Nanoscale probing of liposome encapsulating drug nanocrystal using Atomic Force Microscopy-Infrared Spectroscopy.

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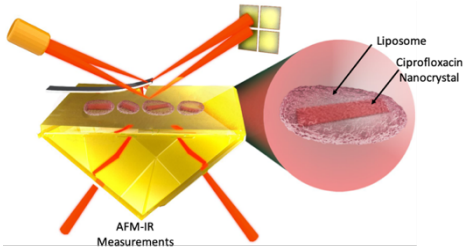
Abstract



Use of liposomes encapsulating drug nanocrystals for the treatment of diseases like cancer and pulmonary infections is gaining attention. The potential therapeutic benefit of these engineered formulations relies on maintaining the physical integrity of the liposomes and the stability of the encapsulated drug. With the significant advancement in the microscopic and analytical techniques, analysis of the size and size distribution of these nanosized vesicles is possible. However, due to the limited spatial resolution of conventional vibrational spectroscopy techniques, the chemical composition of individual nanosized liposome cannot be resolved. To address this limitation, we applied atomic force microscopy infrared spectroscopy (AFM-IR) to assess the chemical composition of individual liposomes encapsulating ciprofloxacin in dissolved and nanocrystalline form. Spatially resolved AFM-IR spectra acquired from individual liposomes confirmed the presence of peaks related to N-H bending vibration, C-N stretching and symmetric and asymmetric vibration of the carboxyl group present in the ciprofloxacin. Our results further demonstrated the effectiveness of AFM-IR in differentiating the liposome containing ciprofloxacin in dissolved or nano-crystalline form. Spectra acquired from dissolved ciprofloxacin had peaks related to the ionised carboxyl group, i.e. at 1576 cm^{-1} and 1392 cm^{-1}, which were

either absent or far weaker in intensity in the spectra of liposomal sample containing ciprofloxacin nanocrystals. These findings are highly significant for pharmaceutical scientists to ascertain the stability and physicochemical composition of individual liposomes and will facilitate the design and development of liposomes with greater therapeutic benefits.

Introduction

Liposomes are popular nanocarriers for drug delivery applications. Liposomal formulations are widely accepted as pharmaceutical products due to their biocompatibility and their ability to encapsulate drugs with complex physical and chemical characteristics.[1, 2] More than a dozen liposome-based formulations have been approved as marketed products and many more are in the final stages of clinical trials. Some of the approved liposome-based formulations are used for the treatment of diseases like cancer, AIDS- related Kaposi's sarcoma, invasive severe fungal infections, hepatitis, influenza and pain management.[1-3] An inhaled liposomal amikacin product was recently approved in 2018 and represents the first to be approved by jet nebulization.[4] The wide popularity of these lipid carriers for drug delivery applications is also due to their ability to alter the release profile, enhance the stability and increase the bioavailability of the encapsulated drugs.[2] Use of liposomes for sustained drug release, prolonging the drug half-life to reduce the systemic drug toxicity and achieving the desired therapeutic benefit is gaining significant attention.

Among the different strategies to achieve sustained release, in situ crystallisation (within liposomes) of encapsulated drug is widely accepted.[5] This approach also facilitates increased drug loading and improved stability of loaded drugs.[3, 6] Drugs such as doxorubicin,[7] topotecan,[8] vinorelbine[9] and ciprofloxacin[10] have been reported to form nanocrystals in situ, either immediately or under the influence of manufacturing process. Ciprofloxacin in particular, does not form crystals immediately and an additional step is needed to induce crystallisation.[10] In our previous study, we induced the formation of ciprofloxacin nanocrystals within the liposomes by freeze-thaw. During the process of freeze-thaw, ice crystals form inside the liposomes and these ice crystals serve as a site of nucleation for crystal growth.[10] Formation of ciprofloxacin nanocrystals resulted in changes in the pharmacokinetics of drug dissolution and release from the liposomes. Compared to liposomes containing ciprofloxacin in a dissolved state, drug release from the liposomes encapsulating nanocrystals (solid state) was slower and remained sustained for a longer

duration.[2, 10] These findings indicate the importance of the physicochemical state of the encapsulated drug within the liposomes. In addition, it is equally important to maintain the stability of encapsulated nanocrystals within the liposomes.

From the application point of view, it is critical to characterise the physicochemical properties of liposomes encapsulating engineered nanocrystals. The average size and size distribution, shape, polydispersity, and surface charge of the liposomes as well as the encapsulation efficiency and solid-state characteristics of the nanocrystals are the most critical physicochemical properties which will influence the stability of, and the drug release characteristics from these liposomes. Multiple suites of techniques may be used to evaluate these characteristics. For example, the size, size distribution and polydispersity measurement can be evaluated using electron microscopy technique, fluorescence microscopy, field flow fractionation, dynamic light scattering, flow cytometry, tunable resistive pulse sensing (TRPS), size exclusion chromatography, atomic force microscopy and nanoparticle tracking analysis.[11] Similarly, the surface charge can be measured using laser doppler velocitometry, zetasizer and TRPS. Measurement of the encapsulation efficiency can be done analytically by measuring the amount of drug released from the liposomes after dissolution in organic media (fully releasing all of the encapsulated drug) or by measuring the amount of free drug present in the supernatant using techniques like spectrophotometry, fluorescence spectroscopy, enzymatic assays, gel electrophoresis, field flow fractionation, or chromatographic methods as high-performance liquid chromatography and liquid chromatography-mass spectrometry method.[11] To characterize the solid state properties of the nanocrystals, techniques such as small angle X-ray scattering (SAXS)[3] and cryo-transmission electron microscopy (cryo-TEM)[2] are often used. A microscopic technique like cryo-TEM can provide valuable information on the physical integrity of the liposomes, their size distribution and presence of encapsulated nanocrystals within the liposomes. However, the chemical composition of individual liposomes cannot be evaluated with this technique. Similarly, the majority of spectroscopic and chromatographic techniques used for chemical characterisation of liposomes are not capable of evaluating the chemical composition at the nanoscale. All the data generated are averaged and only represent the bulk scale chemical composition of the sample. This averaged information about the sample physical and chemical characteristics are highly valuable. However, definitive prediction of the stability and chemical composition of a sample at the individual liposomal level cannot be ascertained. This is a major limitation and can be

overcome only by using techniques that can physically and chemically characterise the liposomes at nanoscale.

Photothermal-induced resonance spectroscopy (PTIR) with the resolution of atomic force microscopy (AFM) can chemically characterise a sample at the nanoscale.[12, 13] This technique has been successfully applied for assessment of nanoscale chemical composition of polymer blends,[14, 15] nanoparticles,[16] drug distribution in polymer blends,[17] cells,[18, 19] bacteria,[20-22] dry powder aerosols,[23] aerosol particle containing bacteriophage[24] and extracellular vesicles.[25, 26] However, use of AFM-IR for analysing the complex nanoscale composition of liposomes encapsulating drug nanocrystals has not yet been reported. AFM-IR allows high resolution probing of multiple liposomes and multiple locations within a single liposome. Results obtained from these analyses will allow researchers to better understand the distribution of drugs and the state of drugs within the liposomes in a sample. Understandings of the precise chemical characteristics of individual liposomes will significantly aid scientists to better design the formulation to optimize the stability and desired therapeutic benefits.

In this study, we present the application of AFM-IR for probing nanochemical and nanomechanical domains across the empty liposomes, liposomes encapsulating liquid ciprofloxacin, liposomes containing ciprofloxacin nanocrystals and liposomes obtained after reconstitution of spray dried powders.

Materials and Methods

Materials

Ciprofloxacin liposomes (control: 50 mg mL^{-1}) and empty liposomes (without encapsulated ciprofloxacin) in a pH 6.0 histidine buffer (Exelead, Indianapolis, IN, USA and Northern Lipids Incorporated (Burnaby, BC, Canada) were kindly gifted by Aradigm Corporation (Hayward, CA, USA). Sucrose, sodium chloride, triethylamine (TEA), magnesium stearate, isoleucine, and adult donor bovine serum were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Nanosep Omega centrifugal filtration devices, 10k molecular weight was purchased from Pall Australia Pty Ltd (Victoria, Australia) and HEPES, free acid from Dojindo (China). The deionised water (DW) used was sourced from Modulab Type II Deionization System (Continental Water System, Sydney, Australia). All the chemicals used in the study were of analytical grade except methanol which was HPLC grade.

Liposome and liposomal powder preparation

Freeze-thaw of liposomes to obtain nanocrystals

Liposomes containing encapsulated ciprofloxacin nanocrystals were produced by the freeze-thaw method reported previously using sucrose as a cryoprotectant.[2] Briefly, liposomes containing ciprofloxacin (50 mg/mL) were diluted four times with 100 mg/mL sucrose solution and DW. The diluted dispersion was transferred to a glass container followed by submersion into liquid nitrogen for 2 min. The frozen dispersion was then allowed to thaw by placing the glass container in a water bath maintained at 25 °C to obtain liposomes with encapsulated nanocrystals.

Spray drying of liposomes encapsulating nanocrystals

After thawing, the formulation was mixed thoroughly using a vortex mixer to ensure homogeneous dispersion of the liposomes. Dispersed liposomes in the suspension containing 3 mg/mL of total solutes and a mass ratio of 2 (sucrose):1 (lipid) were further mixed with 2 % w/w magnesium stearate and 5 % isoleucine. This mixture was pumped into the spray dryer (B-290 mini spray-dryer, Büchi Falwil, Switzerland) at a feed rate of 1.4 mL/min to obtain a spray dried powder of the liposomes. Spray drying parameters were set at an inlet air temperature of 50 °C and an outlet air temperature of 33-35 °C, atomizer setting of 742 L/h and aspirator setting of 35 m^3/h. Spray dried powders were collected and stored under controlled humidity (<15%) until further characterisation.

Assessment of drug encapsulation

To determine the change in the percentage of ciprofloxacin encapsulated in the liposomes, the concentrations of free drug in the control sample, after freeze-thaw and after reconstitution of the spray dried powder sample (1 mg/mL in normal saline) were measured and compared to the total ciprofloxacin. Aliquots of 400 μL from each sample were transferred to Nanosep Omega centrifugation devices (Pall Australia Pty Ltd, Victoria, Australia) composed of modified polyethersulfone membrane filters (molecular weight cut-offs of 10,000). These devices have been successfully applied to separate free ciprofloxacin from ciprofloxacin encapsulated within the liposomes.[27] All the samples were centrifuged at 10,000 rpm (8100 × g (gravitational force)) for 18 min. The filtrate was diluted 20-fold with DW and loaded into HPLC vials for quantitative analysis of free ciprofloxacin. In-order to measure the total concentration of ciprofloxacin (free and encapsulated) in the

formulation, 1 mL of each uncentrifuged sample (control sample, freeze-thawed and reconstituted spray dried powder sample) was diluted with 9 mL of 80% methanol to solubilize liposomes. Following the solubilisation of the liposomes, samples were centrifuged for 15 min at 13,400 rpm to pellet any insoluble components and the supernatant was diluted 4-folds with DW to allow for ciprofloxacin quantitation.

Ciprofloxacin content was measured using previously reported method.[27] In general, HPLC device (Shimadzu, Japan) was loaded with a Phenosphere-Next C-18 column (5 mm, 4.6 × 150 mm, Phenomenex, USA) as a stationary phase at 35 °C, while a mixture of 0.5 % TEA in water, pH 3.0 and 100 % methanol (78: 22 v/v) was used as the mobile phase. The isocratic elution of mobile phase was performed at a flow rate of 0.9 mL min^{-1}. Using a 277 nm wavelength, ciprofloxacin was detected and quantified. By comparing the free drug content to the total amount of drug, the percent encapsulation was established.

Assessment of liposomal size distribution and morphology

Cryogenic transmission electron microscopy (cryo-TEM)

Prior to imaging, the formulations containing empty liposomes and liposomal ciprofloxacin after freeze-thaw were diluted 10 times with DW, while the spray dried powder formulation was reconstituted to achieve a ciprofloxacin concentration of 1 mg/mL. An aliquot of 3 µL was transferred to a glow discharged Lacey formvar/carbon grid (ProSciTech, Australia) in a chamber controlled at 22 °C and 85 % RH. These grids were blotted for 1 s on a filter paper using a blot force of -1. Blotted grids were dipped into liquid ethane using a Vitrobot (Thermo Fisher Scientific, USA) or a Leica EM GP device (Leica Microsystem, Germany) to vitrify the samples. The vitrified samples were stored in liquid nitrogen prior to cryo-TEM imaging. All the images were acquired using a JEOL 2100 instrument (JEOL, Japan) or a Talos Arctica TEM (Thermo Fisher Scientific, USA) operating at 200 kV.

Dynamic light scattering (DLS)

The particle size distribution of the liposomes (empty and containing encapsulated ciprofloxacin nanocrystals) and the reconstituted spray dried powder formulation were analysed using Malvern Zetasizer Nano ZS (Malvern, UK) with the following instrument parameter settings: temperature 23 °C, viscosity 0.887 cP, refractive index 1.34, intensity set point 300 kHz, channel width 10 µs, scattering angle 90° and run time of 5 min. All the

measurements were done in triplicate allowing for a determination of the mean and standard deviation.

Atomic force microscopy (AFM- Tapping mode)

The liposomal dispersion obtained after removal of free ciprofloxacin was collected from the nanosep device (see method for assessment of drug encapsulation). The dispersion was diluted 1:10 in ultrapure water (5 µL) and was placed on top of zinc selenide prism for 3 min. The remaining liquid was removed from the zinc selenide prism and left to dry at room temperature for 120 min. The samples were scanned in tapping mode using a silicon nitride cantilever with a spring constant of 40 N m^{-1} (EXT125, AppNano, Mountain View, CA) at a scan rate of 0.50 Hz and a resolution of 500 × 200 points. Images were processed using Analysis Studio (Anasys Instruments).

Chemical analysis of the liposomes

Nanoscale AFM-IR measurements

Nanoscale IR measurements of the liposomal samples casted on zinc selenide prism (see method for tapping mode AFM) were analyzed using an AFM-IR instrument (nanoIR, Anasys Instruments) as per a previously published protocol.[19] Four IR background spectra were collected in the mid-infrared region from 1000 to 1800 cm^{-1} before the acquisition of AFM-IR spectra. These spectra were averaged and normalized for signal intensity calibration. Furthermore, a cantilever ringdown signal was optimized at its frequency center of above 200 kHz and frequency window of 50 kHz as the band-pass filter. Laser hot spot was optimized at 1000, 1250, 1350, 1550, 1650 and 1750 cm-1. These wavenumbers correspond to the regions where liposome, sucrose and ciprofloxacin may show IR absorbance. It is recommended to optimize the laser at five to eight different wavenumbers corresponding to the chemical composition of the sample to achieve the best signal to noise ratio during the spectral acquisition. Following the signal optimization, a minimum of ten AFM-IR spectra were collected for each sample from 1000 to 1800 cm^{-1} at a spectral resolution of 2 cm^{-1} using 256 co-averages and a laser power of 60 %. A silicon nitride cantilever (EXC450 tips, AppNano, CA, USA) with a nominal spring constant of 0.5 N m^{-1} was used for all measurements. Data analysis was performed using Analysis Studio software. Smoothing of the spectra using the 'Savitzky–Golay' function was achieved using the polynomial function of 2 and 5 numbers of points.

Bulk scale Fourier transform infra-red spectrometry

The chemical composition of the ciprofloxacin (50 mg/mL), empty liposomes, liposomes containing encapsulated ciprofloxacin (in liquid and nanocrystalline form: all diluted 4 fold with DW) and sucrose solution (100 mg/mL in DW) were assessed by collecting FTIR spectra in attenuated total reflectance (ATR) mode using a Bruker Tensor 27 FTIR equipped with Bruker BioATR II (Bruker optics). All spectra were collected over the mid-infrared region of 1800 to 1000 cm^{-1} at a spectral resolution of 2 cm^{-1} and the co-average of 512 scans with a background spectrum of water collected prior to each sample measurement.

To obtain the chemical analysis of the other excipients, magnesium stearate and isoleucine, the FTIR spectra of raw powder was collected by ATR technique using a Bruker Lumos FTIR (Bruker optics). Absorbance spectra were acquired in the mid-IR range 1800 to 600 cm^{-1} at a spectral resolution of 4 cm^{-1} and the co-average of 500 scans.

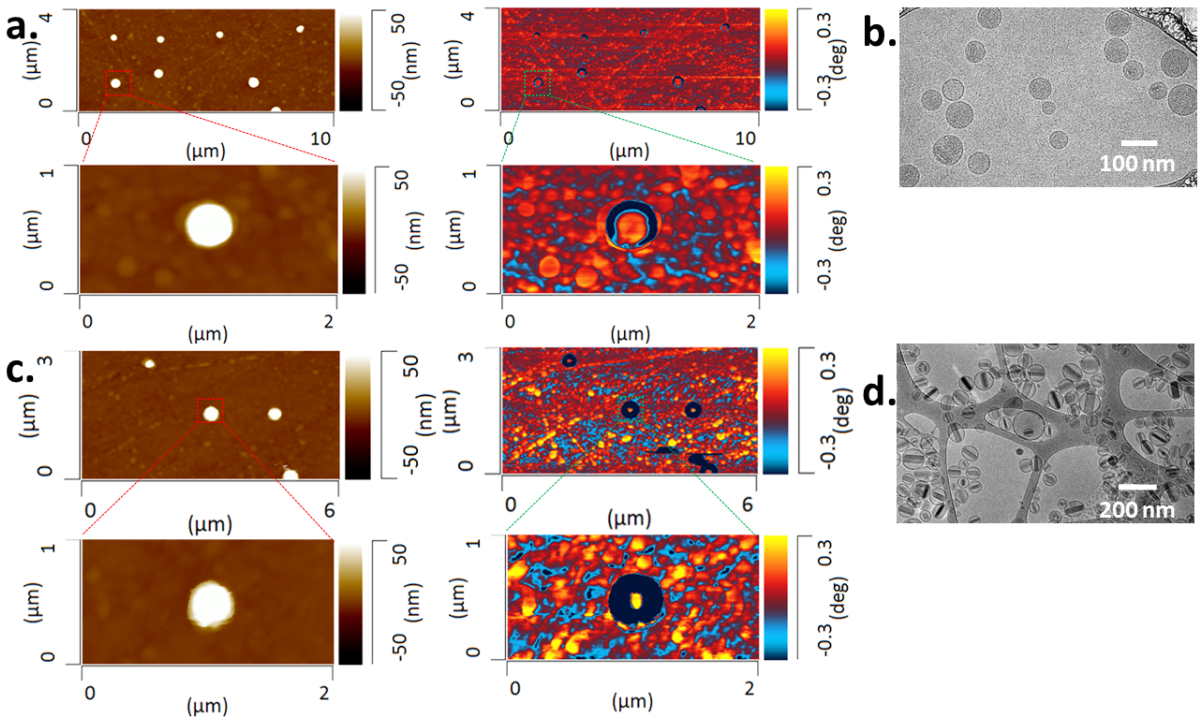


Figure 1. (a) AFM image acquired in tapping mode of liposomes encapsulating dissolved ciprofloxacin with a zoomed in inset; height image (left) and phase image (right). (b) CryoTEM image of liposomes encapsulating dissolved ciprofloxacin. (c) AFM image acquired in tapping mode of freeze-thaw liposomes encapsulating ciprofloxacin nanocrystals with a zoomed in inset; height image (left) and phase image (right). (d) CryoTEM image of liposomes encapsulating ciprofloxacin nanocrystals.

Results and Discussion

Assessment of liposomal size distribution and morphology

CryoTEM and AFM images acquired from empty liposomes (**Figure S1a**), control liposomes encapsulating soluble ciprofloxacin (**Figure 1 a,b)**, after freeze-thaw (**Figure 1 c,d)** and after spray drying and reconstitution (**Figure S1b**) confirmed that the liposomes have circular morphology in general. DLS measurements confirmed that these liposomes were monodisperse with a polydispersity index of 0.1 to 0.2 and ranged from 97 to 130 nm in size (Table 1). CryoTEM images of the liposomes (following freeze-thaw **(Figure 1d)** and after reconstitution of spray dried powders **(Figure S1b)** confirmed the presence of an encapsulated elongated ciprofloxacin nanocrystal within most liposomes. AFM-phase images of a liposome encapsulating ciprofloxacin in the dissolved state **(Figure 1a)** and a liposome containing nanocrystalline ciprofloxacin **(Figure 1c)** demonstrated unique nanomechanical distribution.

Empty liposomes had uniform mechanical properties across all of the individual vesicles. In contrast, liposomes with dissolved ciprofloxacin and those containing a nanocrystal showed two distinct mechanical phases. This result confirmed the presence of two components within the liposome. The lipid component was softer (blue color) in comparison to the stiffer encapsulated drug (red color). Liposomes containing dissolved ciprofloxacin showed that most of the internal space of the liposomes was occupied by the drug **(Figure 1a)** whereas, in the case of liposomes containing nanocrystalline ciprofloxacin, drug was confined in the center of the liposome **(Figure 1c)**.

Results further confirmed that with the formation of nanocrystals, the size of liposomes increased, and the shape of the liposomes also was elongated to a 'rugby ball shape'.[3] The size increase of 4-5 nm was observed in liposomes which were not subjected to spray drying. However, their mean size increased by 20 to 30 nm after the liposomes were spray dried and subsequently reconstituted **(Table 1)**. The change in liposome size may be due to stress related to the freeze-thaw process or subsequent to that, droplet formation during the spraying or drying processes. During the freezing stage, liposomes are subjected to mechanical and osmotic stress due to the formation of ice crystals and a steep increase in encapsulated solute concentration, respectively, prior to complete freezing. This process may lead to rearrangement of liposomes to larger structures as reported in the case of pegylated liposomes subjected to a freeze-thaw cycle.[28] Like freezing, spray drying may

also lead to increased liposomal size because of mechanical stress induced during atomization (spraying) and osmotic stress during the drying cycle.[29] However, the damage induced by these stresses can be minimized by including sucrose in the formulation utilising its cryo and lyoprotectant properties.[29, 30] Our earlier study highlighted that the stability of these liposomes can be optimized by increasing the sucrose concentration. In this study we used the recommended mass ratio of 2 (sucrose):1 (lipid) to ensure the stability of these liposomes after spray drying.[29]

Table 1: Size distribution of liposomes by dynamic light scattering for original (control), freeze-thawed (FT) and freeze-thawed then spray dried samples (FTSD). Mean [SD], n=3.

Sample type (sucrose: lipid) (w/w)	Particle size (nm)	Polydispersity index
Empty (0:1)	96.83 [2.1]	0.07 [0.01]
Control (0:1)	101.5 [3.0]	0.10 [0.03]
FT (2:1)	104.1 [1.6]	0.10 [0.01]
FTSD (2:1)	131.3 [7.3]	0.20 [0.06]

Assessment of drug encapsulation

The results from the encapsulation efficiency assessment confirmed that the percentage of encapsulated drug within the liposomes was 78.2 % for the liposomes after freeze-thaw and then spray drying, 98.7 % for the liposomes after freeze-thaw alone and 98.9 % (**Table 2**) for the liposomes before subjecting them to freeze-thaw or spray drying. The encapsulation efficiency values suggest that when liposomes are subjected to multiple steps of cooling and heating some amount of encapsulated drug can leak out. As observed in our case, ~20 % of the drug leaked out from liposomes which were subjected to the combination of the freeze-thaw and spray drying processes. Complete degradation of liposomes may occur if they are subjected to multiple heating and cooling cycles due to the alteration in the hydration state of the phospholipids.[31] However, the liposomes remained intact and retained both their structure and drug encapsulation following freeze-thaw, indicating that the liposome composition possessed good stability. The insignificant loss in drug content even after subjecting these liposomes through the freezing and heating step is due to the cryo and lyoprotective effect of the sucrose used in the formulation.[29]

Table 2: Encapsulation efficiency (EE) of ciprofloxacin nanocrystals inside liposomes in liquid form (Control and Freeze-thawed (FT) 2:1) and dry powder form (Freeze-thawed then spray dried (FTSD) 2:1). Mean [SD], n=3.

Sucrose: lipid (w/w)	EE (%)
Control (0:1)	98.9 [0.10]
FT (2:1)	98.7 [0.13]
FTSD (2:1).	78.2 [1.20]

Nanoscale infrared spectroscopy on liposomes

To characterise nanochemical domains within the liposomes, liposomes with and without encapsulated ciprofloxacin (liquid or nanocrystalline) were probed using AFM-IR. AFM-IR spectra acquired from individual empty liposomes **(Figure 2a)** confirmed that these liposomes were primarily composed of hydrogenated soy phosphatidylcholine (HSPC) and cholesterol.[29, 32] Peaks associated with HSPC were confirmed at 1060 and 1088 cm^{-1} ν(C-O-C), 1236 cm^{-1} ν(PO_2^-) and at 1468 cm^{-1} (–CH_2 scissoring vibration). Similarly, peaks related to cholesterol were confirmed at 1132 cm^{-1} (-CH stretching) and 1732 cm^{-1} (–C=O stretching).[33]

After assessing the chemical composition of empty liposomes, AFM-IR spectra were acquired from liposomes encapsulating dissolved ciprofloxacin **(Figure 2b)**. Spectra obtained confirmed the presence of peaks related to ciprofloxacin at 1626-1631 cm^{-1} (N-H bending vibration of quinolone moieties), 1576 cm^{-1} and 1392 cm^{-1} (C=O asymmetric and symmetric vibrations respectively), 1720 cm^{-1} (C=O stretching of carboxyl group), and 1268 cm^{-1} (C-N stretching). The presence of peaks at 1720 cm^{-1}, 1576 cm^{-1} and 1392 cm^{-1} confirmed that within the liposomes the carboxyl group of ciprofloxacin exists as a zwitterion. The peak at 1720 cm^{-1} corresponds to the unionised form, whereas peaks at 1576 cm^{-1} and 1392 cm^{-1} represent the ionised carboxyl form.[34]

In contrast, spectra acquired from liposomes encapsulating ciprofloxacin nanocrystals (after freeze-thaw) **(Figure 3a, b)**, confirmed that the majority of the carboxyl groups existed in the unionised form. Peaks related to the ionised carboxyl group; i.e. at 1576 cm^{-1} and 1392 cm^{-1}, were either absent in the spectra or far weaker in intensity than the ones observed in the liposomal sample containing dissolved ciprofloxacin. The presence of the other peaks

associated with ciprofloxacin; i.e., at 1628 cm^{-1} and 1272 cm^{-1} were also confirmed from the spectra. However, these spectra were much sharper and narrower in comparison to that for the dissolved ciprofloxacin sample.

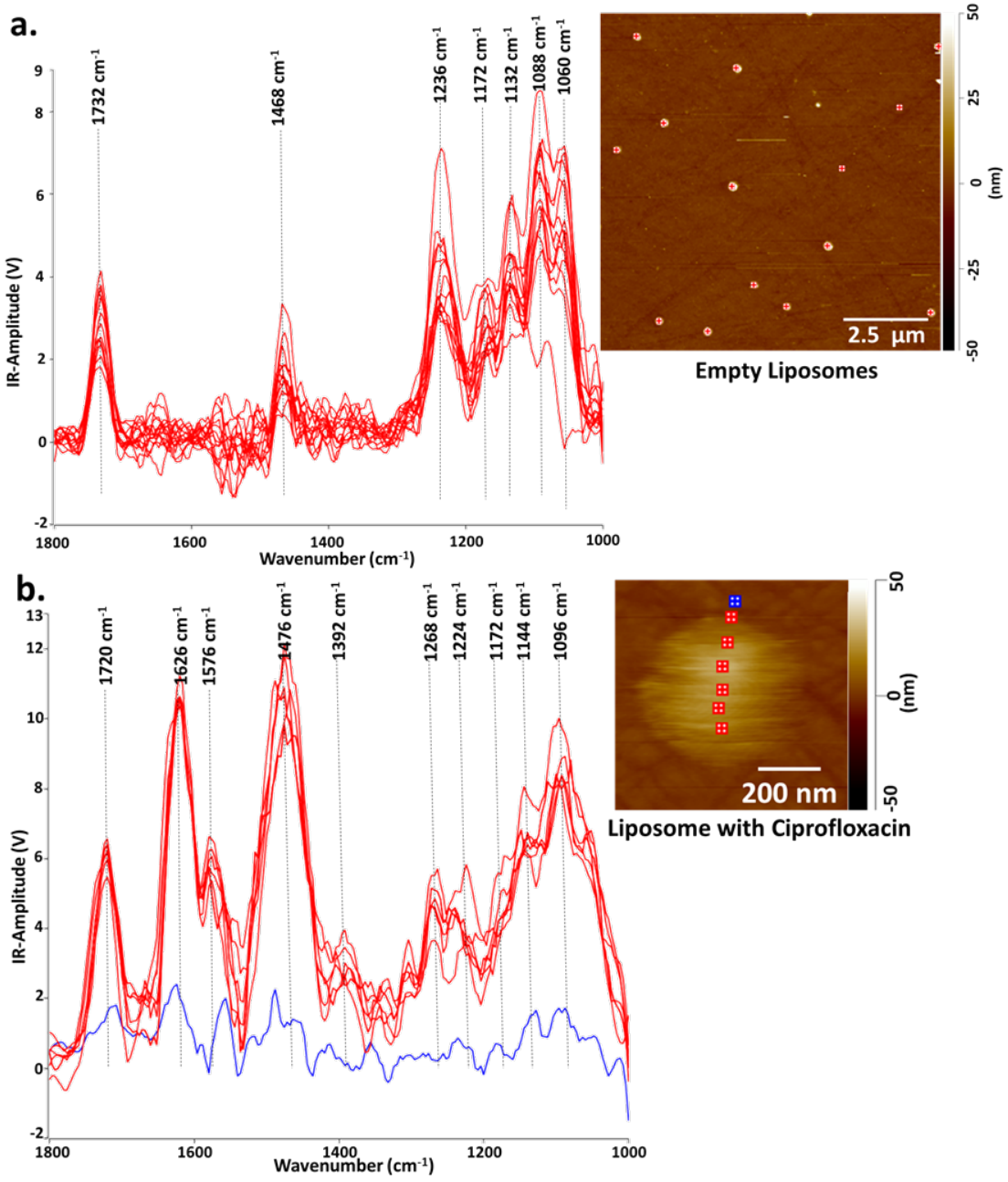


Figure 2. (a) Contact mode AFM image of empty liposomes along with AFM-IR spectra. (b) Contact mode AFM image of liposomes encapsulating dissolved ciprofloxacin along with AFM-IR spectra.

The results confirmed that the ciprofloxacin within the liposomes exists in a crystalline form.[34] This result agrees with the cryoTEM images and small angle x-ray scattering experimental results, where the presence of crystalline ciprofloxacin within the liposome was confirmed.[3] In addition, it has been reported that crystalline samples have sharper IR peaks than those acquired from amorphous samples.[34] These results further demonstrate the

sensitivity of AFM-IR to characterise the chemical composition of a liposome both at the surface and below the surface. This finding agrees with a previous report where AFM-IR was successfully applied to evaluate the subsurface chemical composition of a polymer-bacteria blend.[35]

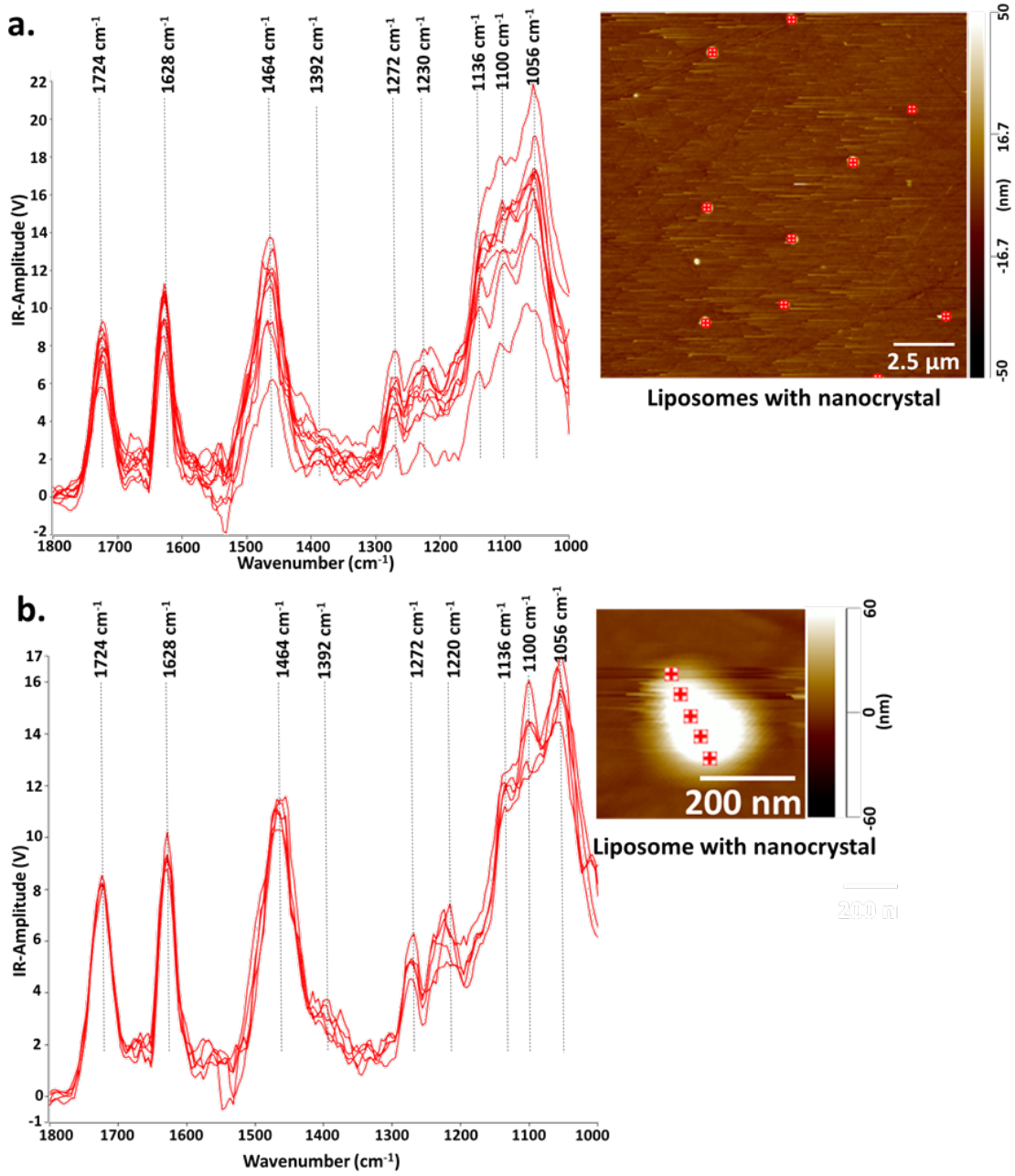


Figure 3. (a) Contact mode AFM image of multiple individual liposomes encapsulating ciprofloxacin nanocrystals along with AFM-IR spectra. (b) Contact mode AFM image of an individual liposome encapsulating ciprofloxacin nanocrystals along with AFM-IR spectra.

Furthermore, to evaluate the impact of spray drying on the nanochemistry of liposomes we acquired AFM-IR spectra from the liposomes obtained after the reconstitution of a spray dried liposomal powder formulation **(Figure 4a, b)**. Spectra obtained from these reconstituted liposomes matched the spectra of the liposomes prior to being spray dried. The

presence of all the peaks related to ciprofloxacin, i.e., at 1724 cm^{-1}, 1628 cm^{-1} and 1268 cm^{-1} were confirmed from the spectra. No significant shift in peak position or decrease in the intensity of the peaks was observed. In addition, the peaks were sharp and peak broadening was not observed confirming the presence of crystalline ciprofloxacin in the liposomes. This indicates that the excipients used including magnesium stearate **(Figure S2a),** isoleucine **(Figure S2b)** and sucrose were compatible with the components of the liposomes and ciprofloxacin. Furthermore, the results highlight that the developed formulation was robust enough to retain the encapsulated drug in crystalline form and maintain the integrity of liposomes despite being subjected to multiple cooling and heating cycles.

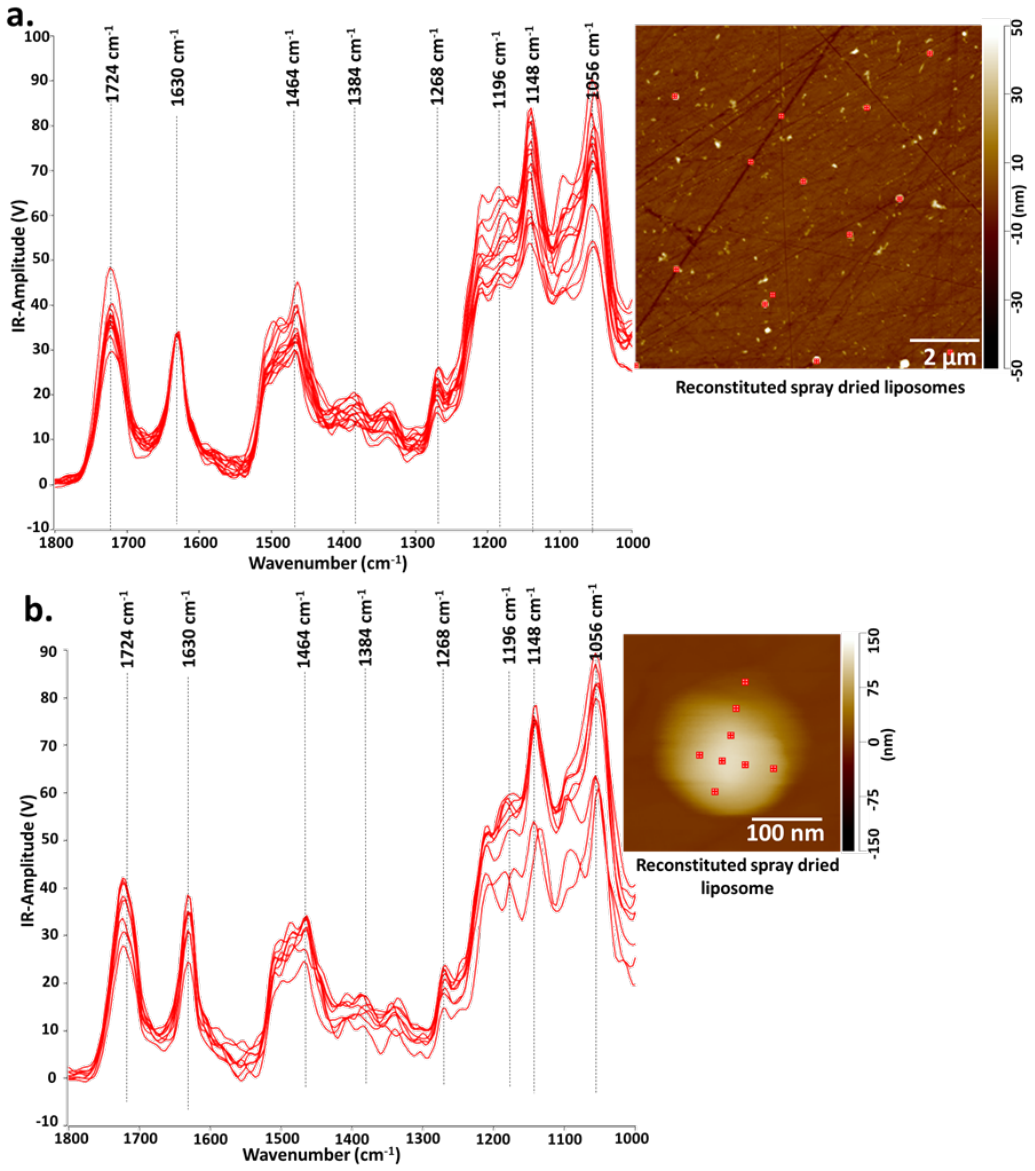


Figure 4. Contact mode AFM images of (a) multiple and a (b) single liposome encapsulating ciprofloxacin nanocrystals obtained after the reconstitution of spray dried powder along with AFM-IR spectra.

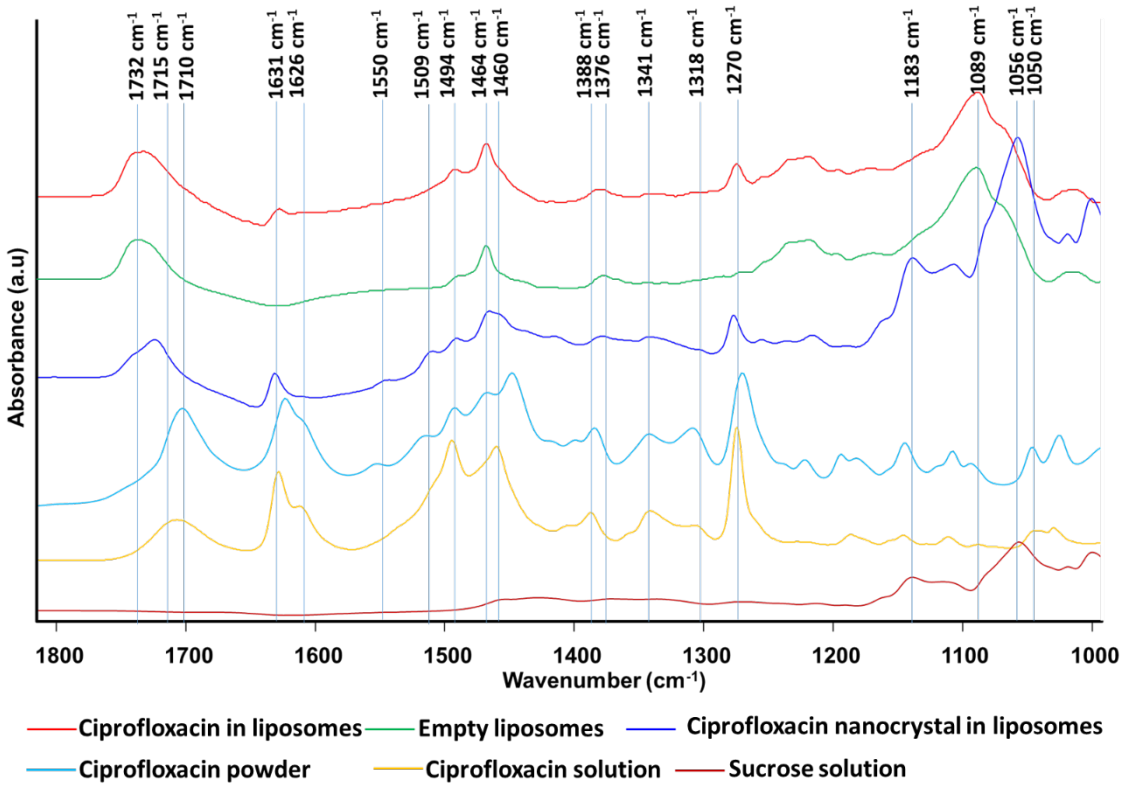


Figure 5. FTIR spectra of liposomes encapsulating dissolved ciprofloxacin, empty liposomes, liposomes encapsulating ciprofloxacin nanocrystals, ciprofloxacin powder, dissolved ciprofloxacin solution and sucrose solution.

Bulk characterisation of the liposomal formulation

We further collected FTIR spectra from the liposomes and the raw materials used to formulate these liposomes for bulk scale chemical characterisation **(Figure 5)**. The presence of peaks related to ciprofloxacin, i.e., at 1631 cm^{-1} and 1270 cm^{-1} were confirmed from the spectra of liposomes containing ciprofloxacin in both the dissolved and nanocrystalline forms. These spectra matched the control spectra obtained from the dissolved ciprofloxacin and ciprofloxacin powder samples. However, spectra obtained from the liposome containing nanocrystal showed greater a similarity with the spectra obtained from ciprofloxacin crystalline powder sample. Well resolved peaks at 1509 cm^{-1} and 1550 cm^{-1} corresponding to the bending vibration of C=C in the aromatic nucleus of ciprofloxacin[36] were confirmed in the spectra obtained from liposome encapsulating nanocrystal and the ciprofloxacin powder **(Figure 5)**. However, these peaks were not well resolved in the case of dissolved ciprofloxacin sample.

Peaks related to unionised C=O stretching of the carboxyl group from ciprofloxacin at 1715-1720 cm^{-1} could not be resolved in the liposomal samples possibly due to the overlapping of peaks of the carboxyl group present in HSPC. However, the majority of the other peaks

related to ciprofloxacin and the sucrose excipient (1056 cm^{-1} and 1183 cm^{-1}) were confirmed from the FTIR spectra acquired from liposomes containing ciprofloxacin as nanocrystals. The results from the bulk chemical characterisation were consistent with the results from the nanoscale chemical characterisation using AFM-IR. These findings further confirm that an individual liposome retains the chemical integrity and AFM-IR can successfully resolve the chemical information from these nanosized liposomes.

Conclusion

AFM-IR has provided unparalleled nanoscale characterisation of the physicochemical composition of individual liposomes encapsulating ciprofloxacin. Nanomechanical and nanochemical measurements on liposomes allowed successful differentiation of empty liposomes, liposomes encapsulating ciprofloxacin in dissolved and nano-crystalline form. Liposomes containing dissolved ciprofloxacin had peaks related to the ionized carboxyl group, i.e. at 1576 cm^{-1} and 1392 cm^{-1}, which were either absent or far weaker in intensity in the spectra of liposomes containing ciprofloxacin nanocrystals. The results further confirmed the capability of AFM-IR in ascertaining the robustness of developed spray-dried liposomal formulation in maintaining the integrity of the liposome and drug in crystalline form.

Although AFM-IR offers many advantages for characterising chemical and mechanical domains of these nanosized liposomes, there are also limitations of AFM-IR for liposomal characterisation. First, optimization of laser power is critical to ensure that the liposomes do not melt or burn during spectral acquisition. Careful consideration and optimization of laser power is recommended to get the best absorbance signal and to avoid sample damage. Secondly, during the AFM image acquisition, some tip dragging was observed. First generation AFM-IR only allows the spectral acquisition while operating in contact mode. This is one of the limitations of this system. Liposomes are fragile and they have soft surface, therefore when imaging is conducted in contact mode, tip dragging may occur. This problem may be avoided by using newer generation AFM-IR instruments which allows spectral acquisition while operating in tapping mode. However, despite these limitations by optimizing the image acquisition parameters (speed of image acquisition) best possible outcomes has been achieved to enable a significant advancement towards our understanding of liposomal formulations. In the future, this research can be extended to study the storage

stability of these liposomes for evaluation of any physicochemical changes in the drug or liposomes. Furthermore, AFM-IR will be applicable to study liposomes encapsulating other antibiotics (e.g., amikacin) and different types of drug, particularly anticancer drugs (e.g., doxorubicin) as they have been marketed as approved products. The nanochemical and nanomechanical properties of liposomes encapsulating these drugs as a single molecule or in combination may be evaluated.

Associated content

Supporting information

CryoTEM images of the empty liposome and liposome obtained after the reconstitution of spray dried powder encapsulating ciprofloxacin nanocrystal; FTIR spectra of magnesium stearate and isoleucine.

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Notes

The authors declare no competing financial interest.

Acknowledgments

This work was financially supported by the Australian Research Council (Linkage Project LP160101498) with Aradigm Corporation as the Partner Organisation, which supplied the stock liquid liposome formulations. The authors acknowledge the facilities and the scientific and technical assistance of Sydney Analytical, a core research facility at The University of Sydney. The authors also thank the facilities of Microscopy Australia at the Electron Microscope Unit, Mark Wainwright Analytical Centre, The University of New South Wales. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Australian Research Council or Aradigm Corporation.

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