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Chitosan and liposomal delivery systems for epicatechin or propyl gallate targeting localized treatment of vulvovaginal candidiasis

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ABSTRACT

Natural polyphenols are promising alternatives to antifungals for novel treatments of vulvovaginal candidiasis (VVC) in an era of antimicrobial resistance. However, polyphenols are poorly soluble and prone to degradation. To overcome their limitations, we propose incorporation in liposomes. The study aimed to develop chitosan and liposome comprising delivery systems for epicatechin (EC) or propyl gallate (PG) as treatment of VVC. EC was selected for its antioxidative properties and PG as an ester of antifungal gallic acid. To improve formulation retention at vaginal site, mucoadhesive chitosan was introduced into formulation as liposomal surface coating or hydrogel due to intrinsic antifungal properties. These polyphenol-loaded liposomes exhibited an average size of 125 nm with a 64 % entrapment efficiency (for both polyphenols). A sustained *in vitro* polyphenol release was seen from liposomes, particularly in chitosan hydrogel ($p < 0.01$ or lower). Viscosity was evaluated since increased viscosity upon mucin contact indicated adhesive bond formation between chitosan and mucin confirming mucoadhesiveness of formulations. Antifungal activity was evaluated by the broth microdilution method on *Candida albicans* CRM-10231. Unlike PG, incorporation of EC in liposomes enabled antifungal activity. Fungicidal activity of chitosan was confirmed both when used as liposomal coating material and as hydrogel vehicle.

1. Introduction

Vulvovaginal candidiasis (VVC) is one of the most frequently diagnosed infections in women seeking gynecological care, affecting approximately 75 % of this population (Conte et al., 2023). Opportunistic overgrowth of *Candida* found in vaginal microflora causes the infection, accompanied, or facilitated, by a disruption in the local vaginal environment. Disruptions can be caused by a decrease in microbial diversity, such as a reduction of *Lactobacilli*, which disturbs vaginal defense mechanisms and increases the pH. Relapses of VVC infection are caused by ineffective treatment, either due to presence of virulence factors, e.g., involved in biofilms, hypha formation or adhesion, or because of suboptimal pharmaceutical formulations (Gonçalves et al., 2016; Kalia et al., 2020; Willems et al., 2020; Atriwal et al., 2021). The recurrent form of VVC, diagnosed as a minimum of four or more relapses per year, presents further challenges in treatment (Satora et al., 2023). Inadequate treatment and recurrence of VVC is increasing, with recurrence rates at 5–8 % within the first two months post treatment (Kalia et al., 2020). If left un- or unsuccessfully treated, vulvovaginal infections can have severe consequences, including miscarriage, stillbirth, infertility, and an increased risk of acquiring new, potentially more serious infections (Vanić et al., 2021). Thus, in recent years, new efforts have been put into improving the treatment of VVC by novel and smart delivery systems (Conte et al., 2023; Martins et al., 2023; Usach et al., 2023) and/or novel antifungal agents from natural sources (Giordani et al., 2020; Sardi et al., 2021; Satora et al., 2023; Bezerra

Abbreviations: VVC, vulvovaginal candidiasis; EC, Epicatechin; PG, Propyl gallate; LMW, low molecular weight; MMW, medium molecular weight; dH_2O, distilled water; EC liposomes, epicatechin entrapped in liposomes; PG liposomes, propyl gallate entrapped in liposomes; Plain liposomes, liposomes without polyphenol; Chitosan-coated liposomes, liposomes coated with chitosan; Liposomes-in-hydrogel, liposomes incorporated within a chitosan hydrogel; PDI, polydispersity index; EE, entrapment efficiency; VFS, vaginal fluid simulant.

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et al., 2024).

Recently, polyphenols have shown promise in treatment of vaginal infections due to their antimicrobial, anti-oxidative and anti-inflammatory properties (Jøraholmen et al., 2019; Abu-Azzam & Nasr, 2020; Giordani et al., 2020; Jøraholmen et al., 2020b). Some polyphenols have also shown encouraging antifungal capabilities (Simonetti et al., 2020; Nguyen et al., 2021; Rossatto et al., 2021; Rayan et al., 2023). However, polyphenols have challenging physicochemical properties seen from a formulation standpoint. They suffer from poor aqueous solubility, rapid enzymatic degradation, and general instability, which in practice makes for poor therapeutic candidates. Therefore, protecting these compounds until arrival at targeted infection site is crucial to achieve desired therapeutic effects (Murthy et al., 2021).

Drug delivery strategies using liposomes, nanosized lipid vesicles, have been proposed as a potential solution to improve the therapeutic index of polyphenolic compounds (Basnet et al., 2012; Jøraholmen et al., 2015; Pandey et al., 2021). Liposomes can improve solubility, protect incorporated compounds from degradation and reduce toxicity (Guimarães et al., 2021). Furthermore, they can enhance the antimicrobial efficacy and enable sustained release of incorporated compounds, which in turn could lower the risk of microbial regrowth and increase exposure time (Ferreira et al., 2021; Murthy et al., 2021). Pursuing a topical administration route may additionally provide an efficient way of circumventing some of the many limitations in the therapeutic use of polyphenols. Topical vaginal drug delivery provides further advantages, including higher local drug concentrations, utilization of lower doses and reduced systemic side effects (Leyva-Gómez et al., 2019).

However, despite the numerous benefits that accompany localized therapy, it also presents challenges. The vaginal mucosal barrier significantly contributes to these challenges by limiting formulation retention after localized application (Vanić et al., 2021). Though efficient in protecting polyphenols, liposomes as suspensions are unsuitable for topical vaginal administration due to their liquid nature and poor retention, thus require either further modifications or subsequent incorporation in a secondary vehicle utilizing mucoadhesive polymers (Jøraholmen et al., 2014; Jøraholmen et al., 2019; Jøraholmen et al., 2020b). Mucoadhesion is considered a viable strategy for increasing retention of localized vaginal formulations upon application, because it improves resistance to being washed out by the mucus turnover and gravity (Smoleński et al., 2021). As such, the addition of advanced mucoadhesive formulations for increased retention is in focus for topical treatment of vaginal infections (dos Santos et al., 2020; Pandey et al., 2021).

Chitosan emerged as an attractive, biocompatible polymer of natural origin, frequently used in both pharmaceutical technology and drug delivery, including for vaginal application (Pellá et al., 2018; Bakshi et al., 2020; Pramanik & Sali, 2021; Khalaf et al., 2023). It is well documented that chitosan exhibits excellent mucoadhesive properties, as assessed through several different methods (Woertz et al., 2013). While the full comprehension of mucoadhesion remains elusive, the theories that attempt to explain it center around different intermolecular bonds, such as hydrogen bonds or electrostatic forces (Pham et al., 2021). The strength of these intermolecular bonds affect formulation viscosity (Sharma et al., 2024). Thus, increased formulation viscosity upon mucin/mucus contact is indicative of the formation or strengthening of these bonds, which may then be used to evaluate mucoadhesion (Rossi et al., 2018). In addition to mucoadhesive properties, chitosan is also well known for exhibiting intrinsic antimicrobial properties (Jøraholmen et al., 2020a; Hemmingsen et al., 2021b). Utilizing chitosan may allow for synergy with antimicrobial abilities of incorporated polyphenol (Iacob et al., 2021; Vanić et al., 2021). Several ways of introducing chitosan and liposomes within a formulation have been previously explored. Some authors included liposomal chitosan-modifications such as coating of the liposomes' surface or using chitosan hydrogel as a secondary vehicle to form liposomes-in-hydrogel formulations (Jøraholmen et al., 2015; Jøraholmen et al., 2019; Liu et al., 2019; Yadav et al., 2024). Adding chitosan to a liposomal formulation may additionally improve the degree of controlled release of active substance, as well as prolong the contact time at site of action (Verlee et al., 2017; Araujo et al., 2021; Nayak et al., 2021).

The liposomal entrapment of polyphenols has shown to enhance their stability in vaginal environment leading to improved therapeutic effect *in vitro* (Jøraholmen et al., 2015; Jøraholmen et al., 2020b). In previous studies, we also discovered that epicatechin liposomes exhibited antioxidative and anti-inflammatory properties (Jøraholmen et al., 2019); moreover, we determined that gallic acid liposomes possess similar antioxidative and anti-inflammatory activities in addition to antifungal activity (Giordani et al., 2020). Gallic acid esters are known to demonstrate enhanced antimicrobial activity compared to gallic acid alone (Leal et al., 2009; Rayan et al., 2023). Consequently, in this work we were interested in investigating both epicatechin (EC) and the gallic acid ester propyl gallate (PG) as novel antifungal agents when formulated with liposome and chitosan comprising delivery systems.

Present work focused on developing both chitosan-coated liposomes and liposomes-in-hydrogel formulations with either EC or PG for future treatment of VVC. We characterized the liposomal formulations and assessed their mucoadhesive capabilities by measuring the viscosity increase upon contact with mucin. Finally, we evaluated the *in vitro* antifungal activity of the formulations against *C. albicans*.

2. Materials and methods

2.1. Materials

Chitopharm™ S – low molecular weight (LMW) and M – medium molecular weight (MMW) chitosan from shrimp (average of 50–450 kDa and 350–600 kDa respectively, degree of deacetylation 75–95 %) were a gift from Chitinor (Tromsø, Norway) and Lipoid S100 (phosphatidylcholine content > 94 %) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Methanol (≥ 99.9 %) and dimethyl sulfoxide (DMSO, ≥ 99.8 %) were purchased from VWR (Fontenay-sous-Bois, France). Ammonium acetate was product of VWR International, (Leuven, Belgium). Acetic acid (≥ 99.8 %), (−)-epicatechin (≥ 98 %), propyl gallate (≥ 98.0 %), sodium chloride, monobasic potassium phosphate, dibasic potassium phosphate, bovine serum albumin, mucin from porcine stomach (Type III, bound sialic acid 0.5–1.5 %, partially purified powder), calcium hydroxide, yeast medium broth (powder), yeast medium agar (powder), and glycerol (86–89 %) were acquired from Sigma-Aldrich (St. Louis, USA). Potassium hydroxide, lactic acid and urea were purchased from NMD (Oslo, Norway).

2.2. Preparation and size reduction of liposomes

All liposomes were prepared via the thin film hydration method as previously described (Jøraholmen et al., 2015). Lipoid S100 (200 mg) and EC (20 mg) were dissolved in methanol. The methanol was evaporated using a rotavapor (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labor-technik, Switzerland) for 1.5 h at 60 kPa and 45 °C resulting in a thin film. The film was rehydrated with distilled water (dH_2O, 10 mL) to form EC liposomes. The same procedure was repeated, replacing EC with PG to form PG liposomes. Plain liposomes (without polyphenol) were made in a comparable manner, with only lipid. All liposomal suspensions were stored in a refrigerator (4–8 °C) overnight prior to further use.

The vesicle size was reduced through stepwise manual extrusion using polycarbonate membranes (Nuclepore Track-Etch Membrane, Whatman House, Maidstone, UK). The membrane diameters were 0.4, 0.2 and 0.1 µm; each size reduction step was repeated five times. Plain liposomes were extruded under the same conditions to be of comparable size.

2.3. Chitosan coating of liposomes

Two separate solutions of chitosan (LMW and MMW) were prepared as previously described (Jøraholmen et al., 2014). In brief, chitosan was added to 0.1 % (v/v) acetic acid in concentrations of either 0.3 % (w/v, LMW chitosan) or 0.1 % (w/v, MMW chitosan) and left at room temperature (22 ± 2 °C) for 48 h before further use. To coat the liposomes, chitosan solution (1.5 mL) was drop-wise added to an equal volume of liposomal dispersion free of unentrapped substance (through dialysis, see section 2.4) over the course of 6 min while under controlled magnetic stirring (500 rpm), it was then left on the stirrer at room temperature (22 ± 2 °C) for 1 h, before placement in a refrigerator overnight (4–8 °C).

2.4. Liposomal characterization

Liposome size was determined with a Malvern Zetasizer Nano ZS Zen 3600 (Malvern, Worcestershire, UK). The liposomes were diluted 1:100 (v/v) with filtered water (0.2 µm) prior to every measurement and measured in triplicates. The zeta potential was determined with the Malvern Zetasizer Nano ZS Zen 3600 (Malvern, Worcestershire, UK) using folded capillary zeta cells (DTS1070). The liposomes were diluted 1:10 (v/v) with filtered water prior to every measurement and measured in triplicates (Jøraholmen et al., 2019). The pH of the water was determined using an Accumet®, Portable pH meter AP115 (Fisher Scientific, MA, USA) at room temperature (22 ± 2 °C).

The EC liposomes (1:500, v/v) and PG liposomes (1:1000, v/v) were dialyzed for 6 h (tube, MWCO: 12–14 kDa; Spectra/Por®4, Spectrum®, VWR International, Fontenay-sous-Bois, France). The liposomal entrapment efficiency of the various polyphenols was determined spectrophotometrically using a Tecan Spark M10 multimode plate reader (Tecan Trading AG, Switzerland) at 270 nm (EC) and 274 nm (PG). A standard curve of each polyphenol in methanol was prepared in the appropriate concentration range for the analysis ($R_2 \geq 0.998$). Entrapment efficiency (EE %) was calculated using equation (1).

$$EE\% = \frac{Pdialyzed}{Ptotal}X100\% \tag{1}$$

where $P_{dialyzed}$ refers to the polyphenol concentration in the dialyzed formulation and P_{total} refers to the polyphenol concentration in the non-dialyzed formulation.

2.5. Preparation of hydrogels

Chitosan hydrogel, with glycerol as a plasticizer, was prepared as described previously (Jøraholmen et al., 2020a; Hemmingsen et al., 2021a). LMW chitosan (0.3 %, w/w) was dispersed in 1 % acetic acid (v/v) and 10 % glycerol (w/w) and gently hand-stirred, to avoid excess air bubbles, at room temperature (22 ± 2 °C) until dissolved. The hydrogel was sonicated in an ultrasonic bath (Branson, Ultrasonic cleaner 5510E-MT, Danbury, USA) for 15 min allowing the removal of potential air bubbles and subsequently left at room temperature (22 ± 2 °C) for 48 h to swell and form hydrogel. This was repeated with MMW chitosan to form 0.1 % (w/w) MMW chitosan hydrogel.

Polyphenol-loaded liposomes (20 %, w/w) were incorporated (hand-stirred to avoid air bubbles) into hydrogels at room temperature (22 ± 2 °C) for 5 min to prepare respective liposomes-in-hydrogel systems (Jøraholmen et al., 2019). Polyphenol-in-hydrogel was prepared in a similar way, as following: EC was dissolved with dH_2O. PG due to solubility, was first dissolved in 100 % dimethyl sulfoxide (DMSO) before dilution with dH_2O until the concentration of DMSO was < 1 %. Further, aliquots of the polyphenol solutions were added to the hydrogels to correspond to the respective polyphenol concentrations in the liposomes-in-hydrogel systems. The concentrations of glycerol in all hydrogels (plain hydrogel, liposomes-in-hydrogel and polyphenol-in-hydrogel) were 10 % (w/w) with a chitosan concentration of either 0.3 % (w/w) LMW or 0.1 % (w/w) MMW.

2.6. Characterization of hydrogels

The pH of all hydrogels was measured using the Accumet®, Portable pH meter AP115 (Fisher Scientific, MA, USA) at room temperature (22 ± 2 °C).

Texture properties of hydrogels were evaluated through utilization of a backward extrusion rig set using a Texture Analyzer TA.XT Plus (Stable Micro Systems Ltd., Surrey, UK) (Hurler et al., 2012). Hydrogel (40 g) was transferred to the rig set container. A 35 mm disk was fixed to the texture analyzer, and compressed into the hydrogel, and redrawn to starting position. The speed was 4 mm s^{-1}, and the starting position was right above the hydrogel surface. The distance and trigger force were 10 mm and 10 g, respectively.

2.7. In vitro viscosity and mucoadhesion assessment

The viscosity of plain, chitosan-coated liposomes or liposomes-in-hydrogels before and after addition of mucin were estimated using the rheological method as described by Hassan and Gallo (Hassan & Gallo, 1990). This analysis was performed with a Thermo HAAKE viscotester 7 Plus, at both room temperature (22 ± 2 °C) and physiological temperature (37 °C). The pH of all final formulations was estimated using the Accumet®, Portable pH meter AP115 (Fisher Scientific, MA, USA) at room temperature (22 ± 2 °C) to ensure pH remained the same for each measurement.

The formation of adhesive bonds between formulation and mucin can be detected as an increase in formulation viscosity that is higher than the total viscosity of both mucin and formulation. Viscosity changes upon contact with mucin (η_{change}) were calculated using equation (2).

$$\eta_{change} = \eta_{total} - \eta_{mucin} - \eta_{formulation} \tag{2}$$

where η_{total} is the total viscosity of formulation mixed with mucin, η_{mucin} is the viscosity of the mucin dispersion and the $\eta_{formulation}$ is the viscosity of the delivery system without the addition of mucin.

A 10 % (w/w) mucin suspension (mucin from porcine stomach Type III, bound sialic acid 0.5–1.5 %, partially purified powder and dH_2O) was stirred at 500 rpm in room temperature (22 ± 2 °C) for 3 h. As vaginal mucus contains 2–5 % mucin glycoproteins (Moncla et al., 2016), this mucin suspension was mixed 1:1 (w/w) with the different formulations (liposomes-in-hydrogel, plain liposomes and chitosan-coated liposomes without polyphenol) on a magnetic stirrer (250 rpm) at room temperature (22 ± 2 °C) for 15 min to a final mucin concentration of 5 % (w/w).

Viscosity measurements were taken for mucin suspension alone (η_{mucin}), formulation alone ($\eta_{formulation}$) and the formulations mixed with mucin (η_{total}). The mucin suspension (η_{mucin}, 10 %, w/w) and the formulations ($\eta_{formulation}$, plain liposomes, chitosan-coated liposomes and liposomes-in-hydrogel) were diluted 1:1 (w/w) to ensure both mucin and chitosan concentration remained the same in all measurements. The viscosity and shear force measurements were performed 1 min after application of shear in the shear range of 20 – 200 s^{-1}. Both viscosity and shear stress were plotted as a function of shear rate and the viscosity change (η_{change}) was calculated.

2.8. In vitro polyphenol release

In vitro release of polyphenols from formulations was assessed through the Franz diffusion cell system (Perme Gear Ink, Diffusion Cells and Systems, Hellertown, PA, USA) utilizing cellophane membrane and 12 mL (1.77 cm^2) acceptor cells as previously described (Jøraholmen et al., 2019). To closer imitate the vaginal environment in this release

study, vaginal fluid simulant (VFS) was prepared, following the procedure originally published by Owen and Katz (Owen & Katz, 1999). In brief, the solution of salts, acid and bovine serum albumin was mechanically stirred at room temperature (22 ± 2 °C) to assure a homogenous mixture and the final pH adjusted to 4.6 by addition of 1 M HCl. The pH of the VFS was determined using the Accumet®, Portable pH meter AP115 as described under section 2.4.

The acceptor chamber (12 mL) was filled with acetate buffer (pH 4.6, 77.1 g/L CH_3COONH_4 and 70 mL/L glacial acetic acid) before the addition of a pre-soaked (acetate buffer) cellophane membrane of appropriate size. VFS (50 μL) was added to donor chamber before the careful addition of liposomal formulations (non-coated liposomes, chitosan-coated liposomes, and liposomes-in-hydrogels, all 550 μL) or controls (polyphenol in either buffer (EC) or DMSO (PG), and polyphenol-in-hydrogel, 550 μL) before the system was sealed. The system was kept under constant heating (Julabo Laboratechnik, F12-ED, Seelback, Germany) at 37 °C under mechanical stirring.

Franz samples (500 μL) were withdrawn from acceptor chamber after 1, 2, 3, 4, 6 and 8 h. The collected samples were replaced by an equal volume of fresh buffer. All collected samples, from the acceptor chamber, as well as remaining sample in donor chamber and the membrane (to control recovery) were either diluted or soaked in methanol and amount of polyphenol was quantified using a Tecan Spark M10 multimode plate reader (Tecan Trading AG, Switzerland) as described in section 2.4.

2.9. Antimicrobial assessment

The antimicrobial activity against *C. albicans* CRM-10231 of liposomal formulations (non-coated liposomes, chitosan-coated liposomes, and liposomes-in-hydrogel) with or without polyphenols, as well as polyphenol controls, were evaluated by the broth microdilution method (Balouiri et al., 2016).

The non-coated liposomes and chitosan-coated liposomes were diluted to match the respective polyphenol concentration in the liposomes-in-hydrogel formulations. The EC was prepared in dH_2O to matching concentration of the hydrogels (EC=260 μg/mL, PG=390 μg/mL). Due to the poor solubility of PG, this polyphenol was first solubilized in DMSO (85 mg/mL) before further dilution in dH_2O to assure that final concentration of DMSO was < 1 %. Plain liposomes and a DMSO control (< 1 %) were assessed alongside the formulations. All concentrations of EC and PG were confirmed before the start of the experiment via UV spectroscopy as described in section 2.4.

C. albicans was aerobically cultured on yeast malt (YM) agar plates at 37 °C for 18–20 h. This was used to prepare the inoculum by first adjusting a sterile saline solution to 0.5 McFarland (1.5 $e10^6$ CFU/mL for *C. albicans*) before a 100-fold dilution in YM broth (Balouiri et al., 2016). Each formulation (50 μL) was diluted in YM broth (50 μL) in a two-fold sequence in 96 well culture plates (Falcon®, Corning Inc., Pisa, Italy). These wells were then inoculated with the previously prepared inoculum (50 μL) to test the inhibition in a known concentration range. Wells containing microbial suspension (50 μL) and YM broth (50 μL) served as growth control. Sterility controls as only sterile YM broth (100 μL), and formulations (50 μL) in sterile YM broth (50 μL) were also included.

Inoculated plates were aerobically incubated at 37 °C for 24 h under constant shaking at 100 rpm. Afterwards minimum inhibitory concentrations (MIC) were determined by comparing the turbidity (OD600) of all wells with that of growth control, utilizing the Tecan Spark M10 multimode plate reader (Tecan Trading AG, Switzerland). To determine the potential microbicidal effect, 25 μL of PBS diluted samples (10^1–10^8) from the wells exhibiting no visual growth and the first well with visual growth were streaked onto YM agar plates and incubated at 37 °C for another 24 h. The minimum microbicidal concentration (MBC) was defined as the minimal concentration that completely inhibited microbial viability (Hemmingsen et al., 2023). Three batches for each formulation were tested in triplicates.

2.10. Statistical evaluation

Results are expressed as mean ± SD. Student t-tests and one-way ANOVA with Tukey's post-test were performed to evaluate significance, which was no less than $p < 0.05$. Microsoft Excel® (Microsoft 365 Business) for windows was used for all statistical analyses.

3. Results and discussion

3.1. Characteristics of liposomal formulations (non-coated and chitosan-coated)

A liposomal formulation intended for localized vaginal delivery requires careful tailoring to assure optimal performance. This involves optimization of liposomal characteristics, such as size, surface charge and load of active ingredient, which are linked to the overall formulation properties (Ensign et al., 2014; Jøraholmen et al., 2020a). The properties of non-coated and chitosan-coated liposomes, with and without entrapped polyphenol, are summarized in Table 1.

For localized vaginal application of nanoformulations, the optimal size remains a disputed topic without consensus. A size range between 200 and 500 nm is proposed as ideal to ensure diffusion of active molecules across the mucus barrier whilst maximizing therapeutic effectiveness (das Neves et al. 2011a; das Neves et al. 2011b). However, liposomal delivery systems for vaginal administration are also reported with significantly smaller diameters (Melo et al., 2020; Tuğcu-Demiröz et al., 2021). We aimed for a vesicle size in the range of 100 to 200 nm and the non-coated liposomes were found to be within this range after extrusion, with an average size around 125 nm (Table 1). A slight increase was seen in the mean vesicle size for the chitosan-coated liposomes, indicating a chitosan presence on the coated surface, which is consistent with previous findings (Jøraholmen et al., 2015; Hamedinasab et al., 2020; Sebaaly et al., 2022). However, all liposomal formulations were still within the desired size range. For topical delivery, a lipid-based colloidal system is considered suitable when its polydispersity index (PDI) is 0.30 or lower (Guimarães et al., 2021). By liposomal extrusion a low PDI below 0.15 was achieved for both non-coated and chitosan-coated liposomes, which indicates a homogenous size distribution. We again confirmed the extrusion technique as an effective means to achieve reproducible liposomal suspensions within the targeted size range.

Zeta potential serves as an indicator of liposomal surface charge, commonly employed to evaluate their stability when in suspension. Additionally, surface charge may be of particular interest considering intended function and application (Midekessa et al., 2020). The plain liposomes (liposomes without polyphenol) exhibited a close to neutral charge with a zeta potential of 0.9 ± 3.0 mV, as expected (Jøraholmen et al., 2022). The incorporation of polyphenol caused a slight anionic shift with a zeta potential of − 3.9 ± 2.3 mV and − 6.2 ± 2.3 mV for the EC and PG liposomes, respectively (Table 1). Due to the lipophilicity of both EC and PG, they are expected to be incorporated within the lipid bilayer. This slight anionic charge can therefore be attributed to interactions between the lipid bilayer and the incorporated polyphenol and charges of the polyphenols themselves (Jyothi et al., 2022). Regardless, the zeta potential remained within the neutral area of ± 10 mV (Wang, 2021) for the non-coated liposomes, as seen earlier (Jøraholmen et al., 2020b). A neutral charge was considered favorable due to the subsequent addition of the cationic chitosan (as a surface coating or hydrogel), avoiding influence on the coating process, incorporation in gel and the release of polyphenol from subsequent formulation (Jøraholmen et al., 2022). Addition of chitosan as a surface coating turned the liposomal zeta potential cationic (Table 1). This increase in zeta potential further indicates a successful surface coating with chitosan. The addition of this cationic charge contributes to necessary mucoadhesive properties in interaction with the anionic structures of the mucins in mucosa (Jøraholmen et al., 2014). Moreover,

Table 1
Liposomal characterization of non-coated and chitosan-coated liposomes, plain or polyphenol loaded. Results are expressed as mean ± SD (n = 3). Plain = no polyphenol, EC=epicatechin, PG=propyl gallate, 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan.

Liposomes	Vesicle size (nm)			PDI[a]			Zeta potential (mV)			EE[b] (%)	
	Plain	EC	PG	Plain	EC	PG	Plain	EC	PG	EC	PG
Non-coated	126 ± 18	126 ± 20	125 ± 22	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.02	0.9 ± 3.0	− 3.9 ± 2.3	− 6.2 ± 2.3	63.9 ± 1.7	63.9 ± 2.6
Coated 0.3 LMW	136 ± 30	145 ± 31	140 ± 31	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.03	12.1 ± 3.1	12.2 ± 3.0	12.4 ± 2.6	65.0 ± 2.9	68.7 ± 4.5
Coated 0.1 MMW	127 ± 21	133 ± 27	129 ± 28	0.10 ± 0.01	0.12 ± 0.02	0.12 ± 0.02	25.3 ± 3.0	15.3 ± 2.4	16.9 ± 2.2	67.3 ± 3.3	70.7 ± 2.4

[a] Polydispersity index.
[b] Entrapment efficiency.

a cationic surface charge is generally preferred in drug delivery systems intended for microbial eradication. This preference is due to the anionic nature of both bacterial and fungal cells, which allows cationic liposomes to have enhanced electrostatic interactions with them (Ramos et al., 2018; Hemmingsen et al., 2023).

The entrapment efficiency of EC and PG was found to be high, with an average liposomal entrapment of 64 % for both polyphenols (Table 1). Moreover, the initial incorporation of polyphenol in liposomes was unchanged after coating with chitosan, indicating that the coating process did not affect the liposomally-associated polyphenols. This is consistent with our previous findings (Jøraholmen et al., 2015).

3.2. pH of liposomal formulations

The vaginal microbiome is highly affected by pH. Innate vaginal pH varies; however, it is estimated to be around 3.5 – 4.5 in healthy women of reproductive age (Leyva-Gómez et al., 2019). A vaginal infection, such as VVC, increases the natural pH (Conte et al., 2023). Locally applied treatment should not further disturb the pH balance in the vaginal environment (Vanić et al., 2021). Additionally, as both texture and viscosity properties of chitosan may be affected by pH, ensuring that the pH of the formulations is stable is important (Richa and Choudhury, 2020). Thus, the pH of all formulations (non-coated liposomes, chitosan-coated liposomes and liposomes-in-hydrogels) was measured (Table 2).

The non-coated liposomes exhibited a pH within the neutral range, as expected. The medium in the colloidal formulation, which was dH_2O at neutral pH, likely contributed to this result. The chitosan-coated liposomes and the liposomes-in-hydrogel were slightly acidic with pH between 5 and 6. This is due to the acetic acid used to solubilize the chitosan in the formulations. Although there is an increase in pH for non-coated EC liposomes when compared to plain liposomes, the pH of the formulations was mostly unaffected by the presence of polyphenol. The low pH of chitosan-modified formulations could ensure that it will not exacerbate the issue of high pH present with VVC, rather, their application could help lower it (Čačić et al., 2023).

3.3. Texture properties of hydrogels

In addition to pH, texture properties influence the suitability of formulations intended for vaginal application. Texture analysis is a straightforward method of confirming hydrogel reproducibility and assess the effects of hydrogel composition, whether it involves the incorporation of liposomes or alterations to the incorporated liposomes' own composition (Hemmingsen et al., 2021a). It has been already shown that the incorporation of liposomes into chitosan hydrogel improves its texture properties (Jøraholmen et al., 2019), hence only the hydrogels containing liposomes were evaluated. Liposomes with surface charge have shown to increase the texture properties and stability of the hydrogel to a greater extent than neutral charged liposomes (Hurler et al., 2012). As our liposomes were neutral, we aimed to use this method to assure reproducibility of the hydrogels and assess potential changes in texture properties based on the incorporation of the polyphenol containing liposomes (non-coated liposomes).

Table 2
pH of liposomal formulations (non-coated liposomes, chitosan-coated liposomes and liposomes-in-hydrogels), plain and polyphenol loaded. Results are expressed as mean ± SD (n = 3). Plain = no polyphenol, EC=Epicatechin, PG=Propyl Gallate, 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan.

Formulation	pH		
	Plain	EC	PG
Non-coated liposomes	7.2 ± 0.1	7.9 ± 0.1	7.0 ± 0.1
Coated liposomes 0.3 LMW	5.4 ± 0.2	5.9 ± 0.3	5.7 ± 0.2
Coated liposomes 0.1 MMW	5.7 ± 0.2	5.8 ± 0.3	5.9 ± 0.3
Liposomes-in-hydrogel 0.3 LMW	5.3 ± 0.2	5.3 ± 0.2	5.1 ± 0.3
Liposomes-in-hydrogel 0.1 LMW	5.3 ± 0.2	5.5 ± 0.2	5.0 ± 0.2

Characteristics such as hardness (g), cohesiveness and adhesiveness (g/sec) of the formulation reveal hydrogel stability (Hurler et al., 2012; Hemmingsen et al., 2021b). Hardness, defined by the maximum force needed to compress the hydrogel, affects its ease of container removal and application to intended site. Cohesiveness refers to the extent of hydrogel deformation after application and adhesiveness indicates the degree to which the hydrogel can adhere after application (Hemmingsen et al., 2021b). These characteristics of the various liposomes-in-hydrogel formulations are presented in Fig. 1.

Phospholipids are known plasticizers, however, the inclusion of liposomes within hydrogel matrix has previously been found to increase hardness and decrease cohesiveness of chitosan hydrogels (Jøraholmen et al., 2019; Hemmingsen et al., 2021a). The texture analysis showed that incorporation of polyphenols had no effect on the hardness, cohesiveness or adhesiveness of the hydrogels. This data supports the robustness of the hydrogels and confirms that the presence of polyphenols did not alter their characteristics. Moreover, the texture properties of the hydrogels were considered suitable for vaginal administration regarding comfort and ease of application (Jøraholmen et al., 2019). Stability of hydrogels as assessed by the texture properties was not included in this study since we have earlier reported that the texture properties of liposomes-in-hydrogels were maintained for at least two months storage (Jøraholmen et al., 2019). Furter, the addition of glycerol is expected to contribute to long term stability of chitosan hydrogel (Hurler et al., 2012). Although the texture analyzer allows for measurements of adhesive or mucoadhesive measurements of the formulation (Jøraholmen et al., 2019), we opted to further investigate mucoadhesiveness by mucus/mucin formulation interactions as defined in 2.7.

3.4. Viscosity and mucoadhesive properties

Rheological and mechanical characteristics, like viscosity, directly impact the formulation performance within vaginal cavity. Characterizing viscosity enables prediction of *in vivo* behavior upon application, including spreadability and retention of formulation within vaginal site (Szymańska et al., 2015).Evaluation of viscosity has been labeled as a "first choice" for *in vitro* mucoadhesive analysis of low viscosity solutions (Machado et al., 2017; Rossi et al., 2018). Therefore, we sought to evaluate and compare viscometrical properties of various liposomal formulations.

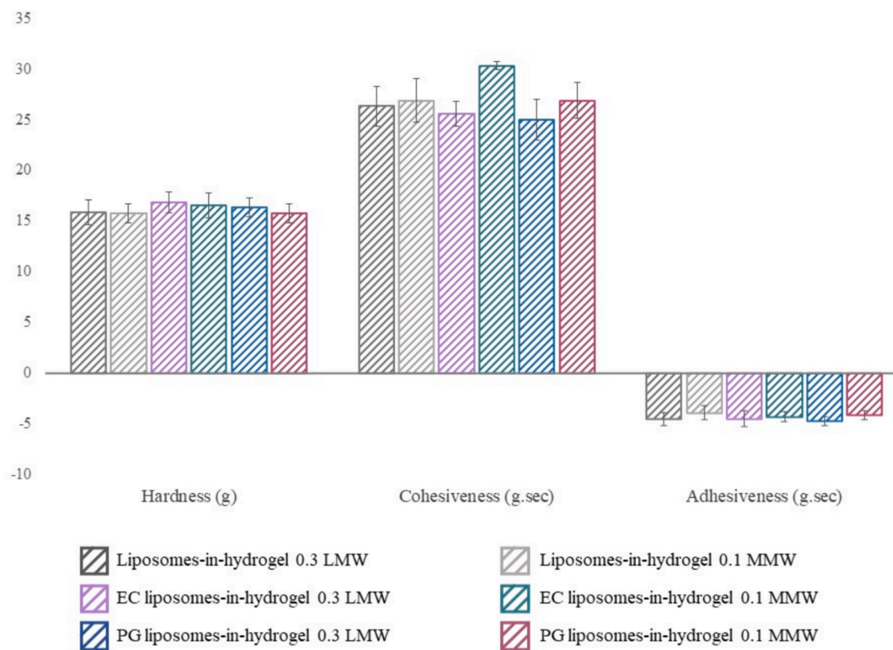

Fig. 1. Texture properties of liposomes-in-hydrogels (Plain, EC or PG loaded liposomes). Results are expressed as mean ± SD (n = 3). EC=Epicatechin, PG=Propyl Gallate, 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan.

To assess mucoadhesion, we employed the viscosity-based technique proposed by Hassan and Gallo (Hassan & Gallo, 1990). This method capitalizes on the formation of mucoadhesive intermolecular bonds between polymer and mucin resulting in heightened viscosity. A rise in viscosity beyond the total of individual components signifies the establishment of these bonds, thereby confirming mucoadhesion (Rossi et al., 2018). As viscosity is highly affected by temperature, we measured all formulations at both room (22 °C, Fig. 2 B and D) and

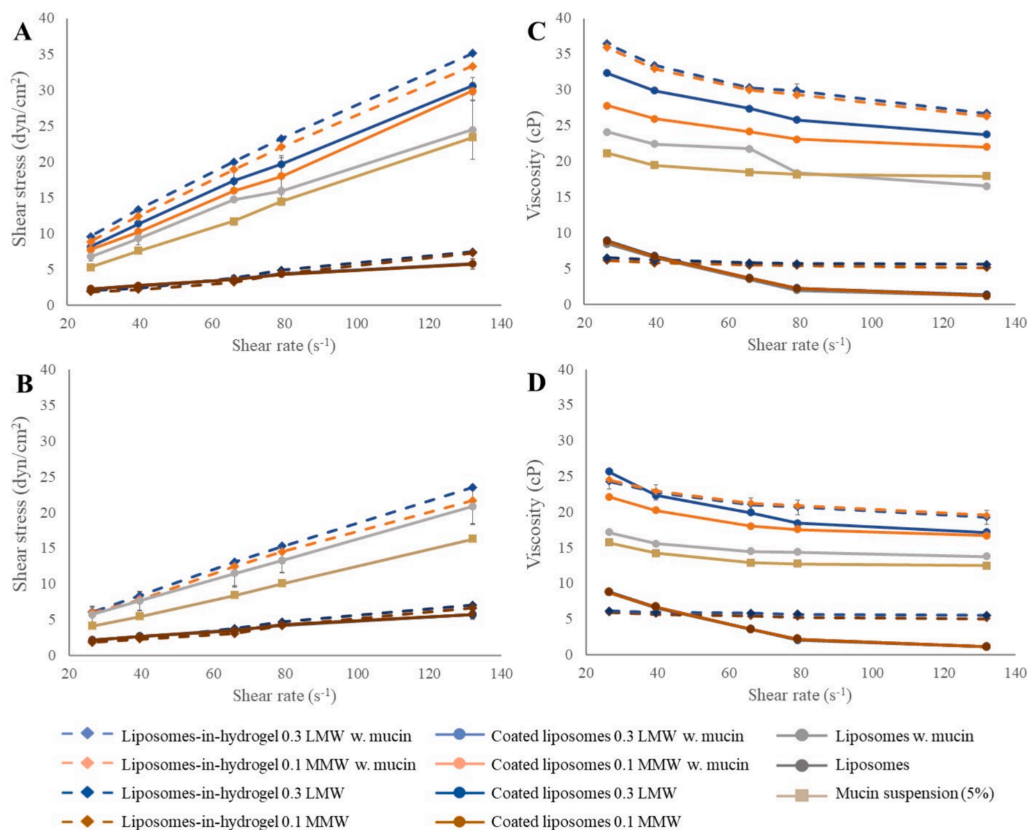

Fig. 2. Rheological characteristics of formulations (non-coated liposomes, chitosan-coated liposomes and liposomes-in-hydrogel) with and without 5 % mucin suspension. A (22 °C) and B (37 °C) = Shear stress as a function of shear rate. C (22 °C) and D (37 °C) = viscosity as a function of shear rate. Results are expressed as mean ± SD (n = 3). 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan.

physiological (37 °C, Fig. 2 A and C) temperature. Since the polyphenols have had a minimal impact on the overall formulations, and the chitosan is presumed to provide the mucoadhesive properties, these measurements were done with polyphenol-free formulations.

As seen in Fig. 2, with increasing shear rate, the shear stress increased while viscosity decreased. All formulations (non-coated liposomes, chitosan-coated liposomes and liposomes-in-hydrogel) demonstrated pseudoplastic flow with typical shear thinning behavior. Semisolid formulations with pseudoplastic properties are considered well suited for local vaginal administration due to their increased flow. This helps in dispensation of formulation from applicator and assists in formulation retention after application (Szymańska et al., 2015).

The formulations remained pseudoplastic in nature even after the addition of mucin. However, presence of mucin revealed a difference in the viscosity between the plain liposomes and the chitosan-coated liposomes, although their viscosity was identical prior to addition of mucin (Fig. 2). The 0.3 % LMW chitosan-coated liposomes exhibited significantly higher viscosity ($p < 0.0005$) than the 0.1 % MMW chitosan-coated liposomes, and the plain liposomes ($p < 0.00006$), once exposed to mucin. Similarly, the viscosity of liposomes coated with 0.1 % MMW chitosan was significantly higher than that of the plain liposomes upon exposure to mucin ($p < 0.0001$). Notably, the difference was most pronounced when measured at low shear rates, though also observed at high shear rates. No difference in viscosity was observed between the hydrogels as the viscosity of 0.1 % MMW and 0.3 % LMW hydrogel remained comparable before and after addition of mucin.

The variation in viscosity can be attributed to the differences in molecular weights and concentration of chitosan, as these factors are known to influence the viscosity of chitosan (Qian et al., 2023). However, considering that the viscosities of both the 0.3 % (LMW) and 0.1 %

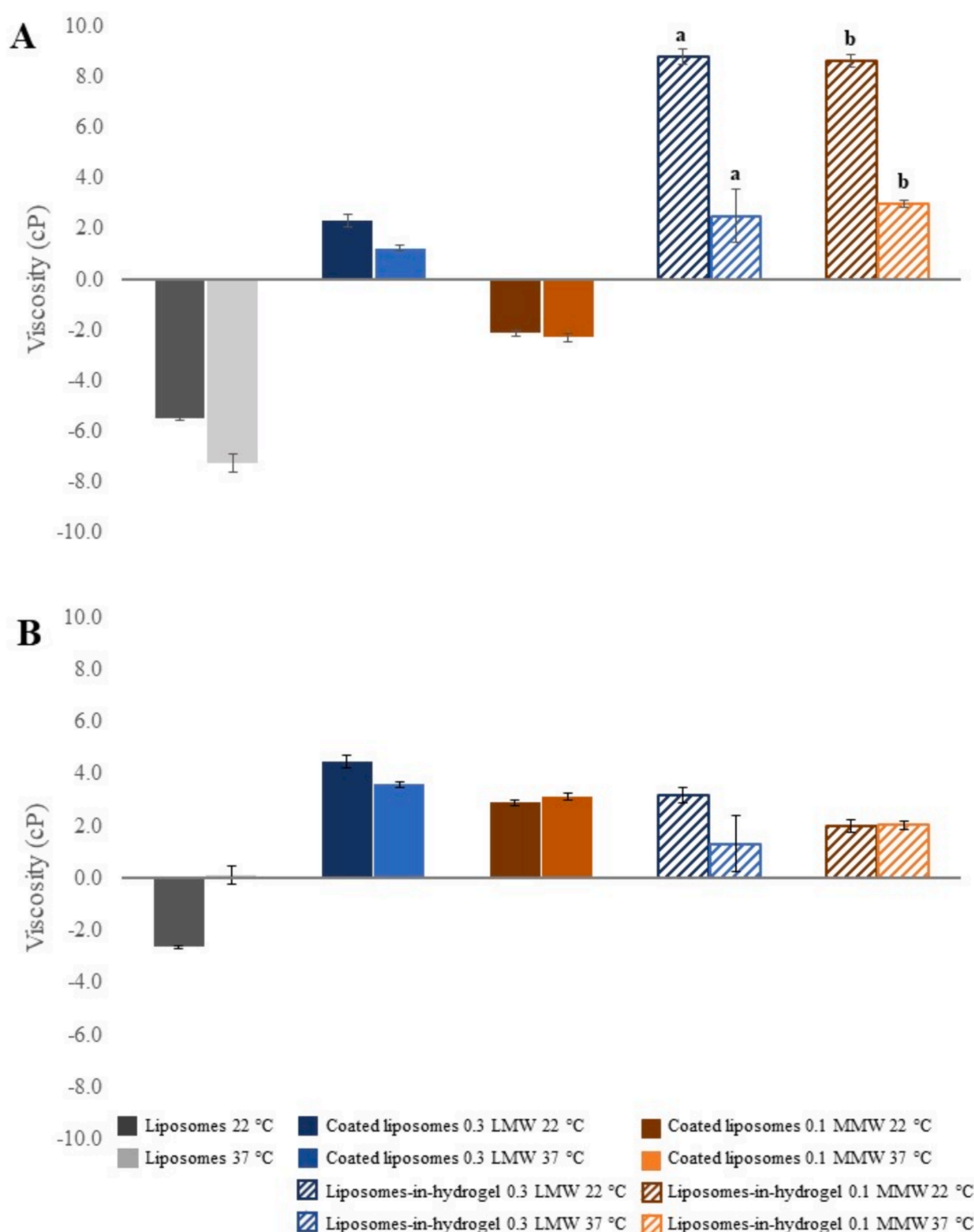

Fig. 3. Viscosity (η_{change}) of formulations without polyphenols mixed with mucin suspension after subtraction of the individual components ($\eta_{formulation}$ and η_{mucin}). A: Low shear (26.4 s^{-1}), B: High shear (132 s^{-1}). Results are expressed as calculated mean ± SD (n = 3). 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan. $^{ab}p < 0.0003$.

(MMW) chitosan-coated liposomes were the same prior to the exposure to mucin, it is plausible that variations in the interactions between mucin and the formulations are responsible for the observed difference in viscosity (Rossi et al., 2018). The formation of adhesive chemical or physical bonds with mucin can also be responsible, as this would directly affect the viscosity.

We observed that all chitosan-modified formulations, when mixed with mucin, exhibited a viscosity (η_{total}) that was greater than the sum of the individual components ($\eta_{formulation}$ and η_{mucin}). The excess viscosity (η_{change}) after subtracting the components according to Equation (2) are shown in Fig. 3 and can be referred to as a positive viscosity synergism (Mackie et al., 2017). This synergy is a product of the formation of adhesive bonds between chitosan and the mucin glycoproteins (Hassan & Gallo, 1990), confirming mucoadhesiveness of chitosan formulations.

In contrast to the other chitosan-modified formulations, the 0.1 % MMW chitosan-coated liposomes exhibited viscosity synergy only at the higher shear rate (132 s^{-1}) (Fig. 3 B). This suggests that, as a coating material, the 0.3 % LMW (w/w) chitosan has superior mucoadhesive abilities compared to the 0.1 % MMW (w/w) chitosan. Liposomes coated with 0.1 % MMW (w/w) chitosan have previously been found to exhibit excellent mucoadhesive properties (Jøraholmen et al., 2015). However, this was estimated using a different method of mucoadhesion evaluation and was not compared with LMW chitosan. The significant difference between the 0.1 % MMW chitosan coating at high and low shear is important due to the low shear that would affect the formulation upon application *in vivo* (Owen et al., 2000).

Another thing of note was the impact of temperature on mucoadhesive properties: the chitosan-coated liposomes maintained their mucoadhesive characteristics regardless of temperature, while the liposomes-in-hydrogel formulations were significantly ($p < 0.0003$) affected by temperature at low shear rate (26.4 s^{-1}) (Fig. 3 A). Increasing the temperature decreased the observed viscosity synergy. Thus, the hydrogels exhibited temperature-dependent viscoelastic behavior, which indicated that their adhesive intermolecular bonds were weakened with rising temperature. This weakening could occur between bonds within the hydrogel itself or between the hydrogel and mucin glycoproteins. Nonetheless, there was no difference in mucoadhesive properties measured between the 0.3 % LMW chitosan-coated liposomes and the liposomes-in-hydrogel at physiological temperature, regardless of shear (Fig. 3 A and B). This suggests that each of these three formulations would exhibit comparable mucoadhesive properties upon application *in vivo*.

The plain liposomes did not exhibit any viscosity synergism after the addition of mucin, rather the total viscosity was less than the individual components (Fig. 3 A and B), as expected. This is likely due to the lack of mucoadhesive polymer since the lipids do not interact with mucin. It seems that the plain liposomes are forming repulsive rather than adhesive bonds with mucin, leading to a reduction in viscosity, and thereby lack of mucoadhesiveness (Mackie et al., 2017).

3.5. Release of polyphenols

Semisolid formulations on the market, available for treatment of vulvovaginal infections, are commonly applied overnight (Sheppard, 2020). The intended mode of administration of our formulations is the same, establishing 8 h retention of our formulation at vaginal site. Consequently, it was crucial to confirm that the liposomal formulations released the encapsulated polyphenol within this treatment window (8 h).

Liposomes are expected to enable sustained release of entrapped compounds, like polyphenols, whilst protecting them from outside degradation (Ferreira et al., 2021). This ability can be enhanced by chitosan modification of liposomes, such as through a chitosan surface coating or their incorporation within a chitosan hydrogel (Hemmingsen et al., 2023). *In vitro* conditions were set to mimic *in vivo* conditions regarding pH, temperature and presence of vaginal fluid simulant.

As shown in Fig. 4, after 8 h the non-coated liposomes released approximately 60 % of EC (A) and 66 % of PG, respectively (B). In comparison, the non-formulated polyphenols (controls) released 81 % and 86 % of EC and PG, respectively. The release from liposomes was significantly slower than the non-liposomal polyphenol controls ($p < 0.02$). Liposomes-in-hydrogel further sustained the polyphenol release; however, there was no significant difference in sustained release between the non-coated and chitosan-coated liposomes.

For the 0.3 % LMW chitosan formulations, the EC liposomes-in-hydrogel (Fig. 4 A) demonstrated a significant slowing in release when compared to non-coated liposomes ($p < 0.001$), chitosan-coated liposomes ($p < 0.003$), and the non-formulated EC control ($p < 0.001$). A similar pattern was observed for PG (Fig. 4 B), where the release from liposomes-in-hydrogel was significantly slower than that from non-coated liposomes ($p < 0.01$), chitosan-coated liposomes ($p < 0.0008$), and the non-formulated PG control ($p < 0.004$).

The release of EC from the 0.1 % MMW chitosan hydrogel formulations (Fig. 4 A) followed a similar pattern, exhibiting a significant reduction in release rate compared to the non-coated liposomes ($p < 0.007$), the chitosan-coated liposomes ($p < 0.01$), and the non-formulated EC control ($p < 0.006$). Similarly, the release of PG from the liposomes-in-hydrogel formulation was significantly slower than that from the non-coated liposomes ($p < 0.005$), the chitosan-coated liposomes ($p < 0.01$), and the control ($p < 0.005$). The sustained release profile of both chitosan-coated liposomes and liposomes-in-hydrogel could prolong contact time at site of action throughout the proposed treatment duration (8 h), increasing the chance of microbial eradication (Araujo et al., 2021; Nayak et al., 2021).

3.6. Antimicrobial evaluation

Polyphenols like EC and PG are poorly soluble and have limited stability in biological environment which affect their therapeutic use (Khayrova et al., 2022; Ousji & Sleno, 2022). Literature reports that liposomal entrapment improves the therapeutic index of polyphenols, including EC, by protecting them from oxidation and early degradation, prolonging release and improving their solubility (Coimbra et al., 2011; Rodrigues et al., 2013; Murthy et al., 2021; Yin et al., 2022). We aimed to evaluate whether incorporation in liposomes could improve the antifungal activity of EC and PG. Therefore, we compared the anticandidal activity of EC or PG either in solution or incorporated within liposomes. Moreover, due to the intrinsic antifungal potential of chitosan, we further investigated whether the addition of chitosan affected antifungal activity, particularly potential synergistic effects.

As a first step, we focused on the MIC to gather information about the tested strain's susceptibility to our formulations, allowing us to directly compare the formulated and non-formulated polyphenols. We additionally investigated the MBC to elucidate whether the polyphenols exhibited fungicidal or fungistatic inhibition of growth (Kowalska-Krochmal & Dudek-Wicher, 2021). Antifungals with a fungicidal action are favored over fungistatic agents because those with fungistatic properties can allow for adaptation and the emergence of resistance (Giordani et al., 2020). The MIC and MBC values are presented in Table 3. Neither EC nor PG exhibited antifungal activities against *C. albicans* when unformulated (polyphenol control). Previous reports have documented two different anticandidal MICs of EC, one within our concentration range (Sachikonye & Mukanganyama, 2016) and the other at a higher concentration against a different strain of *C. albicans* (Jonathan et al., 2013). At the tested concentrations, EC alone did not inhibit *Candida* growth. Formulating EC in liposomes resulted in a mild antifungal effect, with an MIC of 294 μg/mL (Table 3). This effect is contributed to liposomal incorporation of EC as plain liposomes did not affect fungal growth (data not shown). The observed antifungal potential of EC when formulated in liposomes is corroborated by the known ability of liposomes to protect and enhance the antimicrobial efficacy of incorporated polyphenols (Murthy et al., 2021). The MBC for the non-

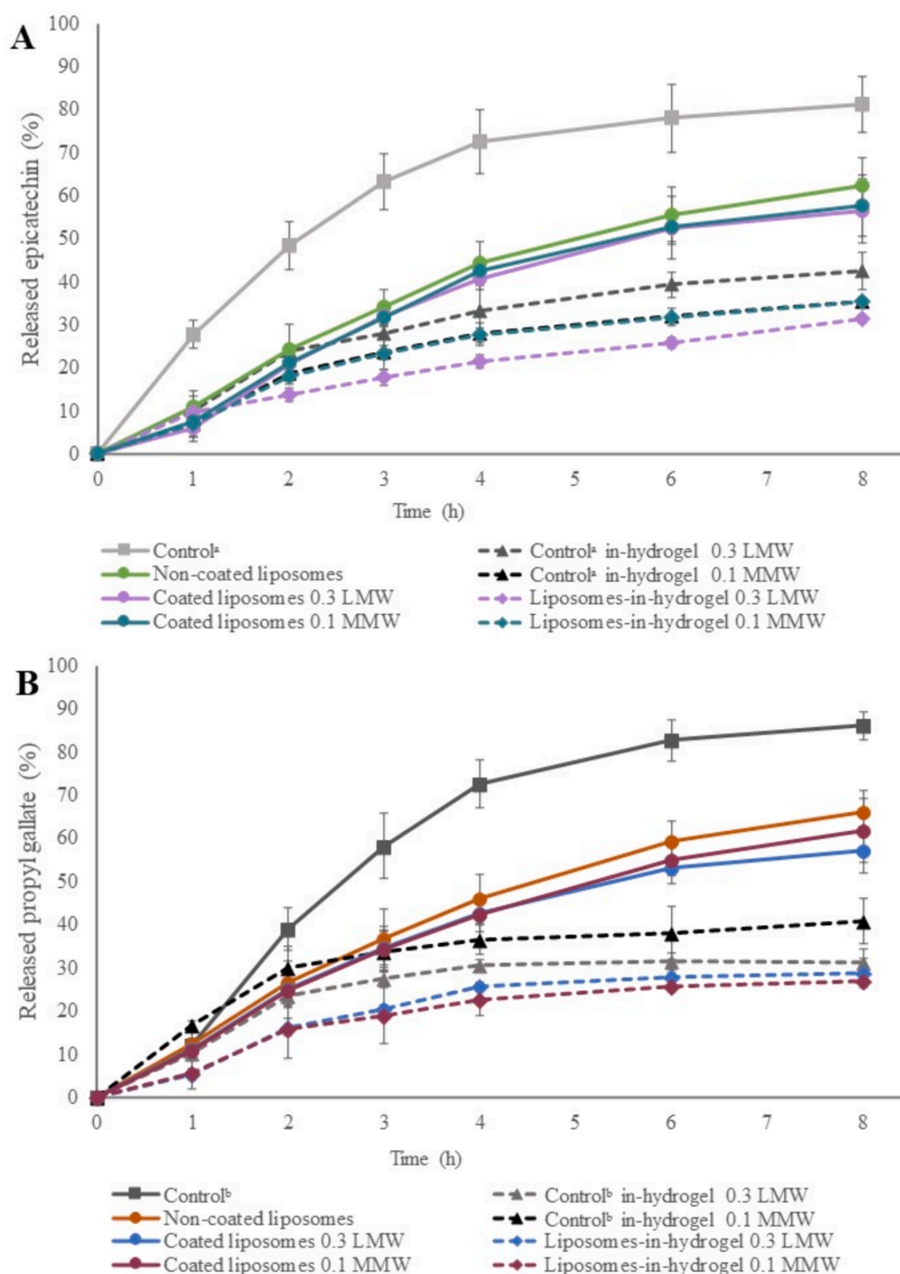

Fig. 4. Polyphenol release from formulations (controls, non-coated liposomes, chitosan-coated liposomes, and liposomes-in-hydrogels) over 8 h. A: EC (epicatechin), B: PG (propyl gallate). Results are expressed as mean ± SD (n = 3). [a]EC dissolved in medium (acetic buffer, pH 4.6), [b]PG dissolved with <1 % DMSO in medium (acetic buffer, pH 4.6), 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan.

Table 3
Antifungal activity (MIC and MBC) of non-formulated and liposomal EC and PG on *C. albicans*. Results are expressed as the polyphenol concentration that visually inhibited fungal growth (MIC) or concentration that completely inhibited fungal viability (MBC) (n = 3).

Formulation	MIC (μg/mL)		–	MBC (μg/mL)	
	EC	PG		EC	PG
Polyphenol control[a]	> 305	NE[b]		> 305	NE[b]
Non-coated liposomes	294	NE[b]		> 294	NE[b]

MIC Minimum inhibitory concentration
MBC Minimum microbicidal concentration
[a] EC (epicatechin) dissolved in dH_2O or PG (propyl gallate) dissolved with < 1 % DMSO in dH_2O.
[b] No effect observed.

formulated EC (polyphenol control) were the same as the MIC, with a value of > 305 μg/mL, whilst the liposomal EC had a higher MBC than MIC at > 294 μg/mL, uncovering that EC is likely not fungicidal at the tested concentrations (Table 3). We were satisfied with improved activity attributed to liposomes and did not repeat testing at higher concentrations.

However, the results for PG were rather disappointing since it failed to eradicate *Candida* (Table 3). Consequently, we continued evaluation on the effect of addition of chitosan using only EC and its formulations. The inclusion of chitosan as a liposomal surface coating significantly improved the antifungal activities of the EC formulations ($p < 0.006$, or lower) (Fig. 5), as did the formulated EC liposomes in both 0.3 % LMW hydrogel ($p < 0.002$) and 0.1 % MMW hydrogel ($p < 0.00005$) (Fig. 5).

The tested strain exhibited a higher susceptibility to the commercial antifungal clotrimazole with an MIC of 0.125 μg/mL (data not shown),

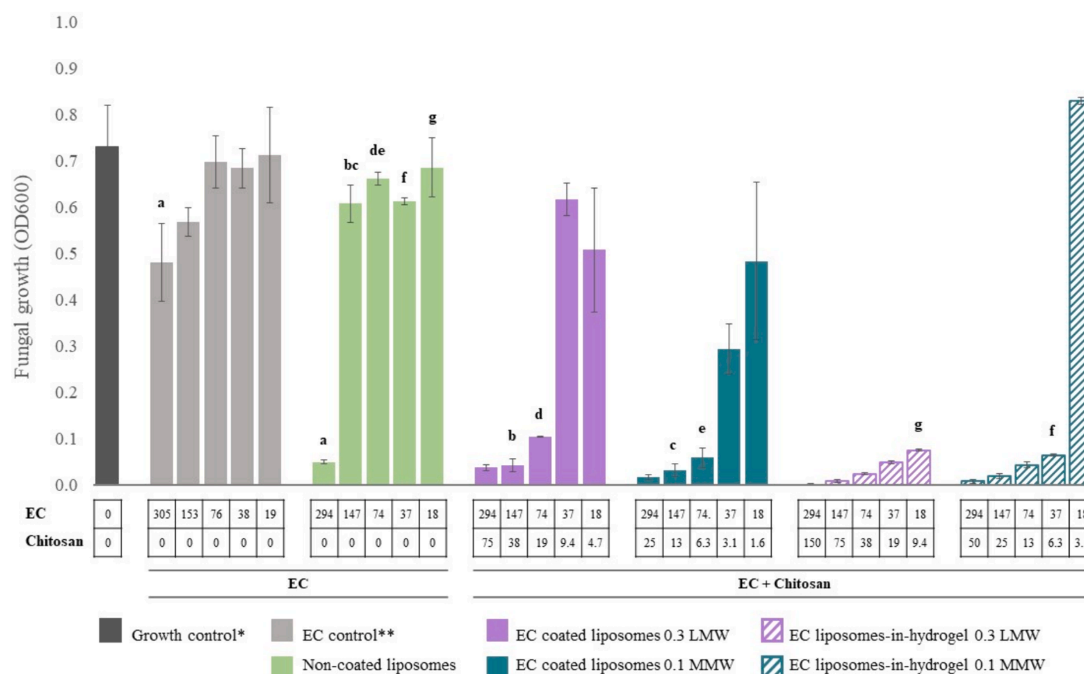

Fig. 5. Inhibition of *C. albicans* CRM-10231 (24 h) in the presence of formulations (non-coated liposomes, chitosan-coated liposomes and liposomes-in-hydrogels) assessed with the micro broth dilution method. A combination of EC and chitosan and the separate components were assessed in equivalent concentrations and formulations. *Growth control was untreated *C. albicans* in YM medium, **EC control was EC dissolved in dH_2O. EC=epicatechin, 0.3 LMW=0.3 % low molecular weight chitosan, 0.1 MMW=0.1 % medium molecular weight chitosan. Results are expressed as mean ± SD (n = 3). Significance level: $^a p < 0.006$, $^b p < 0.0004$, $^c p < 0.001$, $^d p < 0.0003$, $^e p < 0.0006$, $^f p < 0.00005$, $^g p = 0.002$.

compared to our formulation. However, treatment of *Candida* with all formulations comprising chitosan concentration above 10 μg/mL (LMW and MMW), resulted in full eradication of *Candida* as measured through the MBC evaluation, confirming fungicidal activity of chitosan. Chitosan's mechanism of antifungal action is currently not fully uncovered, though it has been largely attributed to the electrostatic difference between anionic fungal cell walls and cationically charged chitosan, similarly to the reported mechanism behind its antibacterial action (Qin et al., 2020). Therefore, chitosan could potentially bind, alter or destroy the surface of fungal cell walls and is considered to be an ideal antifungal (Qin et al., 2020). Considering the synergy between fungistatic EC liposomes and fungicidal chitosan, we found neither statistical difference between the chitosan-coated liposomes with or without EC, nor between the liposomes-in-hydrogel with and without EC liposomes (data not shown). Due to this lack of synergy observed between EC and chitosan, the antifungal activity of chitosan formulation can be contributed to the intrinsic activity of the polymer itself, though this activity was not inhibited by the addition of EC liposomes. A delivery system such as ours, comprising polyphenol, liposomes and chitosan, should assure antimicrobial efficacy, without compromising the advantages of the individual components (Murthy et al., 2021). Moreover, previous studies have shown that liposomal EC expressed substantial anti-inflammatory effect, that is considered beneficial when treating vaginal infections (Jøraholmen et al., 2019). The finding can be seen as a positive step in development of EC formulations comprising liposomes as carrier attributing solubilization and protection, and chitosan implying antifungal potential.

A similar lack of synergy between chitosan formulations and its incorporated antifungal has been reported earlier. da Silva and colleagues incorporated propolis and fluconazole into chitosan nanoparticles to improve treatment of VVC. Their formulation performed comparably to marketed antifungal miconazole cream when tested *in vivo,* with a twenty times lower dose of fluconazole compared with miconazole. However, they observed no statistical difference between the chitosan nanoparticles alone and nanoparticles with only propolis, or with both fluconazole and propolis (da Silva et al., 2023). Similarly, Sato and colleagues attempted to improve retention and efficacy of VVC treatment by incorporating hypericin-loaded nanostructured lipid carriers into a chitosan hydrogel. Their hydrogel significantly reduced the fungal load; however, this activity was seen both with and without the lipid carriers with hypericin (Sato et al., 2023). Thus, the antifungal activity is attributable to the chitosan (as either nanoparticle or hydrogel), similarly to our findings.

The differences in antifungal abilities seen between our chitosan-coated liposomes and the liposomes-in-hydrogel could be concentration related (Fig. 5). Originally, the liposomes-in-hydrogel have the same chitosan concentration as the coating material. However, as the coating material is diluted 1:1 during the coating process the chitosan concentration in chitosan-coated liposomes is halved. For the MMW chitosan, a concentration of 6.3 μg/mL of chitosan was found to be antifungal regardless of whether chitosan was employed as coating material or hydrogel. This, however, was not the case for LMW chitosan, as the LMW hydrogel demonstrated superior antifungal activity compared to the LMW surface coating considering the chitosan concentration. The liposomes-in-hydrogel with LMW chitosan was antifungal at 9.4 μg/mL of chitosan and the LMW chitosan-coating at 19 μg/mL, respectively (Fig. 5). This difference observed between MMW and LMW chitosan could be explained by the difference in molecular weight, which affects its antifungal activity (Confederat et al., 2021). For example, LMW chitosan has shown the ability to penetrate the fungal cell wall, leading to an inhibition of the DNA/RNA and protein synthesis (Ke et al., 2021). Chitosan, when dissolved and forming a hydrogel network, could be readily available to act on this route, especially when the network is disrupted though serial dilution. As a coating material the chitosan is adhered to the liposomal surface, which may inhibit its movement across the fungal cell wall. These results highlight that for the antifungal activity it is important to consider both the chitosan concentration in the final formulation and the molecular weight.

These findings could be further explored for localized treatment of VVC or other vulvovaginal infections. Our aim was to evaluate initial potential of our novel polyphenol formulations to improve treatment of VVC. This involved evaluating mucoadhesion and confirming antifungal activity. Nevertheless, some vulvovaginal infections are mucosal, which introduces additional complexities worth exploring further. One example is biofilm formation, another is formulation activity either on or in the presence of bacteria which would be present *in vivo*. Additionally, the presence of vaginal fluid, and how that effects both mucoadhesion and overall antifungal abilities merits further investigation. Utilizing alternative methods for susceptibility testing, like the checkerboard technique, could potentially demonstrate synergy between chitosan and polyphenols across a spectrum of concentration levels. Lastly, chitosan offers several other beneficial activities, such as anti-inflammatory activity, making it a promising subject for further research in areas beneficial for vaginal applications.

4. Conclusion

We successfully developed promising candidates for the localized treatment of VVC, in the form of chitosan-coated liposomes and liposomes-in-hydrogel delivery systems containing either EC or PG. Liposomes allowed for high encapsulation levels of EC or PG and provided a prolonged release of the polyphenols that was extended by the addition of chitosan. Both chitosan-coated liposomes and liposomes-in-hydrogel formulations exhibited rheological properties appropriate for vaginal application. Upon contact with mucin, chitosan formulations demonstrated an increase in viscosity, confirming their mucoadhesiveness. The EC liposomes showed antifungal activity against *C. albicans* CRM 10231, whereas the PG failed to prevent the growth of *Candida* at tested concentrations. The intrinsic fungicidal activity of chitosan was confirmed both when used as liposomal coating material and as hydrogel vehicle. These findings establish both natural polyphenols and chitosan as beneficial substances for vaginal application in the treatment of vulvovaginal infections.

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CRediT authorship contribution statement

Silje Mork: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Mona Johannessen:** Writing – review & editing, Methodology. **Nataša Škalko-Basnet:** Writing – review & editing, Supervision, Methodology. **May Wenche Jøraholmen:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, 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

Data will be made available on request.

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