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Chitosan-based sponges containing clotrimazole for the topical management of vulvovaginal candidiasis

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ABSTRACT

Vulvovaginal candidiasis (VVC) persists as a worrying women's healthcare issue, often relying on suboptimal therapeutics. Novel intravaginal dosage forms focusing on improving patient acceptability and featuring improved biopharmaceutical properties could be interesting alternatives to available antifungal products. Different formulations of sponges based on chitosan (Ch), with or without crosslinking and co-formulated with poly(*N*-vinylcaprolactam) (PNVCL), were produced for the topical administration of clotrimazole (CTZ) and further tested for physicochemical properties, drug release, cytotoxicity and antifungal activity. Results showed that high amounts of CTZ (roughly 30–50 %) could be incorporated into sponges obtained by using a simple freeze-drying methodology. Cross-linking of Ch with ammonia affected the morphology and mechanical features of sponges and shifted the release profile from sustained (around 20 % and 60 % drug released after 4 h and 24 h, respectively) to fast-releasing (over 90 % at 4 h). The combination of PNVCL with non-crosslinked Ch also allowed tuning drug release, namely by increasing the initial amount of CTZ released in simulated vaginal fluid (roughly 40 % after 4 h), as compared to sponges featuring only non-crosslinked Ch. All formulations displayed low toxicity to cell lines derived from the female genital tract, with viability values kept above 70 % after 24 h incubation with sponge extracts. These also allowed maintaining the rapid onset of the antifungal effects of CTZ at minimum inhibitory concentrations ranging from 0.5 to 16 μg/mL for a panel of six different *Candida* spp. strains. Overall, proposed sponge formulations appear to be promising alternatives for the safe and effective management of VVC.

1. Introduction

Vulvovaginal candidiasis (VVC) remains one of the most prevalent infection of the female genital tract. The disease is caused by yeasts of the genus *Candida* and affects around three quarters of all women at least once during their lifetime (Sobel, 2007). It is typically a mild and treatable condition, but genitourinary symptoms can be severe, prolonged in time and relapse upon halting of suppressive treatment with antifungals (Sobel, 2016). This may lead to considerable decrease in the quality-of-life of women affected by the disease (Aballea et al., 2013). Oral and topical (intravaginal) treatments are available and based on different drugs (mostly azoles) and dosage forms. In the case of vaginal administration, products in the market are usually in the form of semi-solid (e.g. creams) or solid (e.g. tablets, suppositories) systems (das Neveset al., 2008). However, these present drawbacks that can impair user acceptability and adherence to treatment, such as messiness and leakage upon administration (mostly in the case of semisolid products), or the inability to sustain drug release. Thus, other dosage forms could be interesting additions to the armamentarium for managing VVC.

Sponges are an interesting vaginal dosage form that have been used for several decades for non-hormonal contraception (Kuyoh et al., 2003). Although formally a solid dosage form, their reticulated, highly porous structure allows inflow of high amounts of local fluids that can dissolve incorporate active ingredients and control release. Sponges

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have been typical based on non-degradable/non-dissolvable materials such as polyurethane foam, thus needing to be removed after use. This inconvenience can be abridged by the use of biodegradable/dissolvable materials that can originate a gel upon contact with vaginal fluids (Conte et al., 2023). Chitosan (Ch), for example, has been used for producing vaginal sponges for the delivery of drugs such as sildenafil for enhancing uterine blood flow (Aboud et al., 2018), or antifungal compounds (tea tree oil or butoconazole) for managing VVC (Shaker et al., 2018; Ge and Tang, 2016).

In this work, we produced various sponge formulations based on biodegradable, gel-forming cationic polymer Ch, alone or in combination with poly(*N*-vinylcaprolactam) (PNVCL). This last polymer is not only water soluble and able to enhance drug solubility, but also presents thermo-responsive behavior, with a lower critical solution temperature around 35 °C. PNVCL transitions into an agglomerated form at body temperature that can help modulating drug diffusion (Etchenausia et al., 2019). Sponges were used for the incorporation and vaginal drug delivery of clotrimazole (CTZ), one of the most used azole drugs for the topical management of VVC. Sponges were further characterized *in vitro* for features such as physicochemical, drug release, cytotoxicity and antifungal activity properties, as relevant to vaginal drug delivery. A schematic representation of the overall experimental approach followed in this work is shown in Fig. 1.

2 Materials and methods

2.1 Materials

Ch (DA = 25 %, MW = 310–375 kDa), *N*-vinylcaprolactam (NVCL) and resazurin were purchased from Merck (Darmstadt, Germany); CTZ, dimethyl sulfoxide (DMSO; 99.9 %), Triton X-100, glacial acetic acid and polysorbate 80 from Thermo Fisher Scientific (Porto Salvo, Portugal); ammonium hydroxide (30 %) and DMSO (99.9 % for PNVCL synthesis) from Neon (Mumbai, India); and 2,2′-azobis(isobutyronitrile) (AIBN) from DuPont (Barueri, SP, Brazil). AIBN was purified in-house by recrystallization in methanol. All other chemicals and reagents were of analytic grade or equivalent and were used as received.

2.2 Cells and culture media

Ca Ski cervical and HEC-1-A endometrial cell lines were obtained from ATCC (Manassas, VA, USA). Different *Candida* spp. ATCC strains, namely *C. albicans* ATCC 90028, *C. albicans* ATCC SC5314, *C. albicans* ATCC 64550, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 2001 and *C. krusei* ATCC 6258, were kindly provided by Dr. Célia F. Rodrigues and Dr. Mariana Henriques from the collection of the Biofilm Research Group (Centre of Biological Engineering, University of Minho, Portugal). RPMI 1640 medium (for mammal cell culture), RMPI 1640 medium (powder; for yeast cell culture), McCoy's 5A medium, Sabouraud Dextrose Agar (SDA), and dextrose were acquired from Thermo Fisher Scientific; Mueller-Hinton Agar, methylene blue and penicillin/streptomycin from Merck (Darmstadt, Germany); fetal bovine serum from Biochrom (Berlin, Germany); and 3-morpholinopropane-1-sulfonic acid (MOPS) from Sigma-Aldrich.

2.3 Synthesis of poly(N-vinylcaprolactam)

PNVCL was synthetized via radical polymerization of NVCL as previously detailed (Sala et al., 2018; Ribeiro et al., 2021). Briefly, 5 g of the monomer were dissolved in 28 g of DMSO (Neon) and placed into a reactor at 70 °C under reflux and an inert atmosphere (nitrogen flux) for 10 min. AIBN (113 mg) was then added as a radical initiator and the polymerization reaction was allowed to occur for 4 h. PNVCL was further purified by dialysis against deionized water using regenerated cellulose dialysis tubing (3.5 kDa MWCO, cylinder diameter 29.3 mm, 10 cm length; Thermo Fisher Scientific, São Paulo, Brazil), and dried in an oven at 60 °C for 24 h.

2.4 Production of sponges

Different types of sponges were produced using Ch, either non-crosslinked (NCL-Ch) or crosslinked (CL-Ch), as the common polymer. NCL-Ch sponges were obtained by dissolving 250 mg of the Ch in 21 mL of acetic acid 0.35 % (*v/v*), placing 1 mL of the resulting solution into plastic cylindrical molds (diameter = 16 mm and height = 6 mm) and freeze-drying it over 24 h. CL-Ch sponges were similarly produced while promoting the sol–gel physical reticulation of the polymer with ammonia. Ch in acetic acid 0.35 % (*v/v*) placed into the molds was treated under a saturated ammonia atmosphere for 72 h. Physical crosslinking was achieved by the neutralization of protonated amine groups from Ch, which leads to the decrease in the apparent charge density of polymer chains (Montembault et al., 2005). The obtained Ch hydrogel was then thoroughly washed with deionized water to remove

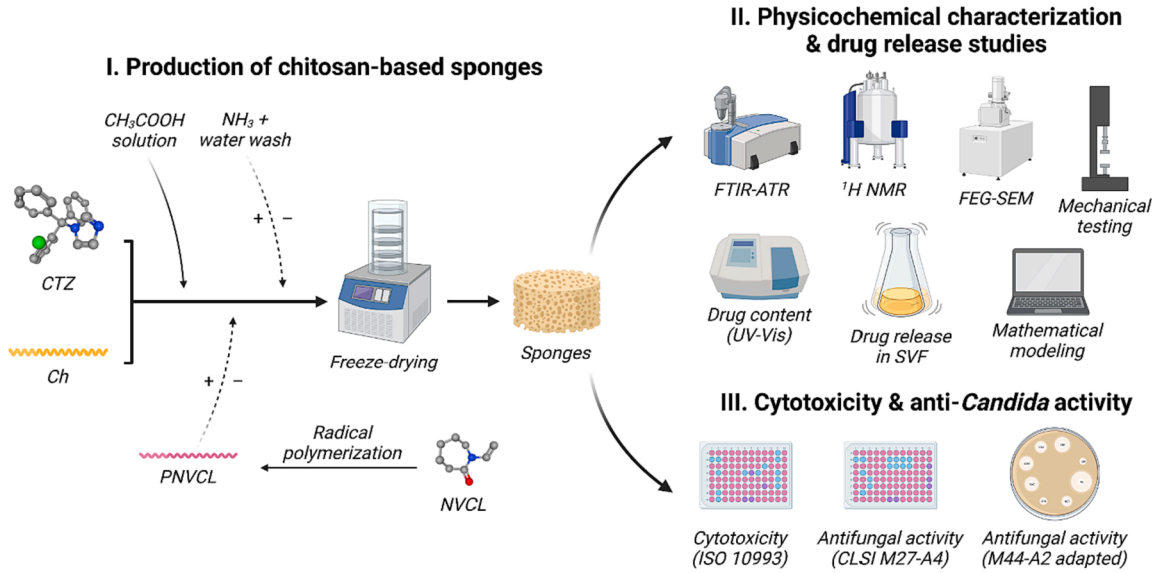

Fig. 1. Schematic representation of the general work flow leading to the production of Ch-based sponges and the methodology used for their characterization. Dashed arrows indicate steps that may or may not be performed during the production of certain CTZ-loaded sponges. Abbreviations are defined in the main text.

ammonium acetate formed during neutralization and excess ammonia, before being freeze-dried. Composite sponges were also prepared by adding PNVCL to the Ch solution in acetic acid at two different Ch: PNVCL weight ratios (3:1 or 1:1) before placing the mixture into molds and freeze-drying. This type of sponges were not submitted to cross-linking with ammonia (NCL-Ch/PNVCL 3:1 or NCL-Ch/PNVCL 1:1 sponges). The incorporation of CTZ into the sponges was achieved by dispersing the drug immediately after the solubilization of the polymer (s) in acetic acid solution at a CTZ:Ch weight ratio of 0.86:1, in order to obtain NCL-Ch/CTZ, CL-Ch/CTZ, NCL-Ch/PNVCL 3:1/CTZ or NCL-Ch/PNVCL 1:1/CTZ sponges.

2.5 Physicochemical characterization of sponges

Chemical characterization of sponges was performed by Fourier-transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) in the wave number region of 400–4000 cm^{-1} (scanning of 32, resolution 4) using a Perkin-Elmer Frontier spectrometer (Waltham, MA, USA), and proton nuclear magnetic resonance (^1H NMR) at a magnetic field of 9.5 T (corresponding to a frequency of 400 MHz for the hydrogen-1 nucleus) using a Bruker Avance III spectrometer (Billerica, MA, USA). Morphological characterization of the sponges was performed by field emission gun scanning electron microscopy (FEG-SEM) using a Supra 35-VP microscope (Carl Zeiss, Oberkochen, Germany). Sponges were further evaluated for their hardness using a TA.XT Plus texturometer (Stable Micro Systems, Godalming, UK) under compression mode, at room temperature. Samples were compressed up to 80 % of their original height at the rate of 1.2 mm/min and a constant force of 0.5 N. The quantification of CTZ content in sponges was performed by extracting the drug with 10 mL of DMSO (Thermo Fisher Scientific) under shaking (100 rpm) for 24 h. The extracts were then filtered using 0.22 μm filters and analyzed by UV spectroscopy at 262 nm. The method was assessed for linearity of the calibration curve ($R^2 = 0.9997$ for the linear regression in the range of 0.1 to 1 mg/mL), limit of detection (0.02 mg/mL) and limit of quantification (0.07 mg/mL). The percentages of association efficiency (AE%) and loading capacity (LC%) were calculated according to the following equations:

$$AE\% = \frac{CTZ_{recovered}}{CTZ_{initial}} \times 100 \quad (1)$$

$$LC\% = \frac{CTZ_{recovered}}{Weight\ of\ sponge} \times 100 \quad (2)$$

where $CTZ_{recovered}$ is the amount of drug recovered from extracts and $CTZ_{initial}$ is the amount of drug placed on each sponge.

2.6 Drug release from sponges

Sponges were tested for the ability to release CTZ in a simulated vaginal fluid (SVF) adapted from Owen & Katz (Owen and Katz, 1999). The fluid surrogate (1,000 mL) contained glucose (5.0 g), sodium chloride (3.51 g), lactic acid (2.0 g), potassium hydroxide (1.4 g), glacial acetic acid (1.0 g), urea (0.4 g), calcium hydroxide (0.222 g), glycerin (0.16 g), and enough sodium hydroxide to adjust pH to 4.2. Additionally, polysorbate 80 was added at 2 % (*w/w*) in order to assure sink condition. The drug release assay was performed by immersing sponges in 50 mL of release medium and maintained at 37 °C under shaking (50 rpm) using a Panasonic MIR-S100 orbital shaker (Kadoma, Japan). One milliliter of medium was collected and replaced with fresh medium at different time between 15 min and 24 h. Samples were filtered using 0.22 μm filters and analyzed by UV spectroscopy at 262 nm for CTZ quantification. The obtained drug release profiles were further fitted to different mathematical models in order to provide insights concerning the general release mechanisms of CTZ from sponges (Siepmann and Siepmann, 2008).

2.7 Cytotoxicity

The effect of CTZ and drug extracts obtained from sponges on the viability of two epithelial cell lines were determined by the resazurin reduction assay, in accordance to ISO 10993-5:2009 (International Organization for Standardization, 2009). Ca Ski and HEC-1-A cells were maintained in RPMI 1640 medium and McCoy's 5A medium, respectively, supplemented with 10 % (*v/v*) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin under standard cell culture conditions (37 °C, 95 % RH and 5 % CO_2). The medium was renewed every 2–3 days and cells used at 80–90 % confluence. Extracts were prepared by incubating sponges in cell culture medium at a surface-to-volume ratio of 3 cm^2/mL for 24 h under orbital shaking (100 rpm) (International Organization for Standardization, 2012).

Cells were placed in 96-well plates at a density of 5000 cells/well for 24 h in medium, before being incubated with extracts for an additional 24 h period. Cells were then washed with phosphate buffered saline (pH 7.4) and further incubated with 10 μg/mL resazurin for 2 h at 37 °C. The fluorescence of supernatants was measured at 590/530 nm and obtained values were used to calculate viability. CTZ at different concentrations, culture medium and 1 % (*w/v*) Triton X-100 were also tested as controls.

2.8 Antifungal activity

The anti-*Candida* activity was determined according to the microdilution broth assay from the CLSI M27-A4 reference protocol (Clinical and Laboratory Standards Institute, , 2017). Different *Candida* spp. ATCC strains were used and maintained in SDA at 37 °C, RH and 5 % CO_2 for 24 h before further use. In the case of sponges, extraction of CTZ was conducted using DMSO (Thermo Fisher Scientific) at a surface-to-volume ratio of 3 cm^2/mL for 24 h under orbital shaking (100 rpm). Extracts were then assayed for determining the content of CTZ and further diluted to 1 % (*v/v*) or less of DMSO with medium.

Candida spp. were dispersed in RMPI 1640 medium supplemented with MOPS (0.165 mol/L; final medium pH = 7.0) and plated in 96-well plates to a final density of 500 to 2,500 cells/well. Yeasts were allowed to incubate for 48 h at 37 °C, RH and 5 % CO_2 in the presence of a 2-fold dilution series of extracts or pure CTZ dispersed in medium. The minimum inhibitory concentration (MIC) was determined upon visual inspection for *Candida* spp. growth, while the minimal fungicidal concentration (MFC) was assessed after plating 20 μL of the content of wells featuring no apparent growth onto SDA and further incubating for 24 h (Facchinatto et al., 2021). The lowest concentration at which no growth was observed was considered as the MFC value.

Whole sponges were also tested for their antifungal activity by adapting the M44-A2 disk diffusion method from CLSI (Clinical and Laboratory Standards Institute, 2009). Briefly, yeasts were plated onto Petri dishes (150 mm in diameter) containing Mueller-Hinton Agar supplemented with 2 % dextrose and 0.5 μg/mL methylene blue. Sponges were trimmed to a cylinder of approximately 9 mm in diameter containing a total amount of 4.5 mg of CTZ, placed on top of the agar medium, and the plate was allowed to incubate for 48 h at 37 °C, RH and 5 % CO_2. Also, Whatman® assay disks (9 mm in diameter) impregnated with 4.5 mg of CTZ and tested for comparison purposes. The antifungal activity was then determined by measuring the diameter of the inhibition zone around sponges or disks. The uptake of water by sponges was also determined by comparing the weight before and after incubation.

2.9 Statistical analysis

Data are presented as mean ± standard deviation (SD) from three experiments, unless mentioned otherwise. Multiple comparisons were performed by one-way ANOVA with Bonferroni post-hoc test using OriginPro 2022 (Originlab, Northampton, MA, USA). Values of $p < 0.05$ were considered as denoting significant differences.

3 Results and discussion

3.1 Production and physical-chemical characterization of sponges

Ch has been extensively tested for the preparation of multiple drug dosage forms (e.g. gels) and delivery systems (e.g. polymer-coated liposomes) for intravaginal use, namely due to its mucoadhesive properties and low cytotoxicity (Andersen et al., 2015; Jøraholmen et al., 2014; Frank et al., 2017; Rossi et al., 2014; Frank et al., 2014; Pradines et al., 2015). Before starting producing sponges, we checked for the chemical identity and the degree of acetylation percentage (DA) of the Ch sample used by ^{1}H NMR. Typical spectra were obtained (*Supplementary Information, Fig. S1*), while the DA was calculated as 26 %, as previously described (Lopez et al., 2020). The successful synthesis of PNVCL was also confirmed by ^{1}H NMR, as evidenced by the disappearance of chemical shifts corresponding to the three protons in the vinyl group involved in monomer bonding formation (*Supplementary Information, Fig. S2*).

Ch-based sponges were successfully obtained by using the above described methodology. These are simple production methods that could be scaled-up. All samples were tested within a few weeks from preparation, but no organoleptic changes (visual and touch feeling) were noted upon up to five months storage at circa 20–25 °C and 40–60 % RH. Even if quantification of the drug content during this extended period was not performed, the dry-state nature of sponges may be effective in circumventing pH-dependent chemical degradation as observed for tablets containing Ch (Knapczyk, 1992), but not for liquid or semisolid formulations containing substantial amounts of water (Knapczyk, 1992; Abdel-Moety et al., 2002; Bachhav and Patravale, 2009; Borhade et al., 2012). Noticeably, sponges could incorporate high amounts of drug, in this case CTZ, which could be interesting if considering active ingredients that need larger quantities to be useful in managing VVC. We also conducted preliminary studies using methylene blue, a highly water-soluble drug showing potential for photodynamic therapy of infections by *Candida* spp. (de Carvalho Leonel et al., 2019), in order to test the ability of sponges for delivering different compounds. Methylene blue was successfully incorporated but testing with additional drugs is required in order to truly assess the versatility of the sponges. As expected, sponges based on NCL-Ch featured AE% for CTZ of 100 % (no washing step occurs during production), with mean LC% of 46 %, 39 % and 30 % for NCL-Ch/CTZ, NCL-Ch/PNVCL 3:1/CTZ and NCL-Ch/PNVCL 1:1/CTZ, respectively (SD below 1 % in all cases; $n = 3$). Curiously, the washing step required for removing excess ammonia before freeze-drying the gel into CL-Ch/CTZ did not affect AE% (102 % ± 9 %) and, thus, allowed achieving high LC% (51 % ± 5 %).

Regarding morphology, sponges featured regular cylindrical shape, corresponding to the mold geometry, except in the case of sponges crosslinked with ammonia (Fig. 2, **a-d**). In this last case, considerable retraction of the Ch matrix was apparent, leading to the formation of rough edges that may be detrimental to its convenient and comfortable intravaginal insertion and use (das Neves et al., 2008). The incorporation of CTZ, even at a high weight drug/Ch ratio of 0.86:1 did not cause changes at the macroscopic level. SEM analysis revealed homogeneous, honeycomb-like porous structure of sponges (Fig. 2, **e-h**), which can favor wettability in physiological media. NCL-Ch featured smaller pore size diameter than CL-Ch (Table 1). The use of ammonia as physical crosslinker also resulted in the formation of a more lamellar microstructure, in line with the findings of Azueta-Aguayo *et al.* for similar formulations (Azueta-Aguayo et al., 2022). The addition of PNVCL led to

Table 1
Pore diameter (mean ± SD) and size distribution (polydispersity index; PdI) of sponges, as assessed by SEM analysis.

Material	Diameter (μm)	PdI
NCL-Ch	168 ± 54	0.10
NCL-Ch/CTZ	177 ± 77	0.19
CL-Ch	346 ± 99	0.08
CL-Ch/CTZ	131 ± 127	0.94
NCL-Ch/PNVCL 3:1	167 ± 63	0.14
NCL-Ch/PNVCL 3:1/CTZ	137 ± 48	0.11
NCL-Ch/PNVCL 1:1	148 ± 59	0.16
NCL-Ch/PNVCL 1:1/CTZ	126 ± 62	0.24

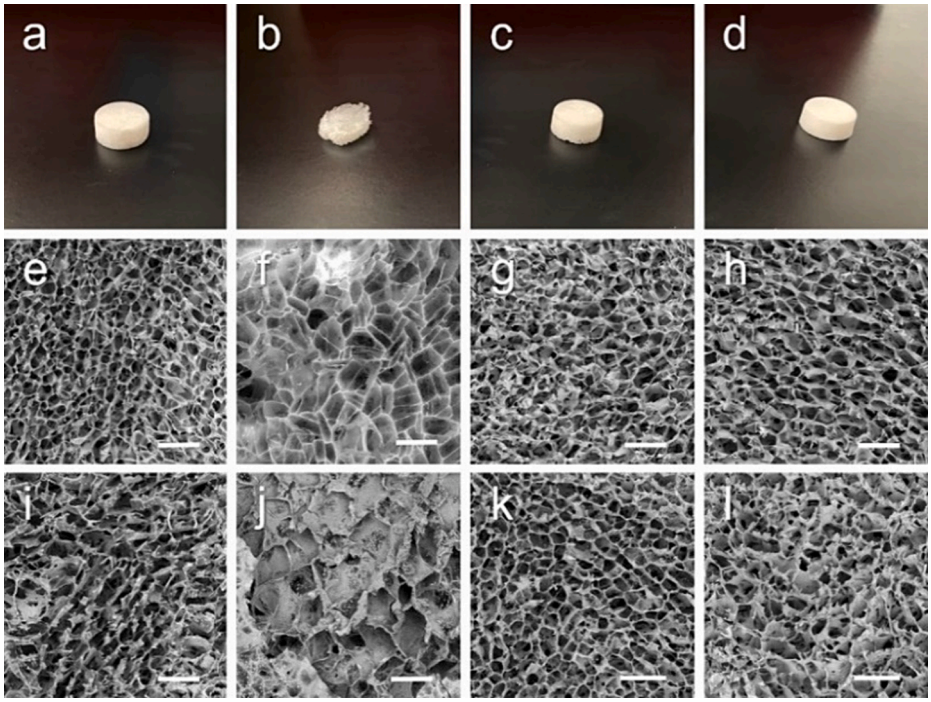

Fig. 2. Morphological analysis of sponges. Macroscopic features of **(a)** NCL-Ch, **(b)** CL-Ch, **(c)** NCL-Ch/PNVCL 3:1 and **(d)** NCL-Ch/PNVCL 1:1; and SEM imaging of **(e)** NCL-Ch, **(f)** CL-Ch, **(g)** NCL-Ch/PNVCL 3:1, **(h)** NCL-Ch/PNVCL 1:1, **(i)** NCL-Ch/CTZ, **(j)** CL-Ch/CTZ, **(k)** NCL-Ch/PNVCL 3:1/CTZ and **(l)** NCL-Ch/PNVCL 1:1/CTZ. **(e-l)** Scale bar = 500 μm.

similar porous structure as compared to NCL-Ch. The incorporation of CTZ had little effect on the porous structure of non-crosslinked meshes but led to considerable decrease in pore size and collapse of the polymer mesh in CL-Ch-based sponges (Fig. 2, **i-l**).

The mechanical properties of produced sponges were measured in order to assess their suitability for vaginal insertion. Solid-state pharmaceutical systems should be soft and compressible under mild forces (e.g. when lightly squeezed in-between two fingers (Fig. 3**a**)), not only to avoid mechanical injury to the vaginal mucosa, but also to be acceptable by potential users (Creatsas et al., 2002). Results for the compressive profile of different sponges are presented in Fig. 3**b**. The relatively low compressive stress values obtained for increasing strain are indicative that sponges are overall soft, and well below those previously described as tolerable for the vaginal mucosa (Rubod et al., 2008; Rynkevic et al., 2017). Compressive stress profiles were similar for all sponges, although CL-Ch featured lower stress values at higher strain. Also, the incorporation of higher amounts of PNVCL slightly increased the stiffness of the polymeric matrix. Interestingly, the incorporation of CTZ did not seem to affect mechanical features of sponges, except for the case of CL-Ch-based sponges, in line with their micromorphology features. Differences in values for the Young's modulus of different sponges at the linear elastic region further support these observations (Fig. 3**c**). The incorporation of CTZ only led to a significant increase in Young's modulus for NCL-Ch-based sponges, while crosslinking of Ch had no impact on such parameter. Higher stiffness was again noticeable for sponges with higher amounts of PNVCL, but the effect was apparently diminished when Ch dominated the matrix of sponges. The absolute differences in Young's modulus were mild and unlikely to substantially impact the overall tactile perception that potential user's may have of the different sponges.

Sponges were further analyzed by FTIR. No apparent chemical interactions occurred between the different materials used for producing sponges, as spectra denoted only absorption bands and peaks that are typical for individual materials (Fig. 4). The FTIR spectrum obtained for CTZ overlapped those previously described in other works (Yasir Siddique et al., 2021; Nematpour et al., 2020). Primary vibrational fingerprints of Ch are clearly illustrated by bands at 1,644 cm^{-1} (C═O asymmetric stretch ν_a of amide I in acetylglucosamine), 1,564 cm^{-1} (mixed N—H and C—N asymmetric stretch ν_a of amide II) and 1,152 cm^{-1} (asymmetric stretch ν_a of C—O—C bridge between saccharide units) (Dimzon and Knepper, 2015). No differences were observed for NCL-Ch and CL-Ch, which suggests that the crosslinking process does not alter the polymer chemical composition. The presence of PNVCL can be ascertain by the characteristic bands around 1,636 cm^{-1} that correspond to C═O (carbonyl) and C—N at 1,479 cm^{-1} (Marimuthu and Murugesan, 2019).

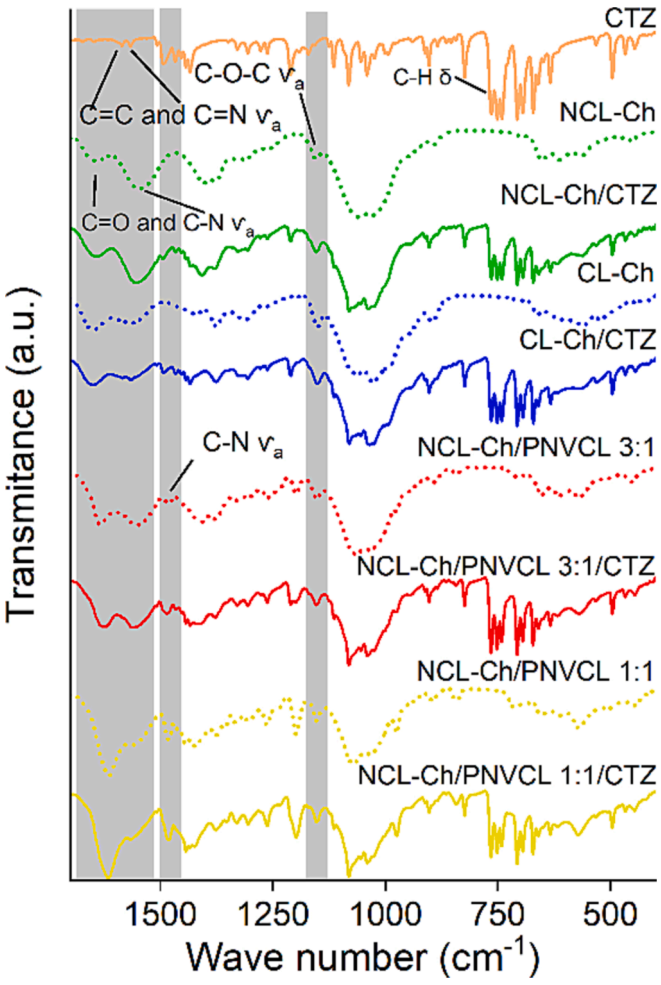

Fig. 4. Representative FTIR spectra of CTZ and sponges. Gray-shaded zones highlight main vibrational signals of Ch (C═O, C—N and C—O—C asymmetrical stretches).

3.2 Drug release from sponges

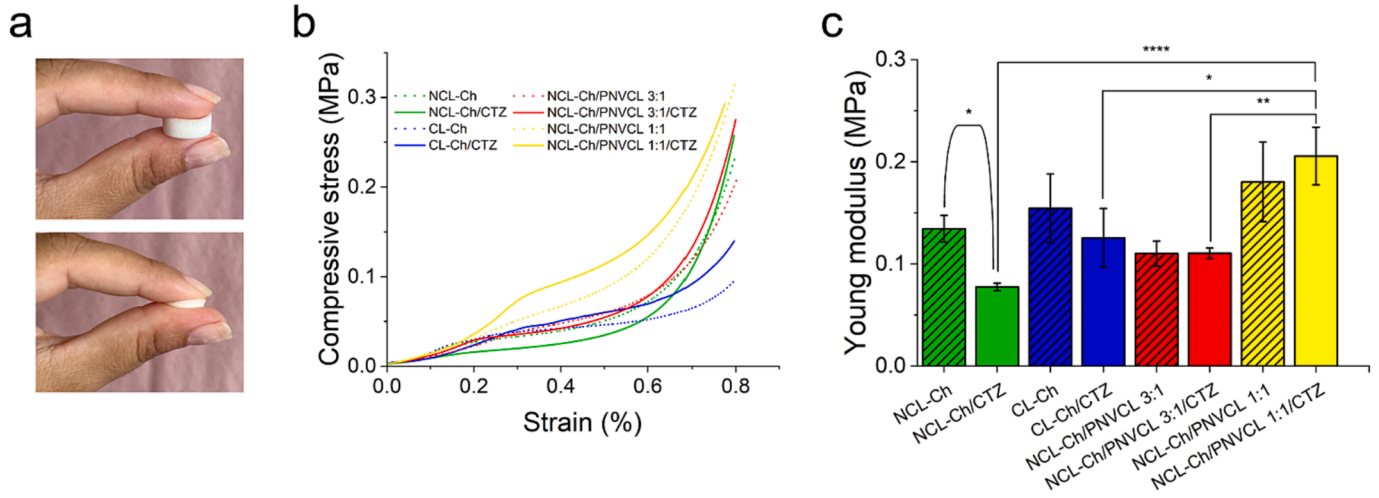

Fig. 3. Mechanical properties of sponges. **(a)** Squeezing of a NCL-Ch sponge between two fingers upon application of gentle force. **(b)** Compressive stress profiles (up to 80 % of initial height); and **(c)** and Young's modulus. (*), (**) and (***) denote p values less than 0.05, 0.01 and 0.001, respectively ($n = 3$).

Upon insertion into the vaginal canal, sponges should be able to quickly take up mucous fluids and release drug content. We studied these features *in vitro* by placing sponges into SVF at 37 °C under mild shaking (50 rpm). The addition of polysorbate 80 at a concentration of 2 % (*w/w*) was required in order to maintain sink conditions throughout the experiments. Sponges were able to rapidly absorb fluid, forming a gel-like body that eroded over time, typically within 4–8 h. *In vivo*, these features could help promoting vaginal spreading and terminal removal of sponges. Drug release profiles for the different sponges are presented in Fig. 5. All formulations appear to feature to some extent biphasic release kinetics, showing faster release up to 4–8 h, which decreased from that point on. This also correlated with the extensive disaggregation of the structure of sponges that was observed in this time window. CL-Ch/CTZ denoted faster and more extensive drug release (nearly complete after 4 h), contrasting with sponges based on non-crosslinked Ch, in particular NCL-Ch. This suggest that the primary amine group of glucosamine (which is involved in the crosslinking process with ammonia) counteracts the release of CTZ, likely due to electrostatic interaction. Moreover, the addition of PNVCL had an apparent effect of promoting faster and more extensive release of CTZ. Despite these differences, all sponges seem to be able to release their drug payload in a fashion that is compatible with their use in the management of VVC.

We further studied how obtained profiles adjust to different mathematical models commonly used for better understanding phenomena that may be involved in drug release (Siepmann and Siepmann, 2008). Drug release profiles of NCL-Ch/CTZ and CL-Ch/CTZ were best fit by the Higuchi (R^2 = 0.977) and Korsmeyer-Peppas (R^2 = 0.807) models, respectively (*Supplementary Information, Table S1*). This suggests that NCL-Ch/CTZ follows nearly diffusive behavior. In the case of CL-Ch/CTZ, the diffusional exponent (n = 0.300) of the Korsmeyer-Peppas model indicates that the main drug release mechanism involved is quasi-Fickian diffusion. Formulations based on NCL-Ch/PNVCL presented higher R^2 values (0.975 for 3:1 ratio and 0.901 for 1:1 ratio) when the Weibull model was used (*Supplementary Information, Table S1*). This indicates that the key mechanism governing release may be a combination of diffusion and matrix solubilization (PNVCL is soluble in water and also likely enhances the apparent solubility of CTZ).

One important note on drug release data relates to the translation of these *in vitro* results to the *in vivo* scenario, namely regarding the available volume of fluids in the vagina. This has been estimated at just up to one milliliter in healthy women (Mitchell et al., 2011), but can be substantially increased in women affected by VVC (Dorjey et al., 2022).

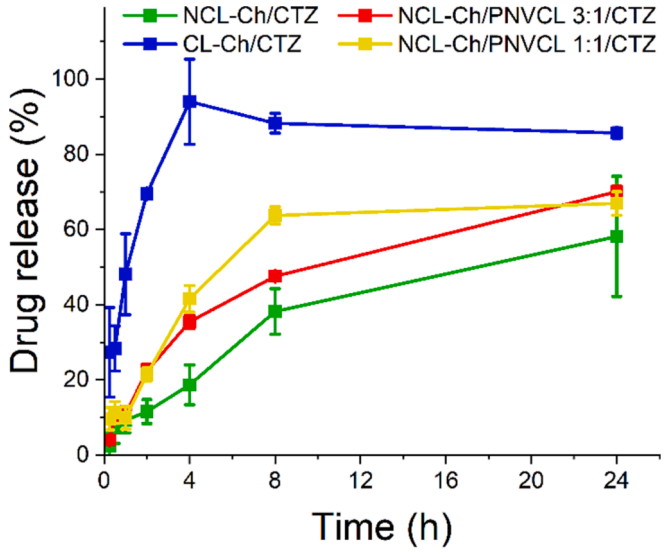

Fig. 5. Time-dependent drug release profiles of sponges. Results are presented as mean ± SD values (n = 3).

Notably, the acidic pH of vaginal fluids is well-known to be maintained during *Candida* spp. infection (Sobel, 1997). The scarce availability of fluids will likely limit the predictability of the hereby presented drug release data.

3.3 Cytotoxicity and antifungal activity of sponges

The effects of sponges on the viability of two genital cell lines was tested in order to early assess potential toxicity issues. HEC-1-A and Ca Ski cells are recognized as relevant *in vitro* models for testing excipients, drug delivery systems and dosage forms intended for vaginal use (Gali et al., 2010; Cunha et al., 2014; Dezzutti et al., 2012). Due to the solid-state nature of tested materials, we used extracts rather than intact sponges to assess viability *in vitro*, according to ISO 10993-5:2009 (International Organization for Standardization, 2009). Results are presented in Fig. 6. Viability of cells exposed for 24 h to extracts was kept at 70 % or above, thus indicating that the sponges (with or without CTZ) should be safe for medical purposes.

We then proceeded to testing the antifungal activity of the sponges, in particular of the drug after release, against a panel of *Candida* spp. with different sensitivity to azoles. Due to the low solubility of CTZ in aqueous media and possible unspecific inhibition of the growth of *Candida* spp. by most organic solvents or surfactants, DMSO (Thermo Fisher Scientific) was used to extract the drug to the maximum extension possible, while keeping its content in final medium dilutions below toxic levels. Results of MIC and MFC were determined according to the clinically ant CLSI M27-A4 reference protocol (Clinical and Laboratory Standards Institute, , 2017), and are presented in Table 2. MIC and MFC values for extracts were generally in line with those for the free drug, with minimal differences (within one to two $\log_2$ dilutions) (Berkow et al., 2020). This indicates that the incorporation of CTZ in sponges does not seem to affect its antifungal activity. The exception was the three-fold increase in MIC values for CL-Ch/CTZ in the case of azole-resistant *C. albicans* ATCC 64550.

Additionally, there was no apparent effect of polymers that might also have been extracted in *Candida* spp. growth, despite the numerous previous reports on the antifungal activity of Ch (Tajdini et al., 2010;

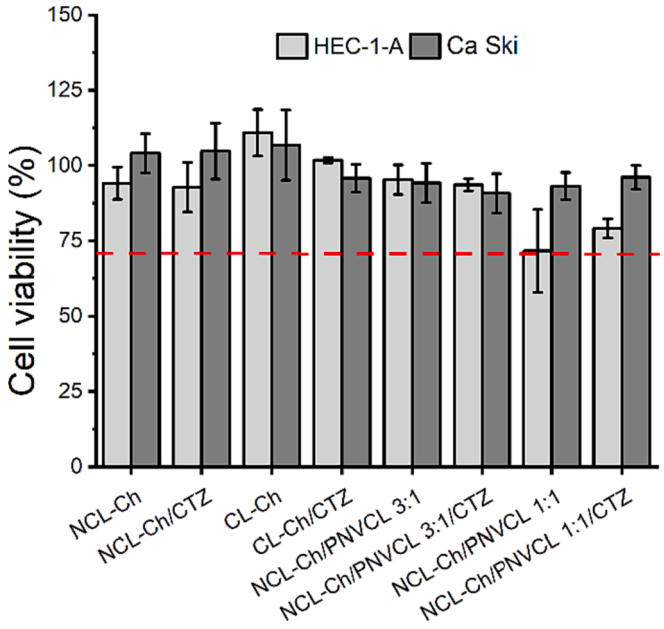

Fig. 6. Viability HEC-1-A and Ca Ski cells upon 24 h incubation with extracts from sponges. Results are presented as mean ± SD values (n = 3). The horizontal dashed red line indicates the 70 % viability threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Anti-*Candida* activity of CTZ extracted from sponges. Results are presented as values of MIC and MFC (expressed in μg/mL; $n = 3$) against different *Candida* spp. strains. Resistance profile to fluconazole determined according to the CLSI M27-A4 reference protocol is provided for comparison purposes (S, susceptible; S-DD, dose-dependent susceptibility; R, resistant).

Strains	Fluconazole susceptibility	CTZ		NCL-Ch/CTZ		CL-Ch/CTZ		NCL-Ch/PNVCL 3:1/CTZ		NCL-Ch/PNVCL 1:1/CTZ	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans ATCC 90028	S	0.5	8	2	16	0.5	16	2	16	2	16
C. albicans ATCC SC5314	S	0.5	32	0.5	>16	0.5	32	0.5	>16	0.5	>16
C. albicans ATCC 64550	R	2	256	8	>32	16	>32	8	>32	8	>32
C. tropicalis ATCC 750	S	8	16	8	32	16	32	8	32	4	32
C. glabrata ATCC 2001	S-DD	2	16	4	>16	8	>16	4	16	4	16
C. krusei ATCC 6258	S-DD	2	4	4	4	4	4	4	4	4	4

Tayel and Moussa, 2010; Arias et al., 2020; Alburquenque et al., 2010; Garcia et al., 2018). Oligomers of NVCL have been reported as possessing activity against *C. albicans* (Lee et al., 2018), but the potential antifungal activity of PNVCL remains elusive. Preliminary testing using extracts obtained from sponges without the incorporation of CTZ also support that Ch and PNVCL used in our study did not show noticeable anti-*Candida* activity (data not presented).

We further complemented the microdilution broth assay with a modified disk diffusion test (Clinical and Laboratory Standards Institute, 2009). Contrary to the former, sponges can be directly used and compared to paper disks routinely used as support medium for drugs in the previous method. The most appealing feature of such approach relates to the possibility to account for drug release kinetics into the agar growth medium in antifungal results. The *Candida* spp. growth inhibition zone diameters for CTZ-loaded sponges are presented in Fig. 7. Values were generally lower for all sponges as compared to paper disks impregnated with the same amount of CTZ (4.5 mg), but agreed in terms of azole susceptibility categories according to the CLSI M44-A2 disk diffusion method (Clinical and Laboratory Standards Institute, 2009): susceptible (≥19 mm) for *C. albicans* ATCC 90028, *C. albicans* ATCC SC5314, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258; dose-dependent susceptibility (15–18 mm) for *C. glabrata* ATCC 2001; and resistant (diameter ≤ 14 mm) for *C. albicans* ATCC 64550. Such results suggest that the sponges are able to somewhat limit the fast release of the CTZ, which could contribute to a more sustained effect *in vivo*. When comparing among different sponges, there was a trend towards larger inhibition zones in the case of formulations containing PNVCL. There was no apparent correlation between these observations and the release data presented above. However, we found that sponges containing PNVCL took up larger amounts of fluid from the agar medium (approximately 11-times the initial weight of sponge) as compared to those of chitosan only (6- to 7-fold). This could justify faster solubilization of CTZ within the sponge matrix and consequently faster release (Soares and Zunino, 2010).

4 Conclusions

In the present study, we have successfully produced and characterized *in vitro* different formulations of Ch-based sponges loaded with CTZ that have the potential to be used in management of VVC. Sponges were able to incorporate high amounts of drug (up to roughly 50 %, *w/w*) and release it in a tunable fashion by adding a water-soluble polymer, i.e. PNVCL. Importantly, sponges – particularly those based on NCL-Ch – seem to present adequate organoleptic, mechanical and low cytotoxicity that are compatible with comfortable and safe vaginal use. Sponges were able to generally retain the ability of the drug to act as a potent anti-infective agent, while the modulation of its release did not appear to have compromised the rapid onset of antifungal action. Overall, the proposed sponge formulations based on NCL-Ch may be an interesting addition to the therapeutic armamentarium of VVC. Additional animal *in vivo* characterization efforts regarding safety and efficacy are required in order to move forward these sponges in the development pipeline.

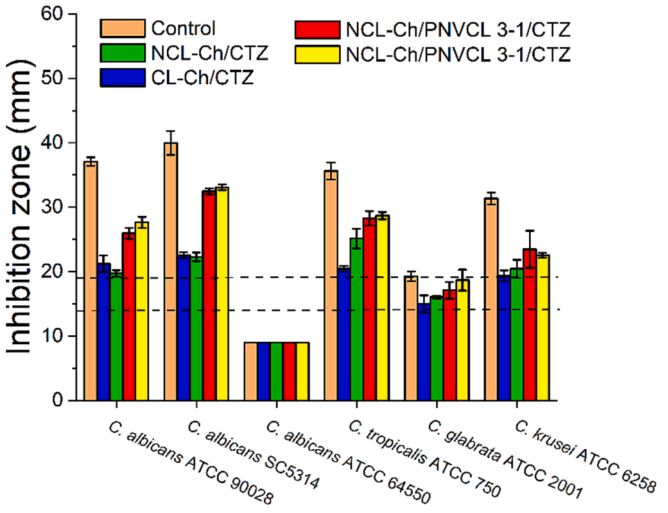

Fig. 7. Anti-*Candida* activity of CTZ-loaded sponges as determined by the disk diffusion method. Results are presented as mean ± SD values ($n = 3$). The horizontal dashed lines indicate azole susceptibility (≥19 mm) and resistance (≤14 mm) threshold levels.

CRediT authorship contribution statement

Fiama Martins: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. **Daniella L. Morgado:** Conceptualization, Investigation, Writing – review & editing. **Bruno Sarmento:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Emerson R. de Camargo:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **José das Neves:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.123508.

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