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Resveratrol liposomes in buccal formulations, an approach to overcome drawbacks limiting the application of the phytoactive molecule for chemoprevention and treatment of oral cancer

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ABSTRACT

Resveratrol is currently considered for chemoprevention and treatment of oral cancer, but unfavorable properties of this molecule hinder its clinical use. The present study deals with preparation of resveratrol liposomes by a method that preserves stability and provides payload high enough for chemoprevention and/or treatment of oral cancer. Different temperatures, resveratrol concentrations and molar fractions, as well as the presence or absence of vitamin C in the hydration media were assayed. Liposome hydrodynamic diameter, polydispersity and zeta potential were evaluated by dynamic light scattering. In vitro cytotoxicity, antioxidant activity, and permeability were evaluated using TR146 cells and PermeaPad® membranes. Liposomes were included in hydrogels and films, and their suitability for buccal application was evaluated by in vitro assays. A temperature of 37 °C was optimal to efficiently produce resveratrol liposomes. Vitamin C was found to influence liposome size, while resveratrol concentration significantly influenced entrapment efficiency. Cytotoxicity assay showed high biocompatibility while potent antioxidant activity, the inclusion of vitamin C reinforcing the latter effect. High permeation across membranes and efficient uptake by cells was proved for resveratrol liposomes. Liposomal hydrogels and films were obtained with payload high enough to produce mucosa concentrations above resveratrol IC50 values reported for cancer cells.

1. Introduction

Resveratrol (RSV) is a plant-derived phytoalexin compound, which is naturally produced in response to environmental stress and pathological invasion, acting as a natural inhibitor of cell proliferation. RSV has inhibitory effects on many processes such as platelet aggregation, vasoconstriction, proliferation of smooth muscle, low-density lipoprotein oxidation, and nitric oxide synthesis [1]. RSV has shown interesting effects on the tumor microenvironment and it is currently considered an option in the prevention and treatment of human cancer [2].

Its biochemical and molecular activities induce apoptosis among cancer cells by increasing pro-apoptotic p53 gene expression and decreasing expression of apoptotic inhibitor Bcl-2 [3,4]. Cancer chemoprevention is based on the use of nutraceuticals or phytochemicals to reverse carcinogenesis before the metastasis phase occurs. This can be achieved by blocking key events of tumor initiation and progression or by inhibition of the ability of cancer cells to migrate to other tissues. It has been demonstrated that adequate treatment during the early stage of cancer could positively affect the carcinogenesis pathways [5]. Numerous phytochemicals are proposed as chemopreventive agents, in particular RSV has recently been under the spotlight due to its wide-spectrum therapeutic properties. Literature data supports RSV as a potent antioxidant, chemopreventive and chemosensitizer agent that enhances anticancer therapies by regulating multidrug-resistant protein expressions, interfering with cell signal pathways and cell cycle regulators [6].

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Despite literature data evidencing RSV effectiveness against various diseases such as diabetes mellitus, metabolic syndrome, obesity, inflammation, cardiovascular, or neurodegenerative diseases and cancer, clinical application is hindered by its instability and unfavorable pharmacokinetic properties. RSV is a non-flavonoid polyphenolic compound with molecular weight of 228.25 g/mol, hydrosolubility of 0.03 mg/mL, and Log P of 3.1 [7] According to the Biopharmaceutical Classification System, RSV is considered a Class II compound [8] showing low solubility and high permeability, the latter allowing this molecule to cross the biological membranes to be widely absorbed after oral administration. Unfortunately, RSV is extensively metabolized in the enterocytes and hepatocytes, leading to glucuronidation and sulphate metabolites with low biological activity [9]. As a result of this, RSV plasma and tissue concentrations are below the IC50 values reported for in vitro cancer cells (5–100 μM) even after administration of very high oral doses [10,11]. Searching of analogue molecules with more favorable pharmacokinetic profile is among current strategies to overcome the RSV drawbacks, but nanotechnology appears as the most promising approach to achieve this goal [12]. Nanotechnology has been applied to produce controlled delivery systems with RSV, including liposomes, polymer nanoparticles, micelles, lipid and inorganic nanocarriers, nanocrystals, nanoemulsions, and bionic drug delivery systems [9]. Several studies have been carried out with liposomes for RSV to be targeted to different cells, such as folate decorated liposomes for osteosarcoma [13] *p*-aminophenyl alfa-D-mannopiranose or germ agglutinin decorated liposomes for brain gliomas [14] mitochondria-targeted liposomes [15,16], deformable and hyaluronic-acid decorated liposomes for epidermal cells [17], fusogenic liposomes for brain-microvascular vessels [18] or pH sensitive liposomes for microenvironment of tumor [19]. Moreover, liposomes have been applied for RSV to increase sensitivity to doxorubicin (DOX) and 5-fluorouracil of colorectal cancer cells [4,20], for docetaxel synergy in prostate cancer [21], and for protective effect against DOX and cisplatin side effects [6,22]. On top of that, RSV liposomes have been assayed for vaginal infection [23,24], periodontal diseases [25] and UV-induced skin damage [26].

Oral cancer represents the most common head and neck cancer, and most cases are histologically defined as oral squamous cell carcinomas (OSCC). The onset of this kind of tumor is multifactorial, starting from changes in the normal mucosa and evolving into cancer lesions that eventually lead to metastasis [27,28]. Despite the great advances in therapeutics over the past decades, the patients' survival rates are still below 50 % in this field [29]. Identification of novel therapeutic agents and innovative approaches, aimed at both treatment and prevention of OSCC, are high concerns among the scientific community. In this particular type of cancer, targeting to oral mucosa is essential to take advantage of the therapeutic properties assigned to RSV. Our hypothesis is that benefits might be achieved in patients prone to suffer buccal cancer or already suffering from OSCC, as far as controlled delivery of RSV at the oral mucosa is accomplished. Liposomes have proved their ability as drug nanocarriers for mucosa membranes [30,31] and physicochemical properties of RSV make it a good candidate to be loaded in liposomes. Accordingly, the aim of the present study was to efficiently produce RSV loaded liposomes suitable for topical application at the oral cavity. A solvent-free procedure that preserves RSV stability has been optimized to obtain RSV liposomes using EPC as the bilayer phospholipid. The produced liposomes have been characterized in terms of size, polydispersity, zeta potential, entrapment efficiency, cell biocompatibility, antioxidant efficacy and in vitro permeability. All these studies focused on assessing the suitability of RSV liposomes to be included in medicines for buccal administration aimed at chemoprevention and enhancement of oral cancer chemotherapy. The novelty of this work was to prepare cholesterol-free liposomes loaded with RSV and to include them in buccal formulations with high payload, as a proposal to overcome biopharmaceutical drawbacks limiting RSV application for chemo prevention and treatment of oral cancer.

2. Materials and methods

2.1. Materials

EPC (60 % egg L-α-phosphatidylcholine) and sodium alginate low and high viscosity (ALV and AHV, respectively) were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany). Tran-resveratrol (RSV), vitamin C, glycerine (G), citric acid monohydrate, xanthan gum and Tween-20® were purchased from Acofarma S.A. (Madrid, Spain). HPLC-grade ethanol and acetonitrile were supplied by Thermo Fisher Scientific (Germany). H_2PO_4, NaOH and Na_2HPO_4 were purchased from PanReac ApplieChem (Darmstadt, Germany). Ultrapure water was obtained with a Wasserlab Automatic Plus System. Cromafil® PETfilters from Macherey-Nagel (Duren, Germany). Human buccal carcinoma cell line TR146 (#151425) was purchased to CancerTools (UK), Dulbecco's Modified Eagle Medium (DMEM, #D5796), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, and 2,7-dichlorodihydrofluorescein diacetate (DCFDA, #D6883) were purchased to Merck KGaA (Germany). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was acquired from VWR (Radnor, Pennsylvania).

2.2. Preparation and characterization of liposomes

Liposomes were prepared from EPC and *trans*-resveratrol (RSV) at 0.3 and 0.5 RSV molar fractions and different RSV concentrations, as shown in Table 1. MilliQ-water or phosphate buffer pH = 7.4 (PBS) with 0.5 % w/w vitamin C were used as hydration media. A solvent free method previously described [32] was optimized to prepare the liposomes. In brief, the proper amounts of EPC and RSV were weighted and easily mixed due to the high lipid solubility of RSV. At room temperature the mixture is a viscous product that was extended on the walls of the glass vessel, then hydration media with 0.5 % w/w vitamin C or without vitamin C was added and mixed until lipid dispersion. The mixtures were placed in a Fisher Scientific FB 15061 ultrasonic bath (50 Hz) for 20 min. Different temperatures (30–40 °C range) were assayed to evaluate the influence of this condition on the efficiency of the process. The resulting suspensions were filtered (8 times across Cromafil® PET filters of 0.45 μm), and the samples were kept at room temperature for 60 min. Then, liposome suspensions were stored at 4–6 °C until use.

Turbidity, viscosity (γ), optical microscopy, as well as pH measurements were carried out as routine controls applied to each batch of prepared liposomes. Turbidity measurement was carried out using a HACH 2100Q turbidimeter calibrated with standard samples in a range of 400–12000 NTU (Nephelometric Turbidity Unit). Samples were properly diluted to ensure turbidity within the calibration range, and inserted in the portable turbidimeter. A glass capillary viscometer was used for viscosity (γ), which was estimated from the time it took for the sample to flow through the capillary marks under the influence of gravity. The time required by the sample to flow through the capillary tube marks was converted to a kinematic viscosity using the equation:

$$\gamma \text{ (Pa x s)} = K \text{ x } t \quad (1)$$

where t is the time (s) and K is the corresponding calibration constant value (k1 = 0.0164 and k2 = 0.012 6 for the first and second mark,

Table 1
Experimental conditions assayed to prepare resveratrol-(RSV) loaded liposomes (EPC:egg-phosphatidylcholine).

	0.3 RSV molar fraction		0.5 RSV molar fraction	
RSV concentration (mg/mL)	1.92	3.83	4.39	8.78
EPC concentration (mg/mL)	14.81	29.61	14.81	29.61
RSV + EPC concentration (% w/w)	16.73 (1.76)	33.44 (3.34)	19.20 (1.92)	38.39 (3.84)

respectively).

In addition to the above routine controls, the next assays were performed in triplicate.

- Hydrodynamic diameter (D_h), polydispersity index (PDI) and zeta potential of liposomes were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Co., Malvern, UK). The analysis was performed at 25 °C and a scattering angle of 173°, after the appropriate dilution with milli-Q with or without vitamin C, to avoid multiple scattering.
- Entrapment efficiency (EE%). Quantification of RSV in samples before and after filtration was carried out by an HPLC technique to evaluate the efficiency of the method to incorporate the RSV into the liposomes. A Purosphere STAR RP-18 endcapped, 50 mm × 4.0 mm, 5 μm column and a mixture of 0.1 % formic acid in water and acetonitrile (70/30 v/v), adjusted to pH 4.2 using triethanolamine, were used. 10 μL were injected with the column at 25 °C and 1 mL/min, flow rate. The UV detector was set at 292 nm (HPLC system with Waters Alliance 2695 separation module, 2998 photodiode array detector and empower processor system). The area under the curve of the RSV peak was registered for detection and further calculation of the RSV concentration. 2 mg/mL RSV stock solutions were prepared using 95 % v/v ethanol or water-ethanol mixture (50 % v/v). Calibration curves of RSV for each solvent were prepared at different concentration ranges and analyzed. The analytical method was validated for specificity, linearity, precision, accuracy and stability (Table S1 in supplementary material).

EE% was estimated according to the following equation

$$EE\% = (Cf/Ci) \times 100 \quad (2)$$

Where Cf is the RSV concentration measured in filtered liposome suspension and Ci is the initial RSV concentration used to prepare the liposomes.

RSV payload (μg/mL; mM) was estimated from corresponding EE% values.

Quantification of EPC in samples before and after filtration was carried out by a colorimetric method based on complex formation between ammonium ferrothiocyanate and phospholipids [33] to check EPC filter retention.

2.3. Studies with TR146 cell line

The TR146 cell line was used to model the human buccal epithelium and to evaluate the antioxidant in vitro effects of RSV liposomes. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37 °C in 5 % CO_2. Culture media was replaced every 2–3 days and at 70–80 % confluence cells were split using 0.25 % trypsin–EDTA. For all studies described TR146 cells were used between passage 16 and 19.

2.3.1. Cell cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used, following ISO 10993–5, to evaluate cell viability in the presence of liposomes. Cells were seeded in 96-well plates with DMEM, using a cellular density of 1×10^4 cells/well, and incubated at 37 °C in a 5 % CO_2 humidified atmosphere for 24 h. After 24 h, the culture medium was removed and replaced with fresh medium mixed with sterile liposome suspension diluted for different RSV concentrations (0.5–200 μg/mL) and maintained at 37 °C in a 5 % CO_2 humidified atmosphere for 24 h. After 24 h the culture medium was removed and replaced with 50 μL of MTT solution (5 mg/mL in PBS). The cells were then incubated for 3 h at a 37 °C in a 5 % CO_2 humidified atmosphere. Thereafter, MTT was removed and 100 μL of dimethyl sulfoxide (DMSO) was added and gently agitated for 15 min at room temperature to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a microplate reader (Multiskan GO—Thermo Scientific). Ethanol 70 % treated cells were used as positive controls (K+) (dead cells), whereas untreated cells (without liposomes) were used as negative controls (K-). The mean cell viability values (n = 3, independent experiments) have been expressed as the percentage of K+.

2.3.2. Reactive oxygen species (ROS) assay

ROS levels were analyzed using the cell-permeable probe 2,7-dichlorodihydrofluorescein diacetate. The DCFDA assay measures the fluorescent 2,7-dichlorofluorescein that results from DCFDA oxidation by ROS. About 2×10^4 TR146 cells were seeded in 96-well plates on the day before the experiments. Cells were incubated with DCFDA (50 μM) in complete medium for 1h at 37 °C, and then treated with the different formulations of liposomes for 1h. Cells incubated with DCFDA, without liposomes treatment, and cells treated with H_2O_2 were included in the assays as negative and positive controls, respectively. The fluorescence produced in the DCFDA assay was measured in a SpectraMax Gemini spectrofluorometer (Molecular Devices) at excitation/emission wavelengths of 485/535 nm.

2.4. Permeation assay

An Erweka vertical diffusion chamber with a permeation area of 2.2 cm^2 was used for this assay. A fixed volume (2 mL) of the liposome suspension was placed in the donor compartment, and a PermeaPad® membrane was mounted to reach occlusive conditions. Then, the chamber was immersed in a vase containing 100 mL of simulated salivary fluid (SSF) [34] of the following composition: Milli-Q water containing 7.7 % (v/v) phosphate buffer pH 7.0, 0.0056 % (w/w) Tween® 20 and 0.08 % (w/w) xanthan gum at 36 °C acting as the receptor compartment. SSF samples were taken at previously programmed times (2, 4, 6, 8, 10, 20, 30, 60, 90, 120 and 150 min) and replaced with the same volume of fresh SSF. RSV was quantified in the withdrawn samples by the HPLC method above described and the cumulative amount in the receptor compartment was estimated. The cumulative amount of permeated RSV was plotted against time, and the slope of the linear part of the curve, representing the steady-state flux rate (Jss), was used for permeability (P) calculation, according to the following equation:

$$P\ (\mu g/cm^2/min) = Jss/A \quad (3)$$

Where Jss is the slope of the linear fraction of the curve, and A is the permeation area.

The similarity factor (f2) was assessed to compare kinetic profiles from samples with and without vitamin C using the following equation

$$f_2 = 50\ log\left\{\left[1 + \frac{1}{n}\sum_{n-1}^{n}(R_t - T_i)^2\right]^{-0.5} * 100\right\} \quad (4)$$

Where n is the number of observations, R_t and T_t the average amount of RSV released from liposomes without vitamin C and with vitamin C, respectively. RSV release profiles were also analyzed based on the following plots: cumulative amount of drug released vs. time for zero order; log % drug remaining vs. time for first order; cumulative % drug release vs. square root of time for the simplified Higuchi model, and the log cumulative % drug released vs. log time for the Korsmeyer-Peppas model [35].

2.5. Buccal formulation (liposomal hydrogels and films)

Liposomal hydrogels and films were prepared using alginates of low and high viscosity (ALV and AHV, respectively) as gelling agent and glycerin (G) as hygroscopic/plasticizer agent. Different combinations of ALV (2–6%w/w) or AHV (2–4%w/w) with G (1–3%w/w) were assayed and the rheological properties (viscosity, extensibility, extrusion

properties) of resulting hydrogels were evaluated.

Hydrogels were prepared by adding the proper amount of alginate to the liposome suspension, the mixtures were agitated until sample homogenization and refrigerated overnight. Then G was added and the resulting mixtures were used for rheology assays and film preparation. Viscosity was measured at 25 °C using a rotational viscometer (801 Nahita). For the extensibility, increasing weights were applied to a fixed amount of hydrogel (0.5 g) placed between two glass plates and the diameter of the spread hydrogel area was recorded. Extrudability through 2–0.5 mm range was tested.

Films were obtained from hydrogels by the film casting method [36]. In brief, 3.5 g of hydrogel were poured into 2 cm side plates and maintained at 30 ± 2 °C for 14 h. Remaining moisture, flexibility and folding resistance of resulting films were tested at the end of drying period.

The experimental procedures above described were carried out protecting the samples from light exposure.

2.6. Electronic microscopy

Liposome suspension and liposomal film were observed by scanning-electron microscopy (SEM) with thermionic emitters using ZEISS microscope. Poly-L-lysine and osmium were used as fixing agents, ketone as the desiccant and gold for the metallic coating.

2.7. Statistical analysis

Statistical analysis and comparison were performed using GraphPad Prim 7, and one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test. Results are described as mean ± standard deviation and data were considered statistically significant at a value of $p < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of liposomes

The preparation of liposomes at temperature above a critical value is a requirement for lipids to hydrate and rearrange into the concentric bilayers forming liposomes. Resveratrol is found in either *cis*- or *trans*-configurations, but the latter is the only active form. Unfortunately, RSV exposure to light, pH > 7 or high temperature activates the pass of *trans*- to *cis*-configuration. Temperatures from 30 °C to 40 °C were assayed in this study and the results revealed that below 35 °C the process was not efficient (EE%<15 %), irrespectively of the RSV molar ratio (0.3 or 0.5), RSV concentration (1.90–8.78 mg/mL) or the presence or absence of vitamin C in samples. Ultrasonic agitation at 40 °C facilitated sample filtration, and produced higher EE% values. Nevertheless, the higher the temperature the greater the risk of isomerization of *trans*-resveratrol to the inactive isomer, therefore a balance between process efficiency and RSV stability was considered and a temperature of 37 °C was selected in our study to prepare the RSV liposomes.

Table 2 shows the main characteristics of produced liposomes. Acidic pHs were found in all cases, irrespectively of RSV molar fraction, RSV concentration and presence or absence of vitamin C, and lower pH values were achieved for samples with vitamin C. For RSV, an acidic medium is advantageous because low pH protects the active form from its isomerization to the cis isomer.

Regarding viscosity, lower values were found in samples with vitamin C, compared to those without the vitamin, irrespectively of RSV molar fraction and RSV concentration, the differences being statistically significant ($p < 0.001$).

Turbidity depends on the number and size of particles suspended in a liquid media and this was selected as the Critical Process Parameter (CPP) related to the liposome hydrodynamic diameter (D_h), which is a critical quality attribute (CQA) of liposomes. As found for viscosity, it was observed that vitamin C influenced turbidity and the samples with vitamin C presented higher values than those without the vitamin, irrespectively of the RSV molar fraction and RSV concentration, the differences showing statistical significance ($p < 0.001$). For D_h, in line with viscosity and turbidity data, results confirm the influence of vitamin C, and higher D_h values were observed for samples with vitamin C (213.26–278.43 nm range) compared to those without the vitamin (95.91–166.86 nm range). Higher osmolarity of samples with vit C might explain the increase of liposome D_h.

A linear relationship between turbidity and D_h was found for 0.3 and 0.5 RSV molar fraction, as shown in Fig. 1. This correlation validates the turbidity of liposome suspension as the CPP related to the CQA of liposomes. Notice the low variability of turbidity measurements, with variation coefficients below 1 % confirming the robustness and reproducibility of the solvent-free method applied here to prepare RSV liposomes. A similar procedure had been previously validated for liposomes of EPC and cholesterol (CHOL) bilayer loaded with sildenafil citrate [32], but this procedure has been applied for the very first time here to prepare EPC liposomes with RSV at low temperature.

PDI and zeta potential were not significantly affected by RSV molar fraction, RSV concentration or vitamin C in samples, although lower PDI values were observed for samples without vitamin C (PDI<0.25) than found for those with the vitamin (PDI 0.26–0.35 range). Negative values of ZP, in the range of −4.68 ± 0.66 mV to −12.56 ± 2.49 mV, were observed in all cases. These results are in line with previous studies performed to characterize the interactions of EPC with vitamin C which reported that the dielectric properties of the lipid bilayer were not

Table 2
Characteristics of resveratrol (RSV) liposomes obtained by a solvent-free procedure (NTU: Nephelometric Turbidity Unit; D_h: Hydrodynamic diameter; PDI: poly dispersion index; ZP: zeta potential).

RSV molar fraction	RSV concentration (mg/mL)		pH	Viscosity (mPa*s)	Turbidity (NTU)	D_h (nm)	PDI	ZP (mV)
0.3	1.92	Without vitamin C	4.39 ± 0.28	1.30 ± 0.11	2949.28 ± 7.94	150.56 ± 2.99	0.23 ± 0.02	−6.36 ± 2.08
		With vitamin C	3.67 ± 0.33	1.27 ± 0.06	6889.16 ± 29.14	278.43 ± 6.51	0.26 ± 0.01	−4.87 ± 0.80
	3.83	Without vitamin C	4.22 ± 0.34	1.78 ± 0.09	2980.39 ± 16.07	166.86 ± 2.45	0.21 ± 0.01	−6.13 ± 0.51
		With vitamin C	3.56 ± 0.18	1.46 ± 0.05	5900.34 ± 35.57	217.73 ± 11.77	0.35 ± 0.06	−5.08 ± 0.44
0.5	4.39	Without Vitamin C	4.37 ± 0.31	1.33 ± 0.07	5946.68 ± 42.10	95.91 ± 0.63	0.23 ± 0.01	−4.68 ± 0.66
		With vitamin C	3.74 ± 0.21	1.18 ± 0.04	11506.06 ± 25.11	270.96 ± 2.54	0.28 ± 0.01	−5.73 ± 0.68
	8.78	Without vitamin C	4.18 ± 0.27	1.58 ± 0.09	5532.83 ± 9.07	102.34 ± 2.17	0.25 ± 0.01	−5.96 ± 0.84
		With vitamin C	3.60 ± 0.24	1.35 ± 0.05	10647.10 ± 22.19	213.26 ± 6.16	0.29 ± 0.01	−12.56 ± 2.49

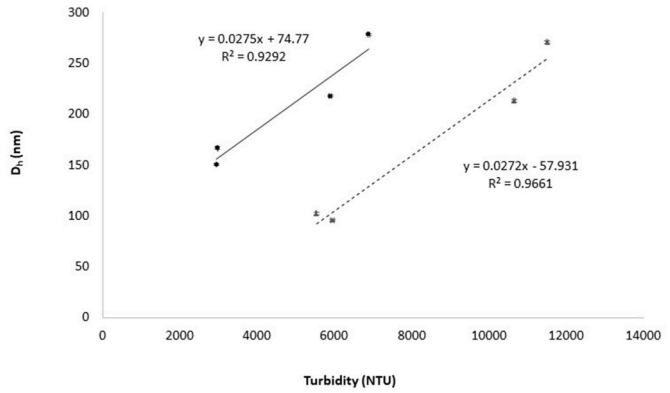

Fig. 1. Linear relationship between turbidity and hydrodynamic diameter (D_h) found for 0.3 (continuous line) and 0.5 (discontinuous line) resveratrol molar fraction.

altered by the vitamin, likely due to conservation of structured water of the phosphate groups in the polar heads of the lipids [37]. Comparison of RSV-EPC liposomes produced here with CHOL-EPC liposomes [38] reveals similar negative ZP values. Some previous studies have shown that RSV incorporates into the hydrophobic core of the EPC bilayer close to the double bonds of polyunsaturated fatty acids, similar to cholesterol location in lipid bilayers [39], while others have shown that RSV also adsorbs onto the membrane surface.

RSV calibration curves in the range of 25–300 μg/mL revealed linearity (r^2 = 0.999), irrespectively of using 95 % ethanol or water-ethanol mixture (Fig. S1 in supplementary material). It was found that RSV quantification in unfiltered liposome samples diluted with water-ethanol mixture produced concentration lower values than expected for the RSV amount initially added. This finding alerted on RSV remaining in the bilayer and not being properly quantified by the HPLC when samples were diluted with the mixture. In fact, same samples diluted with 95 % ethanol and vortexed before analytical quantification lead to RSV concentrations fitting the theoretical values. Accordingly, the later condition was applied for RSV analytical quantification in liposome samples. The results (Fig. 2) showed that EE% progressively

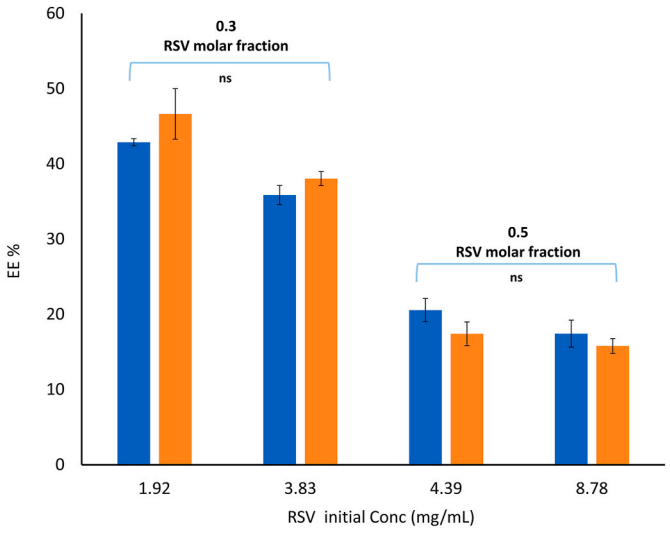

Fig. 2. Entrapment Efficiency (EE%) for liposomes prepared from different resveratrol (RSV) fractions and resveratrol concentrations without vitamin C (blue bars) or with vitamin C (orange bars). Statistical comparison revealed no significant differences (ns) between samples with or without vitamin C, irrespective of RSV initial concentration and molar fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

decreased as RSV concentration increased and the highest values (43.78 ± 0.47 % for liposomes without vitamin C and 47.63 ± 3.35 % for liposomes with vitamin C) were achieved for the lowest RSV concentration and molar fraction (1.92 mg/mL and 0.3, respectively).

Regarding EPC, comparison of concentration in liposome samples before and after filtration reveled that the phospholipid was not retained by filtration, but all incorporated in the liposomes.

This finding points to a limited number of RSV molecules able to be packed into the lipid bilayer of the liposomes, the rest remaining in the dispersion media and retained by filters used for extrusion. Literature data [40] reported that RSV adsorbs on the membrane surface and then inserts and assembles into the bilayer until reaching the appropriate RSV/lipid ratio. According to our results, RSV molar fractions ≥0.3 lead to RSV excess retained in the filter. According to this finding, molecular packing restriction for RSV in lipid EPC bilayer seems to be different that reported for CHOL, the latter able to incorporate into lipid bilayers up to 0.5–0.6 M fraction [41].

RSV payload in liposome samples was estimated from the corresponding EE%, and payload in the range of 0.81–0.90 mg/mL (equivalent to 3.59–3.98 mM) were obtained, irrespectively of the initial conditions applied, this likely due to the above commented restriction for RSV molecules to be packed in the lipid bilayer. Despite this limitation, the RSV payload was high enough for buccal formulations to achieve concentrations in oral mucosa above the IC50 values (5–100 μM) reported for cancer cells [11]. Fig. 3 shows a SEM image from liposome suspension with the highest EE% selected for liposomal films.

3.2. Studies with TR146 cell line

3.2.1. Cell cytotoxicity

Results from the MTT assay are shown in Fig. 4. For the conditions tested, mean cell viability >70 % was observed in all cases and no statistically significant differences were found when compared to negative control (100 % cell viability).

Our results prove that proposed liposomes are biocompatible and safe at RSV in vitro concentrations up to 200 μg/mL (88 mM), this value being much higher than those reported for pharmacological effects linked to RSV benefits. Previous studies have shown that RSV a concentration range of 10–100 μM selectively targets cancer cells including OSCC, head and neck, increasing apoptosis, while such effects are not observed in normal cells [42–44].

3.2.2. Intracellular reactive oxygen species (ROS) assay

TR146 cell line that mimics human buccal epithelium was selected for ROS assay. As shown in Fig. 5, intracellular ROS were significantly

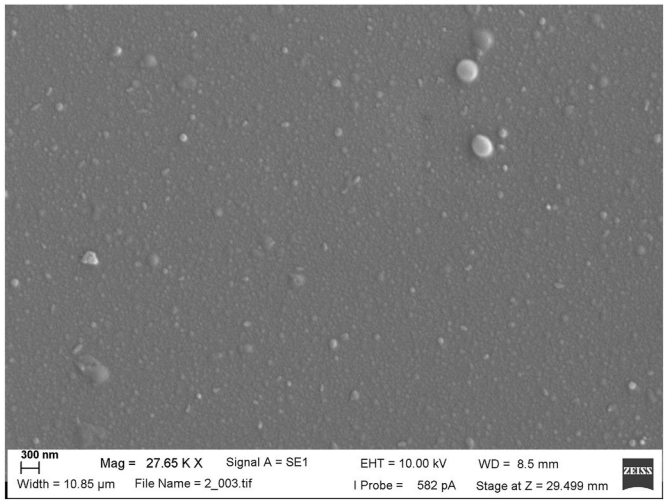

Fig. 3. Scanning electron microscopy (SEM) images of liposome suspension.

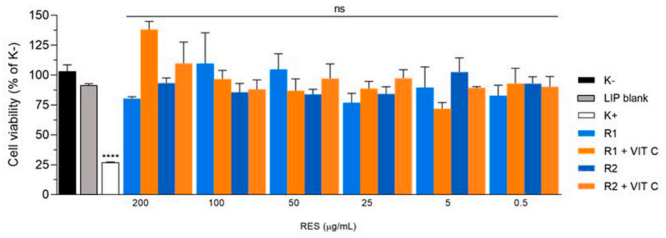

Fig. 4. Results from the cytotoxicity assay with TR146 cells. Effects of resveratrol liposomes without vitamin C are shown in blue bars and those with vitamin C in orange bars. R1 and R2 correspond to liposomes prepared from 0.3 to 0.5 resveratrol molar fraction (ns = no significant differences). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

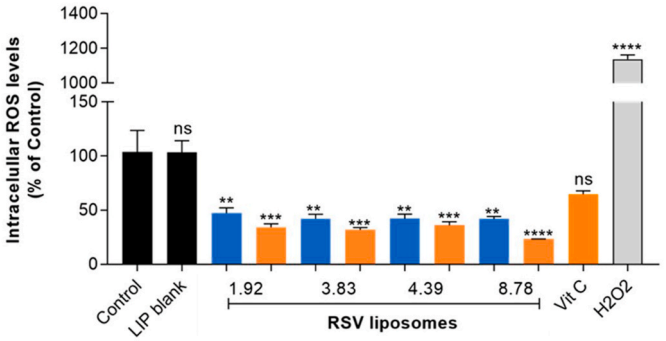

Fig. 5. Intracellular levels of reactive oxygen species (ROS) in TR146 cells treated with resveratrol liposomes. The results are expressed as percentage of untreated cells (Control). Blue and orange bars indicate the mean ± SD for liposomes without and with vitamin C, respectively. (n = 3; **p < 0.01; ***p < 0.001; ****p < 0.0001). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reduced by RSV liposomes showing remarkable antioxidant effects that were not significantly affected by RSV molar fraction or RSV concentration used for liposome production. As previously commented, RSV payload of liposome samples obtained after were all in the narrow range of 0.81–0.90 mg/mL (equivalent to 3.59–3.98 mM).

Vitamin C reinforced the antioxidant effects of RSV liposomes, and lower ROS levels were observed for samples with the vitamin compared to those without vitamin. Regarding liposomes prepared from different RSV concentration, statistically significant differences among them were not found. Despite relevant differences among initial RSV concentration used for liposome production, the final payload was in the narrow range of 0.81–0.90 mg/mL (equivalent to 3.59–3.98 mM) in all cases, this explaining that differences in ROS results among liposomes prepared from different concentrations were not relevant. Results from ROS assay obtained here confirm the interest in encapsulating RSV in liposomes to improve the cell exposure to the phytoactive molecule. Besides acting as efficient nanocarriers, liposomes protected RSV from enzymatic metabolism and facilitated the achievement of effective intracellular concentrations in TR146 cells.

3.3. Permeation assay

The permeability assay was carried out with liposomes produced from 0.3 M fraction and 1.98 mg/mL RSV concentration with or without vitamin C. PermeaPad® membranes were used in this study because these have proven functionality in predicting drug transport across biological barriers, including buccal epithelium [41]. Fig. 6 shows the mean cumulative amounts of RSV quantified in the receptor compartment of the diffusion cell (individual values in Fig. S2 of supplementary material).

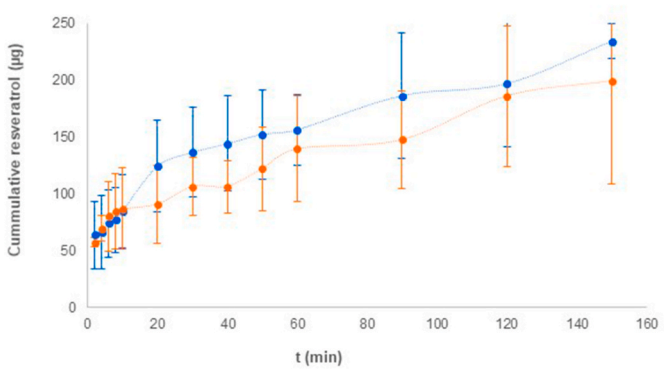

Fig. 6. In vitro release profiles obtained with PermeaPad® membranes for resveratrol liposomes without vitamin C (blue line) and with vitamin C (orange line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

According to similarity factor (45.14 %) and permeability values estimated from Jss, (1.42 ± 0.90 and 0.59 ± 0.66 μg/cm^2/min for samples without and with vitamin C, respectively), the presence of vitamin C produced a significant slower permeation rate compared to samples without the vitamin, this likely due to the higher D_h value observed for liposomes with vitamin C. In any case, the ability of RSV liposomes to efficiently permeate the membrane was proved and these results, together with those obtained from ROS assay with TR146 cells, confirm the multi-functionality of liposomes which showed the ability not only to transport but also to protect RSV from the enzymatic cell activity.

Results from kinetic analysis of curves are shown in Table 3. First order model that describes concentration dependent release was the model that fitted the data the worst, while Higuchi and Korsmeyer Peppas models best fitted the experimental curves. Higuchi's model describes the drug release based on Fickian diffusion as a square root of time, while Korsmeyer-Peppas analysis allows for mechanism discrimination based on the exponent value (N) obtained from experimental data. N values > 0.5 denote non Fickian diffusion and N values < 0.5 point to Fickian diffusion. Results from Higuchi (r^2 = 0.97) and Korsmeyer Peppas analysis (N = 0.31 or 0.22 for liposomes without and with Vit C, respectively) both agree on Fickian diffusion to be the mechanism most likely involved in RSV release from liposomes prepared here.

3.4. Buccal formulation

As proof of concept, liposomes prepared from 0.3 RSV molar ratio and 1.92 mg/mL RSV concentration with vitamin C were formulated as hydrogels and films. Liposomal hydrogels presented smart aspect and favorable rheology. The weak value of zeta potential observed in liposomes makes them prone to aggregation. Nevertheless, a homogeneous distribution of liposomes in the hydrogels was observed, this likely due to the viscosity that hinders liposome diffusion and aggregation. Hydrogels showed favorable rheology for topical application and "on site" retention, as well as easy extrudability through diameter ≤1 mm and good extensibility (extensibility results in Fig. S3 of supplementary material). For films, a remaining moisture in the range of 18–20 % lead

Table 3
Result from in vitro release profiles data fitting to different kinetic models (r = correlation coefficient; N = exponent value).

	Zero Order	First Order	Higuchi	Korsmeyer-Peppas	
	r^2	r^2	r^2	r^2	N
Without Vit C	0.9460	0.9200	0.9681	0.9339	0.3153
With Vit C	0.9381	0.8791	0.9715	0.9619	0.2230

to flexibility and fold resistance (≥15) suitable for buccal application. Moreover, the RSV payload permits to achieve concentrations in the oral mucosa above the IC50 values reported for cancer cells with small hydrogel amounts (<2 g) and comfortable size films (≈1 cm^2). Fig. 7 shows the aspect of RSV liposomal hydrogel and film (6 % LV alginate and 2 % glycerin) selected for best appearance and mechanical properties.

Fig. 8 shows a SEM image of the selected liposomal film. Not a smooth surface but rather bumps are observed, the presence of liposomes embedded in the alginate-glycerin matrix justifies the observation.

Previous studies on RSV liposomes have been performed and reported in literature, but this is the first time that liposomes without CHOL, made of EPC and RSV have been described and efficiently produced by a solvent-free method at low temperature. This is also the first time that RSV liposomes are proposed and characterized in terms of their suitability for buccal administration aimed at chemoprevention and/or treatment of oral cancer. In vitro studies are not enough to prove clinical efficacy of medicines, but these are the first stretch of a long road towards clinical application. If properly designed, in vitro studies provide essential information to discriminate and facilitate further in vivo assays. The present study was designed to obtain RSV liposomes preserving the stability of the active molecule and also to mimic buccal epithelium for in vitro evaluation of RSV liposomes. Permeapad® membranes and TR146 cell line were used since literature data support their suitability as in vitro tool for drug-permeability profiling in mucosa tissues [45,46] and in vitro model to buccal drug delivery studies [47–49], respectively.

Additional preclinical and clinical studies to confirm the benefits of RSV topical application at the buccal mucosa are forward steps to confirm the hypothesis that RSV liposomal buccal formulations may overcome some of the RSV limitations that currently hinder its clinical use. Long-term stability is another challenge to be addressed in future projects.

4. Conclusions

Liposomes made of EPC and RSV had been successfully produced by a solvent-free procedure based on component mixture and ultrasonic agitation at 37 °C. Turbidity-D_h relationships supports turbidity as a critical process parameter related to D_h, a critical quality attribute of liposomes, and this confirms the reliability of the solvent-free procedure applied here to prepare biocompatible, safe and high loaded RSV liposomes. Liposomes produced with vitamin C showed D_h in the range of 213.26–278.43 nm, higher than those found for liposomes without vitamin C, but still compatible for buccal tissue targeting. Efficient permeation across PermeaPad® membranes and TR146 cells uptake was observed for RSV liposomes. RSV protection from cellular metabolism was also proved, with the final result of relevant antioxidant effects that were reinforced by vitamin C. Alginates of high and low viscosity were used to prepare liposomal hydrogels and films that showed rheological properties suitable for buccal application. The payload permits to achieve in vitro RSV concentrations above reported IC50 for oral cancer cells with small amounts of hydrogel and comfortable sized films. This is the first study on production and in vitro characterization RSV-EPC liposomes focused on their suitability for buccal administration aimed at chemoprevention and/or treatment of oral cancer.

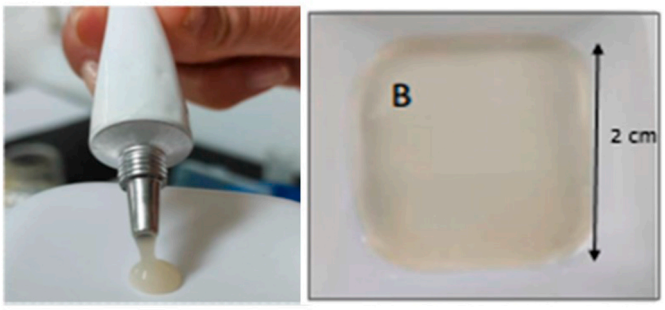

Fig. 7. Alginate hydrogel and film (B) containing resveratrol liposomes.

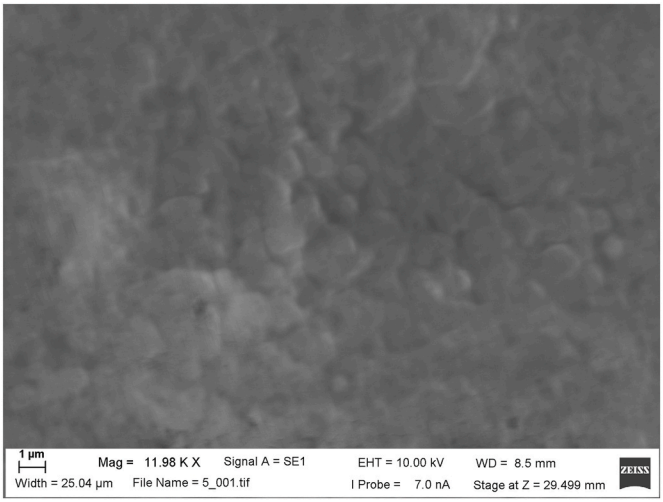

Fig. 8. Scanning electron microscopy (SEM) images of liposomal film sample.

Supplementary Material: complementary information can be found in Table S1 and Figs. S1–S3. Table S1: Results from the validation of the analytical method used for RSV quantification in liposomal samples. Fig. S1: Calibration curves obtained for RSV samples prepared with 95 % ethanol or 50 % ethanol/water mixture; Fig. S2: Individual cumulative RSV curves obtained from the permeation assay carried out with vertical diffusion chambers using PermeaPad® membranes without vitamin C (blue lines) or with vitamin C (orange lines); Fig. S3: Result from the extensibility assay performed with RSV liposomal hydrogels.

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CRediT authorship contribution statement

Maria José de Jesús Valle: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Alexandra Mabel Rondon Mujica:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition. **Aránzazu Zarzuelo Castañeda:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Paula Coutinho:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Ana Catarina de Abreu Duarte:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Amparo Sánchez Navarro:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2024.105910.

References

[1] M.J. Banez, M.I. Geluz, A. Chandra, T. Hamdan, O.S. Biswas, N.S. Bryan, E.R. Von Schwarz, A systemic review on the antioxidant and anti-inflammatory effects of resveratrol, curcumin, and dietary nitric oxide supplementation on human cardiovascular health, Nutr. Res. 78 (2020) 11–26.
[2] I.R.S. Vieira, L. Tessaro, A.K.O. Lima, I.P.S. Velloso, C.A. Conte-Junior, Recent progress in nanotechnology improving the therapeutic potential of polyphenols for cancer, Nutrients 15 (14) (2023) 3136.
[3] D. Delmas, A. Lancon, D. Colin, B. Jannin, N. Latruffe, Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer, Curr. Drug Targets 7 (4) (2006) 423–442.
[4] J.Y. Jang, E. Im, N.D. Kim, Mechanism of resveratrol-induced programmed cell death and new drug discovery against cancer: a review, Int. J. Mol. Sci. 23 (22) (2022) 13689.
[5] G. Mohan Shankar, M. Swetha, C.K. Keerthana, T.P. Rayginia, R.J. Anto, Cancer chemoprevention: a strategic approach using phytochemicals, Front. Pharmacol. 13 (2022) 12.
[6] S. Mirzaei, B. Ranjbar, S.H. Tackallou, A.R. Aref, Hypoxia inducible factor-1α (HIF-1α) in breast cancer: the crosstalk with oncogenic and onco-suppressor factors in regulation of cancer hallmarks, Pathol. Res. Pract. 248 (2023) 154676.
[7] A. Amri, J.C. Chaumeil, S. Sfar, C. Charrueau, Administration of resveratrol: what formulation solutions to bioavailability limitations? J. Contr. Release 158 (2012) 182–193.
[8] E.S. Ha, W.Y. Sim, S.K. Lee, J.S. Jeong, J.S. Kim, I.H. Baek, D.H. Choi, H. Park, S. J. Hwang, M.S. Kim, Preparation and evaluation of resveratrol-loaded composite nanoparticles using a supercritical fluid technology for enhanced oral and skin delivery, Antioxidants 8 (11) (2019) 554.
[9] C. Li, Z. Wang, H. Lei, D. Zhang, Recent progress in nanotechnology-based drug carriers for resveratrol delivery, Drug Deliv. 30 (1) (2023) 2174206.
[10] D.J. Boocock, G.E.S. Faust, K.R. Patel, A.M. Schinas, V.A. Brown, M.P. Ducharme, T.D. Booth, J.A. Crowell, M. Perloff, A.J. Gescher, W.P. Steward, D.E. Brenner, Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent, Cancer Epidemiol. Biomarkers Prev. 16 (6) (2007) 1246–1252.
[11] B. Ren, M.X.Y. Kwah, C. Liu, Z. Ma, M.K. Shanmugam, L. Ding, X. Xiang, P.C.L. Ho, L. Wang, P.S. Ong, B.C. Goh, Resveratrol for cancer therapy: challenges and future perspectives, Cancer Lett. 515 (2021) 63–72.
[12] M.K. Gupta, V. Sansare, B. Shrivastava, S. Jadhav, P. Gurav, Comprehensive review on use of phospholipid based vesicles for phytoactive delivery, J. Liposome Res. 32 (3) (2022) 211–223.
[13] W.T. Zhu, X.F. Zeng, H. Yang, M.L. Jia, W. Zhang, W. Liu, S.Y. Liu, Resveratrol loaded by folate-modified liposomes inhibits osteosarcoma growth and lung metastasis via regulating JAK2/STAT3 pathway, Int. J. Nanomed. 18 (2023) 2677–2691.
[14] D. Kong, W. Hong, M. Yu, Y. Li, Y. Zheng, X. Ying, Multifunctional targeting liposomes of epirubicin Plus resveratrol improved therapeutic effect on brain gliomas, Int. J. Nanomed. 17 (2022) 1087–1110.
[15] J.H. Kang, Y.T. Ko, Enhanced subcellular trafficking of resveratrol using mitochondriotropic liposomes in cancer cells, Pharmaceutics 11 (8) (2019) 423.
[16] T. Tsujioka, D. Sasaki, A. Takeda, H. Harashima, Y. Yamada, Resveratrol-encapsulated mitochondria-targeting liposome enhances mitochondrial respiratory capacity in myocardial cells, Int. J. Mol. Sci. 23 (1) (2021) 112.
[17] S. Franzé, F. Rama, P. Rocco, M. Debernardi, V. Bincoletto, S. Arpicco, F. Cilurzo, Rationalizing the design of hyaluronic acid-decorated liposomes for targeting epidermal layers: a combination of molecular dynamics and experimental evidence, Mol. Pharm. 18 (11) (2021) 3979–3989.
[18] T. Wiedenhoeft, S. Tarantini, Á. Nyúl-Tóth, A. Yabluchanskiy, T. Csipo, P. Balasubramanian, A. Lipecz, T. Kiss, A. Csiszar, A. Csiszar, Z. Ungvari, Fusogenic liposomes effectively deliver resveratrol to the cerebral microcirculation and improve endothelium-dependent neurovascular coupling responses in aged mice, Geroscience 41 (6) (2019) 711–725.
[19] S. Ba, M. Qiao, L. Jia, J. Zhang, X. Zhao, H. Hu, D. Chen, Construction of hierarchical-targeting pH-sensitive liposomes to reverse chemotherapeutic resistance of cancer stem-like cells, Pharmaceutics 13 (8) (2021) 1205.
[20] P. Dana, N. Thumrongsiri, P. Tanyapanyachon, W. Chonniyom, P. Punnakitikashem, N. Saengkrit, Resveratrol loaded liposomes disrupt cancer associated fibroblast communications within the tumor microenvironment to inhibit colorectal cancer aggressiveness, Nanomaterials 13 (1) (2022) 107.
[21] W. Zhang, K. Zhang, A transcriptomic signature for prostate cancer relapse prediction identified from the differentially expressed genes between TP53 mutant and wild-type tumors, Sci. Rep. 12 (1) (2022) 10561.
[22] A. Alanazi, I. Yunusa, K. Elenizi, A.I. Alzarea, Efficacy and safety of tyrosine kinase inhibitors in advanced non-small-cell lung cancer harboring epidermal growth factor receptor mutation: a network meta-analysis, Lung Cancer Manag 10 (1) (2021) LMT43.
[23] M.W. Jøraholmen, M. Johannessen, K. Gravningen, M. Puolakkainen, G. Acharya, P. Basnet, N.S. Basnet, Liposomes-in-hydrogel delivery system enhances the potential of resveratrol in combating vaginal Chlamydia infection, Pharmaceutics 12 (12) (2020) 1203.
[24] M.W. Jøraholmen, P. Damdimopoulou, G. Acharya, N. Škalko-Basnet, Toxicity assessment of resveratrol liposomes-in-hydrogel delivery system by EpiVaginalTM tissue model, Pharmaceutics 14 (6) (2022) 1295.
[25] J. Shi, Y. Zhang, X. Zhang, R. Chen, J. Wei, J. Hou, B. Wang, H. Lai, Y. Huang, Remodeling immune microenvironment in periodontitis using resveratrol liposomes as an antibiotic-free therapeutic strategy, J. Nanobiotechnol. 19 (1) (2021) 429.
[26] H. Abbas, R. Kamel, Potential role of resveratrol-loaded elastic sorbitan monostearate nanovesicles for the prevention of UV-induced skin damage, J. Liposome Res. 30 (1) (2020) 45–53.
[27] C.P.V. Cousseau, B.P. Sorroche, R. de Jesus Teixeira, A.C. de Carvalho, M. E. Melendez, R. de Castro Capuzzo, A.C. Laus, L. Sussuchi da Silva, N. Soares de Menezes, A. Lopes Carvalho, L.M. Rebolho Batista Arantes, miR-99a-5p as a biomarker for lymph node metastasis prediction in oral squamous cell carcinoma patients, Head Neck 45 (10) (2023) 2489–2497.
[28] A.K. Markopoulos, Current aspects on oral squamous cell carcinoma, Open Dent. J. 6 (1) (2012) 126–130.
[29] D.E. Johnson, B. Burtness, C.R. Leemans, V.W.Y. Lui, J.E. Bauman, J.R. Grandis, Head and neck squamous cell carcinoma, Nat. Rev. Dis. Prim. 6 (1) (2020) 92.
[30] S.G. Antimisiaris, A. Marazioti, M. Kannavou, E. Natsaridis, F. Gkartziou, G. Kogkos, S. Mourtas, Overcoming barriers by local drug delivery with liposomes, Adv. Drug Deliv. Rev. 174 (2021) 53–86.
[31] M.J. De Jesús Valle, P. Coutinho, M.P. Ribeiro, A. Sánchez Navarro, Lyophilized tablets for focal delivery of fluconazole and itraconazole through vaginal mucosa, rational design and in vitro evaluation, Eur. J. Pharmaceut. Sci. 122 (2018) 144–151.
[32] M. De Jesús Valle, P. Gil González, M. Prata Ribeiro, A. Araujo, A. Sánchez Navarro, Sildenafil citrate liposomes for pulmonary delivery by ultrasonic nebulization, Appl. Sci. 8 (8) (2018) 1291.
[33] J.C.M. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1) (1980) 10–14.
[34] J. Ali, J.B. Lee, S. Gittings, A. Iachelini, J. Bennett, A. Cram, M. Garnett, C. J. Roberts, P. Gershkovich, Development and optimisation of simulated salivary fluid for biorelevant oral cavity dissolution, Eur. J. Pharm. Biopharm. 160 (2021) 125–133.
[35] I. Singh, S. Arora, V. Rana, G. Arora, K. Malik, Formulation and evaluation of controlled release matrix mucoadhesive tablets of domperidone using Salvia plebeian gum, \"J. Adv. Pharm. Technol. Research\"\" (JAPTR)\" 2 (3) (2011) 163.
[36] J.O. Morales, J.T. McConville, Manufacture and characterization of mucoadhesive buccal films, Eur. J. Pharm. Biopharm. 77 (2) (2011) 187–199.
[37] F. Corrales Chahara, S.B. Díazb, F.A. Ben Altabef, P.E. Alvarez, Chemistry and Physics of Lipids, 2019, pp. 60–69.
[38] H. Farzaneh, M. Ebrahimi Nik, M. Mashreghi, Z. Saberi, M.R. Jaafari, M. Teymouri, A study on the role of cholesterol and phosphatidylcholine in various features of liposomal doxorubicin: from liposomal preparation to therapy, Int. J. Pharm. 551 (1–2) (2018) 300–308.
[39] A.R. Neves, J.F. Queiroz, S. Reis, Brain-targeted delivery of resveratrol using solid lipid nanoparticles functionalized with apolipoprotein E, J. Nanobiotechnol. 14 (1) (2016) 27.
[40] D. Meleleo, Study of resveratrol's interaction with planar lipid models: insights into its location in lipid bilayers, Membranes 11 (2) (2021) 132.
[41] I. Mardešić, Z. Boban, W.K. Subczynski, M. Raguz, Membrane models and experiments suitable for studies of the cholesterol bilayer domains, Membranes 13 (3) (2023) 320.
[42] V. Singh, R. Singh, P.K. Kujur, R.P. Singh, Combination of resveratrol and quercetin causes cell growth inhibition, DNA damage, cell cycle arrest, and apoptosis in oral cancer cells, Assay Drug Dev. Technol. 18 (5) (2020) 226–238.
[43] M. Bostan, G.G. Petrică-Matei, N. Radu, R. Hainarosie, C. Dragos Stefanescu, C. C. Diaconu, V. Roman, The effect of resveratrol or curcumin on head and neck cancer cells sensitivity to the cytotoxic effects of cisplatin, Nutrients 12 (9) (2020) 2596.
[44] M. Fukuda, Y. Ogasawara, H. Hayashi, K. Inoue, H. Sakashita, Resveratrol inhibits proliferation and induces autophagy by blocking SREBP1 expression in oral cancer cells, Molecules 27 (23) (2022) 8250.
[45] A.C. Jacobsen, S. Nielsen, M. Brandl, A. Bauer-Brandl, Drug permeability profiling using the novel Permeapad® 96-well plate, Pharm. Res. (N. Y.) 11 (6) (2020) 93, 37.
[46] C. Butnarasu, O.V. Garbero, P. Petrini, L. Visai, S. Visentin, Permeability assessment of a high-throughput mucosal platform, Pharmaceutics 15 (2) (2023) 380.

[47] P.F. Jacobsen, D.J. Jenkyn, J.M. Papadimitriou, Establishment of a human medulloblastoma cell line and its heterotransplantation into nude mice, J. Neuropathol. Exp. Neurol. 44 (5) (1985) 472–485.
[48] B.J. Teubl, M. Absenger, E. Fröhlich, G. Leitinger, A. Zimmer, E. Roblegg, The oral cavity as a biological barrier system: design of an advanced buccal in vitro permeability model, Eur. J. Pharm. Biopharm. 84 (2) (2013) 386–393.
[49] T. Klemetsrud, A.L. Kjøniksen, M. Hiorth, J. Jacobsen, G. Smistad, Polymer coated liposomes for use in the oral cavity – a study of the in vitro toxicity, effect on cell permeability and interaction with mucin, J. Liposome Res. 28 (1) (2018) 62–73.