearrangements occurring on a cell surface *in vivo*. Forces between cells and forces involve in the folding of membrane proteins have thus became measurable (59). On the other hand, SAM can be used for the formation of a Hybrid Bilayer Membrane (HBM) which results in the deposition of a SAM on a gold surface constituting an hydrophobic surface on which small unilamellar vesicles spontaneously absorb. (7) (34)

In a general manner, artificial lipid membranes for *in vitro* applications have opened the way to study the electrochemical phenomena occurring across the cell membrane through mimicking this cell membrane. The electro-active components are peptides or proteins embedded to the lipid matrix and constituting channels, pumps, receptors and other carriers. Sensing devices developed from these membranes and based on electrochemical techniques would thus combine the sensitivity of electronic devices and the selectivity of biomolecules. (60)

The formation of a lipid bilayer tethered to a support allowing sensing and signal transduction is essential for the creation of membrane-based biosensors. This bilayer has to be able to integrate membrane embedded peptides, channel proteins, transporters and/or other membrane proteins. For most applications to lipid membrane biosensors, the lipid barrier has a passive role and the focus is on the incorporated proteins (2). Usually, the transduction is performed through an electrochemical impedimetric system. Both carriers and channel forming peptides are easy to incorporate functionally to a membrane. For this

reason, they are regularly found in lipid based biosensors. In contrast, channel forming proteins and carriers, which are bigger and thus bulkier, are harder to insert in a functional manner. So a fewer number of sensors using this approach have proven their effectiveness. (61)

Currently only a limited number of lipid membrane-based biosensors are commercially available. But this biomimetic approach is the focus of several research groups and the pharmaceutical industry. The number of lipid biosensors on the market should rise in the next few years. Membrane proteins being a very large class of molecules representing about 30% of all cell proteins, it makes these sensing devices highly desirable. (9)

1.3.1 Design of lipid membranes for biosensors

In a general manner, methods for the adsorption of proteins at surfaces is of high interest for very various applications in fields ranging from stain removal to food processing through to therapeutic purposes. (62)

Different strategies can be employed in order to obtain a lipid bilayer usable for biosensing applications. Nevertheless two methods of the electrostatic layer-by-layer (LbL) and the Langmuir-Blodgett (LB) techniques are the most widely utilized (31) (63).

The LbL technique was first described in 1991 (64) whereas the LB films have been known for 80 years (65). In the LbL protocol, layers of positive and negative charged polyelectrolyte molecules are alternately absorbed from an aqueous solution onto the surface of the support. A multilayer film is thus obtained with as many bilayers as desired. Several parameters play a key-role on the determination of the number of layers such as pH, ionic strength and the concentration of the lipids in the aqueous solution. The resulting film is resistant to washings by an aqueous solution.

The LB technique consists in a dipping of the solid support from the air to an aqueous phase allowing the transfer of an insoluble lipid membrane from the interface onto the support. A multilayer can be obtained as well by repeating the immersion step.

During the formation of a film to be used in a biosensor, three important requirements for the sensing surface have to be kept in mind including the molecular recognition of the target, the transduction and the detection of the signal.

By the means of the two techniques explained above, a wide range of material can be immobilized onto solid supports. In both cases, the control of the thickness of the film is easy and the molecular organization is simple to tune which make them convenient for biosensing purposes.

Inverted micelles are a more recently developed approach leading to the formation of very stable lipid bilayers. This technique, also named vesicle spreading, is mostly used for medium- or high-throughput screening of membrane proteins purposes (1) (2) (66).

Different types of membranes are produced according to the technique, lipid species, and support used. The three main sorts of lipid membranes can be summarized on a schema (Figure 7). It has been shown that the lipids can have different structural characteristics according to the surface used to support the biomimetic membrane. (4) (59) (61)

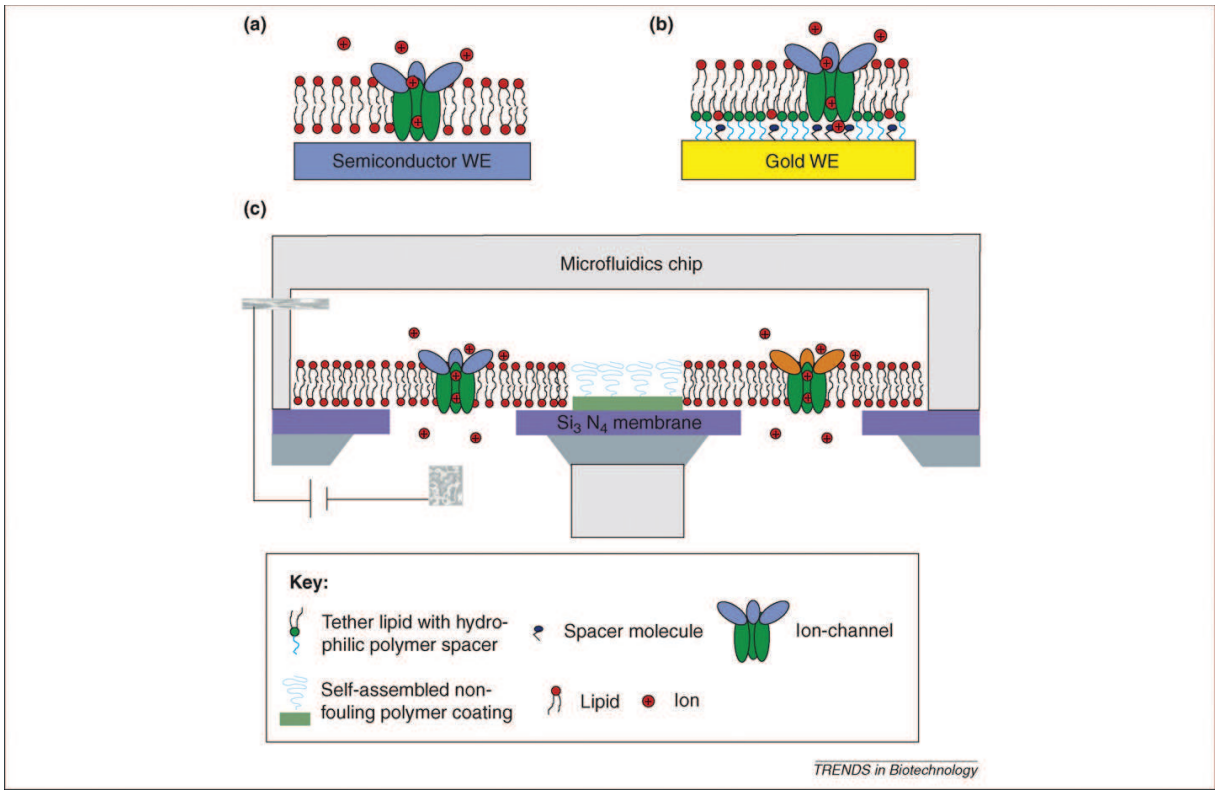


Figure 7 Illustration of Erik Reimhult and al. (4) of different lipid membranes for biosensors (a) Supported lipid bilayer on hydrophilic support. The support acts a as a working electrode (WE) for electrochemical measurements. (b) Tethered supported lipid bilayer. The lipids are tethered to a gold WE (c) Free-spanning membrane or black lipid membrane. It has free liquid access on both sides to accommodate large proteins and to easily conduct electrochemical measurements.

Lipid membranes are attractive because they provide a natural environment for the immobilization of molecules such as hormones, enzymes, antibodies, antigens or other proteins. Therefore, these molecules keep their functionality, sensitivity and specificity and can be analyzed in a near-native environment (35). Moreover lipid bilayers show a low

bioactivity limiting the risk of spurious signal. This, amongst other reasons, makes them ideal for the design of biosensors. (67)

1.3.1.1 Solid Supported membrane

This type of lipid bilayer was first exploited to understand the requirements for T cell activation and the interaction between the cholera toxin and its intestinal receptor GM1 ganglioside (35) (68). They have thus notably been successfully used to determine the role of cell adhesion in immunological responses and replace antigen-presenting cells (69). Different types of support can be used:

- Solid planar supports
- Microspheres

In a general manner, a supported membrane can be defined as a single and continuous phospholipid bilayer on a solid or polymeric substrate. The support will constitute the working electrode in every supported lipid bilayer (SLB)-based biosensor. The measurement is made by simply connecting one wire to the underlying gold electrode to which the membrane is anchored and the return wire to another surface in the bathing solution. The flux of material across the membrane does not need to be large in order to ensure the proper functioning of the biosensor. (1)

Whether we are dealing with planar surface or microspheres, the biomimetic membrane can be linked to its support in different ways:

- Direct deposit: the lipid bilayer is very close from the support which can potentially inactivate a membrane protein embedded in the membrane by generating interactions between support and proteins.
- Cushion supported membrane: a polymer forms a cushion between lipid bilayer and solid support. The risk of inactivation of membrane protein is thus reduced.
- Tethered membranes: this particular type of biomimetic membrane-based biosensor will be reviewed in details afterward (section 1.3.1.3).

Both cushion and tethering techniques have been developed to avoid the issues of membrane proteins inactivation faced with a classic direct deposit. They confer to the sensor a higher level of stability and portability (2).

These different types of SLB are illustrated on Figure 8.

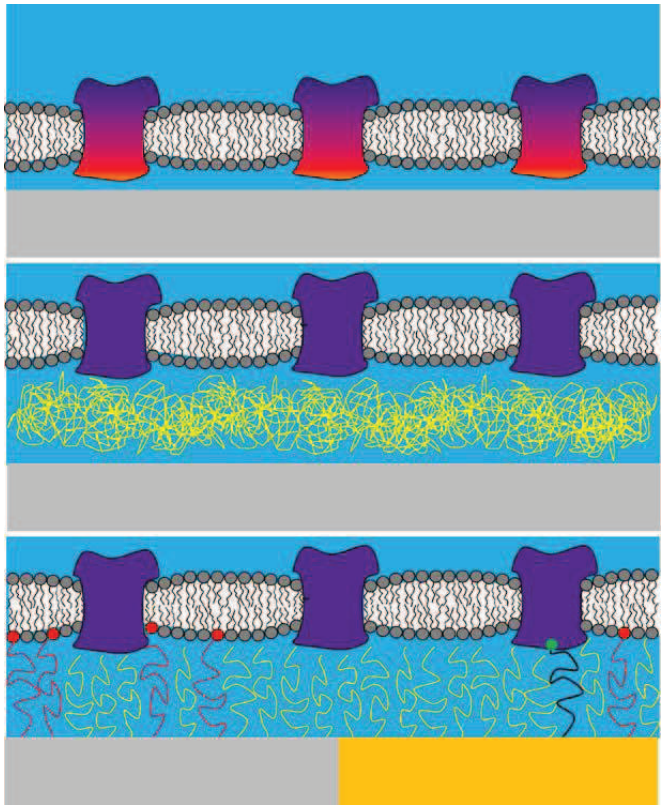


Figure 8 Illustration of Claus Hélix Nielsen (1) showing the different types of supported bilayer. In each example, the solid support also plays the role of working electrode. a) Direct deposit of the bilayer onto the surface of the support. b) Cushion supported membrane. c) Tethered membrane.

The hydrophilic heads of the lipids of the upper leaflet face the solution, whereas the ones of the bottom leaflet face the support's surface. The three critical properties that SLB share with biomembranes make them very attractive for the design of biosensors. Those properties are lateral fluidity, surface motility and self-healing ability. The stability is maintained as long as the biomembrane is kept hydrated. The mobility is essential to study cell mechanisms, and pathogen attacks as well as the immune response (62). Indeed, all these processes rely on the two-dimensional fluidity of the cell surface. The fluidity also found in SLB allows the lateral rearrangement of ligands and thus optimizing thereby their specific binding to a receptor. The lateral fluidity is the feature that differentiates SLB from other lipid membranes. Paradoxically it also represents a difficulty in that the membrane components can permanently mix. To thwart this disadvantage, lateral diffusion barriers can be established that partition the lipid bilayer into several well-defined spaces. (70) (71) (72)

As seen previously, the membrane support might be planar, as in most cases, or composed of many microspheres. The support whether flat or spherical can be made from different materials including silica, glass, mica, polymer or gold. For example, the first SLB ever generated had a mica, glass, quartz or silicon oxide support (73) (74). Gold in particular

has been shown as an ideally suited support for the formation of well oriented self-assembly layers. Microspheres for biosensors are mostly made of silica. On each of them a lipid bilayer has been formed resulting in a biomimetic microsphere with a cell-like structure. Silica microspheres present very interesting properties such as chemical inertia, low autofluorescence, or ease of functionalization which make them attractive for biosensing applications. Unlike liposomes, microspheres have a narrow size of distribution as well as a controllable shape. They can be washed simply and show a high level of stability. Moreover, the microspheres can be suspended in a tube, making the transport of the sample to the sensing surface easier and better as compared to planar support systems. Besides due to their large surface, the biomimetic spheres are readily adaptable to multiplexed tests. (8)

Within the last decades, the emphasis has been increasingly placed on the design of biosensors based on supported membranes (4). The aim is to achieve the design of a biosensor able to detect a target that does not necessarily directly interact with the SLB. Most of the time, the transduction system used is electrochemical or optical or a combination of both. For multiplexing, impedance spectroscopy is the most commonly used technique (33). Several proofs of concept of such technologies have already been published. For instance, a SLB biosensor has been successfully used to detect *Staphylococcus enterotoxin* B in milk through an immunosensing method. Examples of membrane proteins functionally integrated to SLB on microspheres for biosensing applications can also be found in the literature. These examples include the human type 3 serotonin receptor, which is a neurotransmitter receptor, or the bacteriorhodopsin, which is a proton pump. But it seems obvious that this cannot be taken as a proof that all membrane proteins would be functionally incorporated. One could surmise that the incorporation of a protein with a cytoplasmic domain would inhibit or restrain the mobility of the lipid bilayer and interfere with the activity of the whole protein. This would be mainly due to the proximity between the lipid membrane and the solid support, with the resulting space being too small to accommodate cytoplasmic hydrophilic domains. This constitutes a severe drawback for the study of membrane protein function.

Regarding multiplexed testing, the supported bilayer systems are particularly adapted due to their two-dimensional mobility. Individual lipid molecules can easily be transferred along the surface of the biosensor which facilitates the binding of a ligand to its specific receptor as stated above (75). But this relocation also allows several ligands within the

membrane to bind to a single hydrophilic protein with multiple binding sites (7). Thus SLB are attractive for the performance of multiplexing assays.

Due to the specific electrical properties of lipid membranes, the slightest modification of the membrane can be easily detected as a change in impedance. Thus, impedance monitoring of biomimetic membranes allows the study of the action of channel-forming peptides and channel-forming proteins. Once the effect of a specific channel on the lipid bilayer has been defined, one could imagine subsequently using the same sort of lipid membrane-based biosensor for the detection of this channel. This type of measurement has already been successfully performed (76). Nevertheless, the limited size of the aqueous space between SLB and support restricts the number of ions that can fit in this space. This limitation of the ion transport leads to a limitation of the electrochemical measurements. Once the osmotic gradient is too high, no ion is transported and no change in the impedance is able to be monitored (61) (77).

SLB biosensors are evidently not the only method that has proven its worth. Patch-clamp, whole-cell sensing or BLM are well-established techniques that did not disappear with the conception of the first SLB biosensors. However, compared to the more "traditional" techniques, lipid membrane-based sensors brought significant benefits such as ease of membrane assembly, control of the lipid membrane design and of the orientation of the incorporated proteins, stability and applicability to a wide range of analytical techniques which make them well suited as cell membrane mimics. They also offer the possibility of biofunctionalizing inorganic solids and polymeric materials and of incorporating a protein under non-denaturing conditions (66). Great progress in the understanding of the structure-activity relationship of membrane proteins has been possible using SLB biosensors and more remains to be done (59). The role of SLB in biosensors can be simply summarized in three points:

- Electrical insulator: ultrathin layers with a high electric resistance
- Matrix for the incorporation of membrane proteins: providing a cell-like environment suitable to proteins operation
- Suppression of nonspecific ligand binding: two-dimensional mobility allowing proteins rearrangement.

When discussing the supported membranes, one has to mention the particular application of the SLB to the design of phantom cells. These biomimetic cells show well-defined

adhesive properties and are very handy to study the lock-and-key and universal forces involved in cell adhesion and locomotion mechanisms (78). Specific lock-and-key forces also play a crucial role in the modulation of allosteric enzyme activity and immunological recognition process. Supported membranes, through phantom cells, are thus useful for the study of the interplay between the two types of forces. (79)

1.3.1.2 Free-spanning membrane

Free-spanning membrane or lipid bilayers on porous support are a derivative of the BLM mentioned above. Indeed, the so-called black lipid membranes are the oldest lipid membrane spanning aperture systems. They were also the first tool to study ion channel functions. But this technique suffers from major drawbacks including a very poor stability (a few hours before collapse of the bilayer) and the requirement for the membrane to soak in solvents incompatible with a number of proteins (4) (80). Consequently, the use of BLM for biosensor purposes is very limited despite the fact that successful examples of bioanalytical uses can be found in the literature (6). This led several research groups to focus on the development of an alternative to BLM but still based on a porous support which ended up with the free-spanning membranes. One of the main steps toward a higher stability was the reduction of the size of the apertures up to the nanometer. In comparison, apertures spanned by BLM were of the millimeter range.

One could think that cushion membranes on solid support could already satisfy the need for space on the inner side of the membrane for transport across the biomimetic membrane. But for the circulation of a large flux of ion, such a design is not sufficient. Free-spanning or free-standing membranes on porous support allow the access from both sides of the membrane which is more convenient for the functional incorporation of large proteins for example. Multiplexed impedimetric measurements in the form of patch-clamp on several single transmembrane proteins can be considered as well. Free-spanning membranes have the great advantage of possessing an unlimited ion reservoir on each side of the lipid bilayer.

The property of being addressable from both sides of the bilayers also makes them closer from the physiological environment (35).

The biomimetic membrane is formed across an aperture. Two main methods have been developed for the assembly of lipids on a surface with gaps: (1)

- The “painting” technique: the lipids are dissolved in a hydrocarbon solution. This solution is then spread over the surface including across the apertures. (53)
- The “folding” technique: the lipids are spread as monolayers on two distinct surfaces separated by air-water interfaces. The monolayers are then folded across the gap. (81)

Free-spanning membranes have been successfully used for the production of nano- and microdevices suitable for biosensing, drug screening or research on membrane proteins. As with SLB the criteria of sensitivity, versatility and ease of membrane assembly are fulfilled.

As for SLB, both planar and spherical supports can be used. Indeed, mesoporous silica microspheres with controllable pore sizes, for instance, have been developed for biosensing application (82) (83). The porous design represents a convenient way to deliver and/or store different molecules such as fluorophores or intracellular biomolecules. This type of design is thus very suitable to the reconstitution of a cell-like environment. (8)

However free-spanning bilayers show weaknesses. The stability reached is lower than with SLB. Their design is tricky in the sense that the support has to be adequately porous to allow substantial vectorial flow and sufficiently dense to sustain the lipid membrane against transmembrane pressure gradients. Another limitation of free-spanning membrane comes from the roughness of the support and the possible variability in the size of the pores. These properties are intrinsic to the support and significantly impact the stability of the membrane (67).For this reason, cushions might be added between membrane and support exactly as in the case of supported lipid bilayers. Moreover other strategies have been implemented to increase the stability of membranes on porous supports (61). At first, polycarbonate and nitrocellulose filters were used which have then left the way for hydrogel polymers. Whereas the first techniques resulted in a limited control over non-electrolyte flows and complex water-transport properties, hydrogels are used to encapsulate the lipid membrane and proteins incorporated within it (1). Another common approach is the use of the S-layer proteins which are found on the outer of *Bacillus sphaericus* bacterium. These proteins form a scaffold encapsulating the biomimetic

membrane (59). The S-Layer-supported membrane thus built mimics the principle of archeal cell envelopes (67). These different methods to increase porous supported-membranes stability are illustrated in Figure 9.

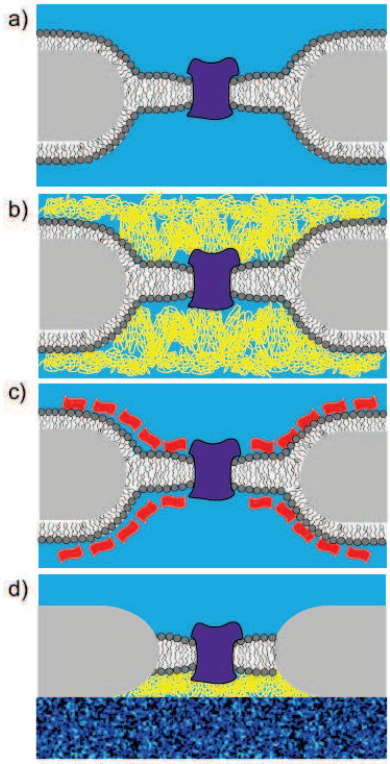


Figure 9 Illustration of Claus Hélix Nielsen (1) showing the different types of porous supported biomimetic membranes. A protein (purple) is embedded to the lipid membrane. a) Free-spanning membrane formed across an aperture.). b) Free-spanning hydrogel-encapsulated membrane formed across an aperture. A hydrogel polymer meshwork (yellow) encapsulates the biomimetic membrane. c) Free-spanning surface (S) layer-encapsulated membrane. The layer of protein or glycoproteins (red) self-assembles into a two dimensional meshwork. d) Free-spanning cushioned membrane.

Free-spanning bilayers, similar to SLB, have to be kept hydrated to avoid the disrupture by drying. Another important feature for the design of the ideal spanning membrane is the absence of solvent. As seen earlier, solvents often interfere with the activity of ion channels and transporters making the membrane requiring them unsuitable for biosensing uses.

To be used in the design of biosensor, one of the greatest challenges faced is the insertion of a membrane protein within a newly formed free-spanning lipid bilayer. Since the dimensions of the apertures are usually in the range from nanometer to micrometer, the exact location where a single protein is incorporated has to be controlled. Otherwise no interpretation of the measurements recorded is possible. Several methods can be employed:

- Insertion straight from solution: for small ion channels mainly, cannot be applied to most molecules (84) (85) (86)
- Fusion of proteoliposome
    - Forming the lipid bilayer (87) (88)
    - Into a preformed bilayer (89) (90)

The second approach seems to be the most promising.

1.3.1.3 Tethered membrane

Tethered membranes are not a new category of supported membranes but a further development of SLB. Tethered bilayer lipid membrane (tBLM) method consists in the tethering of the lipid membrane to a gold electrode through the covalent attachment of hydrophobic molecules with a hydrophilic linker. The gold electrode plays the role of working electrode.

The decoupling of the lipid bilayer from the electrode surface using short hydrophilic spacers is one of the most significant improvements in the field of solid-supported membranes. The space between membrane and support is increased in the tBLM due to the presence of spacers. As a result membrane proteins are more easily integrated and importantly retain functionality because the risk of undesired interaction with the support is substantially reduced. However, the reservoir on the inner side of the membrane is still limited in comparison with porous supports. Thus the monitoring of ion transport is restricted. (4)

The inner leaflet of the membrane is covalently tethered to the surface of a gold surface through a spacer group. The approach is seen schematically below (Figure 10). A family of synthetic compounds is available which posses sulfur groups reactive with gold. The compound binds to the patterned thin gold film which is coated on a plastic slide. The same molecule also contains a sequence of polar ethylene glycol groups that provide an aqueous layer at the support surface and secondly a hydrocarbon group that behaves as an ionic barrier and mimics the property of the lipid bilayer. Indeed, as the molecules bind they pack together and progressively form into a two dimensional lipid bilayer film. This bilayer is way more stable than an unsupported cell membrane or liposome.

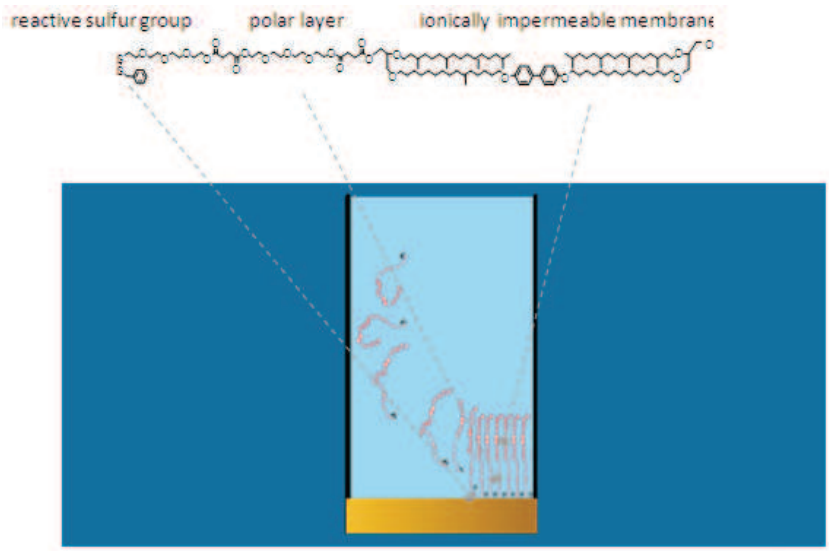


Figure 10 Schematic diagram lent by Bruce Cornell of the formation of a tethered membrane Although the schematic shows the membrane as totally tethered to the gold surface, in most instances spacer groups are tethered at the surface and the rest of the membrane back filled to form a mixture of tethered and mobile molecules. This provides flexibility to allow for the incorporation of proteins such as ion channels and these proteins to relax to their optimal structures.

It should be noted that gold supports are the most widespread surfaces for the design of tBLM, although silicon substrates can also be used (91). Combined to a FET transducer, a direct readout of the currents circulating across the membrane would be achievable (80). Silicon surfaces are compatible with most of the transduction systems for biosensing. But gold stands for the "gold standard" material as it has been extensively investigated and well-defined (33). Supported tBLM devices on gold surfaces have already been widely developed (92). Membranes assembled on gold supports have been shown to bring key-advantages such as ease of fabrication, reproducibility, availability of materials, chemical stability and flexibility (61) (57).

Thus in most cases, both the tethering surface and the return electrode are pure gold which at the applied measurement voltages of 20-50mV has no electrochemistry. This provides a capacitive coupling between the electrons in the gold and the ions in solution (93).

In these coupling capacitors, one plate is formed by the gold charged with electrons and the other by ions in solution crowding at the gold surface. This approach overcomes many problems associated with the silver-silver chloride technique. In particular, proteins can be damaged by silver ions and it is a big advantage when using capacitive coupling since there are no metal ions in solution. Also the approach avoids signal-drift due to variations in electrochemical potential which is dependent on both temperature and trace amounts of ionic impurities.

tBLM are prepared in two stages. The first stage is to coat the gold electrode with tethered lipid and tethered spacer molecules (Figure 11). These respectively anchor the lipid bilayer to the electrode surface and provide space to permit the mobile lipid to adjust to the

requirements of the bilayer and to allow conformational relaxation of any incorporated protein (94). Spacer groups act as a cushion maintaining the bilayer off the gold surface which compensates for the surface roughness effects. They also constitute an ion reservoir on the internal side of the lipid membrane.

The ratio of the tethering fraction to the mobile fraction may be adjusted to permit the incorporation of proteins of varying size. Usually the larger the protein's molecular weight, the lower the fraction of tethers that are used to allow space within the membrane to accommodate large proteins (Figure 12). Aside from the density or fraction of tethering, the chemical composition and length of the spacer molecules are also modular. The right balance has to be determined for each protein.

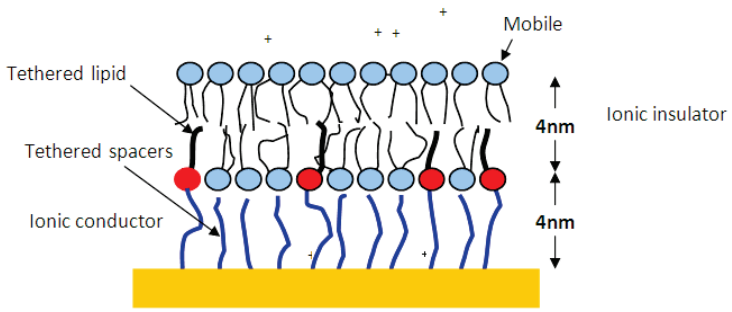


Figure 11 Schematic provided by Bruce Cornell of a fluid, tBLM. An insulating layer 4nm thick is tethered to the gold surface via a 4nm thick polar, "reservoir layer" between the membrane and the gold surface. A fraction of the tethered lipid spans half the 4nm thickness of the bilayer and act as anchors. A component is tethered but does not penetrate the bilayer and act as a spacer for the tethers and a cushion on which the bilayer is supported.

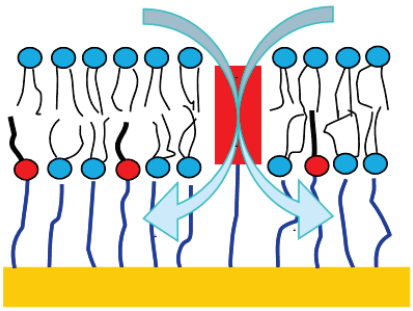


Figure 12 Schematic provided by Bruce Cornell of a fluid, tethered lipid bilayer membrane containing an ion channel. The a.c. excitation causes ions to migrate through the channel between the outer bathing solution and the inner space adjacent to the gold electrode. The hydrophilic layer at the gold surface allows ions to disperse across the entire gold surface. This permits the gold surface to develop a capacitance that is 7-10 times greater than the membrane capacitance which provides an impedance

To summarize, a tethered biomembrane is made of three indispensable components: (5)

- A solid substrate: gold electrode
- A tethering layer
- A lipid bilayer

Within the lipid bilayer can be incorporated hydrophilic peptides and membrane proteins. The tBLM then forms an electrically insulating platform allowing the individual monitoring of a protein's function and activity. (60)

The membranes thus synthesized have the ability to resist factors that would disrupt liposomes or hydrolyze the acyl chain attachments in another context. (95)

Only a fraction of the membrane lipids are tethered which stabilizes the whole structure and provides it a resistance to the insertion of random material. This increased stability is another property specific to tBLM. It results in a longer lifetime of several months against several days for the classic SLB and free-spanning biomimetic membranes. Tethered biomembranes provide a very unique environment in the respect of the minimum requirements for the preservation of membrane proteins in an adequate orientation.

Functionalized tBLM can be used for the design of intelligent molecular devices. They can be applied to almost any sensing application such as blood typing, bacteria, viruses and other biomarkers (e.g. hormones, enzymes, or antibodies) detection (63). Further, practical applications have already been the subject of several publications (96) (97). Membrane proteins embedded in a flexible membrane as are the tBLM can be envisioned as actual sensing units.

Main features	Solid supported membranes		Free-spanning membranes
	Classic	Tethered	
Sensitivity	✓	✓	✓
Versatility	Applicability to a wide range of proteins except large proteins	Any range of proteins	Any range of proteins
Ease of membrane assembly	✓	✓	✓
Lateral fluidity	✓	Limited by the tethering groups	✓
Stability	If kept hydrated	Lifetime of several months if kept hydrated	✗
Self-healing	✓	✓	✓
Functionality of the protein	Possible interaction with support	✓	✓
Reservoir	Limited size	Large size	Unlimited, Massive flux allowed
Geometry	Planar or microspheres	Planar	Planar or microspheres
Multiplexing	✓	✓	✓
Characteristics of the support	Roughness	✓	Roughness, Possible variability of the pores
Additional features	Cushion, Tethering	✗	Cushion, Hydrogel-encapsulation, S-Layer proteins-encapsulation
Measurements		Single proteins monitoring	Single proteins monitoring, Access from both sides BUT need to know the exact location of incorporation

Table II Summary of the main characteristics for each type of biomimetic lipid membranes used in biosensors. ✓= well-fulfilled criterion or good. = moderately-fulfilled criterion or medium. ✗= criterion not fulfilled or low.

1.3.2 Therapeutic applications of membrane-based biosensors

Over the last ten years, the interest in biosensing devices has been steadily growing with research focused on higher biocompatibility and the development of new materials, ideally for chip-based chemical analysis and bioanalysis. For this purpose, the manufacturing of cheap robust biomimetic lipid membrane, such as described above, is very opportune. (98)

Lipid membrane-based biosensor using SPR transduction systems have emerged as a major tool for the study of biomolecular interaction. Their value as drug discovery and protein-

ligand interaction screenings technology has been proven and demonstrated in a number of publications. (99)

Aside from the protein-ligand interactions, the study of lipid-protein interactions is also a main concern. As seen previously, membrane proteins represent more than 50% of drug targets, but the understanding of their characteristics and functions remains limited. Only a few of them have been fully described, including gramicidin. The main difficulty is the requirement of the isolation of the protein in a native-like environment. The lack of suitable methods to study membrane proteins restricts the discovery of new drugs (100).

Lipid membrane-based biosensor can also be used for diagnostic purposes. The use of a lipid membrane with specific membrane proteins incorporated within the bilayer can be used for the detection of pathogens and other biomarkers of diseases. Their use as diagnostic tools is unlimited since they can be designed to detect a vast number of target molecules. This particular feature of biomimetic membranes makes them highly attractive and thus many research groups are focusing on new diagnostic applications.

Many biomolecules have been widely investigated through lipid membrane systems for their recognition and immobilization which includes amongst others: nucleic acid bases, amino acids, sugars, peptides, enzymes, leading for some to the development of biosensors (57) (101).

Herein we summarized the most investigated therapeutic applications of lipid bilayers. Nonetheless the list is not exhaustive as the possible targets for diagnostic and the protein-ligand and protein-lipid interactions to study are vast if not infinite.

1.3.2.1 Incorporated proteins

Ion channels

Ion channels are also very attractive targets for lipid membrane studies and developing biosensors. Indeed, ion channels take part in many physiological processes. Their incorporation within lipid bilayers would provide a way to study them and perform electrophysiological measurements such as impedance measurements in order to better understand of their physiological functions and their therapeutic potential. As ion channels constitute main drug targets, it is crucial to fully understand their mechanism and function

for potential drug discovery. Moreover, the high affinity of these channels for particular specific ligands makes them very attractive for the manufacture of ion channel-based biosensing systems. (102)

As the native environment of ion channels is the cell membrane, all the above uses can be considered with the integration of the protein within a supported lipid bilayer. Patch-clamp is the "gold standard" method to monitor ion channel proteins, but it requires a substantial investment in terms of time (training) and money (equipment). This leaves room for alternative techniques such as artificial lipid bilayer platforms which allow an impedimetric measurement monitoring of the proteins as well but also the engineering of ion channel-based biosensors.

Furthermore it has been shown that the reconstitution of ion channels in simple free-standing bilayer membranes was a good method to study their activity and envision protein-ligand screening, drug discovery and biosensor design. (103) (103)

WATER CHANNELS

Major intrinsic proteins (MIPs) are pore forming integral membrane proteins found amongst others in red blood cell membranes (104). Aquaporins constitute the majority of this family of proteins and play a major role in osmotic processes that allow the rapid diffusion of water along osmotic gradients. Unlike classical carriers, these specialized water channels address a very particular transport as it is decoupled from conformational changes in the proteins. In 2009, Claud Hélix Nielsen reported that 450 members of the MIP family had been identified. The question was then to define which part of the transport was mediated by the pore formed by the protein and which part was due to the lipid membrane matrix. It was thus established that the solute, the specific protein and the supporting membrane were all key-factors influencing the transport. Further studies based on a lipid bilayer support have been accomplished afterward in order to determine the properties of MIPs more precisely. The ultimate purpose of this research is the development of biosensors made of artificial lipid membranes incorporating MIPs for applications based on the concept of up-concentrating selected permeants. (2)

ION CHANNEL SWITCH (ICS)

ICS are used for the design of biosensors in order to detect the presence of a particular biomarker. It consists of the incorporation of two classic ion channels in a tethered lipid bilayer. A mobile ion channel is incorporated in the upper leaflet, in contact with the sample, and a fixed ion channel in the lower leaflet near the solid support. This assembly of two channel permits the measurement of ion fluxes across the membrane. A generic receptor such as an antibody fragment, a DNA probe, an enzyme or any cell receptor to target a specific compound is linked to the mobile outer leaflet channel. Another receptor of the exact same type is also linked to another fraction of the lipid upper layer.

When the target is introduced, the two receptors cross-link which does not allow the formation of an ion-conducting pore by the dimerisation of the two ion channels, as shown on the following figure (Figure 13). Thus, a drop in the electrical conductance is generated across the membrane. Usually, streptavidin is used as a linker between receptor and channel or receptor and lipids. (95)

Also, a decrease of the conductance can be interpreted as the presence of the specific targeted biomarker in the sample submitted to the test.

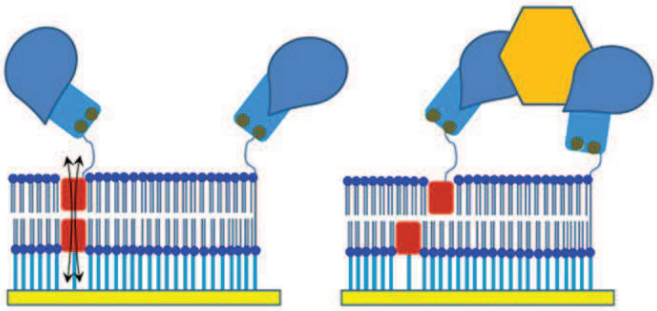


Figure 13 Schematic of Bruce Cornell for SDx Tethered Membranes (131) of the key elements of an ICS biosensor. The yellow hexagon represents the target element.

Such a biosensing device is very attractive since it can be suitable for any type of biomarker as long as a relevant receptor exists. An unlimited number of sensing applications arises from ICS technology. Some significant assays have already been achieved, including examples presented thereafter, and many others are and should obviously be considered in the near future.

ANTIBODIES

As seen in the section 1.1.2.2, immunosensing remains the "gold standard" regarding biosensor techniques. In fact, immunosensing can be regarded as a particular case of ligand binding detection.

As with any antibody-based biosensor device, the aim is to identify a specific antigen with a high sensitivity, rapidity, easy to use, with if possible only a small volume of sample in order to permit an accurate diagnostic within the shortest time. Those characteristics fit well with the definition of an ideal POC testing device. A rapid identification of the pathogen makes possible the initiation of an adequate treatment, a better support of the patient, likely a better compliance, and increases the chance of a positive outcome for the patient. These criteria are actually sought-after for every kind of biosensors, but more easily reachable with antibody-based biosensor.

For instance, antibodies can be used as the "receptor" part in ICS biosensor techniques. It constitutes one of the investigated ways to link an antibody to a lipid membrane, but it is obviously not the only one (3). Some techniques incorporate the antibody directly within the lipid membrane (105).

A basic example of accomplishment of a lipid bilayer membrane-based immunosensor is the detection of Staphylococcus enterotoxin B (SEB) in a sample of milk with a supported lipid membrane device including biotinylated anti-SEB antibodies (106).

A few decades ago, lipid membranes have also been used to elucidate antibody-antigen interactions (107).

ANTIBIOTICS

Through the use of a lipid membrane-based technology, gramicidin is the most well-studied molecule. For this reason this antibiotic stands as a model for the investigation of its class of peptide in the lipid environment. Several other peptides of the same range of size (12 to 50 amino acids residues) are believed to have the same antibiotic activity (63). Moreover this activity is supposed to be related to the interaction peptide-cell membrane. But before considering any further drug development based on such peptide, this specific

interaction and its mechanism need to be demonstrated. These peptides with a plausible antibiotic activity, also called antimicrobial peptides (AMPs), share two main characteristics: positively charged and hydrophilic regions separated from hydrophobic domains. Due to these properties, they are able to interact with bacterial membranes which could partially explain their mechanism of action. Based on the example of the gramicidin peptide, the use of the artificial lipid membrane is a tool of choice to perform further research and maybe, in a near future, to develop new drugs (33) (95) (102).

Furthermore, the good knowledge of gramicidin is a way to test new biomimetic membrane design. Indeed, any lipid bilayer system can be tested by the incorporation of a gramicidin peptide followed by impedimetric measurement. If the results obtained conform to those expected, that is to say the result usually achieved by measuring gramicidin activity, then the system can quickly be assessed as functional or not. Gramicidin stands as a reference to test the functionality of artificial lipid membranes.

Another type of the AMPs, the magnainins, has been successfully studied using an artificial lipid bilayer support. Magnainins show antibacterial and antiparasitic actions and is therefore an interesting potential drug. (33) (108)

The class of the CNY21 bacterial peptides has also been studied by the mean of lipid membrane. The bacteriocidal activity of these petides and its mechanism have been thereby elucidated. (109)

INTEGRINS

Integrins are cell surface receptors mediating the attachment of the cell with other cells or the extracellular matrix proteins like collagen and vitronectin (110). Integrins also have a role in cell signaling. They are made of two subunits α and β which are not covalently attached.

Tests of incorporation of integrins within tethered membranes have been successfully performed. Subsequently further tests, such as the screening of new unknown ligands, have been possible using integrin-functionalized lipid membranes (5).

Obviously, all these experiments are executed in order to achieve a better understanding of physiological mechanisms and in the hope of discovering potential future drugs.

1.3.2.2 Detected protein

Thyroid-stimulating hormone (TSH)

When the hypothalamus releases thyrotroponin-releasing hormone (TRH), the pituitary gland is triggered to produce and secrete TSH. The TSH itself causes the thyroid gland to release two hormones: triiodothyronine (T3) and thyroxine (T4). These two hormones act at several physiological levels including the basal metabolic rate, regulation of bone growth, protein synthesis, fat and carbohydrate metabolism, neuronal maturation, cell development and differentiation. Subsequently, a lack as well as an excess of T3 and T4 can cause significant clinical disorders and are respectively named hypo- and hyperthyroidism. The measurement of the TSH blood level is performed to determine if the thyroid gland is deficient or not in the case of an abnormal level of T3 and T4. (111)

TSH has been successfully used for the demonstration of the efficiency of an ICS biosensor to detect a large analyte (28kD). (95)

No ICS biosensor has yet been manufactured for the measurement of TSH level. Indeed, this particular biomarker would require a quantitative measurement and not simply a qualitative one as the hormone is present in the blood even in a non-pathological context. Currently it has not been achieved with an ICS biosensor.

Ferritin

Ferritin is an ubiquitous intracellular protein. Its role is to store, transport and release iron in a controlled manner. It is also one of the largest soluble proteins (450 kDa). Ferritin is globular with 24 subunits and found in both prokaryotes and eukaryotes organism. Besides its main role in iron transport, ferritin also plays in the immune response, with an increase of its concentration in the presence of an infectious agent, and in the stress response, also with an increase. As with TSH, both a low and a high level can be a problem. A low level generally corresponds to a situation of anemia, whereas an elevated level can be the marker of an iron overload disorder or an infection. (112)

Ferritin, as well as TSH, was successfully used for the demonstration of ICS efficiency to detect large analytes. (95)

DIGOXIN

Digoxin is a cardiac glycoside used in the treatment of different cardiac problems, mainly atrial fibrillation and atrial flutter. It belongs to the class of drugs with a narrow therapeutic range and presents many uncomfortable but common adverse effects like nausea, vomiting, diarrhea, loss of appetite. Other and more disturbing effects are also commonly reported by patients including dizziness, insomnia, agitation or depression.

It owes its numerous side effects to its narrow therapeutic range. For this reason, the serum digoxin concentrations (SDC) are monitored on a regular basis. (113)

Digoxin, was used for the demonstration of the ability of ICS biosensors to detect a specific target. But this time, the aim was to prove the efficiency for small analytes as digoxin is only a 781 Da protein. (95)

Quantitative measures were undertaken in proportion to the admittance measured. But to date, ICS biosensors are not used for the routine follow-up of the SDC, since an accurate result was not attained routinely.

DNA

The detection of DNA by biosensors, specifically named genosensors, is an application of major interest. As seen previously (section 1.1.2.2), they can provide useful diagnostic tools allowing the detection of microorganism genomes for instance. This application is in fact a quick way to diagnose an infectious disease.

As for all the POC devices, the main characteristics required for the identification of an infectious agent are the quickness of response, the requirement of only a small amount of sample, and the portability.

DNA-functionalized tethered membranes can be realized using the ICS technology. Indeed DNA probes specific to a bacteria or a virus can be used as the receptors. This technique should provide a rapid answer as to whether there is a specific pathogen is present or absent in the sample tested. But this is only an example of lipid membrane-based biosensor

for the detection of nucleic acids. Other ways to hybridize and detect DNA on lipid membrane proved their effectiveness (3) (114) (115). In 1997, a regenerable system providing a fast response with a small amount of sample was thus shown as successful (116). Then in 1998, the same research group, CG Siotourou and al, demonstrated the efficacy of their new DNA biosensor which had the combined advantages of sensitivity, rapidity, portability (117).

Lipid bilayer membranes have also been used for the study DNA-lipid interactions. The understanding of these mechanisms is of major interest for gene delivery and drug carrier applications. (118)

ANTIBODIES

Although antibodies are usually used as the receptor part, linked to a lipid bilayer, the contrary is also possible. Devices have been developed for the detection of specific antibodies by immobilizing a certain type of antigen or hapten into a lipid bilayer membrane. The lipid membrane thus functionalized mimics an antigen-presenting cell. Consequently, when a sample of blood or saliva for instance is brought together with the functionalized lipid membrane, if the antibody type corresponding to the antigens or hapten is present in the sample, the antibodies will specifically bind to their ligand. A transduction system has to be set up to permit the detection of this antibody-ligand interaction. Once again, one could develop such a technology using a ICS biosensor system or a similar technique.

Successful assay of various antibodies through the use of lipid membrane device have been performed during the last few years. (119) (120) (121)

This type of device is very useful for diagnostic purposes. Instead of detecting the infectious agent, as with DNA or antigen detection, it is the immune response that is detected. The benefits envisaged are the same for all three techniques: a rapid, cheap, easy and sensitive diagnostic for an improvement of the patient support.

A special application is the detection of auto-antibodies. Auto-antibodies are produced by both cancers and autoimmune diseases (35). The binding of antibodies to their lipid membrane-immobilized target is very useful as it permits the determination of the

pathology and the severity of the disease years before the onset of clinical symptoms (122). Detection assays for auto-antibodies have been performed for several diseases including lung cancer, Alzheimer and Lupus (123) (124) (125).

1.4 NON BIOSENSORS LIPID MEMBRANE APPLICATIONS

Lipid bilayer membrane-based biosensors constitute the main *in vitro* applications of lipid bilayer membranes. However, other utilizations were, are and will be investigated. One could easily imagine that such an interesting structure able to mimic cell membranes should be applicable to many interesting other uses.

The cell membrane is the core of many types of intra- and extra-cellular exchanges that provide communication pathways. In short, it has a major role in the cell life. For this reason, the understanding of its functionality, structure, mechanisms, interaction with biomolecules and other membranes is fundamental. Artificial cell membranes naturally represent an ideal model for these studies.

Peptide-functionalized lipid bilayers can be used for the immobilization of cells and for the combination of these immobilized cells with devices. Neuronal cells have thus been combined with transistor interfaces. Although the construction of such devices is still at an early stage, they have become the workhorse of several research groups and in the near future should appear as commercially available bioelectronic materials. Moreover the immobilization of cells on the devices also allows studies of the properties of different cell-types (57).

Generally, biomimetic lipid membranes form a good way of building bioinspired interfaces. They comprise the most important requirements for the manufacture of such interfaces, that is to say the suppression of non-specific protein absorption and of the biological responses derived from it, the ease of fabrication, the ability to immobilize biomolecules, the maintenance of higher biofunctions and the high capturing ability of the target biomolecules. The ability to manufacture interfaces capable of controlling the interactions with cells is of substantial interest for various medical purposes including cell and tissue engineering and nanobioengineering. (3)

Lipid interfaces have been developed for different other type of uses, including cell sorting, cell preservation or the manufacture of cell-driven nanomachines. (126)

Artifical cells mainly owe their construction to the existence of an artificial lipid bilayer. The lipid vesicle is the main element as it constitutes the envelope, which provides the skeleton. Then different proteins, peptides and ions can be added to the vesicle to introduce the possibility of cell-like activities. Artificial cells are designed to perform different tasks including therapeutic applications. They are an ideal model for the study of cell structure and function but also to study the pharmacological or toxic effect of potential drug or pathogen molecules. Different levels of complexity can be brought in according the feature added to the lipid bilayer. Artificial cells will be reviewed later in this report (section 2.3). (56)

In fact, apart from diagnostic applications mainly performed through biosensing systems, the lipid bilayer also has a key role in drug research. Versatile objects based on lipid membranes have been designed which permit the study, *in vitro*, of the interactions of drug with membrane-bound receptors, but also drug targeting and the mechanisms of penetration and permeation of cell membranes. (127)

The major factor of investigating the mechanism of drug actions, biomimetic lipid membrane drew the attention of research group focusing on *in silico* modeling. Computational technologies were thus developed. The aim of *in silico* modeling is to create stimulations of membrane binding and transport or to become able to predict the reaction of a lipid bilayer to a specific drug, and hence its permeability. Computational modeling would also be suitable for drug delivery research. (128)

Such a methodology is cheap and would enhance drug design and development. Due to the current advancement of sciences in both the fields of biology and informatics, it is now possible to consider the creation of a credible computational system. Before synthesizing and employing any molecule, its interest and relevance as a potential drug could be tested *in silico* which could save a lot of time and money for public and private research groups.

2. IN VIVO THERAPEUTIC USES OF LIPID MEMBRANES

The use of biomimetic lipid membranes is convenient to study the properties of biological cell membranes. This is particularly the case for studying membrane proteins incorporated in the lipid membranes, for ligand-receptor specific binding interactions, and to discover new drugs by performing *in vitro* assays. But the property of biomimicry of natural cell membranes also makes possible the use of lipid bilayer for *in vivo* applications as they are biocompatible by definition. Biomimetic lipid membranes could thus be used to cover various needs like drug delivery, bioartificial organs, encapsulation tools to allow the biocompatibility of exogenous medical equipment (9). Over the last 40 years numerous ways to use lipid bilayers have been considered and many devices using lipid membrane technologies are already commercially available. The *in vivo* therapeutic applications of lipid membranes are reviewed thereafter.

2.1 DELIVERY SYSTEMS

The main concerns in pharmacotherapy are the ease of administration, safety and efficacy of the drug treatments (131). Affordability is also a major issue. With the goal of solving these concerns, research started to focus on better drug delivery systems. The most popular system thus developed is the liposome which mimics the plasma membrane of cells but is lacking the internal cellular contents (132). Other vesicle-shaped carriers have been developed including virosomes, which mimic a virus envelope, and polymersomes, which mimic cell membrane but are composed of synthetic copolymers.

As they are made of a lipid membrane and the most popular carrier, liposomes are the system on which we will focus.

In a general manner; drug carriers can be used for different purposes, including: (132)

- Solubilize a drug that usually requires a high concentration of toxic emulsifying agents

- Use of long circulating vehicles for site-specific accumulation especially in tumors where the microvasculature is more abundant and more permeable, also know as the Enhanced Permeability and Retention (EPR) effect

- Create a high concentration of a drug at or near the site of action.

The ideal carrier would quantitatively retain the drug until it reaches the target site, and then release the drug at the desired rate.

Drug delivery is a fast growing market which covers different therapeutic sectors such a diabetes, oncology or infectious diseases. This market can be segmented in function of the route of administration as seen on Figure 14.

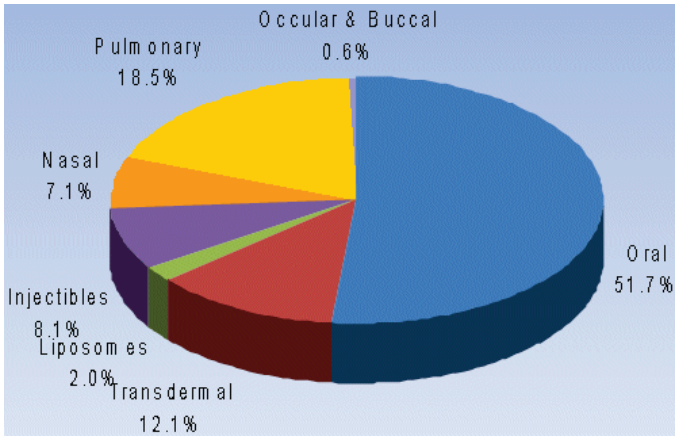


Figure 14 Illustration of Sylvia Miriyam Findlay (149) showing the segmentation of the global drug delivery market in 2007.

As seen on this chart, with 51.7% of the market the oral route is the privileged route for drug delivery formulations, which is not surprising at is it the classic route for conventional medications.

2.1.1 Description of the liposomal system

The first liposomes were developed during the 1960s to be used as models for the study of cell membranes. Then in the 1970s, some researcher considered then as potential means of delivery for cytotoxic drugs (131).

In the late 1980s, liposomal delivery was showing great promise with the commercialization of three different liposome-formulated drugs: doxorubicin and daunorubicin, two chemotherapeutics, and amphotericin B, which is an anti-fungal

medication. The progression was then slowed because of the need to optimize the characteristics of drug loading within liposomes and release from the liposomes (128).

In around 1998, five new intravenous drug formulations based on liposomal delivery were approved by the Food and Drug Administration (FDA). These drugs correspond to the same molecules as the three above:

- DOXIL, Daunosome: doxorubicin and daunorubicin
- Abelcet, Amphotec, Ambisome: amphotericin B.

The market of these five products was valued at $250 million in 1999 (135).

During the last two decades, considerable advancements have been achieved regarding the use of liposome as drug delivery vehicles (56) (132).

Liposomes are also very attractive for cosmetical purposes, they constitute a common component for cosmetic formulations (136).

Different type of liposomes can be manufactured according to the method of preparation. Also named lipid vesicles, liposomes are polymolecular aggregates of lipids. The lipids are bilayer-forming amphiphilic molecules with hydrophilic head groups and lipophilic tails, as explained in section 1.3.1. Lipids in an aqueous solution have the property of naturally forming a self-closed continuous lipid bilayer. Under balanced osmotical conditions, the shape adopted by the lipid assembly naturally tends to a sphere. The hydrophilic part of each lipid is in contact with the aqueous solution, on the inner and outer of the vesicle, whereas the hydrophobic part forms the interior in between the two hydrophilic leaflets of a bilayer. The spherical vesicle can contain one or more lamellae of lipid bilayer depending on the production technique used, in order to form multi-, oligo- or unilamellar liposomes. The size of the vesicles fluctuates between about 20 nm to several mm with a range of 20 to 200 nm for the unilamellar vesicles. (134) (135)

Multivesicular vesicles (MVV) can also be formed. They consist of an entanglement of vesicles of various sizes within or next to each other to form an agglomerated larger vesicle.

Liposomes are also described as lyotropic liquid crystals. (140)

In the following illustration (Figure 15) are shown the different type of liposomes.

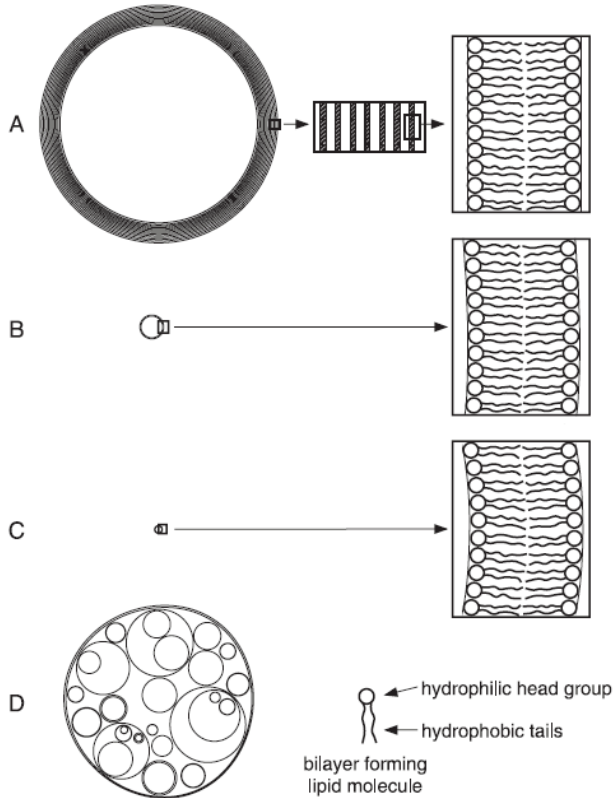


Figure 15 Illustration of P Walde (134) schematically representing the different types of liposomes. (A) A multilamellar vesicle (MLV), typical diameter of 1 µm. (B) A large unilamellar vesicle (LUV), typical diameter of 100 nm. (C) A small unilamellar vesicle (SUV), typical diameter of approximately 30 nm. (D) A multivesicular vesicle (MVV).

The inside of the liposomes is an aqueous core which is roughly chemically composed of the same solution as the solution used to prepare the liposomes. They might be anionic, neutral or cationic depending upon the surface charge.

A liposoluble drug molecule will be entrapped into the lipid bilayer of liposome, whereas a water-soluble molecule will be entrapped inside the lipsome, in the aqueous core, or into the channels between the successive bilayers in the case of a MLV.

A liposome containing a protein is named proteoliposome. The term proteinliposome can also be found.

The most common mechanism of release of the drug from the liposome is endocytosis. Three other mechanisms of interaction with cells can be involved for the release of the liposome content: adsorption, fusion and lipid exchange (141). As a consequence the active molecule is released within cells and can perform its pharmacological action(s). Thus a molecule that could not naturally diffuse through the lipid bilayer is delivered in the

intracellular medium. On the other hand, hydrophobic molecules will be embedded into the cell membrane during the process of endocytosis.

The spleen and liver play a major role in the clearance of the liposomes that do not interact in some way with cells or tissues of the body.

Lipid vesicles are a very varied and flexible class of drug carrier systems. Encapsulation inside liposomes shows major advantages including a possibility of targeted release, which reduces the toxicity and side effect of a drug and increases its efficiency at a same dosage.

The reason why liposomes were first investigated as drug carrier was because of their properties of biomimicry and non-immunogenicity. They permit the compartimentalization of the drug: a proteoliposome can be regarded as a functionalized compartment. As biocompatible element, liposomes theoretically allow: (132)

- A facilitation of drug uptake
- A protection of the drugs: solubilization, decreased exposure to elimination sites and enzymatic or acid attacks
- A protection of the organism: lower exposition to the drug so lower side effects, lower toxicity
- A mean to control drug targeting: site-specific delivery
- Masking of the taste of a drug for patient comfort.

But this is not always the case and not always as efficient as hoped. For example, the toxicity cannot be entirely eliminated which results in a wider therapeutic window due to the remnant partial toxicity. However, this is still an improvement in efficiency of delivery toxic drugs.

Although, the parameters influencing lipid and liposome properties as well as drug-lipid and drug-liposome interactions remain to be widely investigated. To be used in drug formulation, these properties need to be controlled. The characteristics of liposomes are still studied to ensure the safety and efficacy of the drug molecule as well as the comfort of

the patient. Lipid vesicles are actually the most intensively studied lipid-based delivery system.

However liposomal encapsulation presents some issues. It is very difficult to assess the rate of drug release in blood and the different tissues. The control of the location, timing and amount of the release is also very tricky and often poor. Consequently it is a delicate balance between drug retention into the liposome, drug targeting and systemic toxicity. Essentially, the understanding of what happens to a liposome once in the body needs to be studied more deeply, in more details. In fact the relation between pharmacokinetics, bioavailability, and toxicity is not simple to elucidate.

As seen previously (section 1.4), molecular dynamic computer simulations are now available and provide new insights into biological processes. It could aid in the collection of data and information that cannot be accessed experimentally.

The encapsulation of hydrophobic drugs with liposomes remains challenging since the improvements of the pharmacokinetic profile are very low when compared to the improvements reached for amphiphilic drugs.

Similarly, hydrophobic drug liposomal formulations still encounter hurdles in terms of efficiency as the drug release is not entirely predictable. One has to consider the use of multiple triggers to increase the bioavailability of hydrophobic molecules at their site of action.

In fact, the pharmacological profile and effectiveness of a liposomal-formulated drug are depending on several criteria: (136)

- Properties of the encapsulated drug: physicochemical properties, including pharmacokinetics and target
- Properties of lipids constituting the vesicle: composition, degree of unsaturation of the fatty acid tail (< 1% for stability of the membrane) and charge

- Properties of the lipid molecular assembly, i.e the carrier: size, charge, pharmacokinetics, biodistribution and drug release rates
- Dosage and route of administration
- Retention strategy and drug loading
- Targeting strategy: possible conjugation of a ligand.

All these characteristics have to be well-thought and well-defined in designing the function of a specific drug and its therapeutic application.

A wide range of small molecules with therapeutic properties have been unsuccessfully studied using liposomal formulations, including various vinca alkaloids, camptothecins, taxanes, and some anthracyclin analogues. But the formulations were either unstable or too stabilized to efficiently release the active molecule. Further studies were then performed that have led to the current improved understanding of the mechanisms of drug clearance and retention of small molecules in liposomes. This makes possible the production of encapsulated molecules showing optimal activities for their specific therapeutic application.

The basic liposomal delivery system can undergo further modifications such as the conjugation of targeting ligands or environment sensitive-triggers. Environment sensitive-triggering systems correspond to a modification of the liposome to make it sensitive to a specific stimulus, which can be a change in pH, light, oxidation/high reducing potential, enzymatic degradation, heat, ultrasound, or radiation. Whereas the attachment of a ligand molecule leads to the increase of the delivery of the carrier at the site of disease, the use of environment-sensitive triggers lead to an increased drug release from the carrier. One can imagine that the existence of triggers able to accomplish both tasks is possible.

In both cases, the aim of these additional features is always the same; specifically increase the bioavailability of the drug at the site of the disease without increasing the toxicity. Indeed, the effects of some specific drugs would clearly benefit from a high concentration at a specific site which should be more easily achievable by using a triggered release system. Molecules that do not easily cross lipid membranes and subsequently cannot extricate themselves from the carrier would also benefit from a triggered release.

A particular type of liposomes for drug delivery is the stealth liposome which is a type of ligand conjugate system. Polyethyl glycol (PEG) polymers are attached to the surface of the vesicle. Their role is to enhance the targeting of the drug and avoid the detection by the Kupffer cells. It has been shown that PEGylation provide resistance to phagocytosis and complement activation and results in enhanced therapeutic profiles. (127) (131)

Immunoliposomes with attached antibodies that are able to recognize and bind to a specific target are also a common type of ligand targeted liposomes. Other classic targeting elements include metabolites, peptide hormones, and growth factors.

Niosomes are another category of targeted drug delivery systems. Unlike most of the liposomal carriers, they are not composed of phospholipids but lipopolyglycerol, which is a synthetic non-ionic molecule discovered in 1978 by Valenberghe and coworkers. Lipopolyglycerol acts like phospholipids from an organizational point of view (131). Niosomes are a type of synthetic analogue of the liposomes. They are able to entrap molecules and are osmotically active and stable. Previous studies show the equality in term of activity of sodium stibogluconate in niosomes and liposomes formulations.

Passive targeting is also sometimes mentioned (143). It occurs when the physical and/or chemical properties of the vehicle naturally shows a higher affinity for the targeted tissue than for any other tissue. In consequence, the drug delivery at the site of disease is enhanced without the need of targeting elements. Actually the EPR effect (described in section 2.1) is a type of passive targeting.

Even so improvements remain to be done as much on the knowledge side as on the technical and practical side. Recently, considerable advances were made in controlling the pharmacokinetics and drug release profiles of drug in liposomal carriers (147). Several major issues need to be solved, on which several research teams are working. Moreover, some difficulties already have a solution. For instance, the addition of both ligand and environment-sensitive triggers could result in an efficient way to realize therapeutically improved hydrophilic drug in liposomal formulations.

2.1.2 Other drug delivery system using lipid membranes

Liposomes and especially unilamellar vesicules stand as a reference and a standard for drug delivery. A large number of publications have been written on the usage of unilamellar liposomes properties and their therapeutic efficacy (135) (144) (145).

Nevertheless, other drug delivery technologies exist that for some of them also use lipid membranes. It is the case for microcapsules, nanosomes and vesosomes. These three drug delivery system are reviewed hereafter.

2.1.2.1 Microcapsules

All the microcapsules can be described as hollow vesicles or capsules, mostly fabricated by molecular assembly. Thus liposomes can be considered as a type of microcapsules.

The LbL molecular assembly is the most commonly employed technique for the fabrication of biomimetic microcapsules. This method permits to control of several crucial characteristics like size, shape, thickness, composition, permeation and incorporation of biomolecules. (132)

There are many variations of microcapsules in terms of composition, size, and applications.

The assembly of biomimetic microcapsules is of major interest for therapeutic applications as well as biomedical research. The major medical application is the development of drug delivery systems by the mean of microcapsules, as seen in the previous part with liposomes (section 2.1.1). They can be useful for diagnostic purposes as well. As drug delivery systems, biomimetic capsules facilitate the transport of substances to predefined sites and unload them in a controlled manner.

Even if several efficient drug delivery systems are already commercially available, there is still enough room on the market for new drug carriers with different functions. This is because several issues remain to be solved, including specificity, stability and bioavailability.

A recently developed type of microcapsule is the hollow polyelectrolyte microcapsule. The aim was the manufacture of robust biomimetic capsules with uniform size, mechanical and chemical stability for the entrapment of the largest proteins possible. The principle is to assemble oppositely charged polyelectrolytes on colloidal templates. Then after removal of the colloidal core, one obtains a hollow polyelectrolyte microcapsule. The nature of the assembly components can be very a range of biomolecules including peptides, DNA, enzymes or even inorganic nanoparticles. (146)

A lipid bilayer can be coated on the microcapsule newly formed. The microcapsule would thus benefit from more possibilities of additional features to increase its biofunctionality. Also the presence of a lipid bilayer would permit an easy incorporation of hydrophilic drug molecules. An extra layer of polyelectrolyte components can be added onto the lipid bilayer. The charge of the surface plays a key-role in the cellular uptake.

The mechanism of formation of a polyelectrolyte microcapsule covered by a lipid bilayer is illustrated below: (Figure 16)

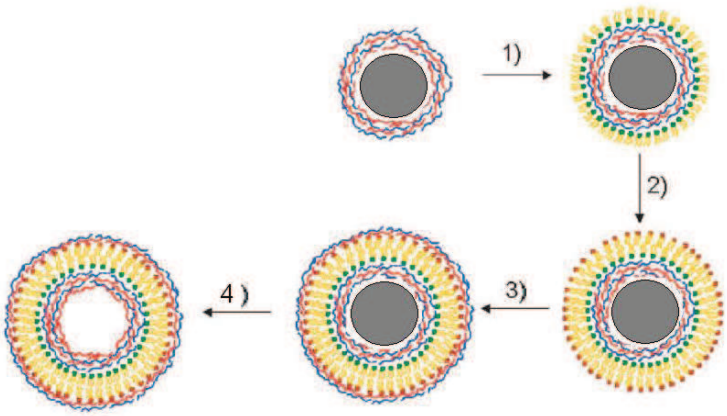


Figure 16 From the illustration of Qiang He (132), schematic representation of the formation of a polyelectrolyte microcapsule covered by a lipid bilayer. Step 1: tethering of the inner lipid leaflet on the molecular assembly. Step 2: addition of the outer layer of the bilayer. Step 3: assembly of an additional layer of polyelectrolytes. Step 4: removal of the core leading to a hollow microcapsule.

One could wonder what the gains of these microcapsules are over more simple liposomal methods. The fact is that microcapsules covered by a lipid bilayer consist in a kind of cushioned-liposomes. The stability of such system is way better, making the lipid membrane less labile.

As for liposomes, and because they are even more stable, these microcapsules need to be functionalized to become sensitive to slight changes in the physiological environment. Otherwise, they cannot selectively release their active molecule at the targeted location. As for liposomes, a targeting ligand can be anchored to the capsule surface.

As with every drug carrier, the microcapsules have several significant advantages over conventional formulations. These advantages are the same as for liposomes, including decreased side effects for a longer duration of activity, a protection of the drug and a targeted release. So far, several studies on various capsule types have been performed. (147) (148)

2.1.2.2 PHOSPHOLIPID NANOSOMES

Nanosomes are small uniform liposomes. Their synthesis requires the use of supercritical fluid technologies. As with liposomes, they are made of phospholipids. Thereby, they are non-toxic, non-immunogenic, and biodegradable since they share the molecular characteristic of biomembranes.

The applicability of using nanosomes for drug delivery is obviously the same as for liposomes, which is to improve the therapeutic action of the simple drug molecule by encapsulating it within a delivery system.

The process of fabrication of a nanosome starts with the solvation of phospholid raw materials in supercritical fluids. Then the whole solution is decompressed ending up by the formation of phopholipid nanosomes.

In the case of a hydrophilic drug, the encapsulation is performed after the formation of the nanosome carriers. As for a hydrophilic drug, this one is co-solvated in supercritical fluids with the lipids. During the decompression, the lipids incorporate the molecule within their bilayers.

The characteristics of the nanosomes, including their size will depend on several parameters: (149)

- Size and design of decompression nozzle: affect the size
- Pressure and rate of decompression
- Interfacial forces between the supercritic fluid phase and the aqueous phase
- Charge distribution of the nanosomes

- Nature of the compound being encapsulated.

As for microcapsules, one can wonder about the advantages of nanosomes over basic liposomes. Despite the fact that they necessitate the involvement a more complicated technique of production, they result in the formation of very uniform vesicles. In term of medical applications and ethical approval, the control of the formulation is a key-factor. A uniform formulation more likely results in a uniform drug content, and thus in a repeatable and expectable therapeutic effect, which is of major importance when dealing with a drug molecule. Also, smaller molecules are cleared from the circulation at a lower rate than bigger molecules like classic liposomes. In consequence, nanosomes could offer a longer circulation lifetime and thus an enhanced cell uptake of the active molecule transported.

2.1.2.3 Vesosomes

A vesosome is a multicompartment drug vehicle. Its structure follows the principle of the multivesicular vesicle (MVV) described above, in section 2.1.1. For this reason, vesosomes are a subcategory of liposomes.

In fact, vesosomes are even closer from the eukaryotic cell structure than the basic unilamellar liposomes usually used for drug delivery applications. The vesosome mimics the nested membrane structures defining of eukaryotic cells that define internal compartments for the nucleus and different organelles. Each compartment is able to accomplish an individual specialized function. For this reason, vesosomes appear to be attractive structures and have been recently studied and developed for drug delivery purposes. (135)

The technique for fabrication of multivesicular vesicles involves the solubilization of phospholipids in ethanol. When a temperature above the gel-liquid crystalline transition temperature is reached, lipids start forming closed vesicles. While a vesicle is closing, it can internalize other vesicles leading to a nested arrangement. Furthermore other elements like biomolecules can be entrapped within the vesicles in the same time. Below is a schematic representation of a typical vesosome (Figure 17).

Additional features can be attached to the structure like PEG-lipids for a better stabilization and targeting ligands or environment sensitive-elements, exactly as for liposomes.

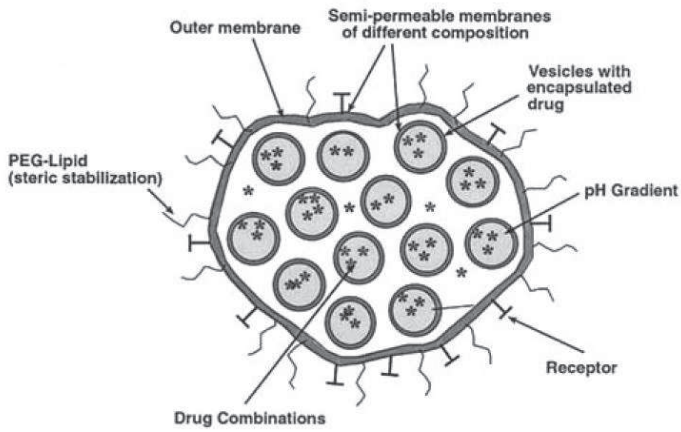


Figure 17 Illustration of ET Kisak (135) showing a schematized drawing of a PEGylated vesosome with attached targeting ligand (receptor).

In vesosomal formulation, the inner compartments are separated from each other as well as from the outer membrane. Each of them can have a different composition in lipids and encapsulate a different substance. Moreover, biological macromolecules can be easily entrapped within vesosomes, whereas with a basic unilamellar vesicle the encapsulation of large molecules is complex. (150)

Each closed bilayer provides a closed environment with its own properties and biochemical characteristics. As a result, the encapsulation of both hydrophilic and hydrophobic drugs in different compartments of the same vesosome is possible.

Additionally, vesosomes provide a better protection than liposomes to the encapsulated drugs against aggressive physiological factors, such as acidification or enzymatic degradation that could lead to a premature release. Every additional lipid bilayer constitutes a supplementary protection against lipolytic enzymes. Consequently vesosomes offer the possibility of an extended release of the molecule and a higher bioavailability.

The progress achieved in liposome development over the last decades is useful for the development of vesosomes since they are very similar. Therefore, the same stabilization and drug loading techniques can be applied to vesosomes. They also benefit from the

numerous studies on the biocompatibility of liposomes. In fact, vesosomes retain all the essential features of unilamellar liposomes but provide additional enhancements.

One considers that the additional difficulty in the assembly of a vesosome is outweighed by the benefits resulting of a multicompartmented structure. Although, clinical studies to demonstrate the effectiveness of vesosomes as drug delivery vehicles still need to be completed.

2.1.3 Therapeutic use of lipid membranes

There are several applications of lipid membrane-based drug delivery systems. Indeed, one could consider the encapsulation of all kind of drug molecules within liposomes or any of the other technologies mentioned above. Nonetheless, for many well-known and well-controlled basic therapies, there is no need for a drug carrier. Drug delivery systems are often required for delicate active substances or particular medical applications.

The following applications that are described are the most noticeable and the better studied. Hence, this list of applications is not exhaustive.

2.1.3.1 Encapsulated drug

Nucleic acids

Nucleic acids are generally used for gene therapy to treat genetic deficiencies and many acquired diseases. These include large DNA molecules like plasmid DNA (pDNA), smaller DNA molecules like oligonucleotides (ODN), and RNA like ribosymes, SiRNA and mRNA. The pDNA has the ability of encoding the other types (antisense ODN, ribozymes and SiRNA) as well as proteins. (151)

Whereas RNA and ODN fragment are relatively short and thus quite easy to encapsule within a carrier, pDNA are bigger which makes them more difficult to transport. In addition, RNA and ODN targets are mainly cytosolic or even if the target is nuclear, they easily pass passively through the nuclear pores. On the contrary, pDNA target always is the cell nucleus since the aim is to generate gene expression but pDNA cannot cross the nuclear pores and need to be actively transported.

Both viral and non-viral delivery options are explored. The use of viral vectors has already been proven as efficient to cure some genetic diseases. But other carriers have shown encouraging results including the encapsulation within cationic liposomes. Trials have been performed with naked nucleic acid sequences, i.e without any delivery system. However the cellular uptake was inefficient and required a large amount of the drug.

Cationic lipids are numerous. Usually, when used to form liposomes, they are combined with a type of neutral lipid. The process of complexing of DNA fragments like pDNA with a cationic vesicle involves electrostatic interactions. The negatively charged nucleic acid sequence binds to the positive lipid membrane surface. The so-formed proteoliposome is called lipoplex. There also exists lipopolyplexes which are combinations of DNA and polymer forming a polyplexes encapsulated in a liposome. The encapsulation of nucleic acids within cationic liposomes is an "active" encapsulating method resulting in high encapsulation efficiency. (152)

Passive encapsulation methods using anionic and neutral liposomes have also been considered. But the rate of encapsulation is only around 3 to 10%, which is not efficient enough.

These mechanisms are not specific to pDNA, and can also be performed for smaller nucleic acid sequences. (127)

Both lipoplexes and lipopolyplexes have shown efficiency as gene delivery systems. The mechanism of delivery is usually the capture of the whole system by the cells: the artificial lipid membrane merges with the anionic cell membrane. The system is then internalized into vesicles within the cells by endocytosis.

This solves the problem of pDNA transport and nucleic acid protection in the circulation. However, there remain the issues of accurate targeting and intra nucleus delivery (152) (153).

One of the greatest challenges of gene therapy is the delivery of the gene-based drug to the desired cell type. For this reason the carrier, after assuring its role of nucleic acid protector within the circulation, has to protect the drug from the cellular endo- and exonucleases once inside the cell, and also release the drug inside the appropriate intracellular compartment.

As seen in the previous part (section 2.1.1), different strategies can be established to control the targeting of a drug molecule. These strategies include the attachment of targeting-ligand or of environment sensitive-elements. This is applicable to the targeting of nucleic acid delivery. However, much work remains to be done in this field.

The delivery system is selected based on several parameters that include the disease to treat, the length of expression (transient or long-term), and the degree of expression (low or high) required for the gene of interest.

Studies have shown that DNA transported by a cationic lipid carrier system can have an immunostimulating effect due to the interaction of the DNA with the blood components, which leads to the activation of the complement system. It results in various toxic effects: inflammatory response, hepatotoxicity, hemato- and serotoxicity (154) (155). Their clearance from the blood is thus rapid. For this reason the use of PEGylation of the liposomes is often applied. The use of vesosomes could also be useful, with the entrapment of lipoplexes within a bigger negative or neutral liposome, hiding the DNA from the blood. The external lipid bilayer could then be submitted to additional modification for stabilization and/or targeting purposes.

Clinical trials on the cationic liposome encapsulation of nucleic acids have demonstrated the safety and non-immunogenicity of the technique. Even so there is still a concern of degree of activity which is lower than with viral vector methods, the liposomal delivery of nucleic acids increase the cellular uptake and the thus the therapeutic efficiency (139). Nonetheless, more clinical studies need to be undertaken to validate the liposomal delivery of nucleic acids.

Currently, viral vectorization of nucleic acids still demonstrates a better therapeutic efficacy than the liposome encapsulation with a higher rate of expression of the gene-based drug. However, viral vectors are subject to a significant degree of safety concerns, which makes attractive the development of alternative techniques such as liposomes with lipoplexes and lipopolyplexes. The basic issues of poor transfection reproducibility need to be solved (135). This is likely due to the low uniformity in size and structure of the liposomes.

Liposomal systems are not the only DNA carrier that has been developed. Peptide carriers, polymers,and polycations such as polylysines, polyarginines, spermine, or dendrimers have

also been studied as nucleic acid complexing systems. As explained above, vesosomes also represent a great opportunity for gene based-drug delivery system development.

The development of all these delivery systems is of major important since it should increase the number of reachable targets for gene therapy.

SIRNA

As explained above, siRNA is a subcategory of the nucleic acids. siRNA has been the specific focus of many research groups over the last decade. However, it is important to keep in mind that gene silencing mentioned hereafter is a type of gene therapy, since siRNA are types of nucleic acids. Thus all the previous details on nucleic acids are applicable to siRNA.

Discovered in the late 1990s, the interest in siRNA keeps growing (151). In fact the discovery was related to the RNA interference (RNAi) which was demonstrated as a very powerful method of gene silencing to the point that they quickly became almost a standard method for the knockdown of targeted genes. This property would be very useful to knockdown pathologically relevant genes. Indeed, some specific pathologies owe their etiology to the upregulation of one or several genes causing their over-expression. (151) (133)

The activity of gene silencing is in fact due to small interfering RNA (siRNA). A siRNA has the ability to naturally target a specific gene and cause its downregulation. But for this interference to happen, the specific siRNA needs to be in the intracellular presence of its specific gene target. As a result, the *in vivo* targeted delivery of specific siRNA is indispensable if one wants to develop an RNAi-based therapeutic application. In fact, the potential future range of applications will more than likely depend on the ability to provide a sufficient amount of bioavailable siRNA at a specific targeted site.

Drug delivery vehicles have thus been developed for the transport and release of siRNA (152). As reviewed previously, for nucleic acids in general, the drug delivery methods using lipid membranes are liposomes, lipoplexes and lipopolyplexes. The parameters that need to be under control are the same as for any other carried drug, including the

protection of the drug molecule (siRNA), cellular uptake, accurate tissue and intracellular localization and endosomal release.

In fact, the intracellular delivery of siRNA is not the only way to achieve gene silencing. The siRNA therapeutic mechanism can also be achieved with the transport and nuclear delivery of pDNA constructs encoding for siRNA.

The success of siRNA was very rapid and the field of RNA interference has been explored expansively. Several siRNA-based drugs have already been successfully submitted to clinical trials and a lot more are already planned to undergo pre- and clinical trials (151) (152) (153) (157) (158). The first one to enter clinical studies, in 2004, was the siRNA Cand5 targeting the vascular endothelium growth factor VEGF for the treatment of patients suffering from age-related macular degeneration. This drug was introduced by Acuity Pharmaceuticals. However, the research for suitable, safe and effective drug delivery systems is still relevant. As explained for the case of nucleic acids, even if liposomes and their derivative provide a safe intracellular uptake and show a correct efficiency of the drug, the activity is lower than with viral vehicles and thus remains to be enhanced.

The improvement of the existing drug delivery systems and the development of new ones specifically adapted to siRNA would be a major step forward in the treatment of diseases based on the aberrant regulation of genes.

ENZYME

Enzymes are present at every level of physiological activity and are essential for the smooth performance of physiological functions. As early as 1970 the use of lipid membrane to carry enzyme was considered with a report on the formation of proteoliposomes containing molecules of lysozyme. (163)

Liposomes have been shown as an efficient system for the delivery of a variety of enzymes. Enzyme-containing lipid vesicles could be useful for various therapeutic applications like enzyme-replacement therapy, but also analytical application type immunoassays. (138)

The enzyme is entrapped in the aqueous core of the lipid membrane if the vesicle is neutral. A number of methods have been developed for the encapsulation of hydrophilic

enzyme within lipid vesicles. Most of these methods end up with the formation of unilamellar vesicles.

Usually, the lipid vesicle is composed of a certain amount of negative or positive lipids. The presence of a charge leads to the effective absorption of the enzyme without the need of any organic solvent during the entrapment process. The enzyme can electrostatically bind on the inside or the outside of the liposome.

The difficult part, as for every drug molecule, is the time of release of the protein. In the case enzymes, they have to be discharged from the liposome before being able to catalyze any extra-liposomal reaction. On the other hand, the purpose can be the catalysis of a reaction within the lipid vesicle. Regarding this particular application, the enzyme should not be released from the vesicle before exerting its action and the substrate has to cross the lipid bilayer to penetrate the liposome. But in a general manner, the enzyme target-reaction occurs outside of the liposome which could be explained by the additional difficulty of having a substrate able to pass through the lipid membrane as its permeability is very low.

According to the particular enzyme to transport and the therapeutic application sought, the choice of the amphiphile and of the assembly and entrapment method will be variable. There is no standard choice.

To date, only a few *in vivo* studies have been completed. However the use of stabilized lipid vesicles has been demonstrated as an advantageous drug delivery system.

2.1.3.2 Therapeutic target

Immunotherapy

Immunotherapy comprises in the induction, enhancement, restoration or suppression of the immune response against a targeted disease or infection. Immunotherapies can be divided into the two categories of activation immunotherapy which promotes the immunity system, and suppression immunotherapy which slows down or inhibits the immunity system.

The major applications of immunotherapy are in the field of cancer, infectious and allergic diseases.

Oligodeoxynucleotides (ODN, see section 2.1.3.1) containing a particular amino acid sequence with a cytosine followed by a guanidine (CpG) have been highlighted for their therapeutic potential as immunotherapy drug for the treatment of the three diseases mentioned above (157).

Clinical trials have been performed with free CpG ODN, but their efficiency was limited due to low stability, toxicity, poor pharmacokinetic characteristics, low specificity and low intracellular uptake.

To face these different issues, strategies of drug delivery were developed including lipid membrane-based methods. As seen earlier, as CpG ODN are nucleic acids, they can be submitted to a passive encapsulation within anionic or neutral liposomes or to an active complexation with cationic liposomes.

The passive encapsulation of CgG ODN in liposomes leads to a low level of encapsulation. On the contrary, with cationic liposome methods the level of encapsulation is elevated. Moreover, lipoplexes show a higher immunostimulary activity in comparison with free CpG ODN (167). However lipoplexes face important issues with a wide range of size, poor uptake reproducibility and a positive charge that can give rise to non-specific interactions with cells or other elements present in the circulation. Another major drawback of lipoplexes is their capacity to generate many side effects, altering different organs, as seen previously (section 2.1.3.1).

Various studies have shown that the liposome delivery of certain CpG ODN sequences is able to increase the circulation lifetime of the molecule which improves its accumulation at sites of diseases. As a result, the immunopotency of the molecule is increased and so is its capacity to generate a prophylactic or therapeutic immune response in a wide range of diseases including infections and cancers (168). On the other hand, other liposome-encapsulated CpG ODN sequences benefit from this accumulation enhancement to exert their inhibiting activity on the immune system and thus induce an immunomodulation, which very useful for the treatment and/or prevention of allergic and autoimmune diseases.

Another way that liposomal formulations can take to improve the activity of CpG ODN-based drugs is to act as drug reservoirs to allow a progressive release of the therapeutic molecule.

Studies have demonstrated that the immunostimulatory effect of liposomal CpG ODN is insensitive to the choice of the route of administration between subcutaneous and intravenous injections. (168)

The delivery of specific targeted antigens through liposomal carrier is another interesting way of creating a desired immunostimulation. This would also be useful for the prophylaxis and the treatment of infectious diseases and cancers.

As for any liposomal-based delivery system, the liposome can undergo some modifications for an increased stability (PEG ligands) and an active targeting (ligands or environment sensitive components). Passive targeting can be considered when the immunotherapy is directed against an infection or a cancer since, in these special conditions, the vascular membrane is more permeable at the site of the disease and allows for a better cellular uptake (169) (170).

Immunotherapies also include vaccinal applications since a prophylactic immunostimulation can be considered as similar to a vaccination. Research for the development of vaccines using liposomes is not new, some teams were already working on it a couple of decades ago (172). Indeed CpG ODN use for vaccinal application has been shown as an efficient and safe adjuvant (166). Nevertheless, cationic liposome formulations are not sufficiently immunostimulatory. Consequently, they are used as adjuvants to improve the vaccinal effect of a more powerful frontline vaccine molecule. Within the last six years, two cationic liposome-based adjuvants have reached the clinical phase (167). In this case, the liposome is used to add to the immunogenicity of peptide or protein antigens. The other way of using liposomal delivery systems like vaccine adjuvant is for the delivery of antigens (168). The field of liposomal adjuvant discovery and development for vaccinal applications is in full growth and more of them should appear in a near future.

CANCER

In the 1970s, liposomes were already studied to develop drug delivery vehicles for transport and release of cytotoxic substances transport and release into malignant cells. By

this time, the positive impact of liposomes on the biodistribution and rates of clearance of cytotoxic molecules was demonstrated and the hypothesis of their capacity to enhance the activity of such drugs was also formulated (134). About 10 years later, liposomal antineoplasic drugs were undergoing clinical trials. These formulations were consisting in the encapsulation of well-known cytotoxic molecules like doxorubicin, daunorubicin or methotrexate (141). The two first ones gave rise to several commercially available products as seen previously (section 2.1.1).

Paclitaxel, a taxane isolated from *Taxus brevifolia*, is a hydrophobic molecule of interest for the treatment of a wide range of cancers including breast, ovarian and lung cancers. Due to its hydrophobicity, paclitaxel activity is improved when the molecule is encapsulated and released only at the site of disease. The use of liposome-based drug delivery system for paclitaxel has been largely studied. Nanosome formulations (section 2.1.2.2) of paclitaxel were also studied, showing in a general manner encouraging results with a lower toxicity and a higher intracellular uptake than the conventional formulation for this drug (paclitaxel in Cremophor EL or Taxol). (176)

Another example of an anticancer drug with low water solubility, camptothecin, drew the attention of research groups, including the United States national Cancer Institute, for its powerful activity against various cancers (leukemia and colon cancer amongst others). Liposomal delivery systems and also nanosome-based delivery systems have been studied for camptothecin, showing encouraging results as well. (150)

Many other cytotoxic molecules have been incorporated into liposomal carriers (179) (133). In each case, the choice of the appropriate delivery system relies on the structure of the drug as well as the properties of the targeted tumor. The next table (Table III) is a list of various anticancer drugs that have been encapsulated into liposomes.

Camptothecins	•Camptothecin •Irinotecan •Topotecan •SN-38 •CKD602 •Lurtotecan
Anthracyclines	•Doxorubicin •Daunorubicin •Mitoxantrone
Vinca alkaloids	•Vincristine •Vinorelbin •Vinblastine
Hydroxamic acid	•LAQ-824
Taxane	•Paclitaxel
Platinium alkylating agent	•Cisplatin
Thymidylate synthase inhibitor	•OSI-7904
Nuceloside	•Cytaribine

Table III List established by Daryl C Drummond and al (133) of various anticancer drugs which have been encapsulated into liposomes as drug delivery systems.

Stealth liposomes are very interesting for cytotoxic delivery purposes. The presence of PEG bound to the surface of the liposome avoids the phagocytosis of the stealth liposomes. Consequently the drug carrier and the drug molecule itself benefit from an extended lifetime in the circulation. This is favorable to an increased concentration of the drug at the site of disease with a reduced toxicity. PEGylation is also applicable to nanosomes, as seen previously. (131)

As far as doxorubicin is concerned, PEGylated liposomal formulations do not display a significantly better antitumor efficacy than non-PEGylated liposomal forms, even if the pharmacokinetic parameters are modified by the presence of PEG.

The mechanism of release of a drug from a liposomal carrier involves two steps. First there is diffusion of the drug delivery system out of the very permeable capillary bed of the tumor, followed by the fusion, adsorption or endocytosis of the liposome into the cell. Once released within the cell, the drug can exert its therapeutic activity.

The cell targeting and uptake can be achievable using a passive strategy in the case of a tumor. Indeed, the intercellular junctions in the capillaries, which are supposed to be tight,

are about a hundred times wider in tumor tissues. According to their size, liposomes cannot cross the intercellular junctions under normal conditions, whereas the large junctions occurring in cancer situations provide the appropriate dimensions for the passage of liposomes (128). In addition, once in the tumor, the liposomes are retained and they have no easy way out. This phenomenon is known as the enhanced permeability and retention (EPR) effect.

Hence, the encapsulation of a drug into a liposomal carrier leads to a lower distribution of this drug in normal tissues and a higher concentration in tumor tissues. The EPR effect is in fact a passive way of targeting a tissue making easier the specific accumulation of an anticancer drug within a tumor (177).

However, the use of an active targeting method could be useful to improve the intracellular delivery of the anticancer drug at the site of disease and consequently enhance the antitumor efficacy of the active molecule (139). Anti-EGFR antibodies presenting liposomes have for example being designed to increase the avidity of tumor cells for the liposomes and enhance the phenomenon of endocytosis and thus the bioavailability of the drug. The rate of endocytosis was demonstrated as a key factor of the antitumor efficacy. Indeed, even though the EPR effect effectively directs the liposomal carrier to the tumor, an active targeting strategy in addition is a good thing to “complete” and improve the specific intracellular delivery in tumor cells and to reduce the non-specific delivery in other cells (178).

The use of a liposomal carrier also significantly decreases the rate of clearance of the drug and hence the lifetime in blood is increased. Early preclinical studies in the field of chemotherapy have clearly demonstrated the correlation between a longer lifetime in the blood and the drug intracellular uptake. Since the lifetime in the circulation is longer, the liposomal system executes repeated passage through the tumor permeable microvasculature, which improves the possibility of extravasation to the tumor site. So besides the EPR effect and the possibility of an active targeting strategy, liposomes also naturally increase the bioavailability of the anticancer molecule by this third means.

FUNGAL INFECTIONS

Amphotericin B (AmB) is the most well-known anti fungal molecule. AmB stands as the "gold standard" for the treatment of the deep-seated systematic fungal infection. Such infections can occur in the central nervous system, the peritoneum, or the genitor-urinary tract. (136)

AmB is an amphiphilic antibiotic with an action that consists of the formation of pores across the cell membranes through which the cytoplamic components leak progressively. This phenomenon results in the cell death. The cytotoxic action of AmB is demonstrated against both mammalian and fungal cells. But the affinity and selectivity of AmB towards the fungal cells is greater.

As see in section 2.1.1, liposomes that encapsulate AmB have been commercially available for more than two decades. The first one was AmBisomet in 1990, by Vestar. The use of liposome as delivery system was shown to enlarge the therapeutic index but also increase the fungicide activity for a lower toxicity (nephro- and neurotoxicity).

As for cancer sites, infection sites have a particular microvasculature resulting in the same EPR effect. For this reason, liposome-encapsulated AmB systems are passively targeted to the site of the fungal infection.

During the 1980s, both *in vitro* and *in vivo* preclinical and clinical studies have demonstrated the efficiency of liposomal AmB (179) (180).

AmB is a leading example in the field of lipid membrane-based drug delivery.

CENTRAL NERVOUS SYSTEM

Many neurological diseases remain very difficult to treat. This is mostly due to the blood brain barrier which is an effective barrier to most drug molecules.

For this reason, the development of drug delivery systems could benefit to neurological therapies (181). For example liposomal AmB could be employed for neurological purposes with the treatment of neurological fungal infections.

INFECTION AND INFLAMMATION BY A FOREIGN BODY

These medical conditions are not limited to a certain part of the body and can affect several organs in parallel.

The particular case of the penetration of a foreign body within the organisms generally requires a surgical removal. But the removal is not always possible and even if it is performed debris can remain in the tissues.

Drug delivery systems are useful tools to treat infection and inflammation secondary to a foreign body injury. Indeed, their ability to target the tissue surrounding or the foreign body itself is great advantage. Nanoliposomes have also been demonstrated as promising candidates (182).

In the section that described fungal infection, we already explained that in infection scenarios the passive targeting of the liposomes through the EPR effect is an operational strategy.

As for any liposome-based formulation, complementary features for an active targeting can be added to the liposomal system.

MYOCARDIAL ISCHEMIA

As seen in the first part (section 1.2), cardiovascular diseases is a major cause morbidity, mortality and disability in industrialized countries. It also represents a huge economic cost.

The most significant cardiac events are myocardial ischemia-reperfusion injuries and acute myocardial infarction. The consequences are disastrous and are mainly due to a phase of ischemia in the cardiomyocytes. Research groups have been working on the possible ways to reduce the risk of heart failures after or even during a period of ischemia.

One of the greatest challenges of drug therapy is the limited access of free drug molecules to the myocardium. Drug delivery systems could be very helpful to reach the cardiomyocytes.

Adenosine triphosphate (ATP) levels in the myocardium fall during a phase of ischemia. ATP is a necessary precursor for the contractile process of the myocardium. During an

extended period of ischemia, due to the reduction in the ATP level, the contractile process cannot be performed efficiently. Consequently, the provision of ATP after and/or during the time of ischemia could help protect the heart from ischemic damage (183).

A delivery system is required for a satisfactory bioavailability of the ATP in the cardiomyocytes because free ATP is quickly cleared from the blood circulation (184). In the circulation, free ATP is metabolized into adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine. Moreover, ATP is a hydrophilic negatively charged molecule which avoids transport from the blood to the intracellular medium.

Liposomal encapsulations of ATP have already successfully passed *in vitro* and *in vivo* studies. Liposomes spontaneously concentrate in the region of myocardial infarction more than the other part of the myocardium (184) (185). This might be due to the presence of an inflammation at this site leading to an EPR effect. Furthermore the lipid membrane of the liposomes could merge with the damaged cell membrane and seal them, thereby alleviating cell death (186).

Enzyme-containing vesicles (section 2.1.3.1) have also been of great interest for the treatment of myocardial infarction. The enzyme of interest is the t-plasminogen activator which catalyzes the dissolution of thrombus. So the release of this enzyme at the site of thrombosis is very useful to prevent a myocardial infection (138).

THROMBOEMBOLIC DISEASES

The most important of these diseases is the deep vein thrombosis (DVT) which is responsible for significant levels of morbidity and mortality. Other thromboembolic diseases include pulmonary embolism, myocardial infarction, stroke, and arterial thrombosis. All those pathologies are responsible for many deaths in industrialized countries.

A radiolabelled urokinase has been encapsulated in liposome. The aim was to improve the half life of this enzyme in the circulation, so it can be effectively used for the detection of thrombi as a method of thromboembolic disease prophylaxis.

The encapsulation was quite efficient since the stability of the drug was increased, the release profile was appropriate, and the thrombus uptake of urokinase was improved. However, this was not sufficient enough to detect a thrombus. (188)

The success of this encapsulation in liposome remains very moderate since the therapeutic application failed.

Low molecular weight heparin is a major anticoagulant commonly used in the treatment of thromboembolic diseases to prevent the extension of an existing thrombus or to prevent the formation of a new thrombus. The formulation of this heparin in flexible liposomes shows encouraging results with a transdermal delivery method. (189)

PULMONARY OXIDATIVE STRESS

This medical condition is involved in many diseases and syndromes and cured by antioxidant therapies.

The most common etiology of pulmonary oxidative stress is the overproduction of reactive oxygene species (ROS): superoxide anion and H_2O_2. Many different triggers can induce this overproduction including an inflammation, any trauma or sepsis, and many other pathological conditions.

Antioxidant therapies include two main drugs, which are superoxide dismutase (SOD) and catalase. These two enzymes catalyze the following reactions:

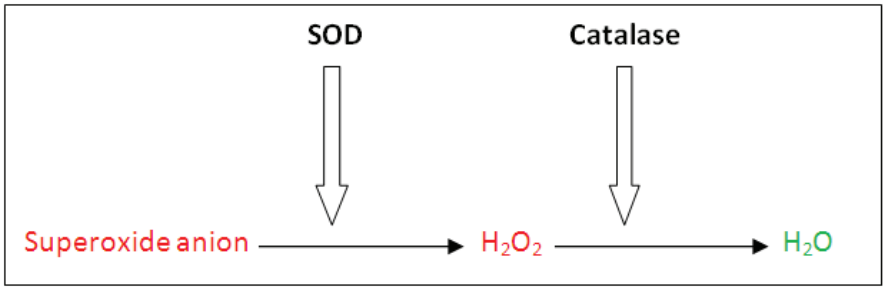


Figure 18 Representation of the reactions catalyzed by the two antioxydant enzymes SOD and catalase.

Free SOD and catalase have a very low protective effect against oxidative stress, if no effect at all. To face this issue, various drug delivery methods have been proposed,

including the encapsulation in liposomes (188) (139). This encapsulation should protect the drug, allow for its release at the site of oxidation and enhance the intracellular uptake.

Preclinical studies have demonstrated that the formulations of SOD and catalase in PEG-coated liposomes have positive effect with an increase of the bioavailability and of the protective effect against oxidation (170). The translation to clinical studies remains to be done.

ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

This disease is caused by the Human Immunodeficiency Virus (HIV). HIV is very contagious and deadly. UNAIDS, the United Nation program on HIV/AIDS, estimated that in 2008 AIDS killed 2 million people and 33.4 million people were HIV positive (190). The need for efficient treatments remains a priority.

In the 1990s delivery systems were developed to face the issues of low drug concentrations at the site of HIV-infection, the toxicity of these drugs towards the host responsible for non-compliance, poor gastrointestinal absorption. In essence, all those factors result in a low bioavailability of the drugs.

Liposomal formulations were thus developed to carry and release various drug molecules including ODN, ribozymes, enzymes, and therapeutic genes. In order to specifically target the HIV-1-infected cells, CD4 antibodies were covalently linked to the liposomes. The activity of inhibition of HIV-1 was shown to be 10 times better when compared with free drugs, and the clearance was also very slower. Mainly PEG-stabilized liposomes were used. The only drawback shown, for all drugs combined, was the necessity to use invasive routes of administration that included intravenous or subcutaneous injections. (191)

To date, the use of liposome encapsulation with CD4 antibodies as targeting ligand is still widely studied (171) (172).

Nanosomes formulations have also been developed. *In vitro* data of betulinic acid in nanosomes thus displays a higher efficacy against HIV-1 at lower concentration than free

betulinic acid molecules (150). Otherwise, nanosomes can be used for the delivery of other anti-HIV drugs, just as for liposomes.

ARTIFICIAL OXYGEN CARRIERS

Allogenic blood transfusion was developed a hundred years ago. Nowadays, blood transfusion has become a very common medical practice since there are large benefits for patient health and welfare. Nonetheless, accidents keep happening which sometimes cause the death of a patient. The main issues are: infection, mismatching of the blood types, immunological response, and short shelf life. With blood transfusion comes the risk of transmission of serious diseases like hepatitis or HIV. To avoid the risks, virus testing is systematically performed on every single sample, but it is expensive.

For a few decades, blood substitutes have been developed that would overcome the problems met with "natural" blood. Hemoglobin (Hb) is one of them. But when administrated as a free drug, hemoglobin generates a high level of toxicity. Hemoglobin is the oxygen carrier molecule naturally contained within the red blood cells. In the 1930s, free hemoglobin was already studied as an oxygen carrier, but due to toxicity and inefficiency, it was not a great success. And the 1970s, after the discovery of liposomes, the first liposomal encapsulated hemoglobin molecules were formed.

Liposomal encapsulation of hemoglobin, to create Hb-vesicles (HbV), was developed to counteract this toxicity. A number of studies demonstrated the safety and efficacy of HbV (196) (197). It was shown that HbV formulations are able to maintain the fundamental parameters of respiration, hemodynamics and tissue oxygenation. This formulation appears as an alternative to the transfusion. There is no risk of mismatching because there is no blood type anymore, no risk of pathogen transmission, and a longer lifetime. Nevertheless, HbV cannot be supplied in a too high dosage, otherwise a transient splenohepatomegaly, a reduced phagocytosis and a modified immune response are observed (198).

ENZYME-REPLACEMENT THERAPY

As seen through these explanations on the different therapeutic targets for liposome-based delivery, the use of enzyme-containing vesicles can be useful for various applications (e.g. cancer, HIV, thromboembolic diseases, pulmonary oxidative stress). This is not surprising since enzymes play a role at every levels of physiological activity (section 2.1.3.1).

Also it is not hard to determine that a lack of functionality of one enzyme can have severe consequences. To solve the lack of functionality, one can encapsulate molecules of the lacking enzyme within vesicles for an *in vivo* delivery at a particular targeted site. The enzymes thus delivered will replace or support the endogenous enzyme. (138)

This type of replacement therapy could be useful for many diseases. For each different enzyme to encapsulate, a personalized liposome-based drug delivery system has to be designed.

2.2. BIOMIMETIC DEVICES AND ARTIFICIAL ORGANS

The different lipid membrane-based delivery systems discussed above (section 2.12.1 Delivery systems) are biomimetic devices since the strategy is to play on their property of cell-like external structure to avoid the immune system.

The lipid membrane-based biosensors reviewed in section 1.3 are a type of biomimetic device as well, developed using lipid membrane for *in vitro* uses. But such devices could also be designed for the in vivo real-time continuous monitoring of physiological function with no need of patient intervention (27). As diagnostic tools, *in vivo* biosensors allow an earlier and more accurate detection of a disease or disorder. This type of device would consist in a “sensor/secretor” system (98). The biosensor would be made of living cells and designed to continuously monitor the level of a certain biomarker. If the level of this specific biomarker was getting below or above predefined limits, then a certain amount of the drug would be released to correct the level of the biomarker, which would provide a quick response.

However, no device has yet been reported as able to perform such a task.

As seen in section 2.1.3.2, lipid vesicles of hemoglobin are an alternative to blood transfusion. These blood substitutes constitute a kind of artificial organ.

Apart from delivery systems, biomimetic devices based on lipid membrane for *in vivo* uses are not very well-documented at the present time. Artificial cells for example are a biomimetic device in development for future *in vivo* use.

The engineering of artificial cells based on a lipid bilayer membrane scaffold aims at mimicking the functionality of natural cells. For this purpose, the artificial cells have to possess some minimal components for the reconstitution of a cell-like activity, with transport activity of various electrolytes and other molecules, metabolism activity, signaling activity. Thus the artificial lipid membrane has to incorporate different components within its bilayer and core including membrane proteins, a source of energy, a replication/transcription/translation and a catalytic system (57). These cells can be specialized to address a particular function.

Regarding the use of artificial cells, their capacity of biomimicry makes them interesting for a lot of purposes. *In vivo*, they could be use to reproduce biological processes and thus be implanted to replace or enhance the function of non-functional or dysfunctional cells (203).

Artificial functionalized lipid bilayer membranes could also be used to partially seal a cell to avoid its death. Liposomes have already demonstrated their efficiency to fulfill this task (189).

In the near future it might become possible to create a tissue exclusively made of artificial cells to be implanted in vivo, for example to replace a dead part of the myocardium after a prolonged ischemic event. Artificial Islets of Langherans could also be produced with specialized artificial cells to be implanted in the pancreas of diabetic patients.

An entire organ made of artificial cells could also be produced for implantation purposes. For instance, the pancreas of a diabetic patient could be substituted with a fully artificial pancreas. Another example could be the grafting of skin-like artificial cell-based tissues for a patient with extreme burns, who is usually now treated with allo- or autografts.

The coupling of the bioartificial organ with a delivery system could facilitate the integration of the bioartificial organ within the body (98).

In addition artificial cells can be used as energy producer, but this specific use will be reviewed in more details in the next part (section 2.3). Artificial cells can also be used for in vitro studies of cell mechanisms or the production of peptides, enzyme and various proteins using the replication/transcription/translation system of the artificial cells.

2.3 BIOPILES AND BIOBATTERIES

As seen in the previous part, one of the many uses of lipid membranes is for the creation of artificial cells. In natural cells, the lipid membrane incorporates many ion pumps and ion channels that create ion gradients across the lipid bilayer membrane. An action potential is thus generated by the cell. One can convert this ion potential into an electric potential, as do electrochemical transducers for example, and thus create a source of electrical energy.

In vivo, the artificial cells would benefit from the natural presence of ions. One can imagine using the conversion of the ion currents into an electrical current that could be used to power another device such as a cardiac implant type pacemaker (204). In fact the artificial cells would provide a biomimetic battery for the device.

Another way of generating a source of energy through artificial cells has been demonstrated by combining two enzymes. Bacteriorhodopsin, which uses light to produce proton gradients, was combined to cytochrome C oxydase, which uses a proton gradient to generate an electron. As a result, the artificial cells, in the presence of light, were able to produce a current (203).

One of the major drawbacks of this method is the need of light to produce a current. For this reason, it is not applicable *in vivo*. Another proton gradient generator, which would not depend on the light, is required if one wants to use such a method to power an implant.

Bacteriorhodopsin was also coupled to an ATP synthase in order to produce ATP, which is the energy currency in cells (57). The aim of this experiment was to produce artificial cells that would not require a supply in ATP. But here again, the system could not be transposed *in vivo* since light is needed.

In another work, a photocurrent was generated using a combination of porphyrin and fullerene (205). This time the lipid membrane used as a support for the incorporation of the protein was a tBLM and not an artificial cell. Following a photoexcitation, an electron transfer occurs from porphyrin to fullerene.

The actual use of artificial cells or other lipid membrane-based devices as batteries or piles for the powering of implant is still at an early experimental stage. But the interest of research groups in the field of bio-production of energy is growing and more methods should be developed.

2.4 Biocompatibility of implants

Previously, we have seen the use of lipid membrane-based implants. Lipid membranes can also be used for the protection of other implants. Due to their property of biomimicry, they are interesting tools to "hide" an implanted device or tissue from the immune system and, thus, improve its biocompatibility. When a foreign body is detected, an immune response occurs with inflammation and possible allergic reaction.

The use of lipid membranes to coat implants in order to improve their biocompatibility has been well demonstrated for all sorts of implants from cardiac to dental (203) (204). In the 1980s, lipid membrane were already envisaged and studied to protect arterial implants from coagulation (204).

Neural implants such as microelectrodes arrays allow for the real-time monitoring of neural activity. The use of a lipid membrane coating has shown encouraging result for the prevention of the formation of scar tissue around the implant that would limit its functionality (206).

The properties of stents can be modulated by a coating. A lipid membrane coating improves the haemocompatibility of the stent and reduces the thrombogenicity (3) (210).

In a general manner, the lipid-based coating of metallic implant is efficient to reduce the risk of bacterial growth and thus the risk of infection of the patients (207). Moreover, lipid membrane coating could lessen the risk of corrosion of the metal and limit the formation of a fibrous tissue around the implant (212).

Lipid membranes are also useful to protect tissue or organs implant. For example, the use of a lipid membrane to encapsulate Islets of Langherans permits the implantation of a replacement pancreas with no need of an immunosuppressive therapy (206). This is very advantageous since immunosuppressive treatments have many unwanted side-effects.

Indeed, lipid membranes for the protection of implants and grafts are efficient and have attracted the attention of research groups for decades. They enhance the biocompatibility of these foreign bodies, which extends their lifetime, stabilizes their activity and could even allow for a reduction of immunosuppressive treatment dosages.

Nevertheless, an important drawback must be acknowledged: once in the body the lipid membrane-coating could undergo a more or less rapid degradation, ending up with a “naked” implant and all the associated complications.

To encounter this problem, the coated could be achieved with artificial cells instead of lipids. This technique is much more complicated since artificial cells have to be built up in a first place. But once in the body, the cells have the ability to repair or regenerate themselves and thus should provide a permanent cover to the implant.

DISCUSSION

Over the last decade, the development of biosensors has been in constant acceleration. Biosensing tools seem to be a very promising way to solve the needs of highly sensitive, fast, portable and inexpensive analytical methods for medical diagnostics. They constitute a credible alternative to classical laboratory analysis using a heavy instrumentation. Although, limitations remain that have to be improved to reach a maximal capacity and efficiency in fulfilling the requirements of POC diagnostic. The prerequisites cited in the introduction of section 1 include amongst others sensitivity, specificity, ease of use, quickness, and automation to lead to the appropriate identification of the biomarkers, sometimes their quantification and thus the determination of the right diagnostic.

So even if biosensors have the potential to revolutionize diagnostics for use in point-of-care testing (POC), this potential still has to be realized. Their capacity and applicability have to be demonstrated on real samples. Here we elucidate the major issues of biosensor development:

- Lack of experiment on real biosamples
- Lack of communication between scientists working on biosensor development and clinicians.

A proper validation on "real life" samples and a standardization of biosensing tests would increase the confidence of health professionals and then make easier the obtaining of regulatory approvals for testing and commercialization. In a general manner, the global medical community shows skeptism with regards to POC analysis tests. Indeed they always find doubtful and questionable both the utility and validity of such tests especially when the results are interpreted by non-professionals. Moreover a better communication between research groups and clinicians would insure a better mutual understanding and thus the selection of adapted and needed applications to develop and the providing of proper samples. If the physicians feel more involved and better understood, they should show a more enthusiastic behavior regarding biosensors.

Other more fundamental points of improvement remain. Among them, the recurring issues include multiplexing as discussed in section 1.1.2.2 as well as low storage stability, difficulties of sterilization and enhancement of the selectivity issues (25).

The argument to know whether multi-use of a biosensor is a good thing or not also remains. The question is to determine if maintenance is not more expensive than just purchasing a new biosensor. One could conclude that it is depending on the particular sensor that is being dealt with and a case-by-case analysis is needed.

Multidisciplinary teams of researchers are currently working on the enhancement and solving of all the issues faced by biosensor devices.

Lipid bilayer membrane-based biosensor, as a subcategory of biosensors for POC diagnostic and research, show their own point of improvement aside from the general issues concerning all sensors. The main problems were the stability and the presence of an inner reservoir, but as explained in section 1.3.1, solutions such as cushioning or tethering have been found as a workaround.

In a general manner, biosensors, based on lipid membrane and others, are very attractive diagnostic devices for POC but also for hospital testing. Their investigation and progresses in their development have been massive over the last two decades, especially the last one. There is no doubt that the market for biosensors will keep growing in the near future. Although substantial work remains to be done to enhance their acceptance by the medical community.

In a broader sense, it is important to realize that the progress made in biosensor development also benefits other fields such as animal diagnostics and environmental surveillance.

As far as veterinary diagnostics is concerned, it is not hard to imagine that, as for human diagnostics, it appeals the same kind of requirements for simple, rapid, easy to use, and cheap POC testing sensors (129). The knowledge acquired for human diagnostic could be transposed to applications in animal health. Moreover, animal disease control and monitoring is useful for both animal and human health. As seen over the last decade with for example pandemic flu from animal hosts, the need of adapted tools to prevent animal to human transmission is becoming evident. The design of biosensors for veterinary diagnostic would be useful.

The monitoring of water and food products can also be performed using adapted biosensors (18). Biosensors for environmental surveillance are being developed to monitor contaminants, respiratory exposure *in situ* or to concentrate trace organic contaminants to assess their toxicity (130).

Lipid membranes could be an interesting support to build sensing tests for environmental or animal sensing purposes.

Beyond the sensing applications, lipid membranes and their ability of mimicking biological cells play a major role in the study of cell functionality and mechanism. The understanding of cells is a great step towards the discovery of potential therapeutic targets and drugs.

Indeed artificial lipid membranes have been demonstrated as a useful technology in drug screening whether or not in the form of biosensor (4). This is not a direct therapeutic use but it is certainly helpful for the future development of therapies.

Another very important use of lipid membrane is in the functionalization of liposomes for sensing applications, which is a new field of research (56). However liposomes are mostly used for *in vivo* drug delivery applications as seen in the second part of this report.

Liposomes and their derivatives, including vesosomes, nanosomes, and microcapsules, have demonstrated their efficiency as drug delivery systems on many occasions and are well accepted. To date, they have been mostly developed to treat infectious diseases and cancer. This is explicable since they show a natural affinity for tumor and infected tissues, owing to the EPR effect. As a consequence, completed with an attractive targeting method to enhance the intracellular uptake of the carried drug, liposomes are particularly interesting for the treatment of these two types of medical conditions.

The derivative options are also quite interesting since they offer an alternative to the basic liposome system. The production of these derivatives, whether as vesosomes, microcapsules or nanosomes, is much more complicated and sometime more expensive especially for microcapsules and nanosomes which require more material and equipment. Nevertheless, this is advantageously balanced by the benefits of these techniques. Nanosomes offer a better uniformity in size and shape and a longer lifetime than liposomes. Vesosomes offer a compartmentalization and thus the possibility of

encapsulating several different molecules, but also a better protection from against enzymatic attacks and immune response. Microcapsules, for their part, have a higher robustness and stability.

However certain drawbacks remain with the use of liposomes as drug carriers. Indeed, most of the formulations are still destined to invasive routes of administration (i.e. intravenous, intracutaneous). One could think that this should be a concern for future work on liposomal systems. Also, the development of a liposomal formulation is a difficult task since there is no standard protocol that could be applied to every drug. With each drug comes a phase or research to determine which encapsulation method would be the most appropriate.

Drug delivery through liposomes or derivatives is the main element of *in vivo* uses of lipid membranes. Nevertheless other very interesting applications have been developed such as the artificial cells, lipid membrane as film to protect implants or grafts, or to design implantable biosensors.

Based on the investigation of biomembrane and cells in a general manner, artificial cells using a lipid bilayer as a scaffold have been synthesized. The *in vivo* and *in vitro* possibilities of applications of such devices are endless. *In vitro* they can be used for drug discovery and drug screening as well as to produce energy, whether ATP or electric current. They are also used to produce artificial tissues or organs for *in vivo* transplants. Their ability to produce energy should shortly find *in vivo* applications. The idea of using artificial cells to power an implant is being considered; even so it has not been practically performed *in vivo*. In fact, the use of artificial cells is just at an early stage but this field should grow quickly in a near future. Evidence of efficiency needs to be shown and more trials have to be performed. One can say that artificial cells have not attained their maximal potential yet.

The biocompatibility of lipid membranes has been proven many times. This is one the reason why they have been envisaged as drug delivery systems in a first place. This biocompatibility also drew the attention of researchers specialized in the field of implants. Indeed, one of the principal issues with implants is their recognition as foreign bodies by the organism which results in a strong immune response. To avoid this immune response,

lipid membranes can be used to “hide” the implants. Such a protective film can also slow down the process of oxidation of metal implants.

Apart from the mechanical implants, lipid membranes have also shown their usefulness to protect a grafted organ or tissue form an immune response. One of the main advantages of this application is the possibility to reduce the dosage of immunosuppressive treatments, which is a big improvement for the quality of life of patients since this type of treatment has many unwanted side-effects.

Another interesting field of application reviewed is the creation of implantable lipid membrane-based biosensors for a continuous real-time monitoring of specific biomarkers. Methods combining a biosensor with a secretion system for an immediate response to the sensing have also been studied. But there is still a long way to go before the creation and commercialization of such devices. For now, they remain at the state of bench-top ideas. However, they would be very relevant for an instantaneous response to the detection of a physiological potentially pathological change.

Lipid membranes have proven their worth in various fields of therapeutic applications from diagnostic to actual therapeutic effects. But this could just be a starting point. Many research teams are working on the development of lipid membrane-based devices. Trials are being performed and more should be planned in a close future. New applications or enhancement of existing applications will certainly be discovered in the next few years.

CONCLUSIONS

The over-arching purpose for this thesis was to review the scientific literature and to consider the ways in which the properties of lipid membranes are exploited to provide the template for biomimetic membranes designed for applications in nanobiotechnologies and for therapeutics. Those biomimetic membranes are designed from an understanding of the structure and function of naturally occurring lipid bilayer membranes. Significantly, 50% of therapeutics are targeted to membrane proteins incorporated into cellular bilayer membranes. We considered the therapeutic applications to include POC medicine, drug encapsulation, and implantable devices, either as the device itself or as a protective membrane for an implant.

Although an important therapeutic use is lipid membrane-based biosensors for POC diagnostics, proof of concept on biological samples is still lacking. Currently, there are very few multidisciplinary teams publishing efficient POC testing devices that provide a rapid and accurate means for diagnostics. For example, the topic "point of care medicine" only stimulated the relatively slow publication rate in Web of Science of 1 every 4 days during 2011. Regarding the *in vivo* uses, the main applications include drug delivery systems that utilize liposome encapsulation, with infectious disease and cancer treatment as the most popular fields for developing such liposomal-formulated drugs. The building of artificial cells, biopiles or artificial organs and tissues is an emerging *in vivo* application for lipid membranes. For example, as biocompatible membranes to protect implants and grafts from the immune system. Lipid membranes have also been imagined for *in vivo* implantable biosensor devices, which would allow for the continuous real-time monitoring of specific biomarkers. But, as for artificial cells, the research in the field of *in vivo* biosensors is still at an early stage. However, compared to POC medicine, those *in vivo* applications of lipid membranes stimulated a much greater publication rate of 1.2 per day during 2011, which was probably driven by the greater industrial and commercial activity in liposomal drug delivery applications.

All of the progress made in the field of biomimetic membranes for human medicine is easily transferrable to animal and environmental applications. It is important for the ongoing development of this field of biomimetic membrane research that veterinary and environmental applications are also considered.

CONCLUSIONS

L'objectif général de cette thèse était de revoir comment les propriétés des membranes lipidiques sont exploitées et servent de modèle pour des membranes biomimétiques utilisées en nanobiotechnologie et thérapeutique. Ces membranes biomimétiques sont conçues d'après la compréhension des structure et fonction des membranes lipidiques naturelles. Fait important, 50% des médicaments ont pour cible l'une des protéines membranaires incorporées dans la bicouche des membranes cellulaires. Nous avons étudié les applications thérapeutiques incluant la médecine « au-point-d'intervention » (« POC »), l'encapsulation de médicaments et les dispositifs implantables.

Bien que les biocapteurs basés sur un système de membrane lipidique pour un diagnostique « POC » soient l'une des applications thérapeutiques importantes, une preuve de concept sur échantillon biologique est toujours manquante. Actuellement, très peu d'équipes multidisciplinaires publient sur des dispositifs efficaces de tests « POC » fournissant un moyen de diagnostique rapide et précis. Par exemple, le sujet « POC médecine » a donné lieu à un taux de publications relativement faible de seulement une tous les 4 jours en 2011 selon Web of Science. Concernant les utilisations *in vivo*, les applications principales comprennent les systèmes de libération contrôlée de médicaments utilisant l'encapsulation liposomale, principalement pour le traitement de maladies infectieuses et cancers. La fabrication de cellules artificielles, biopiles ou encore organes et tissus artificiels est une application *in vivo* émergente des membranes lipidiques utilisée, par exemple, pour la protection d'implants ou greffons contre le système immunitaire. Des membranes lipidiques ont également été imaginées comme biocapteurs implantables *in vivo* pour le suivi en continu et en temps réel de biomarqueurs spécifiques. Mais, de même que pour les cellules artificielles, la recherche dans ce domaine est à un stade précoce. Cependant, en comparaison avec la médecine « POC », ces applications sont sujettes à un meilleur taux de publications de 1,2 par jour en 2011, ce qui est probablement dû à l'importante activité industrielle et commerciale des systèmes liposomaux d'administration de médicaments.

Tous les progrès faits en matière de membranes biomimétiques pour la médecine humaine sont facilement transférables à des applications animales et environnementales.

Il est important pour le développement de la recherche sur les membranes biomimétiques à venir de prendre en considération ces deux dernières applications.

VU ET PERMIS D'IMPRIMER

Grenoble, le 14/3/2012

LE DOYEN

Professeur Christophe RIBUOT

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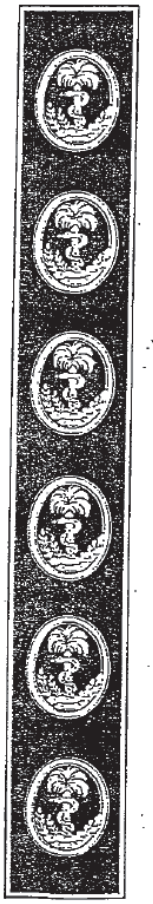
Serment des Apothicaires



Je jure, en présence des maîtres de la faculté, des conseillers de l'ordre des pharmaciens et de mes condisciples :

D'honorer ceux qui m'ont instruit dans les préceptes de mon art et de leur témoigner ma reconnaissance en restant fidèle à leur enseignement.

D'exercer, dans l'intérêt de la santé publique, ma profession avec conscience et de respecter non seulement la législation en vigueur, mais aussi les règles de l'honneur, de la probité et du désintéressement.

De ne jamais oublier ma responsabilité et mes devoirs envers le malade et sa dignité humaine ; en aucun cas je ne consentirai à utiliser mes connaissances et mon état pour corrompre les moeurs et favoriser des actes criminels.

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses. Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque.

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APPLICATIONS NANOBIOTECHNOLOGIES ET THERAPEUTIQUES DES MEMBRANES EN BICOUCHES LIPIDIQUES

Summary

This thesis reviews the scientific literature and considers the ways to use biomimetic membranes for nanobiotechnological and therapeutic applications. About half of the drugs are targeted to membrane proteins incorporated into natural cellular bilayer membranes. We considered the therapeutic applications to include POC medicine, drug encapsulation and delivery, and implantable devices, either as the device itself or as a protective membrane for an implant. Lipid membrane-based biosensors for POC diagnostic represent the most important *in vitro* use of biomimetic membranes. Even so, proof of concept on biological samples is still lacking for these devices. Moreover, only a very few multidisciplinary teams are publishing on efficient POC testing devices that provide a rapid and accurate means for diagnostics. On the other hand, the main *in vivo* applications include drug delivery systems through liposome encapsulation. This field is already well documented. The building of artificial cells, biopiles or artificial organs and tissues is an emerging *in vivo* application of biomimetic lipid membranes. Another type of application is the use of lipid membranes for in vivo implantable biosensor devices, which would allow for the continuous real-time monitoring of specific biomarkers. As for artificial cells, the research in the field of in vivo biosensors is still at an early stage. Besides the discoveries and developments in the field of biomimetic lipid membranes for human medicine are easy to transfer to animal and environmental applications. This underlines the fact that veterinary and environmental applications are also considered for the ongoing development of this field of biomimetic lipid membrane research.

Key-words : nanobiotechnology, therapeutic, lipid bilayer, biomimetic membrane.

Résumé

Cette thèse fait une revue de la littérature scientifique et prend en considération les différents moyens d'utiliser les membranes biomimétiques pour des applications nanobiotechnologiques et thérapeutiques. Environ la moitié des médicaments ont pour cible des protéines membranaires incorporées dans les bicouches des membranes cellulaires naturelles. Nous avons établi que les utilisations thérapeutiques incluent les outils « au point d'intervention » (POC), l'encapsulation et la libération de médicaments, et des outils implantables, en temps que l'outil lui-même ou comme membrane protectrice pour la protection d'un implant. Les biocapteurs basés sur les membranes lipidiques pour diagnostic POC représentent l'utilisation *in vitro* la plus importante des membranes biomimétiques, même si une preuve de concept sur des échantillons biologiques manque toujours. De plus, seules quelques équipes multidisciplinaires publient sur des outils de test POC efficaces qui fournissent des moyens de diagnostique rapides et précis. D'un autre côté, la principale application des membranes lipidiques biomimétiques in vivo comprend les systèmes à libérations modifiée par le biais de l'encapsulation liposomale. Ce domaine est déjà bien documenté. La construction de cellules artificielles, biopiles et organes et tissus artificiels est une application *in vivo* émergente des membranes biomimétiques. Un autre type d'application est l'utilisation des membranes lipidiques pour des biocapteurs implantables qui permettrait le suivi continu et en temps réel de biomarqueurs spécifiques. De même que pour les cellules artificielles, la recherche dans le domaine des biocapteurs *in vivo* est toujours à un stade précoce. En outre, les découvertes et développements dans le domaine des membranes lipidiques biomimétiques pour la médecine humaine sont faciles à transférer aux applications animales et environnementales. Cela souligne le fait que les applications vétérinaires et environnementales sont également étudiées pour le développement à venir de la recherche dans le domaine des membranes lipidiques biomimétiques.

Mots-clés : Mots clés : nanobiotechnologie, thérapeutique, bicouche lipidique, membrane biomimétique.