International Journal of Pharmaceutics 677 (2025) 125667

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Mucoadhesive polymer-coated liposomes as a promising approach to counteract bacteria responsible for aerobic vaginitis

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ARTICLE INFO

Keywords:
Polymer-coated liposomes
Azithromycin
Retention studies
Antimicrobial activity
Aerobic vaginitis

ABSTRACT

Aerobic vaginitis is an infectious disease characterized by the overgrowth of abnormal vaginal microflora. Conventional local dosage forms are not always effective, due to their inadequate drug release and residence time within the vaginal cavity. Therefore, this study aimed to develop azithromycin (AZT)-loaded liposomes, coated with two mucoadhesive polymers, chitosan (CS) and sodium hyaluronate (HYA), to increase the drug's stay at the site of infection and to control its release. Liposomes were developed through the thin film hydration method followed by extrusion and subsequently added to the polymer solution. Later, they were characterized by their size, surface charge, morphology, and encapsulation efficiency. Furthermore, mucoadhesive properties and drug release behavior were investigated at different pH values, e.g., 4.5 and 7.4, mimicking the physiological and pathological conditions, respectively. Finally, antimicrobial tests and *in vitro* permeation studies were carried out. Results showed size and surface charge variations of coated LP with respect to the uncoated ones, confirming the success of the coating process. LP possessed a good capacity to encapsulate the drug. Among all the formulations, CS-LP demonstrated superior control of drug release and greatest mucoadhesive properties at both tested pHs, as well as the highest drug accumulation inside the vaginal tissue, maintaining at the same time AZT antimicrobial effect. Overall, CS-LP could be proposed as a promising nanocarrier for AZT vaginal delivery, *in virtue* of its ability to achieve locally a sustained release of drug, helping to lower the dosage and administration frequency, and consequently improving treatment efficacy.

1. Introduction

Aerobic vaginitis (AV) is a vaginal infectious condition, first defined as a clinical entity by Donders and colleagues in 2002 (Donders et al., 2017), registered in 7.9–23.7 % of women reporting vaginal complaints. Moreover, it is also a common cause of vaginal discharge in reproductive-age women (4.1–8.3 %) which increases the risk of negative pregnancy outcomes (Ma et al., 2022). AV is characterized by an increase of Gram-positive cocci, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and enterococci, and Gram-negative microorganisms, such as Enterobacteriaceae (particularly, *Escherichia coli*) (Donders et al., 2002). These microorganisms are indicative of colonization by enteric bacteria due to an alteration of the vaginal microbiota.

In healthy-state conditions, the vaginal ecosystem is characterized by the dominance of lactic acid-producing bacteria, mainly *Lactobacillus spp.*, which maintain the acidic pH of vaginal fluids (3.5–4.5) (das Neves et al., 2014). In the case of AV lactobacilli decrease, sometimes to the point of disappearing, resulting in a severe decrease in vaginal lactic acid concentration. In addition to increasing vaginal pH (usually > 6.0) (Han et al., 2015), AV is also characterized by vaginal discharge, inflammation and epithelial atrophy.

A therapeutic strategy for AV should include a local or oral antibiotic characterized by an inherent activity against the majority of enteric bacteria and poor interference with the vaginal microbiota, eventually associated with steroids for the inflammatory phenomenon and estrogens for atrophy (Tempera and Furneri, 2010). Local therapy has been recently favored over oral approaches as it maximizes the drug's concentration at the action site, avoids systemic adverse effects and reduces

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https://doi.org/10.1016/j.ijpharm.2025.125667
Received 3 March 2025; Received in revised form 29 April 2025; Accepted 29 April 2025
Available online 30 April 2025

the risk of developing bacterial resistance.

An ideal formulation for local administration should be able to remain in the vagina for a long time, assuring an adequate dose of the drug for a prolonged period. This approach, using the most traditional vaginal dosage forms (tablets, suppositories, semisolids and solutions), is not always possible due to the inadequate spreading of the drug over vaginal surfaces, its low penetration to the submucosal layers, and the possibility of discharge of the formulation because of vaginal fluid dynamics and self-cleaning action of the vagina. As a result, a high frequency of drug administration is often required to achieve therapeutic effects, resulting in poor patient compliance and high treatment costs (Palmeira-de-Oliveira et al., 2015).

To overcome these drawbacks, an interesting strategy is represented by the encapsulation of the drug in nanosystems such as liposomes which offer the possibility of improving the therapeutic efficacy by increasing the drug solubility and eventually controlling its release. Moreover, the association of liposomes with polymers able to interact with mucus can provide enhanced retention on the mucosa and an intimate contact between the drug and the mucosal tissue (Caramella et al., 2015; Pandey et al., 2020).

Among the different mucoadhesive polymers, two of the most used in vaginal formulations are chitosan (CS) and hyaluronic acid (HYA). CS is a naturally abundant cationic polymer which has also received growing attention for vaginal drug delivery because of its excellent mucoadhesive properties (Andersen et al., 2017; Nayak et al., 2021). HYA is an anionic, non-sulfated glycosaminoglycan, showing anti-inflammatory, protective and moisturizing effects (Gerton and Mann, 2021; Harrer et al., 2021; Vasvani et al., 2020). Until now, some research articles focused on the development and characterization of vaginal liposomes coated with CS (Falavigna et al., 2020; Jøraholmen et al., 2020, 2015, 2014; Yadav et al., 2024) or HYA (Abruzzo et al., 2021b). However, at the current state of the art, no studies have been published concerning the comparison between CS and HYA-coated liposomes intended for vaginal application. In fact, up to this time the reported studies investigated the differences between vaginal liposomes coated with CS and other different polymers such as carbopol, pectin or hydroxypropylmethylcellulose (Andersen et al., 2017, 2013; Refai et al., 2017). The only research papers, reporting a comparison between CS and HYA-coated vesicles, regarded the treatment of other diseases and routes of administration. Specifically, Badran and co-workers proposed transferosomes (liposomes enriched with specific amounts of surfactants) coated with CS or HYA for ocular infections, while Wu and colleagues evaluated similar vesicles as a systemic formulation for cancer (Badran et al., 2024; Wu et al., 2023).

Concerning the treatment of AV, currently, there is no internationally standardized therapeutic protocol and therapy is mainly based on antibiotics active against the aerobic pathogens that characterize this infection. Azithromycin (AZT) is a potent, broad-spectrum macrolide antibiotic recommended for the treatment of respiratory, skin and soft tissue infections. It is usually available as a dihydrate (MW 785 g/mol) and its low solubility in water ($\cong$ 0.1 mg mL^{-1}) (Aucamp et al., 2015) represents one of the main drawbacks to obtaining effective treatments.

To the best of our knowledge, up to now, very few studies have been published on the use of AZT-loaded liposomes for the local treatment of vaginal infections. Čačić evaluated the potential of AZT-liposomes incorporated into CS hydrogel for the local treatment of AV (Čačić et al., 2023), by assessing mucoadhesion and *in vitro* drug release both at pH 4.5 and the antimicrobial activity. Vanić et al. developed AZT-loaded liposomes, ie, conventional, propylene glycol and deformable propylene glycol liposomes (Vanić et al., 2019). In this latter study, the authors evaluated the drug release at different pH conditions, the drug accumulation inside the porcine vaginal mucosa and the antimicrobial activity against the microorganisms responsible for cervicovaginal infections.

Indeed, the novelty of this work involves two main aspects. To expand the current findings regarding the AZT vaginal use, we investigated for the first time the possibility of delivering it to the vaginal mucosa through liposomes coated with CS and HYA. Liposomes could increase AZT solubility and promote prolonged release, thus leading to high local concentration. Moreover, the association of liposomes with mucoadhesive polymers could increase their retention within the vaginal cavity. Secondly, CS and HYA- coated liposomes were insightfully compared in order to establish their potential for vaginal drug administration, taking into account several properties such as mucoadhesive ability, drug release and retention inside a vaginal tissue as well as their antimicrobial activity.

2. Materials

Ultrapure chloroform was purchased from Carlo Erba (Milan, Italy). Lactic acid (80 %) was obtained from Polichimica SRL (Bologna, Italy). Sodium hyaluronate (HYA, molecular weight = 800–1200 kDa, pKa = 2.9) was sourced from Farmalabor (Canosa di Puglia, Italy). L-α-phosphatidylcholine from egg yolk (PC, purity ~ 60 % of L-α-phosphatidylcholine), cholesterol (CHOL), azithromycin dihydrate (AZT), chitosan (CS, MW 150 kDa, deacetylation degree 96–98 %, pKa = 6.3), mucin type II from porcine stomach and all other chemicals of analytical grade were obtained from Sigma-Aldrich (Milan, Italy).

Buffer solutions were prepared as it follows: 2.38 g/L $Na_2HPO_4 \times 12$ H_2O, 0.19 g/L KH_2PO_4 and 8.00 g/L NaCl for buffer at pH 7.4 (PBS, buffer simulating the pH of vaginal cavity under pathological conditions); 13.61 g/L KH_2PO_4 (0.1 M) for buffer at pH 4.5 (buffer simulating the physiological pH of the vaginal cavity) (Cook and Brown, 2018; Donders et al., 2017; Falavigna et al., 2020; Jøraholmen et al., 2015; Li et al., 2009, p. 200). The mobile phase was based on a mixture of a buffer (KH_2PO_4 0.01 M adjusted at pH 7.5 with 10 M KOH), methanol and acetonitrile (10/50/40, v/v/v).

2.1. Methods

2.1.1. Preparation of liposomes

Liposomes were prepared using the thin film hydration method as previously described (Abruzzo et al., 2024b) with some modifications. Briefly, PC (70 mg), CHOL (30 mg) and AZT (30 mg) were dissolved in a mixture of $CHCl_3$-CH_3OH (5 mL, 9:1 v/v) in a round-bottom flask. Subsequently, the organic mixture was evaporated using a rotary evaporator (Laborota 4000, Heidolph Instruments, Germany) at 210 rpm under reduced pressure (−0.8 bar) at 60 °C for 90 min. The obtained dried lipid film was hydrated with 10 mL of PBS by rotating the flask at 210 rpm for 60 min. Then, the resulting suspension was extruded 15 times through a polycarbonate membrane with a pore size of 100 nm (LiposoFast manual syringe extruder, Avestin Europe GmbH, Mannheim, Germany) with the final aim of reducing the vesicle size and homogenizing the sample. Unloaded liposomes were prepared as previously described without the addition of the drug.

2.1.2. Liposome coating with CS and HYA

For the preparation of polymer-coated liposomes, the extruded suspension obtained as described in the previous section was mixed with 0.2 % (w/v) polymeric solution. Specifically, CS was solubilized in lactic acid 1.6 % (v/v) at 400 rpm for 24 h; before coating, the pH was adjusted to 5.6 with NaOH 5 % (w/v). Besides, HYA was directly dissolved in lactic acid 1.6 % (v/v), adjusted to pH 5.6 with NaOH 5 % (w/v) at 400 rpm overnight. The polymeric solution was added dropwise under continuous stirring (700 rpm) to the extruded suspension (1:1, w/w) and stirred for 90 min. Uncoated liposomes were obtained by mixing the suspension with lactic acid 1.6 % (v/v) adjusted at pH 5.6 with NaOH 5 % (w/v). After their preparation, in the presence of the drug, uncoated liposomes, CS and HYA-coated liposomes were named LP, CS-LP and HYA-LP, respectively. Samples without AZT were named unloaded LP, unloaded CS-LP and unloaded HYA-LP. All formulations were stored at + 4–8 °C for 24 h before further use. The pH of the final liposomes

suspensions was measured with pH meter (MicroPH CRISON 2000, Modena, Italy).

2.1.3. Determination of vesicle size, polydispersity index (PDI) and zeta potential

Particle size and PDI of the prepared formulations were determined at 25 °C by PCS (photon correlation spectroscopy) using a Brookhaven 90 Plus instrument (Brookhaven Instruments Corp., Holtsville, NY, USA) with an He-Ne laser beam at a wavelength of 532 nm (scattering angle of 90°). Moreover, liposomes were also characterized for their zeta potential at 25 °C by using a Zeta Potential Nicomp 380 ZLS (Santa Barbara, California, USA). Before each analysis, liposomes were dispersed in ultrapure water (18.2 MW cm, MilliQ apparatus by Millipore, Milford, MA, USA) with a 1:1000 (v/v) dilution.

2.1.4. Morphological characterization

Quantitative single-particle morphometry and nanoindentation were performed via Atomic Force Microscopy (AFM) as described elsewhere (Abruzzo et al., 2024b). The only difference with respect to the procedure reported previously is that liposomes with a positive ζ-potential were deposited on the cleaned borosilicate glass substrate immediately after air plasma treatment, while those with negative ζ-potential were deposited after poly-L-lysine coating as normal (Ridolfi et al., 2023). AFM images and nanoindentation curves were analyzed with Gwyddion (Nečas and Klapetek, 2012) and custom Python scripts as described elsewhere (Ridolfi et al., 2020).

2.1.5. Determination of encapsulation efficiency

Encapsulation efficiency was measured following the dialysis method as described before (Abruzzo et al., 2024b). Specifically, a 5 cm-long dialysis tube with a molecular weight cut-off of 6–8 kDa (Spectra/Por 1 Dialysis Membrane, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) was filled with 0.5 mL of each formulation. Then the tube was submerged in 50 mL of PBS pH 7.4 and stirred at 300 rpm for 120 min at 25 °C. Afterwards, the external phase was withdrawn and analyzed through the HPLC method previously published (Abruzzo et al., 2022). To calculate the encapsulation efficiency (%EE) the following equation was used:

$$\%\,EE = \frac{(\text{Total amount of AZT} - \text{Amount of AZT in the external phase})}{(\text{Total amount of AZT})} \times 100$$

2.1.6. In vitro drug release studies

In vitro AZT release from liposomes was investigated by using Franz-type static glass diffusion cells (15 mm jacketed cell with a flat ground joint and clear glass with a 12 mL receptor volume; diffusion surface area of 1.77 cm^2), equipped with a V6A Stirrer (PermeGear Inc., Hellertown, PA, USA) and a surrounding jacket, able to maintain the temperature at 37 ± 1.0 °C. 0.5 mL of each formulation was placed inside the donor chamber, while a mixture of KH_2PO_4 0.1 M pH 4.5/ethanol (80:20, v/v) or PBS pH 7.4/ethanol (80:20, v/v) was used as receiver medium. These two different pH values were selected to investigate the influence of physiological or pathological pH, respectively, on drug release behavior (Donders et al., 2017; Falavigna et al., 2020; Jøraholmen et al., 2015; Li et al., 2009; Cook and Brown, 2018). A pre-soaked cellulose nitrate filter with a pore size of 0.22 μm (Sartorius, Goettingen, Germany) was placed between the donor and the acceptor chambers. Aliquots (0.2 mL), containing the released drug, were collected at predetermined time intervals (30, 60, 120, 180, 240, 300, 360 min) from the receiver chamber and immediately restored with the same volume of fresh medium. The samples obtained were adequately diluted and analysed through HPLC. A control (CTRL) consisting of AZT solution (1.5 mg/mL) in a mixture of water and ethanol (40:60 v/v) was also assessed. The release of AZT over time was determined as a percentage of the Mt/M0 ratio, where Mt represents the drug amount released at each time and M0 the total AZT included in liposomes. Two calibration curves of AZT were obtained, dissolving AZT in a mixture of PBS pH 7.4/ethanol (80:20, v/v): methanol (1:1, v/v) or in KH_2PO_4 0.1 M pH 4.5/ethanol (80:20, v/v): mobile phase (1:1, v/v). Both curves showed good linearity ($R^2 = 0.999$) and were set up with drug concentrations ranging from 0.005 mg/mL to 0.05 mg/mL.

2.1.7. Mucoadhesion properties

The ability of formulations to interact with mucin was investigated by following a previous method with slight changes (Abruzzo et al., 2021a). Briefly, mucin was dispersed overnight (400 rpm) in a buffer at pH 4.5 or pH 7.4 (0.08 % w/v) to mimic the physiological and pathological pH values, respectively. Mucin dispersion was then centrifuged for 20 min at 7500 rpm and the supernatant was collected. Coated and uncoated liposomes were diluted 1:25 (v/v), mixed with the supernatant containing mucin (3:1 v/v), vortexed and then incubated at 37 °C for 120 min. The turbidity of the sample was measured at 650 nm by UV–Vis spectrophotometry (UV-1601 Shimadzu Corp., Australia). As references, the absorbances of the mucin solution and liposomes alone were also measured. The mucoadhesive behavior is directly correlated to the % increase in turbidity at 650 nm and calculated as follows:

$$\%\,\text{Turbidity increase} = \frac{(ABS_{\text{liposomes+mucin}} - ABS_{\text{mucin}})}{ABS_{\text{liposomes}}} \times 100$$

Mucoadhesion properties of liposomes were also assessed by measuring the displacement of a liposome-based lyophilized disc on an agar-mucin inclined plane (Abruzzo et al., 2024a). Specifically, the uncoated and coated liposome suspensions (4 mL) were placed in a 10 mL beaker, frozen overnight at − 20 °C, and finally lyophilized at 0.01 atm and −45 °C (Freeze Dryer ALPHA 1–2, Christ, Milan, Italy). Agar (1 % w/v) and mucin were dispersed in water at 100 °C (0.5 % w/v) and stirred at 800 rpm for 5 min. Then, the dispersion was poured to fill a Petri dish (8.8 cm diameter) and left for 2 h at room temperature to equilibrate. Before placing the lyophilized LP on the dish, three predetermined areas were hydrated with 25 μL of buffer at pH 4.5 or 7.4. Then the lyophilized formulations were positioned on these hydrated areas and after an adhesion time of 3 min, without any external pressure, the Petri dishes were inclined with an angle of 60°, as reported in several vaginal mucoadhesion assessments (Abidin et al., 2024; Pacheco-Quito et al., 2022). A buffer at pH 4.5 or pH 7.4 was dripped at 1 cm from the upper portion of the disc with a flow rate of 0.25 mL/h to simulate vaginal discharges (Thapa et al., 2022). The displacement of formulations after 6 h was recorded.

2.1.8. Antimicrobial activity

The antimicrobial activity of AZT and loaded liposomes was evaluated against Gram-positive (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* BC101, *Enterococcus faecium* BC105, *Streptococcus agalactiae* SO102) and Gram-negative (*Escherichia coli* ATCC 11105) bacteria (Siroli et al., 2017). All strains were routinely grown in Brain Heart Infusion medium (BHI, Difco, Detroit, MI), at 37 °C. Minimal Inhibitory Concentrations (MIC) of AZT and loaded liposomes were determined following NCCLS standard guidelines (Cockerill, 2012). Briefly, MIC were assessed by a microdilution assay carried out on a 96-well plate. Bacterial suspensions were prepared at a concentration of 10^6 CFU (colony forming units)/mL in BHI broth; a suspension containing all bacterial strains together (final concentration 10^6 CFU/mL) was also prepared. Free AZT and AZT-loaded formulations were 2-fold serially diluted in BHI broth, reaching the concentrations 0.05–30 μg/mL (0.005–3 μg/mL for *S. agalactiae* SO102), and added to the bacterial suspensions. MIC values were determined after 24 h of incubation at 37 °C.

2.1.9. Liposome stability

Liposome physical stability was kept under observation for a storage

period of 60 days, at + 4–8 °C. After 7, 14, 30 and 60 days, aliquots of liposome suspension were diluted as mentioned above in Section 2.1.3 and samples were characterized for their size and PDI by using PCS. Any trace of sedimentation was also monitored over the storage period. Finally, the antimicrobial activity was also assessed after 60 days of storage, following the protocol described in Section 2.1.8.

2.1.10. Drug retention inside vaginal mucosa

To assess AZT retention inside vaginal mucosa, a porcine model was used. Porcine vaginal tissue was obtained from a freshly excised pig vagina, provided by a local abattoir (CLAI, Faenza, Italy) and immediately taken to the laboratory. Mucosa was isolated from the underlying tissue, washed with saline buffer 0.9 % (w/v), carefully cut to an appropriate size and frozen in aluminum foil. Before the experiments, the mucosa was left to defrost for 15 min and placed between the donor and acceptor chamber of the Franz cell. As a receiver phase, 12 mL of PBS pH 7.4/ethanol (80:20, v/v), maintained at 37.0 ± 1.0 °C, was used. 0.5 mL of each formulation or control (prepared as described in section 2.1.6) have been placed in the donor chamber. Samples of 0.2 mL were withdrawn after 1, 3 and 6 h from the receiver chamber, replaced with fresh medium and analyzed through HPLC. At the end of the experiment, the remaining liposome suspension was removed, and the mucosal surface was cleaned with 0.5 mL of methanol. Both aliquots were centrifuged at 10,000 rpm for 15 min, filtered and analysed through HPLC to assess the amount of free AZT in the donor chamber. Later, the mucosa was removed from its location, covered with 5 mL of methanol and left stirring overnight to extract AZT retained within itself. Finally, the tissue was removed and the solution was centrifuged at 10,000 rpm for 15 min, filtered and analysed through HPLC. Results are shown as the percentage of the drug amount inside the donor compartment, the mucosa and the acceptor chamber.

2.1.11. Statistical analysis

All the experiments were performed in triplicate. Data are expressed as mean ± SD. *t-test* and Two-way ANOVA followed by Tukey correction for multiple comparisons was used to determine the statistical significance of the studies. All statistical analyses were performed using GraphPad Prims version 9.5.0 for Windows (GraphPad Software, San Diego, CA, USA, www. Graph pad. com) and the criterion for statistical significance was $p < 0.05$.

3. Results and discussion

3.1. Determination of vesicle size, polydispersity index (PDI) and ζ potential

To achieve successful local treatment of vaginal infections, drug delivery systems should possess specific properties like the ability to deliver an adequate amount of the drug in a sustained manner together with the characteristic of being retained for a long time inside the vaginal cavity. In our former study AZT-loaded liposomes and niosomes were prepared for the treatment of skin infections and results demonstrated that liposomes were able to provide a sustained release of AZT (Abruzzo et al., 2024b). Within this study, to fulfill the above-mentioned goal, the previously developed liposomes had been coated with two different mucoadhesive polymers, CS and HYA, by following an easy preparation method, which simply consisted of the addition of the polymer solution to the liposome suspension. The presence of a mucoadhesive coating composed of CS or HYA on the liposome surface could impact in a different way on vesicle characteristics, affecting their size, zeta potential and ability to control the drug release and simultaneously enhance its retention time inside the vaginal cavity.

The obtained uncoated and coated LP showed pH values around 6, which were compatible with the environment of the vagina (pH ~4-6) (Pedersen et al., 2014). The size, PDI and ζ potential of all the formulations are summarized in Table 1.

The determination of vesicle size remains one of the most important properties, especially for vesicles intended for vaginal drug delivery. As is known, the size generally affects the ability of the vesicles to release the drug, as well as their capability to be retained inside the mucosal tissue. A range between 200 and 500 nm is reported as ideal to improve drug retention inside vaginal mucosa (das Neves et al., 2011); on the other hand, the presence of a larger surface area, as in the case of small vesicles, can determine a rapid release of the drug and consequently its quick leakage from the vaginal cavity (Jøraholmen et al., 2015).

As shown in Table 1, uncoated LP were characterized by a mean diameter lower than 500 nm. Conversely, the presence of CS and HYA provided a higher size compared to the uncoated LP (both for unloaded and loaded vesicles; $p < 0.05$), thus indicating the successful surface coating of LP with the polymers. Similar results were also reported in previous works in which different kinds of liposomal formulations were coated with polymers, like CS and HYA (Abruzzo et al., 2020; Castangia et al., 2015; Jøraholmen et al., 2015; Mork et al., 2024; Sebaaly et al., 2022). Moreover, the presence of CS or HYA influenced the final size of the formulations. Particularly, CS-LP presented a higher size than HYA-LP ($p < 0.05$), probably due to different interactions occurring between the polymers and the phosphatidylcholine. Finally, loaded LP showed an increase in size ($p < 0.05$) compared to the respective unloaded ones (except for CS-LP) as a consequence of the inclusion of AZT, in agreement with previous findings (Abruzzo et al., 2024b; Abruzzo et al., 2022). Although the coating with the two employed polymers has led to the formation of vesicles characterised by size out of the ideal range, the presence of these polymers on liposome surface could determine a sustained drug release and an increased drug residence time inside the vaginal cavity. Prolonged drug release and retention time would be a rationale for lowering the dose and the frequency of administration needed to induce the antimicrobial effect, thus consecutively improving the therapy efficacy and patient compliance.

Regarding PDI, which represents a measure of the width of unimodal size distributions, a value around or below 0.3 was registered for all the formulations, thus demonstrating the homogeneity of the samples (Danaei et al., 2018).

Another important parameter for liposome characterization is represented by the ζ potential, which could suggest how the presence of positive/negative polymers on the phospholipid bilayer could affect the superficial charge of the vesicle.

In addition, ζ potential gives predictive information about vesicle stability. As a matter of fact, ζ potential values between −30 and + 30 mV do not assure vesicle stability, as repulsion forces in these conditions are limited (Németh et al., 2022). Another important feature influencing liposome stability is related to the polymer coating as it has been reported that coated LP could also remain stable for a specific period due to steric hindrance (Abdellatif et al., 2020; Feng et al., 2020). As can be seen from Table 1, uncoated LP without AZT showed negative ζ

Table 1
Size, PDI and ζ potential values of uncoated and coated-LP with (loaded) or without (unloaded) AZT.

	Uncoated-LP		CS-LP		HYA-LP	
	Unloaded	Loaded	Unloaded	Loaded	Unloaded	Loaded
Size (nm)	368.0 ± 20.3	444.2 ± 34.4	864.8 ± 70.1	975.9 ± 117.9	514.4 ± 13.1	609.9 ± 31.9
PDI	0.318 ± 0.019	0.301 ± 0.009	0.298 ± 0.017	0.340 ± 0.011	0.307 ± 0.045	0.314 ± 0.016
ζ potential (mV)	−49.8 ± 1.1	−44.7 ± 3.1	+14.7 ± 5.6	+25.1 ± 5.1	−59.7 ± 0.5	−50.7 ± 3.0

potential, attributed to the presence of phosphate groups of phosphatidylcholines, which were negatively charged at pH 6 (pH value of the final formulation). Moreover, the encapsulation of the positively charged AZT provided a shift towards less negative values ($p < 0.05$) (pKa of the drug equal to 8.5) (Abruzzo et al., 2024b), except for CS-LP. CS and HYA influenced the surface charge of the final vesicles. The positive charges of CS switched the ζ potential value of liposomes from negative to positive thanks to the presence of the polymer on their surface. On the other hand, HYA-LP possessed a more negative ζ potential with respect to the uncoated ones ($p < 0.05$) because of the negatively charged carboxylic groups of the polymer. The change in ζ potential when polymers were employed to coat liposomes was also reported by different previous studies (Mork et al., 2024; Abruzzo et al., 2020; Yadav et al., 2024; Andersen et al., 2013; Franzé et al., 2018) and can represent further proof of the coating process of liposomes.

3.2. Morphological characterization

Unloaded samples of uncoated-LP, CS-LP and HYA-LP were observed via single-particle AFM morphometry as previously described for AZT-loaded liposomes and niosomes (Abruzzo et al., 2024b). Fig. 1 reports the particle diameter and contact angle (CA) distributions for each LP formulation. In previous studies, we demonstrated that the CA of individual particles is linearly correlated with their mechanical stiffness: the higher the CA, the stiffer the particle (Ridolfi et al., 2020).

Uncoated-LP were observed to have diameters in the 50 ~ 300 nm range, with an average diameter of 137 ± 90 nm (N = 101 individual particles). Both coated-LP formulations showed increased diameters, ranging in the 100 ~ 800 nm range for CS-LP and the 50 ~ 500 nm range for HYA-LP; average diameters were 270 ± 138 nm for CS-LP (N = 44) and 153 ± 95 nm for HYA-LP (N = 107). The apparent discrepancy between average sizes obtained via AFM and PCS can be partially reconciled when considering that the formers are geometric, while the latter are hydrodynamic (and thus intrinsically larger). However, both techniques agree on the general size distribution trend across the three formulations.

The CA distributions are virtually coincident for all LP formulations, with averages of 95 ± 24° (uncoated-LP), 101 ± 19° (CS-LP), and 96 ± 21° (HYA-LP), suggesting that HYA and CS coating do not significantly impact LP nanomechanics. However, it should be noted that CA comparisons are only significant if samples are deposited on the same substrate, and CS-LP was deposited on a different substrate than uncoated-LP and HYA-LP (see section 2.1.4). To circumvent this limitation, we performed single-particle nanoindentations on individual vesicles. All LP formulations showed the linear contact mechanics regime typical of intact vesicles (Ridolfi et al., 2020), but their stiffness values were measured to be coincident within error, again suggesting that all LP samples shared similar mechanical characteristics.

3.3. Determination of encapsulation efficiency

Encapsulation efficiency (EE) allows for the determination of the

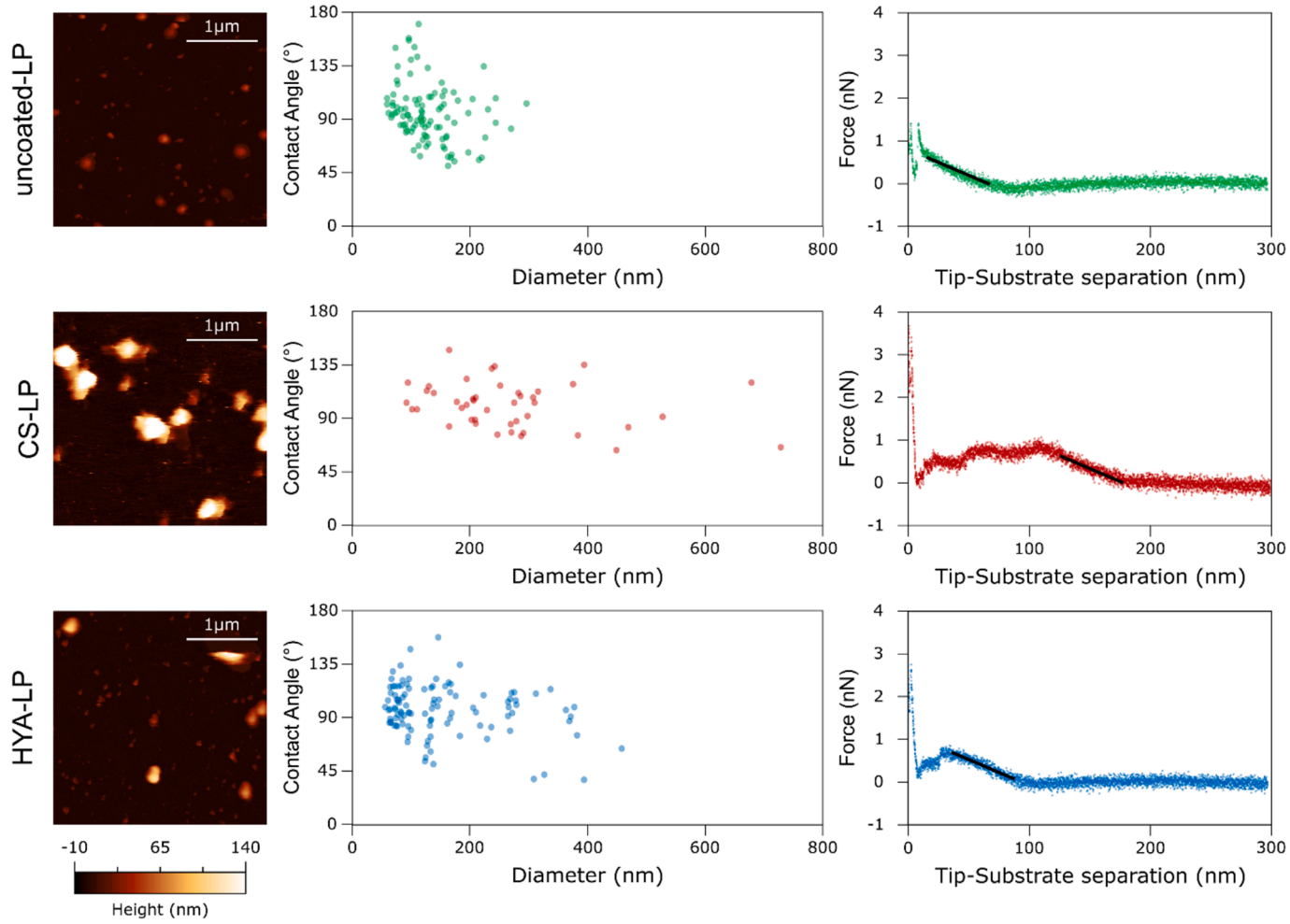

Fig. 1. (Left): representative AFM micrographs. All images are 3x3 μm. (Middle): single-particle AFM morphometry. Each dot represents one individual LP plotted at its measured equivalent spherical diameter and contact angle. (Right): representative single-particle nanoindentations, evidencing very similar linear slopes upon contact. The slope of all three solid black lines correspond to a stiffness of 1.27 pN/nm and closely follows the contact regimes of all three formulations.

vesicles' ability to include a drug inside their structure and evaluates the impact of the free or entrapped drug on its release. A low EE value indicates the presence of a great amount of free active molecules, which can be immediately released. On the other side, greater EE values imply the presence of a higher amount of drug inside the formulation, thus controlling its release over time through the partitioning process across the multiple bilayers. As anticipated in the previous section, prolonged drug release is a desired property of drug delivery systems intended for vaginal application, allowing to reduce the dosage and the administration frequency. Additionally, the possibility of encapsulating the drug inside a nanocarrier can permit to increase of its solubility, especially in the case of lipophilic active molecules. When CS or HYA were employed as coating, a slight decrease in EE% value (44.6 ± 3.2 % and 42.3 ± 2.6 % for CS-LP and HYA-LP, respectively) was registered ($p < 0.05$) with respect to the EE% of the uncoated one (56.9 ± 3.4 %) and no significant difference was observed between CS-LP and HYA-LP ($p > 0.05$). The lower EE% values of coated LP compared to the uncoated ones were probably related to both polymers' capacity to improve drug solubility thanks to ionic or weak interactions established with the drug. Refai and co-workers have observed a reduction of EE% of coated liposomes compared to uncoated ones, and they have reported that some active molecules probably escaped the vesicles during the coating process (Refai et al., 2017). However, the impact of coating on the vesicles' ability to incorporate the drug remains controversial as stated in previously published papers, which reported different outcomes regarding this issue. Castangia and colleagues, for example, did not observe any significant difference in the EE% values of the licorice extract and glycyrrhizin between liposomes and hyalurosomes (Castangia et al., 2015). In other studies, the coating process did not influence the incorporation of different polyphenols (Jøraholmen et al., 2015; Mork et al., 2024). On

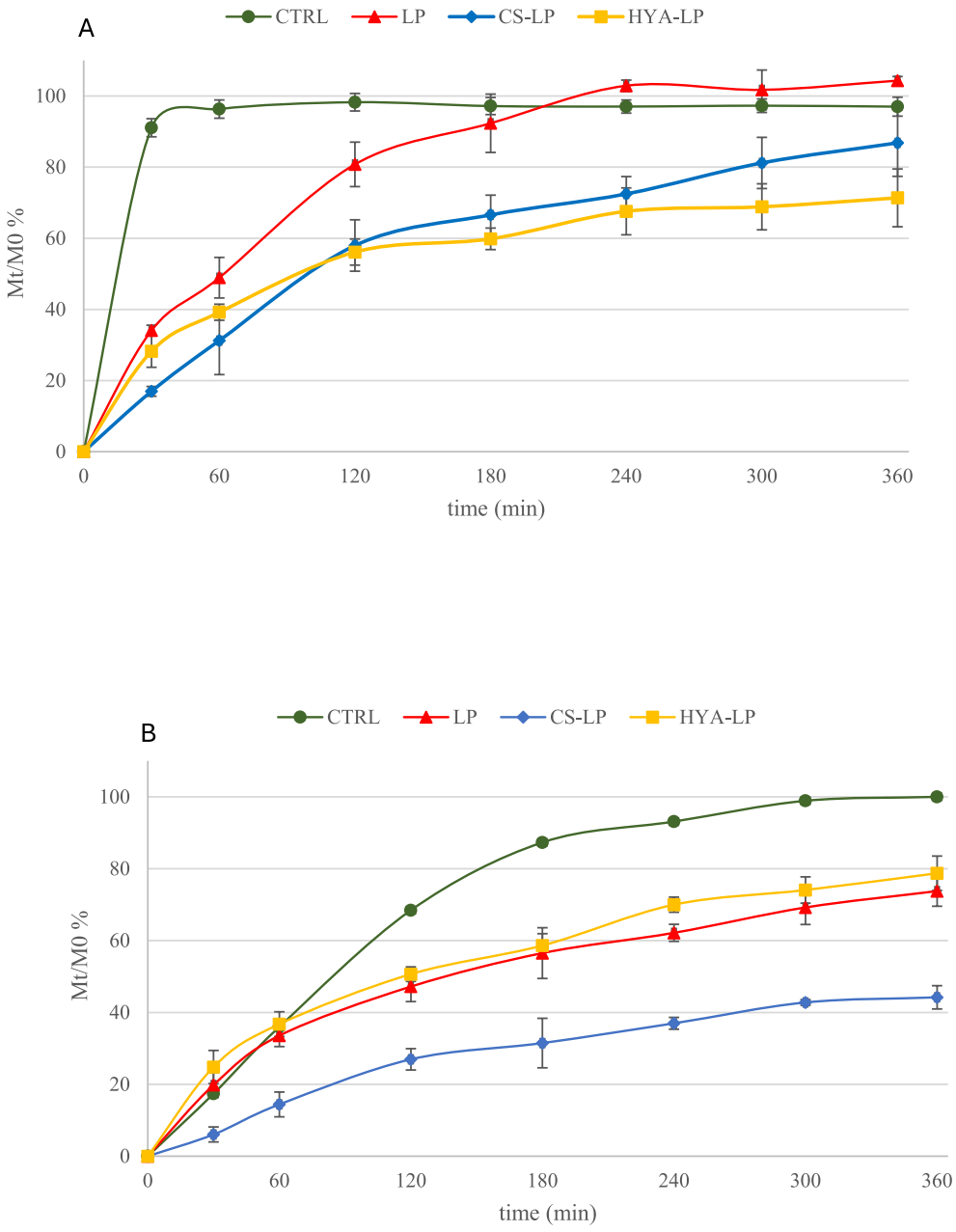

Fig. 2. A and 2B. Cumulative drug amount (expressed as percentage Mt/M0, where Mt represents the amount of AZT released at each time and M0 the total AZT mass) released from the control (CTRL), uncoated (LP) and coated (CS-LP, HYA-LP) liposomes at pH 4.5 (A) and 7.4 (B) plotted as a function of time. Data are expressed as means ± SD, n = 3.

the contrary, Abdellatif et al. observed an increase in EE% of sertaconazole nitrate in pectin-coated liposomes compared to the uncoated ones, attributing this result to pectin's ability to hinder the drug diffusion or to the presence of the drug physically trapped between the pectin layer and liposome surface (Abdellatif et al., 2020).

In all the cases, an increase in drug solubility was reached, as the final liposome suspensions allowed to solubilize AZT up to 1.5 mg/ml (AZT solubility in water equal to 0.14 ± 0.02 mg/mL; (Abruzzo et al., 2022). The possibility to achieve a greater amount of drug in the solubilized form represents a relevant benefit to improve the drug availability at the vaginal site and to achieve the final therapy efficacy.

3.4. In vitro drug release studies

In vitro drug release tests were performed at two different pHs, 4.5 and 7.4, to evaluate liposome ability to control drug release at the physiological and pathological pH values, respectively (Jøraholmen et al., 2015; Li et al., 2009).

Fig. 2A and B show the *in vitro* profiles of AZT released from the CTRL, uncoated and polymer coated LPs at pH 4.5 (A) and 7.4 (B). After 120 min, at both pH conditions, the CTRL determined a more rapid drug release with respect to liposomes ($p < 0.05$), due to the presence of free drug which was immediately available to diffuse towards the receptor. Specifically, the maximum amount of released drug was reached after 120 min at pH 4.5 (98.3 ± 2.5 %) and 300 min at pH 7.4 (98.9 ± 3.7 %). The difference observed between the tested pHs can be mainly related to the drug solubility under the different pH values. AZT solubility in buffer at pH 4.5 or pH 7.4 was measured by dispersing an excess amount of drug in the buffers and keeping the suspensions under agitation (300 rpm) for 48 h at 37 °C. Undissolved AZT was removed by centrifugation (Microspin 12 Centrifuge, Biosan, Latvia) at 10,000 rpm for 15 min and filtration of the dispersion through a 0.2 μm pore-size cellulose acetate filter. Subsequently, an exact volume was rationally diluted and drug concentration was detected by HPLC. AZT (pKa ~ 8.5) was characterized by a higher solubility at pH 4.5 (22.46 ± 1.49 mg/mL) than at pH 7.4 (0.79 ± 0.02 mg/mL) ($p < 0.05$). The higher solubility of the drug at pH 4.5 could speed up its repartition towards the receptor chamber achieving the maximum more rapidly than at pH 7.4.

On the other side, in comparison with the CTRL, a lower drug release was observed when AZT was encapsulated inside the uncoated LP. More specifically, two phases of drug release were observed. The first one was characterized by a burst effect (after 120 min at both pHs) inherent in the amount of free drug outside the vesicles and able to diffuse rapidly. The second sustained phase was caused by the partitioning process between the phospholipid bilayers which the drug underwent before reaching the receptor chamber. Moreover, the drug was completely released uniquely at pH 4.5 (after 240 min 102.9 ± 1.6 %), while at pH 7.4 the same formulation reached only 73.8 ± 4.2 % at the end of the study. This difference was once again attributable to the drug solubility at pH 4.5 and 7.4.

Comparing coated liposomes with respect to the uncoated ones, even though coated liposomes were characterized by a higher amount of free drug (see EE% results), a more sustained drug release was immediately reached than the uncoated ones ($p < 0.05$, except for HYA-LP at pH 7.4). As mentioned before, the surface area of the different liposome formulations can have an impact on drug release profiles: coated LP were characterised by greater dimensions and consequently a lower surface area in comparison to the uncoated LP, determining the release of a lower amount of drug over time. Moreover, these findings were linked to the presence of polymer on the liposome surface which slowed down the diffusion of the drug across the vesicle structure, in agreement with previous results. Particularly, Li and co-workers attributed the slower drug release of polymer-coated LP to the formation of an intense shell on the liposome surface which restricted the fluidity of the lipids bilayer and led to a decrease in the membrane permeability (Li et al., 2009). Another study reported that CS-coated liposomes can prolong the release of resveratrol to a greater extent than non-coated liposomes (Jøraholmen et al., 2015). Refai and colleagues assessed that the presence of a thick coating determines a long diffusion path which contributes to slowing down drug release (Refai et al., 2017).

Nevertheless, at each point of the drug release profile at pH 7.4 no significant difference was measured between uncoated and HYA-LP ($p > 0.05$). This was probably due to the presence of HYA negative charges, which adversely affected its ability to coat the LP surface, making HYA-LP release behavior similar to that obtained from uncoated LP. Likewise, Castangia and co-workers developed liposomes and hyalurosomes for the delivery of licorice extract; they performed *in vitro* release studies in water and they did not observe any significant difference between the release of phenols from the different formulations (Castangia et al., 2015).

Among all the formulations, CS-LP allowed to obtain the greatest control of drug release over time at both pHs. The possibility of reaching a sustained drug release is fundamental to reduce the dosage and the frequency of administration, thus increasing patient compliance (Araujo et al., 2021). Additionally, it is worth mentioning that these advantages could contribute to maximizing the antimicrobial efficacy, avoiding drug overuse which is the main cause of the antimicrobial resistance overspread.

3.5. Mucin-liposomes interaction and mucoadhesion properties

Besides the ability to control drug release, the effectiveness of a vaginal drug delivery system is strictly related to its mucoadhesive property which can permit to increase drug residence time inside the vaginal cavity and at the same time improve the therapeutic efficacy.

Within this study, the mucoadhesive ability was evaluated by employing two different methods. The first one is based on the measurement of the turbidity increase obtained when the formulation was mixed with a mucin solution: an increase in turbidity was related to a high interaction between liposomes and mucin, and consequently to a good mucoadhesive property of the formulation. Fig. 3 shows the results obtained from this method. As can be seen, at both tested pHs no turbidity increase was observed for uncoated LP, thus demonstrating a lack of interaction with mucin. On the other hand, the presence of the two different polymers on the vesicle surface influenced their ability to interact with mucin. Specifically, CS-LP provided an increase in turbidity in comparison to the uncoated LP ($p < 0.05$). In the case of HYA-LP, no significant difference was obtained compared to the uncoated LP ($p > 0.05$). This behaviour can be related to the surface charge of the formulations. As a matter of fact, the negative ζ potential of LP and HYA-LP limited the interaction with mucin, which is abundant in negative charges of sialic acid residues and sulphates, and consequently hindered their mucoadhesion property. Our results were in agreement with previous findings reporting the formation of repulsive forces between liposomes and mucin and thereby a lack of mucoadhesiveness (Mackie et al., 2017). Conversely, the positive charges of CS-LP facilitated the interaction with the negatively charged groups of mucin, in accordance with previous results (Jøraholmen et al., 2015; Yadav et al., 2024). These results demonstrated the superiority of CS-LP in the interaction with mucin over the uncoated-LP and the HYA-LP.

Mucoadhesive ability was also investigated by measuring the movement of liposome-based lyophilized discs on an agar-mucin inclined plane. A high displacement of the disc corresponds to a low mucoadhesion property. Once again, a different behaviour was observed between uncoated and CS-LP or HYA-LP. In fact, at the end of the study at both pHs, LP and HYA-LP-based discs travelled 4.7 cm. On the other hand, CS-LP disc remained in the application area and no displacement was measured. Results obtained with this second method confirmed the outcomes highlighted through the first one. To conclude, among all the formulations CS-LP were characterized by the best mucoadhesive property *in virtue* of their capacity to interact with the mucin. Hence, CS-LP can be a promising nanocarrier able to counteract the resistance to

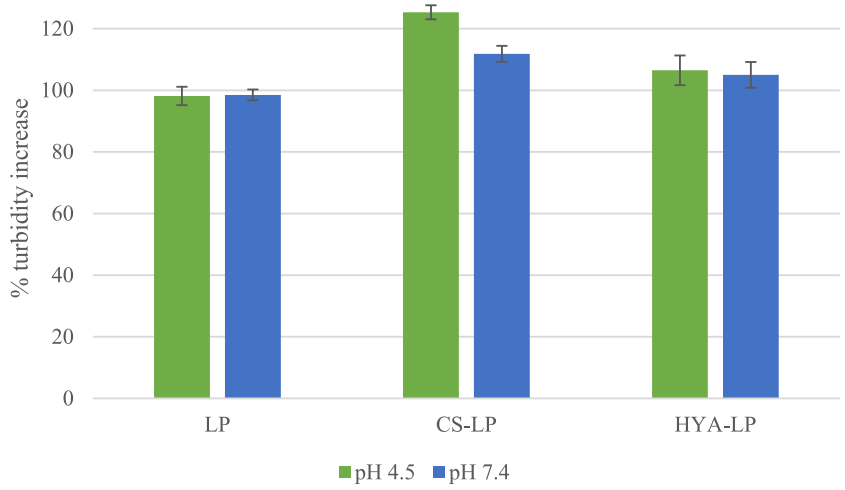

Fig. 3. % Turbidity increase obtained in the presence of mucin suspension and uncoated and coated LP at pH 4.5 and 7.4.

being washed out by the mucus turnover and increase the drug residence time at the infected vaginal site. The latter property could contribute to maximizing the drug's antimicrobial effect over a long period of time.

3.6. Antimicrobial properties

To determine the antimicrobial activity of free AZT and loaded liposomes, Gram-positive (*S. aureus* ATCC 29213, *E. faecalis* BC101, *E. faecium* BC105, *S. agalactiae* SO102) and Gram-negative (*E. coli* ATCC 11105) bacteria commonly found in AV were used. Besides the pure cultures, a suspension containing all the strains together was also included, to mimic the polymicrobial feature of AV. MIC values were determined by microdilution assay after 24 h of incubation and reported in Table 2. In any case, loaded liposomes retained antimicrobial activity, although slightly different MIC values were found for different microbial strains. *S. aureus* ATCC 29213 susceptibility to AZT (MIC 1 μg/mL) is in accordance with previously reported data (Abruzzo et al., 2024b; Abruzzo et al., 2022), and two-fold MIC values were registered for loaded liposomes. A similar behavior was observed for *E. faecium* BC105, for which AZT loaded in liposomes showed two-fold MIC values (15 μg/mL) with respect to free AZT (8 μg/mL). Regarding *E. faecalis* BC101, *S. agalactiae* SO102 and *E. coli* ATCC 11105, no difference in AZT efficacy was registered between free drug and loaded liposomes. In EUCAST guidelines, no breakpoint for resistance in *Enterococcus* spp. and *E. coli* has been reported ("The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 13.1, 2023. https://www.eucast.org.," n.d.); however, a MIC $\geq$ 32 μg/mL have been proposed as the AZT resistance breakpoints in some *Enterobacteriaceae* (Gomes et al., 2019). AZT loaded in liposomes showed two-fold MIC values (30 μg/mL) with respect to free AZT (15 μg/mL) when it was tested towards the polymicrobial suspension. In all cases no difference was observed between CS-LP and HYA-LP ($p > 0.05$). The observed two-fold increase in MIC for loaded liposomes agrees with previous studies (Abruzzo et al., 2024b) and can be explained by considering the *in vitro* drug release profile, since the encapsulation of AZT inside liposomes caused a sustained release of AZT, differently from free AZT which is immediately available to elicit antimicrobial activity.

Table 2
Minimal inhibitory concentrations (MIC, μg/mL) of AZT and loaded liposomes.

Bacterial strain	AZT	LP	CS-LP	HYA-LP
S. aureus ATCC 29213	1	2	2	2
E. faecalis BC101	2	2	2	2
E. faecium BC105	8	15	15	15
E. coli ATCC 11105	1	1	1	1
S. agalactiae SO102	0.75	0.75	0.75	0.75
Polymicrobial mix	15	30	30	30

3.7. Stability studies

All the prepared formulations were stored at 4–8 °C for 60 days and their stability was evaluated by monitoring any change in size and PDI. As can be observed from Fig. 4, all the developed liposomes, both coated and uncoated, showed no significant variations in terms of size. No change in PDI was also measured (data not shown). These results demonstrated that all the formulations remained stable for the period of observation. As mentioned before, the negative values of ζ potential observed for both uncoated and HYA-LP allowed the maintenance of size, thanks to the repulsive forces occurring between the vesicles. On the other hand, for CS-LP the presence of a thick layer of the polymer with its steric hindrance can prevent fusion phenomena between vesicles, guaranteeing their stability over time. Moreover, from a qualitative evaluation of all formulations it was possible to assess that in no case, sedimentation occurred before 60 days. For a more comprehensive stability evaluation, antimicrobial tests were also performed after 60 days from liposome preparation: both coated and uncoated liposomes loaded with AZT showed the same antimicrobial activity assessed soon after preparation. This result is significant since it confirms the retention of AZT antimicrobial activity over time.

3.8. Drug retention inside vaginal mucosa

Another key factor affecting the effectiveness of a vaginal drug delivery system is connected to its ability to retain the drug inside the mucosal tissue, where it should exert its therapeutic action. For this reason, *in vitro* studies were performed to investigate the ability of the uncoated and coated liposome to ensure AZT accumulation inside the vaginal mucosa. Moreover, the measurement of the drug amount able to diffuse towards the receptor chamber is helpful to estimate formulation ability to escape systemic absorption, which should be avoided for topical treatment of vaginal infections. As a matter of fact, great systemic absorption could determine a low efficacy in the local therapy, worsening both the side effects and the antimicrobial resistance phenomenon.

Fig. 5 shows the percentage amount of AZT inside the mucosa, the donor and receiving chamber from the CTRL and the tested formulations after 6 h. As shown, the CTRL, based on an ethanol/water mixture of AZT, allowed the diffusion towards the receptor of 17.4 ± 1.7 %, while in the donor chamber a greater percentage of AZT was found (58.9 ±

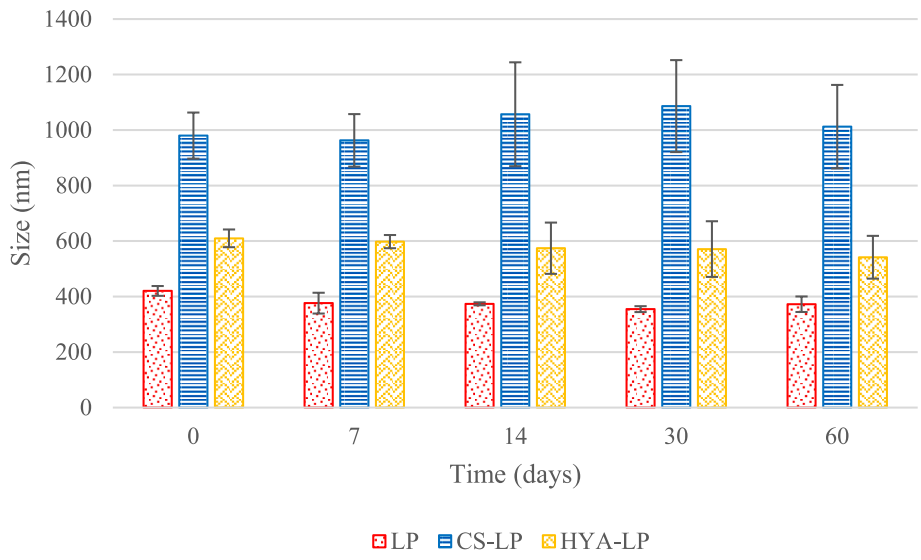

Fig. 4. Size variation of LP, CS-LP and HYA-LP during 60 days of storage at + 4–8 °C. Data are expressed as means size (nm) ± SD, n = 3.

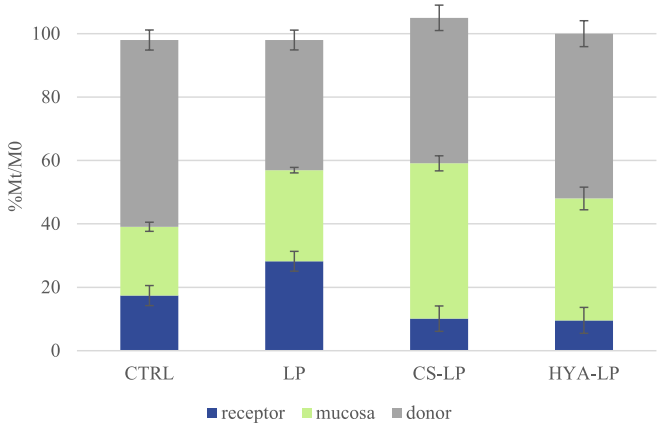

Fig. 5. Percentage amount of AZT in the receptor compartment, within the porcine vaginal mucosa and in the donor compartment obtained after 6 h from the application of control sample (CTRL), uncoated (LP) and coated (CS-LP, HYA-LP) LP. Data are expressed as means ± SD, n = 3.

3.2 %). The encapsulation of the drug inside the uncoated LP determined an increase in the drug amount in the receptor chamber (28.2 ± 3.3 %) and consequently a reduction in drug amount in the donor (41.1 ± 3.1 %) ($p < 0.05$). This result can be correlated to the uncoated LP property to promote drug diffusion across the tissue *in virtue* of their lipidic nature able to penetrate membranes and transport the loaded drugs through the tissues (De Jesús Valle et al., 2022; Yu et al., 2021). Interestingly, the coating with polymers allowed for reducing the drug diffusion in the receiver chamber (CS-LP and HYA-LP reduced the amount of drug permeated 1.7 and 1.8 times more than control, respectively and 2.8 and 2.9 times more than the uncoated LP, respectively, $p < 0.05$). These results can be attributed to different properties of the coated LP, like the vesicle size and the drug release. As described in the previous sections, coated LP showed a greater size and a slower drug release rate with respect to the uncoated ones, which both determined a lower diffusion of AZT across the mucosa. For topical treatment of vaginal infection, it is desirable to limit the amount of systemic exposure of the user to the antimicrobial drug, to avoid side effects, increase patient compliance and reduce the antimicrobial resistance.

As regards the presence of AZT inside the mucosa, uncoated LP favored the accumulation of the drug inside the tissue rather than the control ($p < 0.05$), probably due to LP ability to transport the drug into the tissue, as mentioned before (De Jesús Valle et al., 2022). Moreover, CS-LP and HYA-LP provided an increase of drug retention with respect to the control as well as to the uncoated LP ($p < 0.05$) (CS-LP and HYA-LP retained the drug 2.3 and 1.8 times more than control, respectively and 1.7 and 1.3 times more than to the uncoated LP, respectively), due to their mucoadhesive properties as mentioned before. Particularly, among the two coated formulations, CS-LP, showing the best mucoadhesive properties, allowed to obtain the greatest retention of the drug inside the mucosa ($p < 0.05$). Our results agreed with previous findings reporting CS coated liposomes ability to maintain high drug accumulation in the vaginal tissue, due to the formulation mucoadhesiveness, responsible for trapping it within the mucosal structure (Yadav et al., 2024).

The retention of an adequate drug amount inside the mucosa is fundamental to obtain an improvement in the treatment of vaginal infections. Indeed, the accumulation of AZT inside the vaginal tissue would form drug reservoir, prolonging the residence time at the site of infection. In this way, it could be possible to maximize the therapeutic efficacy of the drug and consequently reduce the drug dosing and administration frequency.

4. Conclusions

AV, infecting more than 70 % of women of reproductive age, has a huge influence on their quality of life due to the high rates of recurrence, the low treatment efficacy and the probability of complications. New local nanocarriers represent innovative platforms for effective and compliant therapies, which can limit the overuse of antibiotics and the spread of bacterial diseases. In this study, two different polymer-coated liposomes containing AZT were successfully developed and compared by highlighting the main differences in terms of mucoadhesive ability, drug release and retention inside a biological tissue as well as antimicrobial properties. As a conclusion of this accurate and insightful comparison, this study firstly allowed us to select the best formulation for vaginal drug administration. Particularly, among all the developed formulations, CS-LP were distinguished by the best controlled drug release and accumulation within the vaginal mucosa, *in virtue* of their size and mucoadhesiveness. Secondly, in the field of AZT vaginal application, these results could contribute to optimizing its local efficacy, by allowing for a reduction in the dosage. Thanks to the latter finding, it could be also possible to limit the antimicrobial resistance phenomenon along with the improvement of patient compliance.

CRediT authorship contribution statement

Sara Lugli: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Angela Abruzzo:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Carola Parolin:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Beatrice Vitali:** Writing – review & editing. **Maria Laura Bolognesi:** Resources, Funding acquisition. **Marco Brucale:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Francesco Valle:** Writing – review & editing. **Teresa Cerchiara:** Writing – review & editing. **Barbara Luppi:** Writing – review & editing. **Federica Bigucci:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Angela Abruzzo reports financial support was provided by EU – NextGenerationEU with funds made available by the National Recovery and Resilience Plan (NRRP) – Partenariati Estesi (PE13 – INF-ACT) – CUP J33C2200287000. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper].

Acknowledgements

The authors would like to thank Chiara Foschi and Melissa Scafa for their valuable contributions. This research was supported by EU within the NextGeneration EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT) - CUP: J33C22002870005.

Data availability

Data are published in the AMS acta repository (DOI: 10.6092/unibo/amsacta/8255).

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