ORIGINAL ARTICLE

Thermosensitive and mucoadhesive hydrogel containing curcumin-loaded lipid-core nanocapsules coated with chitosan for the treatment of oral squamous cell carcinoma

Ana Ortega[1] · Ayana Brêtas da Silva[1] · Leidyana Moraes da Costa[2] · Kelly Cristine Zatta[1] · Giovana Ravizzoni Onzi[1] · Francisco Noé da Fonseca[3] · Silvia Stanisçuaski Guterres[1,2] · Karina Paese[1]

Abstract

Buccal drug administration may be chosen as a medication route to treat various diseases for local or systemic effects. This study proposes the development of a thermosensitive hydrogel containing curcumin-loaded lipid-core nanocapsules coated with chitosan to increase mucoadhesion, circumventing several limitations of this route of administration. Hydroxypropylmethylcellulose and Poloxamer® 407 were incorporated for hydrogel production. Physicochemical characterization parameters, such as particle size distribution, mean diameter, polydispersity index, zeta potential, and morphology, were analyzed. Spherical homogeneous particles were obtained with average diameter, of 173 ± 22 nm for LNCc (curcumin lipid-core nanocapsules) and 179 ± 48 nm for CLNCc (chitosan-curcumin lipid-core nanocapsules). A PDI equal to 0.09 ± 0.02 for LNCc and 0.26 ± 0.01 for CLNCc confirmed homogeneity. Tensile analysis and washability test on porcine buccal mucosa indicated higher mucoadhesion for hydrogels in comparison to the nanocapsules in suspension, remaining on the mucous membrane up to 8 h (10.92 ± 3.95 µg of curcumin washed for H-LNCc and 28.41 ± 24.47 µg for H-CLNCc) versus the latter, which remained washed on the membrane for 90 min only (62.60 ± 4.72 µg for LNCc and 52.08 ± 1.63 µg for CLNCc). The irritant potential (IR) of the formulations was evaluated by the hen's egg chorioallantoic membrane test (HET-CAM), with no irritation phenomena observed. Formulations were tested for their efficacy in an in vitro model against oral squamous cancer cell line, showing a significant reduction in cell viability on all tested groups. These findings demonstrated that the proposed nanosystem is mucoadhesive and has potential to deliver buccal treatments.

Keywords Curcumin · Lipid-core nanocapsules · Mucoadhesive · Oral squamous cell carcinoma (OSCC) · Thermosensitive hydrogels

Abbreviations

LNCc   Curcumin lipid-core nanocapsules
CLNCc   Chitosan-curcumin lipid-core nanocapsules
CLNC   Chitosan-coated lipid-core nanocapsules

✉ Karina Paese
karina.paese@ufrgs.br

Ana Ortega
anagab.ortega@gmail.com

1 Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre 90610-000, Brazil

2 Programa de Pós-Graduação em Nanotecnologia Farmacêutica, UFRGS, Porto Alegre 90610-000, Brazil

3 Embrapa Suínos e Aves, 89715-899 Concórdia, Brazil

Introduction

The buccal administration route is characterized for its high vascularization, and it can be used to administer anti-inflammatory and anticancer drugs. Since it is a highly vascularized region, it allows both local and systemic pharmacological effects. Histologically, the mucous membrane that lines the oral cavity is composed by epithelial cells, which are highly hydrated due to the permanent contact with mucus that provides lubrication and protection [1]. The mucus layer is a medium composed mainly by water (90%), lipids, inorganic salts, and some proteins, such as mucin, which is one of the most important one due to its gel-like structure. However, this administration route has several limitations, for example, the rapid clearance (approximately 6 h) related to the constant mucus renewal, swallowing as a reflex mechanism, limited surface area, and the presence of the mucus itself that also binds to drugs as result

of the chemical interaction between hydrogen bonds and the amino groups from mucin [2, 3]. Considering these limitations, an ideal formulation for oral drug delivery should remain for longer periods within the buccal cavity to allow its proper and controlled release.

Lately, natural products have attracted the attention for therapeutic agents, since they present fewer side effects than some traditional drugs. Among these products, curcumin, a yellow polyphenol isolated from the rhizome of *Curcuma longa Linn* (*Zingiberaceae*), exhibits multiple therapeutic properties, from cytotoxic activity against numerous tumor cell lines to biological activities such as antioxidant, wound healing, anti-inflammatory, and antibacterial effects [4, 5].

Thus, based on its biological potential, curcumin was chosen as a model substance in the present study. Moreover, according to the biopharmaceutical classification, curcumin is a class IV molecule with low solubility and low permeability. Regarding its hydrophobic nature, it does not solubilize properly in physiological fluids and, consequently, this characteristic impairs its bioavailability. Moreover, this polyphenol presents photosensitivity and chemical instability at both alkaline and neutral pH, and its optimum pH range is 3.0–6.5 [6].

For this reason, nanotechnology is an interesting approach to overcome the limitations of phytochemicals, such as curcumin, by means of delivery nanosystems to optimize their application, since they can both increase the chemical stability of active ingredients and enhance their solubility in physiological fluids. Furthermore, nanoencapsulation can control the release rate, making the treatment more efficient and with less harmful effects, and also prevent enzymatic degradation of the active compounds [5]. Among these new nanosystems for drug delivery, particularly lipid-core nanocapsules (LNCs) have gained special interest as nanocarriers to target a specific organ or tissue, making them more effective in a way that allows reducing the dose administration [7].

Considering the topical use of curcumin-loaded lipid-core nanocapsules on mucous membranes, the incorporation of mucoadhesive materials becomes relevant. For this purpose, chitosan has been successfully applied in the pharmaceutical area due to its biodegradability and biocompatibility. It is widely found in nature and consists of a cationic polymer derived from chitin, which is deacetylated and turns into a non-water-soluble polymer, but is soluble in weak acidic medium [8]. Its mucoadhesion mechanism is mainly related to the chemical interaction between the cationic charge of chitosan and the anionic charge of mucin, forming a double electron layer that creates the binding force [9]. Moreover, amino and hydroxyl groups of the chitosan chain react via hydrogen bonding [2]. It also weakens the tight junctions of the mucosa, since it interacts with calcium channels

on mucous membranes by chelating calcium ions, hence increasing the permeation for macromolecules. There is experimental evidence showing that chitosan allows higher permeation of drugs and active substances on epithelial tissue of the mucosa in vitro [7]. Previous studies from our research group have already developed lipid-core nanocapsules coated with chitosan, which exhibited higher stability in comparison with free drugs [10]. Additionally, some findings show that cationic polymers have a direct effect on the encapsulation efficiency and increase drug absorption [11, 12].

Taking into account the buccal route limitations, an interesting formulation should present easy administration and proper mucoadhesiveness. Literature has reported the use of Poloxamer® 407 for developing hydrogels that jellify in situ, since this polymer has the property of reversible gelling when exposed to a thermal stimulus [13]. It is chemically composed by a block copolymer with segments of polypropylene oxide and polyethylene oxide, and acts as a thermoreversible agent on concentration from 10 to 20% (w/v). Moreover, the transition temperature can be modulated according to the concentration of the polymer. The main advantage of using an in situ hydrogel is the higher contact with the mucosa, because the polymer suffers transition from liquid (sol) to semisolid (gel) and becomes more viscous, allowing a higher controlled release [14]. In addition, hydroxylpropyl methylcellulose (HPMC) is a hydrophilic cellulose derivative classified as a first-generation mucoadhesive agent, non-ionic, non-irritating, and resistant to enzymes [2, 15]. Its hydroxyl groups interact with the amino groups of mucins via hydrogen bonding [2]. Furthermore, this polymer has been used to modulate the release profile for drugs by volume expansion when exposed to an aqueous environment [16].

In this context, the purpose of this study was to develop a mucoadhesive and thermosensitive hydrogel containing curcumin-loaded lipid-core nanocapsules coated with chitosan and to evaluate in vitro its potential for the treatment of oral squamous cell carcinoma (OSCC).

Materials and methods

Materials

Chemicals

Poly (ε-caprolactone) (PCL)-Mw 14,000 g mol^{-1}, sorbitan monostearate, curcumin (purity > 65%), low molecular weight chitosan, and mucin were purchased from Sigma-Aldrich (Germany). Grape seed oil was purchased from Importadora Química Delaware (Porto Alegre, Brazil); lipoid® S75 from Lipoid (75% phosphatidylcholine) (Germany); polysorbate 80 from Vetec (Rio de Janeiro, Brazil);

sodium lauryl sulfate, sodium hydroxide, and sodium chloride from Dinâmica (São Paulo, Brazil); Poloxamer® 407 from BASF (USA); hydroxypropyl methylcellulose (HPMC); and Methocel™ K 100 LV from Colorcon (USA). The other solvents and reagents were analytical and/or pharmaceutical grade.

Cell culture

Dulbecco's modified Eagle's medium (DMEM)/Ham's F12, L-glutamine, sodium bicarbonate, and ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO); fetal bovine serum (FBS), fungizone (Amphotericin B), and 0.5% trypsin/EDTA solution from Gibco BRL (Carlsbad, CA); and gentamicin from Schering do Brazil (Rio de Janeiro, Brazil). The 96-well and 24-well culture plates were purchased from TPP (Tissue Culture Test Plates-TPP, Trasadingen, Switzerland), and the oral squamous cell carcinoma culture cell line (SCC-25) was obtained from the American Type Collection Culture, ATCC (Rockville, MD, USA).

Chicken fertilized eggs and porcine oral mucosa

Chicken fertilized eggs were gently donated by the Avian Facility for Teaching and Research (Department of Zootechnics, UFRGS, Porto Alegre, Brazil). Porcine oral mucosa was provided by a local slaughterhouse (Frigorífico Ouro Do Sul, Harmonia, Brazil).

Preparation of lipid-core nanocapsules

Curcumin-loaded lipid-core nanocapsules were prepared by self-assembling, using a previously described technique based on interfacial deposition of preformed polymer method with some modifications [17]. Briefly, the organic phase consisted of 100 mg of PCL-Mw 14,000 g mol^{-1}; 160 µL of grape seed oil, previously dissolved in 24 mL of acetone at 40 °C, added to 38 mg sorbitan monostearate; 10 mg of curcumin and 90 mg of Lipoid® S75 (dissolved in 3 mL of ethanol). This organic phase, after complete solubilization, was injected into the aqueous phase, containing 78 mg of polysorbate 80 and 50 mL of ultrapure water, under constant magnetic agitation at 40 °C. The nanocapsule suspension was evaporated under vacuum (Rotaevaporator R-114, Buchi, Flawil, Switzerland) to a final volume of 10 mL. This formulation was named LNCc. Subsequently, 0.6% (w/v) of chitosan solution, previously dissolved in 1% v/v acetic acid and centrifuged at $1537 \times g$ for 10 min to remove insoluble particles, was dropped in the nanocapsule suspension under magnetic agitation for 2 h, resulting in a final curcumin concentration of 0.9 mg mL^{-1}. This formulation was named CLNCc [17, 18].

Physicochemical characterization of lipid-core nanocapsules

Particle size measurements

The particle size distribution profile and particle mean diameter of the nanocapsules were determined by three complementary techniques: laser diffraction (LD) (Mastersizer® 2000, Malvern Instruments, UK), photon correlation spectroscopy (PCS) (Zetasizer® nano-ZS, Malvern Instruments, UK), and nanotracking analysis (NTA) (NanoSight LM 10 & NTA 2.2 Analytical Software, NanoSight Ltd., Amesbury, UK).

First, the LD technique was performed. For this analysis, the samples were added to the disperser unit filled with distilled water. The diameter was expressed as a mean pondered diameter D (4,3) and the polydispersity via Span was determined according to Eq. 1. In the PCS technique, samples were diluted $500 \times$ in filtered ultrapure water (0.45 µm, Millipore®). The particle diameter and uniformity were expressed in z-average and polydispersity index (PDI), respectively.

$$\text{Span} = \frac{D_{v0.9} - D_{v0.1}}{D_{v0.5}} \quad (1)$$

where $D_{v0.9}$, $D_{v0.1}$, and $D_{v0.5}$ are the diameters of 90%, 10%, and 50% from the cumulative distribution curve, respectively. For all samples, the characterization was performed in triplicate.

Moreover, NTA measurements were performed with samples diluted $10,000 \times$ in filtered ultrapure water (0.45 µm, Millipore®). A camera attached to the microscope captured the reflection of the nanocapsules and tracked their Brownian motion for more than 60 s to calculate the diameter and the concentration of the number of particles, using the Stokes–Einstein equation. All analyses were performed in triplicate.

Zeta potential

Zeta potential values were determined using the electrophoretic mobility (Zetasizer® nano-ZS, Malvern) after diluting each sample ($500 \times$) in 10 mM NaCl solution (previously filtered with 0.45 µm, Millipore®). The measurements were conducted at 25 °C and performed in triplicate.

Determination of pH

The pH was determined by direct measurement, using a calibrated potentiometer (B474; Micronal, São Paulo, Brazil).

Analytical methodology for curcumin quantification

Curcumin was quantified via HPLC, using a Shimadzu LC chromatographic system (Tokyo, Japan), composed of a CBM0-20A system control, LC-20AT pump, degasser DGU-20A, auto-injector SIL-20A, and a UV detector SPD-20AV. Phenomenex Gemini C18 (150 mm × 4.6 mm × 5 μm) column was used as stationary phase. The mobile phase consisted of acetonitrile and acetic acid 0.5% (v/v) solution, isocratic elution (53:47), and a flow rate of 0.7 mL min^{-1}. Curcumin was detected at 360 nm at a retention time of 9.9 min. For quantification, a standard curve of curcumin was established in the range of 0.01–0.05 mg mL^{-1}. Additionally, the method was validated regarding specificity, linearity, accuracy, and precision intra- and inter-day, according to the Brazilian Health Regulatory Agency (Anvisa) legislation (RDC 166/2017) (Brazil, 2017).

Drug content and encapsulation efficiency

Curcumin content was determined using the analytical method validated in the "Analytical methodology for curcumin quantification" section. Encapsulation efficiency (EE%) was determined by means of the difference between total curcumin in the formulation and the free curcumin content (not encapsulated). Total curcumin in the formulation was determined after treating 150 μL of the formulation with acetonitrile and acetic acid 0.5% (v/v) solution (70:30) in a 5-mL volumetric flask to ensure the extraction of the active substance contained in the lipid-core nanocapsules. It was then submitted to 5 min of ultrasound and then centrifugation (4120 × g for 5 min). The ultrafiltration-centrifugation technique was used to determine the free curcumin. The ultrafiltration was performed using a microtube centrifugal filter (cutoff 10 kDa, Microcon, Merck) containing 400 μL of C-LNCc. After centrifugation of the nanocapsules (1537 × g for 10 min), the amount of free curcumin was quantified in ultrafiltrated. EE% was calculated according to Eq. 2:

$$EE\% = \frac{C_t - C_l}{C_t} \times 100 \tag{2}$$

where EE% is the encapsulation efficiency; C_t is the total curcumin concentration in the nanocapsule suspension. C_l is the free curcumin concentration quantified in the ultrafiltrated.

Morphology

Particle morphology was analyzed by transmission electron microscopy (TEM), using an electron microscope (Jeol, JEM 1200-ExII, Tokyo, Japan) operating at 80 kV. For the analysis, each nanocapsule suspension was diluted 10× in filtered

ultrapure water (0.45 μm, Millipore®). The samples were placed on 400 mesh grids and uranyl acetate (2% w/v) was added as a contrast agent. These grids were kept in desiccators 24 h prior to the analysis. Analyses were carried out at the Electron Microscopy Center, UFRGS (Porto Alegre, Brazil).

Determination of in vitro release profile

Dialysis bag technique was used to determine in vitro drug release profile. The bags (cut-off 12–14 kDa, Sigma-Aldrich) were filled with 2 mL of LNCc or C-LNCc and after immersed in 80 mL saliva medium pH 6.0 ± 0.1 and absolute ethanol (8:2, v/v). The medium was composed of a set of salts (monosodium phosphate, sodium chloride, potassium thiocyanate, monopotassium phosphate, potassium chloride, and sodium bicarbonate) and was buffered with phosphoric acid 85% (w/v) [18] to simulate in vivo conditions. Considering that the saturation concentration of curcumin was 61.89 μg mL^{-1}, 96% v/v ethanol was added to ensure sink conditions. The volume of formulation and medium used in the experiment took into account the maximum concentration of curcumin released in the medium for 100% release (22.50 μg mL^{-1}).

The dialysis was carried out under controlled and maintained temperature (37 °C) and agitation. Samples (1 mL) from the dissolution medium were collected during predetermined times (0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, and 48 h) added to ensure sink conditions. After collecting every sample, the same volume of fresh medium (1 mL) was added to the system to keep the volume constant and maintain the dialysis process.

The release profiles of C-LNCc and LNCc were plotted and modeled mathematically using Scientist® 2.0 software (Micromath®, EUA). Semi-empirical models were used, considering the equations of order zero (Eq. 3), first-order monoexponential (Eq. 4), and first-order biexponential (Eq. 5). Curcumin quantification in the release medium was also validated according to the parameters of specificity, linearity ($r = 0.9966$), and the detection and quantification limits (0.12 and 0.42 μg mL^{-1}, respectively) [17].

$$C = 100 - C_0 \, kt \tag{3}$$

$$C = 100 - C_0 e^{-kt} \tag{4}$$

$$C = 100 - [Ae^{-\alpha t} + Be^{-\beta t}] \tag{5}$$

Production of thermosensitive hydrogel

The thermosensitive hydrogels were prepared with the addition of both polymers in the nanocapsule suspensions, using the cold method with some variations [19]. At first, the

mixture was kept in ice and the thermoreversible gelling polymer Poloxamer® 407 (14% w/w) was added. After, HPMC (1.5% w/w) was added under moderate heating (below 40 °C) with the use of manual dispersion to avoid destabilizing the nanocapsule suspension. The resulting thermosensitive hydrogels containing uncoated and coated nanocapsules were named H-LNCc and H-CLNCc, respectively.

Thermosensitive hydrogel characterization

The thermosensitive hydrogels (H-LNCc and H-CLNCc) were characterized for the particle size distribution profile by LD and PCS, following the methodology described in the "Particle size measurements" section; pH value by potentiometry (10%, w/v in solution); zeta potential by electrophoretic mobility; and drug content by HPLC. The measurements were performed in triplicate. Similarly, to the nanocapsule suspensions, the morphology of the nanoparticles incorporated in the hydrogels was evaluated by TEM. The samples were treated as shown in the "Morphology" section and analyzed at the Central Laboratory of Microscopy and Microanalysis–LabCEMM, PUCRS (Porto Alegre, Brazil), using an electron microscope (FEI Company, Tecnai G2 20 S-TWIN) operated with a 200 kV.

Determination of the sol–gel temperature

The transition point, defined as the temperature that the hydrogel, turns its state from sol to gel, was measured in a Peltier rotational rheometer (ARES G2 T.A Instruments, Surrey, England) using a parallel plate geometry with a 50.0-mm diameter. The analysis was performed at temperatures from 20.0 to 45.0 ± 0.1 °C, at a heating rate of 5.0 °C/min, a frequency of 1.0 Hz, and a strain amplitude of 0.05%. A previous strain amplitude sweep test was performed to determine the experimental conditions of linear the viscoelastic behavior. The $T_{sol-gel}$ was defined using temperature sweep tests as the temperature at which the loss modulus (G″) was halfway between the values of this parameter for the solution and the gel. Samples' results were analyzed, using an Ares G2 Software: TA Instruments Trios v5 [20].

Mucoadhesion evaluation

Washability profile

To evaluate the resistance of the formulation to keep attached on the oral mucosa against salivary flux over time, the washability test was performed using manual modified Franz cell system. Pieces of porcine buccal mucosa (2.5 × 3 cm) were obtained, and the experiment was performed, comparing CLNCc, LNCc, H-CLNCc, and H-LNCc. The formulations (300 mg for hydrogels and 200 μL for nanocapsules) were placed in contact with the mucosa surface for 25 min to assure complete interaction [21]. Salivary pH of 6.0 ± 0.1 was used as washing solution [9, 22] and the wash flow was 0.4 mL min^{-1}. Aliquot portions of the washing solution were collected at 5, 10, 20, 30, 45, 60, and 90 min for nanocapsules; for the hydrogels, both formulations were collected at the same prior intervals; however, further sampling was also carried out at 120, 180, 240, 270, 300, 360, 420, and 480 min.

Prior to quantification, curcumin was extracted from the aliquot portions and the procedure depended on the formulation type (nanocapsule suspension or hydrogel). For the former, 1000 μL of acetonitrile and acetic acid solution 0.5% (v/v) (70:30) was added to the sample (500 μL), followed by vortex agitation for 1 min and 5 min sitting in an ultrasound bath. For the latter, 1500 μL of the mixture acetonitrile and acetic acid solution was added to the sample (500 μL), with vortex agitation for 2 min and ultrasound for 10 min. Quantification was performed by high-performance liquid chromatography (HPLC), following the methodology previously described in Sect. 2.3.4. At the end of the washability assay (90 min for nanocapsules or 480 min for hydrogels), the amount of curcumin permeated through the buccal mucosa was evaluated. For this purpose, the medium contained in the receptor compartment of the Franz cell was collected and quantified by HPLC.

Tensile analysis of nanocapsule suspensions and hydrogels

Mucoadhesion properties were determined using a texture analyzer (TA.XT Plus Texture Analyzer, Hamilton, MA, USA). The conditions applied in the test were as follows: height probe of 700 mm; speed of 2 mm s^{-1}; applied force of 0.2 N; pre-test and post-test speed of 2 mm s^{-1}. For the evaluation, mucin discs were used as surface models and were prepared by compression, with an average weight of 190 ± 10 mg. Next, mucin discs were placed on the probe with double-sided adhesive tape and were hydrated with 80 μL of ultrapure water at 37 °C for 1 min. The water excess was removed with absorbent paper. After, the samples and the mucin discs were set in contact for 600 s. The mucoadhesive strength measure was obtained from the evaluation of the debonding distance and the work of mucoadhesion. Taking into account the variability of the analysis, the tests were performed in sextuplicate [16].

Determination of irritant potential

The irritation score (IS) was assessed using the hen's egg chorioallantoic membrane (HET-CAM), which is an in vitro method that uses fertilized hen eggs. The irritancy potential of formulations can be detected by observing vascular changes that occur in the chorioallantoic membrane of the

egg after exposure. This method aims to determine possible signs of irritation, such as vasoconstriction, hemorrhage, and coagulation. In this experiment, the fertilized eggs were incubated under controlled temperature (37 °C) and 60% relative humidity. The test was performed on the 10th day of incubation, when the egg reaches its irritation phase.

Eggshells ($n = 6$/group) were opened carefully in the air chamber and the white membrane was removed, and 300 μL of the nanocapsule suspensions (LNCc and CLNCc) was applied on the chorioallantoic membrane (CAM); for the hydrogels (H-LNCc and H-CLNCc), the same volume was diluted 1:1 with ultrapure water to enhance visibility due to the opacity of the formulations during the analysis and to guarantee a proper spread among the CAM, and to avoid gelation in one point, the final solution (600 μL) was applied on the CAM. After 20 s of exposure, the CAM was rinsed with NaCl 0.9% (w/v) to remove the samples and to facilitate observation due to their opacity. The test was performed by cautiously observing the CAM during 300 s after removal of the formulations. All the effects observed in each egg (vasoconstriction, hemorrhage, and coagulation) were registered and the time points of the first occurrence of irritation were monitored.

In addition, in order to evaluate the effects of the polymers used in the production of nanocapsules (PCL and chitosan) on the possible irritation caused by the formulations, a nanoemulsion containing curcumin (NEc) and hydrogels containing nanoemulsion (H-NEc) were produced. A hydrogel without nanocapsules was also produced (H–H$_2$O) to assess the effects of gel polymers (HPMC and Poloxamer® 407). Positive controls (0.1 M NaOH and 0.1% (w/v) sodium lauryl sulfate (LSS), $n = 3$) and negative control (0.9% NaCl, $n = 3$) were also performed. The irritation score of each group was calculated using the following equation (Eq. 6).

$$IS = \frac{5 \times (301 - hemorrage\ time)}{300}$$
$$+ \frac{7 \times (301 - vasoconstrition\ time)}{300} \quad (6)$$
$$+ \frac{9 \times (coagulation\ time)}{300}$$

The irritation score has a scale and classifies the samples according to each result as non-irritant (0–0.9), slightly irritant (1–4.9), moderately irritant (5–8.9), and extremely irritant (9–21) [20].

In vitro cytotoxicity of curcumin lipid-core nanocapsules

Cell culture

Squamous cell carcinoma line SCC-25 was used to evaluate the in vitro cytotoxicity effects of the proposed nanocapsule formulations (LNCc and CLNCc) [23]. Cells were cultured in high-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FB serum, penicillin (100 U mL^{-1}), streptomycin (100 mg mL^{-1}), and 400 ng mL^{-1} hydrocortisone, maintained at 37 °C, 95% relative humidity, and 5% CO$_2$ in air. For comparative purposes, a DMSO curcumin solution (0.9 mg mL^{-1}) and blank (without curcumin) coated nanocapsule formulation (CLNC) were also evaluated.

Mitochondrial activity evaluation (MTT assay)

Cytotoxicity evaluation of the formulations was determined using the MTT (3-(4,5 dimethylthiazol-2-thiazyl)-2,5-diphenyl-tetrazolium bromide) assay. This method provides a quantitative measurement of cells with metabolically active mitochondria by detecting mitochondrial reduction of a tetrazolium bromide salt. MTT forms a purple formazan product (chromophore) whose absorbance can be determined by spectrophotometry. Briefly, the oral squamous cell carcinoma culture cell lines (SCC-25) were seeded (5×10^3/well) in 96-well plates and cultivated until reaching 80% confluence. The plates were treated as follows: LNCc, CLNCc, and CUR (free curcumin dissolved in 1% (v/v) DMSO) at 5, 10, and 20 μM final curcumin concentrations. For the treatment with CLNC formulation, similar volumes to those used in the treatments with nanocapsule formulations containing curcumin were used, so that the cells could be exposed to the same amount of blank nanocapsules. All nanocapsule formulations were prepared under aseptic conditions. Moreover, control cultures were performed without treatment.

After 24, 48, and 144 h (6 days) of treatment, the formulations were removed, the cells were washed, and MTT solution (0.5 mg mL^{-1}) was added to the cells and incubated for 3 h at 37 °C. The supernatant was then aspirated, and formazan crystals were solubilized in DMSO (100 μL). The absorbance was measured at 570 nm in a PerkinElmer EnVision 2013 multimode plate reader (PerkinElmer®). This absorbance was directly proportional to the number of cells with active mitochondria. The results were expressed as the percentages of the viable cells to the control (considered as 100%), according to the following equation [24]:

$$Cell\ viability(\%) = \frac{Abs_s}{Abs_{control}} \times 100 \quad (7)$$

where Abs$_s$ is the absorbance of cells treated with different formulations (samples) and Abs$_{control}$ is the absorbance of control cells (incubated with cell culture medium only).

Statistical analysis

The results obtained were expressed as mean ± standard deviation. Data were analyzed by one-way analysis

of variance (ANOVA) followed by post hoc analysis for multiple comparisons (Tukey test) using GraphPad Prism 5.0. Differences with $p < 0.05$ were considered statistically significant.

Results and discussion

Physicochemical characterization of the curcumin lipid-core nanocapsules

The nanocapsule suspensions presented a milky aspect, yellowish color due to the curcumin and the Tyndall effect respectively. The suspensions were developed using grape seed oil as an alternative to other oils for curcumin formulations due to its solubility. Moreover, the grape seed oil has antioxidant activity previously described in several in vivo models. In spite of being an alternative for nanocapsule formulations, the grape seed oil can affect the particle size distribution profile [25, 26]. Taking that into consideration, the mean diameter of the nanostructures was analyzed with different complementary techniques.

In order to establish the nanotechnological quality of the formulations, it is essential to evaluate their physicochemical characteristics. This evaluation was performed by comparing the LNCc and CLNCc formulation parameters (Table 1). The median diameter of the equivalent sphere D (4,3) was determined by laser diffraction and showed a diameter of 173 ± 22 nm and 179 ± 48 nm for CLNCc and LNCc, respectively. This result is similar to the data obtained from a previous study, where the D(4,3) for curcumin nanocapsules obtained was 198 ± 6 nm [17]. PCS results showed a significant increase ($p < 0.05$) of z-average after coating with chitosan, as the z-average was 158 ± 10 nm for LNCc and 200 ± 19 nm for CLNCc. A previous study has reported that the diameter increased after coating with chitosan [18]. Both distribution profiles obtained from LD (Fig. 1), and PCS (data not shown) showed homogeneous formulations with distribution in the nanometric range. The homogeneity of the LNCc formulation was confirmed by the PDI value below 0.2, but after chitosan coating, a decrease in homogeneity was observed since the PDI of the CLNCc formulation was significantly ($p < 0.05$) higher than LNCc. Moreover, the Span was significantly lower after chitosan coating, probably due to the sensibility difference between the two techniques.

To confirm if the nanocapsules were coated with chitosan, zeta potential measurements were helpful. LNCc presented zeta potential equals to -18.60 ± 0.50 mV, being the negative value probably related to the anionic charges from Lipoid S75® on the formulation [18]. However, CLNCc presented a zeta potential of $+19.00 \pm 3.18$ mV (Table 1), and this inversion of zeta potential suggests the chitosan coating [25]. The chitosan coating can improve the mucoadhesive

**Table 1** Physicochemical characteristics of the LNCc and CLNCc formulations

Parameters	LNCc	CLNCc
D (4,3) (nm)	173 ± 22	179 ± 48
Span	1.55 ± 0.28	$1.30 \pm 0.24^*$
z-Average (nm)	158 ± 10	$200 \pm 19^*$
Mean (nm)	140.54 ± 5.10	$161.25 \pm 6.58^*$
D(10) (nm)	98.64 ± 3.57	$115.78 \pm 5.08^*$
D(50) (nm)	136.10 ± 5.31	$153.33 \pm 0.66^*$
D(90) (nm)	184.56 ± 6.23	$213.38 \pm 12.24^*$
PDI	$0.09 \pm 0.02^*$	$0.26 \pm 0.01^*$
Zeta potential (mV)	-18.60 ± 0.50	$+19.00 \pm 3.18^*$
pH	5.58	4.03
Curcumin concentration (mg mL^{-1})	0.90 ± 0.09	0.86 ± 0.03
Particle number (particle mL^{-1})	$4.27 \pm 0.12 \times 10^{13}$	$3.30x \pm 0.66 \times 10^{13}$
Encapsulation efficiency (%)	N.D	99.88

Results obtained after physicochemical characterization of the nanocapsules in suspension. *LNCc* curcumin-loaded lipid-core nanocapsules, *CLNCc* chitosan-coated curcumin-loaded lipid-core nanocapsules, *N.D.* not determined. Values represent mean $\pm$ standard deviation ($n = 3$). $^*p < 0.05$

potential of nanoformulations, which is especially important when the site of administration is the buccal mucosa. This application is interesting because there is a lack of therapeutic options that use this route of administration, aiming to improve its retention time on mucous membranes. Also, the polycationic nature of the formulation (due to chitosan coating) allows interaction with the anionic residues present in the mouth cells' surfaces [27]. Regarding pH, C-LNCc is more acidic (Table 1) due to the fact that the coating solution contains acetic acid 1% (v/v) [18].

Laser diffraction (LD) method is a widely used technique for the physicochemical evaluation of materials mainly due to its wide analysis range (nano and micrometric scale) and rapid measurements. LD uses Mie theory to calculate the particle size distribution. DLS and NTA techniques determine particle size with more accuracy with size ranges of 0.3 nm–10 µm and 20–1000 nm, respectively. Both techniques are based on the Brownian movement and the Stokes–Einstein equation. The NTA technique also evaluates multiple populations on the nanometric scale and determines the particle number size of developed formulations [28]. NTA results complement the previously mentioned techniques. Average values for D(10), D(50), and D(90) showed an increase of the diameter after coating ($p < 0.05$) (Table 1) and a homogenous distribution before and after coating. Additionally, this technique measured the particle counts contained in each formulation, showing no significant difference between both uncoated and coated

Fig. 1 **a** Particle size distribution obtained by laser diffraction of the LNCc (curcumin-loaded lipid-core nanocapsules) and CLNCc (chitosan-coated curcumin-loaded lipid-core nanocapsules). Results were expressed as percentage of volume of particle size in µm; **b** LNCc morphology analyzed by transmission electron microscopy (TEM); **c** CLNCc morphology analyzed by transmission electron microscopy (TEM) (scale bar = 100 nm)
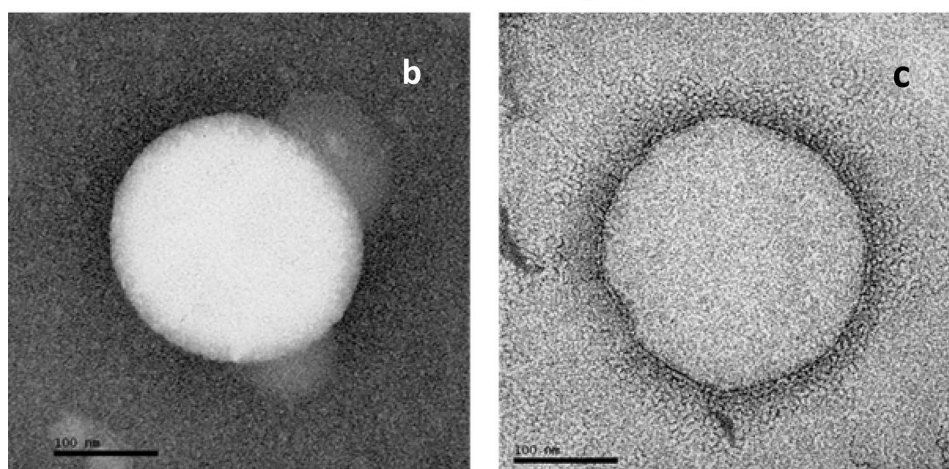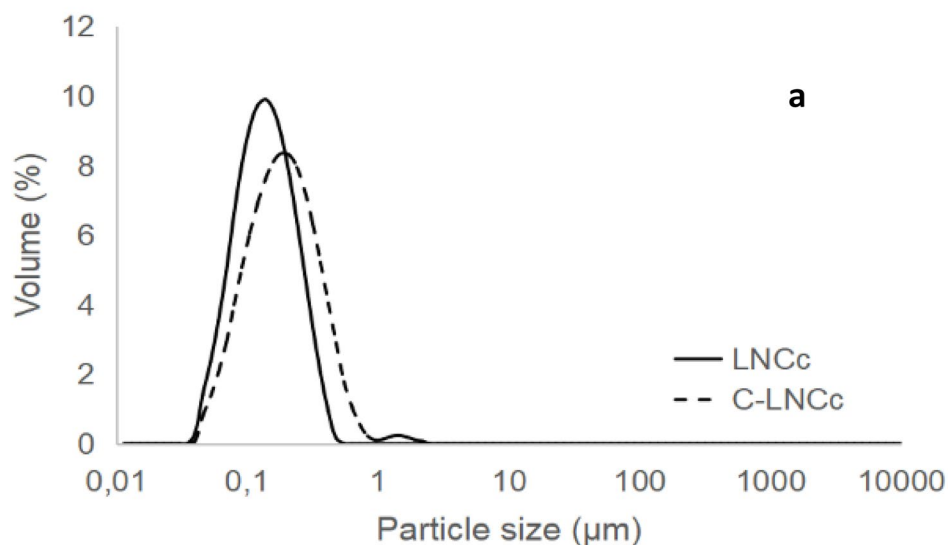
nanocapsules ($p > 0.05$). Spherical nanocapsules were observed by TEM with a particle diameter that confirmed the results obtained by the different techniques previously presented (Fig. 1).

Coradini et al. (2014) characterized lipid-core blank nanocapsules (without curcumin) to evaluate any interference caused by curcumin. No significant difference was observed between the physicochemical parameters, indicating that this delivery system fulfills the nanotechnological criteria, and therefore is suitable for encapsulating lipophilic active substances, such as curcumin [17].

The analytical method developed for curcumin quantification was specific, linear ($r = 0.9986$, $n = 3$) within the range of 0.01–0.05 mg mL^{-1}, and with intra-day (CV% = 0.92) and inter-day (CV% = 0.86) precision. Quantification and detection limits were 2.94 and 0.88 µg mL^{-1}, respectively. Drug content was maintained after coating ($p > 0.05$) and encapsulation efficiency for CLNCc was close to 100% (Table 1), confirming the high affinity of curcumin for the core of nanocapsules [17, 18].

Determination of in vitro release profile

In vitro release profile aimed to predict the velocity of curcumin diffusion from the nanocapsules, using simulated saliva and ethanol (8:2) as the release media to mimic the conditions in the buccal cavity, to help solubilize curcumin and to assure sink condition. Both groups, LNCc and CLNCc, were tested ($n = 4$) to discriminate any possible effect of chitosan coating on the curcumin release profile. The cumulated quantity of released curcumin after 48 h was about 9% (0.0809 mg mL^{-1}) (Fig. 2). The superposition of the curcumin release profiles from coated to non-coated nanocapsules indicates that the coating did not affect the release profile, which can be confirmed by the value of release velocity constant (k) that remained the same after coating ($p > 0.05$) (Table 2).

The controlled release of curcumin from nanocapsules was previously presented by Coradini et al. [17] and Yallapu et al. [29]. However, these authors observed around 20% of curcumin release for the same period. This
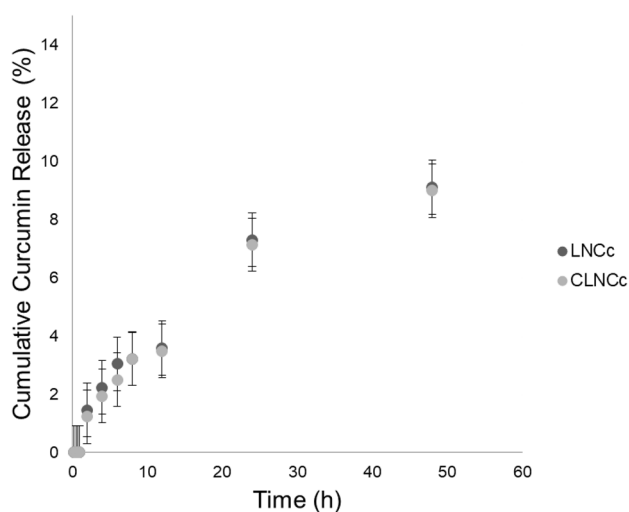
Fig. 2 Drug release profile of LNCc (curcumin-loaded lipid-core nanocapsules) and CLNCc (chitosan-coated curcumin-loaded lipid-core nanocapsules). Values represent mean ± standard deviation ($n = 3$). The superposition of the curcumin release profiles from coated and non-coated nanocapsules indicates that the coating did not interfere on the release profile

significant difference in the release could be related to the composition of the used medium, since these studies have used ultrapure water with polysorbate 80, whereas in this study the medium was composed of a buffered solution (high salt content) with no surfactant. Previous studies have confirmed the nanoencapsulated curcumin efficiency against oral squamous cells carcinoma in vitro, in concentration lower than the release obtained after 48 h of experiment (0.0809 mg mL^{-1}). Lee et al. [23] evaluated the curcumin effect against oral squamous cell carcinoma on H314 and ORL115 lines and observed that in 24 h, 50 μM (0.0184 mg mL^{-1}) and 105.5 μM (0.0388 mg mL^{-1}) of curcumin inhibited 50% of cells, respectively. Meanwhile, in 48 h, 25.5 (0.0093 mg mL^{-1}) and 69.8 μM (0.0256 mg mL^{-1}) of curcumin was able to inhibit the same percentage of cell growth on those cell lines.

Table 2 Parameters obtained from in vitro release modeling on the semi-empirical first-order monoexponential model

Parameters	LNCc	CLNCc
k (% h^{-1})	0.00239 ± 0.00014	0.00233 ± 0.00028
MSC	1.70 ± 0.08	1.86 ± 0.41
r	0.9436 ± 0.0047	0.9440 ± 0.2300

Results of the parameters obtained after mathematical modeling, the most suitable for this release profile was first-order monoexponential model. Values represent mean ± standard deviation ($n = 4$). *LNCc* curcumin-loaded lipid-core nanocapsules, *CLNCc* chitosan-coated curcumin-loaded lipid-core nanocapsules, *k (% h^{-1})* release rate constant, *r* correlation coefficient, *MSC* model selection criteria

The most suitable mathematical model for the curcumin release profile was first-order monoexponential considering the best parameters of correlation coefficient (r) and model selection criteria (Table 2). The first-order monoexponential fit means that the amount of curcumin in the formulation influences the amount of curcumin released, and its release occurs at one velocity. Additionally, this model indicates that the active substance is mostly encapsulated within the lipid core of the nanocapsules, corroborating the results of encapsulation efficiency.

Hydrogel physicochemical characterization

The cold method was chosen to avoid high temperatures that could destabilize the nanocapsules. Meanwhile, the addition of HPMC was under controlled temperature and the order of the incorporation was to facilitate the dispersion. The polymers' concentrations were tested starting from previous study references reporting a proper ratio under some modifications [30]. The nanocapsules incorporated in hydrogels presented a particle size distribution similar to the nanocapsules in suspension (Fig. 3). Moreover, the other physicochemical parameters evaluated were also maintained after the inclusion of Poloxamer® 407 and HPMC in the nanocapsule suspensions to obtain the hydrogels (Table 3). The maintenance of the particle size is important for the proper interaction and permeation of the nanoparticles in the buccal mucosa. Zeta potential decreased after the production of the hydrogel, probably due to Poloxamer® and HPMC (non-ionic polymers) added to the formulation; the initial characteristic of inversion of the zeta potential related to the presence of chitosan was maintained but in lower extent. No degradation of curcumin was observed during the production of the hydrogels since the drug content was around 100% for both groups (Table 3).

Table 3 Physicochemical characteristics of H-LNCc (hydrogel containing curcumin-loaded lipid-core nanocapsules) and H-CLNCc (hydrogel containing chitosan-coated curcumin-loaded lipid-core nanocapsules)

Parameters	H-LNCc	H-CLNCc
D (4,3) (nm)	196.33 ± 3.79	183.33 ± 12.66
Span	1.42 ± 0.56	1.61 ± 0.93*
z-Average (nm)	171.63 ± 4.23	199.23 ± 2.54*
PDI	0.13 ± 0.02	0.12 ± 0.02
Zeta Potential (mV)	−5.48 ± 0.68	5.95 ± 2.06*
pH (10% w/v)	6.52 ± 0.12	4.45 ± 0.26*
Curcumin concentration (mg.g^{-1})	0.91 ± 0.08	1.02 ± 0.05

Results of characterization of the hydrogels H-LNCc and H-CLNCc. Values represent mean ± standard deviation ($n = 3$). Statistical analysis: one-way ANOVA followed by post-hoc comparisons (Tukey Test) ($p < 0.05$). *Significant different obtained between groups

Fig. 3 **a** Particle size distribution obtained by laser diffraction of the H-LNCc (hydrogel containing curcumin-loaded lipid-core nanocapsules) and H-CLNCc (hydrogel containing chitosan-coated curcumin-loaded lipid-core nanocapsules). Results were expressed as percentage of volume of particle size in µm; **b** H-LNCc morphology analyzed by transmission electron microscopy (TEM); **c** H-CLNCc morphology analyzed by transmission electron microscopy (TEM) (scale bar = 100 nm)
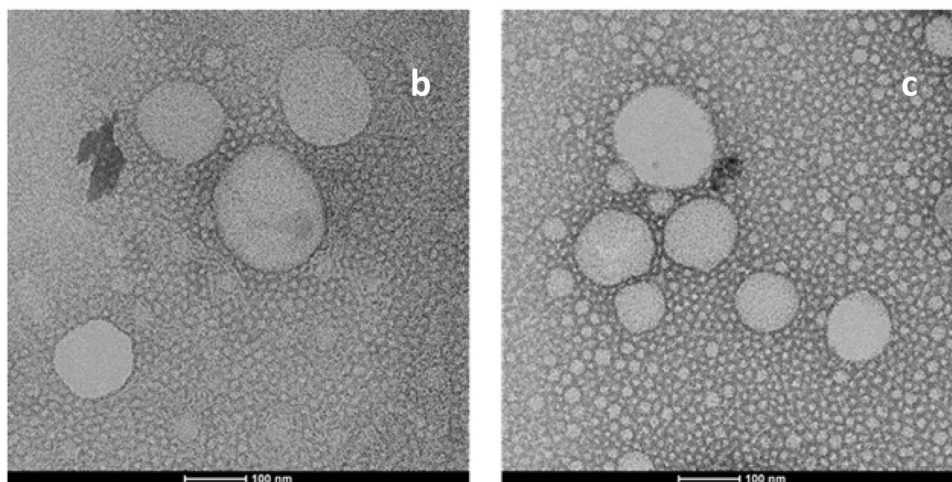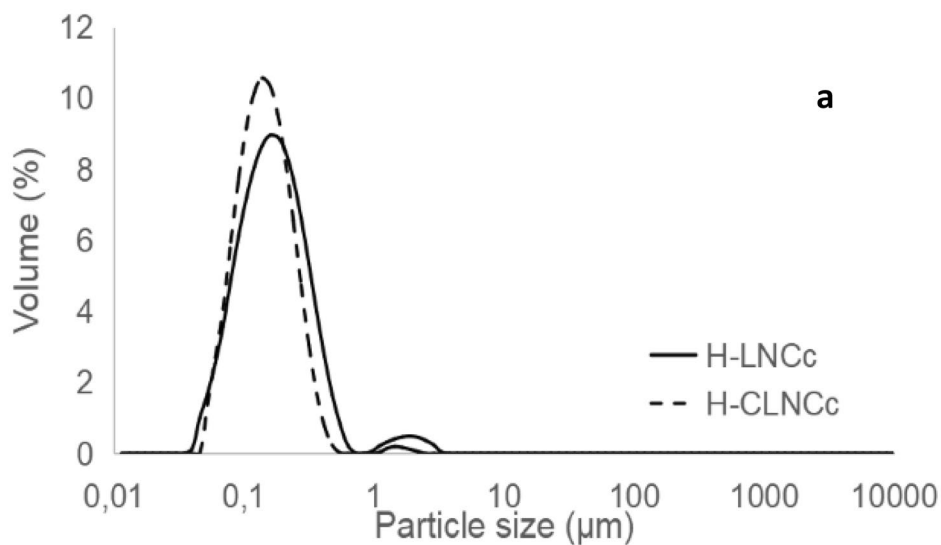
No morphological alteration of the nanocapsules (LNCc and CLNCc) was observed after incorporation in hydrogels (Fig. 3). Paese et al. [31] developed a hydrophilic gel containing benzophenone-3-loaded nanocapsules for topical use as a sunscreen and the nanoparticles remained with the same characteristics after carbomer incorporation in the suspensions. Moreover, Siqueira et al. [32] developed hydrogel containing hydroxyethylcellulose benzophenone-3-loaded nanocapsules and observed the same particle morphology when analyzing the hydrogels by TEM. This result corroborates the stability of nanocapsules after incorporation in hydrogels.

The sol–gel transition temperature ($T_{sol-gel}$) of the developed hydrogels was evaluated. For comparative purposes, a formulation containing the same concentrations of Poloxamer® 407 and HPMC with no nanocapsules was also evaluated (H–H$_2$O). No significant difference was observed between all groups. For the H-LNCc and H-CLNCc formulations, the transition temperatures were 27.07 °C and 28.66 °C, respectively. The presence of nanocapsules in the gel containing 14% (w/w) Poloxamer® 407 and 1.5% (w/w) HPMC did not influence the sol–gel transition temperature, considering that the H–H$_2$O formulation temperature was at 27.47 °C (Fig. 4). Moreover, the chitosan coating slightly increased the sol–gel transition temperature. This can be explained by the presence of acetic acid that is used to solubilize chitosan and acts on this parameter by reducing the hydrogen bond strength among Poloxamer® 407 densely packed units [33]. The concentration of Poloxamer® 407 in the formulations has been associated with an inversely proportional effect on the temperature of $T_{sol-gel}$, according to some previous studies [20]. In addition, the three temperatures of $T_{sol-gel}$ are in the appropriate temperature range, which is between 25 and 37 °C. The temperature range for in situ gelling should be higher than 25 °C, to avoid manufacturing problems, and lower than 37 °C, as a sol–gel transition temperature higher than body temperature would maintain poloxamer in its liquid state after administration [34].
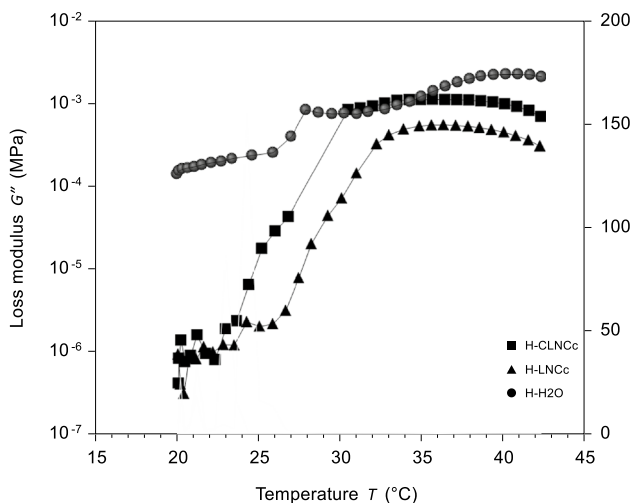
Fig. 4 Sol–gel transition temperature ($T_{sol-gel}$) obtained by means the point where the curve inflection occurs when the plot of temperature as a function of loss modulus G″. H-LNCc (hydrogel-containing curcumin-loaded lipid-core nanocapsules), H-CLNCc (hydrogel-containing chitosan-coated curcumin-loaded lipid-core nanocapsules), and H–H₂O (hydrogel-containing water)

Mucoadhesion test

Washability profile

The washability profile evaluated the resistance of the formulations against a determined flux to analyze its potential for adhesion on a surface, such as a standard mucosa. The washed curcumin content was 52.08 ± 1.63 µg for CLNCc and 62.60 ± 4.72 µg for LNCc, after 90 min, equivalent

to 35.19% and 34.78% of washed curcumin, respectively (Fig. 5). The washability of curcumin from both LNCc and CLNCc suspensions did not differ statistically. Although the literature indicates that the interaction between the cationic charges of chitosan would interact with the anionic charges of the mucous membranes [26], no notorious interaction and consequent increase in mucoadhesiveness related to the coating of the nanocapsules with chitosan was observed in this study.

After 480 min, the amount of curcumin washed from the H-LNCc was 10.92 ± 3.95 µg and the formulation H-CLNCc was 28.41 ± 24.47 µg, equivalent to 9.54% and 3.72%, respectively. Comparing the LNCc and CLNCc suspensions and their respective hydrogels, a statistical difference was observed ($p < 0.05$) between the paired formulations (LNCc/H-LNCc and CLNCc/H-CLNCc), indicating greater resistance of the hydrogel in comparison to the liquid suspensions. This property may be attributed to the mucoadhesive effect of HPMC by physical adsorption. When HPMC gets in contact with aqueous medium, it forms a film that interacts with the mucus layer. Thus, the HPMC chains interpenetrate the chains of mucin, promoting the entanglement of the chains by Van der Waals forces and hydrogen bonds [16].

At the end of the washability experiment, the amount of curcumin permeated through the mucosa was evaluated. The gel containing the chitosan-coated suspension (H-CLNCc) showed the highest amount of curcumin, 52.45 ± 8.98 µg (17.61%) in the receptor medium ($p < 0.05$), compared to the H-LNCc formulation, with 18.13 ± 10.24 µg (6.28%), and both had greater permeation in comparison to the suspensions, with 0.31 ± 0.06 µg and 0.29 ± 0.04 µg for LNCc and CLNCc, respectively (equivalent to 0.17% and 0.20% of
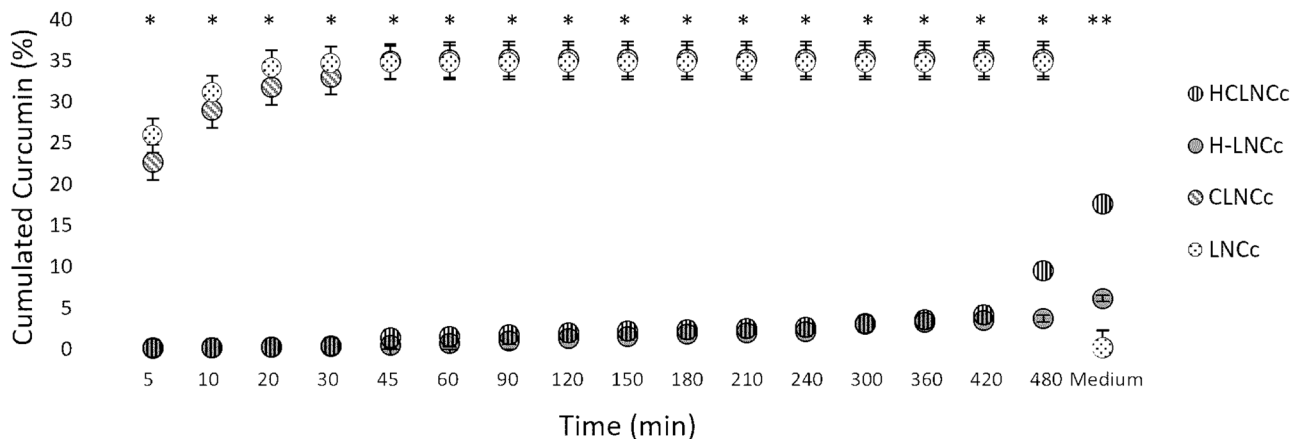

Fig. 5 Curcumin washability test on simulated salivary medium of the formulations. This test was performed with a manual modified Franz cell. Groups H-CLNCc (hydrogel-containing chitosan-coated curcumin-loaded lipid-core nanocapsules), H-LNCc (hydrogel-containing curcumin-loaded lipid-core nanocapsules), CLNCc (chitosan-coated curcumin-loaded lipid-core nanocapsules), and LNCc (curcumin-loaded lipid-core nanocapsules)

were collected in specific time intervals. Values represent mean ± standard deviation ($n = 4$). Statistical analysis: One-way ANOVA followed by post hoc comparisons (Tukey Test) ($p < 0.05$). *Significantly different from the nanocapsule suspensions formulations and their respective form as hydrogels; **H-CLNCc different from H-LNCc ($p < 0.05$)

permeated curcumin for each group). These results are probably due to the ability of the hydrogels to adhere properly to the mucosa, providing a longer retention time (480 min) of the formulations on the mucosa. In contrast, the nanosuspensions were completely washed within 90 min. Moreover, the higher permeation observed for the hydrogel containing CLNCc may be related to the presence of chitosan, which facilitated the process of penetration and permeation in the porcine buccal mucosa by transient opening of the intercellular junctions of the mucosa [35]. One explanation is that chitosan triggers the opening of the tight junctions by acting on the calcium channels, favoring its interaction.

dos Santos Chaves et al. [7] performed a washability test to determine the effect of using nanocapsules for the delivery of carvedilol via sublingual administration by comparing the washability profiles of two prototypes of lipid core nanocapsules, using polymers PLC and Eudragit RS 100 with the free form (non-encapsulated). As for the nanocapsules, the Eudragit RS 100 showed lower washability percentages at almost all-time intervals. No statistical difference was observed in the receptor medium after completing the experiment. Conversely, the samples collected in the experiments indicated higher washability of the free form, significantly different from that obtained by both nanocapsules.

Tensile stress analysis

After the interaction time between the formulations and the mucin disk, the distance traveled until the detachment occurred was greater ($p < 0.05$) for the hydrogels than the suspensions (Table 4). In this case, the area under the curve, related to the force required to detach the probe and the distance traveled by it, corresponds to the mucoadhesion work. The results showed that the hydrogels presented higher mucoadhesion in comparison to the nanocapsule suspensions. These findings corroborate those observed in the assessment of washability, since the mucoadhesion was greater for hydrogels, demonstrating again that in this case HPMC hydrogel exerts a greater influence on mucoadhesion compared to chitosan coating. As for the Poloxamer® 407, its effect on mucoadhesion is weak; however, this thermosensitive polymer after gelation attributes higher viscosity after the hydrogel is administered in situ, due to its high molecular weight, approximately 12.6 kDa [14]. As it is stated, the molecular weight of the polymeric chains is directly proportional to its mucoadhesion properties, and it has been observed in formulations with this effect, when higher concentrations of Poloxamer® were applied [36]. Thus, the addition of HPMC in this delivery system is essential to assure a higher retention of the formulation on mucosa due to its viscosity, allowing a sustained release of the active compound overtime. Meanwhile, Poloxamer® achieves gelation extending the residence time of the hydrogel for proper

Table 4 Results of mucoadhesion obtained from tensile analysis of the different formulations

Formulations	Debonding distance (mm)	Work of mucoadhesion (mN·mm)
LNCc	23.93 ± 0.56	3559.95 ± 558.19
CLNCc	22.85 ± 2.04	3537.46 ± 300.06
H-LNCc	36.34 ± 9.26*	5221.33 ± 1339.39*
H-CLNCc	35.22 ± 8.65*	5393.54 ± 683.97*

Results of the tensile analysis using a texturometer. LNCc (curcumin-loaded lipid-core nanocapsules), CLNCc (chitosan-coated curcumin-loaded lipid-core nanocapsules), H-LNCc (hydrogel-containing curcumin-loaded lipid-core nanocapsules), H-CLNCc (hydrogel-containing chitosan-coated curcumin-loaded lipid-core nanocapsules). Values represent mean ± standard deviation ($n = 6$). Statistical analysis: one-way ANOVA followed by post hoc comparisons (Tukey test) ($p < 0.05$). *Significant different from the value obtained between the nanocapsule suspentions and their respective hydrogels

absorption, improving the overall effect on buccal mucosa [37].

Fathalla et al. (2022) developed and optimized an in situ hydrogel using Poloxamer® 407 as a gelling agent for ocular purposes. The authors tested the most optimal concentrations of polymers to deliver L-carnocine for corneal wound healing, considering the parameters of work of mucoadhesion as critical for the patient compliance, as they retain the drug on the surface reducing the frequency of the administration. Poloxamer® 407 was tested at 18%. As part of this optimization, the addition of mucoadhesive polymers, such as chitosan, was tested in concentrations of 0.5 and 1.5%, resulting in higher work of mucoadhesion in comparison to the control [38]. This increase was dependent of the chitosan concentration values of 21% and 64%, respectively. In this case, the use of a cationic polymer has favored the interaction with mucin via electronic bonding.

In vitro study of irritation (HET-CAM assay)

The HET-CAM assay was performed to evaluate the irritation potential of the different formulations. Positive controls were classified as extremely (0.1 M NaOH, IS: 13.23 ± 0.35) and moderately (1% (w/v) SLS, IS: 6.65 ± 0.28) irritant. In contrast, the negative control did not show any reactions (hemorrhage, coagulation, and vasoconstriction), which validated the experiment. All formulations were classified as non-irritant (Table 5). Although the nano-loaded hydrogels were classified as non-irritant, their IS was different from zero, whereas for the nanosuspensions, the IS was equal to zero. Considering this premise, a blank hydrogel (H–H₂O) was prepared to evaluate the influence of Poloxamer® 407 and HPMC polymers by removing the suspensions of the nanocapsules and replacing them with water. For the H–H₂O formulation, the IS was 0.23 ± 0.56, suggesting that the

hydrogel polymeric base was related to the values observed for the nano-loaded hydrogels. Despite the non-zero IS, it is worth mentioning that the hydrogels were classified as non-irritant, corroborating previous findings on formulations containing Poloxamer® 407 are interesting because they proved to be non-irritant when in contact with biological membranes [39].

A similar thermogelling system was developed for ophthalmic use. The formulation was composed of timolol maleate prescribed for glaucoma therapy. The polymers used were Poloxamer® 127 (9%), and chitosan (0.25%), present as a coating agent for the nanocapsules. The average IS of the formulations was equal to 0.33, classifying this system as non-irritant on the HET-CAM scale [40].

Cytotoxicity assays

Considering that the proposed drug delivery system was designed for buccal administration, we tested the formulations on SCC-25 cells, which are related to oral squamous cell carcinoma, an oral disease with considerable prevalence and morbidity worldwide. In addition, some studies have demonstrated the in vitro antiproliferative effect of curcumin on epidermoid mouth cancer cells [17, 21, 41]. Thus, in order to establish the biological effects of curcumin after nanoencapsulation, in vitro evaluations were performed.

When the SCC-25 strain was exposed to the tested formulations at a curcumin concentration of 5 μM, it did not show any statistically significant difference among all groups

Table 5 Irritation Score of the different formulations obtained by the hen's egg chorioallantoic membrane test (HET-CAM)

Groups	IS	Classification
NaOH	13.23 ± 0.35	Extremely irritant
LSS	6.65 ± 0.28	Moderately irritant
NaCl	0.00 ± 0.00	Non-irritant
LNCc	0.00 ± 0.00	Non-irritant
CLNCc	0.00 ± 0.00	Non-irritant
NEc	0.00 ± 0.00	Non-irritant
H-LNCc	0.51 ± 0.91	Non-irritant
H-CLNCc	0.78 ± 0.97	Non-irritant
H-NEc	0.28 ± 0.69	Non-irritant
H-H$_2$0	0.23 ± 0.56	Non-irritant

Values represent mean ± standard deviation ($n = 5$). Classification: non-irritant (0–0.9), slightly irritant (1–4.9), moderately irritant (5–8.9), and extremely irritant (9–21). NaOH (sodium hydroxide), LSS (sodium lauryl sulfate), NaCl (sodium chloride), LNCc (curcumin-loaded lipid-core nanocapsules), CLNCc (chitosan-coated curcumin-loaded lipid-core nanocapsules), NEc (curcumin nanoemulsion), H-LNCc (hydrogel-containing curcumin-loaded lipid-core nanocapsules), H-CLNCc (hydrogel-containing chitosan-coated curcumin-loaded lipid-core nanocapsules), H-NEc (hydrogel-containing curcumin nanoemulsion), and H-H$_2$O (hydrogel-containing water)

evaluated during all the treatment times. In contrast, when comparing the concentrations of 10 μM and 20 μM, there was an effect on cell viability. There was no cytotoxic effect after 24 h of exposure for all tested groups in all tested concentrations. Conversely, a significant reduction in cell viability was observed after 48 h of treatment, when comparing chitosan-coated, non-coated nanocapsules and free curcumin, while the lowest viability percentage was observed for the third group in comparison to the control group.

To assess the influence of exposure time on the performance of each treatment, 48- and 144-h evaluations were performed. In these time periods, a significant difference was observed between concentrations of 10 μM and 20 μM. Statistical difference was observed after 48 h, as both concentrations were significantly different from control, being 20 μM more effective. This suggests a dose-dependent cytotoxic effect. An interesting aspect to consider is that curcumin in solution was more effective than all the nanocapsules formulations (Fig. 6). This result can be attributed to the curcumin release profile from the nanocapsules produced since it takes about 48 h to release approximately 9% of curcumin. Thus, this may explain the lower performance of the nanocapsules in relation to curcumin in solution, which is more readily available to cells due to the lack of polymeric barriers for diffusion.

Furthermore, chitosan-coated blank lipid-core nanocapsules (CLNC) also presented a significant decrease in the cellular viability when treated at volumes equivalent to curcumin concentrations of 10 μM and 20 μM. This nanoparticulate system presented intrinsic cytotoxic activity and a possible explanation for this phenomenon is related to the presence of chitosan, which is a biopolymer that has been reported as proliferation inhibitor for several cell lines and selectively with low toxicity against normal human cells [42].

Mazzarino et al. [43] evaluated the cytotoxic effect of curcumin-loaded nanoparticles, coated and non-coated with chitosan, for 24, 48, and 72 h with concentrations from 1 to 100 μM on SCC-9 oral cancer cell lines. The authors found similar results in relation to the cytotoxicity of the formulations compared to the free drug. The greatest decrease in cell viability was observed in cells treated with free curcumin. After 72 h of treatment, cell viability was 90% for cells treated with 100 μM curcumin associated with the coated nanoformulation compared to 45% viability in the group treated with free curcumin [43], indicating that this significant difference might be promoted by the drug encapsulation that retards its release to the cells. It is important to mention that these results can vary according to the experimental conditions used.

It was not possible to reproduce the conditions regarding the solubility from lipid-core curcumin nanocapsules to the group of free curcumin, since the concentration
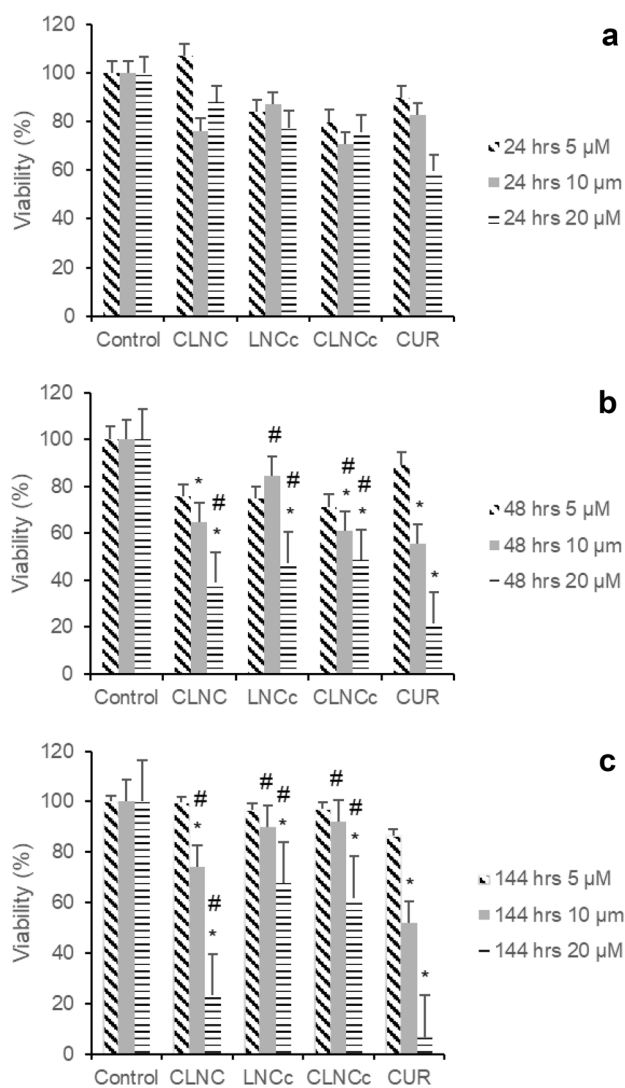
Fig. 6 Cell viability of OSCC cell line by MTT analysis. SCC-25 cells were seeded in a 96-well plate at the following final curcumin concentrations: 5, 10, 20 μM. The plates were incubated for **a** 24 h, **b** 48 h, and **c** 144 h. Groups evaluated: CLNC (blank lipid-core nanocapsule), LNCc (curcumin-loaded lipid-core nanocapsule), CLNCc (chitosan-coated curcumin-loaded lipid nanocapsule), and CUR (curcumin solution in 1% (v/v) DMSO). Values represent mean ± standard deviation ($n = 6$) Statistical analysis: one-way ANOVA followed by post hoc comparisons (Tukey test) ($p < 0.05$). *Significant different from the control group; #significant different from CUR

of curcumin carried on the developed nanosystem was 0.09 mg mL^{-1}. Curcumin is practically insoluble in water, having a low value of 0.00078 mg mL^{-1}[44]. Estimating that the value of curcumin solution on water is approximately around 100 times lower than the concentration of these lipid-core curcumin nanocapsules, the low affinity with water would not permit a curcumin solution at desired concentration. Thus, the dissolution of the active compounds in DMSO guarantees the solubilization required

for the viability test by providing curcu min the availability to act on SCC-25 cell lines. Although the ideal medium to simulate in vivo conditions is water or buffer PBS, when performing these assays, solubi lity is a key parameter to be considered.

Lin HY et al. (2012) evaluated the cytotoxic effect of curcumin microemulsions for delivery in buccal membranes on SCC-25 strain lines by dissolving curcumin in water. As expected, the result of viability remained unaltered along the time, indicating that no activity was performed due to the practically insoluble curcumin in this medium (Data now shown) [45]. In fact, one of the main advantages of the nanoencapsulating active substances and drugs is to enhance apparent solubility, allowing lipophilic compounds with low solubility and permeability to perform better in their site of action [46].

Conclusions

One of the main concerns about buccal administration is the salivary flow that reduces the drug retention time on the buccal cavity. As a strategy to overcome this particular aspect, this study proposed a mucoadhesive hydrogel containing nanocapsules. In this context, the chitosan-coated curcumin-loaded lipid-core nanocapsules presented the following characteristics of quality control expected for nanometric formulations: average diameter of about 200 nm, homogeneous diameter distribution, spherical morphology, content, and encapsulation efficiency close to 100%. The chitosan coating did not influence the curcumin release profile and the results showed that curcumin was dispersed in the core of the nanocapsules. After incorporating HPMC and Poloxamer® 407 to obtain hydrogels, the physicochemical characteristics of the nanocapsules were maintained.

The results obtained for the produced hydrogels demonstrated their sol–gel conversion at a temperature below 37 °C. In addition, all formulations (nanosuspension or hydrogel) were classified as non-irritant. Moreover, in relation to mucoadhesion, the chitosan coating did not present a significant increase in the interaction of the nanocapsule suspension on the porcine buccal mucosa. In contrast, the addition of Poloxamer® 407 and HPMC significantly increased the mucoadhesion on the porcine mucosa, and the hydrogel containing the chitosan-coated formulation had greater permeation of curcumin compared to the uncoated formulation. Although the nanoencapsulation did not improve the in vitro effects of curcumin on SCC-25 cells, the overall findings indicate the benefits of associating two different technologies (nanoencapsulation and hydrogel development) to obtain a promising formulation with desired characteristics for the treatment of epidermoid oral cancer, using the buccal mucosa as an administration route.

Declarations

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