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Curcuminoids-loaded liposomes: influence of lipid composition on their physicochemical properties and efficacy as delivery systems

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ABSTRACT

An extract of *Curcuma longa* containing mainly curcumin was included in liposomes formulated with phospholipids differing for the length of the acyl chains, the charge of the polar headgroup or the degree of unsaturation in the presence and in the absence of cholesterol. In fact, even if it is well known that liposomal curcuminoids are more stable and can be solubilized in aqueous media, a systematic approach that correlates the molecular structure of liposomes components to the physicochemical and biological behavior of the vescicles including curcumin and its derivatives is not described. Size and zeta potential of liposomes, the location and the antioxidant activity of the solute in the bilayer and its effect on lipid packing were evaluated and correlated to the pharmacological activity of liposomal extract against a methicillin susceptible *Staphylococcus Aureus* strain ATCC 29213. The relation between the physicochemical and biological features of formulations points out that the rigidity of the bilayer and liposomes charge are crucial parameters in the interactions of liposomes with the biological environment. In particular, the synergism between curcumin antibacterial properties and the positive liposomes surface brings to a significant reduction of the minimal inhibitory concentration of the liposomal drug with respect to the one of the free extract thus enlarging the prospectives of its pharmacological exploitation.

1. Introduction

The curcuminoids are diarylheptanoids that confers the typical orange coloration to the rhizomes of the plant *Curcuma longa L.* (tumeric). Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) and its analogs devoid of one or both the methoxy groups (*i.e.* demethoxycurcumin and bisdemethoxycurcumin, respectively) are the three main curcuminoids (Fig. 4) present in extracts of *Curcuma longa*.

Even if their total amount and their relative ratio vary, curcumin is generally the more abundant compound. It has been extensively investigated for its biological and pharmacological activities, such as antioxidant, neuroprotective, anticancer and antimicrobial effect. [1,2] Unfortunately the low water solubility of curcuminoids [3] together with their chemical and photochemical instability [4] has hampered their pharmaceutical applications. To circumvent these limitations their inclusion in many drug delivery systems such as liposomes, cyclodextrins, biopolymers, dendrimers, nanogels or their conjugation with metallic nanoparticles was successfully investigated. [5] Most of these systems improved their stability and solubility in aqueous media with a significant increase of their bioavailability and, as a consequence, of their biological effect. Anyway, despite the huge number of investigations on the biological and pharmacological effects of curcumin and its derivatives, studies describing with a systematic approach the relation between the molecular structure of the components and the properties of the drug delivery systems and their efficacy are relatively scarce.

Here we report an investigation on the inclusion of E100 colorant, a *Curcuma longa* extract (CUR) containing high quantities of curcuminoids (curcumin 83 %, demethoxycurcumin 8% and bisdemethoxycurcumin 2% w/w) on liposomes composed of unsaturated (1,2-dioleoyl-*sn*-glycero-phosphocholine, DOPC) or saturated phosphocholines (PC) differing for the length of the alkyl chains (Fig. 5), namely 1,2-dimyris-

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Table 1
Cumulant based average diameters or NNLS main peak diameters (nm) and average zeta potential values (mV) of investigated liposomal formulations in the absence of CUR. Estimated standard deviations (ESD) are reported in parenthesis. Similar results were obtained in the presence of CUR.

Formulation	D_H (ESD)	PDI	Zeta Potential
DPPC	110 (±4)[a]	0.18	−8 (±3)
DPPC/chol 6.7/3.3	170 (±6)[a]	0.21	−11 (±4)
DPPC/CPC 6.7/3.3	133 (±15)	0.44	53 (±2)
DPPC/CPC /chol 6.7/3.3/3.3	117 (±10)	0.17	48 (±3)
DMPC	111 (±14)	0.28	0 (±2)
DMPC/chol 6.7/3.3	162 (±21)	0.38	−6 (±4)
DOPC	122 (±13)	0.26	−5 (±3)
DOPC/chol 6.7/3.3	114 (±16)	0.18	−6 (±1)

[a] From a NNLS intensity weighted distribution presenting a minor population of about 5% around 1 μm.

Table 2
Entrapment efficiency and residual % of CUR (determined by HPLC) at pH = 8.6 after 3 h. The values reported are averaged over three measurements.

Formulation	% E.E. fluorescence	% E.E. HPLC	CUR residual % (pH = 8.6 after 3 h)
DPPC	87 ± 3	77 ± 5	90 ± 3
DPPC/chol 6.7/3.3	58 ± 2	48 ± 4	71 ± 2
DPPC/CPC 6.7/3.3	78 ± 5	67 ± 5	22 ± 5
DPPC/CPC /chol 3.3/3.3/3.3	48 ± 4	36 ± 2	85 ± 4
DMPC	82 ± 3	75 ± 3	95 ± 3
DMPC/chol 6.7/3.3	62 ± 2	50 ± 6	70 ± 2
DOPC	78 ± 6	75 ± 2	67 ± 6
DOPC/chol 6.7/3.3	72 ± 3	57 ± 5	72 ± 3

Table 3
Fraction *f* of CUR accessible to I^- or MV (used as quenchers) and quenching constant, *K*, obtained by fluorescence quenching experiments of CUR containing liposomal formulations. Reported values correspond to the average values over at least three independent measurements and the errors correspond to the standard deviation among the different measurements (errors in *f* determination are not reported because are <5%).

Formulation	*f* (I^-)	*K* [M^{-1}] (I-)	*f* (MV)	*K* [M^{-1}] (MV)
DPPC	0.05	1005 ± 30	0.08	301 ± 28
DPPC/chol	0.22	917 ± 35	0.27	183 ± 43
DPPC/CPC	0.04	4400 ± 115	0.88	10 ± 43
DPPC/EPC/chol	0.22	3757 ± 130	–	–
DMPC	0.07	1205 ± 30	0.22	53 ± 33
DMPC/chol	0.15	920 ± 30	0.42	93 ± 23
DOPC	0.12	242 ± 40	0.20	202 ± 26
DOPC/chol	0.22	310 ± 30	0.65	93 ± 35

toyl-*sn*-glycero-phosphocholine (DMPC) or 1,2-dipalmitoyl-*sn*-glycero-phosphocholine (DPPC) and mixed cationic liposomes composed of DPPC and its cationic analogue 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine chloride salt, (cationic PC, CPC) in the presence and in the absence of 33 % of cholesterol (chol).

The aim of this study was to deepen our knowledge on the interaction of lipid bilayers with CUR in the attempt to correlate its biological activity to the properties of liposomes as drug delivery systems. We evaluated the influence of the chemical structure of lipids on the entrapment efficiency (E.E.) of the three components of CUR and on their stability at physiological and basic pH by HPLC measurements. In fact, in solution they can be present as keto and enol tautomers in a ratio depending on the condition; [6] they are characterized by different stability, solubility and, in some cases, biological activity [7,8]. We previously demonstrated that the three free curcuminoids show a different pH-dependence of degradation rate (because of different intramolecular charge distribution) in water-methanol mixtures:[9] this solvent mixture was chosen to simulate their environmental conditions when embedded in the apolar region of supramolecolar systems to prevent their alkaline degradation [10,11].

Size and zeta potential of the formulations and the localization of the CUR in the bilayer was also assessed. Fluorescence quenching measurements allowed to evaluate the accessibility of CUR, the fluorophore, to the quencher molecule, *i.e.* the proximity of CUR to liposomes surface, and to better understand their interaction with lipid bilayer and the influence of lipid composition. Through differential scanning calorimetry (DSC) measurements the influence of CUR on the packing of the bilayer, besides its positioning, was also evaluated. Moreover, the antioxidant activity of free and liposomal CUR was evaluated and compared. This property, often overlooked in studies concerning liposomal drugs of natural origin, can be crucial because it is strictly related to their pharmacological activities [12–16]. Antimicrobial efficacy of liposomal CUR on methicillin sensible *Staphylococcus Aureus* (MSSA) ATCC 29,213 allowed to estimate the influence of the bilayer composition on the properties of the extract and also to appraise an eventual synergistic effect.

2. Experimental section

2.1. Materials

DOPC, DMPC, DPPC and CPC, the cationic PC, used for liposome preparation were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Phosphate buffered saline (PBS) tablets (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH = 7.4 at 25 °C), chol, Mueller Hinton, KI, Na_2SO_3, methyl viologen dichloride hydrate (MV), dialysis tubing cellulose membrane D 9527 (medium diameter of 27 mm and molecular weight cut-off = 14,000), CH_3COONa, H_2O_2 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and HPLC-grade methanol and acetonitrile were obtained from Sigma Aldrich. Double deionized water was prepared using a Milli-Q filtration/purification system (Millipore, Bedford, MA, USA). Basic buffer at pH = 8.6 was prepared using borax and boric acid. CUR was obtained from a provider of food ingredients. MSSA reference strain from the American Type Culture Collection (ATCC 29,213) was used as control organism.

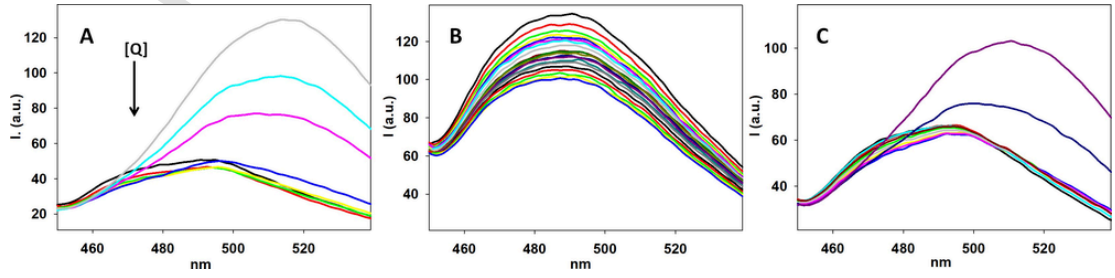

Fig. 1. Fluorescence emission spectra obtained upon the addition of (A) MV to DPPC/CPC/chol liposomes, (B) KI to DPPC/EPC/chol liposomes and (C) MV to DPPC/chol liposomes. The intensity of the fluorescent signal decreases by subsequent addition of the quencher, as indicated in the panel A.

Table 4
Thermodynamic parameters and cooperative unit (CU) of liposomal formulations (MLV) with and without CUR obtained by DSC measurements. Error in the case of the obtained temperatures and ΔH are within 0.5 °C and 1 kJ/mol, respectively (averaged over three measurements).

Formulation	pretransition		main transition		
	T (°C)	ΔH_m(kJ/mol)	T(°C)	ΔH_m(kJ/mol)	CU
DMPC	15.1	3.17	24.1	19.1	96
DMPC + CUR	–	–	24.0	21.7	84
DOPC	–	–	−20.7	36.6	–
DOPC + CUR	–	–	−20.0	38.9	–
DPPC	34.8	2.2	41.7	32.1	160
DPPC + CUR	–	–	41.4	31.8	134
DPPC/CPC	–	–	38.9	26.6	157
DPPC/CPC + CUR	–	–	39.2	30.0	122

2.2. Methods

2.2.1. Liposome preparation

Lipid films were prepared on the inside wall of a round-bottom flask by evaporation of solutions containing the proper amount of PC (dissolved in $CHCl_3$) and CUR at 100:1 M ratio in the presence or in the absence of 33 M percentage of chol (dissolved in $CHCl_3$). The obtained films were stored overnight under reduced pressure (0.4 mbar), then PBS was added to obtain a 1 mM lipid dispersion. In the case of zeta potential measurements PBS buffer 15 mM was used to reduce the Joule-heating effect. The solutions were heated at 50 °C and vortex-mixed, then the suspensions were sonicated for 4 min at 72 W (cycles 0.5 s) under cooling condition of an ice-water bath, using a Hielscher UP100-H ultrasonic processor with microtip probe (7 mm). To remove unentrapped CUR dialysis exchanging 4 times the external medium PBS solution (25 fold the liposome dispersion volume) in two hours was carried out.

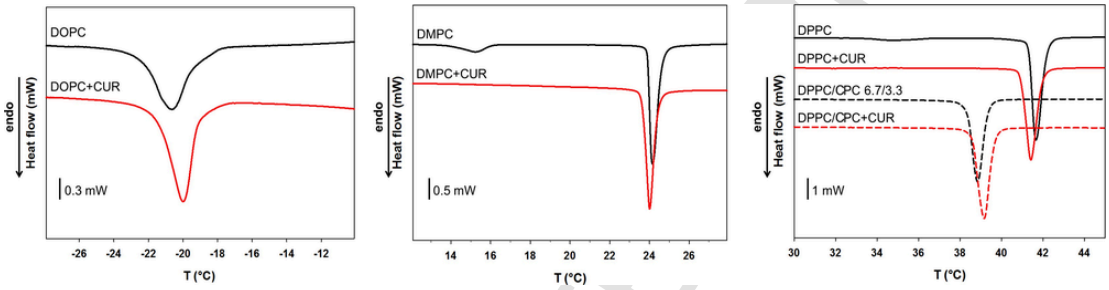

Fig. 2. Thermograms of MLVs with and without CUR. Scan rate is 1 °C/min.

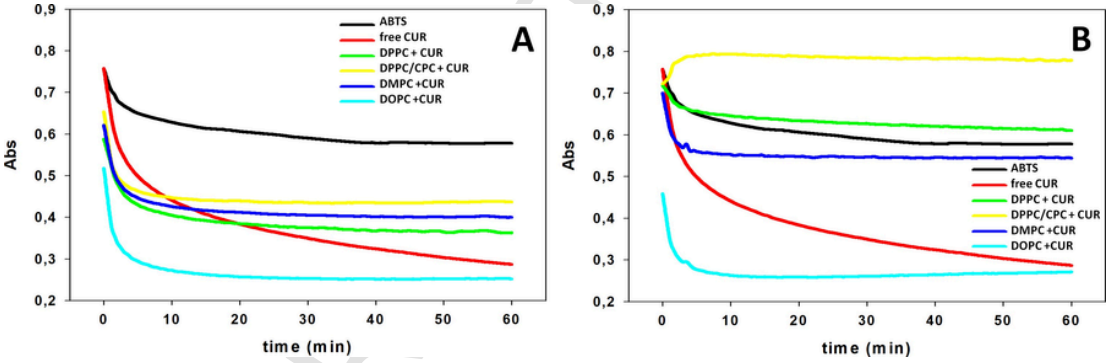

Fig. 3. Kinetic measurements of $ABTS^{\cdot+}$ degradation, measured through the absorption decrease at 417 nm over time in the absence (black trace) or in the presence of free CUR $5.5 \cdot 10^{-8}$ M (red trace) or of the same amount of CUR included in DMPC (blue trace), DPPC (green trace), DPPC/CPC (yellow trace) or DOPC (cyan trace) formulation in the absence (A) or in the presence (B) of cholesterol (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

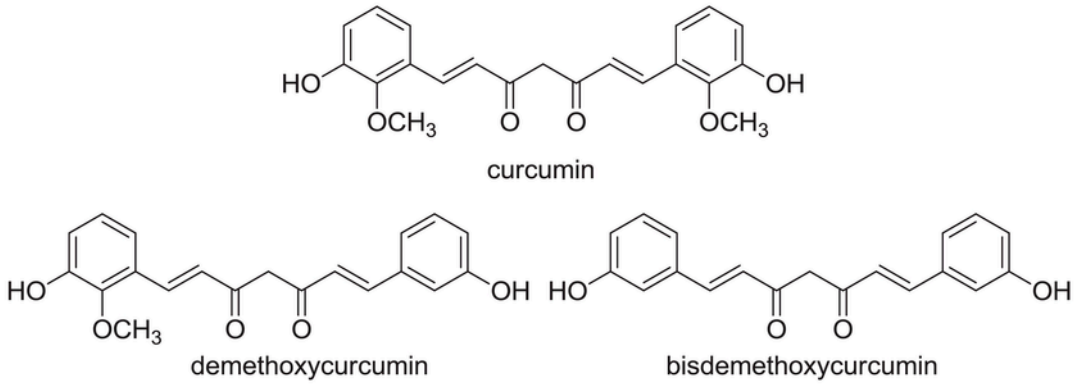

Fig. 4. Molecular structures of curcuminoids.

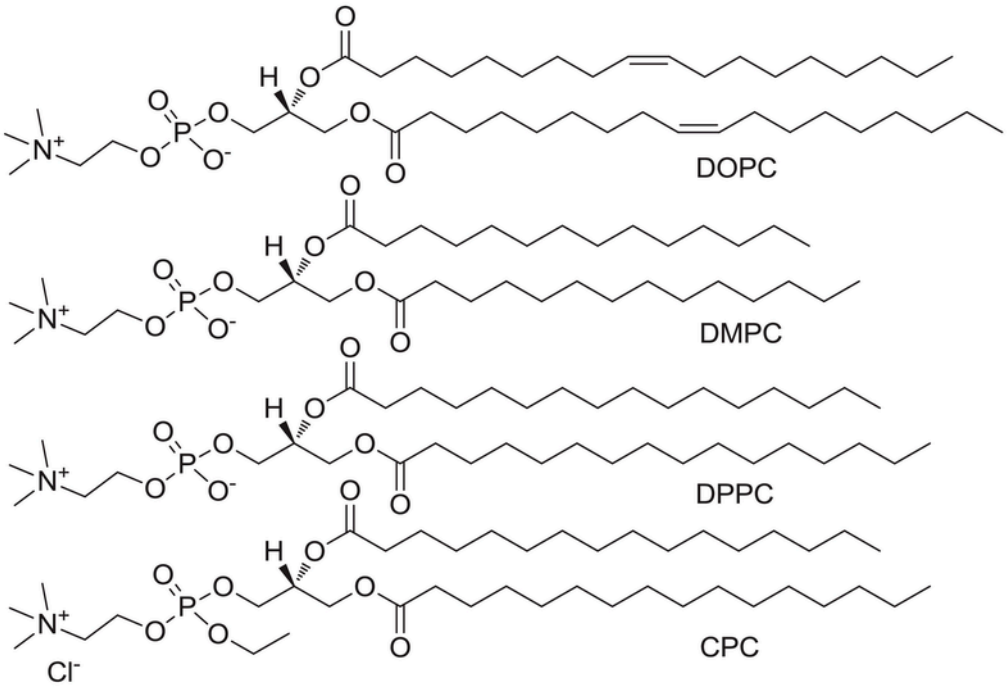

Fig. 5. Molecular structures of PC used in liposomes formulations.

2.2.2. DLS and zeta potential measurements

DLS measurements and electrophoretic mobility by means of the laser Doppler electrophoresis technique were carried out at 25 °C on 1 mM liposomes solutions using a Malvern Nano-ZetaSizer instrument equipped with a 5 mW HeNe laser (λ 632.8 nm) and a digital logarithmic correlator. The normalized intensity autocorrelation functions were measured at an angle of 173° at 25.0 ± 0.1 °C and analyzed by the cumulant method and the non-negative least square (NNLS) algorithm. For each sample, hydrodynamic diameters (D_H) were achieved by cumulant and NNLS analysis. Starting from the diffusion coefficients D, the diameters were estimated by using the Stokes–Einstein relationship $D_H = kT/3\pi\eta D$, where kT is the thermal energy and η the solvent viscosity. The D_H values reported are averaged over at least three measurements. The cumulant zeta average or the NNLS main peak values were used. Only the NNLS intensity weighted distributions were considered. The cumulant polydispersity indexes (PDI) were also estimated. Laser Doppler electrophoresis technique allowed measuring the electrophoretic mobility necessary to estimate the zeta potential of the formulations. The Doppler shift in the Zetasizer Nano series was analyzed by phase analysis light scattering (PALS) implemented with M3 (mixed mode measurement). Low voltages were applied to avoid eventual Joule heating. Zeta potential values were inferred from the electrophoretic mobility data under the Smoluchowsky approximation. The values reported in this manuscript are averaged over 3 consecutive measurements for each sample.

2.2.3. Evaluation of EE and degradation of CUR

The amount of CUR in solution before and after dialysis was measured on solutions composed of 100 μL of liposomes suspension and 500 μL of CH_3OH by HPLC measurements using a chromatographic system (Waters Corporation, Milford, MA) consisting of a Model 600 pump, a 600 pump controller module, a 717 Plus auto-sampler, and a 996 photodiode array detector. The E.E. for each formulation was evaluated by the ratio between the area of the peak corresponding to each CUR component before and after dialysis. The stability of CUR was evaluated by comparing the area of the peak corresponding to each CUR soon after the dialysis and after 3 h of incubation at room temperature in PBS buffer and in borax/boric acid buffer at pH = 8.6. The E.E. of each formulation was also evaluated by recording fluorescence (λ_{exc} = 425 nm, λ_{em} = 485 nm) spectra on a Perkin Elmer LS 50 spectrofluorimeter spectra before and after dialysis to remove free CUR. The determination of each E.E. was performed exploiting a calibration line (5 points) using the Sigma Aldrich standards of the three active principles contained in CUR in the range of the concentration tested.

2.2.4. Fluorescence quenching measurements

The localization of CUR in the bilayer was carried out by fluorescent quenching experiments on a Perkin Elmer LS 50 spectrofluorimeter. All fluorescence experiments (λ_{exc} = 425 nm, λ_{em} = 485 nm) after dialysis were carried out at room temperature on solutions with absorbance lower than 0.1 (liposomes 0.1 mM) to minimize inner filter effects. Small aliquots of 2 M potassium iodide and 0.1 mM Na_2SO_3 solution or of 1 M MV solution were added to liposomes formulations previously dialyzed to eliminate the free CUR as described above and diluted to obtain [CUR] 0.75 μM.

The collected data were graphed following the modified Stern-Volmer equation $I_0/(I_0\text{-}I) = 1/(f \bullet K \bullet [\text{quencher}]) + 1/f$ and the parameters K and f were obtained from the linear fitting of the curve.

2.2.5. Determination of thermotropic properties of liposomes

Differential scanning calorimetry (DSC) measurements were carried out on 30 μL of MLV using a Mettler Toledo DSC 3 calorimeter. Aluminum pans of 40 μL and a PBS-filled pan as reference were used. Liposomes (1 mg/10 μL, ≈148 mM in total lipids) were prepared in PBS in the presence and in the absence of CUR. Two heating scans were recorded at the rate of 5 °C/min and two subsequent heating scans were recorded at the rate 1 °C/min. Under the experimental conditions, reproducible thermal recordings were obtained. Uncertainty on temperatures is ± 0.1 °C and on ΔH is ± 0.5 kJ/mol.

2.2.6. Evaluation of antioxidant activity of CUR by $ABTS^{\cdot+}$ methodology

Four aqueous solutions were prepared as reported in literature [17]: (A) CH_3COONa 0.4 M and NaCl 150 mM; (B) CH_3COONa 30 mM and NaCl 150 mM; (C) glacial acetic acid 0.4 M and NaCl 150 mM; (D) glacial acetic acid 30 mM and NaCl 150 mM. An acetate buffer at pH 5.8 was obtained mixing 235 mL of A solution and 15 mL of C solution; an acetate buffer at pH 3.6 was obtained mixing 18.75 mL of B solution and 231.25 mL of D solution. A solution at pH 5.5, was prepared by mixing 28 mL of the buffer at pH 5.8 and 250 mL of the buffer at pH 3.6. An $ABTS^{\cdot+}$ solution 10 mM was prepared solubilizing 0.2745 g of ABTS diammonium salt in 50 mL acetate buffer at pH 3.6: the radical cation was generated when 14 μL of H_2O_2 35 % (w/v, final concentration 2 mM) were added and left stirring in the dark for one hour and stored in the dark at 4 °C. 25 μL of $ABTS^{\cdot+}$ reagent solution were rapidly added to 2450 μL of acetate buffer at pH = 5.5 containing a proper amount of dialysed liposomes, depending on the E.E. of each system, and PBS (total PBS volume 250 μL, final concentration in cuvette of CUR: $5.51 \cdot 10^{-8}$ M and of $ABTS^{\cdot+} = 9.17 \cdot 10^{-5}$M). We followed the variation of the maximal absorbance at 417 nm over 60 min.

2.2.7. In vitro antimicrobial susceptibility tests

The antimicrobial susceptibility of *S. aureus* ATCC 29,213 to CUR loaded in liposomes freshly prepared was determined in accordance with the CLSI guidelines [18]. Briefly, in the wells of a 96-well microtiter plate that contained 50 μL of two-fold serially diluted free CUR and liposomal CUR in cation-adjusted Mueller–Hinton Broth were added 50 μL of 10^6 CFU/mL bacterial suspension in 0.9 % NaCl solution. Culture medium and bacterial suspension without any compounds were added respectively in separate wells as sterility control and positive control. Microtiter plates were incubated 18 h at 35 °C. Spectrophotometric measurements at 595 nm by a microplate reader iMark, BioRad (Milan, Italy) were carried out to quantify the growth in each well. The minimum inhibitory concentration (MIC) for liposomal CUR (so CUR inside liposomes) was defined as the concentration of CUR that reduces growth by 80 % compared to untreated bacteria. Each MIC value reported corresponds to the median of 3 independent experiments.

3. Results and discussion

3.1. DLS and zeta potential measurements

The formulations showed similar diameters in the presence and in the absence of CUR. In general, distributions with single polulations were obtained except for DPPC liposomes, for which a minor population featuring a D_H value around 1 μm was also present. In Table 1 we reported the average diameters obtained by the cumlant analysis for the sample showing a single population and the main peaks for those presenting a bimodal distributions. Zeta potentials close to neutrality were obtained for all the formulations with the exception of liposomes containing CPC, a cationic lipid (Table1).

3.2. Evaluation of E.E. and degradation of CUR

The E.E observed was quite high in all cases and decreases adding chol to the formulations (Table 2). This evidence can be due to the high rigidity and packing that chol confers to the bilayer thus partially hampering the inclusion of CUR. After 4 days about the 10 % of entrapped CUR is leaked upon storage. The good agreement betwen the results obtained using the two methods supports their consistency and validity. Moreover, our investigation demonstrates that CUR fluorescence can be used to determine its E.E. in liposomes with good reliability without necessarily recurring to HPLC, that is very accurate, of course, but also more time and solvent consuming. The stability of liposomal CUR was also evaluated: after 3 h at pH 7.4 (PBS buffer) no differences were observed whereas at pH = 8.6 (borax/boric acid buffer) CUR partially degraded CUR (between 10 and 30 %). This result demonstrates that the loading of CUR in liposomes protects the active principle to the degradation besides to increasing its solubility in water; in fact is reported in literature that CUR normally degrades at neutral pH and more at basic pH [9,19,20]. The prevention of CUR alkaline degradation when included in lipid bilayer can be attributed to the stabilization of the *bis-keto* tautomer, a limitation of the accessibility of the *keto-enol* moiety to water or OH^- ions or pKa changes promoted by the host environment [9]. The only exception was observed in the presence of CPC: in this case at pH = 8.6 only the 22 % of CUR remains after 3 h, probably because in this formulation a less organized packing of the bilayer occurs; this characteristic allows CUR to interact more easily with the bulk solution.

3.3. Fluorescence quenching measurements

Fluorescence quenching measurements are reported in Table 3. To better investigate the location of CUR we used two different collisional quenchers: I^-, that is anionic, spherical and more specific for the lipid bilayer with respect to MV [21], that is planar, aromatic and bears two positive charges. Fraction *f* of CUR accessible to the quencher was generally very low for all the formulations, thus CUR can be approached by the quencher at very short distances. This result indicates that CUR is mainly located in the apolar region of the bilayer. In the presence of chol we observed an increase of *f* in all cases probably beacuse of the higher rigidity of the membrane with respect to the corresponding formulations devoid of chol. In fact, it is reasonable to hypothesize that a high compaction and a low fluidity of the bilayer can limit the penetration of CUR in the hydrophobic region of the bilayer. In the case of DPPC/CPC liposomes and MV, an anomalous high value of *f* was observed, result that induces to hypothesize a CUR location near the polar headgroups. In the presence of chol *f* could not be obtained because soon after the first addition of MV to liposomes solution the emission peak was shifted to higher values and its intensity increased (Fig. 1A). On the other hand, upon the addition of KI to the same sample the expected variation in the emission spectra occurs (Fig. 1B) and a low *f* value was obtained, thus indicating that the behavior observed with MV is strictly related to the quencher used. The same trend was observed in all cases using MV, but only after several additions of quencher. As a consequence, the data relative to the measurements in which the concentration of MV was too high were discarded before applying the Stern-Volmer equation. In Fig. 1C the fluorescence spectra obtained upon the addition of MV to DPPC/chol liposomes are reported as an example. This evidence indicates that when MV interacts with lipid bilayer it, especially at high concentration, induces a lipid rearrangement that causes a relocation of CUR in a different region of the bilayer with consequent variation of its emission. Obviously this effect is more evident with cationic liposomes than with neutral formulations because of the electrostatic repulsion with the positively charged quencher: evidently, despite the electrostatic repulsion with cationic liposomes, MV approaches enough to the bilayer to induce a significant lipid rearrangement. It is possible that the same phenomenon, to a lower extent, could have affected the measurements also in the case of the other formulations, especially those containing chol, even if inducing variations not appreciable at naked eye. As a consequence, after the elaboration this phenomenon could bring to a *f* value higher than the real one. This effect seems to be directly dependent on the fluidity of the bilayer of the formulation (low for DPPC liposomes, intermediate for DMPC liposomes and high for DOPC ones, Table 3). This is not surprising because in the case of a less rigid bilayer the perturbation due to the presence of MV is more effective.

Quenching constants *K* were mainly high in all cases with I^-, whereas in the case of MV the values obtained were sensibly lower indicating a less favorable interaction/contact between CUR and the quencher molecule. The highest *K* were observed in the experiments relative to CPC containing liposomes and I^- and were due to positive electrostatic interactions between the cationic liposomes and the anionic quencher and probably also due to the low lipid packing of the bilayer (that make CUR located near the headgroup more accessible) favoring its contact with CUR.

3.4. Thermotropic properties of liposomes

Thermodinamic parameters and thermograms of the investigated liposomal formulations are reported in Table 4 and in Fig. 2, respectively.

No big differences, neither changing the composition of the bilayer nor adding CUR, were observed in Tm values: probably CUR components, that are molecules with a flexible structure, can intercalate themselves in the bilayer without disturbing the lipid packing, in good agreement with what observed upon fluorescence quenching experiments. Considering that the amount of CUR with respect to lipids is very low it is reasonable that the effect of its presence on the main transition is not so evident. The addition of CUR caused a disappearance of the pretransition where present, so part of the CUR probably is also located near the glycerol scaffold, analogously to chol that is positioned at the lipid–water interface with its steroidal skeleton buried deep in the hydrocarbon region [22]. It's interesting to observe that also in the absence of CUR, the presence of the 33 % of CPC lowered the main transition temperature and caused the disappearance of the pretransition. The positive charge of this lipid disturbs the organization of the headgroups region. As expected, in all cases the cooperative unit (CU in Table 4) relative to the main transition in the presence of CUR decreased; this result can be explained considering that CUR, as suggested by quenching experiments, is mainly located in the apolar region of the bilayer, thus can disturb the cooperativity of the process. Surprisingly, on the other hand the ΔH associated to the transition increases in all cases when CUR is included in the bilayer with the exception of DPPC liposomes. Evidently, due to its apolar nature, CUR establishes favorable van der Waals interactions with lipid chains that thus lead to an increase of the enthalpy variation.

3.5. Evaluation of the antioxidant activity of liposomal CUR

The antioxidant activity of liposomal CUR was assessed following a procedure reported in the literature: [31] each formulation was added to a solution of $ABTS^{\cdot+}$, a radical cation stable in the absence of antioxidants, that confers to the solution a green color. When $ABTS^{\cdot+}$ interacts with an antioxidant compound it is reduced and the solution becomes colourless at a rate dependent on the antioxidant activity of the system. The dependence of the peak related to $ABTS^{\cdot+}$ on composition of liposomes containing CUR was evaluated following the absorbance value at 417 nm as a function of time and was compared to the one of free CUR and of $ABTS^{\cdot+}$ alone (Fig. 3).

Our results confirm that CUR is a good antioxidant and when it is included in liposomal formulations it exerts its activity more quickly than free CUR. Anyway, even if the regeneration of ABTS from $ABTS^{\cdot+}$ is fast, the curves related to liposomes containing saturated lipids reach a plateau value higher than the one observed in the presence of free CUR, whereas in the case of DOPC liposomes the absorbance values after 1 h are comparable to those observed with free CUR. These evidences can be explained considering that in the case of saturated lipids CUR the packing of the bilayer is very high and $ABTS^{\cdot+}$ can not reach the antioxidant molecules located deeper in the bilayer. On the other hand, the starting value of absorbance in the curve relative to CUR included in liposomes formulated with unsaturated DOPC is significantly lower with respect to all other samples. Being the bilayer more fluid, the reaction between $ABTS^{\cdot+}$ and CUR is so fast that in the few seconds of mixing that precede the beginning of the measurement is almost complete and $ABTS^{\cdot+}$ can the most part of CUR in the bilayer. In the case of liposomes containing saturated lipids and chol, that stiffens the bilayer, the fraction of CUR accessible to $ABTS^{\cdot+}$ further decreases (Fig. 3B) confirming the reliability of our hypothesis. An exception to this behaviour was observed with DPPC/CPC/chol liposomes because the curve relative to regeneration of ABTS increases instead of decreasing. This anomalous result puts in evidence that in this peculiar case a phenomenon not easy to explain causes an increase of the scattering of the solution that covers the effective decrease of absorbance due to the diminishing of the concentration of $ABTS^{\cdot+}$. It is possible that the negatively charged sulphonic moieties of the ABTS molecules (pKa ≈2) [23], interacting with the positive charges on liposomes surface, act as linkers and induce agglutination with consequent increase of the optical density of the solution. Probably the presence of chol, besides rigidifying the bilayer, also causes a slightly different lipid organization that brings to a different exposure of the charged headgroups of CPC that is more suitable for the interaction with the anionic groups of ABTS (either in its oxidized or radical form). Anyway, it is evident that for this sample the kinetic of the process related to $ABTS^{\cdot+}$ scavenging activity of liposomal CUR can not be evaluated.

3.6. Evaluation of the antimicrobial activity of liposomal CUR

CUR included in liposomes shows a MIC equal to 7 μg/mL in the range 3.5–14 μg/mL on MSSA only if included in liposomes containing CPC. The presence or absence of chol in the liposomal formulations loaded with CUR does not affect significantly the antimicrobial activity. The same formulations induce a reduction of bacterial growth of about 30 % already at a CUR concentration equal to 0.88 μg/mL. On the other hand, all liposomes devoid of CUR (including the cationic ones) did not show any antibacterial activity, like free CUR, at all the concentrations tested. This is not surprising because MIC value of free curcumin on MSSA bacterial strains is 125–250 μg/mL according to literature reports [24,25]. These results put in evidence the synergistic effect of CUR and of cationic liposomes, the only ones able to make CUR effective against bacteria at concentration at which it is ineffective if administered as free drug. Also the low lipid packing in the headgroup region of these formulations, as suggested by DSC measurements, together with their positive charge (that favors the interaction with bacterial membrane) plays a role in their efficacy as CUR delivery system.

4. Conclusions

All the investigated liposomal formulations are able to load and protect CUR from degradation besides increasing its solubility in water. The findings of this study confirm the influence of the structure of liposomes components on the physicochemical features of the aggregates they form and on the location and on the antioxidant capacity of a solute included in their bilayer. The differences obtained using I^- or MV as quenchers underline that for each system and each physicochemical characterization the choice of the right probe and/or protocol is fundamental in determining the reliability of the obtained data. Our results point out that the rigidity of the bilayer and the charge of liposomes play a pivotal role in determining liposomes properties and their ability to interact with the biological *milieu*. The synergistic effect of cationic charge of liposomes and CUR lowers its MIC with respect to the one of free CUR thus enlarging the potential of this natural molecule that displays several pharmacological properties. Moreover, our work puts in evidence that the choice of the proper lipid components

is fundamental for exalting the pharmacological property of the liposomal drug and for the success of the delivery system.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.
CRediT authorship contribution statement
Sara Battista: Resources, Methodology, Investigation, Writing - original draft. **Anna Maggi Maria:** Methodology, Investigation. **Bellio Pierangelo:** Investigation. **Galantini Luciano:** Validation, Writing - review & editing. **Antonio D'Archivio Angelo:** Validation, Writing - original draft. **Celenza Giuseppe:** Validation, Writing - original draft. **Colaiezzi Roberta:** Investigation. **Giansanti Luisa:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] K.G. Goozee, T.M. Shah, H.R. Sohrabi, S.R. Rainey-Smith, B. Brown, G. Verdile, R.N. Martins, Examining the potential clinical value of curcumin in the prevention and diagnosis of alzheimer's disease, Br. J. Nutr. 115 (3) (2016) 449–465.

[2] K.I. Priyadarsini, The chemistry of curcumin: from extraction to therapeutic agent, Molecules 19 (2014) 20091–20112.

[3] Y.J. Wang, M.H. Pan, A.L. Cheng, L.I. Lin, Y.S. Ho, C.Y. Hsieh, J.K. Lin, Stability of curcumin in buffer solutions and characterization of its degradation products, J. Pharm. Biomed. Anal. 15 (1997) 1867–1876.

[4] H.H. Tønnesen, Solubility, chemical and photochemical stability of curcumin in surfactant solutions. studies of curcumin and curcuminoids, Pharmazie 57 (2002) 820–824.

[5] N. Ghalandarlaki, A.M. Alizadeh, S. Ashkani-Esfahani, Nanotechnology-applied curcumin for different diseases therapy, Biomed Res. Int. (2014), 394264-394287.

[6] I.K. Priyadarsini, Photophysics, photochemistry and photobiology of curcumin: studies from organic solutions, bio-mimetics and living cells, J. Photoch. Photobiol. C: Photochem. Rev. 10 (2009) 81–95.

[7] D. Yanagisawa, N. Shirai, T. Amatsubo, H. Taguchi, K. Hirao, M. Urushitani, S. Morikawa, T. Inubushi, M. Kato, F. Kato, K. Morino, H. Kimura, I. Nakano, C. Yoshida, T. Okada, M. Sano, Y. Wada, K. Wada, A. Yamamoto, I. Tooyama, Relationship between the tautomeric structures of curcumin derivatives and their αβ-binding activities in the context of therapies for alzheimer's disease, Biomaterials 31 (2010) 4179–4185.

[8] S.C. Gupta, S. Prasad, J.H. Kim, S. Patchva, L.J. Webb, I.K. Priyadarsini, B.B. Aggarwal, Multitargeting by curcumin as revealed by molecular interaction studies, Nat. Prod. Rep. 28 (12) (2011) 1937–1955.

[9] A.A. D'Archivio, M.A. Maggi, Investigation by response surface methodology of the combined effect of pH and composition of water-methanol mixtures on the stability of curcuminoids, Food Chem. 219 (2017) 414–418.

[10] M.H.M. Leung, H. Colangelo, T.W. Kee, Encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis, Langmuir 24 (2008) 5672–5675.

[11] X. Chen, L. Zou, J. Niu, W. Liu, S. Peng, C. Liu, The stability, sustained release and cellular antioxidant activity of curcumin nanoliposomes, Molecules 20 (2015) 14293–14311.

[12] M. Suwalsky, M. Jemiola-Rzeminska, C. Astudillo, M.J. Gallardo, J.P. Staforelli, F. Villena, K. Strzalka, An in vitro study on the antioxidant capacity of usnic acid on human erythrocytes and molecular models of its membrane, Biochim. Biophys. Acta 1848 (11 Part A) (2015) 2829–2838.

[13] L. Saso, O. Firuzi, Pharmacological applications of antioxidants: lights and shadows, Curr. Drug Targets 15 (13) (2014) 1177–1199.

[14] H.L. Lin, T.-C. Chang, S.-T. Chang, A review of antioxidant and pharmacological properties of phenolic compounds in Acacia confusa, J. Tradit. Complement. Med. 8 (4) (2018) 443–450.

[15] A. Bast, G.R.M.M. Haenen, Ten misconceptions about antioxidants, Trends Pharmacol. Sci. 34 (8) (2013) 430–436.

[16] P. Bellio, F. Brisdelli, M. Perilli, A. Sabatini, C. Bottoni, B. Segatore, D. Setacci, G. Amicosante, G. Celenza, Curcumin inhibits the SOS response induced by levofloxacin in Escherichia coli, Phytomedicine 21 (4) (2014) 430–434.

[17] O. Erel, A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation, Clin. Biochem. 37 (4) (2004) 277–285.

[18] CLSI, Performance Standards for Antimicrobial Susceptibility Testing, 27th ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2017, CLSI supplement M100.

[19] L.C. Price, R.W. Buescher, Kinetics of alkaline degradation of the food pigments curcumin and curcuminoids, J. Food Sci. 62 (1997) 267–269.

[20] A.A. D'Archivio, M.A. Maggi, F. Ruggieri, Extraction of curcuminoids by using ethyl lactate and its optimisation by response surface methodology, J. Pharm. Biomed. Anal. 149 (2018) 89–95.

[21] M. Langner, S.W. Hui, Iodide penetration into lipid bilayer as a probe of membrane lipid organization, Chem. Phys. Lipids 60 (2) (1991) 127–132.

[22] D. Marquardt, N. Kučerka, S.R. Wassall, T.A. Harroun, J. Katsaras, Cholesterol's location in lipid bilayers, Chem. Phys. Lipids 199 (2016) 17–25.

[23] S.L. Scott, W.J. Chen, A. Bakac, J.H. Espenson, Spectroscopic parameters, electrode potentials, acid ionization constants, and Electron exchange rates of the 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonate) radicals and ions, J. Phys. Chem. 97 (25) (1993) 6710–6714.

[24] S.-Y. Teow, K. Liew, S.A. Ali, A.S.–B. Khoo, S.–C. Peh, Antibacterial action of curcumin against Staphylococcus aureus: a brief review, J. Trop. Med. 2853045 (2016) 10.

[25] H. Gunes, D. Gulen, R. Mutlu, A. Gumus, T. Tas, A.E. Topkaya, Antibacterial effects of curcumin: an in vitro minimum inhibitory concentration study, Toxicol. Ind. Health 32 (2) (2016) 246–250.