Expert Opinion





# Atomic force microscopy: a tool to study the structure, dynamics and stability of liposomal drug delivery systems

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Much work has been done during the past few decades to develop effective drug delivery systems (DDS), many of which are based on nanotechnology science. Liposomes are the most attractive lipid vesicles for drug delivery. The multifunctional properties of liposomes have a key role in modifying the bioavailability profile of a therapeutic agent. Different analytical techniques can be used to describe liposomes, not least applied scanning probe microscopy (SPM) techniques. Atomic force microscopy (AFM) seems to be one of the most effectively applied SPM techniques. This review article outlines the applications of AFM in evaluating the physical characteristics and stability of liposomal DDSs. Other well-known microscopy techniques used in evaluating liposome physical characteristics are also mentioned, and the contribution of AFM to evaluating liposomal stability is discussed. Among the advantages of AFM in examining the physicochemical properties of liposomal DDSs is its ability to provide morphological and metrology information on liposome properties. AFM thus appears to be a promising tool in technological characterization of liposomal DDSs.

**Keywords:** atomic force microscopy, drug delivery, liposome, stability

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## 1. Introduction

A drug is defined as an agent that alters bodily functions and can be administered in a variety of ways (i.e., inhaled, injected, or administered orally, rectally or vaginally). The profile of a candidate drug comprises of properties such as the following: solubility in aqueous media, lipophilicity, permeability, stability in biological milieu, degree of ionization and metabolism. The ADME profile of a drug is based on data concerning its Absorption, bioDistribution, Metabolism and Excretion (ADME) in animal models while its biopharmaceutical properties include pharmacokinetics and pharmacodynamics, bioavailability and protein binding behavior. The fate of a drug within the body depends, however, not only on its chemical structure and its mode of action, but also on the physical and functional properties of the carrier, which subsequently affect the drug's effectiveness [1]. New biomaterials based on the concept of nanotechnology could be used to produce nanodevices and nanoparticles that possess essential functions and properties due to their nanosize and surface properties. In past decades, nanocarriers composed of biocompatible and biodegradable materials have attracted much attention for their potential use as controlled release systems. Such nanocarriers include liposomes, lipid nanotubes, nanospheres, lipid emulsions, circular peptides, chitosan, viral nanocarriers and nucleic acid nanostructures.

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The dimensions and tolerances of these carriers are in the range of 0.1 to 100 nm [2].

The physical stability of nanocarriers depends on their size and size distribution, ζ potential, release properties, fluidity, elasticity and vesicle shape. The structural components that determine membrane stability include permeability, fusogenicity and fluidity. Size, size distribution and shape of nanocarriers are governed by the preparation method.

Liposomes are the most attractive lipid vesicles for drug delivery. They are spherical structures composed of one or more lipid bilayers, which enclose aqueous space(s). Liposomal technology is one of the fastest growing scientific areas contributing to drug delivery, cosmetics, and food technologies, while their structure and functions could simplify studies on living cells.

Atomic force microscopy (AFM), also known as scanning force microscopy (SFM), is one of the most commonly applied scanning probe microscopy (SPM) technique for liposome description. It was invented by Binning and colleagues and can produce high resolution images [3]. Liposomal surface properties can be detected and measured by using AFM, while their adhesive characteristics can also be evaluated. Materials like cells, proteins and biomolecules linked to the liposomal surface, for recognition of specific cell targets, can also be probed [4].

## 2. Drug delivery systems

Colloidal drug carriers are systems that have a dimension between 1 nm and 1000 nm, at least in one direction [5], and whose physicochemical characteristics render them effective vesicles. The properties of colloidal drug carriers are attributable to their high surface area, and their stability can be influenced by the surrounding medium. The usefulness of colloidal particles as drug delivery systems (DDSs) is dependent on their complete physical characterization.

Control of the carriers' physical properties is crucial for drug delivery purposes, and the nature of structural units used needs to be taken into account. Furthermore, apart from lipophilicity problems, toxicity issues associated with a carrier should also be considered. Materials used for preparing microstructure or nanostructure forms for drug delivery can be categorized into the following groups:

1. biological: lipids, peptides, polysaccharides
2. polymeric: polylactic acid, polyglycolic acid, dendrimers
3. silicon based: silicon, silicon dioxide
4. carbon based: carbon
5. metallic: gold, silver, palladium, platinum

The structures prepared from these materials are: i) liposomes and solid lipid nanoparticles; ii) nanoemulsions, micelles and dendrimers; iii) nanopores and nanoneedles; iv) nanotubes and fullerenes; and v) nanoshells. The most attractive nanocarriers already used as DDSs in pharmaceutical market are liposomes [1].

## 3. Liposomes

Liposomes were first discovered during an attempt to create lipid–water systems that behave as biomembranes. They were first called 'amphisomes' due to their amphiphilic character and their phospholipid structure, but the term 'liposomes' was found to be more relevant for describing both their character and composition. Liposomes belong to the class of biocolloids and can affect the physical properties (i.e., release, solubility, pharmacokinetics) or ADME profile of an encapsulated drug.

Liposomes can be classified according to the number of lamellae, their size and the preparation method. The main classes of liposome are: multilamellar vesicles, oligolamellar vesicles, small unilamellar vesicles and large unilamellar vesicles (LUV). A more detailed classification is presented in the article by Mozafari *et al.* [6].

Liposomes consist of amphiphilic molecules, mainly phospholipids. The physicochemical behavior of phospholipids is greatly influenced by the coexistence of hydrophilic and hydrophobic regions in their structure. Phospholipids also exhibit extended polymorphism and show lyotropic liquid-crystalline phase sequences [7]. The most abundant lipids in liposomes are phosphatidylcholines, in which a glycerol bridge links a hydrophobic tail to a hydrophilic polar head group. When in an aqueous environment, phosphatidylcholines form bilayers due to their molecular tubular shape. Other polar lipids–such as sphingolipids, cholesterol or other sterols, and lipid-conjugated polymers–can also be introduced in the liposomal bilayer [8].

Much work has been done on the physical characteristics, structure and lipid composition of liposomes that affect their size, size distribution, osmotic behavior, surface charge, morphology and elasticity. Their polymorphism, due to the lyotropic liquid crystalline state of their lipidic components, is responsible for phase transition phenomena and affects the thermodynamic and thermotropic behaviour of the 'liquid crystalline vehicles'. The interpretation of mesophases from gel to liquid crystalline state upon heating or cooling offers substantial knowledge for rationally designing DDSs. The dynamics and the microscopic and submicroscopic properties of liposomes (such as fluidity), however, are studied separately from the macroscopic properties (i.e., ion trapping and release characteristics, etc.) [9].

In biological environments, the behavior of a liposome is influenced by its membrane fluidity, surface charge, degree of hydration, size, size distribution and the preparation method. The biocompatibility and biodegradability of liposomes are essential characteristics for their therapeutic use. Recent developments in liposome technology have permitted the introduction of new strategies for controlling their physicochemical properties, stability and pharmacological effects after administration [8].

### 3.1 Stability of liposomes

The physicochemical parameters of liposomes are related to their stability, biodistribution and cellular uptake. As far as the term 'stability' is concerned, it is important to note that during the manufacturing process the chemical, biological, optical and physical status of liposomes must be controlled [8].

Chemical stability is related to the lipid composition of liposomes, especially to the unsaturated fatty acids that are part of the phospholipid structure. The oxidation process of phospholipids affects bilayer permeability because it alters the packing order in the bilayer. This problem can be circumvented by adding antioxidants or by controlling the preparation process with an inert gas.

Biological stability concerns the microbiological charge of liposomes and includes controls for viruses and bacteria yeasts in raw materials and final liposomal formulations. *In vitro* and *in vivo* tests could reveal the reactivity, interactability, toxicity, safety and biological responses of liposomes with and without an encapsulated drug. Coating liposomes with inert hydrophilic polymers, such as polyethylene glycol (PEG), can increase their stability. Polymer-coated liposomes are called sterically stabilized liposomes, or STEALTH liposomes™ (Sequus Pharmaceuticals), and exhibit an increased half-life in blood due to reduced interactions with blood proteins [2].

Optical stability of liposomes can be monitored by electron microscopy, after negative staining for suspensions or the freeze fracturing process, that is. This technique provides indications on the size, shape, morphology and number of lamellae of liposomes.

Physical stability of a liposomal preparation is related to its thermodynamic status, which determines the potential instability of the system after administration. The following parameters should be controlled: size and size distribution, ζ-potential, release properties (related to the dynamics of the lipidic membrane such as fluidity and elasticity), thermal behavior (related to the phase transition of the lipid bilayer) and, finally, the shape and morphology of vesicles.

In summary, the basic parameters affecting liposomal stability are:

- preparation method
- storage conditions
- biological behavior

### 3.2 Methods of characterizing liposomes

Liposome characterization is an important issue, and many analytical techniques can be employed. Thermodynamic, hydrodynamic, mechanical, chemical, microscopic, spectroscopic, diffraction, scattering, theoretical, computing and chromatographic techniques have being used in order to test the physical and chemical characteristics of liposomal formulations, such as lamellarity, permeability, encapsulation efficiency, degree of degradation and stability over time.

The most common techniques for studying the physical characteristics of liposomes are: thermal analysis techniques (TA), dynamic light scattering (DLS), also known as Photon Correlation Spectroscopy (PCS), non-flame atomic spectroscopy (NFAAS), microscopic techniques such as transmission electron microscopy (TEM), cryogenic TEM (Cryo-TEM) scanning electron microscopy (SEM) [10], freeze fracture electron microscopy (FFEM) [11], scanning tunnelling microscopy (STM) [12], scanning force microscopy (SFM) like atomic force microscopy (AFM) [13], lateral force microscopy (LFM) [14], Cryogenic atomic force microscopy (Cryo-AFM) [15], near-field scanning optical microscopy (NSOM) and magnetic resonance force microscopy (MRFM), Infrared and RAMAN spectroscopies, Nuclear Magnetic Resonance (NMR), Electron Paramagnetic Resonance (EPR) small-angle neutron scattering (SANS), small-angle X-ray scattering (SAXS) [5].

## 4. Microscopic techniques to study the morphology, dynamics and stability of liposomes

### 4.1 Optical microscopy

Optical microscopy is commonly used for direct real-time observations of lipid vesicle shape transformations, but horizontal resolution is limited with this technique due to the diffraction limit of light [16]. High-intensity dark-field microscopy is used to obtain real-time high-contrast images of liposomes with micrometer dimensions in aqueous solutions and to characterize the interactions between liposomal membranes and surfactants [17]. Among other optical methods, optical trapping of liposomes is useful in order to manipulate the molecules for sampling, mechanical testing, spectroscopic observation and chemical analysis [18]. Optical forces can also be used to cause submicrometer deformation in giant unilamellar liposomes and for measuring their bending rigidity and shear modulus [19].

In the past two decades, numerous technical approaches and major breakthroughs have taken place during attempts to achieve an optical resolution in the nanometer scale. A substantial improvement in resolution has been achieved with fluorescence microscopy. Multicolor confocal microscopy [20] and three-dimensional confocal fluorescence [21] are used for studying plasma half-life and biodistribution and dynamic processes such as fusion, fission, and expulsion of liposomes [22,23]. However, the diffraction barrier crumbles if features or distinct volumes of the object differ in an optical property such as the emission or absorption spectrum, the polarization, the excited state lifetime etc [24].

### 4.2 Electron microscopy

Electron microscopy is commonly used to establish the morphology, size and stability of liposomes. TEM, SEM, reflection electron microscopy and scanning transmission electron microscopy are techniques that provide information

concerning vesicle shape, bilayer thickness and the distance between bilayers in multilamellar vesicles and could potentially be used to describe phenomena such as fusion and aggregation. The first report describing electron microscopy observations was by Bangham and Horne and was published in 1964 [25]. Papahadjopoulos and Miller subsequently described the dynamics of small unilamellar vesicles in 1967 [26]. The limitations of electron microscopy methods include the need for high vacuum environment operation and the special sample treatments required, which induce irreversible change or damage in the sample.

### 4.3 Scanning probe microscopy

SPM is a powerful analytical technique that produces high-resolution images of a surface. This technique allows the visualization of single biological molecules, such as proteins and nucleic acids, and their complexes with lipid membranes. STM, a type of SPM, can be used to measure liposomal thickness, which can subsequently be used determine the shape, diameter and bilayer thickness of liposomes [27,28]. Among the advantages of SPM are the high spatial resolution and the variety of operating conditions this technique can be used in, such as vacuum, air and liquid. Furthermore, when this technique is used liposomes do not suffer from the structural perturbations that are triggered by the high vacuum conditions and the staining process used in other techniques. Nevertheless, SPM is an invasive method because probe forces are applied to the sample, which may induce deformation. Improvements in operation modes have been made and probe modifications have been developed, however, to minimize the friction in the tip-sample interface. AFM is the applied SPM technique most commonly used to describe liposomes.

### 4.4 Atomic force microscopy

AFM and SFM are very-high-resolution types of scanning probe microscopy that can create three-dimensional micrographs with resolution down to the nanometer and Angstrom scales. The first AFM device was invented by Binnig, Quate and Gerber in 1986 – 4 years after the invention of STM [29]–through modification of one of the STM instruments [3]. Today, AFM is the most widely used type of SPM due to the broad variety of combinations of environment (i.e., air, vacuum, liquid) and sample types (i.e., semiconductor, organic, biological) that can be investigated [30-32].

AFM uses a tip (probe) attached to a flexible cantilever of a specific spring constant, which is responsible for signal transduction. The mechanism of this technique is based on the detection of repulsive and/or attractive surface forces between the atoms of the sample and those of the tip (probe), which scans the surface of the sample [33]. During tip scanning of the sample surface, the cantilever deflects in the *z*-direction due to the surface topography while a small laser detects any bending or twisting of the cantilever. This optic mechanism enables detection of forces generally between $10^{-7}$ and $10^{-12}$ N [34].

AFM can operate in different modalities: repulsive (contact) and attractive (non-contact) mode [35,36]. In repulsive mode, the tip is in constant close 'physical contact' with the surface of the sample during the scanning process. In attractive mode, a stiff cantilever oscillates within the attractive region, meaning that the tip is quite close to the sample but does not touching it. The forces between the tip and the sample are quite weak, so it is more difficult to measure. Tapping mode (also known as intermittent-contact mode) is similar to non-contact mode except that the vibrating cantilever tip is brought closer to the sample so that at the bottom of its travel it just barely hits, or 'taps', the sample. The intermittent-contact motion of the tip eliminates lateral or shear forces, which are present when the non-contact mode is used and could deform or scrape the sample [37]. Tapping mode has become an important AFM technique, as it overcomes some of the limitations of both the contact and the non-contact modes. Limitations can arise due to the thin layer of liquid that forms on most sample surfaces in an ambient imaging environment, for example in air or some other gas. The amplitude of the cantilever oscillation in tapping mode is typically on the order of a few 10's of nanometers, which ensures that the tip does not get stuck in this liquid layer. The amplitude used in non-contact AFM is much smaller, as different forces are being measured. As a result, the non-contact tip often gets stuck in the liquid layer unless the scan is performed at a very slow speed. In general, it has been found that intermittent-contact mode is more effective than non-contact mode for imaging large scan sizes that may include great variation in sample topography. Additional measurement modes such as lateral force microscopy [38] and force modulation microscopy [39] have been developed to examine elastic surface properties.

In addition to taking topographic measurements, AFM can also provide force–distance curve measurements by force-spectroscopy application [40]. AFM devices can record the amount of force felt by the cantilever as the probe tip is brought close to and even indented into a sample surface and then pulled away. This technique can elucidate local chemical and mechanical properties such as adhesion and elasticity, and even the thickness of adsorbed molecular layers or bond rupture lengths [41,42].

AFM is ideally suited to characterizing nanoparticles. The technique offers three-dimensional visualization and both qualitative and quantitative information on many physical properties including size, morphology, surface texture and roughness [43]. In recent years, it has been demonstrated than an AFM probe can capture, handle and re-deposit target nanocarriers or biomolecules on substrates by applying a tip apex on a solid substrate [44] or by coating the tip with organic monolayers like polydimethylsiloxane (PDMS) [45] or oligoethylene glycol derivatives [46]. Chemically functionalized tips can also be used for chemical sensing and for

measuring specific interactions with biomolecules [47], and as a biosensor to study surface derivatization [48].

AFM has advantages over electron microscopy because it can generate three-dimensional surface images, whereas electron microscopy provides a two-dimensional projection sample. High resolution AFM is comparable in resolution to STM and TEM. Unlike electron microscopy, AFM does not require special sample treatments nor a vacuum environment, but can operate in both air and liquid [30-34]. Additionally, it is possible to study samples at low temperatures by using cryogenic AFM [49].

AFM is, however, an invasive technique, as the probe tip interacts with the sample. It is possible, therefore, that lateral (i.e., shear) forces will distort features in the image. The forces of interaction can be extensive in air due to capillary forces from the adsorbed fluid layer on the sample surface [50]. The combination of lateral forces and high normal forces can result in reduced spatial resolution and scraping of soft samples (i.e., biological samples, polymers, silicon) [51]. An operation mode such as tapping mode applies lower forces, however, virtually eliminating lateral forces, and induces less damage to soft sample in air [37]. The disruption of soft images (e.g., when using biological samples) is significantly less likely when tapping mode is used in a liquid environment, as high adhesion forces are reduced due to the water film on samples. During tapping mode scanning, the vertically oscillating tip alternately contacts the surface and lifts off, generally at a frequency of 50,000 to 500,000 cycles *per se*cond. The value of the resonance frequency depends on the type and shape of the cantilever and varies according to the square root of its spring constant. Many chemical [52,53] and shape [54] modifications to probe tips have been developed in order to limit sample damage and topographical artifacts.

## 5. Studies of morphology, stability and dynamics on liposomal DDSs using AFM

### 5.1 Atomic force microscopy studies of the morphology and stability of liposomal drug delivery systems

To study the suitability of liposomes as DDSs, the geometry, size and size distribution are key parameters to evaluate, as are both *in vitro* and *in vivo* stability, encapsulation efficiency, and finally applicability in the pharmaceutical field. A variety of TEM techniques have been developed for this purpose. At present, freeze fracture electron microscopy and cryogenic TEM are the approaches most commonly used to describe the entrapment of drug into liposomes [55,56], but these techniques require stringent and laborious sample treatments. Negative staining [57], one of the methods used, can produce artifacts due to the staining processes and the analysis conditions. AFM is routinely used to study the interaction between drug and lipid membranes and to understand the properties of biomembranes and the correlated processes, such as cell adhesion, membrane fusion and drug membrane interaction [58-60]. Clarification of lipid bilayer reorganization that occurs after drug incorporation and its effect on the permeability and fluidity of the bilayers provides useful information on the encapsulation efficiency and stability of the liposomal formulation.

Additionally, AFM can obtain visual information on the protein–drug carrier conjugation in a fast and reliable manner. Anabousi *et al.* [61] imaged the binding of transferrin to liposomes, which takes place via three different coupling methods. This process might prove useful for producing DDSs that specifically bind to transferrin receptors, which are overexpressed on cell types of therapeutic interest (i.e., tumor cells). AFM showed that liposomal morphology changes after transferrin conjugation, with small globular structures appearing on their initially smooth surface, and their diameter also increased. Furthermore, conjugation of polymers (e.g., PEG) on the surface of liposomes prevents rupture upon mica adsorption, thus indicating an increase in stability. Casals *et al.* [62] investigated the covalent conjugation of fibrinogen onto liposomes, because it has been reported that liposomes enhance fibrinogen adsorption on thrombogenic surfaces. After mica adsorption the liposomes ruptured, thus converting into supported planar bilayers. Formation of an additional lipid layer is an indication of fibrinogen conjugation on liposomes. Furthermore, Ho *et al.* [63] studied antibody conjugation on liposomes, for biosensor applications, by using tapping mode AFM. Antibody conjugation generated protrusions on the liposomal surface that changed from smooth to rough.

Zhao *et al.* [60] studied the effect of cholesterol on the molecular interactions between an antineoplastic drug (paclitaxel) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) by using the lipid monolayer at the air–water interface as a model of the biological cell membrane. AFM studies on the morphology and roughness of the mixed monolayer confirmed that cholesterol enhances the intermolecular forces between paclitaxel and DPPC and produces an area-condensing effect, thus increasing the stability of the monolayer. This research may provide useful knowledge for optimizing the liposomal formulation of paclitaxel and other anticancer drugs.

The morphological changes of liposomes and the effect of such changes on the stability of the interaction between the Am-b-CD/DNA complex and liposomes were examined with AFM by Tavares *et al.* [64]. The use of anionic liposomes for efficient and safe DNA transfection is an appealing alternative method of gene delivery, as was observed in the findings of Patil *et al.* [65]. AFM images showed that complex incorporation in liposomes did not change their shape, but positively affected their size distribution and stability, as no tendency towards spreading on the substrate was observed.

Ruozi *et al.* investigated the influence of liposome phospholipid composition, both on encapsulation and on

oligonucleotide carrier capacity *in vitro* [66]. Liposomes composed of neutral and/or cationic lipids with different molar ratios were complexed with 50-fluorescein-conjugated 29-mer phosphorothioate oligonucleotide (PS-ODN). The interaction was evaluated using AFM, and the authors found that the liposomes with the cationic lipids interacted with the nucleic acid to form a dense irregular structure in which the lipid bilayer completely embedded the PS-ODN. The liposome/PS-ODN molar ratio used influenced the tendency of the liposomes to aggregate in large structures.

Another study on liposomal stability, performed by Azevedo *et al.* [67], demonstrated the stability of liposomes containing 5-fluorouracil in a gel formulation.

In our recent studies we used AFM to compare the morphology and stability of egg phospatidylcholine (EPC)/dipalmitoylphosphatidylglycerol (DPPG) liposomes of different sizes produced with two different methods: the thin film hydration method (TFHM) and the novel heating method (HM) [68]. The size of liposomes was reduced by extrusion through polycarbonate membranes (pore sizes 200 nm and 100 nm). AFM was performed in tapping mode by using a Nanoscope diInnova (TappingMode™; Veeco Instruments, Inc., Santa Barbara, CA) with an Innova scanner possessing a maximum range of 100 × 100 × 7.6 μm. Phosphorus-doped silicon tips with a nominal constant range from 20 N/m to 80 N/m were used. Scanning speed was optimized between 1.0 Hz and 2.0 Hz, depending on the scan size. Liposomes were deposited on freshly cleaned muscovite mica, washed with water and analyzed immediately with intermittent contact.

Liposomes prepared with the TFHM method (200 nm) tended to collapse on a mica surface to produce supported lipid bilayers (SLBs), whereas liposomes produced with the HM method retained a spherical shape and appeared stable. Liposomes 100 nm in size prepared with both methods and scanned with the same image force of the AFM tip showed different morphology. Reviakine and Brisson [69] provided many details about vesicle adsorption on mica and demonstrated that only vesicles with sizes below a critical rupture radius remained intact. In our studies, liposomes prepared with the HM method revealed a convex shape (**Figure 1**) that remained stable through the first hour of deposition. On the other hand, liposomes prepared with the TFHM method presented various structures, including convex shape, planar shape and 'concave' shape characterized by a depressed central portion and a higher outline (**Figure 2**). This characteristic shape modification has also been reported in many previous works [70].

### 5.2 Atomic force microscopy studies of the rigidity and bending modulus of liposomal drug delivery systems

The mechanical properties of liposomes are key parameters for shape and stability and have an important role in processes such as fusion and adhesion. A crucial property of liposomes is the bending rigidity, which is closely related to the activities of liposomes and the gel–liquid phase transition of the liposome bilayer. The most popular method to determine the bending rigidity of unilamellar liposomes is micropipette aspiration, in which the bending modulus is deduced from the surface tension of the sucked projection [71]. Alternatively, optical tweezers can be used to calculate bending rigidity and shear modulus [72,73] by measuring submicrometer deformation of giant unilamellar liposomes under optical forces. These experimental techniques are limited, however, to giant unilamellar liposomes of at least 10 microns in diameter.

AFM can be used to probe the micromechanical properties of drug-encapsulating liposomes, as the technique allows force–deformation measurement of submicron liposomes. A variety of force measurements on liposome rigidity and elastic modulus have been published. Many mathematical models have been generated using force curve analysis in order to calculate Young's modulus and the bending modulus of adsorbed vesicles. Elastic properties of liposomal vesicles can be used to determine the success of drug encapsulation.

Ramachandran *et al.* [74] examined cisplatin encapsulation in liposomes. Quantitative analysis of AFM force mapping revealed an increase in liposome stiffness after cisplatin encapsulation. Young's modulus (*E)* calculations were based on a Hertz–Sneddon model [75]. Similar local elasticity measurements on vesicles containing amyloid β, a peptide involved in protein conformational diseases (e.g., Alzheimer's disease, Parkinson's disease, type II diabetes, bovine spongiform encephalopathy), demonstrate stiffness increase after calcium uptake [76]. The size and shape of liposomes after amyloid β incorporation changed from small and spherical to large and disk shaped.

AFM can also be used for studying the physicochemical properties of layers of immobilized liposomes, which can be used in drug delivery and diagnostics [76]. Brochu and Vermette [77] used tapping mode AFM to image layers of intact liposomes immobilized on NeutrAvidin-coated PEG-Biotin:HApp layers on borosilicate glass. Immobilizing liposomes loaded with a therapeutic agent and able to retain their integrity on biomaterials can lead to drug delivery systems for local administration. Young's moduli calculations on such layers were obtained from force-distance measurements, using colloidal probe tips and a modified Hertz model derived from Dimitriadis *et al.* [78]. Similar studies by Tarasova *et al.* [79] demonstrated the elastic deformation of affinity-bound liposomes under various stress conditions and the reduction of interfacial friction in aqueous environments after PEGylation. Vermett *et al.* [80] also reported that immobilized PEGylated liposomes can be significantly compressed without rupturing.

The rigidity of liposomes is one of the most important properties affecting drug delivery effectiveness, as assessed by particle stability, release profile of encapsulated drug, blood circulation time and depends on vesicle size, phospholipid

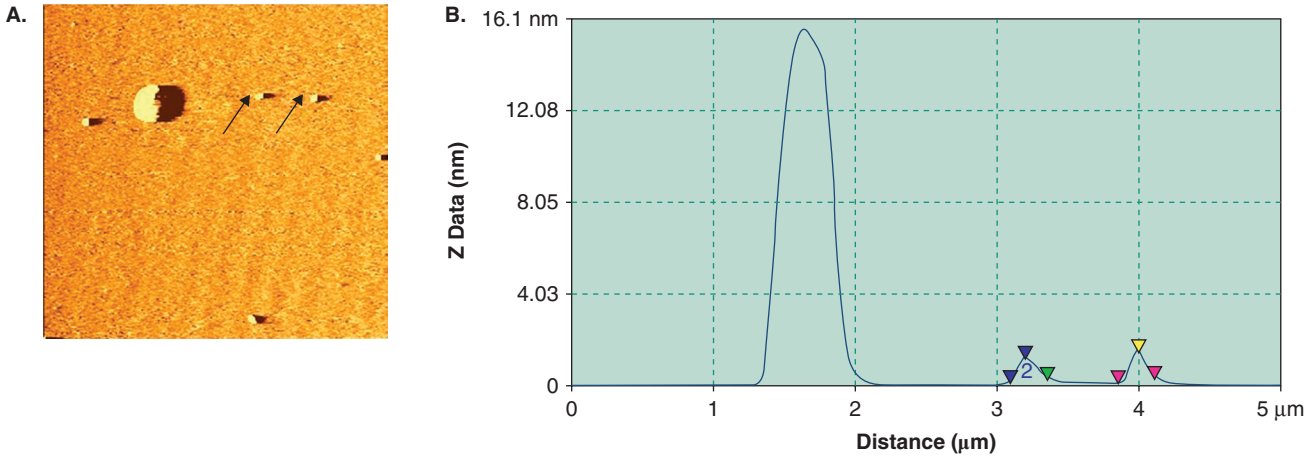


**Figure 1. Tapping mode atomic force microscopy image of liposomes 100 nm in size produced by the novel heating method.** **A.** The convex shape of liposomes prepared by this method. These liposomes remained stable throughout the first hour of deposition. **B.** A cross section of the height and diameter of the convex shape liposomes, indicated by triangles.

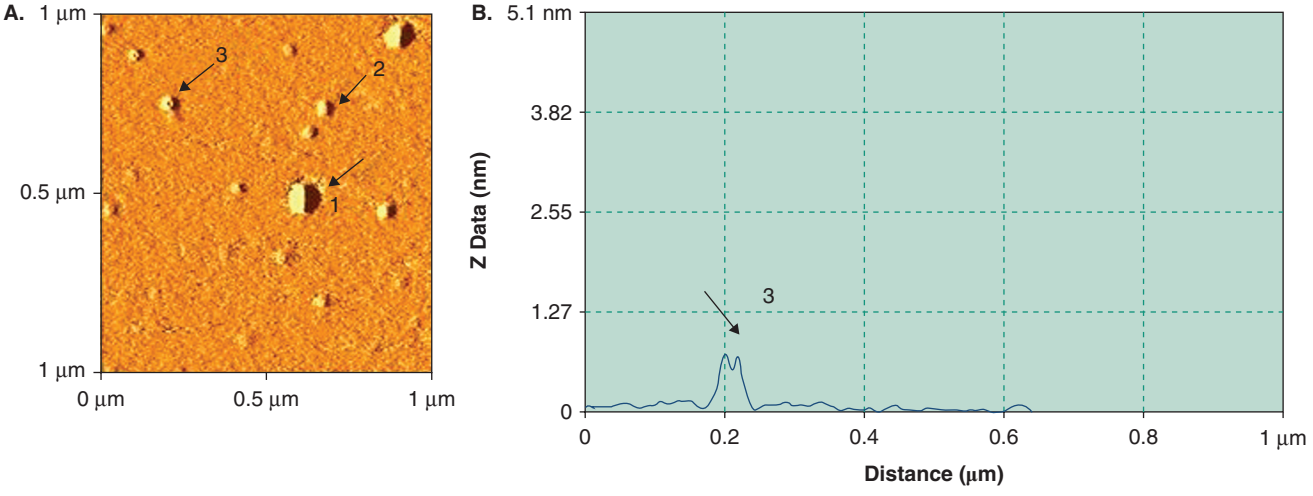


**Figure 2. Tapping mode atomic force microscopy image of liposomes 100 nm in size produced by the traditional thin film hydration method. A.** The various structures of the liposomes prepared with the thin film hydration method, including convex shape (arrow 1), planar shape (arrow 2) and 'concave' shape characterized by a depressed central portion and a higher outline (arrow 3). **B.** A cross section of the height and diameter of the 'concave' shape liposome indicated by black arrow 3.

composition and cholesterol incorporation. Specifically, the rigidity of liposomes decreases with an increase in size or a decrease in transition temperature of the liposomal bilayer [81], whereas cholesterol incorporation changes the packing order of the bilayer thus influencing its fluidity. Delorme and Fery [82] demonstrated that AFM can be used to calculate directly the rigidity of DPPC liposomes immobilized on a silicon substrate by using Hertz model and Reissner model [83,84]. Nakano *et al.* [81] demonstrated a unique method for evaluating liposomal rigidity using AFM and DLS. This approach involved imaging the change in liposome height against liposome diameter (*H*/*P*) after mica adsorption. AFM calculates the value of height (*H*), whereas particle size (*P*) is measured with DLS.

Liposomal stability can be enhanced by covalent attachment of biocompatible polymer systems, such as PEG or peptides [85,86]. Liang *et al.* [87] used AFM to study the morphology and stability of small unilamellar egg yolk phosphatidylcholine liposomes modified with the Pluronic™ copolymer (BASF, Inc., North Mount Olive, NJ), also known as poly(oxyethylene)–poly(oxypropylene)–poly(oxyethylene) (PEO–PPO–PEO). Copolymer incorporation significantly changed the morphology and topography of the liposomes and increased the bending modulus, which relates to liposomal stability and bilayer strength. The molecular weight and chain lengths of the PEO and PPO components of PEO–PPO–PEO determine the morphology of liposomes on mica substrate. Furthermore, Li *et al.* [88] demonstrated

the formation of globular, nonaggregative liposomes with increased stability upon polymer incorporation.

We have recently studied the effect of thiol-reactive lipid (Mal-SA) encapsulation on liposome elasticity [89]. Thiol-reactive liposomes composed of EPC/DPPG/Mal-SA were prepared using the TFHM (**Figure 3A**). An increase in image force of the AFM tip, produced by decreasing the set point in tapping mode, deformed liposomes without Mal-SA. These liposomes acquired a concave shape with extremely depressed centre (**Figure 3B**). When the image force was decreased, the liposomes almost returned to their initial shape (**Figure 3C**), thus revealing elastic deformation under high compressive forces. Liposomes with Mal-SA were damaged, however, and plastically deformed under the same conditions (results not shown). Rupture of liposomes resulted in a rather smooth lipid film, easily distinguished from the mica surface. An AFM image obtained 5 min later showed semi-fusion between the two liposomes that were initially in contact. In both cases, force curves measurements (results not shown) showed that the elastic and plastic properties of liposomes could be estimated by observing the size of the elastic/plastic indentation left by AFM tip.

### 5.3 Atomic force microscopy studies of the dynamics of liposomal drug delivery systems

Direct real-time observation of dynamic transformations of lipid vesicles will bring new biophysical information on cellular responses into the field of medicine (e.g., photodymanic therapy). By using fluid tapping mode AFM, Thomson *et al.* [90] observed liposomes interacting, in a cationic dynamic fashion, with positively charged aminopropylsilane mica. Liposomes slowly collapse on mica and rearrange into flat patches with the hydrophobic chains exposed, whereas they remain intact on aminopropylsilane-mica. The tip induced fusion of cationic liposomes at lower magnification images. Cationic liposomes are attractive as DNA carriers in gene therapy, as they avoid the immunogenic problems associated with viral delivery, are simple to prepare and can carry DNA with practically no size limitation. A number of studies found that the size and shape of the liposome–plasmid DNA complex is correlated with its *in vitro* transfection efficiency [58,91-94]. Transfection efficiency can be influenced by lipid composition, vesicle size and size and shape of the complexes. Kawaura *et al.* [92] demonstrated the effect of cholesterol-derivative incorporation on complex size and consequently on transfection efficiency, with complexes of moderate size proving to be the most effective. Ruozi *et al.* [93] studied the effect of the fusogenic co-lipid DOPE (1,2-dioleyl-sn-glycerol-3-phosphatidylethanolamine) on transfection efficiency of liposomes consisting of dimethyldioctadecylammonium bromide (DDAB), phosphatidylcholine (PC), a phospholipid derivative of polyethylene glycol (DSPE-mPEG) and DOPE, complexed with the plasmid pCMVβ. Incorporation of DOPE resulted in compact aggregates of large diameter with increased stability and transfection efficiency that completely embedded DNA molecules. Induction of liposome fusion by plasmid DNA has been reported by Almofti *et al.* [94]. This phenomenon leads to the formation of homogeneous lipid particles that encapsulate DNA. The biophysical characteristics of these particles depend on the ± charge ratio. The transfection efficiency of complexes formed in the presence of amyloid β [95] depends on the shape rather than the size of the complex.

We have also observed using tapping mode AFM the fusion of liposomes, as induced by the AFM tip. **Figure 4** shows AFM images of EPC/DPPG/Mal-SA liposomes prepared by the HM. With increased tip force, disruption of lipid bilayers was observed (results not shown). AFM scanning with the initial image force showed spontaneous reorganization of lipid bilayers, creating semispherical separated liposomes. Liposome shape, directly after fission, is the result of the coupling between Gaussian curvature and the bending energy of liposomes [96].

Jass *et al.* [97] studied with tapping mode AFM proteoliposome coalescence (fusion) onto solid supports and the mechanism of SLB formation on hydrophilic and hydrophobic surfaces. The effect of $Ca^{2+}$ in increasing the rate of the process was also observed.

Ramachandran *et al.* have characterized liposomal drug formulations [74]. This group examined the dynamic interaction between A2780 cells and cisplatin-encapsulating liposomes, studying the size, drug encapsulation, stability, effectiveness and cellular uptake of liposomes via endocytosis, as well as the release kinetics, by repeating the encapsulation study at different time intervals. Smaller liposomes proved more effective in causing death of cancer cells.

AFM has been used to characterize and study lipid bilayer symmetry in multicomponent SLB systems. By using AFM imaging, the dynamic of conversion from a mixed symmetric to an asymmetric lipid bilayer can be observed. Goksu *et al.* [98] showed that carefully designed experiments along with real-time AFM imaging can provide quantitative details on the mechanisms and factors controlling vesicle rupture, domain shape and size, phase transformations, and some model biological interactions. Kinetic analysis of their AFM data revealed that lipid flip-flop at the interface between symmetric and asymmetric domain regions was controlling the rate of conversion. Giocondi *et al.* [99] also performed a real-time study on the time-dependent topology of gel-phase domains in SLBs composed of a mixture of DOPC and DPPC. In this study, the gel and liquid crystal phases were distinguished by thickness differences. Finally, Choucair *et al.* [100] studied peptide–membrane interactions, which revealed the specified interaction of the peptide Aβ (1 – 42) only with the gel phase of DPPC domains in phase separated DOPC/DPPC lipid bilayers.

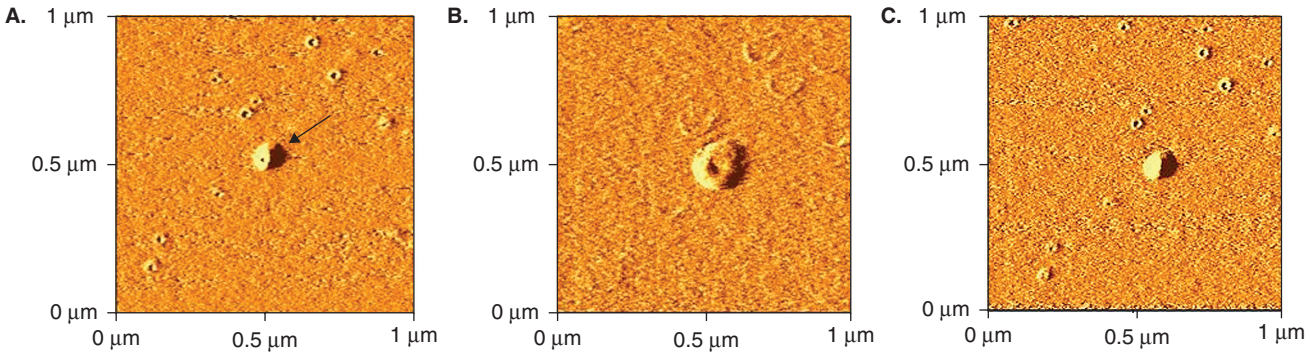


**Figure 3. The effects of thiol-reactive lipid (Mal-SA) encapsulation on liposome elasticity. A.** Thiol-reactive liposomes composed of egg phospatidylcholine, dipalmitoylphosphatidylglycerol and Mal-SA, as prepared using the thin film hydration method. The black arrow indicates convex shaped liposomes. **B.** Atomic force microscopy characterization of liposomes without Mal-Sal under increasing tip image force. These liposomes acquired a concave shape with extremely depressed centre. **C.** After a decrease in tip image force, the liposomes almost return to their initial shape, thus revealing elastic deformation under high compressive forces.

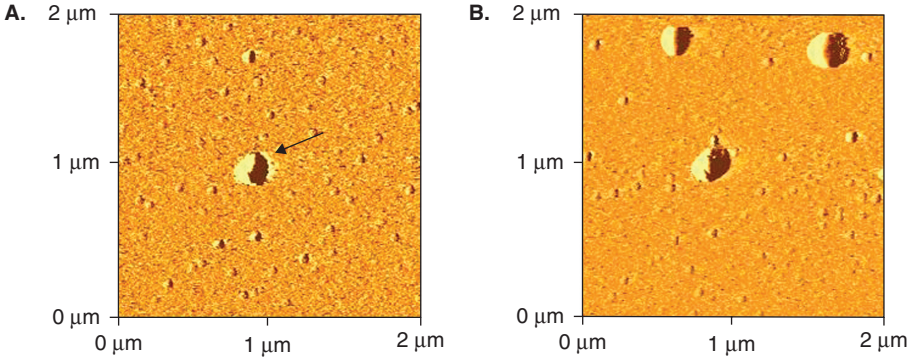


**Figure 4. Fusion of thiol-reactive lipid (Mal-SA) encapsulated liposomes prepared with the heating method. A.** Liposomes composed of egg phospatidylcholine, dipalmitoylphosphatidylglycerol and Mal-SA, as prepared using the heating method. With high compressive tip force, the lipid bilayers were distorted (results not shown). **B.** Atomic force microscopy scan using the initial image force shows spontaneous reorganization of the lipid bilayers, creating semispherical separated liposomes.

## 6. Conclusion

Due to their high loading capacity and their flexibility to accommodate photosensitizers with variable physicochemical properties, liposomes can be used as carrier and delivery agents for a great variety of molecules. The factors affecting drug entrapment and retention in liposomes or vesicles, however, are not completely understood. AFM is a microscopic technique used to examine the morphological characteristics and stability of liposomes encapsulating drugs or liposomes with ligands incorporated in their lipid bilayer. AFM provides information on the interaction between the lipid carriers and the active compounds and the elastic, chemical and adhesion properties of the carriers. Additionally, AFM can be used to examine the dynamic interaction of drug-encapsulating liposomes with cells or surfaces and to observe shape transformations and transitions. Selective fusion and plastic deformation of liposomes can also be induced. Recently, AFM has been applied for capturing, handling and redepositing target nanoparticles or biomolecules on substrates by using chemical functionalized tips. The rapid evolution of the AFM technique will allow the optimization of current applications and the design of new effective DDSs.

## 7. Expert opinion

During the continuous research for optimized liposomal DDSs, a variety of techniques have been used for the physicochemical characterization of liposomes in terms of lamellarity, permeability, encapsulation efficiency, degree of degradation and stability over time. Among them, AFM has made its mark as a topographic measurement tool. AFM has the ability to create three-dimensional micrographs with resolution down to the nanometer and Angstrom scales and is also able to probe the nanomechanical properties of

liposomes in various environments (e.g., vacuum, air, liquid). This technique is an essential tool for measuring binding forces and electrostatic forces, thus providing information on liposome stability and local adhesive or elastic (compliance) properties, without causing structure perturbations. Moreover, operation costs, laboratory space and complexity are reduced with AFM compared to SEM and TEM.

AFM is a technology in its infancy and will be develop more and more as a spectroscopic and topographic tool. In near future, AFM is likely to be come into widespread use for studying both the pharmacokinetic properties and the pharmaceutical effectiveness of liposomal drug formulations and will contribute in optimizing the ADME profile of an encapsulated drug. The necessity of reducing the adverse effects of a therapeutic agent while increasing its therapeutic effectiveness led the interest of the scientific community towards the development of formulations for targeting a drug to a specific site. Active targeting of liposomes involves the conjugation onto the liposomes surface of specific ligands (i.e., monoclonal antibodies, vitamins, peptides, proteins, aptamers) that interact with target cells. The study of this interaction is of great importance for the effective design of DDSs. AFM could make a crucial contribution to the study of physico-chemical interactions between liposomes and biomolecules. Through using appropriately modified tips, AFM could also be vital in directly measuring the binding forces of individual ligand–receptor pairs. By using this approach, the rupture length of the ligand–receptor bond could be calculated. Additionally, binding and rupture forces during DNA hybridization can be observed by immobilizing a single-stranded DNA on a surface and examining this DNA using a complementary sequence on a spherical probe attached to the AFM cantilever. Probing ligand-targeted liposomes with cantilever tips onto whichever specific biomolecules have been chemically conjugated can provide knowledge on the ligand–biomolecule interaction and a better understanding of the mechanism of action of ligand-targeted liposomes.

## Declaration of interest

The authors declare no conflict of interest and have received no payment in preparation for this manuscript.



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