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Research Paper

Phycocyanin liposomes for topical anti-inflammatory activity: in-vitro in-vivo studies

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Abstract

Objectives The aim of this work was to investigate the anti-inflammatory activity of C-phycocyanin (C-PC) on skin inflammation after topical administration and the influence of liposomal delivery on its pharmacokinetic properties.

Methods Liposomes of different size and structure were prepared with different techniques using soy phosphatidylcholine and cholesterol. Vesicular dispersions were characterised by transmission electron microscopy, optical and fluorescence microscopy for vesicle formation and morphology, dynamic laser light scattering for size distribution, and Zetasizer for zeta-potential. C-PC skin penetration and permeation experiments were performed *in vitro* using vertical diffusion Franz cells and human skin treated with either free or liposomal drug dispersed in a Carbopol gel.

Key findings The protein was mainly localised in the stratum corneum, while no permeation of C-PC through the whole skin thickness was detected. Two percent C-PC-encapsulating liposomes showed the best drug accumulation in the stratum corneum and the whole skin, higher than that of the corresponding free 2% C-PC gel. Moreover, skin deposition of liposomal C-PC was dose dependent since skin accumulation values increased as the C-PC concentration in liposomes increased. The topical anti-inflammatory activity of samples was evaluated *in vivo* as inhibition of croton oil-induced or arachidonic acid-induced ear oedema in rats.

Conclusions The results showed that C-PC can be successfully used as an anti-inflammatory drug and that liposomal encapsulation is effective in improving its anti-inflammatory activity.

Keywords cutaneous delivery; dehydrated–rehydrated vesicles; inflammation; liposomes; phycocyanin

Introduction

Phycobiliproteins are water-soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae. In these organisms, they act as accessory pigments for photosynthetic light collection. On the basis of their colour, phycobiliproteins are classified into two large groups – phycoerythrins (red) and phycocyanins (blue). C-phycocyanin (C-PC) is one of the main biliprotein constituents of blue-green algae, such as *Spirula* (*Arthospira) maxima*.

At present C-PC is used as a nutrient-dense source in food,[1,2] as a colorant in cosmetics and food[3,4] and in biomedical applications.[5]

The antioxidant and anti-inflammatory properties of C-PC have been described by several authors. In fact, Romay *et al*.[6,7] showed that C-PC is an efficient scavenger of oxygen free radicals and also reacts with other oxidants of pathological relevance. Moreover, the effective capability of C-PC to inhibit CCl_4-induced lipid peroxidation in rat liver *in vivo* as well as the contribution of the chromophore, phycocyanobilin to the antioxidant activity has been demonstrated.[8] Benedetti *et al*.[9] demonstrated the ability of a natural extract from a blue-green alga enriched with C-PC to protect normal erythrocytes and plasma against oxidative damage *in vitro* and the involvement of C-PC in the antioxidative protection of the extract. C-PC extract was also shown to reduce leukotriene B_4 levels in arachidonic acid-induced mouse-ear inflammation test.[10] Furthermore,

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Reddy *et al*.[11] pointed out that the hepatoprotective, anti-inflammatory and anti-arthritic properties of C-PC is due to its selective cyclo-oxygenase (COX)-2 inhibition. C-PC was shown to inhibit nitrite production and inducible nitric oxide synthase (iNOS) induction and tumour necrosis factor (TNF)-α formation and to attenuate nuclear NF-κB activation in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.[12] Recently, the ability of C-PC to accelerate wound healing by a urokinase-type plasminogen activator-dependent and independent mechanism was described.[13]

Despite the great amount of research on the anti-inflammatory properties of C-PC and the knowledge that proteins can act in the sub-cutaneous tissue,[14] C-PC has never been studied following topical application. Topical application of peptides and proteins can give several advantages over other traditional routes. Indeed, it avoids the effects of both gastric degradation and hepatic first-pass metabolism, it presents a large surface area for absorption, it has relatively low proteolytic activity and the skin is undoubtedly one of the most easily accessible organs of the body.[15] However, the skin is very effective as a selective penetration barrier and the outermost skin layer, the stratum corneum (SC), provides the main barrier function as a consequence of its specialised 'brick and mortar' structure. The multilayered membrane allows no drug to pass readily, but nearly all penetrate to some extent via passive diffusion. However, to be delivered passively through the skin, drugs need to have an adequate lipophilicity and also a relatively low molecular weight (< 500 Da). Peptides and proteins are generally large and hydrophilic and, hence, are poorly absorbed. Therefore, numerous strategies for formulation optimisation have been developed to overcome the skin's barrier function in a reversible manner. These include penetration enhancers to transiently reduce the skin barrier function, chemical modifications to improve drug hydrophobicity, and encapsulation into suitable carriers. Encapsulation within vesicular carriers, such as liposomes, niosomes, tranferosomes and ethosomes, has been proposed for dermal and transdermal drug delivery since these carriers can aid macromolecules to overcome the skin barrier.[16–23] Indeed, liposomes and niosomes are known to increase skin hydration and behave as skin permeation enhancers.

The aim of this work was to study the in-vivo anti-inflammatory property of C-PC on skin inflammatory disease models after topical administration and to evaluate the influence of liposomal encapsulation on its pharmacokinetic properties.

In this work, the effects of C-PC entrapment in liposomes on the in-vitro (trans)dermal delivery and in-vivo anti-inflammatory activity of the hydrophilic protein C-PC was studied.

Materials and Methods

Materials

Soy phosphatidylcholine (PC), cholesterol (Chol) and all the other products were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). C-Phycocyanin (C-PC; 97% purity) extracted from the microalgae *Arthrospira maxima* was a gift from Dr Cheyla Romay. Male rats (OF1), 20–25 g, were purchased from the National Center for Laboratory Animal Production, Havana, Cuba. Human skin specimens were obtained from abdominoplasty surgery and kept at −18°C until used.

Vesicle preparation

Multilamellar vesicles (MLVs) were prepared according to the thin film hydration method. PC and Chol in chloroform solution were mixed in an equimolar ratio. The lipid mixture was deposited as a thin film in a round-bottom flask by roto-evaporating the chloroform under vacuum. The vacuum was applied for one hour to ensure total removal of trace solvents. The film was hydrated with phosphate-buffered saline solution (PBS, pH 7.0) or with C-PC solution (35 mg/ml) at room temperature by mechanical shaking for 1 h. The final lipid concentration was 32 μmol/ml in all cases.

Empty MLVs were sonicated to obtain sonicated vesicles (SVs) in a Soniprep 150 apparatus (MSE, Crowley, UK), under a nitrogen stream for 8 min (99 times for 5 s) at room temperature. C-PC was encapsulated in SVs by mixing them with the same volume of C-PC solution in PBS (35 mg/ml).

Dehydrated–rehydrated vesicles (DRVs) were prepared from empty MLVs according to the procedure described by Kirby & Gregoriadis.[24] Briefly, MLVs were hydrated with de-ionised water and sonicated. Encapsulation of C-PC was achieved by mixing the obtained SVs with the same volume of a 35 mg/ml C-PC solution in PBS and shaking using a vortex mixer for 5 min. Then, vesicles were frozen with liquid nitrogen (–196°C) for 10 min and freeze-dried overnight in a freeze-drier (Advantage XL; Virtis, NY, US). Liposomes were previously sampled into 8R-type borosilicate vials containing 2.0 ml per vial. The shelf temperature was fixed to −50°C, and the pressure in the chamber was 50 mTorr. At the end of the freezing-drying process the chamber was filled with nitrogen gas, the vials were closed until further treatment and then reconstituted by controlled rehydration in distilled water.

Vesicle suspensions were purified from the unentrapped C-PC by centrifugation (Mikro 200 Hettich) at 14 000 rev/min for 20 min at 4°C. Supernatant was removed by a syringe filter and pellets were washed three times with PBS (pH 7.0). The supernatant was analysed for C-PC content by the UV/visible method described below. Encapsulation efficiency (E%), expressed as a percentage of the total amount of C-PC entrapped in the studied formulations at the end of the preparation procedure, was determined spectrophotometrically after disruption of the purified vesicles with 1% Triton X-100 in PBS (pH 7.0) according to the following equation:

$$E\% = 100 \times [\text{C-PC in the purified liposomes}]/[\text{C-PC total amount in vesicular suspensions}] \quad (1)$$

The C-PC content of samples was analysed at 615 nm using a UV-visible spectrophotometer (UV-Visible; Hitachi-U200). All suspensions were prepared under yellow light and kept in the dark at all times.

Vesicle characterisation

Vesicles were characterised by transmission electron microscopy (TEM), optical and fluorescence microscopy for

vesicle formation and morphology, by dynamic laser light scattering (DLLS) for mean size and polydispersity index (PI), and by Zetasizer (ZS) for zeta-potential.

A drop of vesicle dispersion was applied to a carbon film-covered copper grid and was stained with 1% phosphotungstic acid. Then samples were examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV.

Optical and fluorescence micrographs were obtained with an optical microscope (Olympus AT70TF) equipped with a camera (Olympus C-2000 zoom). C-PC fluorescence was observed with a bandpass filter set for protein.

Liposome size distribution was determined by DLLS (N4 plus; Beckman Coulter) at 25°C. Samples were scattered (633 nm) at an angle of 90°. Data were fitted by the method of inverse 'Laplace transformation' and Contin.

Liposome zeta-potential was determined by Zetasizer (Nanoseries; Malvern, UK) at 25°C. Data were worked out by Smoluchowsky equation.

Gel preparation

Carbopol 940 (0.5% w/w final concentration) was dispersed in glycerine (5% w/w final concentration) and distilled water. The mixture was stirred for 1 h. Triethanolamine was added to obtain pH 5.6 with gentle stirring to avoid inclusion of air. Finally, empty or C-PC-loaded DRVs (0.5, 1, 2% w/w), or C-PC PBS solution was incorporated.

In-vitro skin permeation studies

In-vitro skin permeation studies were performed using vertical diffusion Franz cells with an effective diffusion area of 0.636 cm^2. The experiments were carried out using human skin specimens obtained from abdominal plastic surgery. The skin, previously frozen at −18°C for a maximum of six months, was pre-equilibrated in PBS solution at +25°C for 12 h before the experiments. A circular piece of this skin was sandwiched securely between the two halves of the cell with the SC side facing the donor compartment. The receiver compartment was filled with 5.5 ml of PBS solution and continuously stirred. To achieve a skin temperature of 32°C (in-vivo value), the receptor fluids were maintained at 37°C by using cell jackets perfused at 37 ± 1°C throughout the experiments. In each donor compartment 10 μl of acetone were placed on the skin surface to simulate the same in-vivo experimental conditions. The tested (50 μl) C-PC gels (5, 10 and 20 mg/ml C-PC) or control free C-PC gels were placed on the membrane surface. Before starting the experiments, the donor cell was sealed with parafilm and the cells were covered with aluminium foil to prevent exposure to light. At hourly intervals up to 8 h, 1 ml of the receiving solution was removed and replaced with an equal volume of pre-thermostated (37°C) fresh PBS solution. The solutions were analysed by a UV/visible spectrophotometer (UV-Visible, Hitachi-U200).

At the end of the permeation study, the skin surface was washed three times with 1 ml of PBS and dried with filter paper. The SC was removed by stripping (20 times) with tapes of Tesa film (Tesa, Hamburg, Germany) and then extracted with 1 ml of PBS for 24 h. The other unstripped skin samples (whole skin) were each soaked in a flask with 1 ml of PBS for 24 h. Then the samples were shaken by sonication in a Soniprep 150 apparatus (MSE) 10 times for 2 min each to extract all the drug accumulated in the skin pieces. Studies were performed in triplicate and the mean values were used for the analysis of the data.

In-vivo topical anti-inflammatory activity

The topical anti-inflammatory activity of samples was evaluated as an inhibition of croton oil-induced[25] or arachidonic acid-induced[26] ear oedema in rats. The rats were housed under a 12-h light–dark cycle with room temperature maintained at 25°C, humidity at 60% and food and water freely available. All animal experiments were performed in accordance with the ethical guidelines for investigations in laboratory animals (European Community Council Directive of November 24, 1986; 86/609 ECC) and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research, Habana, Cuba. Male rats (OF1), 20–25 g, were used for the current study. Inflammation response was induced on the right ear surface (about 1 cm^2) by application of 15 μl of an acetone solution containing the irritant – 75 μg of croton oil (CO) or 2 mg arachidonic acid (AA). The left ear remained untreated as control. After acetone evaporation, the rat ear tissue (0.283 cm^2) was topically treated with 20 μl of C-PC liposomal gel (0.5, 1 or 2%), or free C-PC (2%) gel, or empty liposomes in Carbopol gel. In each group treated with arachidonic acid two reference rats were treated with indometacin (Ind) (1%) gel. In each group of rats treated with croton oil two reference rats received triamcinolone (Tri) (1%) gel. Four hours (CO-oedema induced) or one hour later (AA-oedema induced), rats were sacrificed by an overdose of ether and a plug (6 mm Ø) was removed from both right and left ear tissue (0.283 cm^2). Oedematous response was measured as the weight difference (mg) between treated and untreated tissues, and the anti-inflammatory activity was expressed as a percentage of oedema inhibition in the treated rats compared with the control rats (Oed I%). After CO-induced inflammation, ear tissue was weighed and assessed for the content of myeloperoxidase (MPO).[27,28] Briefly, tissue was minced and homogenised in 50 mM KH_2PO_4–K_2HPO_4 buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) to obtain 50 mg of tissue per ml. The homogenate was freeze–thawed three times to release the content of neutrophil granules. After centrifugation, samples of the supernatant were taken for determination of MPO enzyme activity spectrophotometrically using hydrogen peroxide as a substrate and O-dianisidine as the hydrogen donor. One unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 22°C. MPO data were collected as absorbance at 460 nm using a UV spectrophotometer apparatus (UV 240 Shimadzu). MPO data are reported as units per gram of tissue or percent reduction with respect to the control group (without treatment) (MPO I%). Three experimental groups of twelve rats were tested for each irritant treatment and experiments were carried out in triplicate. All the rats of each group were investigated simultaneously, thus all procedures were done in parallel.

Statistical analysis of data

Data analysis was carried out with the software package Microsoft Excel, version 2003. Results are expressed as mean ± standard error (SEM). Pharmacological data were analysed using the Stat Soft program 2003. One way analysis of variance was used to substantiate statistical differences between groups, while the Student's *t*-test and Duncan test were used as post-hoc tests for comparison of the other data. $P < 0.05$ was taken as the minimal level of significance.

Results

Vesicle characterisation

To achieve a liposomal formulation suitable to enhance (trans)dermal delivery of C-PC, as well as its residence time on the skin, we prepared and characterised three different liposomal dispersions. Liposomes were prepared with an identical lipid composition (PC-Chol 16 : 16 μmol/ml) and C-PC concentration (35 mg/ml), but they differed in structure. More precisely, we prepared multilamellar vesicles (MLVs), sonicated vesicles (SVs) and dehydrated–rehydrated vesicles (DRVs) by using different methods. Indeed, MLVs were prepared by mere mechanical shaking for 1 h, while SVs were obtained by means of the energy of a sonication process applied to MLVs. Finally DRVs were prepared from SVs following a dehydration–rehydration procedure.[24]

Vesicle formation and morphology were confirmed by TEM and optical microscopy. In particular, Figure 1 shows a fluorescence photomicrograph of unpurified C-PC-loaded DRVs. As can be seen, fluorescence microscopy confirmed the presence of the fluorescent protein inside and outside the liposomes. In Table 1 the physicochemical properties of the vesicles are shown. All liposomes showed a high negative zeta-potential value. Vesicle mean size and polydispersity index decreased from MLVs to SVs to DRVs. All formulations showed a good encapsulation efficiency (32–49%). Overall, DRVs showed the best properties in terms of size distribution (PI = 0.2) and entrapment efficiency (49 ± 5%) and they were chosen as carriers for the topical delivery of C-PC.

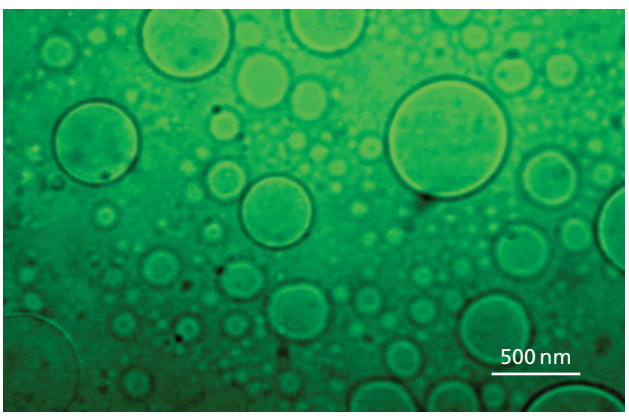

Figure 1 Fluorescence micrograph of unpurified C-phycocyanin loaded DRVs.

Table 1 Physico-chemical properties of phycocyanin entrapping liposomes

Liposomes	Mean Size (nm)	PI	Zeta-potential (mV)	E%
MLVs	979 ± 98	0.538	–50 ± 7	32 ± 7
SVs	657 ± 67	0.385	–43 ± 6	36 ± 4
DRVs	591 ± 73	0.216	–58 ± 9	49 ± 5

PI, polydispersity index; E%, encapsulation efficiency; MLVs, multilamellar vesicles; SVs, sonicated vesicles; DRVs, dehydrated–rehydrated vesicles. Size, zeta-potential and E% are expressed as mean ± SEM, $n = 9$.

In-vitro skin permeation study

The in-vitro permeation study was carried out using human skin and vertical Franz diffusion cells, in non-occlusive conditions. To increase the biliprotein residence time on the skin and to make administration easier, free (2% w/w, control) or DRV-encapsulated C-PC was dispersed in a Carbopol gel at a final drug concentration of 0.5, 1 or 2% w/w. No permeation of C-PC through the whole skin thickness was detected in this study using both controls and liposomal formulations but the protein was able to accumulate into the SC and whole skin as can be seen from Figure 2, which shows the cumulative amounts of C-PC retained in the SC and in the whole skin per unit of surface area at the end of the experiments (8 h). However, the protein accumulation values into the skin were quite low, reaching approximately 3% of the applied dose. The protein was mainly localised in the SC and only a small amount was found in the inner skin layers.

Nevertheless, 2% C-PC-encapsulating liposomes showed the best drug accumulation into the SC and the whole skin, higher than that of the corresponding free 2% C-PC gel ($P < 0.05$). In addition, skin accumulation values increased as the C-PC concentration in liposomes increased. As can be seen from Figure 2, the 1% liposomal C-PC gel showed drug accumulation values (whole skin and SC) statistically equivalent to those of the free 2% C-PC gel ($P > 0.05$).

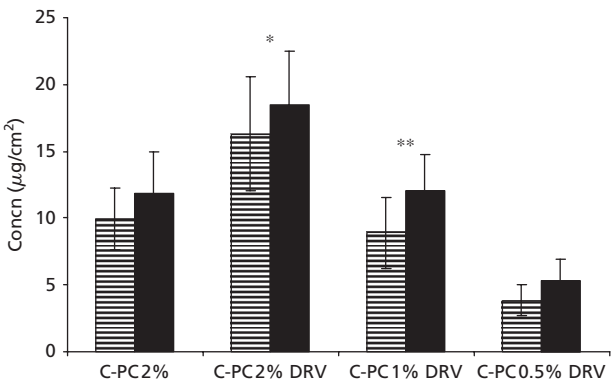

Figure 2 In-vitro accumulation in human skin of C-phycocyanin (C-PC) from different gel formulations (free C-PC and dehydrated–rehydrated vesicles (DRVs)) after 8 h treatment. Striped columns, stratum corneum; solid columns, whole skin. The applied dose of each formulation was 50 μl of gel (0.5, 1, 2% C-PC) for 0.636 cm^2 of skin. Bars represent the SEM, $n = 18$. *$P < 0.05$ compared with free CP-C 2% gel; **$P > 0.05$ compared with free CP-C 2% gel.

In-vivo topical anti-inflammatory activity

The influence of DRV encapsulation on the topical delivery of C-PC was also tested *in vivo* by comparing the anti-inflammatory activity of the free and the liposomal protein, using two different inflammation models, the arachidonic acid (AA)-induced rat ear swelling test and the croton oil (CO)-induced ear oedema in rats.

In the AA-induced rat ear swelling test, the anti-inflammatory effect of C-PC was evaluated by conventional ear thickness measurements. We compared the effects of the free and liposomal C-PC with those of indometacin (1%) by evaluating the oedema inhibition percentage (Oed I%) (i.e. the percentage of oedema reduction in the treated rats compared with control rats) (Table 2). Carbopol gel alone, as well as empty liposomes, showed no anti-inflammatory activity ($P > 0.05$ in comparison with AA). In the tested samples, indometacin gel (1%) showed the highest Oed I% value (95.18), which was 2-fold higher than that of 2% free protein gel (46.90). However, C-PC induced an anti-inflammatory activity that was dose-dependent when the protein was tested in the liposomal gels. In fact, it increased with protein concentration (2% > 1% > 0.5%), and was improved by liposomal encapsulation, although the 0.5% liposomal gel showed a non-significant activity ($P > 0.05$ in comparison with AA). Nevertheless, 2% C-PC-encapsulated liposome gel showed an Oed I% value 1.46 times that of free C-PC at the same concentration (2%; $P < 0.05$) and was not far away from that of the potent non-steroidal anti-inflammatory drug (NSAID) indometacin. Moreover, 1% C-PC liposome gel (Oed I% = 50.60) was as active as the free 2% C-PC gel (Oed I% = 46.90).

In the CO-induced ear oedema in rats, we compared the drug activity as a function of Oed I% and myeloperoxidase reduction (MPO I%). We evaluated C-PC formulations in comparison with triamcinolone, a corticosteroid (Table 3). In these experiments the Carbopol gel alone and empty liposomes did not show any anti-inflammatory activity. Triamcinolone was the most active compound with a 100% MPO activity reduction. However, C-PC also showed a good anti-inflammatory activity, which was enhanced by its liposomal encapsulation. In fact, 1% C-PC liposomal gel was at least as active as the free 2% protein. The dose-dependent activity of the protein was also confirmed by these experiments: C-PC Oed I% and MPO I % improved as protein concentration in liposomes increased and a good MPO I% (26%, $P < 0.05$) was found for the lowest tested C-PC concentration (0.5%). As shown in Tables 2 and 3, oedema inhibition of C-PC after AA inflammation was a little higher than that measured in the CO-induced inflammation.

An excellent correlation between the results obtained using the two different inflammatory models was observed ($r^2 = 0.9984$; $y = 1.5385x - 4.5419$). Similarly, an excellent correlation between the reduction of the CO-induced ear oedema in rats and the MPO activity was also detected ($r^2 = 0.9951$; $y = 0.4237x - 2.7573$).

Table 2 Topical anti-inflammatory activity of free and liposomal C-phycocyanin (C-PC) gel assessed by arachidonic-acid-induced ear oedema in rats

Sample	Oedema (mg)	Oed I %
Control (no treatment)	6.90 ± 0.40^{a}	n.a.
Acetone	7.10 ± 0.36^{a}	n.a.
Arachidonic acid	15.20 ± 1.20^{b}	n.a.
Gel	15.00 ± 1.00^{b}	n.a.
Empty liposome gel	14.70 ± 1.10^{b}	5.09
1% Indometacin gel (reference drug)	7.30 ± 0.20^{a}	95.18
2% C-PC gel	11.30 ± 1.10^{c}	46.90
2% C-PC liposome gel	9.50 ± 0.30^{d}	68.67
1% C-PC liposome gel	11.00 ± 1.10^{c}	50.60
0.5% C-PC liposome gel	13.99 ± 1.40^{b}	35.64

Oed I %, percent oedema inhibition; n.a., not applicable. Oedema value is expressed as mean ± SEM, $n = \geq 9$. $^{a–d}$Similar letters show no significant difference between groups; different letters show significant differences between groups ($P < 0.05$). Incubation time was 1 h.

Discussion

The results reported in Table 1 confirmed that vesicle properties were dependent on the preparation method and therefore on the energy and time employed in the vesicle formation process.[29] Indeed, inspection of the mean size

Table 3 Topical anti-inflammatory activity of free and liposomal C-phycocyanin (C-PC) gel assessed by croton-oil-induced ear oedema in rats

Sample	Oedema (mg)	Oed I%	MPO activity (U/mg tissue)	MPO I %
Control (no treatment)	6.80 ± 0.40^{a}	n.a.	0.17 ± 0.07^{a}	n.a.
Acetone	6.88 ± 0.36^{a}	n.a.	0.42 ± 0.05^{b}	n.a.
Croton oil	13.20 ± 1.20^{b}	n.a.	2.83 ± 0.27^{c}	n.a.
Gel	13.00 ± 1.00^{b}	n.a.	2.80 ± 0.14^{c}	n.a.
Empty liposome gel	12.80 ± 1.10^{b}	4.85	2.60 ± 0.20^{c}	7.14
1% Triamcilone gel	7.35 ± 0.42^{a}	91.40	0.16 ± 0.02^{a}	100.00
2% C-PC gel	$10.23 \pm 0.53^{d*}$	45.40	1.50 ± 0.10^{f}	50.00
2% C-PC liposome gel	9.35 ± 0.69^{c}	60.15	1.25 ± 0.10^{e}	60.27
1% C-PC liposome gel	$10.00 \pm 0.59^{d*}$	49.00	1.44 ± 0.12^{f}	52.25
0.5% C-PC liposome gel	11.63 ± 0.60^{e}	24.53	2.13 ± 0.20^{d}	26.31

Oed I %, percent oedema inhibition; MPO, myeloperoxidase; MPO I%, percentage reduction in MPO activity; n.a., not applicable. Oedema value and Oed I% are expressed as means ± SEM, $n = \geq 9$. Incubation time was 4 h. $^{a–f}$Similar letters show no significant difference between groups; different letters show significant differences between groups. $^{*}P < 0.05$, compared with 2% C-PC liposome gel ($n = 12$).

showed that an increase in the preparation energy entails a smaller vesicle size. Moreover, MLVs had the highest PI as well as the lowest E%, while DRVs had the best results in terms of PI and E%, as a consequence of the higher energy supplied in their preparation.

Mean E% ranged from 32% (MLVs) to 49% (DRVs), which are good loading yields for a hydrophilic macromolecule.[29–31] Zeta-potential values were very similar for the three formulations and the small differences (Table 1) could depend on the different C-PC E% and vesicle dimensions. The values were always negative and ranged from −43 to −58 mV, which indicates a good stability of all vesicular dispersions. Previously, it was reported that small unilamellar vesicles are better carriers for the delivery of different drugs locally to the skin.[32–35] Since C-PC-loaded DRVs, obtained from SV dispersions, had shown the best physico-chemical properties (smallest mean size, high homogeneity, highest E%), in this work we selected these formulations as a potential delivery system for the topical application of C-PC.

It is known that conventional liposomes themselves do not penetrate intact through the skin but they facilitate deposition of liposome-associated drug into the skin.[32,36] Therefore, liposomes are potential suitable carriers for site-directed delivery of proteins by the topical route and can provide an adequate local drug concentration in subcutaneous diseased tissue without systemic distribution. A first conclusion, based on data reported in Figure 2, is that C-PC was unable to reach the receptor compartment but, in these experimental conditions (8 h, pH 7.0 and 20 mg/ml), it was able to accumulate into the SC and whole skin. C-PC was mainly localised in the SC and only a small amount was found in the inner skin layers. Nevertheless, 2% C-PC-encapsulating liposomes showed the best drug accumulation into the SC and the whole skin, higher than that of the corresponding free 2% C-PC gel ($P < 0.05$). Moreover, skin deposition of liposomal C-PC was dose dependent.

Therefore, results obtained in this work have shown that liposomes are able to improve C-PC penetration and deposition into the skin. Comparison of skin retention obtained by 2% free C-PC and 1% C-PC-encapsulated liposomes show the same accumulation values ($P > 0.05$), thus showing the capability of these carriers to enhance cutaneous deposition of the tested protein. These results confirm the potential value of liposomes for topical therapy since they have been shown to improve drug penetration and deposition in the outermost skin layers. Several mechanisms have been proposed to explain vesicle–skin interaction.[32,34] Results obtained in this work could be due to the ability of the conventional liposomes to interact with SC intercellular components.

The influence of DRV encapsulation on the topical delivery of C-PC was also tested *in vivo* by comparing the anti-inflammatory activity of the free and the liposomal protein, using two different inflammation models. At first, the anti-inflammatory effect of C-PC was studied by conventional ear thickness measurements, using the AA-induced rat ear swelling test, which is a suitable model for screening inhibitors of lipoxygenase (LOX) or COX.[37] In AA-induced rat ear inflammation, we compared the therapeutic properties of the free and liposomal C-PC with those of the conventional well-known NSAID indometacin (1%). The anti-inflammatory activity of the samples was evaluated from the oedema inhibition percentage (Oed I%), which expresses the percentage of oedema reduction in the treated rats compared with control rats. As expected, Carbopol gel alone did not show any anti-inflammatory activity and neither did the empty liposomes. It is of note that the anti-inflammatory activity of C-PC in the liposomal gels was dose dependent, increased with protein concentration and was improved by the liposomal encapsulation. Indeed, 2% C-PC-encapsulating liposome gel showed a good anti-inflammatory activity (68.7%) not far away from that of the potent NSAID indometacin. Moreover, 1% C-PC liposome gel was as active as the free 2% C-PC gel, showing that liposomal encapsulation improves anti-inflammatory activity of the protein. This result is in agreement with in-vitro skin permeation studies that showed an improved cutaneous delivery of C-PC when liposomally encapsulated. In fact, a good correlation between the in-vitro whole skin accumulation and in-vivo CO Oed I % can be found ($r^2 = 0.986$; $y = 2.6281x + 11.853$), thus showing that the enhanced skin deposition of C-PC is accompanied by an increased anti-inflammatory activity.

To better investigate the anti-inflammatory activity of C-PC-encapsulated liposomes, we also used an alternative model of tissue inflammation and drug efficacy detection: the CO-induced ear oedema in rats. CO is an irritant natural oil that induces oedema and increases the activity of MPO, a specific polymorphonuclear leucocyte (PMN) enzyme, following topical administration. It is well known that application of a single dose of CO to rat ears induces an acute inflammatory reaction consisting of erythema, oedema and PMN infiltration. PMNs release MPO, which causes formation of irritant hypochlorite and water.[38] In these experiments we compared the drug activity as a function of Oed I% and myeloperoxidase reduction (MPO I%). The level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue.[39] In this work, we used MPO activity in inflamed ear tissue as a quantitative and sensitive assay of drug anti-inflammatory efficiency. In fact, recent observations suggest that quantification of MPO activity could be a useful laboratory technique to detect tissue inflammation.[40,41] In the CO inflammation test, we tested C-PC formulations from parallel experiments with a corticosteroid, triamcinolone. Again, the Carbopol gel alone and the empty liposomes did not show any anti-inflammatory activity. As expected, triamcinolone was the most active compound with a 100% MPO activity reduction. However, in this test C-PC also showed a good anti-inflammatory activity, which was enhanced by liposomal encapsulation. In fact, 1% C-PC liposomal gel was as active as the free 2% protein. The dose-dependent activity of the liposomal protein was also confirmed. Oedema inhibition of C-PC after AA-induced inflammation was a little higher than that measured in the CO-induced inflammation, possibly because C-PC is an inhibitor of both COX and LOX.[7]

Previous studies have demonstrated the ability of C-PC to prevent acetic-acid-induced gastric ulceration. Anti-ulcerogenic activity (and MPO inhibition) of the protein suggests that C-PC

could interfere with action or production of the eicosanoids.[40] It has also been shown that C-PC reduces inflammatory mediators (leukotriene LTB_4, and prostaglandin E_2) produced in the AA-induced mouse ear inflammation.[6] Moreover, Reddy *et al*.[11] demonstrated that C-PC is a selective inhibitor of COX-2. Results obtained during this work using the rat skin oedema model seem to confirm a relative specificity of C-PC protein in the inhibition of COX.

It is of note that an excellent correlation between the results obtained using the two different inflammatory models was observed, suggesting that a similar mechanism of action is involved. Among the mechanisms mentioned above, the reduction of MPO activity could play a significant role.

Conclusions

Results obtained in this work have shown that liposomes can be a good carrier for the topical delivery of anti-inflammatory proteins such as C-PC. The used conventional phospholipid vesicles were capable of improving anti-inflammatory activity in a dose-dependent fashion. Indeed, liposomes were able to produce the same anti-inflammatory response as the free protein by using half the C-PC dose. Correlation between in-vitro and in-vivo test also showed that this is the consequence of enhanced C-PC skin delivery obtained by using the liposomal carrier. Since skin drug delivery is strongly dependent on several factors related to vesicle composition and morphology, research on innovative vesicular formulations is in progress with the aim of further improving C-PC cutaneous delivery and studying the mechanisms involved in vesicle–skin interaction.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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