The influence of millimeter waves on the physical properties of large and giant unilamellar vesicles

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Abstract Exposure of cell membranes to an electromagnetic field (EMF) in the millimeter wave band (30–300 GHz) can produce a variety of responses. Further, many of the vibrational modes in complex biomolecules fall in the 1–100 GHz range. In addition to fundamental scientific interest, this may have applications in the development of diagnostic and therapeutic medical applications. In the present work, lipid vesicles of different size were used to study the effects of exposure to radiation at 52–72 GHz, with incident power densities (IPD) of 0.0035–0.010 mW/cm², on the chemical-physical properties of cell membranes. Large unilamellar vesicles (LUVs) were used to study the effect of the radiation on the physical stability of vesicles by dynamic light scattering. An inhibition of the aging processes (Ostwald ripening), which usually occur in these vesicles because of their thermodynamic instability, resulted. Giant unilamellar vesicles (GUVs) were used to study the effect of the radiation on membrane water permeability under osmotic stress by phase contrast microscopy. In this case, a decrease in the water membrane permeability of the irradiated samples was observed. We advance the hypothesis that both the above effects may be explained in terms of a change of the polarization states of water induced by the radiation, which causes a partial dehydration of the membrane and consequently a greater packing density (increased membrane rigidity).

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1 Introduction

Interaction of cell membranes with electromagnetic fields (EMF) in the millimeter wave (MMW) band (30–300 GHz) can produce a variety of biological effects; for reviews see references [1–3]. These have included increased activity of Ca^{2+} -activated K^{+} channels, reversible externalization of phosphatidylserine molecules, increased lateral pressure of phospholipids monolayer, and changes of action potential in the neuronal plasma membrane. The mechanisms underlying these effects are not clear; nevertheless, radiations in this frequency range are widely used in Eastern European countries in diagnostic and therapeutic medical applications [4–6].

On the other hand, many of the vibrational modes in complex biomolecules, such as RNA and DNA, fall in the 1–100 GHz range [7]. In this frequency range, there are significant parts of the rotational modes of water molecules [8–12], fluctuations of atoms in organic molecules and hydrogen bonds. Biologically relevant processes can therefore be initiated using radiation in this frequency domain [13].

For optimal effectiveness of these bio-medical applications, one must choose appropriate power levels of this radiation that must, however, be well below the safe exposure limit of 1 mW/cm² to avoid heating of the biological sample [14, 15]. In fact this radiation is strongly absorbed by dipole-oriented molecules, such as water and phospholipids and a superficial—0.1–0.5 mm depth—heating of the tissue media can occur [16].

Recently, physical changes of cell-sized lipid vesicles, such as elongation, induced diffusion of fluorescent dye di-8-ANEPPS and increased attractions between vesicles were demonstrated when these vesicles were subjected to 53.37 GHz radiation [17]. The action of the field on charged and dipolar residues, located at the membrane-water interface, is thought to be the major factor determining the overall perturbation of these giant vesicles. This hypothesis seems to be reinforced by results recently obtained on multilamellar lipid vesicles subjected to 52–72 GHz radiation. In this case, changes to the main phase transition process by ²H-NMR spectroscopy analysis were reported [18].

In order to improve the understanding of these phenomena, we report in the present work an evaluation of changes in the chemical-physical properties of lipid membranes, induced by 52–72 GHz radiation on large and giant unilamellar vesicles (LUVs and GUVs) made of 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC). We have chosen LUVs as a model system as their physical stability over time has been extensively studied [19–21]. Aging processes in DLPC vesicles can occur mainly through exchange of amphiphile monomers between vesicles of different sizes (curvature), leading to a change in the size distribution [19–22] that can be measured with dynamic light scattering (DLS).

While this aging process is, however, ruled out in GUVs whose curvature energy is very low compared to LUVs (resulting in nearly zero membrane curvature), these giant vesicles are very suitable for evaluating osmotic permeability [23]. In fact, because of their size, GUVs better reflect the properties and behavior of cell plasma membranes and allow for direct manipulation and observation of lipid membrane resulting, indeed, in a very practical tool to study the response of membranes to external perturbations e.g., locally applied forces [24] and electric fields [25, 26]. For this reason, we have chosen GUVs to analyze the membrane response to osmotic stress under irradiation conditions.

2 Materials and methods

2.1 Chemicals

DLPC was purchased from Avanti Lipids. D-glucose was from Fluka (Switzerland). Chloroform, of the highest quality commercially available, was purchased from Sigma-Aldrich. The water used in all sample preparations was purified by a Millipore Milli-Q Cartridge system (EMD Millipore Corporation, Billerica, MA, USA).

2.2 Large and giant unilamellar vesicles preparation

The procedure for obtaining extruded large unilamellar vesicles (LUVs) has been previously described [27–29]. Briefly, a DLPC lipid film was hydrated with high-purity water—to avoid any undesirable osmotic effects—at a final concentration of 10 mM and subjected to three sonication-vortex-mixed cycles. After ten freeze-and-thaw cycles, vesicles were extruded ten times through each of the three polycarbonate membranes (Whatman) having decreasing pore sizes of 400, 200, and 100 nm, respectively. The extrusion was carried out under nitrogen gas pressure in a 10-ml Thermobarrel Extruder T.001 (Lipex Biomembranes, Vancouver, Canada) [30] at 25 °C; this temperature is above the main phase transition temperature of DLPC so that the phospholipid bilayers can be considered to be in their liquid crystalline state; the resulting LUVs appeared to be translucent. Lipid concentration was determined in triplicate by phosphorous analysis. Samples were diluted (ten times) to perform dynamic light scattering (DLS) measurements. The millimeter wave (MMW) exposures of LUVs were always carried out on the day of their preparation.

Giant unilamellar vesicles (GUVs) were also prepared from DLPC using the electroformation method [31]. Briefly, phospholipids were dissolved in chloroform:methanol (9:1 v/v) at a concentration of 0.5 mg/ml. Approximately 2 μ l of this solution was carefully deposited with a Hamilton syringe on two different areas of two parallel platinum wires mounted in an electroformation chamber (extensively described in Bucher et al. [32]). The lipid films were then dried by storage overnight in a desiccator under vacuum ($\sim$ 8 mbar). The chamber was then put on the stage of an inverted microscope (NIKON) fitted with 60 $\times$ objective and ELWD 0.3 lenses to provide the phase contrast mode. Pure water (1.2 ml) was added into the chamber and the platinum wires were connected to the frequency generator (model AFG3022, Tektronix). A voltage at 10 Hz, gradually increasing from 0 to 2 V (peak-to-peak) in 0.5 V steps every 5 min, was applied between the two electrodes. The formation of giant vesicles was observed after approximately 2 h; some vesicles reached a size of more than 50 μ m in diameter.

All steps of the GUVs electroformation and MMW exposures were performed at 25 °C; as previously mentioned, this temperature is above the main phase transition temperature of DLPC, thus the phospholipid bilayers can be considered to be in their liquid crystalline state.

2.3 Size distribution measurements of GUVs

The microscope was equipped with an optical condenser and a single-lens reflex camera (NIKON); the images were acquired using analysis control software and presented as collected, i.e., no image processing was applied.

Vesicle size distribution measurements were obtained from the acquired images by using software provided by the manufacturer. To this aim, different zones on each platinum wire were selected and the vesicle size was measured in the direction parallel to the wire. To account for all the vesicles present on the selected region, the focus plane was gradually changed until no further vesicles could be seen. Because the measurement of vesicle size distribution was time-consuming, in preliminary experiments we estimated the minimum number of vesicles required to obtain a reliable distribution (about 80). For each vesicle size distribution, about 100 vesicles were then counted, requiring only about 5 min for the measurement.

Vesicle size distribution was measured after 40 and 80 min from the start of the irradiation and also at the end of the irradiation time (4 h). Twenty minutes after stopping the MMW radiation, vesicle size distribution was measured again.

2.4 Osmotic stress in GUVs

Osmotic stress experiments were carried out by adding 150 μl of a 10 mM solution of glucose on GUV samples inside the electroformation chamber (the final volume was 1.2 ml and the final glucose concentration 1.25 mM). Vesicle size distribution in sham-exposed samples (4 h since vesicle preparation) was measured 5 and 30 min after glucose addition. GUV samples were also induced to osmotic stress during the irradiation (4 h after starting the MMW exposure) and vesicle size distribution was measured 5 and 30 min after glucose addition.

2.5 Dynamic light scattering measurements of LUVs

Dynamic light scattering (DLS) measurements of LUVs were performed using a 90 Plus/BI-MAS Multi-Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY) equipped with a 15-mW, linearly polarized HeNe laser light source with wavelength $\lambda_0 = 488 \text{ nm}$ (Spectra-Physics, Model 124B). A Neslab RTE-5DD refrigerated, circulating bath was used to maintain a constant temperature of $25.0 \pm 0.1^\circ\text{C}$ in the test tube containing the LUV samples and surrounding liquid. For each sample, data were acquired—typically for a duration of 10 min—for five runs at a scattering angle of 90° .

In dynamic light scattering, the time autocorrelation function of the scattered light $g(t)$ is measured and used to extract the size distribution of suspended particles $G(\Gamma)$. For monomodal, polydisperse distributions of particles, $g(t)$ can be expressed as (1):

$$g(t) = \int_0^\infty G(\Gamma) e^{-\Gamma t} d\Gamma \quad (1)$$

In this work, the method of cumulants was used to obtain the size-particle distribution function $G(\Gamma)$. In the cumulant analysis, no assumption is made about the form of $G(\Gamma)$. The exponential in (1) is expanded in a Taylor series around the mean value and the resulting series is integrated to give an expression of the logarithm of $g(t)$ as a polynomial in the delay time. The coefficients of the powers of t are the cumulants of the distribution. In practice,

the first couple of cumulants provide the first two moments of the distribution, (2) and (3), respectively, which coincide with the average and the variance of the distribution:

$$\Gamma = Dq^2 \quad (2)$$

$$\mu_2 = (D^{2*} - D^{2*}) q^4 \quad (3)$$

where D is the diffusion coefficient of the particles and q is the magnitude of the scattering wave vector that depends on λ_0 , q and the refractive index of the solvent. D is related to the particle effective diameter (hydrodynamic radius) d by the Stokes–Einstein relation, $D = k_B T / 6\pi\eta d$, where k_B is Boltzmann's constant and η is the dynamic viscosity.

From the diffusion coefficient D , the polydispersity is calculated according to (4):

$$\text{Polydispersity} = \mu_2 / \Gamma^2 \quad (4)$$

Polydispersity is a dimensionless parameter that is close to zero for monodisperse samples, small for nearly all monodisperse samples (0.020–0.080) and larger for broader distributions. Effective diameter and polydispersity are intensity-weighted parameters. Since the size distributions analyzed here were broad and unimodal, the lognormal distribution by weight was chosen to obtain weight-average results from the cumulant analysis. This data treatment provides the geometric mean and geometric standard deviation of the particle size distribution.

2.6 Experimental procedure

Irradiation experiments were conducted at an incident power density (IPD) of 0.0035–0.010 mW/cm², using a conical horn antenna (IMG 56/76, MicroMedTech, Nizhny Novgorod, Russia) having a maximal diameter (D) of 34 mm.

LUV suspensions (1 ml) were exposed in wide-band mode between 52 and 72 GHz for up to 4 h. The geometry of the irradiation was with a normal incidence of the field to the liquid surface (Fig. 1); the distance of the center of the sample volume to the horn antenna aperture was 8 cm.

The test tube containing the LUVs suspension was immersed in a water bath at 25 °C for 2 h prior to the start of the irradiation. Under these conditions, DLPC vesicles were well above their main phase transition temperature ($T_m = -1.8$ °C), therefore the vesicles can be considered to be in their fluid phase. LUV suspensions were subjected to either MMW radiation or sham exposure, i.e., when the MMW radiation was turned off. The suspension of LUVs was carefully stirred before collecting samples for DLS measurements. In this way, measurements were averaged over the whole sample and the non-uniformity of vesicle exposure in different positions of the suspension was taken into account. Overall irradiation experiments and DLS measurements were carried out in controlled temperature conditions at 25.0 ± 0.05 °C.

GUV samples were also exposed in wide-band mode between 52 and 72 GHz and simultaneously observed under a microscope using the same experimental setup previously adopted for 53.37-GHz irradiation of GUVs [17]. Briefly, the exposures were carried out directly under the microscope in the electroformation chamber placed on its stage. The center of the sample volume to the horn antenna aperture was 8 cm and the incidence angle was 45° (Fig. 2). The overall experimental setup was located in a temperature-controlled room (≈ 25 °C), which governed the exposure temperature. GUVs were subjected to either

Fig. 1 Diagram of the exposure setup for irradiation of LUV₁₀₀ by multiscan IMG 56/76 including the horn antenna, the exposure chamber, and the water bath. The sample, contained in a 2.3 cm × 2.3 cm × 2.3 cm poly(methylmethacrylate) (PMMA) box, was immersed in a water bath at 25 °C. Dimensions: D = 34 mm; R = 8 cm

MMW radiation or sham exposure—when the MMW radiation was turned off—for up to 4 h. All vesicles were monitored in real-time during the irradiation in the formation chamber. The vesicles were attached to the platinum wires and thus located in a medium thickness of about 100–200 μm . This allowed studying the effects the radiation in a rather uniformly exposed volume of vesicle suspension.

Table 1 summarizes the main physical characteristics, preparation methods, and exposure setup for the two (LUVs and GUVs) biological models used in this work.

2.7 Temperature measurements

To evaluate the heating effect of MMW irradiation, temperature measurements were carried out, in a first attempt, using a variable temperature controller unit (Bruker VT2000) equipped with a thermocouple probe having a resolution of 0.1 °C. The probe tip of the thermocouple was placed at ~ 1 mm below the upper surface of the liquid containing either LUVs or GUVs.

In a second attempt, the surface temperature of the suspension was measured with a non-contact infrared thermometer (Raynger MX4 from Raytek, Germany) with adjustable emissivity and equipped with a thermopile detector resulting in a spectral response in a range of 8–14 μm and a resolution of 0.1 °C. The close focus option was used to select a sample surface area as small as 6 mm. The double-bright circular laser sighting system allowed us to point the IR detector in different positions of the sample surface. The infrared thermometer was placed 35 cm away from the exposure chamber. Temperature measurements, over 4 h of MMW exposure, were recorded every 250 ms in order to reveal any rapid thermally transient events. The variation and distribution of the temperature on the surface of the aqueous solution were measured with an uncertainty of 2%.

Fig. 2 Diagram of the experimental setup used for the exposure of GUVs to 52–72-GHz radiation, including the horn antenna and the exposure chamber. Dimensions: $D = 34$ mm; $R = 8$ cm. The sample was exposed from the top. *Inset*: GUV samples in water solution and particulars of the reference system to measure vesicle size. The vesicle diameter was measured in the direction parallel to the wire. Bar is $20\ \mu\text{m}$

2.8 Statistical analysis

Statistical analysis was performed on the rough data collected by either DLS (N , number of counts $> 1,400$) or light microscopy ($N \geq 100$). Data were averaged over three independent experiments. The unpaired Student's t test was used for the evaluation of the difference between the mean of sham and MMW-exposed samples. The paired Student's t test was used for the evaluation of the difference between the mean of the same sample before and after different treatment (e.g., exposure for different times, osmotic stress, and osmotic stress with exposure). The chosen confidence limit was $p = 0.05$.

3 Results and discussion

3.1 Stability of giant unilamellar vesicles

In a first series of experiments we carefully checked if the GUV preparations had good stability, a prerequisite for successful investigation of the effects induced by MMW radiation. For this purpose, we monitored any changes in the GUV's shape for more than 2 h and measured the size distribution of vesicles at least twice. No variation in shape, size, or number of vesicles was observed. The inset panel of Fig. 2 shows the vesicle size measured in the direction parallel to the wire.

Table 1 Comparison between physical parameters, preparation methods, and exposure setups for LUVs and GUVs

Model system	Average size (μm)	Lipid composition	Preparation method	Physical parameter monitored	Observation method	MMWs exposure setup			Exposure distance ^a (cm); Angle of incidence ^b (degrees)
						Wide-band mode, Frequency range (GHz)	Conical horn antenna Max diameter (cm)	IPD (mW/cm ²)	
LUV	0.100	DLPC	Extrusion [14–16]	Physical stability over time (aging effect)	Dynamic Light Scattering	52–72	3.4	12.5	0.0035–0.010 8; 90 (Fig. 1)
GUV	>20	DLPC	Electro-formation [18]	Water permeability (induced by osmotic stress)	Optical Microscopy	52–72	3.4	12.5	0.0035–0.010 8; 45 (Fig. 2)

^aSet as the distance between the antenna output and the open surface of the liquid; ^bset as the angle between the propagation direction of the radiation and the plane defined by the liquid surface

3.2 Effects of MMWs on GUVs size

In order to assess whether MMW radiation would affect vesicle size distribution, we performed a population study of GUVs rather than single-vesicle observations. A typical vesicles size distribution of GUV suspension is reported in Fig. 3a. The same size distribution was observed 4 h after vesicle preparation (sham sample) confirming vesicle stability over the observation time. No significant differences in the vesicles size distributions were observed between the sham and irradiated samples after 4 h (Table 2). Thus, it can be concluded that irradiation does not affect the vesicle size distribution of a stabilized GUV suspension.

Fig. 3 **a** Size distribution of GUVs in pure water (sham sample). **b** and **c** Vesicle size distribution measured 5 min (**b**) and 30 min (**c**) after the addition of a glucose solution 1.25 mM. **d**, **e** Vesicle size distribution measured after 4 h from starting the vesicle exposure to MMWs and 5 min (**d**) or 30 min (**e**) after the addition of a glucose solution 1.25 mM. The data shown are representative of three independent experiments

Table 2 GUV size parameters before and after MMWs exposure, and under osmotic stress induction for the sham and MMW-exposed systems

GUV size parameter ^a (μm)	Sham	MMW	Sham		MMW	
	4 h of exposure	4 h of exposure	Minutes under osmotic stress induction		Minutes under osmotic stress induction	
			5	30	5	30
Mean	23 ± 9	24 ± 8	20 ± 8	16 ± 6	22 ± 8	21 ± 10
Min	7 ± 2	6 ± 3	9 ± 3	7 ± 2	8 ± 2	7 ± 3
Max	49 ± 3	50 ± 4	37 ± 4	28 ± 3	48 ± 3	47 ± 4

^aValues are the average over three independent experiments

3.3 Effects of MMWs on GUVs under osmotic stress

In order to study the effect of irradiation on the permeability to water of GUV membranes, a transmembrane flux of water was induced by the application of osmotic stress and this process was studied under both sham and irradiation conditions.

The osmotic pressure was induced by the addition of 1.25 mM glucose solution (final concentration) (Fig. 3b, c, d, e). Five minutes after the addition of the glucose solution to the sham-exposed sample, vesicle shrinkage occurred with a significant ($p = 8 \times 10^{-6}$) decrease of vesicle diameter (Fig. 3a versus b and Fig. 4). After 30 min, the shrinkage effect became so large as to lead to a 30% decrease of the mean vesicle diameter compared with that of the initial vesicle suspension ($p = 6 \times 10^{-28}$) (Fig. 3a versus c and Fig. 4). It is worth noting that the osmotic pressure mainly affects the larger vesicles. Indeed, the diameter of the large vesicles was significantly reduced, from 49 ± 3 to 37 ± 4 after 5 min and from 49 ± 3 to 28 ± 3 after 30 min (i.e., $p = 0.0004$ and $p = 0.00002$, respectively). The diameter of the smaller vesicles was unaffected (Table 2).

Interestingly, if the osmotic pressure was applied to the vesicle suspension during irradiation, no significant shrinkage effect was observed, neither after 5 min ($p = 0.8339$) nor after 30 min ($p = 0.2486$) (Fig. 4 and Table 2). Furthermore, the vesicle size distribution was not affected under irradiation (Fig. 3a versus d and e).

These results clearly show that vesicle shrinkage, which is markedly induced on sham vesicles by osmotic pressure, is strongly inhibited in vesicles exposed to irradiation. This

Fig. 4 Shrinkage effect estimated by the mean diameter of sham and MMW exposed GUV samples after some time since the application of osmotic stress. Asterisks indicate statistically significant differences with respect to the control vesicle suspension at $p < 0.001$

means that irradiation has a dramatic effect in reducing membrane permeability to water. This reduction could be due to a decrease of the membrane fluidity of GUVs [33].

3.4 Average size and polydispersity of exposed LUVs

In the inset panel of Fig. 5, a representative size-distribution of a LUV suspension is reported. LUV suspensions presented broad unimodal size distributions—the average effective diameter was 113.80 nm and the average polydispersity was 0.14 nm.

In order to evaluate the effects of MMW radiation, LUV samples, from three different preparations, were subjected to exposure of 4 h (Fig. 5). Two parameters such as the mean diameter and the polydispersity of vesicles were considered. Figure 5 shows the relative increase of the mean vesicle diameter x_i at time t_i , normalized toward the mean vesicle diameter at time $t_0 = 0$, x_0 (initial vesicle size in fresh preparation), calculated according to (5):

$$\frac{x_i - x_0}{x_0} \quad (5)$$

The normalized diameters of both the sham and irradiated vesicles exhibited an increasing trend for the whole time over which DLS observations were performed (18 h). This trend is related to the aging process of the vesicles [19–22]. It is interesting to note that two distinct growing rates are clearly visible in the sham vesicle suspensions: an initial one, more rapid, which occurred within a few hours from sample preparation and a following long-term one that appeared 18 h after the preparation of vesicles. This observation is in agreement with literature data (see Zhdanov and Kasemo [21] and references [4–6] therein) that attribute the first stage to the supersaturation of lipids following the preparation of vesicles and the second one to monomer ripening that, for amphiphiles with a high monomer solubility such as DLPC (monomer solubility = $9 \cdot 10^{-8}$ M, data from Avanti Polar Lipids), occurs mainly through exchange of amphiphile monomers from small vesicles to larger ones [21, 34].

Fig. 5 Relative increase of the effective vesicle diameter as a function of time for the exposed (red diamond) and sham (blue square) LUV samples. Arrows indicate the time t_0 , at which the radiation exposure started and the time (4 h) at which the radiation was switched off. Samples were subsequently monitored 1 and 14 h after the end of the exposure. Error bars denote the standard deviation obtained from three independent experiments. Inset: a representative size-distribution of LUVs is reported

From Fig. 5 it can be seen that the first stage of LUVs aging was not affected by irradiation, since no significant difference in the mean vesicle diameter was observed between sham and irradiated samples after 4 h. In contrast, after 18 h, the mean diameter of the sham samples was significantly ($p = 0.0033$) larger than the irradiated ones, 0.133 ± 0.014 and 0.082 ± 0.008 , respectively, indicating that irradiation induces a delay effect in the kinetic of the second stage of vesicle aging. This stage is driven by the large curvature energy of the smaller vesicles that tend to release their amphiphile monomers in favor of the larger vesicles with smaller curvature [20, 22]. The growth of larger vesicles at the expense of smaller ones occurs via lipid desorption, diffusion, and incorporation of lipid molecules from the solution into the external lipid layer. Desorption from this layer, jumps between the external and internal layers and the rate of growth of vesicles are determined by the relative kinetic mechanisms [21]. All of these mechanisms are affected by the chemical characteristics of the transported lipid molecules and by the composition and lipid packing density of the donor and acceptor vesicle bilayers (see [21] and references [5, 6] therein). It is likely that irradiation has some impact on one of the above mechanisms and that the final effect is the slowing down of the second stage of vesicle aging. We hypothesize that irradiation could affect mainly the interface processes involved in the exchange of lipids from one vesicle to another, which are controlled by the lateral packing density of the bilayer lipids.

An increase of polydispersity, which approximately doubled its value in the first 5 h in both the sham and irradiated vesicle suspensions, was observed (data not shown). Despite the significant difference in the mean vesicle diameters between the sham and irradiated samples observed after 18 h, no effects of irradiation on the polydispersity either in the first hours or after long-term observation resulted. This can be explained by the fact that irradiation did not influence the first stage of the aging process over which the most significant increase of polydispersity occurs (data not shown).

It seems, therefore, that despite the differences in size between the vesicles studied, the effects induced by irradiation on LUVs and GUVs can be explained with a common mechanism, i.e., an increase in membrane rigidity, as membrane rigidity is correlated to changes in the packing of the phospholipids that are associated with pressure stress [35].

Our hypothesis seems reinforced by the fact that lateral surface pressure is influenced in a noticeable way on phospholipid monolayers by short-term exposure to 60-GHz radiation [36]. Further, a decrease of the orientational order parameter of water in dimyristoylphosphatidylcholine multilamellar vesicles induced by 52–72 GHz has been also reported [18]. These overall effects could be interpreted by a common mechanism, i.e., a reduction of the membrane hydration. Indeed, (1) membrane dehydration results in a reduction of the area per lipid molecule in the plane of the bilayer (A), which in turn, causes an increase of the lateral pressure (τ), as expressed by (6):

$$\tau = K_\alpha \frac{|A - A_0|}{A_0} \quad (6)$$

where K_α (constant slope in the elastic limit of deformation) is the isothermal elastic area compressibility modulus and A_0 ($> A$) is the stress-free value of A in excess of water [37]; (2) reduction of the water orientational order parameter can be interpreted as an increase of the fraction of free or loosely bound water to the membrane [18, 38].

Interestingly, it has been suggested that 61.2-GHz radiation, modulated by 4 Hz, could induce cell dehydration, which, in turn could have a pain relief effect [39]. It is known that cell pathology is accompanied by cell over-hydration. Neuronal hydration causes an

increase in excitation, resulting in an increase of the number of ionic channels functioning in the membrane. The over-hydration induced over-excitation of neurons was suggested as the main mechanism of pain (nociceptive) signal generation.

This topic is of great interest in view of potential applications of radiation at 35–78 GHz in therapeutic treatment. Moreover, the possibility that our observed effects might be related to the vibrational/librational modes of water and/or membrane components can stimulate further work in establishing a more precise link between the target morphology and the physical parameters of the radiation pulse.

3.5 Temperature measurements

To evaluate the heating effect of MMW irradiation, we carefully measured temperature changes and heating rates in the liquid containing either LUVs or GUVs. Figure S1A shows the temperature profile of the LUVs suspension in the inner layers of the aqueous medium as a function of the exposure time. These measurements were performed with a thermocouple probe (probe tip 0.5 mm) located at about 1 mm below the liquid surface. The temperature oscillated randomly around the set point (25.0 °C) within ± 0.2 °C and no systematic changes were detected neither in the first instants of exposure (first 30 s, see inset) nor for long-term exposure (up to 4 h). This result is consistent with the very low power densities incident on the sample (Table 1) and the fact that, in the downward exposure setup used, about half of the IPD is reflected back [9] and the fraction transmitted into the sample exponentially decays as a function of the propagation direction with a rate that depends on its depth of penetration in the aqueous medium (δ). Assuming that δ ranges from about 0.35 mm up to about 0.40 mm in the frequency range studied [9], it can be calculated that 90% of the power density is absorbed within the first 0.4 mm of the suspension. However, as the presence of the sensor in the liquid could perturb the MMW field distribution [40], a remote temperature recording is desirable in order to obtain correct surface-temperature dynamics data on the convection process during MMW irradiation [41]. Therefore, a second measurement of potential heating patterns on the surface of the suspension was performed by using a noncontact, MMW-immune infrared thermometer. The temperature of selected areas (6 mm in diameter) on the sample surface was measured by detecting the infrared radiation coming from the most superficial layers of the suspension. In Fig. S1B, a representative temperature-vs-exposure time profile of the chamber-containing LUVs is shown. The temperature was monitored during 1 h of sham exposure and successively during 4 h of MMW irradiation: also in this case, no thermal increment of the sample surface was detected, irrespective of the area selected on the sample. The temperature profile of the first 30 s of exposure (inset in Fig. S1B) shows that no significant temperature rise was detected even in the first instants of exposure, when neither convection nor conduction phenomena can take place.

In addition, simulation data, previously performed [17] on similar exposure chambers, demonstrated that the local specific absorption rate (SAR_{AVG}) showed a maximum value of 0.48 ± 0.05 W/kg in the upper layers of the sample and a minimum value of 0.12 ± 0.01 W/kg, close to the bottom of the exposure chamber. However, it is worth noting that although in [17] the exposure setup was similar to that used in the present work, vesicle irradiation was performed at 53.57 GHz and at an incident power density at least ten times larger than that used in this study. In our case, indeed, the intensity was so low as to cause no local heating of the sample (Fig. S1).

4 Conclusions

In this paper, we have studied the effects of wide-band millimeter waves, MMWs (52–72 GHz and incident power densities (IPD) in the range 0.0035–0.010 mW/cm²), on the physical properties of membranes (i.e., physical stability and permeability) by monitoring vesicle size and size distribution changes on biomimetic systems of different sizes. The physical stability over time of 100-nm-large unilamellar vesicles (LUVs) exposed to MMWs was monitored by dynamic light scattering. An inhibition of the aging processes (Ostwald ripening), which usually occur in these vesicles because of their thermodynamic instability, has been observed. Moreover, giant unilamellar vesicles (GUVs) have been used to study the effect of millimeter waves on membrane water permeability under osmotic stress, by monitoring the size distribution with a phase contrast microscope. In this case, a decrease in membrane water permeability was observed in the irradiated samples. Even though LUVs and GUVs are very different in size and chemical-physical parameters, we conclude that the resulting MMW-induced effects, such as the inhibition of the ripening process and the decrease of the membrane water permeability most likely arise from partial dehydration of the membrane as a common mechanism.

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