Imaging of liposomal drug delivery systems by atomic force microscopy

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Over the past four decades, the research focused in the development of new liposomal drug delivery systems exponentially grew as a result of their proven efficacy in carrying pharmacological active molecules to target specific sites. In fact, the drug loading into liposomes both increases the drug’s bioavailability through the prevention of unfavourable chemical reactions triggered by enzymatic attacks as allows a sustained release. Several studies highlighted that physical characteristics of lipids such as their geometry, size and surface charge strongly influences the encapsulation efficiency as well the in vitro and in vivo stability. These characteristics can be accessed by atomic force microscopy (AFM) as well as the biomechanical properties of liposomes with and without drug encapsulated. In this chapter, the crucial contribution of AFM to study the dynamic behaviour of liposomes is reported, including a discussion about challenges and limitations needed to overcome as the collapse of liposomes induced by the AFM-tip.

Keywords: liposome, AFM, drug encapsulation, viscoelastic measurement

1. Introduction

Liposomes are considered promissory as vehicles for development of biosensors, drug delivery, cell adhesion inhibitors and carriers for the introduction of genetic material into cells (1 and references therein). Liposomes can be defined as a colloidal association of amphiphatic lipids that spontaneously organize themselves in closed concentric structures, or vesicles, in aqueous solutions. Considering their lower toxicity and higher biocompatibility and biodegradability, these systems are already used in clinical medicine as carriers to encapsulate and transport several drugs or even biosensing systems. According to Singer-Nicolson fluid mosaic membrane model (2), the biological membrane is a dynamic and fluid structure, whose basic constitution is a phospholipid bilayer with incorporated proteins, which diffuse freely in the cell membrane plane. The hydrophilic polar heads of the phospholipids bilayer are outer disposed occupying the two surfaces (intra and extracellular) and the hydrophobic tails are thus oriented to each other. The lipids of the bilayer are movable, often changing their position within a layer (3). On the external face of the cell membrane, carbohydrates are linked to phospholipid heads (glycolipids) or to proteins (glycoproteins), which are believed to have molecular recognition capabilities. The biomembrane’s ultrastructure presents an asymmetric arrangement, because it has proteins linked to the membrane surface (extrinsic proteins) and proteins partially or wholly embedded in the bilayer (intrinsic proteins). These proteins may work as enzymes, substances transporters or signals receptors of the external environment. The relative proportions of proteins and lipids vary with the type and function of cell membrane. Besides, this model proposes that lipids and proteins diffuse freely in the plane of the cell membrane (4).

Lipid membranes structures are difficult to study because of the considerable variety of lipids, with different physical properties such as cross section, fluidity, electrical charge, molecular weight and the polymorphic nature of the lipid arrangement. Furthermore, the covalent bonding of proteins and carbohydrates increases the complexity of the membrane structure (5). The in vivo lipids role extends beyond structural division, involving also molecules in cell signalling pathways (5) and in maintenance of the differences in electrolyte concentration and electrical field gradient between the extracellular environment and the cytoplasm. Biomembranes are essential to life regulation mechanisms throughout many cell interfaces. Moreover, cell membranes composition can change quickly to respond to environmental stimuli (6). Due to the complexity and heterogeneity of cell membranes, mechanisms and functions of lipid-biomolecule interactions have been often investigated using simplified models called biomimetic systems.

The plasma membrane of a cell is composed by a bilayer of glycerophospholipid molecules. A single glycerophospholipid molecule is composed of two major regions: a hydrophilic head and a hydrophobic tail. When phospholipids are mixed with water, a self-organization of the molecules occurs, where the hydrophobic portion is directed toward the centre and may form various types of systems, including: i) Micelles: aggregates with hydrophobic chains oriented inwards and the remaining hydrophilic groups at the surface in contact with water molecules, creating a water free environment. Essentially spherical, micelles can be also disc-shaped, cylindrical and ellipsoidal; and ii) Liposomes: vesicles obtained from phospholipids dispersion in an aqueous environment; which may comprise one or more lipid bilayers interspersed with aqueous surroundings. These features can be used to mimic biological membranes that are present in the organelles of living cells, e.g. mitochondria, golgi apparatus, nucleus cell, lysosomes and endoplasmic reticulum.

Liposomes can be formed by sonication or mechanical dispersion of phospholipids in an aqueous solution and may be composed of one or more concentric lipid bilayers separated by an aqueous medium. These systems are often used to
encapsulate active drugs or to incorporate proteins (7–9) and have proved to be useful as controlled models to investigate the permeability features of biological membranes (10). Phospholipid vesicles were first described in 1965 by Alec Bangham and coworkers (9). Immediately after the Bangham work, liposomes showed up as simplified systems for biological membranes research. Since Gregory Gregoriadis studies, in 1971, liposomes have been widely investigated (8,11–13) in what concerns their physical properties (14–16), preparation (17,18), formation and fusion mechanisms (19,20), membrane transport (21) and characterization methods. Apart from its utility in physical chemistry, these biomimetic models have been extended to the medicine field (22,23), such as for encapsulate bioactive agents (24) or to interact with living cells (25), for vaccines (26) and for veterinary goods (27) production, for erythrocyte substitution (28–31) and for cosmetic applications (32). The liposomes may also be used as pharmaceutical transporters, e.g. in cancer therapy (33), by introducing exogenous molecules which are carried in their “water bags” or within the lipid bilayer, into living cells, i.e., liposomes containing soluble molecules that after fusion with the cell membrane release their contents into the cell cytoplasm (34). In 2005, Michel et al. have successfully assembled intact vesicles interlayered with polyelectrolytes layers onto solid supports, extending these models’ ability to be used as drug reservoirs and in well-designed sensors and devices (35).

Liposomes can be prepared from natural or synthetic phospholipids. The first can be extracted from biological material of living cells; e.g. phosphatidylcholine (lecitin) phospholipid can be removed from egg yolk or soybeans. These natural phospholipids are used as the main lipid components for the production of liposomes because they are quite abundant in cell membranes. They may vary concerning the length, composition and saturation level of the hydrocarbon chains. Synthetic lipids, despite having a well-defined lipid composition of their hydrocarbon chains, may have greater or lesser number of carbon atoms, equal or different and saturated or unsaturated fatty acid chains. Besides, they possess a greater homogeneity allowing a better understanding, characterization and manipulation of their behaviour in opposition to natural phospholipids.

Currently, liposomes are not only applied to the biomedical field (e.g. diagnostic tests, blood transfusion in the absence of an appropriate donor, or detoxification through the use of chelating agents), but their applications have been extended to industry, cosmetics, agriculture (fertilizer stabilization), livestock (dairy maturation of milk), purification, recovery, catalysis, energy conversion processes (36) and sensors.

Summarizing, liposomes can be defined as a colloidal association of amphipathic lipids that spontaneously organize themselves in closed concentric structures or vesicles. They can be extracted from natural lipid mixtures or extracted and purified from synthetic lipids that are commercially available. These vesicles can be classified by their size, lamellae number, lipid formation, stability and preparation mode. The liposomes types that are commonly used as membrane model systems, are: multilamellar vesicles (MLV), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV). SUVs are usually obtained by extrusion of multilamellar liposomes through porous polycarbonate membranes at high pressure or by sonication of lipid aqueous suspensions (37). The liposome category is essentially determined by their preparation method which should always be selected carefully, since its composition, number of layers, size distribution and encapsulated volume considerably influence the applications in view.

The key factors for vesicles’ characterization are size, lamellae number and lipid bilayers constitution. Various parameters such as charge, stability, curvature of the bilayer or bilayers, phase membrane and formation of lipid domains depend on the phospholipid composition, the presence of sterols, the proportion of these components and the insertion of exogenous molecules into their bilayers (36). The liposomes true conformation may be also crucial for specific applications. Moreover, control and monitoring of physicochemical properties of liposomes as size distribution, encapsulation efficiency, biodistribution and stability over time, is central to achieve therapeutically effective nanocarriers or sensing devices. For example, physical properties of proteins and lipids as cross section, fluidity and electric charge, are altered in cancer cells, compromising anticancer drug accumulation inside of cells (33). Drugs can get into cells through various mechanisms as diffusion, transport and endocytosis, so as expected, the efficacy of a drug is compromised in cancer cells by the fact that these cells have a different exposition and number of membrane-bound cell surface receptors due to the suffered alterations in membrane morphology and elasticity, which difficult drug penetration comparative to in healthy cells. Beyond, the physical constraints of cancer cell membranes, the therapeutic activity of the drug in a tissue is also conditioned by its poor water solubility and chemical instability coming from the attack of enzymes of the bloodstream. Some of these issues can be circumvented by the encapsulation of the drug within biocompatible materials (lipids, polysaccharides, polyclations) designed in different type of nanocarriers as liposomes, micelles, nanoparticles, and dendrimers.

Atomic Force Microscope (AFM) has been used to imaging liposomes with the objective to characterize their morphology and the intra and inter chemical/physical interactions. AFM is a technique invented by Binnig, Quate and Gerber in 1986 (38), which is widely employed nowadays to characterize conductive and non-conductive surfaces at high resolution, in different environments (liquid, air, vacuum). Particularly, in biomolecules such as liposomes, AFM is the only technique able to assess the morphological characteristics under various physiological conditions. Several parameters like temperature, pH or salt composition can be optimized during the AFM measurements creating a biological environment to study the liposomes. The technique involves the use of a cantilever bearing a sharp tip at its end, which suffers attraction and repulsion forces when it is a few Angstroms from the surface (39). Generally, in the AFM apparatus a laser beam is focused in the upper side of the cantilever, reflected by it, and detected afterwards by a
four quadrant photodetector. The beam reaches the photodetector and the position of the tip below the cantilever is determined from the relative position of the reflection in the photodetector. This will vary according to the surface embossed. During the interaction between the tip and the sample there are several types of acting forces as van der Waals forces, electrostatic forces or surface tension forces, with the former being the dominant. Depending on the forces magnitudes involved, the AFM can operate in contact, non-contact or dynamic modes (40–42).

In contact mode, the cantilever tip scans the surface through a feedback loop that maintains a constant ‘“setpoint” deflection between the cantilever and the surface while the scanner is moving across the surface area. The force between tip and surface is kept constant during this operation. In bio-samples, this mode is less used to study the topography because it is liable to scratch the surface. However, it is powerful to analyze surface electrical properties with a sub-nanometer resolution (43) and to study adhesion or mechanical properties of liposomes using force spectroscopy measurements and single molecule recognition (44).

Dynamic and non-contact modes are more appropriate for bio-imaging since they are able to scan the surface without damaging it, thus preserving the morphological properties. In dynamic mode, an oscillating cantilever tip taps the solid surface only for a short time lapse, providing a high resolution and avoiding the drag of the tip across the surface. Further details of materials properties can be obtained with this mode using the phase signal (1,45), or an harmonic contribution (46) of the cantilever oscillation. In particular, this mode is useful to investigate the interaction between the liposomes and other molecules, when the tip is in contact with the surface. The tip can also be functionalized with other molecules which interacts with liposome creating a receptor-ligand complex (1,47).

In the non-contact mode the cantilever tip oscillates with smaller amplitude (a few Angstroms) from the surface to maintain a constant distance between tip and surface only for a short time lapse, providing a high resolution and avoiding the drag of the tip across the surface. Further details of materials properties can be obtained with this mode using the phase signal (1,45), or an harmonic contribution (46) of the cantilever oscillation. In particular, this mode is useful to investigate the interaction between the liposomes and other molecules, when the tip is in contact with the surface. The tip can also be functionalized with other molecules which interacts with liposome creating a receptor-ligand complex (1,47).

AFM can be also used to obtain three-dimensional images of liposomes surface, or to carry out force measurements giving information about their rigidity and stability, as discussed below. Considering that liposomes are hydrodynamic vesicles, some researchers performed their AFM studies in water in tapping mode in order to decrease the lateral forces and prevent the scraping of biological samples. Researchers have also been modifying chemically the AFM tip and tested tips with different shapes (conical, triangle) in order to eliminate tip underlying issues to AFM as the arising topographical artefacts and the collapse of liposomes during scanning (48,49) as illustrated in Figure 1.

Recently, AFM has been combined with optical imaging techniques which enables the spectroscopic discrimination of different species in a biological sample. In particular, fluorescence microscopy has proven to be a powerful tool for selective and specific visualization of single molecules and monitor the dynamics of living cells. The combination of these two techniques is important to study the interaction of liposomes and cellular structures (50,51).

This review was raised within the scope of AFM characterization of liposomes with incorporated molecules, evaluation of the biomechanical properties of liposomes and to probe lipid-biomolecules interactions. Although, this thematic has been already revised into some extent (52,53), the number of papers recently published as well as new important achievements about this subject requires further reporting.

2. Topology and size distribution of liposomal drug delivery systems

The characterization of liposome by AFM started in middle of 90’s decade with the work of Storrs et al (1) who synthesized and characterized a new class of paramagnetic polymerized liposome (PPL) particles. This study allowed to determine the size of the PPL particles which presented diameter values of 30-200 nm in the case of unextruded liposomes and of 100-150 nm in the case of liposomes extruded through filters with nominal pore diameters of 100 nm.

In fact, the first studies are usually associated to the determination of the size of liposomes. For example, Kawara et al (54) used AFM to determine the size of liposomes prepared with mixtures of cationic cholesterol derivatives and 1,2-dioleoyl-sn-glycero-3- phosphatidyl ethanolamine (DOPE) to study gene transfection mediated by cationic liposomes which contain a cationic cholesterol derivative with a different spacer arm. It was concluded that vesicles made of the liposome/DNA complex had various diameters depending on each cationic cholesterol derivative with a different spacer arm and the diameter of the liposome/DNA complex was well related to the transfection activity of plasmid pSV2CAT DNA to a cultured cell line (NIH3T3). Moreover, vesicles with moderate diameters (from 0.4 to 1.4 μm) are most effective for gene transfection of plasmid pSV2CAT DNA into the target cell. Vermette et al (55) used AFM to image surface-bound liposomes containing PEG-biotinylated lipids being the surface composed by NHS-PEG-biotin molecules already covalent binding onto a surface amine groups by carbodiimide chemistry (AApP:PEl:PEG-Biotin:NeurAvidinE layers). The AFM results revealed a density of liposomes well below close packing. As the topographic images were obtained at high load force to observe the liposomal structures, the immobilized liposomes...
were perturbed by the AFM scanning tip since the immobilized liposomes were smaller in height than their diameter measured by dynamic light scattering measurements. Two years after the same authors published a new article where also AFM compression and fluorescent dye release techniques were used to analyse anchored liposomes (56). In their work, authors refer that surface-immobilized liposome layers are of interest for various potential applications such as localized drug delivery, being their characterization challenging. Those techniques allowed to observe intact liposomes immobilized onto solid supports or substrates via three different hydrogel interlayer schemes and biotin/avidin binding. In the measurements, an AFM modified cantilever was used by attaching a spherical silica colloidal particle to the AFM cantilever. The resultant compression curves were indicative of soft compressible layers of a size compatible with that of intact liposomes in opposition to the sharp force curves obtained in case of lipid bilayers formed upon liposome disruption. It was also shown that the release rate of the surface-bound liposomes is \(\sim 2.5\) times that of liposomes in solution but again independent of the attachment mode. Therefore, one should pay attention to this type of details when measuring or interpreting AFM topographic liposomes images. In fact, both contact and tapping modes were able to capture soft samples but the indentation and the vertical force deform the surface of soft or elastic materials (30,57). To avoid these effects, Ruozi et al (31) used the non-contact mode to characterize vesicle colloidal systems concluding that AFM analysis is a rapid, powerful and relatively non-invasive technique which can provide information on morphology, size and size distribution, on possible liposomes aggregation processes occurring during their storage. In addition, AFM measurements in aqueous medium allow the observation of liposomes under physiological conditions. These authors also verified that the AFM images of liposomes should be obtained within 10 min after deposition on mica supports as their integrity is lost only in few minutes after deposition. In many studies, AFM is used to reveal liposome topography and size distribution, which in most of the cases is in disagreement with the results obtained by cryo-TEM imaging or dynamic light scattering (DLS), respectively, considering the tendency of liposomes to flattening few minutes after deposition (58). This thematic will be further discussed in section 5 (see Immobilization of intact liposomes section).

![Topography images](image)

**Topography images**

**DPPG Liposomes**

a) b) c)

**Fig. 1.** Topographic AFM images a) and b) with 0.5×0.5 \(\mu\)m\(^2\) area of DPPG cast films deposited onto a silicon substrate. c) The height profile obtained from topographic image b) shows a liposome, on left, with a diameter of \(\sim\)100 nm and two levels, on right, with about 5 nm corresponding two to DPPG lipid bilayers deposited one above the other.

Farkas et al (59) observed that the mean size and size distribution of a targeted nanoparticle delivery system (NDS) strongly influences the intrinsic stability and functionality of this molecular complex, affects its performance as a systemic drug delivery platform and ultimately determines its efficacy towards early detection and treatment of cancer.
Surface-bound proteins can be also observed through AFM analysis of proteoliposomes. In fact, Mašek transformation of Gel Phase domains in phospholipid bilayers without affecting their integrity. incorporated was also analysed by Tian two-dimensional crystal-like structure on liposome surfaces. The structure of liposomes with and without drugs rgp120 (derived from HIV-1 Env). The obtained results revealed the tendency of rgp120 to form microdomains with proteins attached by metallochelation, including histidine (His)-tagged recombinant green fluorescent protein and glycero-3-[[N(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl](nickel salt) to formulate proteoliposomes with characterized a mixture of egg phosphatidyl choline and nickel-chelating lipid DOGS–NTA–Ni (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) and a fluid-phase bilayer of egg PC (EPC:Chol:DSPE-PEG2000), by AFM is shown in Figure 2. These results revealed that the incorporation of QD took place by hydrophobic self-association within the membranes and showed also that the encapsulation of hydrophilic small molecules in the internal aqueous phase of the L-QD hybrids present different degrees of carboxyfluorescein (CF) release in buffer and serum, depending on the type of lipid used. Hydrophilic and hydrophobic drugs can be internalized into the bilayer membrane or in the aqueous core of liposome. Nonetheless, drug entrapment in a liposome is conditioned by drug-to-lipid mass ratio and, in some cases, can be problematic since affects the bilayer reorganization, in terms of the size, fluidity and elasticity of the bilayers. Concerning these drawbacks, some authors entrap water soluble drug-cyclodextrin complexes in the liposome aqueous cavity for transdermal drug delivery (61–63). Literature is controversial regarding the effect of cyclodextrin in liposome size. Some authors did not observe any difference in liposome size after inclusion of drug-cyclodextrin complexes comparatively to the liposome with the drug alone (64), while, other achieved AFM scanning images proving that drug-cyclodextrin-liposome system is a heterogeneous sample, because of the large variations in particle size (three distinct subpopulations), contrary to drug-liposome systems that shows a homogenous size distribution (65,66).

Vázquez-González et al imaged hyaluronic acid-encapsulating liposomes deposited on cellulose and on stratum corneum in order to elucidate how the integrity of liposomes designed to deliver topically drugs is affected when these systems interact with skin. Topographic AFM images showed that the addition of penetration enhancers to liposomal formulations, that improve tissues drug penetration and the release of hyaluronic acid by destabilizing liposome integrity and decreasing adhesion forces. This research allowed to conclude that the liposomes morphology and adhesion forces are dramatically affected by the nature of the support and by the penetration enhancers and also that the liposomal cargo unloading is dependent of adsorption mechanism onto the substrates (67).

Herbig et al (68) have been demonstrated by AFM that the cell penetrating peptides pVEC and W2-pVEC can induce transformation of Gel Phase domains in phospholipid bilayers without affecting their integrity.

Surface-bound proteins can be also observed through AFM analysis of proteoliposomes. In fact, Mašek et al (69) characterized a mixture of cationic liposomes with pectin solution. The AFM images revealed a direct evidence for association of cationic liposomes on the pectin chain. It should be also referred that the obtained AFM images of pectin showed a chain-like structure with a small number of branches while those of liposomes showed a spherical form.

Since its components undergo significant reorganization during multiple self-assembly stages, it is essential to monitor the size and stability of the complex throughout the NDS formulation, in order to ensure its potency and manufacturability prior to entering clinical trials. The work of those authors showed that scanning probe microscopy (SPM) and dynamic light scattering (DLS) techniques are adequate to obtain quantitative and reliable size measurements of the NDS, and to investigate the impact of variations in the NDS formulation or self-assembly process in the size, structure and functionality of the complex with various therapeutic and diagnostic agent payloads. These combined SPM and DLS methods, when implemented at an early stage of the NDS formulation, present a potential measurement approach to facilitate drug discovery and development, optimization and quality control during manufacturing of the NDS.

The AFM technique is also the adequate technique to explore self-assembled nanocomplexes, for example, Srimornsak et al (60) characterized a mixture of cationic liposomes with pectin solution. The AFM images revealed a direct evidence for association of cationic liposomes on the pectin chain. It should be also referred that the obtained AFM images of pectin showed a chain-like structure with a small number of branches while those of liposomes showed a spherical form.

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The effect of medium conditions on liposomes without and with incorporated molecules can be also easily analyzed by AFM. One of these conditions is the pH. Zhou et al. (71) synthesized a pH- and thermo-sensitive berberine hydrochloride liposome modified by the poly(Nisopropylacrylamide-co-methacrylic acid-co-octadecyl acrylate) and characterized the morphology and size by AFM. The results indicate that the size of copolymer–liposome depends on the mass ratios of the copolymer to soya bean lecithin (SPC) and on solution pH value. The observed sizes can reach a maximum value at phase transition pH (pH*), being this maximum value is affected by the mass ratios of the copolymer to SPC and by copolymer properties. The maximum release of berberine hydrochloride (BH) from the copolymer–liposome was seen to occur at phase transition temperature (Tm) and pH* of the copolymer–liposome. The obtained results also illustrated that BH released from the liposome is due to the phase transition of the copolymer–liposome, where both Tm and pH* acted as phase transition switches of the, and both spatial and temporal releases can be carried out by adjusting the temperature, pH value and release time.

The role of pH is also significant in the case of recombinant adenovirus (Ad) which is promising towards gene therapy. In fact, it has been shown that the artificial envelopment of adenovirus within lipid bilayers decrease the immunogenicity and hepatic affinity of naked Ad in vivo. This also resulted in a significant reduction of gene expression, which is attributed to poor endosomal release of the Ad from its artificial lipid envelope. Bossche et al (72) explored the artificial envelopment of Ad within pH-sensitive DOPE:CHEMS bilayers and showed that AFM can easily give information about the stability of Ad. These authors demonstrated that the artificially enveloped viral vectors exhibit good stability at physiological pH, but immediately collapse and release naked Ad virions at pH 5.5.

Preparation methods such as mechanical extrusion and sonication play a key role in controlling the liposome on surfaces and yet the effects of vesicle preparation method on vesicular properties and integrity (e.g., shape, size, distribution and tension) remain incompletely understood. Cho et al (73) prepared vesicles composed of 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine (POPC) lipid by either extrusion or sonication, and investigated the effects on vesicle size distribution over time as well as the concomitant effects on the self-assembly of solid-supported planar lipid bilayers. These authors observed that the sonicated vesicles offer more robust control over the self-assembly of homogenous planar lipid bilayers, whereas extruded vesicles are vulnerable to aging and must be used soon after preparation.

Other application of AFM is the analyses of the liposomes interactions with other corporal fluids. For example, Nguyen et al (74) analysed the potential of liposomes as a drug delivery system for use in the oral cavity, conducting experiences of adsorption of charged liposomal formulations to hydroxyapatite (HA), a common model substance for the dental enamel and measured by AFM the aggregation reactions of liposome–parotid saliva mixtures. These studies allowed to conclude that saliva constituents interact with liposomes in such a way that an appropriate liposomal drug delivery system intended for use in the oral cavity seems to be dependent on the liposomal formulation. In this case, negatively charged DPPC/DPPA-liposomes seem to be most suitable for use in the oral cavity since they were found to be the less reactive with the components of parotid saliva.

3. Bending moduli and phase transition of liposome

Stability and efficacy of drug entrapment is dependent of physicochemical properties of liposomes, such as size, lipid composition, conformation of lipid headgroups, surface charge and thermodynamic phase of lipid bilayer membrane. AFM is a powerful technique used to correlate the thermodynamic behaviour of liposomes with the mechanical stress applied during the force spectroscopy analysis. Phase transitions of phospholipids can be grouped in crystalline, gel, and fluid liquid crystalline membrane phases, depending on water content, temperature, molecular structure and composition of the particular phospholipid (head group, length of tails (acyl groups), saturation level, cholesterol amount) and ambient conditions as well. Cellular uptake of liposomal drug delivery systems by endocytosis is conditioned by mechanical rigidity (bending modulus) of liposomes, which, in turn, is affected by the phase state of membrane and the liposome fluidity (75). DOPC/DOTAP (liquid-disordered phase) has similar size (~42 nm of diameter) and zero potential (~46 mV) as DPPC/DOTAP (solid-ordered phase), however it has a higher bending modulus, fact that contributes to a higher uptake of this type of liposomes by cells (76). The quantification of bending moduli requires stable liposomes on supports to perform AFM force curve measurements. The immobilization of 1mg/mL bovine serum albumin (BSA) on glass substrates favours hydrophobic and van der Waals interactions between liposomes and support rather than electrostatic interactions, fact that enables the observation of various intact and spherical liposomes in aqueous medium. DOPC/DOTAP (90/10) and DPPC/DOTAP (90/10) liposomes adopted a liquid-disordered and solid-ordered phase, respectively, fact that proves that BSA coating prevents liposome collapse independently of membrane phase state. BSA treatment was also effective regardless of the membrane rigidity (content of saturated lipids and cholesterol), membrane surface charge and PEG modification, allowing the quantification of the bending modulus (77). Same authors concluded that the bending modulus is dependent of membrane phase state, increasing in the order of liquid disordered > liquid ordered > solid ordered thermodynamic phases. Although the DPPC/DOTAP (90/10) and HSPC/DSPE-PEG (94.7/5.3) had different zeta potential values and, the latter one, was PEG-modified, they adopt a solid ordered phase membrane having similar bending moduli. Commonly, cholesterol is
added to liposomal drug delivery systems containing unsaturated lipids in order to increase membrane rigidity and decrease membrane permeability, which gives rise to stable liposomes with a suitable drug storage and release in vivo.

However, some authors observed that the addition of cholesterol to liposomes containing saturated lipids has the opposite effect, achieving an inverse correlation between the membrane bending modulus and the permeability of liposomal membrane to the hydrophilic drug: calcein (78).

Structural changes induced by the entrapment of melittin, an anti-tumor agent, on two negatively charged-lipid bilayers deposited on polarized gold electrodes, has been revealed by AFM imaging. Although the lipids have the same surface charge, the prolonged exposure to 10 µM melittin solution induces the collapse of DMPG liposomes and leads to a formation of pores when penetrate into DMPS bilayer. The Young’s modulus of the DMPG and DMPS liposomes were 25 and 34 MPa, respectively, meaning that the packing density of the molecules and the elastic properties of liposomes are different, fact that can affect the ability of melittin to penetrate the lipid bilayer, phenomenon schematically illustrated in Figure 3 (79).

Fig. 3. Schematic illustration of the influence of the charge and lipid packing on the ability of a drug to penetrate lipid bilayer over time exposure. a) Negatively-charged liposomes, as DMPG, allow the penetration of melittin, a drug that creates pores in the lipid membrane, leading to desorption of DMPG from gold substrate after longer exposure. b) The action mode of melittin in the negatively-charged DMPS is different since these liposomes had molecules more densely packed which difficult the insertion of drug into their membrane. In this case, melittin adsorbs on top of the lipid film which prevents the formation of pore-like defects and consequently, not affect the residence of liposomes on gold substrate.

Fig. 4. AFM curves measurements can reveal the occurrence of interaction between SP-D and carbohydrate. During approach (red dashed line), no interaction will be observed until the carbohydrate-functionalized tip gets into surface contact where binding between SP-D and carbohydrate occurs. Upon retraction (black line), the force is building up due to the elastic response of the linker molecules.

4. Ligand–receptor interactions and membrane structure

The AFM and dynamic force spectroscopy techniques can be also be applied to biomedical research, specifically within immunology and liposome-based drug delivery entities to gauge interactions at the single-molecule scale. In 2007, Thormann et al (80) demonstrated that it is possible to quantitatively assess the interaction between proteins and their binding to specific ligands and membrane surfaces. Those authors studied the specific interaction between lung surfactant protein D (SP-D) and various carbohydrates by measuring the stability of a SP-D/carbohydrate bond when
subjected to an applied force being the SP-D is anchored on a solid support and the carbohydrate ligands are attached to the AFM-tip. Corresponding force curve is shown in Figure 4 which was obtained when the AFM-tip coated with saccharide ligand was approached to a substrate on which SP-D is covalently attached by polymer linkers. This figure also shows that when the binding between the protein and ligand takes place, the cantilever is retracted from the surface and an increasing force on the bond gradually builds up. Moreover, during the cantilever retraction (upper curve), the force as a function of tip–surface separation distance is characterized by the elastic response of SP-D and the linker molecules. This elastic response together with the retraction velocity of the cantilever defines the loading rate. A force of 65 pN was measured in this case. Successive measurements allowed also determining histogram of unbinding forces based on more than 2000 approach/retraction cycles and 200 unbinding events of single SP-D/mannose bonds.

5. Immobilization of intact liposomes

The research of topology and mechanical properties of liposomal drug delivery system adsorbed onto solid substrates using AFM is quite challenging due to the competition phenomenon existing between the liposomal-membrane bending and the substrate that are responsible for the deformation and alterations of bending rigidity of vesicles (81,82). Ideally, the force should be strong enough to immobilize the liposomes onto substrates but, at the same time, weak enough to prevent the collapse of liposomes.

When liposomes reach smooth surfaces (silicon (83,84) or mica (85,86)) there is a tendency to the systems start to break and spread throughout the surface forming supported lipid bilayers (SPBs) or fused vesicle patches (87). The work of Reimhult et al. (88) and Wang et al. (39), showed that the adsorption of intact vesicles of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) occurs in unfunctionalized gold (Au) substrates, but is hampered when vesicles are functionalized with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(-2-pyridyldithio)propionate] (DSPE-PEG-PDP) to establish Au-thiolate bonds at the surface. This proves that the substrate chemistry plays a role in vesicle adsorption and SPBs formation. Another interesting conclusion is the fact that, the vesicles attachment to the substrate through chemical functional groups, decreases the external force induced by the AFM tip required to induce the liposomes collapse, see Figure 5a), as the concentration of functional groups and the osmotic gradient increase (89,90). In addition, SPBs formation process is strongly influenced by calcium ions (91), temperature (92), vesicle composition (81,93) and surface topography (94). Combining AFM and quartz crystal microbalance (QCM) techniques was prove nanotopographic features of substrate (95).

The adsorption of liposomes on hydrophobic surfaces (gold substrates) or in rough nanostructured layer-by-layer substrates is an attractive strategy for the maintenance of the liposome integrity. In recent studies, 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DPPG) liposomes with a diameter of 25 nm were adsorbed onto polyelectrolyte multilayer cushions by layer-by-layer technique, showing that the kinetics of immobilization of intact liposomes on these polymer cushions is highly dependent of surface roughness. In fact, the amount per unit area of DPPG liposomes in rough surfaces was of 51±1 mg/m², a value above the estimated for a planar DPPG bilayers (5±1 mg/m²), suggesting that the liposome rupture is hindered by surface roughness (96,97). In fact, the immobilization of phosphocholine liposomes on two surfaces having the same charge (negatively-charged), differing only to the extent that one was a rigid surface (mica) and other was a smooth surface (mica covered with a chitosan-alginate multilayer masking film), renders more stable liposomes, see Figure 5b). For the latter case, this is due to the ability of alginate molecules freely move accommodation the shape of liposomes, which relieve the tension on liposomes responsible for their collapse (98).

Other strategy to immobilize liposome is the surface-bound cytomimetic assembly based on chemically selective and biocompatible immobilization and further modification of intact liposome as described Ma et al (99). The preparation of spherical liposomes is critical for developing tools for targeted gene and drug delivery applications in biotechnology and medicine, however, it has only been demonstrated in solution phase until now (100). To obtain spherical liposomes, these authors reported tethering of spherical 1,2-dioleoyltrimethylammoniumpropane liposome–gold nanoparticle (DOTAP–AuNP) on amine terminated monolayer by simple electrostatic interaction on gold transducer, see Figure 5b). Cuddling of cationic liposome by AuNP prevents spherical vesicle fusion in both liquid and solid phases, an essential criterion required for gene and drug delivery applications.

Fig. 5 – a) Conversion of liposomes into supported lipid bilayers by AFM tip; b) immobilization of liposomes on rough surfaces; and c) chemical functionalization of liposomes and substrates.
6. Conclusion

Atomic force microscopic measurements in non-contact or dynamic modes was shown to be indispensable to characterize liposomes deposited on surfaces allowing to know about, morphology, size distribution and interactions between drugs or sensing molecules. A variety of liposomal formulations consisting in the design of thermos-, pH-, enzyme-, or photo-sensitive liposomes have been tested for successfully deliver encapsulated drugs to cells. The analysis of the dynamics of these systems is crucial to reveal the cell internalization process of liposome and entrapped drugs, namely, factors controlling vesicle rupture. Although the characterization of intact liposomes on surfaces revealed to be fundamental to evaluate the drug delivery variables effects, the major difficulty found by the researchers concerns with the liposomes opening when adsorbed on flat surfaces. Nonetheless two strategies have been highlighted to maintain liposome intact: I) the adsorption of liposome should be performed on rough surfaces or II) use chemical functionalization of liposomes and substrates to avoid liposome rupture.

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