Doctoral Thesis

Interactions of lipidic assemblies with metal oxides and brush-like polyelectrolytes

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Publication Date:
2005

Permanent Link:
https://doi.org/10.3929/ethz-a-005165023

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INTERACTIONS OF LIPIDIC ASSEMBLIES WITH METAL OXIDES AND BRUSH-LIKE POLYELECTROLYTES

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of

DOCTOR OF SCIENCES

presented by

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Zurich, August 2005
Abstract

Lipidic systems, such as liposomes or supported phospholipid bilayers (SPBs) are used in a wide range of biological and biotechnological applications. For example, liposomes are used as carrier systems in genetic engineering and drug delivery, and can also serve as nanofactories, inside which enzymatically catalysed reactions can occur in a protected environment. When allowed to interact with a surface under appropriate conditions, they can give rise to supported bilayers. SPBs are very useful for studying processes such as cell adhesion and cell-cell interactions, protein-lipid interactions, and protein crystallisation. SPB-based applications in biosensor and biomaterial areas are under intense investigation, driven by the unique properties of SPBs that include a degree of biological inertness, electrical insulating properties, and the ability to serve as matrices for surface-immobilisation of transmembrane proteins. Due to their planar structure, they are excellent candidates for investigation with surface-sensitive techniques.

The first part of this thesis focuses on the interactions of vesicles with titanium oxide, a material that forms spontaneously on metallic titanium upon exposure to
Abstract

Titanium is widely used in biomedical applications: its mechanical properties and biocompatibility, conferred by the oxide present on its surface, make it the material of choice for various implants (i.e. artificial hip and knee joints, dental prosthetics, vascular stents, heart valves). Furthermore, the high refractive index of titanium oxide is advantageous in biosensor applications based on optical detection methods. In both application field mentioned above, novel surface modification strategies leading to biointeractive interfaces (that trigger specific responses in biological systems) are continuously sought. In this work, we investigate the interactions between TiO$_2$ and liposomes containing the acidic phospholipid phosphatidyl serine (PS), and report, for the first time, the conditions needed for the formation of supported phospholipid bilayers of various compositions on TiO$_2$. The main requirements for SPB formation were the presence of calcium ions in solution and a PS content of at least 20%. Detailed investigations of the properties of these SPBs revealed that ~ 98% of the fluorescently labelled PS present in these bilayers is immobilised, while the zwitterionic phospholipid, phosphatidyl choline, is mobile. Both components were found to be mobile in SPBs of identical compositions prepared on silica. To explain these results, we proposed that, on TiO$_2$, PS is trapped in the proximal leaflet of the bilayers, forming TiO$_2$-Ca$^{2+}$-PS complexes. The presence of bound calcium on the titanium oxide surface was confirmed by $\zeta$-potential measurements. The implications of these results for the bilayer formation mechanism and biocompatibility of TiO$_2$ are discussed. The resulting new insight and knowledge represent an important first step towards the design of TiO$_2$-based biointeractive interfaces that reproduce a cell membrane-like environment. An extensive comparison with available data on the formation of SPBs on other surfaces, such as silica and mica is made and parallels are found for some of the properties. In this context, SPB corrals on SiO$_2$/TiO$_2$ patterned substrates were created, by taking advantage of the unique ability of liposomes to distinguish between different surfaces.
In the second part of the thesis, the interactions of vesicles and SPBs with a soft material (poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG)) were investigated. PLL-g-PEG is a graft copolymer that carries polyethylene glycol (PEG) side chains grafted to a polycationic poly(L-lysine) backbone. Interactions between lipid membranes and charged macromolecules—such as DNA and proteins—play an important role in many cellular and biotechnological processes. Examples include the binding of peripheral proteins to the surface of membranes, DNA-phospholipid interactions (relevant for gene delivery studies), and the formation of SPBs on polyelectrolyte cushions. The studies in this field have essentially focused on polyelectrolytes of simple structure, i.e. homopolymers (linear or branched) or random copolymers. Because a number of membrane-binding proteins are either modified hydrophobically or form membrane-inserting domains, significant research efforts have also focused on polymers bearing hydrophobic moieties. In contrast to that, research regarding the interactions between liposomes and block copolymers or graft copolymers containing only hydrophilic moieties has been limited. Such systems are however interesting to investigate, due to the particular properties resulting from the grafted polymer architecture, which cannot be achieved with linear polymers (i.e. the resistance to protein adsorption of polymers grafted with poly(ethylene glycol) side chains). In the present work, it was found that, at low ionic strength, highly grafted PLL-g-PEG decomposed "flat" lipidic systems (e.g., SPBs or micrometer-sized multilamellar vesicles), while more curved membranes (such as vesicles of 50-200 nm diameter) were immune to decomposition. No decomposition of either flat or curved lipidic membranes was observed at high ionic strength. A comparison between PLL-g-PEG copolymers with different architectures suggested that the decomposition of flat lipidic membranes is due to the steric (entropic) repulsion that arises between the PEG chains of highly grafted PLL-g-PEG copolymers adsorbed on a flat surface.

Given that PLL-g-PEG is known to impart resistance to non-specific protein
adsorption to the negatively charged surfaces it adsorbs to, and given that smaller vesicles are not disrupted by the polymer, the effectiveness of a PLL-g-PEG coating in protecting the underlying lipidic membrane from being decomposed by enzymes, detergents, or solvents, was evaluated. This was done by exposing polymer-coated vesicles to a membrane-decomposing enzyme and monitoring which effects the latter had on the lipidic membrane. On the vesicle membranes, the polymer was found not to reproduce the excellent protein resistance it exhibits on metal oxide surfaces and on polymeric microspheres. The high curvature of the vesicles investigated—because of its dramatic influence on the PEG chain density in the adsorbed copolymer—along with the different stiffness of vesicles, microspheres and oxide surfaces, was again proposed to explain the differences observed.
Riassunto

I sistemi lipidici—quali i liposomi o i doppi strati fosfolipidici su supporto planare (DSF)—vengono usati in una vasta gamma di applicazioni biologiche e biotecnologiche. I liposomi trovano applicazione come vettori nell’ingegneria genetica e nella somministrazione di farmaci, oppure possono servire da "nanofabbriche" all’interno delle quali possono avvenire, in ambiente protetto, delle reazioni catalizzate da enzimi. Quando i liposomi interagiscono con determinate superfici in condizioni appropriate, essi possono formare dei doppi strati fosfolipidici. I DSF sono molto utili nello studio di processi quali le interazioni tra cellule, l’adesione di cellule su superfici, le interazioni tra proteine e lipidi e la cristallizzazione di proteine. Grazie alla loro struttura piana, sono eccellenti candidati per essere investigati con tecniche analitiche di superficie. Intensi studi dedicati alle applicazioni basate sui DSF sono in corso nei campi dei materiali biocompatibili e dei biosensori, motivati dalle proprietà uniche dei DSF. Tra queste si annoverano proprietà elettriche isolanti, un certo grado di inerzia biologica e la capacità di servire da supporto per immobilizzare le proteine native della membrana cellu-
L'interazione dei liposomi con il diossido di titanio (TiO\textsubscript{2}) è un argomento di rilevante interesse in ambito di applicazioni biomediche. Il titano, una sostanza largamente impiegata nelle protesi biomediche, ha la capacità di formare il titanio ossidato (TiO\textsubscript{2}), che garantisce una superposta biocompatibilità e proprietà meccaniche. L'ossido di titanio è utilizzato in protesi articolari, protesi dentali, valvole cardiache e apparati vascolari.

In questo lavoro, si studiano i meccanismi di formazione di DSF (doppi strati) contenenti fosfatidilserina (FS). I requisiti per la formazione di DSF includono una presenza di ioni di calcio e un contenuto di FS almeno pari al 20%. L'FS presente in questi DSF è immobilizzata, a differenza di un altro fosfolipide, la fosfatidilcolina, che è mobile.

Recenti studi hanno dimostrato che sul TiO\textsubscript{2} è bloccata la FS, formando complexes di TiO\textsubscript{2}-Ca\textsuperscript{2+}-FS. Queste implicazioni hanno contribuito al nostro understanding sul meccanismo di formazione dei doppi strati su supporti planari e sulla biocompatibilità dell'ossido di titanio.
di TiO\textsubscript{2}. Inoltre, un ampio confronto è fatto con i dati disponibili sulla formazione di DSFs su altre superfici, quali il diossido di silicio o la mica e viene evidenziata la presenza di paralleli per alcune delle proprietà esibite da questi sistemi. In questo contesto, dei "coralli" di DSF sono stati generati su supporti strutturati esibenti un contrasto SiO\textsubscript{2}/TiO\textsubscript{2}, approfittando della capacità unica dei liposomi di distinguere fra superfici diverse.

Nella seconda parte della tesi, è stato studiato un esempio delle interazioni tra sistemi lipidici (vescicole e DSF) con un materiale sottile, il poli(L-lisina)-graft-poli(etilene glicolo) (PLL-g-PEG). Il PLL-g-PEG è un copolimero che ha innestate delle catene laterali di polietilene glicolo (PEG) ancorate alla poly(L-lisina). Le interazioni fra membrane lipidiche e macromolecole caricate elettricamente, come ad esempio il DNA e le proteine, svolgono un ruolo importante in molti processi cellulari e biotecnologici. Esempi includono l’ancoraggio di proteine estrinseche sulla superficie della membrana cellulare, le interazioni del DNA con i fosfolipidi (importanti per la consegna di geni all’interno delle cellule) e la formazione di doppi strati su superfici ricoperte di polielettroliti. È stato scoperto che, a basse concentrazioni ioniche, l’interazione di membrane piane con il PLL-g-PEG ne causa la loro decomposizione, mentre nessun effetto distruttivo è stato osservato né per le strutture lipidiche altamente curve, né per le strutture piane in presenza di un’alta concentrazione ionica nella soluzione. Un confronto fra copolimeri di PLL-g-PEG con varie quantità di catene laterali di PEG per unità di lisina ha indicato che questa decomposizione è una conseguenza della repulsione sterica fra le catene di PEG che si instaura quando del PLL-g-PEG altamente innestato si adsorbe su di una membrana lipidica piana.

Poiché è noto che il PLL-g-PEG impartsce alle superfici caricate negativamente su cui si adsorbe una notevole resistenza all'adsorbimento non-specifico di proteine, e poiché le vescicole di piccole dimensioni non vengono distrutte dal
polimero, una serie di vescicole sono state rivestite di uno strato di PLL-g-PEG con l’obiettivo di proteggerle dalla decomposizione causata da enzimi, detersivi, o solventi. L’efficacia dello strato protettivo di PLL-g-PEG è stata valutata studiando l'effetto che un enzima che attacca specificatamente le membrane lipidiche ha sulle vescicole in presenza ed in assenza di PLL-g-PEG. Si è trovato che il polimero adsorbito sulle membrane delle vescicole non è in grado di riprodurre l’eccellente resistenza alle proteine che esso mostra quando è adsorbito sia sugli ossidi di metallo, sia su delle microsfere polimeriche. L'alta curvatura delle vescicole studiate è stata anche qui proposta come causa delle differenze osservate, in concerto con le diverse rigidità di vescicole, microsfere polimeriche e superfici di ossido metallico.
# Table of Contents

ABSTRACT ................................................................. i  

RIASSUNTO ............................................................. v  

CHAPTER 1 Introduction .............................................. 1  

1.1 Lipids and their assemblies ........................................ 1  
   1.1.1 Lipidic systems resulting from the deposition of liposomes to surfaces ........................................ 4  
   1.1.2 Support-induced effects in supported phospholipid bilayers .... 5  
   1.1.3 Properties of the solid supports ............................... 7  
1.2 Polyelectrolytes ...................................................... 10  
   1.2.1 Interactions of polyelectrolytes with lipidic membranes ........ 10  
   1.2.2 Poly(L-lysine)-graft-poly(ethylene glycol) .................. 11  
1.3 Biocompatibility considerations ................................. 13
CHAPTER 2 Materials and Methods ...........................................17

2.1 Materials .............................................................................. 17
  2.1.1 Chemicals, polymers and proteins .................................... 17
  2.1.2 Phospholipids ................................................................. 19

2.2 Vesicle preparation protocols ................................................. 21
  2.2.1 Extruded unilamellar vesicles (EUVs) ................................. 21
  2.2.2 EUVs loaded with a fluorescent dye ................................. 22

2.3 Vesicle leakage studies by fluorimetry ................................. 22

2.4 Supported phospholipid bilayer (SPB) formation protocols ........ 23

2.5 Substrate preparation, characterisation and cleaning .............. 24
  2.5.1 Substrates for fluorescence microscopy, atomic force microscopy and quartz crystal microbalance .............................. 24
  2.5.2 Patterned samples ............................................................. 25

2.6 Instrumental techniques ....................................................... 25
  2.6.1 Quartz crystal microbalance with dissipation monitoring (QCM-D). 25
    2.6.1.1 Principle of the technique ............................................ 25
    2.6.1.2 Protocols ................................................................. 31
  2.6.2 Fluorescence microscopy (FM) ........................................... 32
    2.6.2.1 Principle of the technique ............................................ 32
    2.6.2.2 Protocols ................................................................. 33
    2.6.2.3 Fluorescence recovery after photobleaching (FRAP) ........ 34
  2.6.3 Atomic force microscopy (AFM) ......................................... 35
    2.6.3.1 Principle of the technique ............................................ 35
    2.6.3.2 Protocols ................................................................. 36
  2.6.4 Dynamic light scattering .................................................. 36
    2.6.4.1 Principle of the technique ............................................ 36
    2.6.4.2 Protocols ................................................................. 40
  2.6.5 ζ-potential ................................................................. 40
    2.6.5.1 Principle of the technique ............................................ 40
    2.6.5.2 Protocols ................................................................. 42
# Table of Contents

## CHAPTER 3 Formation and Properties of Supported Phospholipid Bilayers on Titanium Oxide Surfaces

3.1 Introduction ......................................................... 45
3.2 Results: homogeneous surfaces ............................................ 46
  3.2.1 Interaction of liposomes with TiO$_2$ ................................. 46
  3.2.2 Lateral mobility of lipids in SPBs formed on TiO$_2$ and their distribution across leaflets ......................... 52
3.3 Discussion ............................................................... 56
  3.3.1 Interpretation of the acoustic response from surface-adsorbed vesicles ........................................... 56
  3.3.2 In the absence of Ca$^{2+}$, there is an energy barrier of electrostatic origin that prevents vesicles from reaching the surface ........................................ 58
  3.3.3 In the presence of Ca$^{2+}$, the adhesion strength of liposomes to TiO$_2$ increases with increasing DOPS content due to the formation of TiO$_2$-Ca$^{2+}$-PS complexes ........................................ 62
  3.3.4 Redistribution of PS during vesicle adsorption and SPB formation on TiO$_2$ .............................................. 63
  3.3.5 PC and PS in supported bilayers formed on SiO$_2$ are distributed asymmetrically ............................................. 66
  3.3.6 Mechanism of SPB formation on TiO$_2$: a comparison to SiO$_2$ and mica .............................................. 67
  3.3.7 The biocompatibility of TiO$_2$ in view of the TiO$_2$–PS interactions ..................................................... 73
3.4 Patterns of supported phospholipid bilayers .............................. 74
  3.4.1 Patterns of SPBs on pre-patterned metal oxide surfaces .............. 74
  3.4.2 Build-up of an example of a biosensor interface via functionalised lipids .............................................. 76
3.5 Summary and conclusions ................................................. 77

## CHAPTER 4 Interactions between Lipidic Systems and PLL-g-PEG

4.1 Introduction ............................................................... 79
4.2 Interaction of PLL-g-PEG with supported phospholipid bilayers and supported vesicular layers ........................................... 80
Table of Contents

4.2.1 Results ................................................................. 80
4.2.1.1 Interaction of PLL-g-PEG with supported bilayers investigated by QCM-D: effect of ionic strength and bilayer composition. . . . 80
4.2.1.2 Interaction of PLL-g-PEG with supported bilayers examined by fluorescence microscopy: effect of bilayer quality and ionic strength .............................. 87
4.2.1.3 Investigating bilayer quality by fluorescence recovery after photobleaching (FRAP) measurements. ......................... 92
4.2.1.4 Interaction of PLL-g-PEG with supported vesicular layers. . . . 94
4.2.2 Discussion. ............................................................ 94
4.2.3 Summary and conclusions ....................................... 99
4.3 Interaction of PLL-g-PEG with unilamellar and multilamellar vesicles in solution ....................................................... 100
4.3.1 Results ................................................................. 100
4.3.1.1 Stability of extruded unilamellar vesicles against PLL-g-PEG. . 100
4.3.1.2 Stability of multilamellar vesicles against disruption by PLL-g-PEG ......................................................... 103
4.3.2 Discussion. ............................................................ 105
4.3.3 Summary and conclusions ....................................... 106
4.4 Enzymatic degradation of liposomes coated with PLL-g-PEG. . . . 107
4.4.1 Results ................................................................. 107
4.4.1.1 Enzymatic degradation of lipidic vesicles investigated by QCM-D ............................................................. 107
4.4.1.2 Enzymatic degradation and leakage of lipidic vesicles investigated by fluorimetry ............................................. 111
4.4.2 Discussion. ............................................................ 112
4.4.3 Summary and conclusions ....................................... 116
4.5 Non-specific adsorption of negatively charged liposomes on PLL-g-PEG layers ....................................................... 116
4.5.1 Results ................................................................. 117
4.5.2 Discussion. ............................................................ 120
4.5.3 Summary and conclusions ....................................... 122
# Table of Contents

APPENDIX $\zeta$-potential of TiO$_2$ Colloidal Particles in the Presence of Ca$^{2+}$ ................................. 123

A.1 Introduction .............................................................. 123
A.2 Results and discussion .............................................. 124

REFERENCES ................................................................. 129

ACKNOWLEDGEMENTS ..................................................... 149

CURRICULUM VITAE ....................................................... 153
CHAPTER 1

Introduction

1.1 Lipids and their assemblies

Phospholipids are the major component of biological membranes. These molecules have an amphiphilic character, with a hydrophilic headgroup and two hydrophobic fatty acid chains that are held together by a glycerol backbone (Figure 1.1). The most frequently occurring natural headgroups are either zwitterionic—such as phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE)—or negatively charged—such as phosphatidyl serine (PS), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidic acid (PA), and cardiolipin (CL).\(^1\) The length of the acyl chains, as well as the number of double bonds along the chain can vary widely. Common examples are myristoyl (chain length is 14 carbon atoms and no double bonds are present), palmitoyl (16 C, no double bonds), stearoyl (18 C, no double bond), and oleoyl (18 C, one double bond).\(^1\) In this study, the lipids used were typically dioleoyl phosphatidyl choline (DOPC) and dioleoyl phosphatidyl serine (DOPS).
Chapter 1

Figure 1.1  Molecular structure of phospholipid molecules. Two acyl chains and a phosphate group carrying a further group (i.e., choline, serine, ethanolamine, inositol,...) are attached to a glycerol backbone. The hydrophobic tails have typically either 0, 1 or 2 double bonds along their chain. The presence of double bonds introduces "kinks" (see also Figure 1.3) in the chain that lower the transition temperature from the gel to the liquid crystalline state. Image adapted from ref. 2.

In aqueous solution the hydrophobic effect\(^3\) causes the phospholipids to self-assemble into supramolecular structures that expose the polar headgroups at the interface with water, while the hydrophobic tails are hidden in the interior. Examples of such assemblies are micelles, lamellar, cubic and hexagonal phases. At low water contents, inverse phases—such as inverse micelles or inverse hexagonal phase—can be stable as well. The type of assembly that is formed is determined by the relative sizes of the headgroup and the tails:\(^4\) a large headgroup (compared to the tails) will result in a conically shaped molecule that assembles into micelles. If, on the other hand, the area of the chain region is larger than the headgroup, inverse micelles are formed. In the case both parts of the molecule have the same diameter, a lamellar phase is formed.\(^1\)
The biologically most important and by far most common phase for phospholipids is the lamellar phase. Spherical structures that have water both in the outside and the inside of the bilayer are called lipidic vesicles (or liposomes, see Figure 1.2). Their interactions with various materials and the structures that can result as a consequence of these interactions are the subject of investigation of this study (see Chapters 3 and 4).

Above the so-called main transition temperature lipidic membranes are in a two-dimensional liquid phase, in which the lipids can diffuse freely in the plane of the membrane. Below this temperature, a gel phase is observed. Phospholipids containing double bonds in their tails generally have lower main transition temperatures compared to phospholipids that do not have double bonds. This is due to the fact that the double bonds induce kinks in the acyl chains, and these kinks make it more difficult to achieve a highly ordered, "frozen" phase (Figure 1.3).
1.1.1 Lipidic systems resulting from the deposition of liposomes to surfaces

When liposomes are deposited on a surface, several structures can be formed. These include densely packed layers of intact vesicles (supported vesicular layers, or SLVs\textsuperscript{5}), supported phospholipid bilayers (SPBs)\textsuperscript{6-9} and supported phospholipid monolayers.\textsuperscript{10,11} The pathway chosen by the vesicles depends on the details of the vesicle-surface interactions. The most immediate example is that SPBs and SLVs are usually observed on hydrophilic surfaces, while supported monolayers spontaneously form when liposomes come in contact with a hydrophobic surface.\textsuperscript{11-15}

The properties of supported lipidic systems and the process of their formation on various forms of silica, mica, glass, silicon nitride, surface-functionalised
Introduction

Over the past two decades, supported phospholipid bilayers have become useful tools for studying processes such as cell adhesion and cell-cell interactions, protein-lipid interactions and protein crystallisation, as well as membrane properties in general. SPB-based applications in biosensor and biomaterial areas are also under intense investigation, driven by the unique properties of SPBs that include a degree of biological inertness, electrical insulating properties, and the ability to serve as matrices for surface-immobilisation of transmembrane proteins.

One practical advantage of supported lipidic systems is the possibility to investigate them with surface-sensitive techniques such as the quartz crystal microbalance (QCM-D), atomic force microscopy (AFM), fluorescence recovery after photobleaching (FRAP), optical waveguide lightmode spectroscopy (OWLS), surface plasmon resonance (SPR), total internal reflection fluorescence microscopy (TIRFM), surface acoustic wave spectroscopy (SAW), surface force apparatus (SFA), impedance spectroscopy (IS), cyclic voltametry (CV), Fourier transform infrared spectroscopy (FTIR) and ellipsometry. In Sections 3.2.1 and 3.2.2 of this work, the properties of supported phospholipid bilayers and supported vesicular layers deposited on TiO$_2$ are investigated by a combination of FRAP and QCM-D.

1.1.2 Support-induced effects in supported phospholipid bilayers

The surface properties of the substrate chosen for depositing lipidic vesicles are very important. They determine if an SPB is formed at all under given solution conditions and can influence several characteristics of the resulting SPBs. Formation of SPB has been reported on surfaces such as SiO$_2$,
glass, quartz, mica, or polymer cushions but only adsorption of intact vesicles was observed on Au, \( \text{Al}_2\text{O}_3 \), Cr, and Pt. On TiO\(_2\), SPB formation does not occur in the case of phosphatidyl choline vesicles, but it is shown in this work to occur in the case of phosphatidyl serine-containing vesicles in the presence of Ca\(^{2+}\) (see Chapter 3). The latter is a new development that resulted in the context of the present work. The details of SPB formation on TiO\(_2\) will be discussed in this thesis in Section 3.2.1, while the properties of such bilayers are investigated in Section 3.2.2.

The presence of a support adjacent to a lipidic membrane can change the characteristics of the latter compared to the case of a free standing membrane in solution, or to a membrane formed on another type of support. As a result, characteristics such as the lipid mobility are influenced. In the case of SPBs formed on TiO\(_2\), this effect is discussed in Section 3.2.2. The lateral mobility of lipids in an SPBs—a property also imparted onto the guest molecules coupled to them—is one of the most important characteristics of these systems that makes them attractive for applications both in fundamental research and in the applied realm. Understanding how lipid mobility is altered due to interaction of the membrane with the support, protein binding or incorporation of immobile hydrophobic anchors—such as immobile lipids or proteins—is an issue important in the context of elucidating how multi-component cell membranes function in vivo, in concert with the associated cytoskeleton and glycocalyx structures.

The support also introduces an asymmetric environment that affects the two leaflets of the SPB differently, resulting in an asymmetry of the properties of the leaflets. Phenomena that were observed in this context include a smaller diffusion coefficient of lipids in the proximal leaflet than those in the distal one, independent (de-coupled) distribution of gel state domains in the two leaflets,
and de-coupling of the main transition temperature in the two leaflets.\textsuperscript{96}

In the case of multi-component SPBs, an asymmetric distribution of lipids across leaflets was reported by several groups\textsuperscript{21,29,31,97} (see also Sections 3.3.4 and 3.3.5 of this work). There are at least two reasons why this aspect of bilayer organisation is important. Firstly, membranes of cells are asymmetric with respect to the distribution of lipids between the two leaflets.\textsuperscript{98-103} This has profound implications for the various functions performed by membrane components, while loss of membrane asymmetry leads to pathologies.\textsuperscript{99-103} There is therefore an interest in model systems capable of replicating the subtle details of lipid organisation in cell membranes for biophysical studies. To successfully develop such model systems based on SPBs, a clear understanding of the effects the underlying support has on lipid organisation is required. Secondly, control of surface properties is a prerequisite for successful development of biosensor platforms. Thus in this case the effects of the underlying support on lipid organisation are also of interest.

\subsection*{1.1.3 Properties of the solid supports}

The surface chemistry plays an important role in the interactions of liposomes with surfaces, and particularly in the SPB formation process (see Section 1.1.2 above and the discussion of the results of this work in Sections 3.3.2 and 3.3.3). This section summarises the properties of the solid supports used in this work, namely titania (TiO\textsubscript{2}) and silica (SiO\textsubscript{2}). The selectivity of vesicles with respect to these two materials, allowed us, for example, to prepare patterns of SPBs on pre-patterned TiO\textsubscript{2}/SiO\textsubscript{2} surfaces (see Section 3.4). Mica as a substrate is addressed as well, since it is a relevant surface in the context of SPB formation and a comparison of our work with results obtained on mica is made at several points throughout the thesis (see, in particular, Section 3.3.6).
Titanium oxide ($\text{TiO}_2$)

$\text{TiO}_2$ can occur in amorphous or crystalline form. The amorphous phase is found mostly in thin native oxide layers (passive films) on the surface of metallic titanium. The most stable crystalline forms of $\text{TiO}_2$ are anatase and rutile (see Figure 1.4). Both types of crystals have a tetragonal structure. At temperatures above 800 °C there is an irreversible transition from anatase to rutile.

![Figure 1.4](image)  
*Figure 1.4* Lattice structures of rutile (a) and anatase (b). At temperatures above 800 °C there is a transition from anatase to rutile. The lattice parameters of rutile are $a = 4.594$ Å and $c = 2.959$ Å, those of anatase $a = 3.785$ Å and $c = 9.514$ Å.  

In contact with water, Ti-OH groups are formed on the $\text{TiO}_2$ surface, at a density of 5-6 up to 10 OH groups per nm$^2$. It was calculated that at physiological pH (i.e. pH 7.4) the amount of deprotonated OH groups is about 20% of the total (an isoelectric point (IEP) of 6.2 was assumed for this calculation). The isoelectric points reported in the literature for this metal oxide vary mostly between 5 and 6.7. It has to be kept in mind, however, that the measurement conditions—in particular the presence of ions that bind to the oxide surface—can influence considerably the measured IEP (see Kosmulski *et al.* for an extensive review of the published IEP values for $\text{TiO}_2$). The effect that the
electrolytes used in this study (e.g., NaCl, CaCl₂) have on the IEP of TiO₂ and on its surface charge is discussed in the Appendix in the context of the interactions of TiO₂ with phosphatidyl serine containing liposomes. In particular, Ca²⁺ ions have been shown to bind to TiO₂ and induce a shift of the IEP of rutile to higher pH¹¹⁵ (see also the Appendix). Other authors have identified the presence of calcium to a depth of 17 nm in the oxide layer after only 5 minutes of exposure of the surface to a CaCl₂ solution.² Additionaly, it has been shown previously by immersion studies that the amount of Ca²⁺ bound to the surface increases continuously over 24 hours¹⁰⁴ (this last result is not directly relevant to our investigations—since the exposure time to CaCl₂ was much shorter—but it underscores nevertheless the affinity of the Ca²⁺ ions for TiO₂).

**Silicon oxide (SiO₂)**

The most common crystalline form of SiO₂ is quartz. Amorphous SiO₂ (such as the thin layer of oxide that grows spontaneously on silicon wafers exposed to air) is commonly referred to as vitreous silica. The fully hydroxylated surface of SiO₂ carries 4-5 Si-OH groups per nm²¹¹⁷,¹¹⁸ This oxide exhibits an IEP < 2, if any;¹¹⁹-¹²¹ therefore, at physiological pH, the surface is negatively charged. In contrast with the case of TiO₂, the adsorption of Ca²⁺ ions on the SiO₂ surface is negligible under the conditions relevant for this work (i.e. pH 7.4 and 100 mM NaCl).¹²²

**Mica**

Muscovite mica, K₂Al₄Si₆Al₂O₂₀(OH,F)₄, is a mineral belonging to the class of the phyllosilicates. It has a layered structure in which adjacent layers are weakly bound by a layer of potassium atoms. In aqueous solution, the potassium
ions dissociate and are exchanged by hydrogen or other cations.\textsuperscript{4,123-125} At pH 7.4 the surface is negatively charged and a charge density of about 0.1 electron/nm\textsuperscript{2} has been reported.\textsuperscript{125} Binding of significant amounts of divalent, ions such as Ca\textsuperscript{2+}, was observed as well.\textsuperscript{124,125}

1.2 POLYELECTROLYTES

1.2.1 Interactions of polyelectrolytes with lipidic membranes

The interactions between lipidic membranes and charged macromolecules—such as DNA and proteins—play an important role in many cellular and biotechnological processes. An example of the former is the binding of peripheral proteins to the surface of membranes, which can induce lipid demixing, alter the phase behaviour, and cause other effects, such as alterations in the membrane curvature.\textsuperscript{41,85,126-130}

Linear polypeptides composed of basic amino acids (especially polylysines) have been widely used as model systems for the interactions between peripheral proteins and lipid bilayers.\textsuperscript{131-143} They were found to induce domain and pore formation, enhance the flip-flop rates of the lipid molecules, alter the phase behaviour of the lipids, influence bilayer stability, etc. These systems are also useful for distinguishing the effects of membrane insertion (occurring for proteins with hydrophobic domains) from electrostatically mediated adsorption.

On the biotechnological side, examples of important processes include DNA-phospholipid interactions (relevant for gene delivery studies; their investigation has also lead to the discovery of novel soft biomaterials\textsuperscript{144-146}), and the formation of supported bilayers on polyelectrolyte-coated surfaces.\textsuperscript{8}

The studies cited above have essentially focused on polyelectrolytes of simple
structure: homopolymers (linear or branched) or random copolymers. Because a number of membrane-binding proteins are either modified hydrophobically (e.g., myristylated\textsuperscript{147} or form membrane-inserting domains,\textsuperscript{148-150} significant research efforts have also focused on polymers bearing hydrophobic moieties.\textsuperscript{151-153} To the best of our knowledge, research regarding the interactions between liposomes and block copolymers or graft copolymers containing only hydrophilic moieties has been limited.\textsuperscript{154} Such systems are however interesting to investigate, due to the particular properties resulting from the grafted polymer architecture, which cannot be achieved with linear polymers. An example of such a property is the resistance to protein adsorption of polymers grafted with poly(ethylene glycol) side chains (see, for example, references 155-159). The influence the polymer architecture on the interactions between such a grafted copolymer (PLL-g-PEG; see Section 1.2.2 below) and lipidic assemblies is discussed in Sections 4.2 and 4.3.

1.2.2 Poly(L-lysine)-\textit{graft}-poly(ethylene glycol)

Poly(L-lysine)-\textit{graft}-poly(ethylene glycol) (PLL-g-PEG)\textsuperscript{160,161} is a graft copolymer that carries polyethylene glycol (PEG) side chains grafted to a polycationic poly(L-lysine) backbone (Figure 1.5).

In this work, we investigated the interactions of PLL-g-PEG with various lipidic systems, including supported phospholipid bilayers, supported vesicular layers (Section 4.2), and uni- and multilamellar vesicles in bulk solution (Section 4.3). This polymer was chosen for the favourable properties it imparts to the negatively charged surfaces it adsorbs to: resistance to non-specific protein adsorption (resulting from the densely packed,\textsuperscript{155-159} extensively hydrated\textsuperscript{162} PEG chains) and the ease with which functional groups can be attached to its structure\textsuperscript{157,163-165}. 
The resistance to protein adsorption that PLL-g-PEG exhibits can find application not only in the passivation of surfaces to prevent bio-fouling, but may in principle also be exploited to prevent—via a coating step—fragile species such as lipids from coming in contact with aggressive chemical and biological agents. Stability is in fact a crucial issue for the use of lipidic systems in biosensor technology\textsuperscript{166} and has motivated several studies on coating liposomes with various polyelectrolytes. The aim was either stabilisation of the liposomes,\textsuperscript{89,135,141,167} or tailored release of their contents in drug delivery applications.\textsuperscript{168} “Stealth” liposomes, containing PEG-bearing lipids, are well known for their ability to evade the body’s defence systems, resulting in an increased circulation time \textit{in vivo}.\textsuperscript{169} A generic polymer coating offers however more versatility, in that it can be used in a modular approach to modify any negatively charged lipidic or polymeric vesicles. Previous work on PLL-g-PEG-coated polystyrene and poly(lac-
tic-co-glycolic acid) (PLGA) microspheres has shown that both protein adsorption and phagocytosis are drastically reduced by the coating.\textsuperscript{170,171} Therefore, in this work (Section 4.4), the performance of PLL-g-PEG coatings in repelling proteins and protecting the underlying vesicle from the environment was tested by using an enzyme that specifically degrades phospholipid molecules.

\section*{1.3 BIOCOMPATIBILITY CONSIDERATIONS}

In recent years, the focus of biocompatibility-related research has shifted from the reduction of negative responses caused by implanted materials in biological hosts to designing interactive biointerfaces capable of evoking specific responses in biological systems (e.g., in the context of improving wound healing, fostering cell differentiation, etc.\textsuperscript{172-174}). In this context there is a growing interest in exploring the properties of surfaces coated with supported phospholipid bilayers,\textsuperscript{46} due to their cell membrane-like structure, biocompatibility, and ease with which various functionalities can be incorporated into them (e.g., receptors for eukaryotic cell attachment,\textsuperscript{62} antibody fragments,\textsuperscript{175} receptors for bacterial toxins,\textsuperscript{86} G-protein coupled receptors,\textsuperscript{176} and nucleic acids\textsuperscript{50,61,177}). Various approaches to patterning SPBs have also been reported.\textsuperscript{49,91,176,178-184}

Titanium is a widely used implant material (Figure 1.6a). It is found in artificial hip and knee joints, dental prosthetics, ventricular assist devices, heart valves, and vascular stents.\textsuperscript{106} The success of titanium in implant technology is to a large extent due to its biocompatibility, conferred by a native layer of oxide that protects the underlying reactive metal.\textsuperscript{185} Furthermore, the high refractive index of the oxide makes it an attractive material for use in biosensor technology (Figure 1.6a).\textsuperscript{186}
Titanium as a biomaterial and its combination with supported phospholipid bilayers (SPBs).

a) Examples of application of titanium oxide in implant and sensor technologies (from left to right): hip joint implants, vascular stents that dilate constricted blood vessels, biosensors based on an optical sensing technique.

b) Implants (for example a vascular stent) coated with lipidic bilayers mimic cell membranes, resulting in a better biocompatibility and the possibility of targeting certain types of cells in the body by incorporating specific receptors in the bilayer.

c) The possibility of forming phospholipid bilayers on TiO$_2$-based biosensors opens new venues in the biosensor research area. For example, optical waveguide lightmode spectroscopy, that so far has not been compatible with SPB technology due to surface limitations, can now be applied.

d) Structure of a supported phospholipid bilayer and of its constituting molecules. A phospholipid consists of a hydrophilic head (phosphatidyl choline or phosphatidyl serine in this study) and two hydrophobic alkyl chains (oleoyl in this study). In aqueous solutions, lipids self-assemble into a variety of phases, of which the lamellar phase is one example.

In this work, we report on the formation of supported bilayers on titanium oxide surfaces and on their properties (Chapter 3), bringing the unique advantages of the supported bilayer methodology to the surface of a medically relevant material—a development that is expected to open new venues in biomaterial and
biosensor research (Figure 1.6). In this regard, the implications of the TiO$_2$-PS interactions that we observed (Section 3.3.3) are discussed in Section 3.3.7 in view of the biocompatibility of TiO$_2$. 
CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals, Polymers and Proteins

Chloroform was purchased from J.T. Baker (Phillipsburg, NJ), sodium dodecyl sulphate (SDS) and Triton-X100 from Sigma (Buchs, Switzerland), the cleaner "Cobas Integra" from Roche Diagnostics (Mannheim, Germany), carboxy fluorescein from Molecular Probes (Leiden, The Netherlands) and streptavidin, as well as the enzyme phospholipase A\(_2\) (PLA\(_2\)) from honey bee venom, from Fluka (Buchs, Switzerland). Green fluorescent protein (GFP) was a gift of Eva Künne- mann (Institute for Molecular Biology and Biophysics, ETH Zurich, Switzerland).

Four different buffers were used throughout this work (Table 2.1—chemicals listed therein were purchased from Sigma or Fluka (Buchs, Switzerland)). The pH was adjusted to 7.4 with a 6 M solution of NaOH. The water used for all
aqueous solutions was from a MilliQ Gradient A10 system (Millipore, Volketswil, Switzerland) and contained less than 5 ppb of organic content.

<table>
<thead>
<tr>
<th>Table 2.1 Buffer solutions.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbreviation</strong></td>
</tr>
<tr>
<td>H1</td>
</tr>
<tr>
<td>H2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Ca(^{2+}) buffer</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>EDTA buffer</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

a. 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid.
b. EDTA (ethylenediaminetetraacetic acid) is a chelator of divalent cations such as Ca\(^{2+}\).

Poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) was synthesised in house from poly(L-lysine) hydrobromide (PLL, 20 kDa, Sigma, Buchs, Switzerland) and an N-hydroxysuccinimidyl ester of methoxy-terminated poly(ethylene glycol) (mPEG-NHS, 2 kDa Shearwater, Huntsville, AL) according to a procedure published previously.\(^{155,156}\) The architecture of the polymer is comb-like, with PLL serving as a backbone for the grafted side chains. The grafting ratio (the number of lysine units per PEG side chain) was determined by proton NMR in D\(_2\)O on a 300 MHz spectrometer.\(^{163}\) Fluorescently labelled PLL-g-PEG was synthesised in-house according to a previously published protocol,\(^{165}\) using a PLL (20 kDa) backbone and both PEG (2 kDa) and PEG-fluorescein (5 kDa) side chains. Poly(ethylene glycol) (PEG, 2 kDa, Fluka, Buchs, Switzerland) and PLL were used in control experiments. All batches of PLL-g-PEG polymers used in
this work, along with their abbreviation and architecture are listed in Table 2.2.

<table>
<thead>
<tr>
<th>Polymer Abbreviation</th>
<th>Grafting Ratio</th>
<th>% of derivatised PEG chains</th>
<th>Mw (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL-g-PEG</td>
<td>3.1</td>
<td>-</td>
<td>59</td>
</tr>
<tr>
<td>PLL-g-PEG</td>
<td>2.9</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td>PLL-g-PEG</td>
<td>3.6</td>
<td>-</td>
<td>57</td>
</tr>
<tr>
<td>PLL-g-PEG</td>
<td>3.4</td>
<td>-</td>
<td>94</td>
</tr>
<tr>
<td>PPB30 (biotinylated)</td>
<td>3.85</td>
<td>27.6 %</td>
<td>98</td>
</tr>
<tr>
<td>PPB30 (biotinylated)</td>
<td>3.3</td>
<td>31 %</td>
<td>102</td>
</tr>
<tr>
<td>PPB50 (biotinylated)</td>
<td>3.3</td>
<td>59 %</td>
<td>116</td>
</tr>
<tr>
<td>PLL-g-PEG(5kDa)</td>
<td>3.4</td>
<td>-</td>
<td>153</td>
</tr>
<tr>
<td>PLL-[g=14.2]-PEG(1kDa)</td>
<td>14.2</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>PLL-g-PEG(fluo)</td>
<td>4.0</td>
<td>25 %</td>
<td>69</td>
</tr>
<tr>
<td>PLL-g-PEG(fluo)</td>
<td>4.8</td>
<td>12 %</td>
<td>48</td>
</tr>
</tbody>
</table>

a. The molecular weight of the PEG side chains is 2 kDa, unless stated otherwise.

### 2.1.2 Phospholipids

The phospholipids used in this work are listed in Table 2.3. They were purchased either from Avanti Polar Lipids Inc. (Alabaster, AL) or from Molecular Probes (Leiden, The Netherlands). The corresponding chemical structures are depicted in Figure 2.1.
Table 2.3  Phospholipids used in this work. An "A" or a "P" in front of the product number indicate that the lipids were obtained from Avanti or Molecular Probes, respectively. The main gel to liquid crystalline transition temperatures, $T_c$, were found in refs. 187, 188, 189 and 190.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
</tr>
</thead>
<tbody>
<tr>
<td>a DOPC</td>
<td>1,2-Dioleoyl-$sn$-Glycero-3-Phosphocholine</td>
</tr>
<tr>
<td>b DOPS</td>
<td>1,2-Dioleoyl-$sn$-Glycero-3-[Phospho-L-Serine] Sodium salt</td>
</tr>
<tr>
<td>c NBD-PC</td>
<td>1-Oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino][dodecanoyl]-$sn$-Glycero-3-Phosphocholine</td>
</tr>
<tr>
<td>d NBD-PS</td>
<td>1-Oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino][dodecanoyl]-$sn$-Glycero-3-Phospho-L-Serine Ammonium salt</td>
</tr>
<tr>
<td>e biotin-X-DOPE</td>
<td>1,2-Dioleoyl-$sn$-Glycero-3-Phosphoethanolamine-N-(Cap Biotinyl) Sodium salt</td>
</tr>
<tr>
<td>f DOGS-NTA-Ni</td>
<td>1,2-Dioleoyl-$sn$-Glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] Nickel salt</td>
</tr>
<tr>
<td>g TRITC-PE</td>
<td>N-(6-Tetramethylrhodamine-thiocarbamoyl)-1,2-Dihexadecanoyl-$sn$-Glycero-3-Phosphoethanolamine Triethylammonium salt</td>
</tr>
</tbody>
</table>

a. The fluorescently labelled lipids most probably have transition temperatures that are slightly lower than those of their unlabelled counterparts, since the presence of a bulky label prevents an ordered, closed packing of the chains from occurring.

b. Value for DOPE without the biotin modification.

c. Value for DHPE without the TRITC label.
**Materials and Methods**

**Figure 2.1** Chemical formulas, molecular weights and excitation/emission wavelengths of the lipids listed in Table 2.3. (e) Modifying the headgroup of a lipid such as PE with an additional functionality (i.e. a spacer carrying biotin) inserts a new chemical bond at the place of the positive charge of the amine group. As a consequence, the originally zwitterionic headgroup is transformed to a negatively charged one.

2.2 **Vesicle Preparation Protocols**

2.2.1 **Extruded Unilamellar Vesicles (EUVs)**

Multilamellar vesicles (MLVs) were prepared by mixing appropriate amounts of lipids dissolved in chloroform and evaporating the solvent with argon. After at least 30 min of drying in a vacuum oven (Bioblock 45001, Fisher Bioblock Scientific, Illkirch, France) connected to an oil-free diaphragm vacuum pump (Model MZ2D, Vacuubrand, Wertheim, Germany), the lipids were re-suspended by vortexing in the appropriate buffer at the desired concentration. Unilamellar vesicles were obtained by extruding MLV suspensions through polycarbonate membranes (Avestin Inc., Ottawa, Canada) with pores of appropriate diameter.
(50-400 nm) using a Lipofast extruder (Avestin Inc., Ottawa, Canada).

2.2.2 EUVs loaded with a fluorescent dye

Vesicles to be used in leakage experiments were prepared as above, with the difference that they were hydrated with a concentrated solution (20 mM; the high concentration causes quenching of the fluorophore) of carboxy fluorescein instead of pure buffer. After extrusion, the excess of dye outside the vesicles was removed through gel chromatography (performed in the dark to avoid bleaching of the fluorophore). This technique separates substances by size. In the column used in the present work (27 cm long, 1 cm thick, and filled with Sephadex G 100 (Fluka, Buchs, Switzerland)), the loaded vesicles were therefore eluted first (starting after about 3 ml of elution volume), followed by the non-encapsulated carboxy fluorescein (starting at more than 10 ml). The presence of the samples in the collected fractions was monitored in real time by a model UV-1 spectrometer connected to a pen plotter (Amersham Pharmacia Biotechnologies, Piscataway, New Jersey).

2.3 Vesicle leakage studies by fluorimetry

The vesicle leakage studies were performed using a LS50B Perkin Elmer luminescence spectrometer (Boston, Massachusetts). Fluorescence was excited at 488 nm. A baseline was first acquired by recording the fluorescence intensity emitted by 2.5 ml of a diluted (0.015 mg/ml) suspension of vesicles loaded with quenched carboxy fluorescein. The stability of the vesicles was then assessed by adding small amounts of either PLL-g-PEG (at a final concentration of 0.1 mg/ml) or PLA$_2$ (final concentration: 4 µg/ml) dissolved in the same buffer as the vesicles and monitoring the fluorescence signal. At the end of all experiments, 10
Materials and Methods

µl of concentrated detergent (Triton-X-100) were added in order to completely
destroy the vesicles and measure the maximum fluorescence intensity that is
obtained when all carboxy fluorescein is released into the bulk solution (the
value obtained might be somewhat lower than expected, due to the incorporation
of carboxy fluorescein molecules into Triton-X-100 micelles and the consequent
concentration-dependent quenching of the fluorescence).

2.4 SUPPORTED PHOSPHOLIPID BILAYER (SPB)
FORMATION PROTOCOLS

SPBs were typically formed by incubating clean substrates with vesicles sus-
pended in the appropriate buffer at 0.5 mg/ml lipid concentration for at least 10
minutes. Excess vesicles were then removed by rinsing with pure buffer.

In situ QCM-D experiments were used to confirm that bilayer formation
occurred spontaneously under the same conditions used for fluorescence micros-
copy experiments. Since QCM-D allows one to determine when the SPB forma-
tion is complete (as can be inferred by the return of the dissipation value to the
baseline level\textsuperscript{14}), the same experiments were also used to estimate the incubation
time needed for bilayer formation when the vesicles suspensions had lipid con-
centrations smaller than 0.5 mg/ml (the kinetics of SPB formation is related to
the vesicular concentration\textsuperscript{17}).

To prepare SPBs for observation in fluorescence microscopy experiments, two
different protocols were used. The standard protocol, or protocol (A), consisted
of incubating the microscopy slides with the vesicles suspension in a right-side-
up configuration and was used for most experiments reported in this thesis. When
specifically investigating the influence of the preparation method on the quality
of the SPBs, the incubation was carried out in a upside-down configuration (pro-
tocol (B)\textsuperscript{7,16}. The rinsing procedure of protocol (B) was modified as well and consisted in exposing the surface to a large excess of ultrapure water under stirring for at least 10 minutes. The water was then exchanged back to the desired buffer prior to observation in the fluorescence microscope. The visual inspection of these SPBs, as well as QCM-D experiments involving rinses at different ionic strengths of pre-formed bilayers, both confirmed that the rinses with ultrapure water didn’t affect the integrity of the SPBs.

\section{2.5 Substrate Preparation, Characterisation and Cleaning}

\subsection*{2.5.1 Substrates for Fluorescence Microscopy, Atomic Force Microscopy and Quartz Crystal Microbalance}

Round glass coverslips (Menzel Gläser, Braunschweig, Germany) to be used in fluorescence microscopy experiments, silicon wafers (WaferNet GmbH, Germany) to be used in AFM experiments, as well as gold-coated quartz crystals for use in the QCM-D experiments were coated with 12 nm of different metal oxides (SiO\textsubscript{2}, TiO\textsubscript{2}, Nb\textsubscript{2}O\textsubscript{5}—in the case of SiO\textsubscript{2} an additional 12 nm thick layer of TiO\textsubscript{2} was introduced as adhesive layer) by reactive sputtering in a Leybold dc-magnetron Z600 sputtering unit at the Paul Scherrer Institut (Villigen, Switzerland) as described previously.\textsuperscript{186}

Before use, all substrates were cleaned in a 2\% SDS solution for at least 30 min, rinsed with ultrapure water, and treated in a pre-heated UV/ozone cleaner (model 135500, Boekel Industries Inc., Feasterville, PN) for 30 min. The quality of the coating was checked by X-ray photoelectron spectroscopy (XPS).
2.5.2 Patterned samples

SiO$_2$/TiO$_2$ patterned substrates were prepared as described in Lussi et al.$^{191}$ and cleaned by first sonicating them 15 min in a series of solvents (toluene, hexane, acetone, 2-propanol, ethanol and water) and then treating them with oxygen plasma for 2 min immediately before the first use. For the subsequent uses, the patterned samples were cleaned with the same procedure as for non-patterned surfaces (soaking for 30 min. in SDS solution followed by 30 min of UV/ozone treatment, see section above).

2.6 INSTRUMENTAL TECHNIQUES

2.6.1 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

2.6.1.1 Principle of the technique

The quartz crystal microbalance (QCM) is a technique that is based on monitoring the resonance behaviour of an oscillating quartz crystal as it is loaded with an overlayer of interest. It has been used since the 1960s to detect the deposition of thin films on the crystal’s surface with a resolution down to ng/cm$^2$.$^{192}$ Originally, the operation of QCMs was either in vacuum or in a gaseous environment, but in recent years several instrumental set-ups were developed that allow for the operation in liquid,$^{193-195}$ establishing QCM as a tool for the study of organic thin films such as polymer layers, proteins or even cells.
Figure 2.2 Principle of operation of the quartz crystal microbalance.

a) Picture of a quartz crystal sandwiched between two gold electrodes (obtained from Q-Sense AB (Gothenburg, Sweden)).

b) The piezoelectricity of quartz causes the crystal to deform in response to the application of an electric field.

c) Periodically inverting the current (AC current) across AT-cut crystals drives them to oscillate in shear mode. The frequency of the driving circuit is chosen in order to excite the resonance frequency of the crystal (drawn in blue; fundamental and 3rd harmonic are shown). When material adsorbs on the top electrode, the antinode of the oscillation is shifted further up, thus inducing an increase of the wavelength and a decrease of the frequency (the adsorbed layer is not drawn to scale, and is assumed to have the same acoustic properties as quartz).

The basic set-up of this technique consists of a single crystal of quartz shaped as a thin disc that is sandwiched between a pair of electrodes (Figure 2.2a). Since quartz is piezoelectric, it deforms mechanically when an electric field is applied (Figure 2.2b). Thus the crystal can be driven to oscillate at its resonance frequency, \( f_0 \), by applying an alternating current. In the case of an AT-cut quartz crystal set up as shown in Figure 2.2 the oscillation occurs in a thickness shear mode (Figure 2.2c).
Materials and Methods

The value of the resonance frequency is determined by the thickness of the crystal, \(d\), and the speed of shear waves in quartz, \(v_Q\):

\[
f_0 = \frac{v_Q}{2d}
\]

Equation 2.1

In 1959 Sauerbrey\(^{192}\) showed that a crystal loaded with a thin layer of material can be treated as a homogeneous quartz plate of the same total mass and a thickness \(d' = d + \Delta d\), where \(d\) is the thickness of the unloaded crystal. The change in the resonance frequency of the crystal before and after loading, \(\Delta f\), is then directly proportional to the mass of the overlayer, \(\Delta m\):\(^{192}\)

\[
\Delta f = \frac{\Delta d}{d} f_0 = -\frac{\Delta m_Q}{\rho_Q \cdot d} f_0 = -\frac{\Delta m}{\rho_Q \cdot d} f_0 = -\frac{2 f_0^2}{v_Q \cdot \rho_Q} \Delta m = -\frac{1}{C} \cdot \Delta m
\]

Equation 2.2

\(\Delta f = f_0' - f_0\) = change in the resonance frequency, or frequency shift
\(\Delta m_Q\) = mass per area of a slice of quartz of thickness \(\Delta d\)
\(\Delta m\) = mass per area of the overlayer loading the quartz
\(\rho_Q\) = density of quartz (2.65 g/cm\(^3\))
\(v_Q\) = speed of sound in quartz (3340 m/s)
\(1/C\) = mass sensitivity of quartz (\(C = 17.7\) ng/(cm\(^2\) · Hz) for a 5 MHz crystal)

The reason why the resonance frequency decreases with increasing mass is illustrated in Figure 2.2c: loading of the crystal causes the antinode of the wave at
the top surface to move further away from the antinode at the bottom surface. The consequence is an increased wavelength, and therefore a reduced frequency. However, since the different acoustic properties of the overlayer are not taken into account, Sauerbrey’s equation (Eq. 2.2) is strictly valid only under following conditions:

(i) the frequency decrease due to loading must be smaller than 2 % of the resonance frequency of the bare crystal;\(^\text{196}\)

(ii) the adsorbed mass must be distributed homogeneously on the sensor surface;

(iii) there must be no slip between the crystal and the coupled overlayer;

(iv) the overlayer must be rigid (that is, no viscoelastic deformation can occur)

Condition (iv) is often found to be violated in the case of biological molecules adsorbed from an aqueous environment.

**Measurement of soft overlayers**

Changes in the resonance frequency as a function of loading of a quartz crystal are not the only type of information that can be gained from a QCM. (Frictional) energy losses originating from the contact of the crystal with the surrounding medium (i.e. a gas, a liquid, or an adsorbed thin film) can be used to gather additional information on the adsorbate. There are two ways for detecting such dissipative processes: the first one consists of recording an impedance spectrum of the crystal with the help of a network analyser (see for example references 196 and 197). This approach allows for the determination of resonance frequency and bandwidth (Figure 2.3). The second method consists of exciting the crystal intermittently and measuring the decay of the oscillations as a function of time. The latter approach is known as the quartz crystal microbalance with dissipation.
monitoring (QCM-D, see Figures 2.3 and 2.4).\(^{193}\)

**Figure 2.3** Contemporary measurement of the resonance frequency and bandwidth (left), respectively resonance frequency and dissipation factor (right). In the case shown on the left, the measurement is done in the frequency domain (e.g., with an impedance analyser), while in the case shown on the right it is done in the time domain (that is, exciting the crystal intermittently and measuring the decay of oscillations; this is the method used for the QCM-D system, see Figure 2.4 for details). The two sets of measurements are related to each other via a Fourier transform. The frequency and bandwidth/dissipation in air (blue) and the frequency and bandwidth/dissipation in liquid (red) are related to each other via the viscosity and density of the liquid.\(^{198,199}\)

In the QCM-D approach, the energy losses are represented in terms of the so-called dissipation factor, \(D\), defined as:\(^{193}\)

\[
D_0 = \frac{1}{Q} = \frac{E_{\text{lost}}}{2\pi \cdot E_{\text{stored}}} = \frac{1}{\pi \cdot f_0 \cdot \tau} \quad \text{Equation 2.3}
\]

- \(Q\) = quality factor
- \(E_{\text{lost}}\) = energy dissipated during one oscillation cycle
- \(E_{\text{stored}}\) = mechanical energy stored in the oscillator
- \(\tau\) = decay time constant of the oscillation

Through the additional information obtained from \(D\) it is possible to distinguish between different conformations of the same material (such as, for example, intact adsorbed vesicles and supported phospholipid bilayers\(^{14}\)).
Figure 2.4  Principle of operation of the QCM-D technique.
a) On the left, a bare crystal in vacuum is driven to oscillate at its resonance frequency by a circuit that is periodically switched off. On the right, the same crystal loaded with a rigid overlayer resonates at a lower frequency. Due to the operation in vacuum and the rigidity of the overlayer, dissipational losses are minimal for both the bare and loaded crystal. In this case the Sauerbrey equation (Eq. 2.2) is valid.
b) If the bare crystal is operated in liquid (left), the resonance frequency will be different than in vacuum or in air and is dependent on the density and viscosity of the liquid. Since both these properties change with temperature, it is necessary to work with a temperature-controlled set-up. Also, in liquid dissipational losses cause the oscillation to decay quickly as the driving circuit is switched off. Adsorption of a viscoelastic rough overlayer (right) both shifts the resonance frequency to a smaller value and increases the dissipation factor D (which means that the oscillation is damped more quickly). In this case, Sauerbrey’s equation is invalid.

The dissipation losses that cause the dampening of the oscillation of the crystal are also responsible for a reduced penetration depth of the shear waves in the medium in contact with the crystal. This puts an upper limit on the thickness of the films that can be sensed by QCM-D. For a 5 MHz crystal operated in water the penetration depth is about 250 nm. For the higher harmonics, the penetration depth decreases approximately as the inverse square root of the overtone number.200

The QCM-D system is particularly useful for investigating fast processes, as it
has a time resolution of ca. 1 s, compared to the 10 s of other systems.\textsuperscript{197} It has
however some drawbacks if quantitative analysis of the data is aimed at. First of all, the number of measurable overtones is limited to the 1\textsuperscript{st} (fundamental), 3\textsuperscript{rd}, 5\textsuperscript{th} and 7\textsuperscript{th}, whereas the use of harmonics higher than n = 7 has been advised, since phenomena such as piezoelectric stiffening become negligible only at higher harmonics.\textsuperscript{197} Additionally, a precise quantitative analysis of the data requires the knowledge of the resonance frequency of the crystal in vacuum (or air),\textsuperscript{197,201,202} a parameter that is normally not considered when interpreting QCM-D data (instead, the frequency of the bare crystal in liquid is subtracted from the frequency of the loaded crystal in the same liquid). For these reasons, quantitative analysis of the QCM-D data was not carried out in this work, and only relative effects were considered.

2.6.1.2 Protocols

The experiments were carried out with a QE301 (electronics unit)/QAFC301 (axial flow chamber) instrument from Q-Sense AB (Gothenburg, Sweden). Crystals were mounted in the flow chamber of the instrument immediately after cleaning and checked for resonance on the fundamental frequency (\~5 MHz) and the overtones (\~3x, 5x, and 7x the fundamental frequency). If all four resonance frequencies could be found, the chamber was filled with the appropriate buffer, and the four sets of resonance frequencies and dissipation factors were recorded continuously. Once the drift in the resonance frequencies and dissipation factors had settled out, they were recorded for a further 10 – 20 min (to acquire a baseline), after which 0.5 ml of temperature-equilibrated sample solution (i.e. a suspension of vesicles, or a polymer solution) were injected into the measurement chamber. Temperature equilibration was achieved by letting 1.5 ml of solution flow through the instrument’s T-loop and allowing it to equilibrate there for 3
min before injection into the measurement chamber. After the adsorption step was considered complete (as judged by the attainment of a stable signal), the excess molecules and/or vesicles were removed by rinsing with pure buffer.

After completion of the measurement, the QCM-D chamber was cleaned by soaking it in Roche cleaner for at least 60 minutes. The QCM-D data (frequencies and dissipation factors recorded as a function of time) was then imported into a spreadsheet program for analysis. The frequency and dissipation factor shifts were calculated by averaging the absolute values of the frequency and dissipation factor over approximately 10 min periods before and after the adsorption step, during which the signals were stable, and subtracting the appropriate average values from one another to obtain the desired shifts. For convenience, all shifts were scaled by the overtone order and only the results obtained on 3rd overtone are shown in the plots included in this thesis.

2.6.2 Fluorescence Microscopy (FM)

2.6.2.1 Principle of the Technique

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength (mostly in the UV/visible spectrum) and to subsequently emit light of longer wavelength after a brief interval. The absorption of photons by the so-called fluorophore molecules excites some of the electrons into higher energetic states. At this point, some of the acquired energy is dissipated either via a molecular internal conversion or through non-radiative relaxation processes (such as conversion to heat), before the electron returns to its ground state by emitting a photon that has an energy equal to that of the absorbed photon minus the energy losses.

The fluorescence microscopy method is well suited for the visualisation of
materials that either fluoresce themselves or that can labelled by binding fluoro-phores to their molecules.

### 2.6.2.2 Protocols

Experiments were performed using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany) equipped both with a 25 mW Argon laser (lines used: 488 nm and 514 nm) and a 1 mW HeNe laser (line used: 543 nm). Either NBD- or TRITC-labelled phospholipids (NBD-PC, NBD-PS or TRITC-PE) or fluorescein-labelled PLL-g-PEG were used as the fluorescent species for all experiments.

The observation of species adsorbed at the surface (SPBs, vesicles, polymer layers) typically began with bleaching a spot on the sample at full laser power. In the case of lipidic systems, recovery of fluorescence was taken to be indicative of bilayer formation, while no recovery indicated the presence of a vesicular layer. In the case of bilayer formation, a more precise characterisation was then carried out by fluorescence recovery after photobleaching (FRAP, see below). SPB samples that where intended for coating with PLL-g-PEG where then rinsed with the buffer in which the adsorption of the polymer would be carried out and incubated for 30 min with a solution of polymer dissolved in the appropriate buffer at a concentration of 0.5 mg/ml. Images were recorded at 2 min. intervals during this time. The size of the images was 92.1 μm x 92.1 μm with a pixel size of 0.09 μm x 0.09 μm. Care was taken to avoid contact of the supported bilayer with air and/or bubbles throughout the experiment to prevent damage to the bilayer. As control experiments, vesicular layers deposited on titanium oxide were coated as well with the polymer, following the same procedure as for SPBs.

To be able to directly correlate the results regarding coating of SPBs with PLL-g-PEG obtained by QCM-D and FM, SPBs formed in the chamber of the
QCM-D instrument were also examined by FM. This was done by preparing a fluorescently labelled SPB as described in the previous subsection, unmounting the SPB-coated crystal from the QCM-D instrument, and transferring it to the confocal microscope for analysis. The SPB was never exposed to air during the transfer.

2.6.2.3 Fluorescence Recovery after Photobleaching (FRAP)

Following the protocol for data acquisition and evaluation published by Berquand et al.\textsuperscript{71} (based on the theory published by Axelrod et al.\textsuperscript{203} and Soumpasis et al.\textsuperscript{204})\textsuperscript{i}, a round spot of 33.8 µm diameter was bleached in the supported bilayers to be investigated. The bleaching time was kept at less than 5% of the characteristic recovery time of the bleached spot, as recommended in the literature\textsuperscript{203} (this is to avoid that too much diffusion occurs already during the bleaching step). In the first minute after bleaching, images were acquired every 5 seconds, in the second minute, every 10 seconds, and from the third through the seventh minutes, every 20 seconds. Further images were recorded after 5 and 10 additional minutes.

The data were evaluated according to the following equation:

\[
f(t) = \frac{F(t) - F(0)}{F(i) - F(0)}
\]

Equation 2.4

where \(F(i)\) is the initial intensity before bleaching, \(F(0)\) the intensity inside the bleached spot at \(t = 0\) and \(F(t)\) the intensity of the recovering spot at time \(t\). \(f(t)\) was fitted with:

\textsuperscript{i} Equation (3) that appears on page 1703 of Berquand \textit{et al.}\textsuperscript{71} contains an error; it is the sum, and not the difference, of the Bessel functions that enters this equation. See Lopez \textit{et al.}\textsuperscript{205} for the correct equation.
Materials and Methods

Equation 2.5

\[ f(t) = \exp\left(-2\frac{\tau_D}{t}\right)\left[I_0\left(2\frac{\tau_D}{t}\right) - I_1\left(2\frac{\tau_D}{t}\right)\right] \]

using \( \tau_D \), the characteristic diffusion time, as a single fitting parameter; \( I_0 \) and \( I_1 \) are modified Bessel functions. Diffusion coefficients were calculated using the relationship \( \tau_D = \omega^2/4D \), where \( D \) is the diffusion coefficient and \( \omega \) is the radius of the bleached area at \( t = 0 \). The mobile fraction was calculated using Equation 2.4 with \( t = 1000 \) s, unless stated otherwise.

2.6.3 Atomic Force Microscopy (AFM)

2.6.3.1 Principle of the Technique

Atomic Force Microscopy\textsuperscript{206} belongs to the group of the Scanning Probe Microscopies (SPM) and can be considered as a development of the Scanning Tunnelling Microscope (STM), which was invented in the early eighties by Binning, Rohrer and Gerber.\textsuperscript{207} An advantage of the AFM over the STM is that it does not require a conductive specimen to work with and is therefore suitable for investigating a wide range of materials. The principle of functioning of an AFM is shown in Figure 2.5.

The sample is mounted on a piezoelectric scanner, while the tip, mounted on a spring cantilever, is stationary. As the scanner translates the specimen in the xy-plane, the deflection of the cantilever is monitored with an optical detection system. The latter consists of a laser beam that is focused on the end of the cantilever via a mirror and is then reflected onto a photodetector. Two operating modes are routinely used to image samples: the "contact mode", in which the tip is in con-
tact with the sample’s surface during imaging, and the "tapping mode", in which the tip is in an intermittent contact with the sample.

![AFM Principle of Operation](image)

**Figure 2.5** Principle of operation of the AFM.

### 2.6.3.2 Protocols

AFM measurements were performed in contact mode with a Nanoscope IIIa (Digital Instruments, USA) using Si$_3$N$_4$ tips mounted on triangular cantilevers with a spring constant of 0.06 N/m. All images were acquired in liquid using a fluid cell (DI). The procedures for incubating the substrate with vesicle solutions, as well as the rinsing procedures were the same as for fluorescence microscopy.

### 2.6.4 Dynamic light scattering

#### 2.6.4.1 Principle of the Technique

*The scattering of light*\(^{208,209}\)

When a beam of light passes through a colloidal dispersion, the particles scat-
ter some of the light in all directions. This happens because of the electromagnetic nature of light, the oscillating electric field of which induces an oscillating polarisation of the electrons present in the illuminated particles. The particles then serve as secondary sources of light that subsequently re-radiate (scatter) the incident light.

A typical set-up for a light scattering experiment is shown in Figure 2.6. An incident beam of monochromatic laser light (with vacuum wavelength $\lambda_i$, wavevector $\mathbf{k}_i$, and polarised in the vertical plane) illuminates the scattering region. The scattered light with wavevector $\mathbf{k}_s$ is then observed at a scattering angle $\theta$ at a (large) distance $R$ from the scattering volume. When no energy losses occur (i.e. light is not absorbed by the sample) the scattering is termed "elastic" and $|\mathbf{k}_s| = |\mathbf{k}_i|$.

![Figure 2.6](image.png)  

**Figure 2.6** Schematic representation of a light scattering experiment. The vector $|\mathbf{Q}|$ is called the scattering vector and is defined as $|\mathbf{k}_i| - |\mathbf{k}_s|$.

**Dynamic light scattering**

The Brownian motion of scattering particles suspended in a liquid causes a non-uniform distribution of the scattering centres and fluctuations of their concentration. Correspondingly, the intensity of the scattered light detected at an angle $\theta$ undergoes temporal fluctuations around a mean value $<I_s>$, the so-called time
averaged scattering intensity.

The kinetics of the intensity fluctuations depend on the diffusion coefficient of the particles, which in turn is related to their size: small and rapidly diffusing particles produce rapid fluctuations, while large and slowly moving particles cause slow fluctuations. The dynamic light scattering technique (DLS) analyses the rapidity of the fluctuations by means of autocorrelation functions, such as the normalised temporal autocorrelation function of the scattered field amplitude, \( g_1(Q,\tau) \), which is defined as follows:

\[
E_s(Q, t) = \text{scattered electric field} \\
I_s(Q, t) = \text{scattered intensity} \\
Q = \text{scattering vector (see Figure 2.6)} \\
\tau = \text{time difference between the correlated points}
\]

\[
g_1(Q,\tau) = \frac{<E_s(Q,t)E^*_s(Q,t+\tau)>}{<|E_s(Q,t)|^2>}
\]

Equation 2.6

\( g_1(Q,\tau) \) is related to the diffusion coefficient, \( D \), of the scattering particles via the Siegert relation:

\[
g_2(Q,\tau) = 1 + |g_1(Q,\tau)|^2 = 1 + e^{-2DQ^2\tau}
\]

Equation 2.7

\( g_2(Q,\tau) \): normalised temporal autocorrelation function of the scattered intensity

Once \( D \) is known, the hydrodynamic radius of the particles can be obtained via the Stokes-Einstein equation:
**Data analysis**

DLS data is usually analyzed by numerically fitting the measured correlation functions with calculations based on assumed distributions. Several analysis methods are available that have to be carefully chosen depending on the properties of the investigated sample.

The cumulants method assumes nothing about the form of the size distribution: it is simply a polynomial fit of the natural logarithm of the normalised correlation function. It is a good method for a first analysis of an unknown sample, as it gives a good idea of the mean size and the spread. It is best suited for monomodal samples.

The regularisation method assumes that the size distribution is an arbitrary, but smooth function, and seeks a non-negative distribution producing the best fit to the experimental data. The method is also known as non-negatively constrained least squares (NNLS) and the perhaps most popular program of this type is called CONTIN. CONTIN is particularly successful at detecting widely spaced bimodal samples, smooth continuous distributions, and the presence of weak secondary peaks.
2.6.4.2 Protocols

Light scattering experiments were carried out with an ALV-5000/E instrument (Langen, Germany), equipped with an Ar laser of 488 nm wavelength. Typically, 1 ml of vesicles suspension at 0.5 mg/ml was placed in a cuvette in the trajectory of the laser beam. To check for angular dependency of the scattered laser intensity (relevant for the larger particles), measurements were performed at $\theta = 40, 60, 80, 90, 100, 120$ and $140^\circ$.

Analysis of the data was generally carried out with the cumulants method (2nd order cumulants). In our case this type of analysis is sufficient, because the samples are fairly monodisperse, especially the smaller vesicle sizes investigated (50 and 100 nm). To confirm the results of the cumulant analysis, the data was fitted to other models as well (either CONTIN or Williams-Watts). No significant difference in the results could be found.

2.6.5 $\zeta$-potential

2.6.5.1 Principle of the Technique$^{211,212}$

Most colloidal dispersions in aqueous media carry an electric charge. This surface charge affects the distribution of ions around the particles and induces the formation of a so-called electrical double layer (Figure 2.7).

When a charged particle surrounded by a cloud of ions moves, only the rigidly bound ions follow the particle, while the less firmly bound ions of the diffuse layer are continuously replaced. The potential at the boundary between the ions that are displaced with the particle and those that stay with the bulk dispersant (that is at the hydrodynamic shear plane) is defined as the $\zeta$-potential and has great practical importance, as it can be determined experimentally and predicts the stability of colloidal suspensions.
Materials and Methods

Figure 2.7 Electric double layer. Directly at the surface are the specifically bound ions that form the inner Helmholtz plane (IHP). These ions can dramatically influence the surface charge of the particles and even reverse it (in this example, a moderately negatively charged surface is made positively charged by the adsorbed cations). The ions in the inner Helmholtz layer are generally dehydrated (small circles). At a slightly larger distance from the surface, at the so-called outer Helmholtz plane is a layer of rigidly bound hydrated counterions (shown as large circles). The inner and outer Helmholtz layer form together the Stern layer. Just outside of the Stern layer is the plane of hydrodynamic shear. The potential at this location is defined as the $\zeta$-potential. Further away from the surface there is a more diffuse layer of coions and counterions that are less firmly associated to the particle. The thickness of the double layer (usually described by the Debye length $1/\kappa$) depends on the concentration and valence of the ions in the suspending medium: the higher the ionic strength, the more compact the double layer.

One important parameter in the evaluation of $\zeta$-potential data is the pH of the suspension and its distance from the so-called isoelectric point (IEP), which is defined as the pH at which the $\zeta$-potential is equal to zero. The closer the pH is to the IEP, the smaller the magnitude of the $\zeta$-potential. Other important parameters are the concentrations of non-specifically bound (or "indifferent") and specifically bound ions. A high concentration of the first species strongly screens the charges, dramatically reducing the magnitude of the $\zeta$-potential at pH far from the IEP. The position of the IEP itself is however not affected. On the other hand,
even a small concentration of specifically bound ions, will not only change the magnitude of the ζ-potential, but also shift the IEP.

A way to determine the ζ-potential experimentally is given by the electro-phoresis technique: when an electric field is applied across an electrolyte, the charged particles suspended therein are attracted towards the electrode of opposite charge. Viscous forces acting on the particles tend to oppose this movement. When equilibrium is reached between these two opposing forces, the particles move with a constant velocity, \( v \). Dividing \( v \) by the electric field, \( E \), one obtains the electrophoretic mobility \( \mu_E \), that is dependent on the dielectric constant, \( \varepsilon \), of the medium, the viscosity, \( \eta \), of the medium, and the ζ-potential:

\[
\mu_E = \frac{v}{E} = \frac{2 \varepsilon \zeta \cdot f(\kappa a)}{3\eta}
\]

Equation 2.9

The factor \( f(\kappa a) \) accounts for the ratio between the particle radius, \( a \), and the thickness of the double layer, \( 1/\kappa \). When the particle is much smaller than the double layer, then \( f(\kappa a) = 1 \) (Hückel limit), while when the particle is much larger than the double layer, as is the case when the ionic strength is high, then \( f(\kappa a) = 1.5 \) (Smoluchowsky limit).\(^{212}\)

### 2.6.5.2 Protocols

ζ-potential experiments were performed with a Malvern Zetasizer 3000HSA (Malvern, UK). The wavelength of the incident laser beam (He-Ne, 5 mW) was 633 nm. The ζ-potential of colloidal suspensions of TiO\(_2\)/anatase particles (270 nm diameter, washed according to ref. 112) was measured either as a function of pH (to determine the IEP) or as a function of ion concentration. The effects of
varying concentrations of either Ca\(^{2+}\) or Na\(^{+}\) ions were investigated. The samples were prepared by incubating the colloids in the appropriate buffer for at least 3h, after which the supernatant was diluted to obtain a transparent suspension (non-transparent samples were found to give unreliable results with the technique we used). The volume-% of colloids in the measured samples is estimated to be ~ 0.1%.
CHAPTER 3

Formation and Properties of Supported Phospholipid Bilayers on Titanium Oxide Surfaces

3.1 INTRODUCTION

In this chapter, the interactions between liposomes and TiO$_2$ are investigated under various conditions. This topic has only recently begun to be addressed$^{19,20,66,67,213,214}$—despite the potential relevance to understanding the biocompatibility properties of titanium oxide.$^{46,106,185,215}$ In this context, the experimental conditions under which supported bilayers are formed on TiO$_2$ are presented for the first time$^i$. The properties of these bilayers are then investigated in detail, focussing on the lateral mobility of the lipids in the SPBs and their distribution across the two bilayer leaflets. A comparison is then made with the well-known cases of SPBs formed on SiO$_2$ and mica. Finally, by combining the knowledge gained in this work regarding SPB formation on TiO$_2$ with the available knowledge about SPB formation on SiO$_2$, SPB patterns were produced by using pre-patterned metal oxide surfaces$^{191,218}$ as substrates.

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$i$. Bilayer formation on TiO$_2$ reported previously by Starr et al.$^{216}$ may actually be due to silica contamination on the surface.$^{217}$
3.2 RESULTS: HOMOGENEOUS SURFACES

3.2.1 Interaction of liposomes with TiO$_2$

*Adsorption of intact liposomes on TiO$_2$*

Research carried out in recent years by several groups has shown that liposomes composed of phosphatidylcholines adsorb intact to titanium oxide surfaces.$^{19,20,66,67,213,214}$ It was demonstrated by a combined AFM and QCM-D study that vesicles adsorb to the surface until very densely packed layers are formed (Figure 3.1), without formation of supported bilayers.$^{66}$ This is clearly different from the situation on SiO$_2$, where SPB formation is observed once a critical surface concentration of vesicles on the surface is reached.$^{14,19,20,23}$

![AFM image of 200 nm DOPC vesicles adsorbed on TiO$_2$ in EDTA buffer (lipid concentration: 0.03 mg/ml; corresponding vesicular concentration: $8 \times 10^{10}$ vesicles/ml). Image size: 2 µm.](image)

**Figure 3.1**  AFM image of 200 nm DOPC vesicles adsorbed on TiO$_2$ in EDTA buffer (lipid concentration: 0.03 mg/ml; corresponding vesicular concentration: $8 \times 10^{10}$ vesicles/ml). Image size: 2 µm.

*Influence of negative charge on vesicles adsorption to TiO$_2$.*

To better characterise the details of vesicle-TiO$_2$ interactions, the effect of the content of DOPS (a negatively charged phospholipid) on vesicle adsorption was
studied in a buffer containing 100 mM NaCl, 2 mM EDTA and 10 mM HEPES (EDTA buffer). The presence of DOPS in the vesicle membrane had a dramatic

Figure 3.2 Influence of the DOPS content on the interactions of lipidic vesicles with TiO$_2$ in the absence of Ca$^{2+}$ ions (vesicles of 50, 100 and 200 nm nominal size were used). For all compositions and sizes, vesicles were found to adsorb intact and form supported vesicular layers.

a) Fluorescence intensities and asymptotic frequency shifts from QCM-D are plotted as a function of surface potential of the vesicles for different vesicle sizes. The DOPS content is shown on the top axis (see Figure 3.6 for the relationship between surface potential and DOPS content). DOPC vesicles cover the surface completely at the concentrations used.$^{66}$ The large scatter of the results at 5% DOPS is likely due to the statistical distribution of DOPS lipids in the single vesicles. Due to electrostatic repulsion, vesicles with a slightly larger DOPS content than the nominal 5% will adsorb in smaller numbers to the surface than those containing fewer DOPS molecules than average. Even small differences in this statistical distribution of lipids between different batches of vesicles will have a large effect on the scatter of the data in the steeper regions of the adsorption curve.
b) In this plot, the data shown in (a) were normalised by the frequency shift (for QCM-D data) and the fluorescence intensity (for fluorescence data) in the absence of DOPS. The normalised plots are well-described by a curve of the form $e^{a(\Psi^{2}−b\Psi)}$, where $\Psi$ is the surface potential of the vesicles, $A = \frac{4 \cdot E^{2} \cdot n \cdot \delta \cdot S}{(k \cdot T)^\frac{1}{2} \cdot \kappa \cdot \Delta}$, $B = \frac{\Psi}{\delta}$, $\Delta = e^{\kappa l} - e^{-\kappa l}$, $\delta = \frac{e^{-\kappa l}}{2}$, $\kappa = \sqrt{\frac{2 \cdot n \cdot E^{2}}{\varepsilon \cdot \varepsilon_{0} \cdot k \cdot T}}$ is the inverse of the Debye length, $\Psi_{s}$ is the surface potential of TiO$_{2}$, $l$ is the distance between the oxide surface and the maximum of the energy barrier, $k$ is the Boltzmann’s constant, $T$ is the temperature, $n$ is the concentration of the electrolyte in m$^{-3}$, $E$ is the elementary charge, and $\varepsilon_{0}$ and $\varepsilon$ are the dielectric permeability of vacuum and water, respectively. $S$ is a fitting parameter which has the unit of area (m$^{2}$). This expression was obtained from eq. (38) in Parsegian and Gingell$^{219}$ in the following manner: the normalised response, $\frac{y_i}{y_0}$, where $y_i$ is the response at a given DOPS content and $y_0$ is the response in the absence of DOPS, is given by $e^{\frac{\Delta G_0 - \Delta G_i}{kT}}$, where $\Delta G_0$ and $\Delta G_i$ are the free energies of interaction between the negatively charged TiO$_{2}$ surface and DOPS-free or DOPS-containing vesicles, respectively. These, in turn, are given by eq. (38) in the paper by Parsegian and Gingell$^{219}$ A satisfactory fit to the data was obtained by varying the value of $S$ while keeping $l \approx 0.8$ nm and $\Psi_{s} = -40$ mV constant (these values were found to give the best fits). $S$ varied slightly with the vesicle size, but this variation was not significant given the experimental errors. For the QCM data, $S$ values in the range of 12.1 – 13.2 nm$^{2}$ were obtained (the solid line in the plot corresponds to a fit with $S = 12.8$ nm$^{2}$), while for the fluorescence data, $S$ was 16.2 – 16.8 nm$^{2}$ (dotted line, $S = 16.4$ nm$^{2}$). This difference is most likely due to the fact that, as it was recently shown$^{66}$, the acoustic response is not a linear function of vesicle surface concentration, and the normalised response will contain other terms (although the fact that the data could be fit at all to the above expression suggests that these terms do not modify the functional form of the dependence). Further quantitative evaluation of the barrier height is impossible, however, since Parsegian’s expressions are only applicable to the cases where $\Psi$ is small or $\kappa l$ is large, neither of which is true in our case.

Influence on the amount of vesicles that adsorbed on the surface: the asymptotic frequency and dissipation factor shifts measured by QCM-D as well as the fluorescence intensities resulting from the formed vesicular layers were found to decrease exponentially as a function of increasing DOPS content for all sizes studied (Figure 3.2a). Negligible values were reached above a DOPS content of ~20%. The surface concentration of vesicles on TiO$_{2}$ in the absence of Ca$^{2+}$ varies therefore, with increasing DOPS content, from a densely packed layer of vesicles (at 100% DOPC, see Figure 3.1) to only very few, isolated vesicles$^{ii}$ (at 20%
DOPS and more, see cartoons in Figure 3.2a). Similar results have recently been reported for other substrates, where cessation of adsorption was observed at a similar DOPS content on mica,\textsuperscript{64} and at a somewhat higher DOPS content on SiO\textsubscript{2}.\textsuperscript{23}

**Influence of Ca\textsuperscript{2+} on the adsorption of negatively charged vesicles to TiO\textsubscript{2}: vesicle decomposition and supported bilayer formation at more than 20% DOPS content.**

Typical QCM-D curves obtained when 100 nm vesicles containing various amounts of DOPS were allowed to interact with TiO\textsubscript{2} in the presence of Ca\textsuperscript{2+} ions (that are known for binding to DOPS\textsuperscript{187,220-225}) are shown in Figure 3.3a. Between 0 and 15% of DOPS the curves show an initial steady decrease (increase) in the frequency (dissipation factor) shift upon vesicle adsorption, followed by saturation at an asymptotic value, indicating the formation of a supported vesicular layer.\textsuperscript{14,20,66} Vesicles containing more negatively charged DOPS in their membranes elicited smaller asymptotic frequency and dissipation factor shifts (Figure 3.3a). In striking contrast to that, the fluorescence intensity remained largely independent of DOPS content in the vesicles under the same conditions (150 ± 8 a.u. and 147 ± 14 a.u. for DOPC:DOPS 90:10 and DOPC vesicles, respectively; Figure 3.3b)\textsuperscript{iii}. These observations are clearly different from those in the absence of Ca\textsuperscript{2+} presented above, where the fluorescence intensity was found to decrease in the same way as the frequency and dissipation factor shifts (Figure 3.2). We therefore conclude that, in the presence of Ca\textsuperscript{2+}, the

\textsuperscript{ii.} At these compositions the preferential adsorption of vesicles on surface defects or along scratches significantly influences the frequency and dissipation shifts measured by QCM-D and may lead to larger values than expected.

\textsuperscript{iii.} These values cannot be compared directly with those in Figure 3.2a, because the two sets were recorded with different settings of the microscope. On the other hand, care was taken to ensure that the microscope settings were identical within each set of experiments (the one reported here and the one reported in Figure 3.2).
number of vesicles on the surface remains approximately constant for compositions ranging from 0 to 15% DOPS.

Figure 3.3 Influence of the DOPS content on the interactions of lipidic vesicles with TiO₂ in the presence of Ca²⁺ ions.

a) Adsorption of intact vesicles vs. supported bilayer formation on TiO₂ followed by QCM-D (changes in the frequency are shown on the left, changes in the dissipation on the right; Adsorption of material causes a decrease in the frequency. Dissipation factors are related to how flexible the layer is). When vesicles of 100 nm diameter are deposited on the bare TiO₂ surface they either stay intact, forming a supported vesicular layer at less than 20% DOPS (large asymptotic frequency and dissipation shifts, dotted and dashed lines), or open up to form a bilayer at 20% or more DOPS (extrema in frequency and dissipation factor indicating that the bilayer formation is preceded by vesicle adsorption and decomposition, solid line). The stages corresponding to each part of the curves are sketched on the right plot. Lipid concentrations of 0.5 mg/ml (DOPC, DOPC:DOPS 85:15) and 0.05 mg/ml (DOPC:DOPS 80:20; this concentration was chosen to slow down the bilayer formation process in order to obtain a clear bilayer formation curve) were used. In the cases where SPBs were formed, the total frequency shift was found to be \((-26.0 \pm 1.3) \times n \) Hz, which corresponds to that reported in the literature for SPBs formed on SiO₂\(^{14}\) (\(n\) is the overtone number). In the cases where supported vesicular layers were formed, the scaled shifts (3rd overtone) were found to be -240 ± 27 Hz (DOPC), -93 ± 47 Hz (DOPC:DOPS 90:10) and -148 ± 29 Hz (DOPC:DOPS 85:15) for the frequency and \((27.8 \pm 0.7) \cdot 10^{-6}\) (DOPC), \((12.1 \pm 4.8) \cdot 10^{-6}\) (DOPC:DOPS 90:10) and \((13.8 \pm 3.1) \cdot 10^{-6}\) (DOPC:DOPS 85:15) for the dissipation factor, respectively. The large
scatter can be explained by the fact that both the size distribution of the vesicles and the accuracy of the DOPS content can vary with the batch of vesicles used.

b) Fluorescence microscopy images of a supported vesicular layer formed on TiO$_2$ from vesicles containing 10 wt-% DOPS. No sign of recovery of the intensity in the bleached spot is observed after 10 min., indicating that the species adsorbed on the surface are not laterally mobile.

c) Fluorescence microscopy images of a supported phospholipid bilayer formed on TiO$_2$ by adding vesicles containing 20 wt-% DOPS (solid line in panel a). The spot bleached in the centre of the image recovers within 10-15 minutes, indicating the presence of a bilayer. The rather grainy appearance of the image might be due both to trapped intact vesicles (possibly exchanging lipids with the surrounding bilayer) and excess lipid material that didn’t arrange itself in a bilayer due to the fast kinetics of bilayer formation at this lipid concentration (0.5 mg/ml). The faster kinetics of SPB formation found for TiO$_2$ (compared to the case of SiO$_2$, see Section 3.3.6) may explain why bilayers formed on TiO$_2$ often appear grainier than those formed on SiO$_2$.

At 20% DOPS, extrema characteristic of supported bilayer formation appear in the QCM-D curves$^{14}$ (Figure 3.3a). SPB formation proceeded via a fusion or a fusion/decomposition pathway and required a critical surface density of the vesicles to commence.$^{14,18,226}$ Formation of supported bilayers under these conditions was confirmed by fluorescence microscopy (Figure 3.3c). An SPB was distinguished from a layer of adsorbed vesicles (supported vesicular layer, or SVL$^5$) by bleaching a spot in the imaged area and recording the intensity inside the bleached spot as a function of time. In the case of an SVL, no recovery was observed (Figure 3.3b), while in the case of an SPB, the fluorescence intensity in the bleached area was found to recover to the initial value (Figure 3.3c). A diffusion coefficient of $(2.3 \pm 0.7) \times 10^{-8}$ cm$^2$/s (n = 12) for the NBD-PC probe in DOPC:DOPS 80:20 bilayers was calculated from the recovery data (for the results obtained using NBD-PS as a probe, see section 3.2.2 below). This is consistent with the values reported in the literature for similar systems on other substrates.$^{216,227}$ An SPB could also be formed in the presence of Ca$^{2+}$ from a mixture containing 80% DOPC, 10% DOPS, and 10% biotin-X-DOPE, since the coupling of biotin to the zwitterionic PE renders this lipid negatively charged (see Figure 2.1 on page 21 for the details of the chemical structure).
3.2.2 Lateral mobility of lipids in SPBs formed on TiO$_2$ and their distribution across leaflets

In order to better understand the lipid-TiO$_2$ interactions and to characterise the properties of phosphatidyl serine-containing SPBs prepared on TiO$_2$, these bilayers were investigated by fluorescence microscopy techniques, focusing on lipid lateral mobility and distribution of lipids across the two leaflets. The results are compared to the properties of SPBs formed on SiO$_2$ under identical conditions.

![Figure 3.4](image)

**Figure 3.4** Mobility of the NPD-PS and NBD-PC lipids in DOPC:DOPS 80:20 bilayers formed on SiO$_2$ and TiO$_2$.
(a) NBD-PS is fully mobile on SiO$_2$: when a spot is bleached in the bilayer, the lipid molecules quickly diffuse laterally and the fluorescence intensity inside the spot recovers to the value it had before bleaching.
(b) NBD-PS is immobile on TiO$_2$: the fluorescence intensity inside the bleached spot does not recover after several minutes.
(c) The actual presence of a supported bilayer on TiO$_2$ (vs., for instance, a vesicular layer) is proven by the fact that when NBD-PS is substituted with NBD-PC, full recovery of the bleached area is observed.
It was shown in Section 3.2.1 above that the formation of supported bilayers on TiO$_2$ from DOPC:DOPS mixtures requires both the presence of Ca$^{2+}$ in solution and a DOPS content of at least 20%. These conditions were therefore used in this study for FRAP experiments with SPBs formed on titania. For comparison, SPBs containing 0, 5, 10 and 20% DOPS formed on silica in the presence of Ca$^{2+}$ were investigated as well.

To study the mobility of both the zwitterionic (DOPC) and negatively charged (DOPS) components of the SPBs, two different fluorescent lipids—NBD-PC and NBD-PS—were used. Since lipid-surface interactions (dominated by the headgroup region) were the focus of these experiments, acyl chain-labelled lipids were chosen. Both labels were found to be fully mobile in supported bilayers formed on silica in the presence of Ca$^{2+}$ (Figure 3.4a, Table 3.1), as expected. Contrary to this observation, when SPBs were formed on TiO$_2$, the fluorescence signal recovered to at most 4% of the original intensity (during the

<table>
<thead>
<tr>
<th>Probe</th>
<th>% DOPS</th>
<th>SiO$_2$ D (10$^{-8}$ cm$^2$/s)</th>
<th>n</th>
<th>TiO$_2$ D (10$^{-8}$ cm$^2$/s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD-PC</td>
<td>0</td>
<td>1.7 ± 0.3</td>
<td>16</td>
<td>_a</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.3 ± 0.2</td>
<td>26</td>
<td>_a</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.1 ± 0.2</td>
<td>13</td>
<td>2.3 ± 0.7</td>
<td>12</td>
</tr>
<tr>
<td>NBD-PS</td>
<td>5</td>
<td>1.6 ± 0.5</td>
<td>6</td>
<td>_a</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.5 ± 0.2</td>
<td>9</td>
<td>_a</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.0 ± 0.1</td>
<td>5</td>
<td>~ 0 b</td>
<td>3</td>
</tr>
</tbody>
</table>

a. Under these conditions, a supported vesicular layer is formed on TiO$_2$, and no recovery is observed.

b. The diffusion coefficient for NBD-PS on TiO$_2$ is vanishingly small, since in this case the fluorescence intensity, at the time scale considered here (400 s), does not recover more than up to 4% of the original value.
first 400 s after bleaching) when NBD-PS was used as a label (i.e., the diffusion coefficient was below $10^{-10}$ cm$^2$/s; Figure 3.4b). Complete recovery of the fluorescence intensity within 400 s was observed, however, when the NBD-PC probe was used instead of NBD-PS under otherwise identical experimental conditions (Figure 3.4c), thus indicating SPB formation. The homogeneous distribution of the two probes on the micron scale (Figure 3.4b,c) furthermore suggests that no phase separation of the lipids has occurred.

It therefore appears that on the TiO$_2$ surface, an SPB is formed in which DOPS molecules are immobilised while DOPC molecules are freely mobile. To confirm this idea, experiments were performed with DOPC:DOPS 80:20 SPBs containing mixtures of the two probes in various ratios (Figure 3.5). The extent of recovery was found to correlate linearly with the contents of NBD-PC (Figure 3.5b).

![Figure 3.5](image-url) Fluorescence recovery after photobleaching for DOPC:DOPS 80:20 bilayers containing mixtures of NBD-PC and NBD-PS lipids in the presence of Ca$^{2+}$. The substrate is TiO$_2$. The amount of labelled lipids (PC + PS) is always 1% of the total lipid.

(a) Normalised fluorescence intensities as a function of time. The extent of recovery of the fluorescence intensity after bleaching at 0 s depends on the amount of NBD-PC in the bilayer. The plots were obtained by averaging over at least 3 FRAP curves for each composition. The lines are fits to the model by Axelrod et al. and Soumpasis.\textsuperscript{71,203-205}

(b) Mobile fractions as a function of NBD-PC content for the data shown in (a). The extent of recovery of the fluorescence intensity correlates very well with the relative amount of NBD-PC in the mixture.
To explain these observations, we propose that most (~ 96 – 98%) of the DOPS is contained in the leaflet proximal to the surface of TiO$_2$ and immobilised via a strong, Ca$^{2+}$-mediated, TiO$_2$-PS interaction (e.g., TiO$_2$-Ca-PS complexes, see Figure 3.8—in this context it is interesting to note how a similar asymmetric distribution of lipids was observed for SPBs prepared in the presence of Ca$^{2+}$ on mica, a surface that is known to bind Ca$^{2+}$ ions). Such TiO$_2$-Ca$^{2+}$-PS complexes may be similar to the trans-bilayer Ca(PS)$_2$ ones found in cochleate structures, where PS is immobilised in a gel phase. The formation of true cochleates can be excluded here, since they are multilayered structures, whereas only a single bilayer was formed in our case according to QCM-D results (see Figure 3.3a). Binding of Ca$^{2+}$ to a single bilayer is not expected to result in the formation of a gel phase or alter the mobility of PS, and indeed, these effects were not observed on silica (Figure 3.4a).

The remaining 2 – 4% of recovery of fluorescence intensity observed (Figure 3.5) is most likely due to PS present in the distal leaflet that is expected to remain mobile even if PS in the proximal leaflet is immobilised.

The presence of immobile PS molecules is expected to restrict the mobility of PC in the proximal leaflet, since the presence of obstacles—in the form of lipid molecules immobilised on the underlying support, by a bound protein, or in gel phase domains—is known to restrict the mobility of the free component. Yet the mobility of PC in these bilayers is, on average, not impaired (Figure 3.4, Table 3.1). This apparent controversy can be reconciled in qualitative terms by considering that the overall diffusion coefficient measured by FRAP contains contributions from both leaflets: the distal leaflet, comprised almost entirely of PC, where its diffusion is not restricted, and the proximal leaflet, where the diffusion of PC is restricted, but which contains less of it. The presence of an outline of the bleached area, persistently observed on TiO$_2$ with the
NBD-PC label even after 300 s of recovery (Figure 3.4c), strongly argues in favour of the presence of a slow-diffusing PC component in these bilayers. This line of argument furthermore supports the conclusion that the distal leaflet is essentially devoid of PS.

3.3 DISCUSSION

The first part of the discussion focuses on the interactions of liposomes with TiO$_2$ in the presence and absence of Ca$^{2+}$. In the second part, the mechanism of bilayer formation on TiO$_2$ is discussed in detail, on one hand by focussing on the role of flip-flop and lipid redistribution across leaflets, and on the other hand by comparing the results of this work with the available knowledge on SPB formation on surfaces such as silica or mica. Finally, some considerations on the biocompatibility of TiO$_2$ are made in view of the TiO$_2$-PS interactions discussed in this work.

3.3.1 Interpretation of the acoustic response from surface-adsorbed vesicles

The acoustic response (frequency and dissipation shifts) of vesicular layers deposited on TiO$_2$-coated QCM-D crystals decreases with increasing amount of DOPS in the vesicle membranes, both in the presence and in the absence of Ca$^{2+}$ (Section 3.2.1). However, while in the presence of Ca$^{2+}$, the fluorescence intensity does not depend on DOPS content, suggesting that the number of vesicles on the surface does not change as a function of DOPS, in its absence a decrease in the number of vesicles adsorbed on the surface is observed by fluorescence. Therefore the causes for the decrease in the acoustic response are quite different in the two cases.
In principle there can be two reasons why the acoustic response from SLVs decreases: a decrease in the number of vesicles adsorbed on the surface or a decrease in their height \textsuperscript{iv} (with no change in their number). We therefore conclude that, while in the absence of Ca\textsuperscript{2+}, the decrease in the acoustic response as a function of DOPS content is due to the diminishing number of vesicle adsorbed on the surface, in its presence, it is due to the diminishing thickness of the SVLs, the number of vesicles within which remains approximately constant. In other words, the height of the adsorbed vesicles decreases as the content of DOPS within them increases.

Comparison of the height of an adsorbed vesicle with the size it has in bulk solution yields the extent of deformation of a vesicle in contact with a surface. The extent of deformation is determined by the effective adhesion strength, given by \( w = \frac{WR^2}{\kappa_b} \), where \( W \) is the value of the vesicle-surface interaction potential at its minimum (Figure 3.7), \( \kappa_b \) is the bending modulus of the bilayer and \( R \) is the vesicle radius—i.e., vesicle adhesion is a result of a competition between vesicle-surface attraction and bilayer rigidity (which opposes bending).\textsuperscript{238} Therefore a larger extent of deformation (smaller height) implies a stronger adhesion (larger values of \( w \)), which can either be the result of an increase in the lipid-surface attraction (characterised by \( W \)), or a decrease in the bending modulus of the bilayer, as a function of DOPS.\textsuperscript{v,238,239} The effect of an increasing lipid-TiO\textsubscript{2} attraction is schematically depicted in Figure 3.7. When the strength of adhesion becomes sufficiently strong (e.g., in this case for vesicles containing at least 20% DOPS), a bilayer is formed (see also Figure 3.3).

\textsuperscript{iv} The dominant contribution to the frequency shift observed by QCM-D is proportional to the mass per unit area of the adsorbed layer. Mass per unit area of a layer is, in turn, the product of layer density and thickness. The former remains approximately constant (close to the density of water). Therefore the differences in the acoustic responses between two layers with similar numbers of vesicles adsorbed are due to the differences in the thicknesses of the two layers.

\textsuperscript{v} According to dynamic light scattering measurements, vesicle size remains nearly constant as a function of DOPS (not shown; the effect vesicle radius has on the frequency shift in the case of surface-adsorbed vesicles is discussed in detail in Reimhult \textit{et al.}\textsuperscript{19} and Reviakine \textit{et al.}\textsuperscript{66})
3.3.2 In the absence of Ca$^{2+}$, there is an energy barrier of electrostatic origin that prevents vesicles from reaching the surface

When Ca$^{2+}$ is absent from solution, fewer and fewer vesicles adsorb to TiO$_2$ as the DOPS content in the vesicles increases (Section 3.2.1). A decrease in the number of surface-bound vesicles can be caused by an energy barrier that prevents vesicles from coming into contact with the surface. The height of such a barrier should be a function of the vesicle surface potential (given by DOPS content and ionic strength,$^{240,241}$ see Figure 3.6) and of the surface potential of the metal oxide. The situation is schematically depicted in Figure 3.7. Indeed, QCM-D and fluorescence data acquired in the absence of Ca$^{2+}$ and normalised by the response in the absence of DOPS collapsed onto similar curves for the response (fluorescence intensity, frequency, or dissipation) vs. surface potential of the vesicles$^\text{vi}$ (Figure 3.2b) that were well-described by an exponential of the form $y = e^{A(\Psi^2 - B\Psi)}$, where $\Psi$ is the surface potential of the vesicles and $A$ and $B$ are defined in the legend of Figure 3.2 (c.f. eq. 38 in Parsegian and Gingell$^{219}$). The absolute value of the term $A(\Psi^2 - B\Psi)$ gives the height of the energy barrier (Figure 3.2, Figure 3.7).

Theoretical analysis of surface-adsorbed vesicles$^{239}$ predicts the existence of a critical adsorption radius, $R_a$, below which vesicles do not adsorb. However, in the presence of an energy barrier, vesicles with $R > R_a$ may be prevented from reaching the surface in a first place. Under such circumstances, $R_a$ will not be observed.

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$^\text{vi}$ The slight difference of the normalised response curves obtained from QCM-D data and fluorescence data are attributed to the non-linearity of the acoustic response of the QCM-D as a function of vesicle surface concentration.$^{66}$
Figure 3.6  Surface potential as a function of mass-% of DOPS in DOPC:DOPS vesicles, calculated according to Eisenberg et al.\textsuperscript{240} and Ohki et al.\textsuperscript{241} in the presence and in the absence of Ca\textsuperscript{2+}. In both cases the surface potential becomes more negative as the DOPS content of vesicles increases.

It is interesting to note that the adhesion of vesicles to the surface remains sufficiently strong for the vesicles that do adsorb to withstand rinsing with the vesicle-free buffer (e.g., in the case of vesicles containing 5\% and 10\% DOPS, Figure 3.2). This implies that the barrier to desorption is higher than the barrier to adsorption. In other words, there remains a short-range attractive component to the interaction between the DOPS-containing vesicles and the surface. Most probably, this component is due to DOPC.

The actual presence of an energy barrier was supported by temperature-dependent QCM-D experiments (not shown): a TiO\textsubscript{2}-coated crystal was first exposed to a suspension of DOPS-containing vesicles at 25 °C. Then the temperature was increased to 35 °C and the system was allowed to equilibrate. After rinsing and cooling down again to 25°C the frequency and dissipation factor shifts obtained
were compared to those prior to heating to 35°C. An increase in the amount of adsorbed vesicles was observed, indicating that the energy put into the system allowed more vesicles to overcome the energy barrier. It was however not possible to quantify these experiments, as the instrument was not sufficiently stable over longer times to allow for several temperature cycles and warrant a satisfactory reproducibility.

![Diagram with energy barriers and vesicle interactions](image)

**Figure 3.7** The results on the interactions between DOPC:DOPS vesicles and the TiO$_2$ surface are summarised in this diagram. It shows what the interaction energy between the vesicles and TiO$_2$ may look like under various conditions. Numbers next to the curves refer to cartoons depicting the relevant situation (shown at the bottom of the figure). Thick lines: the situation in absence of Ca$^{2+}$ for vesicles with low (solid line) and high (dashed line) content of DOPS; vesicles need to overcome an energy barrier in order to reach the surface. The height of the barrier is a function of DOPS content and its location is given by the value of $l$ (see also Figure 3.2). If the barrier is sufficiently high (as is the case for $> 20\%$ DOPS), no adsorption occurs (cartoon 1). There remains a short-range attractive component to the vesicle-surface interactions, however, which prevents the vesicles that do adsorb (e.g., vesicles containing less DOPS) from desorbing (cartoon 2). We attribute this attractive interaction to that between TiO$_2$ and DOPC (dotted curve; no distinction is made between the situations in the presence and in the absence of Ca$^{2+}$ for reasons of clarity), and therefore the minima in these three curves are drawn to be of similar depth ($W$ refers to the value of the vesicle-surface interaction potential at...
the minimum\(^{239}\)). It is currently not possible to ascertain how this short-range attraction depends on DOPS contents. Whether an energy barrier exists also in the case of DOPC – TiO\(_2\) interactions is also not known at present, but in any case it is of the order \(kT\).

In the presence of Ca\(^{2+}\) (Figure 3.3; curves and cartoons 3 and 4), the energy barrier is lowered or abolished. Furthermore, there is an increase in the adhesion strength between the vesicles and the surface as a function of DOPS content in the vesicles. This is due to the formation of TiO\(_2\)-Ca\(^{2+}\)-PS complexes that are the more numerous the more DOPS is present in the vesicles (this is reflected in the diagram by the minimum of the vesicle-surface interaction potential getting deeper from curve 3 to curve 4). It is also possible that the increase in the adhesion strength is caused by a decrease in the bending modulus of the vesicles or by a combination of the two factors (not shown). In either case, it results in the corresponding increase in the vesicle deformation (c.f. cartoons 2 and 3) and finally leads to bilayer formation (cartoon 4).

The presence of statistical variations in the number of DOPS molecules in each vesicle may affect the adsorption process of DOPS-containing vesicles to TiO\(_2\) in the absence of Ca\(^{2+}\). For example, at 10% average DOPS concentration, a fraction of the vesicles will have a slightly higher DOPS content, while other vesicles will contain less DOPS than average. The vesicles that adsorb then correspond to the fraction with the lowest DOPS content (and therefore the lowest electrostatic repulsion from the TiO\(_2\) surface). As the mean DOPS content increases, the fraction of vesicles with sufficiently low DOPS content becomes smaller, and therefore fewer vesicles are observed to adsorb.

When, on the other hand, a Ca\(^{2+}\)-containing buffer is used, densely packed supported layers of vesicles containing 5 or 10% DOPS can be formed (Figure 3.3b). Thus, one clear role of Ca\(^{2+}\) is to reduce or abolish the energy barrier that arises due to electrostatic repulsion between the negatively charged oxide and vesicle surfaces. This conclusion is supported by the results reported in the Appendix, where it is shown that the \(\zeta\)-potential of TiO\(_2\) colloids at pH 7.4 is influenced dramatically by the presence of Ca\(^{2+}\) ions: in the absence of Ca\(^{2+}\) the \(\zeta\)-potential is of the order of – 35 mV, while in the presence of the cation its value drops to almost zero (Figure A.2). Considering that the surface potential of DOPS-containing vesicles also becomes less negative in the presence of Ca\(^{2+}\)
Chapter 3

(Figure 3.6), it follows that the electrostatic repulsion between TiO$_2$ and vesicles in the presence of 2 mM Ca$^{2+}$ must be very small, or even non-existent. Hence, no energy barrier to adsorption is present (or it is very small) and only the attractive component, due to the DOPC-TiO$_2$ interaction, remains.

3.3.3 In the presence of Ca$^{2+}$, the adhesion strength of liposomes to TiO$_2$ increases with increasing DOPS content due to the formation of TiO$_2$-Ca$^{2+}$-PS complexes.

The presence of a densely packed SVL is the first requirement for SPB formation to proceed by the fusion or fusion/decomposition pathway$^{14,18,226}$ but it is not sufficient, as shown in references 5, 14, 18, 20, 66, and 67; see also Figure 3.3a. An adequate adhesion strength, $w$, is required as well in order for the vesicles to deform sufficiently for the bilayer formation to commence. In the case of vesicles adsorbed on TiO$_2$, this occurs when Ca$^{2+}$ ions are present and the DOPS content in the vesicle membrane is at least 20%. Thus, the presence of both Ca$^{2+}$ and PS has the effect of increasing the adhesion strength of the vesicles to TiO$_2$.

Ca$^{2+}$ has previously been shown to significantly affect the process of SPB formation from EggPC vesicles on mica$^{18}$ to which it is known to bind$^{124,125}$ Similarly, Mg$^{2+}$ was found to have a comparable effect on the behaviour of EggPC vesicles on –OPO$_3$ and –OSO$_3$ modified gold surfaces, to which it also binds$^{242}$.

It should be noted that in this last study, the divalent cations were added asymmetrically to the vesicles suspensions (i.e. they were present only on the outside of the vesicles), while in the present work, as well as in all references by Reviakine et al.$^{18}$ and Richter et al.$^{21,23,64}$ the divalent cations were present on both sides of the vesicle membrane.

The FRAP results showing immobile PS on the TiO$_2$ surface indicate that the joint effect of Ca$^{2+}$ and PS on the adhesion strength may be due to the formation
of strong TiO$_2$-Ca$^{2+}$-PS complexes that sequester DOPS at the contact area between an adsorbing vesicle and TiO$_2$ (see Section 3.2.2 and Figure 3.8; the presence of adsorbed Ca$^{2+}$ on the TiO$_2$ surface is confirmed by the results shown in the Appendix). The more DOPS is present in the vesicle, the stronger is the interaction with the surface, and therefore the larger the adhesion strength (and deformation) of the vesicle will be. A similar phenomenon was reported for vesicles immobilised on functionalised surfaces either via hybridisation of complementary DNA strands,$^{243}$ or via the biotin/avidin system:$^{244}$ the more anchoring groups between the vesicles and the surface, the more deformed the vesicles were found to be.$^{243}$ In the case of biotinylated vesicles immobilised on an avidin-coated gold surface, the increasing deformation of the vesicles as a function of biotin functional groups lead ultimately to vesicle rupture and SPB formation.$^{244}$

### 3.3.4 Redistribution of PS during vesicle adsorption and SPB formation on TiO$_2$

In solution, vesicles used to form SPBs are expected to have an approximately symmetric distribution of lipids across the two bilayer leaflets. On the other hand, we have concluded from our data that bilayers formed on TiO$_2$ have a highly asymmetric distribution of lipids (Section 3.2.2). This means that a redistribution of lipids across leaflets occurs either during the bilayer formation process or in the SPB itself after its formation is complete. The latter of these two possibilities is not likely in our case, since lipid redistribution is favoured by the presence of defects in the bilayer,$^{31}$ which are more abundant during SPB formation than in a completely formed SPB (defects are expected to reduce the activation energy associated with transfer of the lipids from one leaflet to the other). Indeed, Richter et al., have found their SPBs to be stable against redistribution once formed.$^{21}$ The difference between their results and those of Käsbauder et
al.,\textsuperscript{29} who observed redistribution as long as 12 hr after bilayer formation was complete, is most probably due to the difference in ionic strengths employed in the two studies. We therefore focus on the bilayer formation stage as the more likely instance when lipid redistribution occurs.

Formation of an SPB begins with the adsorption of intact vesicles to the surface (Figure 3.8), irrespectively of the pathway by which an SPB is formed (via a supported vesicular layer intermediate or by rupture of individual vesicles)\textsuperscript{vii.5,17,18,226,239} If the membrane of an adsorbing vesicle contains two components, and one of them interacts with the surface much stronger than the other, during the adsorption process the strongly-interacting component will become enriched (trapped) within the contact area between the vesicle and the surface (Figure 3.8; see also Simson \textit{et al.}\textsuperscript{36} for a discussion of this phenomenon). In an extreme case, few molecules of the strongly interacting component (e.g., a specific target, or an impurity) may be enough to immobilise a vesicle on a surface,\textsuperscript{49,243} while the extent to which vesicle deforms upon adsorption will depend on the amount of the strongly interacting component, and not only on vesicle size and bilayer bending modulus.\textsuperscript{239}

Moreover, concentration of one type of lipid in the contact area (Figure 3.8) will result in a discontinuity in the composition of the membrane, and therefore in a discontinuity in the bending modulus at the edge of the contact area. This may facilitate pore formation and thus provide an additional pathway for lipid redistribution across the two leaflets at the stage of vesicle adsorption.

\textsuperscript{vii. If both pathways are available, the faster one will be observed in practice (see, for example, ref.\textsuperscript{64}). Under the experimental conditions employed in this study, bilayer formation proceeds via an SVL intermediate, which does not rule out the possibility that individual vesicles can rupture.}
Figure 3.8  Re-distribution of lipid molecules across the two leaflets during SPB formation TiO$_2$. Top left. A vesicle containing 20% of homogeneously distributed DOPS (black headgroups) approaches the surface. Bottom left. Once the vesicle is in contact with the surface, DOPS in the outer leaflet of the vesicle becomes trapped within the vesicle-surface contact region in TiO$_2$-Ca$^{2+}$-PS complexes. Bottom right. Most (96 – 98%) of PS is immobilised in the proximal leaflet of the SPB that is finally formed. This implies that flip-flop of DOPS lipids from the distal to the proximal leaflet has occurred.

Another possibility to keep in mind is that lipid redistribution (flip-flop) at the moment of vesicle adsorption might initiate vesicle rupture and the subsequent bilayer formation, instead of simply occurring after vesicle decomposition has been initiated by another mechanism (for example, when the critical vesicle surface concentration is reached). Indeed, flip-flop was reported to be the cause of vesicle disruption for charged vesicles in contact with oppositely charged polyelectrolytes, when the amount of charged lipids was higher than 20-30%.$^{139}$ In our case, therefore, flip-flop (induced by the presence of Ca$^{2+}$ on the TiO$_2$ surface, see Appendix) might promote the rupture of vesicles that are only moderately deformed and that would be otherwise stable.
3.3.5 **PC and PS in supported bilayers formed on SiO$_2$ are distributed asymmetrically**

Close inspection of the diffusion coefficients observed in SPBs containing 0 to 20% DOPS prepared on silica (Table 3.1) reveals that the diffusion coefficient of NBD-PC decreases as the DOPS content of the bilayer increases. An opposite trend is observed for NBD-PS. A similar phenomenon has previously been reported for eggPC:brainPS 90:10 bilayers formed on glass, where NBD-PS was observed to diffuse at \((1.0 \pm 0.07) \times 10^{-8} \text{ cm}^2/\text{s}\) versus \((0.8 \pm 0.05) \times 10^{-8} \text{ cm}^2/\text{s}\) for NBD-PC.\textsuperscript{41} Considering that it has previously been shown that the mobility of PC in the proximal leaflet of SPBs formed on SiO$_2$ is reduced,\textsuperscript{28} these trends may suggest that PC is depleted from the distal leaflet and accumulates in the proximal leaflet of these SPBs, while the reverse is true for PS. The driving force for this asymmetry is the combination of electrostatic repulsion between the negatively charged SiO$_2$ surface and the negatively charged PS headgroup, and attraction between SiO$_2$ and PC.

In this context it is interesting to note that even small amounts (6%) of a negatively charged fluorescent dye were found to destabilise vesicles adsorbed on glass with respect to leakage of molecules encapsulated in the vesicle interior.\textsuperscript{25,26} Lipid redistribution at the stage of vesicle adsorption, discussed above, may, in fact, be responsible for this observation.

We must caution, however, that the effects observed in this and some of the other studies may depend on the properties of the surface used for SPB preparation: asymmetric lipid distribution was not observed in SPBs prepared on rough glass surfaces\textsuperscript{245} (probably due to a thicker and less structured layer of water sandwiched between the surface and the SPB\textsuperscript{28}).

\textsuperscript{viii.} The possibility that the integrity of the bilayer may be compromised during vesicle adsorption and the need to study this process has been raised in a previous study by Reviakine et al.\textsuperscript{18} It has since been addressed by Johnson and Schönherr in their work.\textsuperscript{25,26}
3.3.6 Mechanism of SPB formation on TiO$_2$: a comparison to SiO$_2$ and mica

A close inspection of the SPB formation curves obtained by QCM-D when supported bilayers are formed on TiO$_2$ reveals that the absolute values of the minimum in frequency ($|\Delta F_{\text{min}}|$) and maximum in dissipation factor ($\Delta D_{\text{max}}$) are significantly smaller than in the case of SPBs formed on SiO$_2$ with vesicles of the same size suspended in the same buffer (see Figure 3.9). Several factors may be responsible for this behaviour:

(i) In the context of the model proposed in Section 3.3.1 for interpreting of the acoustic response of surface-adsorbed vesicles, smaller values of $|\Delta F_{\text{min}}|$ and $\Delta D_{\text{max}}$ may be due to a larger deformation of the vesicles adsorbed on TiO$_2$ (than those adsorbed on SiO$_2$) before the decomposition to an SPB begins. It is interesting to note that in the absence of Ca$^{2+}$ a larger deformation of DOPC vesicles is observed on SiO$_2$ than on TiO$_2$.\(^\text{19}\) When Ca$^{2+}$ is present, the deformation of DOPC vesicles on TiO$_2$ is even smaller than in its absence—despite the fact that only ~ 0.1% of DOPC molecules are expected to be occupied by Ca$^{2+}$ ions under our experimental conditions\(^\text{246,247}\)—due to the binding of Ca$^{2+}$ to the TiO$_2$ surface (see Appendix). This can be deduced from the QCM-D data, which show that DOPC vesicles adsorbed in the presence of Ca$^{2+}$ give rise to a larger frequency shift than vesicles adsorbed in its absence (240 ± 27 Hz compared to 177 ± 17 Hz, c.f. the behaviour of 100 nm DOPC vesicles in Figure 3.3 and Figure 3.2). Given that a fully packed SVL is already formed in the absence of Ca$^{2+}$,\(^\text{66}\) this indicates that a thicker layer is formed in the presence of Ca$^{2+}$ than in its absence. Thus, the adhesion strength of DOPC vesicles to TiO$_2$ is weakened by Ca$^{2+}$. This result is consistent with the binding of Ca$^{2+}$ to TiO$_2$ reported in this work (Section 3.2.2 and Appendix) and conjectured in the past.\(^\text{116}\)
Figure 3.9  QCM-D plots for the formation of SPBs on SiO$_2$ and TiO$_2$ (blue curves: frequency shifts; pink curves: dissipation factor shifts). Left: DOPC vesicles of 100 nm size are deposited on SiO$_2$ (lipid concentration: 0.1 mg/ml). Right: DOPC:DOPS 80:20 vesicles of 100 nm size are deposited on TiO$_2$ (lipid concentration: 0.05 mg/ml). The absolute values of the minimum in frequency and the maximum in dissipation are clearly smaller in the case of TiO$_2$. Both experiments were carried out in Ca$^{2+}$ buffer. (It was shown by Richter et al.\textsuperscript{23} that $|\Delta F_{\text{min}}|$ and $\Delta D_{\text{max}}$ on silica depend on the composition of the vesicles. In particular, their value in the presence of Ca$^{2+}$ is larger for DOPC:DOPS 80:20 vesicles than for DOPC vesicles. Therefore, for vesicles of the same composition (DOPC:DOPS 80:20) deposited on both SiO$_2$ and TiO$_2$, the differences between the two sets of $|\Delta F_{\text{min}}|$ and $\Delta D_{\text{max}}$ are expected to be even more pronounced than what appears from this Figure.

With increasing DOPS content, the deformation of the vesicles decreases on SiO$_2$, both in the presence and the absence of Ca$^{2+}$.\textsuperscript{23} On the other hand, our results show that on TiO$_2$ in the presence of Ca$^{2+}$, the vesicle deformation increases with increasing DOPS content (Section 3.2.1). These opposite trends can be explained with the different binding affinities of the two metal oxide surfaces for Ca$^{2+}$ ions: as the vesicles become more negatively charged (their overall charge is still negative even though Ca$^{2+}$ binds to PS, see Figure 3.6), they are less attracted by the negatively charged SiO$_2$ surface, but more attracted by the Ca$^{2+}$-bearing TiO$_2$ surface (see Appendix). The latter is supported by the fact that surface-Ca$^{2+}$-PS complexes (with the consequent immobilisation of PS in the proximal leaflet) are only formed on TiO$_2$ (Figure 3.4) and by the opposite
asymmetric distribution of lipids in SPBs formed on the two surfaces (see Sections 3.3.4 and 3.3.5).

The stronger attraction of the more DOPS-rich vesicles to the Ca\textsuperscript{2+}-covered TiO\textsubscript{2} surface will cause them, above a certain DOPS content, to reach the threshold deformation needed for the rupture of an isolated vesicle. Compositional inhomogeneities from vesicle to vesicle in the solution may cause a fraction of vesicles (those containing more DOPS than average) to rupture individually on TiO\textsubscript{2} even for compositions with a lower mean DOPS content. The remaining vesicles will stay intact until the critical surface concentration is reached. The two mechanisms of rupture of individual vesicles and of a concerted decomposition at the critical surface concentration of vesicles may therefore coexist during the formation of SPBs on TiO\textsubscript{2}. Additionally, it has to be kept in mind that vesicle deformation is not the only mechanism involved in the rupture of DOPS-containing vesicles: flip-flop of the lipids is known to cause the disruption of vesicles in suspension when the amount of charged lipids flipping to the outer membrane leaflet is too high\textsuperscript{139}. Indeed, flip-flop was found to occur in concomitance with SPB formation on TiO\textsubscript{2}, giving rise to an asymmetric distribution of lipids in the SPB (see Section 3.3.4 for details).

(ii) Alternatively, the critical surface concentration of vesicles needed to trigger SPB formation\textsuperscript{14,17} might be smaller on TiO\textsubscript{2} than on SiO\textsubscript{2}. The model discussed in (i) assumes that the surface coverage needed for SPB formation is identical on both surfaces. If this is not the case, then the different $|\Delta F_{\text{min}}|$ would point to a smaller number of vesicles on TiO\textsubscript{2} than on SiO\textsubscript{2} at the moment of SPB formation. To distinguish between these two possibilities, a direct determination of the surface coverage would be needed, for example by AFM. Another way to estimate the surface coverage is to analyse the kinetics of vesicle adsorption: since the process is diffusion-limited, the surfaces of SiO\textsubscript{2} and TiO\textsubscript{2} are occupied
by the same number of vesicles at each given time (provided the concentration of vesicles in solution is the same). Hence, the time at which rupture starts can be related to the surface concentration of vesicles. Indeed, for SPBs formed from DOPC:DOPS 80:20 vesicles of 50 nm diameter and a lipid concentration of 0.5 mg/ml, $t(|\Delta F_{\text{min}}|)$ was found to be $15 \pm 7$ s for TiO$_2$ and $37 \pm 7$ for SiO$_2$, indicating that on TiO$_2$ bilayers start to form at a smaller surface concentration than on SiO$_2$. It has to be kept in mind, however, that hypothesis (i) and (ii) are not mutually exclusive, because vesicles that are deformed to a greater extent (hypothesis i) will start interacting laterally at a lower surface concentration, thus leading to SPB formation at lower coverages (hypothesis ii).

Summarizing the considerations (i) and (ii) discussed above, it has to be noted that the opposite trends in the deformation behaviour on the SiO$_2$ and TiO$_2$ surfaces as a function of DOPS content are independent of our interpretation of QCM-D data. In fact, the trend to increasing deformation on TiO$_2$ in the presence of Ca$^{2+}$ is evident from the fact that there is a transition from SVLs to SPBs as the DOPS content increases. Conversely, on SiO$_2$ in the absence of Ca$^{2+}$ there is a transition from SPBs to SLVs with increasing DOPS content. In the presence of Ca$^{2+}$ this is not observed, however the vesicle adsorption kinetics slows down with increasing DOPS content. These facts support our model (Section 3.3.1) and indicate that hypothesis (i) is very likely. The possibility of individual rupture of vesicles is very likely as well, considering that rupture of isolated vesicle containing 20% DOPS was observed experimentally on mica.

In this context, it is instructive to compare the results obtained here with those published recently on the details of SPB formation on mica and silica (Table 3.2).
Table 3.2  Comparison of the results obtained for the deposition of PC and PC:PS vesicles on SiO$_2$, mica and TiO$_2$ surfaces, in presence and absence of Ca$^{2+}$ (in the studies by Richter et al.$^{23,64}$ and Keller et al.$^{17}$ small unilamellar vesicles (SUVs) were used; in the study by Jass et al.$^{15}$ vesicles were prepared by detergent depletion techniques; in the remaining studies, extruded unilamellar vesicles were used either exclusively$^{67}$ or in addition to SUVs$^{18,19}$). Vesicle sizes are given in parenthesis (the size of SUVs is typically 25 nm).

<table>
<thead>
<tr>
<th>Composition</th>
<th>SiO$_2$</th>
<th>EDTA Ca$^{2+}$</th>
<th>EDTA</th>
<th>mica Ca$^{2+}$</th>
<th>EDTA</th>
<th>TiO$_2$ Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>• SPB formation (all sizes)</td>
<td>• SPB formation (all sizes)</td>
<td>• Intact vesicles$^a$ (SUVs-150 nm)</td>
<td>• SPB formation$^{18,64}$ (SUVs-200 nm)</td>
<td>• Intact vesicles$^{20,66}$ (SUVs-200 nm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Isolated vesicles are stable$^{17}$ (SUVs)</td>
<td>• Isolated vesicles are stable$^{23}$ (SUVs)</td>
<td>• Slight desorption upon rinsing$^{64}$ (SUVs)</td>
<td>• Isolated vesicles are stable$^{18,64}$ (SUVs-200 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Adsorbed vesicles are very deformed$^{19}$ (SUVs-200 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC:PS</td>
<td>• SPB formation up to 33% PS$^{23}$ (SUVs)</td>
<td>• SPB formation up to 33% PS$^{23}$ (SUVs)</td>
<td>• Small amount of intact vesicles at &lt; 20% PS$^{64}$ (SUVs)</td>
<td>• SPB formation up to 80% PS$^{64,248}$ (SUVs)</td>
<td>• Small amount of intact vesicles at &lt; 20% PS$^{67}$ (50-200 nm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Isolated vesicles rupture$^{b,15}$ (200-400 nm)</td>
<td>• Isolated vesicles rupture$^{b,15}$ (200-400 nm)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Intact vesicles at 33-50% PS$^{23}$ (SUVs)</td>
<td>• Restructuring$^c$ at &gt; 50% PS$^{23}$ (SUVs)</td>
<td>• No adsorption at &gt; 20% PS$^{64}$ (SUVs)</td>
<td>• Isolated vesicles containing 20% PS$^{64}$ rupture within minutes to hours$^{64}$ (SUVs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Restructuring$^c$ at &gt; 50% PS$^{23}$ (SUVs)</td>
<td>• Restructuring$^c$ at &gt; 50% PS$^{23}$ (SUVs)</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>• No adsorption at &gt; 66% PS$^{23}$ (SUVs)</td>
<td>• No adsorption at &gt; 20% PS$^{64}$ (SUVs)</td>
<td>• Increasing adhesion strength with increasing DOPS content$^{67}$ (100 nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Redistribution of PS across leaflets of SPB (flip-flop)$^{21}$ (SUVs)</td>
<td>• Redistribution of PS across leaflets of SPB (flip-flop)$^{21}$ (SUVs)</td>
<td></td>
<td></td>
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<td></td>
<td>• Redistribution of PS across leaflets of SPB (flip-flop)$^{21}$ (100 nm)</td>
<td>• Redistribution of PS across leaflets of SPB (flip-flop)$^{21}$ (100 nm)</td>
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Since calcium is known to bind to mica\textsuperscript{124,125}, the behaviour of vesicles on mica is expected to be similar to that on TiO\textsubscript{2}. In fact, Richter \textit{et al.}\textsuperscript{64} have also observed a smaller $|\Delta F_{\text{min}}|$ on mica than on silica, for DOPC:DOPS 80:20 vesicles suspended in a Ca\textsuperscript{2+}-containing buffer. Thus, Ca\textsuperscript{2+} seems to increase the adhesion strength of DOPS-containing vesicles on mica in the same way as it does on TiO\textsubscript{2}. Indeed, Richter \textit{et al.}\textsuperscript{64} found that the acoustic response, as a function of PS, due to vesicles adsorbed on silica and on mica in the presence of Ca\textsuperscript{2+} exhibited opposite trends: the more DOPS was contained in the vesicles, the larger was the vesicle deformation on mica, but the smaller was the vesicle deformation on silica. Redistribution of DOPS across the leaflets of the SPB due to flip-flop in the presence of Ca\textsuperscript{2+},\textsuperscript{21} as well as the cessation of vesicle adsorption in the absence of Ca\textsuperscript{2+} at 20\% DOPS content\textsuperscript{23} also occur on both mica and TiO\textsubscript{2} (see also Table 3.2). The latter result indicates that, when Ca\textsuperscript{2+} is absent, the electrostatic repulsion between negatively charged vesicles and both mica and TiO\textsubscript{2} is much stronger than between the same vesicles and silica (for which cessation of adsorption does not occur until 66\% DOPS\textsuperscript{23}). Additionally, vesicles were seen to rupture individually on mica (at 20\% DOPS, in the presence of Ca\textsuperscript{2+}), on a time scale of minutes to hours.\textsuperscript{64} The latter supports the conjectures presented above, indicating that it is likely that vesicle decomposition induced via the critical vesicular coverage coexists with the rupture of isolated vesicles, both on mica and TiO\textsubscript{2}.

Some major differences between TiO\textsubscript{2} and mica can, however, be observed as

\begin{itemize}
\item a. Only for extruded vesicles smaller than the critical rupture radius (~ 75 nm) and for SUVs at concentrations lower than 0.5 mg/ml\textsuperscript{18,64}
\item b. The vesicles used were composed of mixtures of DPPC, DPPE (zwitterionic), DPPG (anionic, 20\% of the total lipids), cholesterol and galactosylcerebroside.
\item c. "Restructuring" refers to a change of conformation of the material adsorbed on the silica surface, to form a soft and highly flexible layer, the detailed properties of which are unknown.\textsuperscript{23}
\end{itemize}
well. In the presence of Ca\(^{2+}\), DOPC and DOPC:DOPS 90:10 vesicles form bilayers on mica but not on titania. This indicates that PC interacts stronger with mica than with titania.

### 3.3.7 The biocompatibility of TiO\(_2\) in view of the TiO\(_2\) – PS interactions

*In vivo*, phosphatidyl serine is contained in the inner leaflets of cell membranes.\(^{98-103}\) Presence of PS in the outer leaflet occurs, e.g., during apoptosis (where it acts as a signal for destruction of the cell by the macrophages\(^{103}\)), and is also involved in the initiation of blood coagulation at the site of injury or at an implant surface.\(^{101, 249}\) It is therefore quite interesting that the material which performs relatively well when implanted into the blood stream\(^{249, 250}\) has the property of sequestering PS (Figure 3.8). In this context it should be mentioned that platelets, which play a key role in the process of blood coagulation, were found to adsorb to implant surfaces within 5 s of exposure to blood.\(^{251}\) This time scale is comparable to that of protein adsorption from blood on implant surfaces—a process that is thought to play a defining role in implant biocompatibility.\(^{46, 251}\)

Since PS immobilisation is a Ca\(^{2+}\)-dependent process (that involves formation of TiO\(_2\)-Ca\(^{2+}\)-PS complexes), it serves as an indirect proof that Ca\(^{2+}\) adsorbs on the TiO\(_2\) surface, as previously suggested.\(^{116}\) This may enhance bone formation around titanium prostheses implanted into the bone (e.g., artificial hip joints and dental implants). In fact, titanium implants coated with Ca-PS-PO\(_4\) complexes were found to enhance osteoblast growth and differentiation, as well as calcium deposition.\(^{252, 253}\)
3.4 PATTERNS OF SUPPORTED PHOSPHOLIPID BILAYERS

3.4.1 Patterns of SPBs on pre-patterned metal oxide surfaces

Richter et al.\textsuperscript{23} have extensively characterised the interactions between DOPC:DOPS vesicles and silica. Comparing their results with those presented in Section 3.2.1 for the titania surface shows that bilayer formation on silica occurs over a much broader range of conditions and does not always require Ca\textsuperscript{2+}. On TiO\textsubscript{2}, SPB formation from DOPS-containing vesicles always requires Ca\textsuperscript{2+}. Moreover, in the absence of Ca\textsuperscript{2+}, liposomes containing 20% DOPS or more do not adsorb to the surface (Figure 3.2). Here it is described how these differences can be exploited for preparing patterns of bilayers on surfaces exhibiting a SiO\textsubscript{2}/TiO\textsubscript{2} material contrast. The preparation of such surfaces with pattern sizes down to 200 nm has been reported previously.\textsuperscript{191,218}

![Diagram of bilayer formation](image.png)

**Figure 3.10** Using the selectivity of lipidic vesicles to prepare patterns of SPBs.
(a) Vesicles containing between 20% and 50% DOPS are added in absence of Ca\textsuperscript{2+} to a substrate that exhibits a SiO\textsubscript{2}/TiO\textsubscript{2} contrast. A bilayer is formed on SiO\textsubscript{2}, while the electrostatic repulsion does not allow the vesicles to adsorb on TiO\textsubscript{2}.
(b) The second type of vesicles, containing more than 20% DOPS, is added to the substrate in the presence of Ca\textsuperscript{2+}. A second kind of bilayer is formed on the TiO\textsubscript{2} regions of the sample. The presence of the first bilayer on the SiO\textsubscript{2} regions prevents vesicle adsorption on those regions.
(c) Fluorescence microscopy image of a TRITC-labelled DOPC:DOPS 80:20 bilayer formed in Ca\textsuperscript{2+} buffer on TiO\textsubscript{2} (red) surrounded by a NBD-labelled DOPC:DOPS 80:20 bilayer formed in EDTA buffer on SiO\textsubscript{2} (green). This image was taken 10 hours after formation of the bilayers and shows no sign of intermixing of the two kinds of bilayers.
The procedure, outlined in Figure 3.10, involves the preparation of a bilayer on the SiO$_2$ regions of the surface in the absence of Ca$^{2+}$ from vesicles containing more than 20% DOPS, which do not adsorb to the titania regions (Figure 3.10a). This is followed by the preparation of another bilayer on titania in the presence of Ca$^{2+}$ (Figure 3.10b). Vesicles used for bilayer preparation in both steps can contain guest molecules, thus imparting the desired, different functionalities on the silica and titania areas of the surface. In the simplest case, shown in Figure 3.10c, two lipids bearing different fluorescent groups were incorporated into the two kinds of vesicles used in the bilayer formation steps. A bilayer containing NBD-PC (green) was formed on the silica surrounding 48 x 48 µm$^2$ TiO$_2$ squares, while a TRITC-PE-containing bilayer (red) was formed within the squares (the opposite distribution of labels has also been successfully achieved, not shown). Fluorescence recovery after photobleaching measurements confirmed that PC lipids in the bilayers formed on both surfaces were mobile. In most cases, a rim of vesicles appeared to be present between the TiO$_2$ and SiO$_2$ surfaces that did not recover when bleached. This could be due to complex surface chemistry and topography at the SiO$_2$/TiO$_2$ interface.

The patterns shown in Figure 3.10c were stable for at least 24 hrs with respect to intermixing of the two kinds of bilayers. This stability is likely due to the presence of a step (of the order of 10 nm) between the two metal oxides$^{191}$ (Figure 3.10a, b) that acts as a diffusion barrier. This raises an intriguing question concerning the smallest height of such a step that is required to prevent intermixing. Adsorbed vesicles at the rim of the patterns could provide an additional diffusion barrier. (Others have used surface scratches or stripes of materials that do not support bilayer formation (such as alumina, titania or partially oxidised poly(dimethylsiloxane)) to form diffusion barriers and separate bilayers by non-bilayer regions$^{90,178,179,254,255}$).
3.4.2 Build-up of an example of a biosensor interface via functionalised lipids.

To test the ability of the SPB corrals (term coined by Groves et al.\textsuperscript{90}), such as those shown in Figure 3.10c, to serve as a platform for the design of “smart”, bio-interactive interfaces based on TiO\textsubscript{2} (similar interfaces based on bilayer patterns formed on a SiO\textsubscript{2}/Au template have been reported\textsuperscript{49}) two types of functional lipids—biotinylated lipids and Ni-chelator lipids\textsuperscript{256}—were incorporated into the vesicles used in the SPB formation steps. This resulted in the formation of a biotinylated bilayer on silica and a Ni-bearing bilayer on TiO\textsubscript{2}. The former was loaded with streptavidin and fluorescently labelled biotinylated vesicles, while the latter with histidine-tagged green fluorescent protein (Figure 3.11 a,b). Some degree of non-specific interactions of the protein with the bilayers not carrying Ni-NTA was observed, in spite of the recognised protein-resistant properties of supported bilayers.\textsuperscript{53} The use of negatively charged (DOPS-exposing) bilayers is thought to be the reason for this result. These bilayers may also have a thrombo-

![Figure 3.11](image.png)

**Figure 3.11** Build-up of an example of a bio-interactive patterned surface.
(a) Lipids carrying various functionalities (biotin, the Ni-chelator NTA, etc.) can be incorporated in the SPB patterns, thus allowing for the specific immobilisation of different molecules (i.e. biotinylated vesicles or his-tagged proteins) on each side of the pattern.
(b) Fluorescence microscopy images corresponding to the situation depicted in panel (a). Left: overview image of biotinylated DOPC vesicles (red) immobilised via streptavidin on a biotinylated SPB. Right: his-tagged green fluorescent protein (green) immobilised on an SPB containing Ni-NTA lipids, surrounded by biotinylated DOPC vesicles (red) immobilised via streptavidin on a biotinylated SPB.
genic effect in the body due to the initiation of a blood clotting cascade. Both these issues might be resolved by adding cationic molecules to the bilayers formed on SiO$_2$ that will sequester the DOPS lipids. DOPS-containing SPBs formed on TiO$_2$, on the other hand, are expected to have a resistance to protein adsorption comparable to that of pure DOPC bilayers, since in this case the DOPS is sequestered in the proximal leaflet and therefore not exposed to the bulk solution.

3.5 **Summary and Conclusions**

The results presented in this study enhance the general understanding of liposome-surface interactions and open a new chapter in the research on TiO$_2$-based biointeractive interfaces. In the presence of Ca$^{2+}$, supported bilayer formation was observed for vesicles containing more than 20% DOPS. A short-range TiO$_2$-DOPS interaction mediated by Ca$^{2+}$ provides the driving force for the bilayer formation. In the absence of Ca$^{2+}$, bilayer formation was not observed, and adsorption of vesicles was prevented by an energy barrier of electrostatic origin.

Close inspection of the properties of SPBs formed on TiO$_2$ revealed that the lipids are distributed asymmetrically across the leaflets, with DOPS being contained to 96-98% in the proximal leaflet and immobilised on the surface by TiO$_2$-Ca$^{2+}$-PS complexes. Flip-flop of lipids at the moment of bilayer formation, when the presence of several pores and defects in the membrane facilitates the inter-leaflet transport of lipids, is proposed as the mechanism responsible for lipid redistribution.

As an application of the knowledge gathered in the first part of the study, supported bilayer corrals that incorporated various functionalities were prepared on TiO$_2$/SiO$_2$ patterned substrates. This patterning method relies on the unique abil-
ity of lipidic vesicles to distinguish between different surfaces. The system described provides substantial versatility with respect to the functionalities that can be incorporated into the SPB corrals. Biotinylated nucleic acids have previously been bound to streptavidin-bearing SPBs and are easy to incorporate into the protocol developed above. Gangliosides, that often serve as receptors for pathogenic bacteria, can be incorporated into the bilayers, thus making possible the design of pathogen-sensing biosensors. Advances in synthetic organic chemistry have produced peptide-bearing lipids that can serve as receptors for cell attachment; and the Ni-chelator lipids/Histidine tagged protein technology provides almost unlimited possibilities for incorporating soluble proteins into the patterning strategy. The use of liposomes with asymmetric lipid distribution between the two leaflets may be required to ensure that the bulky headgroups of the guest lipids do not interfere with the SPB formation process. More important perhaps is the possibility of integrating hydrophobic moieties—from lipophilic drugs to transmembrane proteins—into the SPBs. The ability to prepare such functional interfaces on the basis of a widely used biomaterial such as titanium constitutes a crucial advance in the research on interactive bio-interfaces.
4.1 INTRODUCTION

In this chapter, the interactions of the polyelectrolyte poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG\textsuperscript{160,161}) with lipidic systems were investigated. It was found that PLL-g-PEG caused the decomposition of lipidic membranes in a fashion that was dependent on the architecture of the polymer, the ionic strength of the buffer and the curvature of the membrane (which ranged from flat SPBs to highly curved vesicles of 50 nm diameter).

On one hand, the effect of PLL-g-PEG on lipidic systems is interesting in the context of the important role that the interactions between lipid membranes and charged macromolecules—such as DNA and proteins—play in many cellular and biotechnological processes. An example of the former is the binding of peripheral proteins to the surface of membranes that can induce lipid demixing, alter the phase behaviour, and cause other effects, such as alterations in membrane curvature.\textsuperscript{41,85,126-130} On the biotechnological side, examples of important processes
include DNA-phospholipid interactions (which are relevant for gene delivery studies and have also lead to the discovery of novel soft biomaterials\textsuperscript{144-146}, and the formation of supported bilayers on polyelectrolyte-coated surfaces.\textsuperscript{8}

On the other hand, the resistance to non-specific protein adsorption that PLL-g-PEG imparts to the negatively charged surfaces it adsorbs to\textsuperscript{155-159} has lead us to evaluate the performance of a PLL-g-PEG coating in protecting the underlying lipidic membrane from a harsh environment (such as solutions containing an enzyme that specifically catalyses the hydrolysis of the ester bond of glycerophospholipid molecules).

### 4.2 Interaction of PLL-g-PEG with Supported Phospholipid Bilayers and Supported Vesicular Layers

#### 4.2.1 Results

**4.2.1.1 Interaction of PLL-g-PEG with Supported Bilayers Investigated by QCM-D: Effect of Ionic Strength and Bilayer Composition**

The quartz crystal microbalance with dissipation (QCM-D) was used to follow in situ and in real time the interactions between PLL-g-PEG and supported phospholipid bilayers (the experimental procedure is outlined in Figure 4.1).
Figure 4.1  Schematic representation of the experimental procedure. (A) A supported phospholipid bilayer is formed on a SiO$_2$ surface by vesicle fusion in Ca$^{2+}$ buffer. A bilayer composed of a DOPC (black headgroups): DOPS (white headgroups, negatively charged) mixture is used in this example; some experiments were performed with SPBs composed of DOPC alone. (B) After rinsing with the appropriate buffer(s), PLL-g-PEG is added and the interaction with the bilayer is monitored by QCM-D and fluorescence microscopy. All the results shown in this section were obtained by using three PLL-g-PEG polymers with different architectures and/or functionalisations: standard PLL(20.kDa)-[g=2.9]–PEG(2k.Da), fluorescently labelled PLL(20.kDa)-[g=4.0]–PEG(2k.Da) (with 25% of fluorescein-terminated PEG chains), and the high grafting ratio polymer PLL(20.kDa)-[g=14.2]–PEG(1.kDa)$^{159}$ (see Table 2.2).

**Stability of Supported Bilayers during the Exchange of Buffers**

The formation of highly negatively charged SPBs on SiO$_2$ was dependent on the presence of Ca$^{2+}$ ions and high ionic strength (here 2 mM CaCl$_2$ and 100 mM NaCl, see also reference 23 and Table 3.2); for consistency, less negatively charged, as well as zwitterionic SPBs were prepared in the same buffer. On the other hand, high ionic strength buffers attenuate the interaction between a polyelectrolyte and a charged surface. Thus, addition of PLL-g-PEG to SPBs was car-
ried out in a low ionic strength buffer. This required a buffer exchange step, from the high ionic strength buffer to the low ionic strength one, before adding the polymer. However, such a step could potentially damage the SPB, because a thin film of the original buffer remains between the substrate and the bilayer and may create electrical and osmotic gradients across the membrane.\textsuperscript{259,260} For that reason, the stability of SPBs to buffer exchanges was investigated first. In the QCM-D experiment shown in Figure 4.2 the Ca\textsuperscript{2+} buffer was exchanged for H1 buffer and back.\textsuperscript{i} The frequency and dissipation factor shifts (\(\Delta F\) and \(\Delta D\)) observed in this case are due to the different properties of the buffers and do not correspond to adsorption or desorption of material. Both the frequency and dissipation factor shifts return to within \(\pm 3\) Hz of their initial values once the exchange cycle is

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig42}
\caption{Stability of supported phospholipid bilayers with respect to buffer exchange. Arrows along the time axes indicate injections. A baseline is acquired in Ca\textsuperscript{2+} buffer. A vesicles suspension in Ca\textsuperscript{2+} buffer is injected in the QCM-D chamber at \(t = 0\) min; the frequency and the dissipation response observed is consistent with bilayer formation.\textsuperscript{14} Once the signals have stabilised, Ca\textsuperscript{2+} buffer is exchanged for H1 buffer in a series of injections at \(t = 16, 30\) and \(42\) min. The procedure is repeated in reverse to return the system to Ca\textsuperscript{2+} buffer (injections at 57, 70, and 80 min). Vesicles are injected once more to ascertain bilayer integrity (injection at 93 min).}
\end{figure}

\textsuperscript{i} The Ca\textsuperscript{2+}-chelator EDTA was not used in these buffer exchanges. The very extensive rinses performed ensured nevertheless that the Ca\textsuperscript{2+} ions were removed almost completely (i.e. only traces were left).
complete (Figure 4.2). Subsequent addition of vesicles to the SPB did not cause any further adsorption. Both these results indicate that the buffer exchange does not significantly perturb the SPBs: bilayer damage would be identified from the QCM-D curves as loss of mass from the surface and subsequent adsorption of further vesicles in the resulting defects.

**PLL-g-PEG adsorption in a low ionic strength buffer (H1 buffer)**

Typical QCM-D curves obtained when PLL-g-PEG was added to zwitterionic and negatively charged bilayers in H1 buffer are shown in Figure 4.3 a and b. In these experiments, an SPB was first formed from a vesicles suspension in Ca\(^{2+}\) buffer, which was used to facilitate SPB formation from negatively charged vesicles. The buffer was exchanged for the H1 buffer, in order to maximise the electrostatic interactions between the SPB and the polyelectrolyte (see above).

**Figure 4.3** Addition of PLL-g-PEG in H1 buffer to zwitterionic (a) and negatively charged (b) bilayers. Arrows along the time axes indicate injections. The various stages of the experiment are indicated with schematic drawings where possible.

a) Zwitterionic bilayer (DOPC). The frequency decreases and the dissipation factor increases upon addition of PLL-g-PEG, indicating copolymer adsorption. However, material is removed from the surface upon both further injection of the polymer solution and rinsing with H1 buffer (thick arrows).

b) Negatively charged bilayer (DOPC:DOPS 95:5). Initial adsorption of PLL-g-PEG is followed by a spontaneous desorption of material (encircled).

For clarity and space reasons, some of the buffer exchange steps have been omitted. *1 and *2 indicate the start- and end-points used to calculate the frequency and dissipation shifts shown in Table 4.1.
Addition of the PLL-g-PEG solution in H1 buffer to SPBs caused transient changes in the frequency and dissipation shifts consistent with polymer adsorption (Figure 4.3). In the case of negatively charged SPBs (Figure 4.3b), this adsorption was followed by desorption. In the case of zwitterionic SPBs, either further injections of the polymer solution or rinses with H1 buffer were required to initiate the desorption process (see arrow in Figure 4.3a), indicating that the desorption was not spontaneous, at least on the timescale of the experiments (typically 60 min).

The differences between the frequency of the bare crystal in buffer and the frequency of the crystal after the final rinse with the same buffer are listed. They characterise the total amount and properties of material adsorbed on the substrate at the end of the experiment. The frequency and dissipation shifts for the formation of supported bilayers in Ca^{2+} buffer were, independently of composition, $\Delta F3/3 = -26 \pm 1$ Hz and $\Delta D3 = (0.12 \pm 0.4) \times 10^{-6}$, respectively. Values with errors are averages of a minimum of three experiments; errors are standard deviations. Values without the standard deviations are single measurements.

The total frequency shifts for the SiO$_2$/zwitterionic bilayer/polymer experiments were similar to those observed in the case of polymer adsorbed directly on SiO$_2$ (Table 4.1, Table 4.2). In the case of the negatively charged SPBs, significantly larger frequency shifts were observed when the polymer was added to the

**Table 4.1** Total frequency (in Hz) and dissipation shifts ($\times 10^{-6}$, in arbitrary units) for the scaled 3$^{rd}$ overtone observed in the QCM-D experiments with PLL-g-PEG in high (EDTA) and low (H1) ionic strength buffers and on supported bilayers of various compositions.

<table>
<thead>
<tr>
<th>Amount of DOPS (mass-%)</th>
<th>0%</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta F3/3$</td>
<td>-33 ± 2</td>
<td>-41 ± 12</td>
<td>-45</td>
<td>—</td>
</tr>
<tr>
<td>$\Delta D3$</td>
<td>1.8 ± 0.1</td>
<td>3.1 ± 2</td>
<td>4.3</td>
<td>—</td>
</tr>
<tr>
<td>EDTA buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta F3/3$</td>
<td>-28 ± 5</td>
<td>—</td>
<td>-29</td>
<td>-26</td>
</tr>
<tr>
<td>$\Delta D3$</td>
<td>2.3 ± 2.2</td>
<td>—</td>
<td>-1.2</td>
<td>0.59</td>
</tr>
</tbody>
</table>

The differences between the frequency of the bare crystal in buffer and the frequency of the crystal after the final rinse with the same buffer are listed. They characterise the total amount and properties of material adsorbed on the substrate at the end of the experiment. The frequency and dissipation shifts for the formation of supported bilayers in Ca$^{2+}$ buffer were, independently of composition, $\Delta F3/3 = -26 \pm 1$ Hz and $\Delta D3 = (0.12 \pm 0.4) \times 10^{-6}$, respectively. Values with errors are averages of a minimum of three experiments; errors are standard deviations. Values without the standard deviations are single measurements.
Interactions between Lipidic Systems and PLL-g-PEG SPBs than to the bare SiO$_2$ surface. On the other hand, the $\Delta$D values for the SiO$_2$/zwitterionic bilayer/PLL-g-PEG experiments performed in H1 buffer were higher than those for PLL-g-PEG adsorption on bare SiO$_2$ in the same buffer, and increased with the proportion of the negatively charged lipids in the SPB (Table 4.1, Table 4.2). The dissipation factor is related to the viscoelastic properties of the layer and its thickness.\textsuperscript{261,262} Thus, upon addition of PLL-g-PEG the surface layer is converted from an SPB to a thicker and/or less compact layer than either an SPB (Table 4.1) or a polymer film alone (Table 4.2). Combined with the observed desorption processes (see above), these results suggest that partial replacement of the SPB by PLL-g-PEG takes place at the surface. The fluorescence microscopy results presented below provide further insight into the nature of this process.

No interaction between 2 kDa PEG and the SPBs could be detected by QCM-D.

**Table 4.2**  Mean values of the frequency and dissipation shifts for the adsorption of PLL-g-PEG to bare SiO$_2$ crystals in high (EDTA) and low (H1) ionic strength buffers. The averages were calculated from a minimum of three experiments and are listed along with the corresponding standard deviations.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Frequency (Hz)</th>
<th>Dissipation factor (1x10^{-6})</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>$\Delta F_{3/3}$</td>
<td>-35 ± 3</td>
</tr>
<tr>
<td>EDTA buffer</td>
<td>$\Delta F_{3/3}$</td>
<td>-26 ± 4</td>
</tr>
</tbody>
</table>

**High ionic strength buffer (EDTA buffer)**

In the case of a high ionic strength, Ca$^{2+}$-free buffer, a small initial adsorption of the copolymer was followed by its partial removal during rinses with the polymer-free buffer. The overall frequency shifts, after the rinses with the Ca$^{2+}$ buffer (for comparison with the initial baseline values acquired in this buffer), were larger than those characterising the bare SPB alone or polymer alone. The overall
dissipation shifts were similar to those of the polymer (Table 4.1, Table 4.2). This indicates that some polymer did adsorb to the bilayer without displacing the latter.

High ionic strength, calcium-containing buffer (Ca\(^{2+}\) buffer)

A typical QCM-D curve recorded in the case of PLL-g-PEG addition to supported bilayers in a Ca\(^{2+}\) buffer is shown in Fig. 4.4 In the case of negatively charged SPBs, the QCM-D curves exhibited a behaviour consistent with adsorption of the polymer. This adsorption was transient however: rinsing with polymer-free buffer caused both the frequency and the dissipation factor signals to return to those of an SPB alone (Figure 4.4a). No exchange of SPB for the polymer had occurred. This conclusion is also supported by the absence of the complex behaviour that was observed in the H1 buffer (see above).

Figure 4.4 Interaction between PLL-g-PEG and SPBs in Ca\(^{2+}\) buffer.
a) The changes in frequency and dissipation that are observed upon addition of PLL-g-PEG to a negatively charged bilayer (in this case: DOPC:DOPS 85:15) are interpreted as polymer adsorption. Upon repeated rinsing, the signals return to approximately the same values (within ± 3 Hz) as before the polymer addition. The asterisk indicates the difference in frequency between SPB and (SPB + copolymer). A similar difference was calculated for the dissipation factor. These are plotted in (b) as a function of the weight percent of negatively charged lipids in the bilayer.
b) The frequency and dissipation shifts due to the adsorption of PLL-g-PEG to supported bilayers (measured as described in (a)) are plotted as a function of the weight percent of negatively charged phospholipids in the SPBs.
The frequency and dissipation factor shifts for the adsorption of PLL-g-PEG on supported bilayers measured before the rinsing steps are displayed in Figure 4.4b. Both correlate well with the amount of negatively charged phospholipid in the SPB, indicating specific interactions between PLL-g-PEG and the negatively charged supported bilayers.

No interaction between PLL-g-PEG and the zwitterionic SPBs could be detected in the Ca\(^{2+}\) buffer.

### 4.2.1.2 Interaction of PLL-g-PEG with supported bilayers examined by fluorescence microscopy: effect of bilayer quality and ionic strength

To gain further insight into the PLL-g-PEG–bilayer interactions, the process was studied by fluorescence microscopy. Depending on the experimental conditions (method by which the bilayer was initially prepared, size of the vesicles used in the preparation, bilayer composition and buffer ionic strength), different responses to the polymer were observed: they ranged from no changes to the bilayer to complete disruption and removal of the latter from the surface (Table 4.3, Figure 4.5).

**Figure 4.5** Various responses of SPBs doped with NBD-PC to the addition of PLL-g-PEG. See Table 4.3 and the text for explanation.
a) No signs of disruption could be observed within 15 min after the addition of PLL-g-PEG in some of the preparations. These are considered to be resistant to the disruption by PLL-g-PEG.
b) In some of the preparations, only a small number of worm-like structures was observed within 15 min after the polymer addition, and their appearance was localised. These were considered to be partially resistant.
c) Completely disrupted samples were significantly damaged by PLL-g-PEG within 15 min.

**High ionic strength buffer (EDTA buffer and Ca\(^{2+}\) buffer)**

When PLL-g-PEG was added to supported bilayers in high ionic strength buffers, the SPBs were always observed to remain intact, and the copolymer was observed to adsorb to the SPBs (Figure 4.6). This response was independent of whether Ca\(^{2+}\) ions were present or absent (Ca\(^{2+}\) buffer or EDTA buffer, respectively) the quality of the bilayers, and their composition (zwitterionic or negatively charged). These results are consistent with the QCM-D observations.

![Figure 4.6 Fluorescence signals arising from a DOPC:DOPS 9:1 bilayer doped with TRITC-PE (green) and from PLL-g-PEG-fluorescein (red) for an SPB alone (a, b) and for an SPB coated with PLL-g-PEG in EDTA buffer after rinsing away the excess polymer (c, d). The images were taken under identical conditions using the laser lines appropriate for each probe, and demonstrate that after the addition of PLL-g-PEG-fluorescein, the previously bare SPB (a, b) remains intact (c) and is coated with the polymer (d).](image-url)
**Low ionic strength buffer (H1 buffer)**

**Effect of bilayer quality**

The exposure of bilayers prepared with protocol (A) ("standard protocol") from 400 nm vesicles, bilayers exhibiting defects—in the form of holes, attached vesicles, or aggregates—or bilayers damaged by contact with air, to PLL-g-PEG in H1 buffer led to their decomposition (Figure 4.7). In the first few seconds to 10 min after addition of PLL-g-PEG, small fragments of the bilayer were observed to come off the surface (Figure 4.7, b and f). These fragments assembled spontaneously into globular and worm-like structures (aspect ratio > 10; less than 0.5 micron in diameter and several microns long, Figure 4.7d). Some of the structures were observed to float in solution, while others remained anchored to the surface. The removal of the SPB from the surface continued for another ~ 10 min (Figure 4.7, c and g), but was never complete: some of the lipid remained at the surface after the disruption, most likely in a complex with the adsorbed polymer. This latter observation is consistent with QCM-D results (see above).

Approximately 30 min after addition of PLL-g-PEG, the elongated structures started to collapse into globular structures (Figure 4.7d). Using fluorescently labelled PLL-g-PEG and TRITC-labelled lipids, it was ascertained that the worm-like structures consisted of polymer-lipid complexes (Figure 4.7h), and that both polymer and the lipids were present at the surface after the disruption (not shown). This latter observation is also consistent with QCM-D results (see above).

Using 50 nm vesicles instead of 400 nm ones, adsorbing them on the substrate in an upside-down configuration, and using an enhanced washing procedure (protocol (B),\(^7,16\) see Section 2.4 on page 23 for details) allowed both zwitterionic and negatively charged SPBs that were partially or completely resistant to
Figure 4.7  Time-lapse sequences of false-coloured fluorescence images depicting the disruption of supported bilayers on SiO$_2$ ($A$-$H$) and the interaction of PLL-g-PEG in H1 buffer with supported vesicular layers on TiO$_2$ ($I$-$L$). The green colour corresponds to the lipids.

($A$-$C$) A DOPC bilayer, doped with 1% NBD-PC, before ($A$), a few seconds after ($B$), and 30 min after ($C$) the addition of PLL-g-PEG in H1 buffer.

($D$) The worms formed during the bilayer disruption were observed to collapse into globular structures (encircled). Two sequential images taken 45 min after PLL-g-PEG addition, 60 seconds apart, are shown. The collapse is instantaneous (< 1 s).

($E$-$G$) A DOPC:DOPS 9:1 bilayer, doped with 1% NBD-PC, before ($E$), 1 min after ($F$), and 5 min after ($G$) PLL-g-PEG addition in H1 buffer.

($H$) A fluorescence image demonstrating the co-localisation of phospholipids (green) and PLL-g-PEG-fluorescein (red) in the worm-like structures and aggregates that form after the disruption of a of DOPC:DOPS 9:1 SPB. The polymer was added in H1 buffer. Some worms appear as double lines (one in green and one in red) in the images (white arrowheads) due to their movement during the time passed between the acquisition of the images in the two channels. Co-localisation was also observed in the case of DOPC bilayers (not shown).

($I$-$J$) Zwitterionic (DOPC) vesicles adsorbed on the surface of TiO$_2$ form a supported vesicular layer ($J$). They are replaced on the surface by the adsorbing PLL-g-PEG ($J$).

($K$-$L$) The copolymer has no effect on the negatively charged (DOPC:DOPS 9:1) vesicles. The average intensity of the images is the same before ($K$) and after ($L$) the addition of the polymer. In both cases, the vesicles remain intact. Formation of worm-like structures is not observed. Vesicles were labelled with NBD-PC.
PLL-g-PEG to be prepared (Table 4.3). While a significant proportion of the bilayers was still disrupted, these experiments indicate that defects in the SPBs play a role in the disruption process.

SPBs containing fluorescent lipids formed on QCM-D crystals were invariably disrupted upon addition of PLL-g-PEG in H1 buffer. This is consistent with the QCM-D results obtained in H1 buffer (Figure 4.3).

**Effect of polymer architecture**

Independent of their quality, both zwitterionic and negatively charged SPBs remained intact after the addition of PLL (20 kDa) (Table 4.3). Moreover, PLL was found to protect SPBs from subsequent disruption by PLL-g-PEG. We infer from this observation that PLL adsorbs to SPBs in H1 buffer—this is in fact consistent with previously published observations. Therefore, PLL-g-PEG is also expected to coat resistant bilayers (see above). The incidence of resistant bilayers in H1 buffer was however not sufficiently high to directly test this assertion.

The effect of the PEG chain density on the ability of the copolymer to disrupt the bilayers was further investigated by using a copolymer with a high grafting ratio (i.e. low density of PEG chains): PLL(20 kDa)-[g=14.2]–PEG(1 kDa). Both zwitterionic and negatively charged bilayers were found to be resistant to disruption by this copolymer.

No effect of the addition of 2 kDa PEG to SPBs could be detected by fluorescence. PEG was also not able to protect the SPBs from subsequent disruption by PLL-g-PEG. This is consistent with what was observed by QCM-D, namely that PEG does not interact with the SPBs (not shown).
Samples were classified into three categories according to the degree of disruption of the bilayer 15 minutes after addition of PLL-g-PEG: resistant (appearance identical to that of the bare SPB, Figure 4.5a), partially resistant (some defects and few worm-like structures present, Figure 4.5b), and non-resistant (Figure 4.5c). For this table, the percentages of occurrence of resistant and partially resistant samples were added.

### Table 4.3  Resistance of SPBs formed on SiO$_2$-coated coverslips to disruption by PLL-g-PEG in H1 buffer.

<table>
<thead>
<tr>
<th>Bilayer composition</th>
<th>Protocol (A)</th>
<th>Protocol (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>43 % 50-nm vesicles, resistant and partially resistant</td>
<td>89 % 50-nm vesicles, resistant and partially resistant</td>
</tr>
<tr>
<td></td>
<td>57 % 50-nm vesicles, disrupted</td>
<td>11 % 50-nm vesicles, disrupted</td>
</tr>
<tr>
<td>DOPC:DOPS 9:1</td>
<td>29 % 400-nm vesicles, resistant and partially resistant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71 % 400-nm vesicles, disrupted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43 % 50-nm vesicles, resistant and partially resistant</td>
<td>50 % 50-nm vesicles, resistant and partially resistant</td>
</tr>
<tr>
<td></td>
<td>57 % 50-nm vesicles, disrupted</td>
<td>50 % 50-nm vesicles, disrupted</td>
</tr>
</tbody>
</table>

4.2.1.3  Investigating Bilayer Quality by Fluorescence Recovery after Photobleaching (FRAP) Measurements

The lateral mobility of the phospholipid molecules in the bilayers was characterised by FRAP. It was found that the diffusion coefficient of the fluorescently labelled NBD-PC probe depended on the composition of the bilayers: faster diffusion was observed in DOPC bilayers than in DOPC:DOPS 9:1 ones (Table 4.4). This is most probably due to the asymmetric distribution of the lipids in SPBs formed on SiO$_2$ (DOPC is found preferentially in the proximal leaflet, where it can be slowed down by the interaction with the substrate, while DOPS is found mostly in the distal leaflet, where it can diffuse freely; see Section 3.3.5 for details). In both cases, the observed values are in good agreement with those found in the literature for NBD-PE, NBD-PC and NBD-PS.
Interactions between Lipidic Systems and PLL-g-PEG

No change in the diffusion coefficients or mobile fraction of the probe in either zwitterionic or negatively charged bilayers was observed upon addition of PLL-g-PEG as long as the bilayers were completely resistant to the addition of the polymer (Table 4.4). Interestingly, the negatively charged bilayers retained their fluidity even after being coated with the polymer. Bilayers that were even partially disrupted by the addition of the copolymer were not analyzed, because they no longer conform to the assumptions used in the analysis of FRAP experiments\textsuperscript{71} due to the removal of lipids from the surface. No correlation was observed between the results of the FRAP analysis and the behaviour of the bilayers upon addition of PLL-g-PEG in H1 buffer (Table 4.5).

Table 4.4 Diffusion coefficients and mobile fractions for NBD-PC probes in DOPC and DOPC:DOPS bilayers before and after coating with PLL-g-PEG in H1 buffer (only fully resistant bilayers were considered). Amount of fluorescent lipids: 1 wt-%. n = number of measurements.

<table>
<thead>
<tr>
<th></th>
<th>before adding PLL-g-PEG</th>
<th>after adding PLL-g-PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D (cm(^2)/s)</td>
<td>M</td>
</tr>
<tr>
<td>NBD-PC in DOPC</td>
<td>(1.7 ± 0.3) (10^{-8})</td>
<td>0.99 ± 0.06</td>
</tr>
<tr>
<td>n=16</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>NBD-PC in DOPC:DOPS 9:1</td>
<td>(1.2 ± 0.2) (10^{-8})</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td>n=22</td>
<td>n=16</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Diffusion coefficients and mobile fractions of uncoated SPBs in H1 buffer, separated according to their response to PLL-g-PEG (n = number of measurements).

<table>
<thead>
<tr>
<th></th>
<th>D (cm(^2)/s)</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC, will disrupted</td>
<td>(1.7 ± 0.7) (10^{-8})</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>n=3</td>
<td></td>
<td>n=3</td>
</tr>
<tr>
<td>DOPC, will NOT be disrupted</td>
<td>(1.9 ± 0.2) (10^{-8})</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td>n=3</td>
<td></td>
<td>n=6</td>
</tr>
<tr>
<td>DOPC:DOPS 9:1, will be disrupted</td>
<td>(1.3 ± 0.2) (10^{-8})</td>
<td>0.90 ± 0.13</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td>n=12</td>
</tr>
<tr>
<td>DOPC:DOPS 9:1, will NOT be disrupted</td>
<td>(1.1 ± 0.1) (10^{-8})</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>n=4</td>
<td></td>
<td>n=3</td>
</tr>
</tbody>
</table>
4.2.1.4 Interaction of PLL-g-PEG with supported vesicular layers.

The effect of PLL-g-PEG on supported vesicular layers prepared under the same conditions as the SPBs but on titanium dioxide (adsorption in Ca\textsuperscript{2+} buffer followed by buffer exchange to H1 before addition of the polymer) was investigated by fluorescence microscopy (Figure 4.7i-l). It was found that neither the zwitterionic nor the negatively charged vesicles were decomposed by the polymer, and no formation of worms was observed. However, the zwitterionic vesicles were displaced from the substrate by PLL-g-PEG, while the negatively charged ones were not. This phenomenon is consistent with the findings of Section 3.3.4, where it was shown that the DOPS in the outer leaflet of vesicles adsorbed on TiO\textsubscript{2} in Ca\textsuperscript{2+} buffer is trapped in the contact area between vesicle and substrate, due to the formation of TiO\textsubscript{2}-Ca\textsuperscript{2+}-PS complexes. DOPS-containing vesicles are therefore more tightly bound to the surface than pure DOPC ones, and are less likely to be displaced by PLL-g-PEG.

4.2.2 Discussion

The disruption of supported bilayers by PLL-g-PEG in a low-ionic strength buffer is an interesting phenomenon. Given that under identical conditions, the copolymer does not induce the decomposition of surface-adsorbed vesicles (Figure 4.7i-l), it illustrates the limitations of supported bilayers as a model system for free-standing lipidic structures – such as cell membranes and vesicle walls. In what follows, the mechanism of this disruption is considered in terms of the influence of polymer architecture, bilayer quality (presence or absence of defects), and the solid support, on the disruption process.

Interactions between charged vesicles and polyelectrolytes, including poly(lysine), have been studied in detail. In particular, adsorption of poly(N-
Interactions between Lipidic Systems and PLL-g-PEG

ethyl-4-vinylpyridinium bromide) (PEVP) to vesicles containing cardiolipin (CL) resulted in the transfer of the latter to the outer leaflet of the membrane due to the enhancement of the flip-flop rate. Vesicles containing more than 20-30 % CL were ultimately destroyed by the polymer. Pure CL liposomes were found to undergo a transformation to multilamellar lipid-polymer complexes upon addition of PLL. High molecular weight PLL was also found to destabilise black lipid membranes composed of pure PS.

In the case of bilayers with lower charge density (such as those used in this study), polycations have been found to adsorb to the vesicle surface without disrupting the latter, but only perturbing the packing of the lipids in the membrane, causing a shift in the gel-to-liquid transition temperature, and causing domain formation for stochiometric ratios of polymer to lipid smaller than one. Thus, disruption of the SPBs by a polyelectrolyte is not expected under the experimental conditions employed in this study (typically 10% of DOPS and excess of PLL or PLL-g-PEG), and neither is domain formation (which could lead to formation of defects in the SPB). Indeed, PLL by itself was not found to cause the disruption of the SPBs (Table 3). Compared to PLL, PLL-g-PEG with a grafting ratio, \( g \) (number of lysine monomers per PEG side chain), of \( \sim 3 \) has an approximately 30% smaller charge density (only the fraction \( 1-1/g \) of the amino groups of PLL remains free, while the rest are involved in the amide linkage to the PEG side chains and are uncharged). Therefore, the PLL-g-PEG-induced disruption of bilayers in a low ionic strength buffer (Fig. 4) does not proceed by a mechanism typical of polyelectrolytes, such as those observed in the studies mentioned above. It is instead related to the copolymer architecture. Upon adsorption of the copolymer to a flat surface, such as that of an oxide or an SPB, the volume available to the PEG chains is significantly reduced compared to that available to them in solution. In the case when the distance between the
grafting points along the PLL backbone, $L$, is smaller than the radius of gyration of the PEG chains, $R_{gPEG}^{265}$—in other words, when $L/R_{gPEG}$ is smaller than one—the PEG chains are laterally compressed upon adsorption.$^{266}$ Simple calculation (taking the lysine monomer length at $\sim 0.355$ nm based on the distance between the $\alpha$-carbon atoms in a typical $\beta$-sheet structure, $R_g$ of 2 kDa PEG at 1.7 nm, and $R_g$ of 1 kDa PEG at 1.1 nm$^{265}$) shows that in the case of PLL(20)-[$g=2.9$]-PEG(2), which disrupts the bilayers, this ratio is $\sim 0.4$, while in the case of PLL(20)-[$g=14.2$]-PEG(1), which does not, the ratio is $\sim 4.5$. Compression of PEG chains incurs an entropic penalty. The chains can expand, and the free energy of the system can be lowered, if the PLL backbone is allowed to curve. This induces curvature in the underlying bilayer to which the copolymer is bound, and thus lifts the bilayer off the surface (Figure 4.8). The natural curvature of the membrane in liposomes may therefore constitute one reason why they are resistant to decomposition by PLL-\textit{g}-PEG (in fact, the polymer did not induce leakage in 50 nm diameter liposomes in solution, see Section 4.3.1.1). Other reasons may include the fact that a portion of the surface area of the liposomes is stored as membrane fluctuations$^{239}$ (these fluctuations are damped by the presence of the solid support$^{267}$) and the ability of the liposomes to change shape.

Better-quality bilayers (it has been reported by several investigators that the use of smaller (i.e., 50 nm instead of 400 nm) vesicles leads to better quality bilayers and improves the reproducibility of experiments with SPBs$^{16,19}$) were less likely to be disrupted by PLL-\textit{g}-PEG (Table 3, Figure 4.5). Exposure of the bilayers to air invariably led to their destruction by the copolymer. These observations suggest that defects play a crucial role in the disruption process. This is once again consistent with the observation that liposomes, which essentially lack defects if prepared from fluid-phase lipids, are resistant to decomposition by the copolymer (Figure 4.7i-l).
Figure 4.8 Interpretation of the bilayer disruption mechanism.
Upon adsorption of the copolymer to a flat surface of an SPB, the PEG chains are compressed. This process is entropically costly, and the free energy of the system is lowered by bending the bilayer and lifting it off of the underlying substrate. The process involves bilayer rupture and is therefore thought to nucleate at pre-existing defects or be facilitated by osmotic and electrical gradients that destabilise the bilayer. When the disruption process is completed, most of the lipids are floating in solution in the form of worm-like lipid-PLL-g-PEG complexes. The copolymer also coats the SiO$_2$ substrate with the remains of bilayer.

The role of defects in the disruption process can be understood if one considers that destruction of the bilayer by any mechanism, including the one outlined above (Figure 4.8), must invariably proceed via bilayer rupture. This is a thermally activated process.$^{268}$ A pre-existing defect will therefore serve as an efficient nucleation centre for the disruption by lowering the activation energy required for the disruption process to commence. As to the source of the defects, some authors have argued that preparing a defect-free SPB by a vesicle fusion procedure is virtually impossible.$^{9,54,82,269}$ Furthermore, buffer exchanges, inherent in the experimental procedure used here, may also have contributed to the formation of defects in the SPB that are below the detection limit of the QCM-D as well as to the destabilisation of the SPB in general: Diederich et al.$^{138}$ have observed that black lipid membranes were more sensitive to destabilisation by PLL in the presence of only 10 mM salt than in the presence of 100 mM salt. Additionally, the exchange of buffers on one side of the bilayer only, may induce
osmotic and electrical gradients across the bilayer.

It is interesting to note that SPBs prepared on QCM-D crystals were invariably disrupted by PLL-g-PEG added in H1 buffer, suggesting that their quality was not as good as of those prepared on SiO$_2$-coated glass slides. Yet it has previously been shown that SPBs prepared on QCM-D crystals are quite effective in preventing non-specific adsorption of proteins to surfaces they cover.$^{53}$ The contradiction between this finding and our result is only apparent, however, because to initiate the disruption process, the defects need not be either large or numerous$^{138}$ – they can be quite small (pinholes); too small for the proteins to adsorb or for the adsorption of a small amount of proteins to be detected. In fact, neither the mobile fraction nor the diffusion coefficient of the fluorescently labelled phosphatidylcholine could be used to predict the behaviour of the bilayers with respect to disruption by PLL-g-PEG (Table 4.5). Large defects are expected to affect both properties. Furthermore, non-specific protein adsorption to the defects in the bilayer is a self-limiting process (no more protein adsorbs once the defects are filled), while the decomposition by PLL-g-PEG is a self-catalytic one (removal of a portion of the bilayer creates a nucleation point for further removal).

The stability of the bilayers to disruption by PLL-g-PEG in high ionic strength buffers (EDTA buffer and Ca$^{2+}$ buffer) can be explained in terms of improved overall bilayer stability in the presence of salt, different conformation of the polymer backbone at a high ionic strength, smaller amount of polymer adsorbed to the surface, or a combination of these factors. Currently it is not possible to differentiate between them. The fact that the copolymer was observed to interact with the bilayers at high ionic strength at all (Figure 4.6) is consistent with the previously published accounts of polymer adsorption to liposomes in the presence of electrolytes.$^{142}$ There is some controversy in the literature with respect to
the binding of ionic polymers to bilayers composed of zwitterionic lipids, such as dimyristoyl phosphatidyl choline (DMPC).\textsuperscript{135} Here we clearly observed disruption of DOPC SPBs by PLL-g-PEG in a low ionic strength buffer (Figure 4.7, a-d) and some adsorption in a high-ionic strength one. This phenomenon has likely the same cause as the adsorption of divalent cations to DOPC bilayers.\textsuperscript{270,271}

The effect of Ca\textsuperscript{2+} on the polymer–SPB interactions deserves special attention. In the case of DOPC bilayers, no interaction between the copolymer and the SPB was observed in the presence of Ca\textsuperscript{2+}, while some adsorption could be detected in its absence (Figure 4.4). Ca\textsuperscript{2+} is known to bind to PC bilayers,\textsuperscript{246,270,271} causing them to become slightly positively charged. Thus the positively charged PLL-g-PEG is expected to be repelled from the SPB under these circumstances. While Ca\textsuperscript{2+} binds to the PS-containing bilayers to a much greater extent than to a purely PC-one, it does not induce a positive charge, but rather ties up phosphatidyl serines in Ca\textsuperscript{2+}(PS)\textsubscript{2} complexes.\textsuperscript{225,232} Binding of a macro-cation, such as PLL-g-PEG, will release the bound Ca\textsuperscript{2+} ions and lead to the dissociation of the DOPS dimers—both entropically favourable processes. The binding is reversible, and upon rinsing with pure buffer, when the concentration of the copolymer in the subphase drops to zero (i.e., the equilibrium between adsorbed and bound PLL-g-PEG is shifted) Ca\textsuperscript{2+} replaces the copolymer again (Fig. 4.4 A).

\section*{4.2.3 Summary and Conclusions}

We have shown that the graft copolymer PLL-g-PEG disrupts supported phospholipid bilayers in a low ionic strength buffer, but not in a high ionic strength one. The copolymer architecture was found to play a significant role in the disruption of the bilayers. This is attributed to the entropic cost associated with compressing the PEG side-chains of the copolymer when it adsorbs to the planar surface of an SPB. The free energy of the system is lowered by inducing curva-
ture in the underlying bilayer, which leads to the removal of the latter from the surface. This process appears to be initiated at the defects present in the SPBs, and indeed preparation methods that improve the quality of the SPBs led to a higher incidence of bilayers resistant to disruption by PLL-g-PEG, even in low ionic strength buffers. Surface-bound phospholipid vesicles were not decomposed by PLL-g-PEG under the same conditions. This observation supports the proposed disruption mechanism and illustrates the significance of the subtle differences between the two systems (supported bilayers and vesicles).

4.3 Interaction of PLL-g-PEG with Unilamellar and Multilamellar Vesicles in Solution

PLL-g-PEG was found to disrupt SPBs in low ionic strength buffer, while supported vesicular layers remained intact (see Section 4.2). It was proposed that the curved surface of the vesicles allows the polymer to accommodate its PEG side chains in a more relaxed conformation than on a flat surface. This explanation is tested in the present work by investigating the stability of vesicles suspensions against decomposition induced by the polymer. The curvature of the investigated vesicles was varied by varying their size from 50 nm (highly curved membrane) to several microns (fairly flat membrane).

4.3.1 Results

4.3.1.1 Stability of Extruded Unilamellar Vesicles against PLL-g-PEG

The size distribution of vesicles suspensions of different compositions and nominal sizes was evaluated by light scattering, before and after exposure to
PLL-g-PEG (Table 4.6). For all samples measured in Ca\textsuperscript{2+} buffer, as well as for the DOPC vesicles measured in H1 buffer, the mean sizes determined after interaction with PLL-g-PEG remained within 2 nm of those measured before exposure to the copolymer. DOPC:DOPS 90:10 vesicles measured in H1 buffer were on average 5 nm larger after interacting with PLL-g-PEG, independently from the original size.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
<th>50 nm</th>
<th>100 nm</th>
<th>200 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsuperscript{2+} buffer</td>
<td>DOPC</td>
<td>65 ± 7</td>
<td>66 ± 8</td>
<td>95 ± 11</td>
</tr>
<tr>
<td></td>
<td>DOPC:DOPS 95:5</td>
<td>65 ± 8</td>
<td>65 ± 9</td>
<td>94 ± 11</td>
</tr>
<tr>
<td></td>
<td>DOPC:DOPS 90:10</td>
<td>58 ± 7</td>
<td>59 ± 7</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>H1 buffer</td>
<td>DOPC</td>
<td>60 ± 6</td>
<td>61 ± 6</td>
<td>97 ± 9</td>
</tr>
<tr>
<td></td>
<td>DOPC:DOPS 90:10</td>
<td>68 ± 7</td>
<td>72 ± 7</td>
<td>103 ± 10</td>
</tr>
</tbody>
</table>

The shape of the size distributions remained constant and, in particular, additional peaks at either larger or smaller sizes were not observed (these would appear if the polymer caused, for example, formation of large aggregates or disruption of large vesicles into smaller ones).

The results obtained by light scattering were confirmed by transmission electron microscopy (TEM, Figure 4.9): the size and shape of 50 nm DOPC:DOPS 90:10 vesicles suspended in H1 buffer remained unchanged after exposure to PLL-g-PEG, indicating that disruption of the vesicles did not take place (the polymer could not be visualised by this technique because of an insufficient contrast compared to water).
Figure 4.9  TEM images of DOPC:DOPS 90:10 vesicles of 50 nm diameter in H1 buffer before (a) and after (b) exposing them to PLL-g-PEG. The polymer is not visible with this technique, as it does not have a sufficient contrast compared to water. However, the comparison of the two images confirms that the vesicles are not disrupted by PLL-g-PEG (size, shape, and size distribution have remained identical).

Stability of vesicles against leakage induced by PLL-g-PEG

Pore formation is a form of destabilisation of lipidic membranes that does not necessarily imply a change in the size or shape of the affected vesicle. This effect is therefore not resolved by a technique such as light scattering, but can be detected by observing the leakage of a quenched dye encapsulated in the vesicle interior (pores could in principle be visible by TEM, but only if they are stable long enough (in the order of seconds)). Leakage studies were therefore carried out to further assess the stability of vesicles interacting with PLL-g-PEG. Vesicles of 50 nm diameter and with 10% charge (DOPC:DOPS 90:10) were found to be completely stable when exposed to PLL-g-PEG (Figure 4.10a). However, if the size of the vesicle was increased to 400 nm, a steady leakage was detected (Figure 4.10b), indicating a size dependence (and therefore a dependence on the curvature) of the membrane disruption induced by the copolymer. The influence of the vesicle charge on the vesicle stability was investigated by using 100 nm vesicles that contained 20% of charged lipids (composition: DOPC:DOPS:DOPE-b 8:1:1). In this case some leakage was detected as well,
which, however appeared to stop within a few minutes (Figure 4.10c).

**Figure 4.10** Fluorescence intensity as a function of time for vesicles that encapsulate quenched carboxy fluorescein (20 mM). The principle of this technique bases on the fact that when a concentrated, quenched dye is diluted (i.e. released from inside a vesicle into the bulk solution) the quenching is suppressed and the measured fluorescence intensity increases. The experiments started by acquiring a baseline, after which PLL-g-PEG was added to the vesicles suspension (1). The decrease of the intensity observed for the 50 nm DOPC:DOPS 90:10 vesicles (a) is due to the dilution resulting from adding a small amount of polymer solution. This decrease is not visible for the larger (400 nm DOPC:DOPS 90:10, (b)) and more charged vesicles (100 nm, DOPC:DOPS:DOPE-b 80:10:10 (c)), because it is overcompensated by the intensity increase due to leakage.

(a) The constant fluorescence intensity indicates that 50 nm DOPC:DOPS 9:1 vesicles are stable in the presence of PLL-g-PEG  
(b) Leakage of 400 nm DOPC:DOPS 9:1 vesicles is visible. The leakage rate is constant over time, and the leakage does not stop after several minutes.  
(c) After an initial increase of the fluorescence intensity at the moment of PLL-g-PEG addition, the curve for 100 nm DOPC:DOPS:DOPE-b 80:10:10 vesicles levels off, indicating that the leakage stops.  

At the end of every experiment, the vesicles were destroyed by adding the concentrated surfactant Triton-X-100 (2), which induces an immediate release of all encapsulated dye.

### 4.3.1.2 Stability of Multilamellar Vesicles against Disruption by PLL-g-PEG

The outer membrane of multilamellar vesicles (MLVs) is rather flat, due to the large size of these objects (in the micron range). MLVs were therefore chosen to further study the influence of the membrane curvature on the interactions
between PLL-g-PEG and lipidic systems. MLVs have also the advantage that they can be sedimented by centrifugation, so that after coating them with PLL-g-PEG, the excess polymer can be easily removed by centrifugation and redispersion of the pellet in polymer-free buffer.

Figure 4.11 TRITC-PE labelled multilamellar vesicles (red) containing 10% DOPS were exposed to fluorescein-labelled PLL-g-PEG (green) in H1 buffer for 15 minutes. The excess polymer was removed by centrifugation and resuspension of the pellet in fresh buffer prior to observation.

(a)-(c) The images show the co-localisation of lipids and PLL-g-PEG, indicating that the MLVs are coated with the polymer. A worm-like structure, composed of both lipids and PLL-g-PEG is observed to extend from the multilamellar vesicle.

(d),(e) Enlarged images of the contours of the MLV. Smaller objects are seen to be coupled to the surface and be wrapped within the polymer coating. Oligo-(uni-)lamellar structures that appear to be budding vesicles are observed as well.

(f) Overview showing several elongated worm-like structures connecting some of the MLVs.

Fluorescently labelled DOPC:DOPS 90:10 multilamellar vesicles (red) that were exposed to PLL-g-PEG (labelled in green) in H1 buffer were found to be
coated by the polymer (see the co-localisation of the red and green labels in Figure 4.11). Several additional effects of the polymer on the MLVs were observed as well: first of all, the same worm-like structures that arise from the disruption of supported bilayers (see Section 4.2) were found to appear here as well. However, in this case they were less numerous and most of the vesicles still appeared, at least macroscopically, intact. A closer inspection of the contours of coated MLVs revealed the presence of smaller spherical objects, that were either in contact with the larger vesicle, or at a small distance, but wrapped inside the same polymer layer as the large vesicle (Figure 4.11d,e). These findings point to a partial disruption of the MLVs as a consequence of the interaction with PLL-g-PEG.

4.3.2 Discussion

The results obtained in this section confirm that the membrane curvature plays a fundamental role when it comes to the stability of lipidic systems against the polyelectrolyte PLL-g-PEG in a low ionic strength buffer, as was suggested in Section 4.2.2. At one end of the spectrum one finds supported bilayers, that are flat and completely disrupted by PLL-g-PEG (Figure 4.7a-h and Figure 4.8). At the other end are 50 nm vesicles, that are highly curved and fully stable in presence of the polymer (Figure 4.9 and Figure 4.10a). Between these two situations there are 400 nm vesicles, that are partially destabilised (i.e. they leak, Figure 4.10b), but still appear macroscopically intact (Figure 4.7i-l) and MLVs, that are more severely damaged, in a similar way as SPBs (cf. Figure 4.7a-h and Figure 4.11). The fact that MLVs are nevertheless not completely disrupted by PLL-g-PEG and macroscopically intact vesicles co-exist with worm-like structures may be due to the large excess of lipids compared to the amount of polymer: in order to completely disrupt a MLV, the polymer must disrupt (peel off) several lamellae. When the excess of polymer available is used up, the disruption process...
stops, and a mixture of "peeled" intact MLVs and disrupted lamellae is observed.

An additional phenomenon that was observed is the transient leakage of 100 nm vesicles containing 20% of negatively charged lipids (Figure 4.10b). Given the high curvature of the membrane for vesicles of this size, a decomposition following the mechanism described above is not expected. The amount of charged lipids is more likely to be the determinant factor in this case: as it was shown by Yaroslavov et al., the adsorption of a polyelectrolyte to oppositely charged vesicles induces flip-flop in the membrane. If the amount of charged lipids is higher than 20-30%, then the vesicles are disrupted as a consequence of excessive flip-flop. In our case, the amount of charge is not sufficient to cause complete disruption, but sufficient to destabilise the vesicle membrane and cause leakage.

4.3.3 Summary and Conclusions

The findings of this Section confirmed that the curvature of the lipidic membrane plays an important role in the interactions between PLL-g-PEG and lipidic systems: small vesicles with a high curvature allow the adsorbing PLL-g-PEG to accommodate the PEG chains in a relaxed conformation, and therefore no perturbation of the lipidic membrane occurs. As the size of the vesicles increases, there is a gradual decrease in the curvature of the membranes and the likelihood of vesicle disruption increases accordingly. This is consistent with the interpretation proposed in Section 4.2 for the disruption mechanism of supported bilayers exposed to PLL-g-PEG (the PLL backbone bends in order relax the compressive stress of the PEG side chains). The amount of charge in the membrane was also found to be a source of instability of vesicles in contact with PLL-g-PEG, due to flip-flop of the charged lipids induced by the oppositely charged polymer.
4.4 **Enzymatic Degradation of Liposomes Coated with PLL-g-PEG**

Small vesicles with a highly curved membrane were shown to be resistant to disruption by PLL-g-PEG. Given the particular properties of this polymer (it bears highly hydrated, densely packed PEG side chains that render it protein resistant), the question arises if a PLL-g-PEG coating would protect vesicles from being decomposed by enzymes, detergents, or solvents. In fact, it was shown by Müller *et al.* that the adsorption of proteins on the surface of PLGA microspheres was dramatically reduced by coating them with PLL-g-PEG. In this Section, the effectiveness of a PLL-g-PEG coating in protecting vesicles from the membrane-active enzyme phospholipase A₂ (PLA₂) is therefore evaluated.

4.4.1 Results

4.4.1.1 Enzymatic Degradation of Lipidic Vesicles Investigated by QCM-D

Biotinylated vesicles were immobilised on the surface of Nb₂O₅-coated quartz crystals *via* the so-called docking sites method (Figure 4.12). The immobilised vesicles were then coated with PLL-g-PEG and exposed to the enzyme phospholipase A₂ (PLA₂) from bee venom. Unprotected vesicles were used as a control (Figure 4.13).

PLA₂ specifically catalyses the hydrolysis of the ester linkage at the sn-2 position of the glycerol unit of PC lipids, leading to the formation of lysophospholipids and fatty acids (i.e. one of the two oleoyl "tails" of DOPC is removed). As a consequence of this reaction, a decrease of the mass sensed by QCM-D is expected, due to leakage of water from the vesicles and/or loss of the products of
the hydrolysis. A typical QCM-D curve confirming the loss of mass in the case of unprotected, bare liposomes is shown in Figure 4.13 (cf. also reference 273).

![Figure 4.12](image.png)

**Figure 4.12** Build-up of the docking sites interface. The first step (1) involves coating of a metal oxide surface with biotinylated PLL-g-PEG, followed by binding of streptavidin to the biotinylated sites (2). Biotinylated DOPC:DOPS:DOPE-b 8:1:1 vesicles of 100 nm diameter are then immobilised on the streptavidin layer (3). The vesicles can optionally be coated with a layer of PLL-g-PEG prior to further investigation (4).

Throughout this section the substrate is always Nb$_2$O$_5$, onto which PLL-g-PEG adsorbs more strongly than on other metal oxides.$^{156}$ The buffer used for this experiment was H1, and the percentage of biotinylated PEG chains 27.6%.

Standard experimental conditions for the results shown in this section, unless stated otherwise: composition of the polymeric interface: PLL-g-PEG/PPB30 95:5 (1.7% biotinylated), deposited at 0.5 mg/ml.; concentrations of the solutions used: streptavidin: 0.01 mg/ml, vesicles (100 nm diameter): 0.5 mg/ml, PLL-g-PEG for coating: 0.5 mg/ml.

Since both the vesicles used and PLA$_2$ are charged species, the ionic strength of the buffer might have an influence on the enzymatic degradation process. Therefore the enzymatic degradation of bare vesicles was performed at various ionic strengths. The results are compared in Figure 4.14: no differences due to the salt concentration in solution could be detected.
Interactions between Lipidic Systems and PLL-g-PEG

Figure 4.13 Enzymatic degradation of bare vesicles immobilised on a docking sites interface. When PLA₂ is added (at 50 µg/ml), a loss of mass from the surface is observed, that corresponds to loss of water from interior of the degraded vesicles and/or loss of the products of the hydrolysis.

Figure 4.14 Normalised frequency shifts for the degradation of bare vesicles by PLA₂. To allow for a better evaluation of the data, the frequency shift caused by the addition of PLA₂ was divided by the frequency shift evoked by vesicle adsorption (step 3 in Figure 4.12). No influence of the ionic strength of the buffer is apparent from the rates of degradation.
Degradation of PLL-g-PEG-coated vesicles by PLA₂.

The protective effect of PLL-g-PEG coatings on vesicles exposed to PLA₂ was tested under various conditions. Since the ionic strength of the buffer can influence the amount of polyelectrolyte adsorbed on the vesicle surface (thus affecting the effectiveness of the coating) the degradation of vesicles by the enzyme was studied both in low (H1) and high (H2) ionic strength buffers (Figure 4.15a). The results were compared with those obtained in the absence of coating shown in Figure 4.14. The influence of the molecular weight of PEG was investigated by using a PLL-g-PEG with PEG chains of 5 kDa instead of the standard 2 kDa (Figure 4.15b). The degradation rate of coated vesicles was found to be identical to the one of bare vesicles for all conditions tested. Therefore no protective effect of the polymers was present, independently of the ionic strength and PEG chain length.

Figure 4.15  Effect of the PLL-g-PEG coating on the enzymatic degradation of vesicles. 

a) Coating vesicles with PLL-g-PEG(2 kDa) does not show any improvement in their resistance against enzymatic degradation.
b) Increasing the PEG molecular weight to 5 kDa also does not improve the stability of the coated vesicles.

The short adsorption step that is observed immediately after the addition of the enzyme can be explained by the adsorption of the latter to the vesicle surface, before the onset of the degradation.
To ensure that the PLL-g-PEG used to coat the vesicles was indeed protein resistant and that it was not degraded by the enzyme, control experiments were performed by adding PLA$_2$ directly to a flat layer of PLL-g-PEG adsorbed on Nb$_2$O$_5$ in H1 buffer. No adsorption at all of PLA$_2$ was detected in this case (not shown), even though this positively charged enzyme was found to adsorb readily to the bare, negatively charged Nb$_2$O$_5$ surface ($\Delta F_3/3 = -24$ Hz).

4.4.1.2 Enzymatic Degradation and Leakage of Lipidic Vesicles Investigated by Fluorimetry

The ability of PLL-g-PEG to shield liposomes from enzymatic degradation was investigated in bulk solution by fluorimetry. The experiment consisted of monitoring the variation of fluorescence intensity after addition of PLL-g-PEG and PLA$_2$ to suspensions of vesicles loaded with a highly concentrated dye (the high concentration causes the dye to be quenched, so that the fluorescence inten-

![Figure 4.16](image)

**Figure 4.16** Fluorescence intensities arising from suspensions of bare and PLL-g-PEG-coated vesicles that encapsulate a quenched dye as they are exposed to PLA$_2$. After addition of the enzyme a fluorescence intensity decrease step is first observed, that is due to the dilution caused by the addition of the enzyme solution. Then the signal starts to increase steadily in both cases, indicating that the vesicles are leaking. Buffer used: H1; polymer: PLL-g-PEG(2).
sity arising from a suspension of intact, loaded vesicles is small). Decomposition of the vesicles causes the quenched dye to leak out in the bulk solution. The dilution results in de-quenching and an increase in the fluorescence intensity.

When the enzyme is added to both PLL-g-PEG(2)-coated and uncoated vesicles, a decrease in the fluorescence intensity is observed at first (Figure 4.16). This is due to dilution of the vesicles suspension by the enzyme-containing solution. However, in both cases the decrease is followed by a steady increase in fluorescence intensity that can be attributed to a continuous leakage of dye from the vesicles. A comparison of the two curves indicates that the slopes of intensity increase are identical, independently of the presence or absence of a PLL-g-PEG coating.

At the end of the experiments the surfactant Triton-X-100 was added to the solution provoking instantaneously a complete decomposition of the vesicles and the release of all encapsulated dye. This gives a reference value for the relative amount of dye that was released during the experiment.

It should be noted that when PLL-g-PEG was added to the samples in order to coat the vesicles, a small leakage was observed, that however stopped within a few minutes (Figure 4.16; for a detailed discussion of this phenomenon, see Section 4.3 above)

4.4.2 Discussion

It was found by two independent methods (QCM-D, Figure 4.13; and fluorimetry, Figure 4.16) that bare vesicles exposed to PLA$_2$ release their encapsulated content. Similar results obtained by QCM-D for surface-immobilised DOPC vesicles have been published recently.$^{273}$ The surprising result was that the enzyme was not prevented by a PLL-g-PEG coating from accessing the vesicle mem-
brane and degrading it (Figures 4.15 and 4.16). The effect was independent of the PEG molecular weight (2 or 5 kDa). This is in contrast with the results of Kaasgaard et al.\textsuperscript{274} that have shown how PEG can effectively prevent avidin from adsorbing on biotin receptors on the membrane of PEGylated liposomes. On the other hand, other authors\textsuperscript{275-280} have reported that the enzymatic degradation by PLA\textsubscript{2} shows a remarkable increase with increasing concentrations of PEGylated phosphatidyl ethanolamine lipids in the vesicle membrane. This behaviour has been associated with electrostatical interactions between the positively charged enzyme and the increased negative charge on the vesicle surface (the PEG modification renders the originally zwitterionic PE negatively charged).\textsuperscript{280} This interpretation might apply in our case as well, since the vesicles used contained 20\% of negatively charged lipids (DOPS and DOPE-b). One has also to keep in mind, however, that even a very small number of PLA\textsubscript{2} molecules reaching the vesicle membrane will lead to the complete destruction of the liposome. This happens because the membrane-degrading action of the enzyme does not stop until all

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{penetration.png}
\caption{Penetration mechanism of the enzyme through the PEG layer.\newline
a) The enzyme reaches the vesicle by diffusing through the PEG chains.\newline
b) The enzyme reaches the vesicle at defective sites in the coating.}
\end{figure}


Lipids are used up. This particularity of the enzyme/phospholipid system renders it more sensitive to small amounts of molecules passing the barrier of the PLL-g-PEG coating (compared for example to the biotin/avidin system\textsuperscript{ii}), offering a powerful method for testing the protective properties of PLL-g-PEG.

In order to reach the vesicle membrane, the enzyme has to go past the polymer coating (which is not expected to be degraded by the enzyme, since the polymer lacks ester linkages). The penetration of small proteins through a brush of PEG chains (Figure 4.17a) has been both predicted theoretically and observed experimentally.\textsuperscript{281,282} This mechanism was found not to apply in the case of flat PLL-g-PEG coatings on Nb\textsubscript{2}O\textsubscript{5}, where no adsorption of the enzyme was observed. Liposomes, however, have a curved surface, which significantly influences the density of the grafted PEG chains in the PLL-g-PEG coating (Figure 4.18). In Sections 4.2 and 4.3 it was shown how the density of the PEG side chains and the curvature of the lipidic membrane are the crucial parameters that govern both bilayer disruption and protein resistance. From that, it follows that the conformation of PLL-g-PEG on the surface of vesicles that are not disrupted by it is such, that the resulting PEG chain density may be too low to provide protein resistance. If the density of PEG chains was still sufficiently high for protein resistance to persist, the vesicles would be disrupted in the same way as the flat bilayers (i.e. through a change in the conformation of the polymer that allows it to accomodate

\textbf{Figure 4.18} Spacing between the PEG side chains on a flat surface compared to the spacing between the PEG chains on a curved surface.

\textsuperscript{ii.} In this case the binding of a molecule (i.e. avidin) on the receptor (i.e. biotin) occupies the site and prevents further molecules from binding as well.
the PEG chains in a more relaxed fashion). Therefore, this low PEG density will have the consequence that the PLA\textsubscript{2} molecules can penetrate more easily the polymer coating and reach the vesicle membrane.

A further mechanism that allows PLA\textsubscript{2} to degrade coated liposomes may consist of PLL-g-PEG sequestering all available negatively charged lipids in patches on the vesicle membrane, leaving the remainder of the vesicle uncoated. Adsorption of a number of PLL-g-PEG molecules that is insufficient to completely coat the vesicle surface—thus leaving uncoated patches—may also be a factor allowing the enzyme to reach the surface at these particular sites (Figure 4.17b).

Elbert et al.\textsuperscript{161} showed that it is possible to obtain a protein resistant PLL-g-PEG coating on fluid lipidic membranes, such as those of red blood cells. The authors point out that an appropriate PLL molecular weight and grafting ratio had to be selected. A successful protection against non-specific protein adsorption was achieved using PLL-g-PEG with a very high molecular weight (i.e. PLL(375)-g[5.6 or 7]-PEG(5)), whereas no protective effect was observed with PLL(20)-g-PEG coatings. The latter result is in agreement with our observations for liposomes. The effect of PLL-g-PEG with a high molecular weight backbone on the protection of liposomes is however not predictable, because of differences between cells and vesicles, such as the size and the presence, in cells, of a cytoskeleton that reduces the thermal fluctuations of cell membranes compared to the membrane of liposomes.

On the other hand, Müller et al.\textsuperscript{170} successfully reduced protein adsorption on micrometer-sized PLGA microspheres by adsorbing PLL(20)-g[3.3]-PEG(2) onto their surface, that is by using the same type of polymer used in the present work. The difference between these results and what we observed here are probably due to the different properties of the two systems, i.e. curvature—with the consequent difference in PEG chain density (see above; the size of the PLGA
microspheres is in the micron range)—and stiffness.

4.4.3 Summary and Conclusions

The effectiveness of PLL-g-PEG at protecting liposomes from being degraded by the membrane active enzyme PLA$_2$ was evaluated under several experimental conditions. It was found that PLL-g-PEG offers no protection for the vesicles, independently of the ionic strength of the buffer and of the length of the PEG side chains (2 kDa or 5 kDa). On the other hand, adsorption of PLA$_2$ to flat Nb$_2$O$_5$ surfaces is suppressed completely by coating the metal oxide with PLL-g-PEG. We therefore concluded that the enzyme reaches the vesicle membrane either because the conformation of PLL-g-PEG on a highly curved surface renders it no more protein resistant, or because uncoated patches are present on the liposome surface, which allow the enzyme to reach the surface. The uncoated patches can be caused by thermal fluctuations of the lipidic membrane, by the lateral mobility of lipids (that can lead to sequestration of charged lipids in patches), or by an insufficient number of polymer molecules adsorbed on the vesicle surface. Since only very few enzyme molecules are sufficient to completely degrade a vesicle, the PLA$_2$/phospholipid system is a particularly sensitive tool for testing the protective properties of a coating such as PLL-g-PEG.

4.5 Non-Specific Adsorption of Negatively Charged Liposomes on PLL-g-PEG Layers

The use of negatively charged vesicles in combination with the docking sites immobilisation method$^{272}$ (Figure 4.12) revealed some unexpected results: non specific adsorption of vesicles on PLL-g-PEG was observed, despite the excellent protein resistant properties of this polymer. In this section this phenomenon is investigated in detail and possible causes are elucidated.
4.5.1 Results

The "docking sites" method combines the reliability of the well-known biotin/(strept)avidin system (one of the strongest non-covalent affinity interactions in nature) with the protein-repellent properties of PLL-g-PEG by introducing biotin functionalities on some of the PEG side chains. The resulting polymeric interface allows for the specific binding of streptavidin at the modified sites, while preventing non-specific adsorption to the background. After loading this interface with streptavidin, biotinylated objects (such as vesicles) can be immobilised.

The build-up of the interface on a QCM-D crystal shown schematically in Figure 4.12 was performed using a polymer containing 27.6% of biotinylated PEG chains (i.e., every third chain) for the first step. For certain applications, this density of biotinylated sites at the surface may be too high, for example when the investigation of isolated, spread out objects is aimed at. In order to increase the distance between biotinylated islands, the biotinylated PLL-g-PEG molecules (from now on abbreviated as "PPB30") were diluted by mixing them with standard PLL-g-PEG in a ratio of 95:5. For the polymer batches used in this study, this results in 1.7% of functionalised PEG chains.

The reduction of the density of the biotinylated PEG chains in the first step of the interface build-up lead to an unexpected result at the moment of coating of the immobilised vesicles (Figure 4.19, black arrow): instead of a frequency shift downwards indicating adsorption (see Figure 4.12), a frequency shift indicating desorption was observed. Possible explanations for such a loss of material are (i) removal of non-specifically bound vesicles by the polymer; (ii) disruption of the vesicles by the polymer, with consequent loss of the encapsulated water; or (iii) replacement of the whole biotinylated PLL-g-PEG + streptavidin + vesicles.
interface by the added plain PLL-g-PEG. Explanation (ii) is unlikely, because vesicles of this size (100 nm) were observed to be resistant to the interaction with PLL-g-PEG (Figure 4.10). Explanation (iii) is in principle possible, but is invalidated by fluorescence microscopy results, which show that vesicles are still present on the surface after addition of the polymer (Figure 4.20). Explanation (i) is therefore the most likely one, and was investigated further by adding negatively charged, biotinylated vesicles directly to PLL-g-PEG (that is without first loading the interface with streptavidin).

Table 4.7 lists the frequency and dissipation shifts obtained from the non-specific adsorption of negatively charged vesicles to PLL-g-PEG in two different buffers: the low ionic strength H1 buffer and the high ionic strength H2 buffer. It clearly shows how DOPC:DOPS:DOPE-b 8:1:1 vesicles do adsorb on PLL-g-PEG even in the absence of the specific biotin/streptavidin interaction. The vesicles can not be removed by simply rinsing with the same buffer used for the
Visualisation of coated vesicles by fluorescence microscopy. Left: TRITC-PE labelled phospholipids. Middle: fluorescein labelled PLL-g-PEG. Right: superposition of both pictures. The presence of both the red and green signals shows that PLL-g-PEG is adsorbing to the immobilised vesicles. A close inspection of the superposition of both pictures reveals the co-localisation of lipids and PLL-g-PEG. (The vesicles used in this experiment had a size of 200 nm).

adsorption. The lower amount of vesicles adsorbed in H2 buffer points to the electrostatic nature of the interaction, which was confirmed by the fact that part of the vesicles adsorbed in H1 could be removed by a subsequent rinse with H2 buffer.

Introducing PLL-g-PEG on top of the non-specifically adsorbed vesicles causes removal of a large amount of them, especially in H1 buffer, where the electrostatic interactions are less screened (Figure 4.21). The removal of vesicles visible in Figure 4.21 reproduces the phenomenon observed in Figure 4.19 (black arrow), indicating that hypothesis (i) (see above) was the correct one.

Table 4.7 Frequency and dissipation shifts from QCM-D experiments in which DOPC:DOPS:DOPE-b 8:1:1 vesicles were added to a PLL-g-PEG layer either in H1 and H2 buffer, after rinsing with the same buffer in which the adsorption was carried out. In the case of adsorption in H1, exchanging the buffer to H2 for a rinse, and then returning to H1 buffer caused a removal of a significant part of the adsorbed vesicles.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>ΔF (Hz)</th>
<th>ΔD (10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in H2</td>
<td>-9.0</td>
<td>3.2</td>
</tr>
<tr>
<td>in H1</td>
<td>-44.9</td>
<td>14.0</td>
</tr>
<tr>
<td>in H1 after H2 rinse</td>
<td>-14.6</td>
<td>6.4</td>
</tr>
</tbody>
</table>
Figure 4.21 Non-specific adsorption of negatively charged liposomes (20% total charge) on a PLL-g-PEG layer in H1 and H2 buffers.

a) The vesicles are not removed by rinsing with buffer, but can be in great part displaced from the surface by adding PLL-g-PEG on top of them. The amount of non-specifically adsorbed vesicles in H2 buffer (high ionic strength) is significantly smaller.

b) Fluorescence image showing a packed layer of DOPC:DOPS:DOPE-b 8:1:1 vesicles of 200 nm size, adsorbed non-specifically on PLL-g-PEG in H1 buffer.

One can therefore conclude that when negatively charged vesicles are immobilised on a PLL-g-PEG-biotin/streptavidin interface, non-specific adsorption occurs to the background, additionally to the specific adsorption to the streptavidin sites. When a PLL-g-PEG coating is added, most of the non-specifically bound vesicles are removed and the surface then consists of specifically bound, coated vesicles, with some remainings of non-specifically bound, coated vesicles (Figure 4.20).

4.5.2 Discussion

The non-specific adsorption of vesicles on PLL-g-PEG layers formed on Nb₂O₅ was found to depend on the amount of negative charges in their membranes. Both purely zwitterionic vesicles,²⁴³ and vesicles that contained a low amount of negatively charged lipids (10% DPPE-biotin) suspended in a high
ionic strength buffer$^{272}$ did not show non-specific adsorption in studies on the controlled immobilisation of vesicles on homogeneous and patterned PLL-g-PEG/PEG-biotin interfaces.$^{243,272}$ This is consistent with QCM-D experiments we performed that revealed no adsorption when 100 nm vesicles containing 10% DOPS were added to PLL-g-PEG in H2 buffer (not shown). Increasing the total amount of charged lipids from 10 to 20% (that is, 10% DOPE-b + 10% DOPS) caused a small amount of vesicles to adsorb on PLL-g-PEG in H2, and an even higher amount of them to adsorb in H1 (Table 4.7). This indicates that negatively charged vesicles interact electrostatically with PLL-g-PEG, most probably with the positively charged PLL backbone.

The Debye lengths, $1/\kappa$, at 10 mM (H1) and 160 mM (H2) of salt are about 3 nm and 0.8 nm respectively. Comparing these numbers to the thickness of the PEG layer in PLL-g-PEG (of the order of about 7-8 nm$^{283}$), it follows that in H1 the separation of vesicles and PLL induced by PEG is of the order of twice the Debye length, allowing the electric double layers of the two species to slightly overlap, apparently resulting in a sufficient interaction energy (one has to keep in mind that the Debye length is defined as the distance from a charged surface at which the electrostatic potential has decreased to $1/e$ of the original value, but that a residual potential is present even past this length).

In the case of H2, the PEG layer thickness is much larger than the Debye length, but a slight adsorption of vesicles containing 20% of negatively charged lipids is observed nevertheless. A possible adsorption mechanism that was postulated for large proteins$^{281}$ involves a partial compression of the PEG layer exerted by an approaching vesicle, which would bring the vesicle and PLL close enough to start interacting. This mechanism is opposed by the steric repulsion exerted by the PEG chains and therefore only a very small amount of vesicles will adsorb (Table 4.7). However, once the vesicles have first adsorbed, the
charged lipids can diffuse laterally in the membrane towards the contact area and remain trapped there, in a similar situation to the TiO$_2$-Ca$^{2+}$-PS complexes reported in Section 3.3.4, providing a sufficient interaction energy to prevent the adsorbed vesicles to be removed by rinsing with buffer.

Most of the non-specifically adsorbed vesicles can however be removed by adding more PLL-g-PEG to the surface (Figure 4.21). This can be explained by a competition between the bulk PLL-g-PEG with the surface-adsorbed PLL-g-PEG for the negative charges on the vesicles. Since the PLL backbone of the bulk polymer is directly accessible (i.e. not sequestered on the metal oxide surface and screened by a layer of PEG brushes) the resulting interaction is much stronger and the vesicles are coated by the PLL-g-PEG in solution. At this point the negative charges of the lipids are compensated by the positive ones of the coating and therefore no interaction can persist with the surface-bound polymer. As a result, the non-specifically adsorbed vesicles desorb from the surface.

### 4.5.3 Summary and Conclusions

We have shown that the ability of the graft copolymer PLL-g-PEG to prevent non-specific adsorption on the surfaces it is adsorbed to can be impaired when highly negatively charged systems are used, such as vesicles containing 20% of negatively charged lipids. The interaction is of electrostatic nature, since the amount of non-specifically adsorbed vesicles was greatly reduced if the ionic strength of the system was increased. We concluded from our results that the negative charges on the vesicles were able to interact with the positive charges on the PLL backbone of the polymer, probably by compressing the PEG chains in order maximise the electrostatic interaction. Finally, competition between surface-adsorbed and bulk PLL-g-PEG for the negatively charged lipids caused most of the non-specifically bound vesicles to be removed from the surface at the moment of coating of the immobilised vesicles.
The results presented in Chapter 3 demonstrate that calcium ions play an important role in the interactions between TiO₂ and negatively charged liposomes. In particular, Ca²⁺ was found to both lower the energy barrier that prevented liposomes from adsorbing, and to mediate specific interactions between the oxide surface and the phosphatidyl serine present in the vesicle membrane. For vesicle compositions at which SPB formation occurred (i.e. at more than 20% negative charge), the negatively charged DOPS in the resulting bilayers was found to be immobilised on the surface in TiO₂-Ca²⁺-PS-complexes. These results were considered as an indirect proof that Ca²⁺ adsorbs on the TiO₂ surface. Others have reached a similar conclusion before.¹¹⁴-¹¹⁶ It is worth noting that references to relevant studies on ion binding in the field of colloidal suspensions¹¹⁴,¹¹⁵ are rarely found when the interactions between biomolecules and TiO₂ are considered. In the review on biological surface science by
Kasemo\textsuperscript{46} the effect of ions at surfaces is discussed in a more general way, namely in the light of the influence that hydrated ions have on the water structure at surfaces. The author argues that the orientation of water molecules in the water shell surrounding a solid surface may be a factor in determining if, for example, an adsorbing protein will be denatured or not.

To investigate these issues further, the effect of Ca\textsuperscript{2+} on the properties of the TiO\textsubscript{2} surface was investigated by measuring the $\zeta$-potential of anatase colloidal particles through electrophoresis. The experimental details are contained in Section 2.6.5.

**A.2 RESULTS AND DISCUSSION**

Binding of cations to a surface is known to shift the isoelectric point (IEP) of the latter to higher pH (conversely, the IEP is shifted to lower pH when anions adsorb).\textsuperscript{212} Thus, determining the IEP of a given surface as a function of solution conditions allows for the detection of surface-binding ions. The adsorption behaviour of Ca\textsuperscript{2+} on TiO\textsubscript{2} was investigated by recording $\zeta$-potential curves as a function of pH at various ionic strengths. The IEPs observed were then compared relatively to each other and with the values reported in the literature (Figure A.1).

The IEPs of our colloidal suspensions of anatase\textsuperscript{1} in the presence of 1 mM and 5 mM CaCl\textsubscript{2} were found to be at about pH = 5.6 and pH = 6.0, respectively (Figure A.1a). The higher IEP at a higher concentration of Ca\textsuperscript{2+} is consistent with binding of the cation. The particular shape of the $\zeta$-potential vs. pH curves that was observed (Figure A.1a) compares well with the results published by Kosmul-

\textsuperscript{i} The question whether it is reasonable to compare data obtained for rutile-TiO\textsubscript{2} with data obtained for anatase-TiO\textsubscript{2} was addressed by Kosmulski \textit{et al.} in an extensive review of the IEP data available in the literature.\textsuperscript{114} The average IEP for rutile in absence of specific binding of ions was found to be 5.4 and that for anatase 5.9. The difference between the two corresponds to half of the standard deviation, and is therefore not substantial.\textsuperscript{114}
ski et al.\textsuperscript{115} and is also consistent with the assumption that Ca\textsuperscript{2+} binds to the titania surface. In fact, such behaviour was reported before for the adsorption of hydrolysable cations (divalent and trivalent metal ions) to oxide surfaces.\textsuperscript{284-286}

Figure A.1 $\zeta$-potential curves as a function of pH for TiO\textsubscript{2} (anatase) colloids suspended in solutions containing various amounts of different salts.

a) Curves recorded in the presence of Ca\textsuperscript{2+} ions. The general shape of the curves—characterized by one IEP and one charge reversal point (CRS)—has been observed before for the adsorption of hydrolysable cations (such as calcium) on oxide surfaces.\textsuperscript{284-286} 

$\Delta$IEP(1): shift of IEP resulting from the presence of 1 mM Ca\textsuperscript{2+} in solution, compared to
samples prepared in 0.5 mM NaCl (data from this work).

\(\Delta \text{IEP}(2)\): shift of IEP between the data of Larson et al.\textsuperscript{113} in an indifferent electrolyte and the data of Kosmulski et al.\textsuperscript{115} in the presence of 1 mM Ca\(^{2+}\).

b) Curves recorded in the absence of Ca\(^{2+}\) ions. The shift to lower pH values of the IEP as a function of increasing NaCl concentration (black to red curves) is probably due to adsorption of chloride ions on the surface. The dashed red, green and black lines are just a guide for the eye in the region close to the IEP. Included in this graph is also the data for rutile colloids in the presence of 0.1 and 1.0 mM KNO\(_3\) by Larson et al.,\textsuperscript{113} showing that the IEPs are essentially independent of salt concentration.

Our IEP values are however somewhat smaller than the IEP of 6.5 reported in the work of Kosmulski et al. The difference might be explained by the different anions present in solution in the two cases: the salt we used was CaCl\(_2\), while Kosmulski et al.\textsuperscript{115} used Ca(NO\(_3\))\(_2\). NO\(_3^–\) ions are known to be indifferent (i.e. they do not adsorb specifically on the surface of TiO\(_2\)), whereas some adsorption of chloride ions to TiO\(_2\) has been reported before.\textsuperscript{288} Consequently, binding of Cl\(^–\) to TiO\(_2\) in the presence of Ca\(^{2+}\) might compensate for the shift to higher IEP resulting from the binding of Ca\(^{2+}\) to the surface. To test this hypothesis, the \(\zeta\)-potential as a function of pH for TiO\(_2\) particles suspended in NaCl solutions of different ionic strength was measured. The IEP was found to shift from 5.1 to 4.0 as the concentration of Cl\(^–\) ions was increased from 0.1 mM, to 10 mM (Figure A.1b), thus confirming our supposition.

Comparing the IEPs we obtained in the presence of NaCl and CaCl\(_2\) at about the same concentrations, one can see, despite the scatter of the data, that there is a shift of about 1 pH unit between the two sets of data. This shift can be attributed to the binding of Ca\(^{2+}\) to the TiO\(_2\) surface (see \(\Delta \text{IEP}(1)\) in Figure A.1). The same type of IEP shift is visible if the data obtained by Kosmulski et al.\textsuperscript{115} in the presence of 1 mM Ca(NO\(_3\))\(_2\) (IEP = 6.5) is compared with the data of Larson et al.\textsuperscript{113} obtained in the presence of 1 mM KNO\(_3\) (IEP = 5.6, see \(\Delta \text{IEP}(2)\) in Figure A.1).
Figure A.2  ζ-potential of anatase colloids as a function of CaCl₂ concentration, measured at pH 7.4 in the presence of 10 mM Hepes buffer. Reversal of the surface charge (from negative to positive) was observed at 3 mM Ca²⁺. Above 15 mM Ca²⁺ the z-potential levels off at a value of about +20 mV, indicating that the TiO₂ surface is saturated with divalent cations. The shape of the curve is logarithmic (dashed line).

Since all experiments involving the interactions of liposomes with TiO₂ were done at pH 7.4 (see Chapter 3) further investigations of the ζ-potential of TiO₂ colloidal particles at this pH were carried out. The focus was on the magnitude and sign of the ζ-potential in the presence of varying concentrations of CaCl₂ (Figure A.2). The pH was kept constant at 7.4 with 10 mM Hepes buffer.

The presence of Ca²⁺ ions was found to have a dramatic effect on the ζ-potential: in the absence of Ca²⁺ the ζ-potential at pH 7.4 is strongly negative. Small amounts of CaCl₂ diminish the magnitude of the potential, which reaches zero at a concentration between 2 and 3 mM Ca²⁺ (Figure A.2). At higher concentrations the sign of the ζ-potential becomes positive, indicating that a sufficient amount
of Ca\(^{2+}\) ions is present on the TiO\(_2\) surface to reverse its charge. Saturation of the \(\zeta\)-potential value was observed above 15 mM Ca\(^{2+}\).

The data point at 2 mM Ca\(^{2+}\) is of particular interest\(^{ii}\) (see Figure A.2), as it is the calcium concentration used for investigating the liposomes/TiO\(_2\) interactions (Chapter 3). The value of the \(\zeta\)-potential is still slightly negative, but very close to zero. A vesicle approaching the surface under these conditions will not be (strongly) repelled electrostatically by the oxide surface, and will adsorb. Considering that the surface potential of the vesicles is also reduced by Ca\(^{2+}\) (Figure 3.6), it is evident that the energy barrier of electrostatic origin that prevents vesicles from adsorbing to TiO\(_2\) in the absence of Ca\(^{2+}\) is greatly diminished, or eliminated, by the presence of the cation, as was proposed in Sections 3.3.2 and 3.3.3 (see also Figure 3.7). From the plots in Figure A.2 and 3.7, a reduction of the repulsive interaction potential of the order of ~40-50 mV can be estimated for vesicles containing 20% DOPS.

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\(^{ii}\) The data shown in Figure A.2 was acquired in the presence of various amounts of CaCl\(_2\) and 10 mM Hepes. The studies on liposome adsorption on TiO\(_2\) were carried out in the presence of 2 mM CaCl\(_2\), 10 mM Hepes and 100 mM NaCl. However, the additional 100 mM NaCl do not seem to influence the \(\zeta\)-potential at 2 mM CaCl\(_2\) (the absolute value was found to be < 5 mV both in the presence and absence of additional 100 mM NaCl)
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It is my pleasure to express my sincere gratitude to the many people who helped to make this thesis successful:

Firstly, I wish to express my special thanks to Prof. Dr. Marcus Textor, who made this work possible by giving me the opportunity to pursue a Ph.D. thesis at the BioInterface Group. Thank you for the freedom and support you gave me in my project and your patience during the times when the results just didn’t seem to make sense (yet).

Dr. Ilya Reviakine for being a great supervisor, who introduced me into the world of scientific research and taught me so many things ranging from how to approach scientific problems, to experimental tricks, to how to be accurate and precise in presenting one’s work. Спасибо for the continuous support and the many enlightening scientific discussions!

Prof. Dr. Fredrik Höök (Lund University, Sweden) and Prof. Dr. Viola Vogel (ETH Zurich) for enthusiastically accepting to co-referee this thesis and for the invaluable critical comments that helped make this work better.
Acknowledgements

Prof. Dr. Ludwig J. Gauckler for having accepted to chair my thesis defence.

Dr. Fabiano Assi for his support and all the scientific discussions we had throughout his stay at LSST.

Dr. Janos Vörös and Dr. Susan DePaul for all the suggestions, discussions and insightful comments. Susan, thank you also for always providing tips and tricks on how to fix the QCM-D even after you left the group.

Prof. Dr. Martien Cohen Stuart (University of Wageningen, The Netherlands) for helpful suggestions concerning the interactions between liposomes and poly-electrolytes.

Prof. Dr. Bengt Kasemo (Chalmers University, Sweden) and Dr. Alexander N. Morozov (Leiden University, The Netherlands) for insightful comments and stimulating discussions around the topics of vesicle-surface interactions and supported bilayer formation.

Dr. Roger Michel and Dr. Didier Falconnet for the production and characterisation of the SMAP samples.

Dr. Gabor Csucs for introducing me to fluorescence microscopy and helping putting together a FRAP set-up, and Prof. Dr. Edgar Stüssi for allowing me to use their confocal fluorescence microscope in Schlieren.

Dr. Stéphanie Pasche, Firat Durmaz, Dr. Ningping Huang and Dr. Marcus Müller for the synthesis and characterisation of the unlabelled PLL-g-PEG molecules used in this study. Dr. Jost Lussi and Dr. Didier Falconnet for providing fluorescently labelled PLL-g-PEG.

Dr. Kurosh Rezwan for his invaluable comments regarding the zeta potential data and for proofreading the Appendix of this thesis.

Guoliang Zhen and Dr. Eva Künemann for providing the GFP protein.

Michael Horisberger and the many LSST volunteers for coating substrates at PSI Villigen.
Prof. Dr. Antonella Rossi for proofreading the Italian version of the abstract.

Dr. Matthias Rudh and Hans Green at Q-Sense AB (Sweden) for the great technical support with the QCM-D instrument.

Dr. Samantha Benito, Dr. Corinne Vébert-Nardin, and Prof. Dr. Wolfgang Meier (University of Basel, Switzerland) for providing access to their light scattering and gel chromatography set-ups, and for helping with the evaluation of the data.

Dr. Roland Keir and Prof. Dr. Peter Schurtenberger (University of Fribourg, Switzerland) for giving me access to their light scattering instrument.

Dr. Stefanie Krämer (ETH Zurich) for letting me use the Zetasizer instrument.

Prof. Dr. Jeff Hubbell (ETH Zurich) for letting me use their fluorimeter and Dr. Ronald Schoenmakers for helping me with its operation.

It was a pleasure to supervise two students, Marta Bally and Karthik Kumar, during their semester and diploma work. By supporting and coordinating their work I have learned a lot, including how to better manage myself. Marta, special thanks to you for the big discovery you made during your outstanding semester thesis!

Finally, I would like to thank all group members at LSST/BiG for the nice working atmosphere and especially those of you who also got the "vesicle bug", for making me feel less exotic with my project.

Last but not least, I would like to thank my family for their support and encouragement over the years.

This work was financed by the Swiss Top Nano 21 program (KTI project # 5493-1 and 6348-1), the EU Framework Program 6 (Project “NANOCUES”), and the European Science Foundation EUROCORES Program “Self-Organized Nanostructures” (SONS, project “NanoSMAP”).
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