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# Dissolved metal ions and mineral-liposome hybrid systems: underlying interactions, synthesis, and characterization

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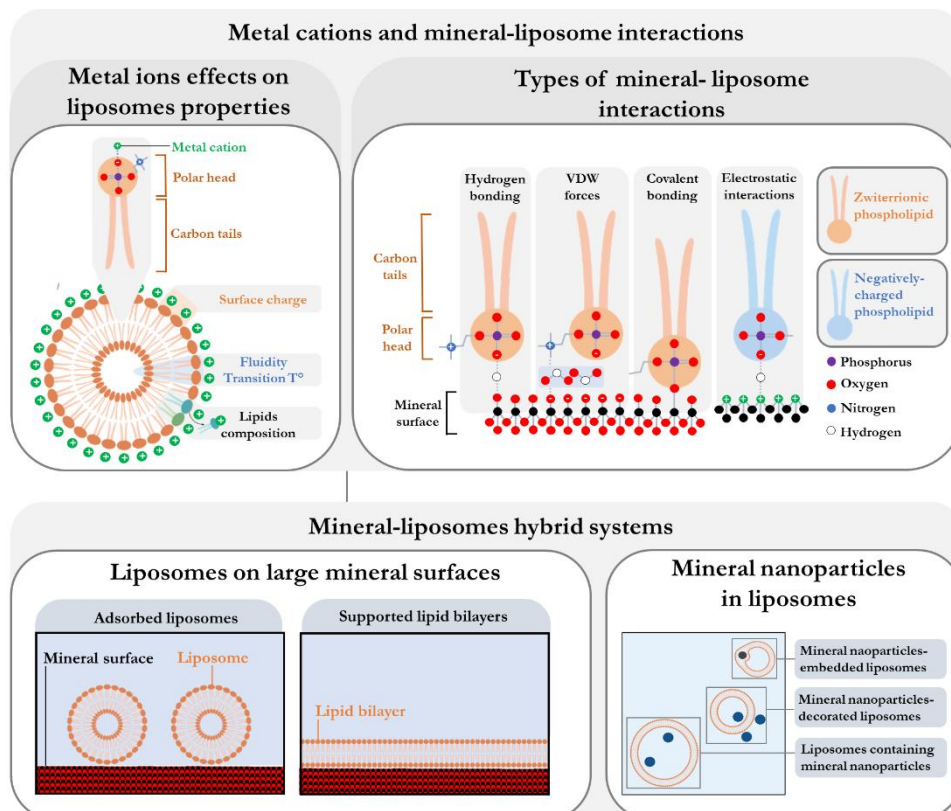
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## Highlights

- Liposomes properties are modified when interacting with dissolved ions and minerals
- Lipid-mineral interactions are broad and lipid and mineral type-specific
- Minerals may enhance liposomes with interesting physico-chemical properties
- Different protocols fit the synthesis of each type of mineral-liposome hybrid system
- Mineral-liposome systems characterization covers a wide range of methods

## Graphical abstract



## Abstract

**Liposomes are versatile lipid-based vesicles with interesting physicochemical properties, making them excellent candidates for interdisciplinary applications in the medicinal, biological, and environmental sciences. The synthesis of mineral-liposome hybrid systems lends normally inert vesicles with the catalytic, magnetic, electrical, and optical properties of the integrated mineral species. Such applications require an understanding of the physicochemical interactions between organic molecules and inorganic crystal structures. This review provides an overview on these interactions and details on synthesis and characterization methods for these systems.**

## 1 – Introduction

The separation of an external and internal environment is fundamental to biochemical metabolism, primarily maintained through the construction of lipid-based membranes found within cells. A simple lipid-based structure, the liposome is a lipid-bilayer based vesicle primarily consisting of phospholipids and fatty acids, constituting a versatile platform with interesting physicochemical properties; being highly tunable, controllable, and customizable, making them excellent candidates for a wide range of interdisciplinary applications.

Lipids are the basic amphiphilic unit constructing liposomes, made of a polar hydrophilic head varying in hydrodynamic size and charge, and one or two hydrophobic carbon tails varying in length and number of unsaturations [1,2]. As such, lipids spontaneously self-organize into lipid bilayer-based vesicles, with the hydrophobic carbon tails facing each other separating the two aqueous media on both sides of an hydrophobic core. The nature of the lipids organizing the membrane influences several liposomal properties: surface charge, fluidity, phase transitions temperatures, permeability, and size [3–6]. While these properties are tunable through lipid composition, pH and temperature [3–6] and enhance liposomes specific features such as its molecular carrying capacity, they are devoid of catalytic, magnetic, electric, and optical properties [7,8].

Minerals are naturally forming organized crystalline materials with a broad range of physicochemical properties applicable to medicine, industry, environmental science, as well as the emergence of life. Liposome behaviors are further modified through their interactions with their local environment, of note is the wealth of reactions between minerals and liposomes. Various minerals are known to integrate within membranes, modifying the chemical, magnetic, or reactive properties of the liposome as a result [7]. The highly malleable nature of liposomes combined to the wide range of capacities offered by mineral crystals allows one to imagine a myriad of hybrid mineral-liposomes systems, some of which are already used in the medical or environmental fields.

Mineral-liposome hybrid systems are extremely interesting in the medical field, both for diagnostic or therapeutic applications. Mineral nanoparticles can enhance the drug carrying duty of liposomes by repressing, initiating, or modulating their fusion in response to a stimulus (light, magnetic field, pH variation) [7]. Controlling stimuli avoids the premature release of the payload by increasing the carrying capacity of the liposomes or by increasing the vesicle properties such as fluorescence, magnetism, or surface charge [7]. Liposomal encasement allows mineral nanoparticles to be soluble in aqueous solutions, increasing their biocompatibility and preventing their aggregation within biological fluids, promoting their penetration into cells [7]. More specifically, (superparamagnetic) iron-oxide nanoparticles (SPIONs)-embedded liposomes, such as magnetite ( $\text{Fe}_3\text{O}_4$ )-embedded liposomes, are not only used as contrast agents for medical magnetic resonance

(MRI) [9] or magnetic particle imaging (MPI), but also for specific magnetic targeting of cancer tumors and payload release in response to specific stimuli [10] (temperature [11], electromagnetic heating [12], magnetic field [13], photothermal activation [14] or light [15]) leading to a significant increase in the accumulation of the liposome-contained drug near the tumor [13,14,16]. For instance, the controlled release of the content of 1,2-dipalmitoylphosphatidylcholine (DOPC) liposomes decorated with TiO<sub>2</sub> nanoparticles can be controlled through the photocatalytic activity of these oxides when exposed to UV-light [15].

Persistent pollutants, both anthropogenic and naturally occurring, threaten water resources and human health. Some minerals are known to display reactive behaviors towards organic contaminants [17] and heavy metals [18], and have been used to degrade or scavenge these pollutants within the aquifer themselves. The modification of liposomes using minerals for environmental purposes has been demonstrated with clay-liposomes formulations, consisting of pesticide-containing vesicles adsorbed onto clay surfaces (e.g. montmorillonite), and are used to prevent pesticide leakage in groundwater [19,20], thus reducing its contamination. The use of these versatile systems can be extended to the removal of pollutants and microorganisms from water and grounds, mainly through adsorption processes, depending on specific formulations [21,22]. The further modification of minerals with liposomes then offers potential benefits to environmental remediation efforts. For instance, complexes formed from the clay bentonite with didodecyltrimethyl-ammonium (DDAB) liposomes yielded a significant efficiency in removal from water of both herbicides (e.g. S-metolachlor, alachlor) and bacteria [22].

On the early Earth prior to the emergence of life (~4-3.8 Ga), minerals could have been the key structures and catalysts leading to the formation and energetic sustaining of the first cell-like biological systems [23–25]. Various minerals have been shown to catalyze lipid vesicle formation [23,26,27] and amino-acid synthesis [28], however their catalytic redox properties also make them interesting candidates as inorganic precursors of current bioenergetic electron transport chain metallo-enzymes [25,29], which allow to produce redox and chemical energy for most of the current biological organisms. Simple liposomes could have constituted the first cell-like systems in the form of vesicles whose formation was induced out of free lipids following contact with mineral surfaces [23,26]. The latter could also have played a role in the selection of those vesicles, by favorizing certain kind of liposomes rather than others. Lastly, liposomes naturally generate compartmentalized systems, separating an internal medium from the external environment, in which some minerals could be used to generate a bioenergetic-like transmembrane cation gradient [23]. The synthesis of such a system could be used to test some minerals ability to sustain protocells bioenergetic requirements.

The building of such systems requires a deep understanding of the physicochemical interactions underlying the relationship between minerals and lipids, both to understand their effects on the intrinsic properties of each component, to understand the potential of such a hybrid system, and to perform its synthesis. This review is written in the context of fundamental knowledge and instructions for building a never-before-seen mineral-liposome hybrid system, including key known interactions between these two components, methods for synthesis and characterization, and current applications. This review follows the key mineral and dissolved metal ions properties and their effects on the properties of and interaction with the liposome/lipid bilayer. First, the effect of metal ions on liposomes is described, as they may be implied in the hybrid system synthesis process, either in the case of a co-precipitation synthesis of the mineral from metal ion precursors, or in the

case of a release of metal ions from the minerals structure. Then, proper solid phase mineral-liposome interactions and hybrid systems are presented, followed by synthesis and characterization methods.

## 2 – Properties and consequences of metal ions-liposomes interactions

Many minerals contain metal ions which, when dissolved, have been shown to alter the surface properties of liposomes (e.g., charge, fluidity, permeability, lipid composition, and stability). These interactions are of importance in two specific cases: when minerals are synthesized through co-precipitation of metal ion precursors in presence of liposomes, which can affect the latter properties and when minerals of certain types release their metal ions in solution, thus impacting the liposomes involved in the mineral-liposome hybrid system. The effect of metal ions on these different liposomal parameters is well known and will be detailed here.

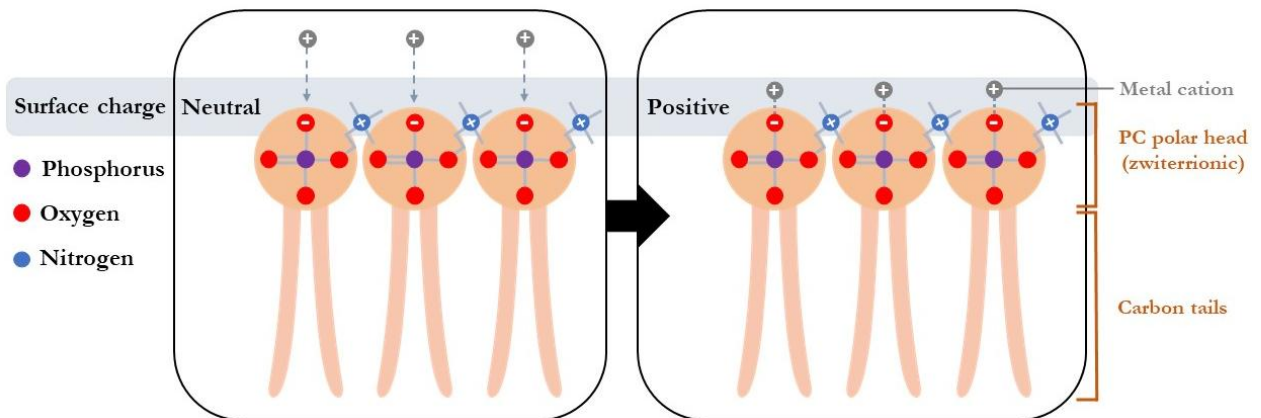
### 2.1 – Effect of metal ions on liposomal surface charge

Several studies [23,30–33] have shown the impact of dissolved metal cations on liposomes surface charge, claiming that this modification is due to the interaction of cations with lipids polar heads negatively charged sites, thus masking them at the liposome surface (**Figure 1**). This effect depends on lipids composition, defining the initial number of negatively charged sites, as well as pH, which influences the protonation of these sites and their accessibility to binding with cations.

Liposomes consisting of phosphatidylcholine (PC), a zwitterionic phospholipid bearing one positive and one negative charge on its polar head, have been studied when interacting with monovalent ( $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cs}^+$ )[30], divalent ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ )[33] and trivalent ( $\text{Al}^{3+}$ ,  $\text{In}^{3+}$ ,  $\text{La}^{3+}$ ) [31] metal cations solutions, across three different studies.

Electrophoretic mobility measurements on monovalent cation-containing liposomes suspensions displayed an increase in liposome surface charge alongside increasing cations concentrations as function of the pH of electrolyte, with surface charge densities shifting from negative to positive and increasing by  $+0,04 \text{ C/m}^2$  and  $+0,03 \text{ C/m}^2$  between pH2 and pH11 for  $155 \mu\text{M}$  of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cs}^+$  or  $\text{Li}^+$  cations solutions, respectively [30]. Similarly, with divalent cations, the zeta potential of PC liposomes measured by dynamic light scattering (DLS) increases from 0 to +6, +7, +10 and +11 Mv at 100 Mm concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$ , respectively, and then concomitantly decreasing to +5 mV at  $1 \mu\text{M}$  [33]. The same trend is again observed with trivalent metal cations, as zeta-potential increases from -10 mV at pH10 to +30-40 mV at pH6, for all cation types [31].

These results show a common trend of surface charge density increase in PC liposomes upon addition of all types of metal cations.



**Figure 1: Effect of metal cations binding to phosphatidylcholine (PC) lipids zwitterionic polar heads on overall liposomal membrane surface charge:** The binding of metal cations on phosphatidylcholine polar heads negatively charged sites, based on their electrostatic compatibility, leads to the masking of these negatives charges and increase of the overall membrane surface charge, going from neutral to positive.

## 2.2 – Effect of metal ions on membrane fluidity, transition temperature and liposomes size

Several studies have shown a synergic effect of divalent metal cations binding on both membrane fluidity, transition temperature and size of liposomes composed of phosphatidic acid (PA), phosphatidylserine (PS) or phosphatidylglycerol (PG) phospholipids bearing either saturated (-DM) or partly unsaturated (-PO) acyl chains.

### 2.2.1 – Metal ions electrostatic binding to liposomal membranes

A study [34] reveals that the binding of divalent metal cations (Ca, Mg, Zn) to DMPA liposomes induce the deprotonation of the DMPA headgroups as demonstrated by a decrease of pH with increasing cations concentrations, ranging from pH7 at 0 mM to pH4 at 10 mM cation concentration. Differential scanning calorimetry (DSC) shows that cation-lipid complexes formation resulting in the latter effect depends on the lipid:cation ratio and pH. Indeed, at pH 3-5, DMPA DSC heating curves pattern reveal only highly ordered 1:1 lipid:cation complexes upon addition of increasing cation concentrations. Meanwhile, at pH7, 2:1 lipid:cation complexes appear in addition to highly ordered 1:1 ones when decreasing the lipid:cation ratio in either  $\text{DMPA:Mg}^{2+}$ ,  $\text{DMPA:Ca}^{2+}$  or  $\text{DMPA:Sr}^{2+}$  complexes, as displayed by the splitting of the transition temperature (i.e. the temperature at which the lipid bilayer switches from one phase to another, for instance from gel to liquid phase) peak towards lower lipid:cation ratios. This is claimed to be due to the binding of cation either to single or double negatively charged DMPA, thus forming either 1:1 or 1:2 individual lipid:cations complexes across the membrane [34] revealing potential single or double deprotonation of the DMPA polar head depending on Ph and ionic strength, allowing electrostatic interactions with cations.

### 2.2.2 – Effect on membrane fluidity

Laudran generalized polarization (LGP) measurements, which determine membrane fluidity, were performed on either saturated (-DM) or partly unsaturated (-PO) negatively charged phosphatidic acid (PA), phosphatidyl-serine (PS) or phosphatidyl-glycerol (PG) liposomes above their respective gel-to-fluid phase transition temperatures. These analyses showed a progressive membrane rigidification of all liposome types upon addition of increasing  $\mu\text{M}$  concentrations of divalent metal cations ( $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) [32,35] as displayed by positive changes in

generalized polarization (GP) values, whereas little effect is observed with liposomes composed of zwitterionic phosphatidyl-choline (PC) lipids. In addition to being observed only in negatively-charged liposomes, revealing the importance of electrostatic interactions in the interplay between polar heads and cations, this effect also depends on both cation type and lipid acyl chain type (saturated or unsaturated). Indeed, it is observed that  $\text{Co}^{2+}$  has a stronger rigidifying effect than  $\text{Ni}^{2+}$ , displayed as higher  $\Delta\text{GP}$  values, on PA and PG liposomes, and  $\text{Mn}^{2+}$  has a stronger effect than  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on PA and PS liposomes, while all cations are bearing the same charge, expressing a cation-type dependent phenomenon. Furthermore, the acyl chains unsaturation level is also impactful, as membranes of fully saturated (-DM) lipids are more sensitive to the rigidifying effect compared to their partly unsaturated (-PO) homologs. For instance,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  cations have the same rigidifying effect on both DM- and PO-PS liposomes but higher effect on DMPA liposomes over POPA. Similarly, acyl chains differences do not impact  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  effect on PA liposomes, but PG and PS liposomes tend to rigidify more after addition of these cations in the case of fully saturated (-DM) lipids type

### 2.2.3 – Effect on transition temperature

Interestingly, transition temperature of liposomes (i.e. the temperature at which the lipid bilayer switches from one phase to another, for instance from gel to liquid phase) tends to increase alongside membrane rigidification. In the same 2018 study [32], Sule et al. claim, using Laurdan generalized polarization, that addition of both  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  tend to increase transition temperature in PA, PG or PS liposome bearing either saturated or partly unsaturated tails. For instance, an optimal effect is observed when 300  $\mu\text{M}$  of  $\text{Co}^{2+}$  or  $\text{Ni}^{2+}$  are added to DMPG liposomes and increase the transition temperature by 5,5°C and 1,5°C, respectively.

Accordingly, the previously mentioned study by Blume et al. showed that the binding of divalent metal cations (Ca, Mg, Zn) to DMPA liposomes modified their thermotropic phase behavior. For instance, at pH3-5, increasing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations to 1:80 DMPA:cation ratios increased transition temperature from 50°C to 60 and 65°C, respectively.

### 2.2.4 – Effect on liposome size

Following the same trend, Sule et al. [32] showed, using Dynamic Light Scattering (DLS) that liposome sizes change alongside membrane fluidity and transition temperature, upon metal cations addition. They demonstrated that addition of metal cations such as  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  led to an increase of their sizes from an average 137,7 nm to 736.7, 817.1 and 849.9 nm, respectively for  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  200  $\mu\text{M}$  cation solutions. Similarly, addition of 150  $\mu\text{M}$   $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  led to 80.4 and 97.3 nm sized DMPA liposomes, respectively, above 54.3 nm pure DMPA liposome size.

The interdependence between liposomes membrane fluidity, transition temperature and size can be explained by the fact that they are commonly influenced by the lipids molecular structure and headgroup charge [3]. Therefore, they depend on the initial lipid composition and the further binding of metal cations, as supported by the results of these studies.

## 2.3 – Effect of metal ions on lipids composition

While the effects of cations on liposomes surface charge, size, fluidity, and transition temperature are dependent on lipid composition, it has been claimed that they can also influence the latter since fatty acids tend to aggregate when divalent cations bind to their negatively charged carboxylic head group [23]. Indeed, the interaction of  $\text{Mg}^{2+}$  cations with phospholipid/fatty acid liposomes composed of a POPC/OA mix led to FA-deprived and PL-enriched membranes by the selective interaction of cations with negatively charged fatty acids rather than the zwitterionic phospholipids. At high concentrations,  $\text{Mg}^{2+}$  cations can then lead to liposomes destabilization, those

with the highest PL content being less sensitive, since POPC is more tolerant to cations, being neutrally charged (zwitterionic). Indeed, the  $Mg^{2+}$

concentration at which vesicles are destabilized increases from 5 Mm for pure FA vesicles to 20 Mm for OA/POPC mix at a 10:1 ratio, and reaches 50 Mm of 1:1 ratios. Similar observations were made with  $Mg^{2+}$  and  $Ca^{2+}$  binding to OA-DOH liposomes, with  $Ca^{2+}$  showing stronger destabilizing behavior since its smaller hydration sphere leads to stronger interaction with fatty acids headgroups[23].

These results reveal that, when building a liposome/mineral system, the effect of dissolved metal ions partly composing the mineral on several key membranes properties must be considered to ensure the stability and correct function of the vesicles. In addition to foreseeing cations influence on liposomes properties such as surface charge, fluidity and size, parameters such as lipids composition, pH and temperature must be finely tuned throughout the experiment to maintain them. These effects depend on the type and concentration of the metal ions partly composing the mineral, and the latter tendency to release them in solution. A knowledge of the atomic composition and crystal structure of the minerals is therefore necessary to understand which metal ions are likely to interact with the membrane and to what extent.

## 3 – Properties and consequences of mineral-liposomes interactions

### 3.1 – Influence of size

Potential mineral-liposomes interactions are mainly determined by the relative size of each component. Liposome sizes can range from tens of nm to several  $\mu m$  for the largest ones while mineral crystals sizes vary from few nm to several micrometers. In the literature, researchers are primarily interested in optimizing liposome size to ensure drug delivery or mineral encapsulation, the latter requiring a high liposome:mineral size ratio. However, in seeking mineral membrane-embedded liposomes, the critical parameter is the lipid bilayer thickness rather than the vesicle diameter. Indeed, in this case, mineral crystal size must match the thickness of the bilayer, which can vary between 4 nm for PC liposomes to 10 nm for the largest phospholipids. If the size of the mineral crystal is smaller than that of the liposome ( $<10$  nm), we can expect them to be present in the internal volume of the liposome or bonded to either side of the membrane in the case of hydrophilic crystals, or incorporated into the lipid bilayer in the case of hydrophobic crystals matching the bilayer thickness [7]. Conversely, when mineral crystal size is above that of the liposomes ( $>1$   $\mu M$ ), the latter can be adsorbed on their surface [23,26,36]. Not only will relative size influence the type of hybrid system that is potentially formed, but it will also determine the mineral crystal-to-liposome ratio in those systems.

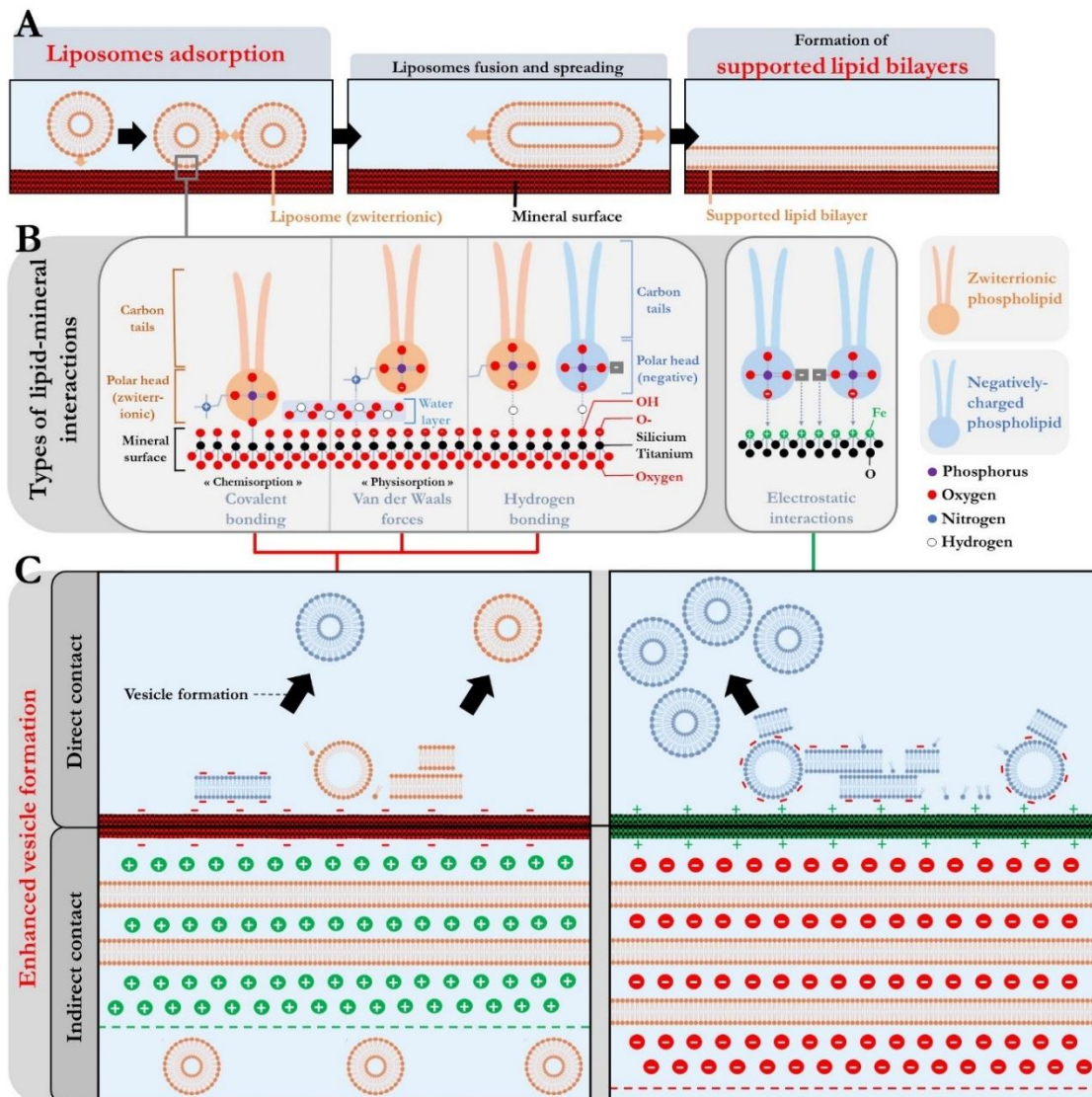
### 3.2 – Influence of electrostatic interactions, Van der Waals forces, hydrogen bonding and covalent bonding

The physicochemical properties of the mineral particles and the liposomes are the most important factors in mineral-liposome interactions. As demonstrated with cations, contact interactions between minerals and liposomes are controlled by their electrostatic properties [1–3,6], but also by Van der Waals forces, hydrogen bonding and covalent bonding; especially depending on pH, ionic strength, and mineral and liposome types, and concentrations [8,23]. These properties were studied in three specific cases resulting from lipid-mineral surface contacts: a) liposome



adsorption, b) formation of supported lipid bilayers (SLB) and c) enhanced vesicle formation on mineral surfaces. The adsorption of different type of lipids, from fatty acids to phospholipids on several types of minerals, from metal oxides to silicate and layered double hydroxides (LDHs) was studied. The type of lipids and minerals involved influence the nature of physical interactions taking place and the type of system formed by this interplay.

In 2016, Liu et al. [8] revealed mineral-dependent interaction differences for PC liposomes with various minerals (e.g. SiO<sub>2</sub>, TiO<sub>2</sub>, Fe<sub>3</sub>O<sub>4</sub>, ZnO) depending on surface charge and chemical structure. Due to these differences, they are either a) adsorbed or b) forming SLBs on the mineral surface, the latter being a consequence of the former after fusion of the liposome on the mineral surface.



**Figure 2: Types of lipid-mineral interactions (electrostatic interactions, Van der Waals forces, hydrogen bonding, covalent bonding) and their consequences on liposome-mineral systems (liposome adsorption, supported lipid bilayers, enhanced vesicle formation) :**

**(A):** From left to right: Zwitterionic liposomes (depicted in orange) are adsorbed on a negatively-charged mineral surface (in black and red) then fuse and spread to form supported lipid bilayers at the contact of the mineral, involving different type of lipid-mineral interactions covalent bonding, Van der Waals interactions and hydrogen bonding, depending on the mineral's nature.

**(B):** Types of lipid-mineral interactions. **Left panel:** Interactions involving oxygen atoms of negatively-charged mineral surfaces (e.g. SiO<sub>2</sub>, TiO<sub>2</sub>) with phosphatidylcholine zwitterionic polar-heads. From left to right: Covalent bonding between an oxygen atom from a TiO<sub>2</sub> crystal's surface and an oxygen atom from the PC polar head following a nucleophilic attack. Van der Waals interactions between deprotonated SiO<sub>2</sub> surface oxygen atoms and PC polar heads nitrogen atoms across a water layer. Hydrogen bonding between mineral surface protonated oxygen atoms and PC polar head's oxygen atoms and negatively charged (e.g. PE) polar head's oxygen atoms. **Right panel:** Electrostatic interactions involving positively-charged mineral surfaces and negatively charged phospholipid polar head's deprotonated oxygen atoms.

**(C):** Direct (**top panels**) or indirect (**bottom panels**) interaction of either free lipids, micelles or lipid bilayers composed of zwitterionic (in orange) or negatively charged (in blue) lipids with negatively (left panels, in red) or positively (right panels, in green)-charged mineral surfaces leads to enhanced vesicle formation compared to mineral-free systems.

**Direct contact: Top-left panel :** Zwitterionic (in orange) and negatively-charged (in blue) lipids interact directly with negatively-charged mineral surfaces based on covalent bonding, Van der Waals forces and hydrogen bonding. These interactions lead to the formation of a lipid matrix adsorbed at the surface of the mineral, serving as a template for lipid bilayers enclosing and formation of either zwitterionic or negatively-charged liposomes. **Top-right panel:** Negatively-charged lipids interact with positively-charged mineral surfaces thanks to hydrogen bonding with higher affinity and rate compared to lipid-negatively-charged minerals interactions. These interactions lead to the formation of a lipid matrix adsorbed at the surface of the mineral, serving as a template for lipid bilayers enclosing and formation of negatively-charged liposomes with a higher formation rate compared to negatively-charged mineral surfaces.

**Indirect contact : Bottom-left panel :** Interaction of zwitterionic phospholipid bilayers (in orange) with negatively-charged mineral surfaces (in red) excludes solvent and positive counterions (green dots) from the proximity of the surface, thus extending the electric double-layer (depicted as a dotted line) and its electrostatic effect 7 nm away from the surface and allowing the vesicle formation enhancement process to occur without direct contact at greater distances, based on electrostatic interaction, hydrogen bonding and van der Waals forces. **Bottom right panel:** With the same principle, interaction of zwitterionic phospholipid bilayers with positively-charged mineral surfaces (in green) leads to a thicker electric double layer (depicted as a dotted line) extended up to 13 to 15 nm away from the surface, where lipid adsorption occurs and leads to vesicle formation enhancement.

### 3.2.1 – Liposome adsorption

Cryo-TEM analysis [8] showed adsorption of DOPC liposomes without further SLB formation on all three types of metal oxides as well as SiO<sub>2</sub> (**Figure 2A**). However, given that the presence of free phosphate ions and high pH (11) inhibits PC liposome adsorption on metal oxides but the latter cannot disrupt an already established adsorption on TiO<sub>2</sub>, a different involvement of the PC phosphate group is proposed for the interaction with either metal oxides or silicate. While adsorption of PC liposomes on silicate has been attributed to on Van der Waals forces across a 1 nm-thin layer of water between lipids headgroups and mineral nanoparticles ("physisorption"), it is proposed that PC adsorption on TiO<sub>2</sub> is based on a covalent bond resulting of a nucleophilic attack from the lipid phosphate towards a Ti<sup>4+</sup> cation from the mineral surface("chemisorption") (**Figure 2B**) [8]. This explains the deleterious effect of high pH on the liposome-TiO<sub>2</sub> interaction, as the Ti center is deprotonated and negatively charged, making it an unfavorable target for the nucleophilic attack [8].

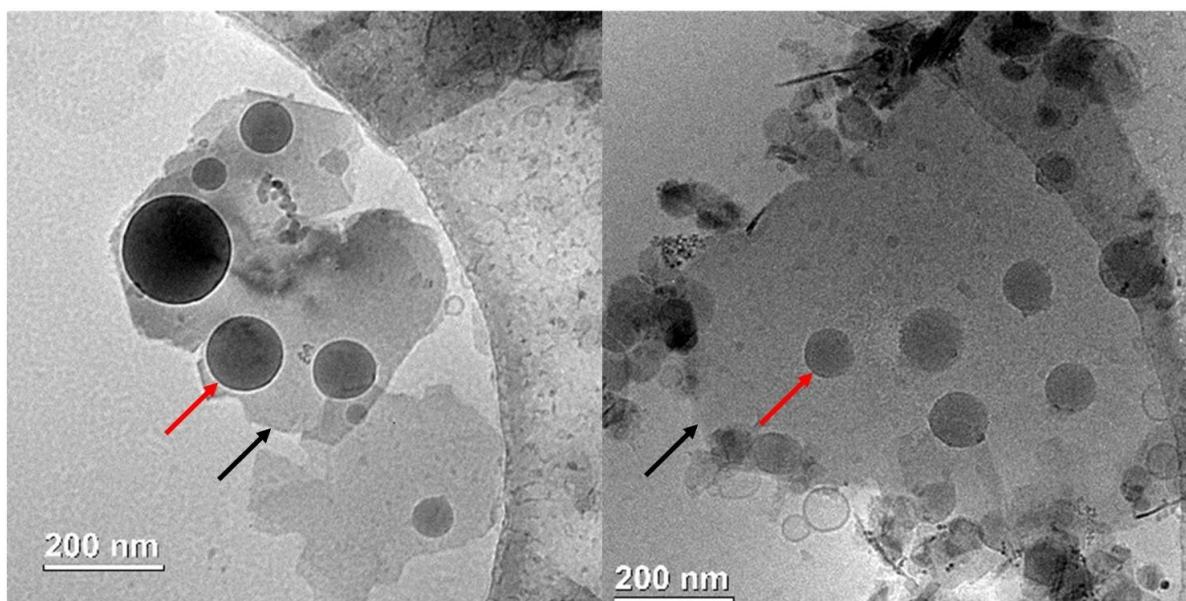


Figure 3: Cryo-transmission electron microscopy image of phosphatidylcholine liposomes (red arrows) of 100-200 nm in diameter adsorbed at the surface of  $\mu\text{m}$ -sized crystals of layered-double-hydroxide (LDH) hydroxycarbonate Green Rust  $\{[\text{Fe}^{2+}_{(1-x)}\text{Fe}^{3+}_x(\text{OH})_2]^{x+}[\text{CO}_3^{2-}_{(x/2)}\text{H}_2\text{O}_{(y)}]^{x-}\}$  (black arrows) (unpublished data).

### 3.2.2 – Supported lipid bilayers (SLB) formation

PC membrane formation on silica ( $\text{SiO}_2$ ) surfaces under the form of SLBs (**Figure 2A**) was shown [8] to rely on electrostatic interactions involving not only Van der Waals forces but also an interplay of ionic strength, pH and divalent cations contributions. Similar phenomena exists with  $\text{TiO}_2$  particles which require higher liposome concentrations and incubation time [37] and decreased pH with additional  $\text{Ca}^{2+}$  [38–40], suggesting the different modes of interaction deciphered on the “liposome adsorption” section.

### 3.2.3 – Enhanced vesicle formation

The lipid adsorbing properties of various minerals (e.g silicates, metal oxides, clays) have been extensively studied for their ability to facilitate vesicle formation, revealing the dynamics of their interactions. The rate of vesicle formation is increased in presence of minerals either from micelles or during rehydration of a dried lipid film, either with direct contact or indirect interaction, and depending on the isoelectric point (IEP) and reactive surface area of the mineral [23]. Mineral-lipids direct contact is thought to involve not only electrostatic interactions, but also Van der Waals forces and hydrogen bonding (**Figure 2B**), as described above, in numerous minerals such as corindon ( $\text{Al}_2\text{O}_3$ ), goethite ( $\alpha\text{-FeOOH}$ ), zincite ( $\text{ZnO}$ ), silica ( $\text{SiO}_2$ ), anatase ( $\text{TiO}_2$ ), montmorillonite ( $(\text{Na,Ca})_{0,3}(\text{Al,Mg})_2\text{Si}_4\text{O}_{10}(\text{OH})_2 \cdot n(\text{H}_2\text{O})$ ) and pyrite ( $\text{FeS}_2$ ), etc. Electrostatic forces are a key factor for the adsorption of lipids since negatively-charged single-chain phospholipids have a higher adsorption and vesicle formation rate on positively-charged minerals (alumina, goethite, zincite) compared to negatively-charged ones (silica, anatase, montmorillonite, pyrite) [27,41,42] (**Figure 2C, top panels**). However, adsorption can happen even if vesicles and minerals hold the same charge thanks to hydrogen bonding between lipid head groups and the mainly neutrally charged surface sites of the

minerals (**Figure 2B**), as well as Van der Waals interactions [43]. Comparable interactions were proposed with Mg<sub>3</sub>Al double-layered hydroxides inducing spontaneous vesicle formation in SDS:DTAB (8:2 ratio) micelle solutions, thanks to the interaction between positively charged LDH surfaces and negatively charged micelles or free amphiphiles, leading to their aggregation and facilitating vesicles formation [36] (**Figure 2C, top panels**). The importance of mineral crystal phases rather than free metal cations in this phenomenon is justified by the vesicle rate formation from 80 mM fatty acids micelles (myristoleic, palmitoleic and oleic acid) interacting with variously sized, charged and shaped mineral suspensions being unaffected by the addition of cation chelators (e.g. EGTA, EDTA) [26].

Furthermore, Van der Waals and electrostatic interactions have been proven to be involved in mineral-promoted vesicle formation enhancement without direct contact, depending on the concentration of zwitterionic phospholipids interacting with rutile, quartz and corundum surfaces [23,26,27,41,42] (**Figure 2C, bottom panels**). In these systems, several free lipid bilayers are adsorbed at the surface of the minerals, coordinated by Van der Waals forces between lipid bilayers and with mineral surfaces. Solvent and counterions usually interacting with the mineral are excluded further away from the surface by the interacting lipid bilayers, thus pushing the electric double layers several nanometers away and extending the effect of the mineral surface electrostatic charge. At low phospholipid concentration (0,65 mM), adsorption is higher on rutile compared to quartz and corundum, which is consistent with the decrease of Hamaker constants, showing a greater contribution of Van der Waals forces at this lipid concentration [44]. Meanwhile, at high phospholipid concentration (1 mM) adsorption is higher on corundum compared to rutile and quartz, following the decrease of those minerals surface charge and showing a greater impact of electrostatic interactions.

### 3.3 – Influence of hydrophobic interactions

The phospholipids constituting liposomes are composed of one or more hydrophobic “tails” that are acyl-chains of varying length and number of unsaturations, connected to a polar hydrophilic “head” based on polar molecule-esterified glycerol 3-phosphates [1,2]. The hydrophobic interaction between fatty acid aliphatic chain “tails” is the primary driving force for the formation of liposomal bilayers in aqueous solution, meaning that any membrane-embedded particle must possess strong enough surface hydrophobic properties to interact with lipids hydrophobic tails. Since minerals may not be intrinsically hydrophobic [45], one solution to incorporate minerals inside the liposomal membrane is to functionalize its surface using amphiphilic molecules. The size of the mineral particle, including the eventual surfactant coating agent, must be matching the lipid bilayer thickness in order to be incorporated into the membrane, or have a size-matching hydrophobic surface [7,46].

While necessary, the functionalization may however impact the liposome properties, as the incorporation of hydrophobic nanoparticles induces several changes related to lipids packing disruptions [46]. For instance, 5 nm hydrophobic oleic acid capped superparamagnetic iron oxide nanoparticles (SPIONs) embedded into DPPC bilayers lead to an increase of its transition temperature, meanwhile incorporation of decanethiol-covered Au-NPs rather lowers the transition temperature at a high lipid-to-nanoparticle ratio with an increase of membrane fluidity by reduction of lipid ordering.

Hydrophobic nanoparticles generate “unzipped” areas corresponding to disruptions in lipids packing inside the membrane, that tend to fuse to decrease deformation free energy, leading to nanoparticles clustered aggregations. It may lead to the formation of pores inside the membrane, increasing liposomes permeability. Then, effects of hydrophobic nanoparticles addition on liposomes properties, especially fluidity, permeability, and transition temperature, must be foreseen [46].

## 4 – Synthesis methods for liposomes-minerals systems

### 4.1 – Hydrophobic functionalization of minerals

Some synthetic hybrid liposome-mineral systems embed vesicles lipid membranes with hydrophobic nanoparticles, as seen with iron-oxide embedded “magnetoliposomes”, used in the medical field for both diagnostic and therapeutic purposes. As described above, functionalizing minerals is necessary to induce lipid-mineral hydrophobic interactions. Functionalization protocols are mineral type-dependent, since the interaction of the coating agent with the surface depends on its atomic composition and structure [9,13,47–49].

Natural clinoptilolite, for example, belonging to the hydrated aluminosilicate porous minerals known as zeolites, has been functionalized by Huang et al. using the interaction of its surface OHs with 3-aminopropyltrimethoxysilane through a covalent bond with oxygen [47]. Another group [48] has developed a method to functionalize the surface of the apatite mineral using lauroyl chloride. Finally it has been demonstrated that superparamagnetic iron oxide nanoparticles (SPIONs), used as contrast agents (CAs) for magnetic resonance imaging (MRI), often intrinsically hydrophobic when synthesized using thermal decomposition of metal precursors [9], is hydrophilic when synthesized through hydrolytic methods such as co-precipitation of metal precursors [49]. In order to be incorporated into liposomal membranes, SPIONs have been functionalised using palmityl-nitroDOPA, claimed to bind to SPIONs surface through its hydrophilic DOPA head and to interact with the bilayers hydrophobic parts through its palmityl carbon chain [13].

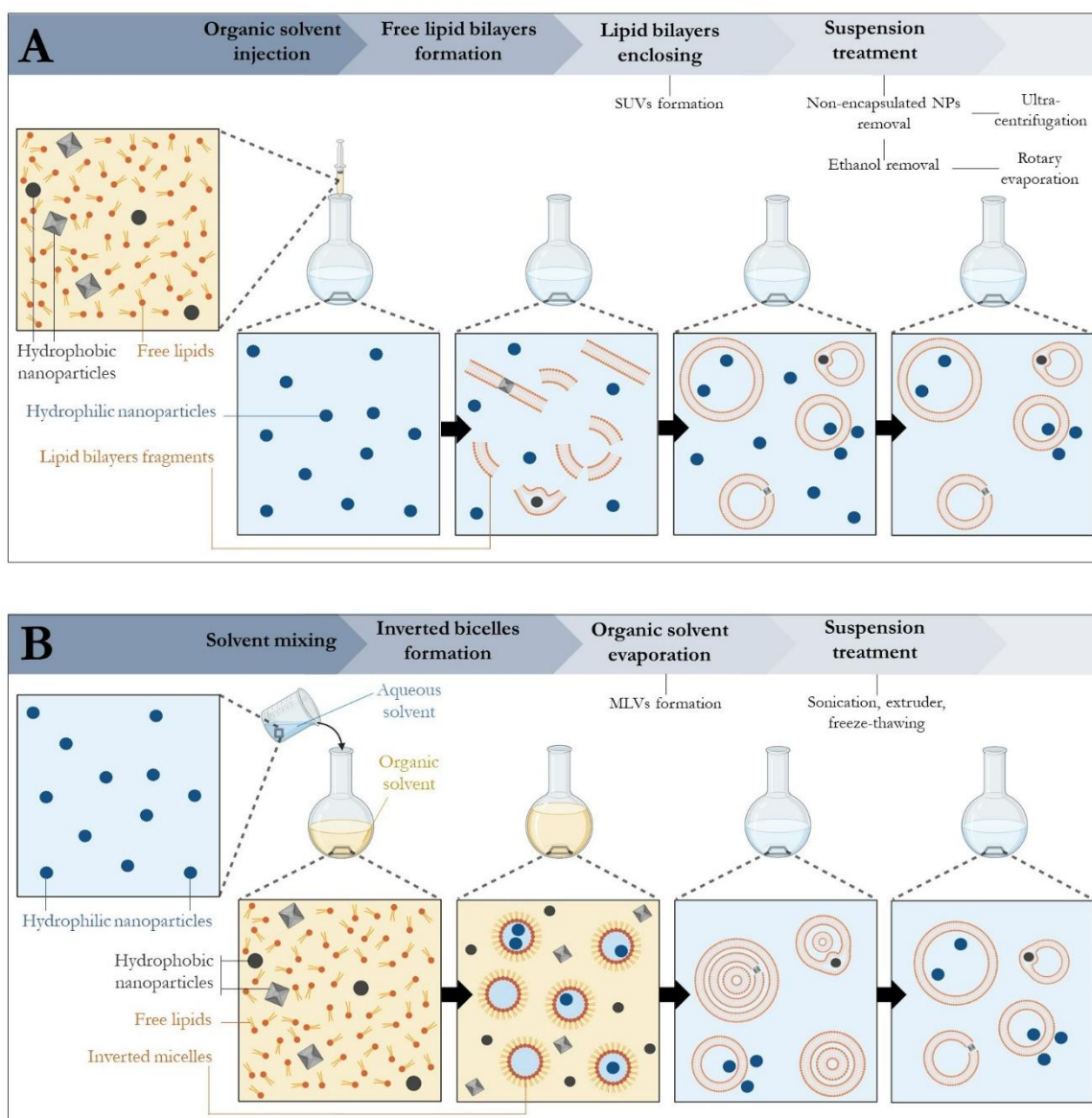
### 4.2 – Synthesis of mineral-liposomes systems

There are several methods for producing liposome-mineral systems, with minerals interacting at the external or internal site of the membrane, embedded into it, or contained in the internal medium of the vesicle [7]. One set of methods relies on the mixing of an organic and an aqueous solvent, each containing one of the two components (liposomes or minerals), another relies on the incorporation of inorganic mineral nanoparticles during the drying-rehydration steps of liposome synthesis, and a final one is based on the co-precipitation of metal-precursors directly inside liposomes. Finally, liposomes can also be adsorbed onto large mineral surfaces in a durable manner.

#### 4.2.1 - Solvents mixing methods

There are two distinct protocols (**Figure 4-1**). The first, called ethanol injection (**Figure 4-1A**), starts with lipids suspended in a water-miscible organic solvent (e.g. ethanol) and then injected under vigorous stirring into a larger volume of water. These lipids then rearrange at the water-organic solvent interface based on their amphiphilic nature and to form phospholipids bilayers naturally closing into liposomes. Depending on their nature, the mineral nanoparticles can be added in the aqueous solvent, if they are hydrophilic, and will be incorporated inside the liposomes, or in the organic solvent, if they are hydrophobic, and will be embedded inside the bilayer.

The second protocol, called reverse phase evaporation (or emulsification) (**Figure 4-1B**), is based on the addition of an aqueous solvent to an organic solvent containing lipids, generating water droplets surrounded by the hydrophilic polar heads of the lipids in a monolayer vesicle system. The organic solvent is then evaporated under reduced pressure to obtain an aqueous suspension of liposomes. Again, mineral nanoparticles are initially suspended in either solvent depending on their nature. At the end of both these protocols, NPs in solution and residual solvent are removed using dialysis, (ultra)-centrifugation or size exclusion chromatography [7]



**Figure 4-1: Solvent mixing methods for hybrid mineral-liposome systems:**

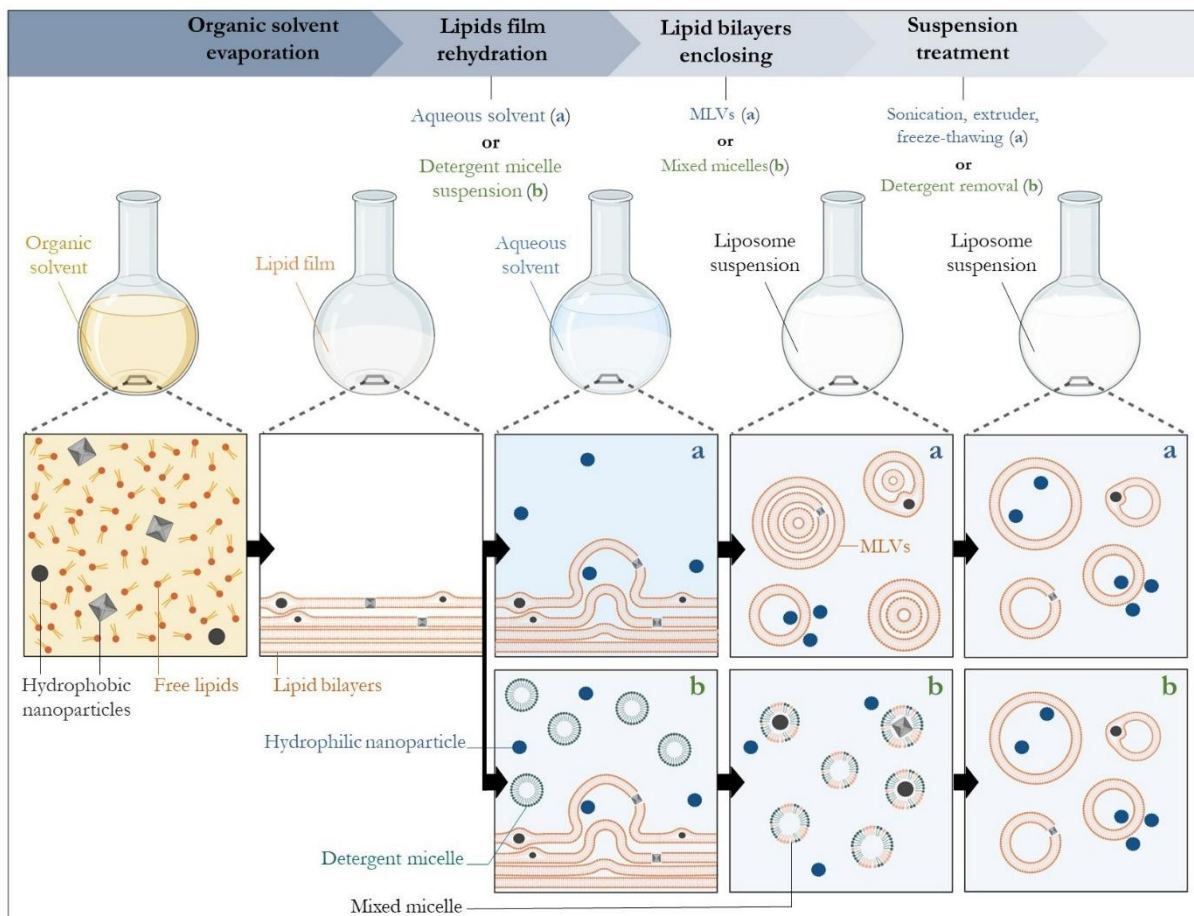
**(A) Ethanol injection method:** A water-miscible organic solvent containing lipids and possibly hydrophobic nanoparticles is injected in a larger volume of aqueous solvent possibly containing hydrophilic nanoparticles, leading to the reorganization of the lipids at the water-organic solvent interface under the form of free lipid bilayers naturally enclosing into unilamellar liposomes. The suspension may however be treated by ultra-centrifugation to remove traces of the organic solvent or ultra-centrifugation to get rid of unincorporated nanoparticles.

**(B) Reverse phase evaporation method:** An aqueous solvent possibly containing hydrophilic metal nanoparticles is mixed with an organic solvent containing lipids and possibly hydrophobic nanoparticles, generating water droplets surrounded by lipids called inverted micelles. Evaporation of the organic solvent leads to the re-organization of the lipids into multi-lamellar vesicles (MLVs) embedded the hydrophobic nanoparticles and/or containing hydrophilic nanoparticles, that can be transformed into unilamellar liposomes through sonication, extruder or freeze-thawing treatment of the suspension.

#### 4.2.2 – Lipid film rehydration methods

An organic solvent (i.e. chloroform/methanol or ethanol) containing suspended lipids is evaporated to obtain a lipids film (**Figure 4-2**). The latter is then rehydrated either with an aqueous solvent (thin-film rehydration method) (**Figure 4-2a**) or with a detergent solution at high critical micellar concentration (CMC) (detergent depletion method) (**Figure 4-2b**), under vigorous shaking [7].

In the first case, lipids bilayers constituting the lipids film are resuspended and reorganized to form liposomes enclosing or embedding mineral nanoparticles [50,51]. It is to note that addition of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  should promote vesicle formation during this step in PC liposomes, based on electrostatic repulsion [52]. In the second case, vortexing and sonication are added to form mixed micelles composed of both lipids and detergent in which mineral nanoparticles are trapped. Detergent is then removed from solution using dialysis, size exclusion chromatography, dilution or BioBeads, forcing detergent molecules to leave the micelles for the solution in order to maintain the equilibrium, inducing the merging of the lipids bilayer fragments until formation of liposomes. Here again, the addition of mineral nanoparticles either in the initial organic solvent before evaporation [12,14,49], or in the rehydration aqueous solvent [51,53–55] which depends on the hydrophilic or hydrophobic nature of the mineral, will define their incorporation either inside the bilayer, or within the internal liposomal volume, respectively [7]. These methods however have a low incorporation rate, and need further steps to get rid of nanoparticles and solvent traces in solution. They also lead to the synthesis of multilamellar vesicles (MLVs) with a broad size distribution, which may be corrected by sonication, extrusion or freeze-thawing.

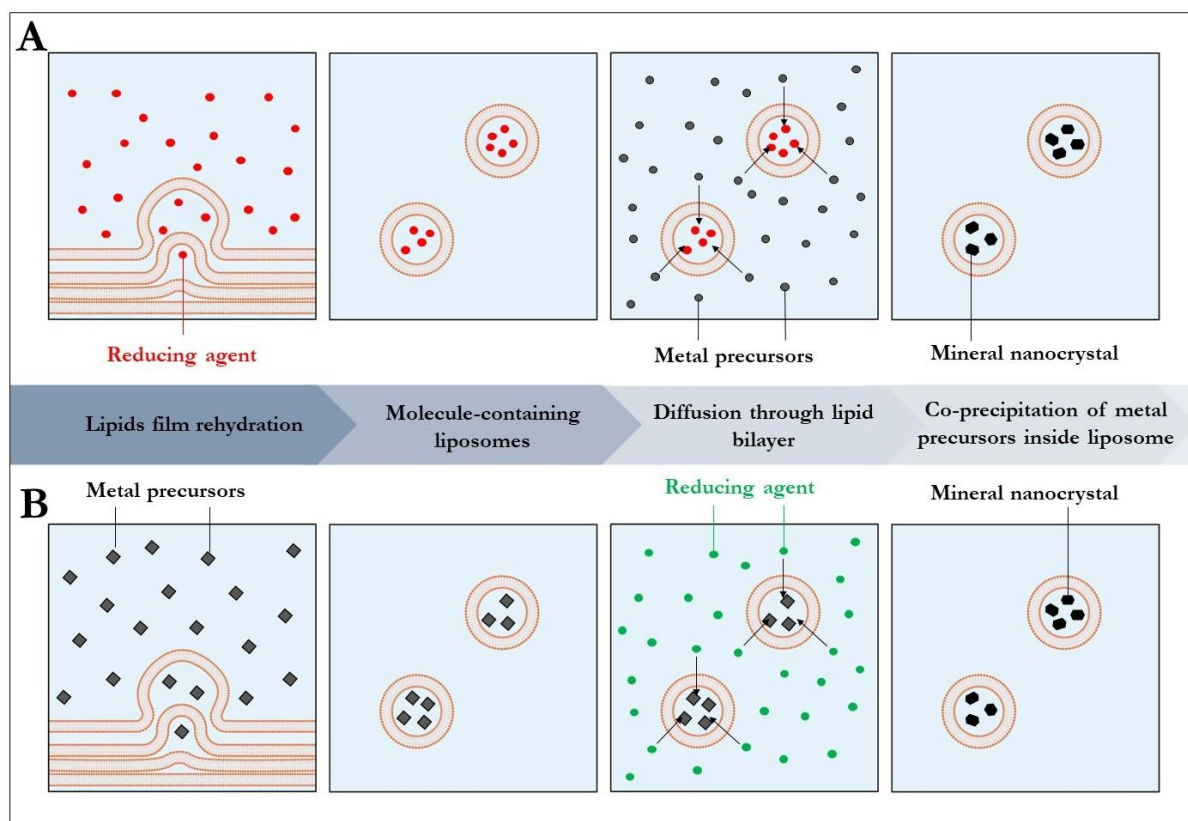


**Figure 4-2: Lipid film rehydration methods for hybrid mineral-liposomes systems synthesis:**

An organic solvent containing lipids and possibly hydrophobic nanoparticles is evaporated in a round-bottom flask to form a lipids film composed of multiple lipid bilayers that is then rehydrated with either an aqueous solvent **(a)** possibly containing hydrophilic nanoparticles or a detergent micelle aqueous suspension **(b)**. In the first case **(a)**, rehydration leads to the swelling of the bilayers that naturally enclose into multilamellar vesicles (MLVs) embedded with hydrophobic nanoparticles and/or containing hydrophilic nanoparticles, that can be transformed into unilamellar liposomes through sonication, extrusion or freeze-thawing of the suspension. In the second case **(b)**, the rehydrated lipid bilayers are mixed with detergent to form mixed-micelles containing hydrophobic nanoparticles. After removal of detergent using dialysis, size exclusion chromatography, dilution or biobeads, unilamellar liposomes are formed embedded with hydrophobic nanoparticles or containing hydrophilic nanoparticles.

### 4.2.3 – Mineral nanoparticles synthesis inside liposomes

In order to produce liposomes containing higher concentration of mineral nanoparticles, this method relies on the co-precipitation and/or reduction of metal precursors in the intravesicular side of the liposome, thanks to the diffusion of active molecules through the lipids bilayer (**Figure 4-3**). For instance, some protocols are proposed where Fe(II) and Fe(III) precursors are encapsulated inside liposomes during rehydration of the lipids, then co-precipitated inside the vesicle thanks to the diffusion of either HO<sup>-</sup> [56] or NaOH [51] from the aqueous solution through the bilayers, resulting in the formation of iron oxide-containing liposomes. A study from Kang et al. showed that this method can be applied to a broad range of minerals, since they were able to produce Au, Ag, Pd, Pt and hybrid-nanoparticles-containing liposomes based, conversely, on the diffusion of metal precursors from the aqueous solution to the inside of the liposomes containing reducing agents [57].





**Figure 4-3: Co-precipitation method for hybrid mineral-liposome systems synthesis:**

**(A)** The lipid film is rehydrated using an aqueous solution containing osmotically inactive reducing agents necessary for the co-precipitation of metal-precursors, leading to the formation of reducing agent-containing liposomes. Addition of osmotically active metal precursors in the suspension, and their diffusion through the lipid bilayers to penetrate the liposome internal medium allows reducing agents to initiate the co-precipitation of metal precursors, leading to the synthesis of mineral crystals directly inside the liposome.

**(B)** The lipid film is rehydrated in an aqueous solution containing the osmotically inactive metal precursors, leading to the formation of metal precursors-containing liposomes. The addition of osmotically active reducing agents in the liposome suspension, and their diffusion through the liposomes membranes allow the co-precipitation of the metal precursors inside the liposome, leading to the synthesis of mineral crystals-containing liposomes.

#### **4.2.4 – Liposome-adsorbing large mineral surfaces**

Preparation of mineral surfaces adsorbing liposome can be done by mixing already synthesized mineral crystals with either free lipids solutions or micelle suspensions, as described in part II-2, or already synthesized liposome suspensions (**Figure 2**). Concerning the latter, for instance, clay-liposomes composed of DDAB (Dimethyldioctadecylammonium) liposomes adsorbed on montmorillonite surfaces for controlled vesicle-contained herbicide release were obtained after mixing a DDAB lipids solution with a clay suspension under stirring, followed by continuous shaking at 20°C during 3 days [19]. Meanwhile, DTPC (1,2-dimyristoylphosphatidylcholine) and DPPC (1,2-dipalmitoylphosphatidylcholine) lipid bilayers adsorption on rutile, quartz and corundum was observed after simple mixing of liposomes and metal-oxides suspensions [44]. In this case, unlike the previous synthesis methods, both liposomes and mineral crystals must be synthesized among mixing, and size matching should be considered, with mineral crystals larger than the vesicles diameter, to prevent the formation of mineral-containing liposome systems.

## **5 – Techniques for characterization of hybrid mineral-liposomes systems**

The characterization of mineral-liposome systems faces several issues, including determining the effect of the interaction on the properties of mineral and liposome, as described above, and observing the overall system without perturbing the solution.

Determining the key mineral and liposomal properties is a first step in understanding the overall hybrid system. These properties include the lipid composition, surface charge, fluidity and size of the liposome, and the size, surface charge and chemical composition of the mineral. Second, it is necessary to elucidate the interaction between minerals and liposomes, the hybrid system it forms and the type of interactions it relies on. The following table (**Table 1**) lists the different techniques used to decipher these different aspects, the information they provide and the associated papers.

**Table 1:** Useful techniques to decipher liposomes, minerals or hybrid liposome-mineral systems key properties.

Component	Technique	Properties and information	Constraints	References
MINERAL	X-ray Diffraction (XRD)	<ul style="list-style-type: none"> <li>Mineral crystal phase, structure, and particle size</li> <li>Miller indexes corresponding to the different atomic plans inside the crystal structure</li> </ul>		[8,10,18,35,51,55,58]
		<ul style="list-style-type: none"> <li>Crystal size distribution</li> <li>Zeta-potential and electrophoretic mobility</li> </ul>	<ul style="list-style-type: none"> <li>Inappropriate for oxidation-sensitive crystals</li> <li>Inappropriate for sedimenting crystals in the case of zeta-potential measurement</li> </ul>	[11,54,54,55]
LIPOSOME	Dynamic Light-Scattering (DLS)	<ul style="list-style-type: none"> <li>Liposome size distribution</li> <li>Surface zeta-potential and electrophoretic mobility</li> </ul>	<ul style="list-style-type: none"> <li>Unable to inform about the lamellarity of the vesicles (i.e. the number of lipid bilayers)</li> </ul>	[13,14,49,59,60]
	Differential Scanning Calorimetry (DSC)	<ul style="list-style-type: none"> <li>Phase transition temperatures of lipid bilayers</li> </ul>	<ul style="list-style-type: none"> <li>Temperature ranges may be inappropriate to maintain the stability of certain high temperature-sensitive minerals</li> </ul>	[12,34,35,61]
	Laurdan Generalized Polarization (LGP) by Fluorescence Spectroscopy	<ul style="list-style-type: none"> <li>Changes in lipids environment polarity displayed by a shift in Laurdans dye fluorescence emission</li> <li>Membrane fluidity</li> <li>Phase transition temperatures</li> </ul>		[32,35,62]
	Small-Angle Neutron Scattering (SANS)	<ul style="list-style-type: none"> <li>Structure factors of the liposome membrane</li> <li>Membrane structure and deformations</li> <li>Lipid bilayer hydrophobic thickness</li> <li>Lipids organization</li> </ul>		[13,60]
	Confocal-fluorescence microscopy	<ul style="list-style-type: none"> <li>Lipids composition and organization within the membrane</li> <li>Membrane dynamics</li> <li>Vesicle formation in contact with mineral surfaces</li> <li>Observation of lipid type-specific localization</li> <li>Observation of liposome formation</li> </ul>	<ul style="list-style-type: none"> <li>Use of specific fluorescence-labelled lipids</li> </ul>	[26]
	Fourier-Transform Infrared Spectroscopy (FT-IR)	<ul style="list-style-type: none"> <li>Vibration frequencies of typical (in)organic moieties</li> <li>Interactions between chemical groups (i.e., cations and polar heads)</li> </ul>		[14,34,48,63]
	Scanning Electron Microscopy (TEM)	<ul style="list-style-type: none"> <li>Interplay between liposomes and mineral crystals</li> <li>Image of the overall liposome-mineral hybrid system</li> </ul>	<ul style="list-style-type: none"> <li>Dehydrated sample and contact with the carbon grid may flatten the liposomes and modify (disrupt or enhance) lipid-mineral interactions compared to the aqueous phase</li> </ul>	[11,14,64,65]
		<ul style="list-style-type: none"> <li>Interplay between liposomes and mineral crystals</li> </ul>	<ul style="list-style-type: none"> <li>Observation of a 2D-projection of a 3D space which then cannot</li> </ul>	[56,60,66]

<b>HYBRID MINERAL-LIPOSOME SYSTEM</b>	Cryo-Transmission Electron Microscopy (cryo-TEM)	<ul style="list-style-type: none"> <li>• Image of the overall liposome-mineral hybrid system in amorphous ice, giving an observation of a close-to-solution state of the system</li> </ul>	give information about the height structure of an element contained in the 300 nm thick ice layer. Makes it difficult deciphering the location of mineral nanoparticles regarding the liposomes and vice-versa.	
	Cryo-EM tomography	<ul style="list-style-type: none"> <li>• Interplay between liposomes and mineral crystals in the three-dimensional space</li> <li>• Series of tilted 2D images used to build a reconstituted 3D model of the overall hybrid system</li> </ul>		[66]
	Atomic Force Microscopy (AFM)	<ul style="list-style-type: none"> <li>• Nanometric size and shape of both components</li> <li>• Liposome-mineral relationship in the overall hybrid system (location, size ratio, repartition)</li> <li>• Vesicle adsorption on mineral</li> <li>• Repartition of hydrophobic or hydrophilic coating of mineral nanoparticles</li> <li>• Surface mapping of either components or of the overall hybrid system at a nanometric-to-atomic scale (vesicles absorbed on mineral surface, specific mineral nanoparticle-membrane interactions, liposome intrinsic structure)</li> <li>• Mapping minerals surface defects due to surfactant coating or penetrating the underlying crystalline structure.</li> </ul>	<ul style="list-style-type: none"> <li>• Dehydrated sample may lead to liposome flattening and modified (disrupted or enhanced) lipid-mineral interactions compared to the aqueous phase</li> </ul>	[9,44,46,67–69]

## Conclusion

The construction of a mineral-liposome hybrid system relies on the many interacting properties of these components, which must be finely tuned to achieve the desired structure and function. While liposome/lipid bilayer properties such as surface charge, membrane fluidity, lipid composition and size should match with both experimental conditions and mineral type, the latter also influences these properties, for instance adding catalytic capacities. Anticipating these type of effects helps to avoid the destabilization of either the liposomes or minerals due to incompatible setup conditions (pH, temperature, cations concentrations) or undesired interactions side-effects. Mineral-liposome/lipid bilayers interactions rely on a fine interplay between electrostatic interactions, Van der Waals forces, hydrogen bonding, covalent bonding, and hydrophilic/hydrophobic interactions. Based on these and on each components properties, several types of systems can be formed: hydrophobic mineral nanoparticles-embedded liposomes, nanoparticles containing liposomes, nanoparticle-surface decorated liposomes or liposomes adsorbed on a mineral surface. Therefore, the type of system formed can, to some extent, be predicted based on the characteristics of minerals and liposomes, and reciprocally, the type of liposomes and minerals can be chosen to obtain a system with the desired properties. To confirm this, the synthesized system can be characterized using microscopic, spectroscopic, calorimetric, and other bio-physical methods. Mineral-liposome hybrid system are of major importance both in applied science, with their numerous medical and environmental applications and in fundamental science, in which they are behind several theories and hypotheses. A more complete understanding of these types of systems, through thorough characterization, is necessary to improve, optimize and extend

their use in the future. By deciphering mineral-liposomal interactions and reactions more complex systems can be imagined, to further explore their medical, environmental, and fundamental potentials.

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### Conflicts of interest

The authors declare no conflict of interest.

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