Investigating the effect of graphene oxide in chitosan/alginate-based foams on the release and antifungal activity of clotrimazole in vitro

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A R T I C L E   I N F O

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A B S T R A C T

Polyelectrolyte complexes (PECs) have been used as the matrix of solid foams for drug delivery. This study aimed at investigating the effect of graphene oxide (GO) and the composition of excipients in chitosan/alginate-based buccal foams on the clotrimazole release and antifungal activities. The investigation has been focused on the interactions of the drug with excipients in the foams, and the changes of ionization degree upon exposure to various media are discussed. The solid foams were prepared by mixing the excipients and clotrimazole via probe sonication, followed by a freeze-drying method. The pH values of the formulations were measured during the foam preparation process to estimate the ionization degree of clotrimazole and the other excipients. The foam matrix was the PECs between the cationic chitosan and anionic alginate. The mechanical strength of clotrimazole-loaded foams was lower than that of drug-free foams due to the positively charged clotrimazole interacting with the anionic alginate and interfering the PECs between chitosan and alginate. Addition of GO in the clotrimazole-loaded matrix made the foams mechanically stronger and contributed to a faster release of clotrimazole from the buccal foams by disrupting the electrostatic interactions between alginate and clotrimazole. However, addition of 1 wt% GO in the formulations didn’t affect the antifungal activity of clotrimazole-loaded foams significantly. A lower amount GO in the formulation may be required for enhancing the antifungal effect, which should be further investigated in future.

1. Introduction

The effect of excipient-drug interactions on the pharmacokinetics of drug molecules and the formulation quality has been discussed previously with focus on the drug permeability and adsorption (Gerber et al., 2018). Some poorly water-soluble drugs are ionizable and their solubility is pH-dependent (Göke et al., 2018; Kalepu and Nekkanti, 2015). The release behavior of an active pharmaceutical ingredient may be affected by the ionization degree of the drug substance and excipients in the formulation. It is challenging to elucidate the changes of the ionic interactions upon the drug release process due to different molecular interactions may occur simultaneously. With the increasing number of poorly water-soluble drug candidates, however, it is very interesting to investigate the molecular interactions of drug substances with excipients to develop new polyelectrolyte formulations.

Local drug delivery to diseased tissues is an effective therapeutic strategy (Joraholmen et al., 2014). The potential of solid foams for local treatment of infections has been reported previously (Hegge et al., 2010; Michailidou et al., 2019). Porous drug carriers showed extended drug release with increased local drug concentration (Lepretre et al., 2009) and an increased effect of antibacterial agents (Song et al., 2015). A “trap and kill” effect to E. coli was observed in a cryogel due to the presence of macropores (Shirbin et al., 2016). The porosity of solid foams endows high surface area (Svagan et al., 2016), high drug loading (Boateng et al., 2010), and the possibilities for controlling drug release. Additionally, the release of drug substances could be controlled by adjusting the pore structure of solid foams (Chambre et al., 2020). The matrix of foams affects both the physical properties and drug release profile of solid foams. Different drug release profile was observed when lyophilized chitosan wafer was used for buccal drug delivery, comparing annealed chitosan wafer to the non-annealed one (Ayensu et al., 2012). Polyelectrolyte complexes (PECs) formed by chitosan and xanthan gum as the matrix significantly improved the rigidity of the foams comparing to that prepared from the individual polymers alone (Liu et al., 2011).

Graphene oxide (GO) is amphiphilic and contains different functional groups, i.e., epoxide, carbonyl, carboxyl, and hydroxyl groups (Kim et al., 2010). Addition of GO in formulations, such as sodium alginate fiber (He et al., 2012) and dry chitosan film (Han et al., 2011),

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increased the tensile strength. Additionally, the presence of GO in the formulations resulted in fast drug release (Islam et al., 2020) and a prolonged antifungal effect in vivo (J.-F. Huang et al., 2016). The aim of the present study was to incorporate GO into buccal foams made by chitosan and alginate as the PEC matrix to investigate the effect of the matrix on the release and antifungal activities of clotrimazole in vitro. Clotrimazole is a weak basic poorly water-soluble drug with a logP of 6.3 (Borhade et al., 2012), it is normally used for the treatment of oral candidiasis caused by Candida albicans (C. albicans) (Arendorf and Walker, 1980). In the present study, the buccal foams were prepared by freeze drying the PEs of chitosan and alginate with/without clotrimazole and/or GO, followed by characterization and evaluation of the foams in vitro.

2. Materials and methods

2.1. Materials

Clotrimazole (≥ 98.0%), graphene oxide suspension (GO, 2 mg/mL), chitosan (molecular weight: 190–310 kDa, 75–85% deacetylated, viscosity of 1% aqueous solution containing 1% acetic acid: 200–800 cP), alginic acid sodium salt (molecular weight: 190–310 kDa, viscosity of 1% aqueous solution containing 1% acetic acid: 200–800 cP), sabouraud dextrose broth (SDB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sabouraud dextrose agar (SDA) was obtained from Thermo Scientific (Waltham, MA, USA). Ethanol absolute (99.96%) for sample preparation and methanol (HPLC-grade) for analysis were obtained from VWR Chemicals (BDH, France). NaH₂PO₄•2H₂O for phosphate buffer was obtained from EMPROVE (Merck, Darmstadt, Germany). The reagents were of analytical grade and used without further purification. Ultrapure water from an Ultra Clear UV system (Evoqua Water Technologies, Pittsburgh, USA) was used throughout the study.

2.2. Preparation method of buccal foams

Buccal foams containing clotrimazole and the excipients chitosan, alginate and GO were prepared by a freeze-drying method as described previously (Michailidou et al., 2019). The PEC of chitosan and sodium alginate was used as the matrix and GO as a functional excipient, the formulations were prepared by mixing appropriate volumes of the individual excipient and the clotrimazole solution (Table 1). In short, the alginate solution (10 mg/mL in H₂O) was mixed with the GO suspension (2 mg/mL) and clotrimazole ethanol solution (8 mg/mL), followed by mixing with the chitosan solution (10 mg/mL in 1% acetic acid) using ultrasonic probe sonication in a pulse mode (2 s/2 s, 2 min, intensity: 100%) (Chemical Instruments AB, Sollentuna, Sweden). The mixtures were added to a flat bottom 24-well cell culture plate (plate: 15.7 mm) by pipetting and weighing, followed by frozen at −20 °C for 4 h and freeze-drying at −55 °C (condenser temperature) for 48 h (Telstar LyoQuest-55, Azbil Telstar Technologies S.L.U., Terrassa, Spain). The obtained buccal foams were placed in seal bags and stored in a desiccator at room temperature up to 3 months prior to usage.

2.3. Evaluation of mechanical properties of buccal foams

The mechanical properties of the foams were evaluated in a compression mode by a texture analyzer (TA. XT plus, Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell (Itinini et al., 2019). The diameter and thickness of the foams were measured using a digital caliper (Linear Tools Limited, Middlesex, UK) prior to the analysis (Fig. S1). The cylindrical foams (diameter: 14.6 ± 0.2 mm, thickness: 8.6 ± 0.8 mm) were placed on the support and a stainless steel cylindrical probe (12.8 mm in diameter) was set to punch the foam at a speed of 3 mm/s with a trigger force of 0.49 N at a test probe speed of 0.2 mm/s. The data were presented as maximum force (N) that the foam can withstand and work of compression (N·mm). The work of compression is the area under the curve, which was calculated by automatic integrated analysis using the Exponent software (TA. XT plus, Stable Micro Systems, Godalming, UK). The compression tests were performed in six repetitions (independent samples).

2.4. Scanning electron microscope (SEM)

The morphology of the bottom and the cross-sectional surface of the buccal foams were observed using SEM (TM3030, Hitachi, Japan). Samples were slightly cut by a scalpel and placed on a metal specimen stub and coated with gold in an argon atmosphere for 15 s prior to the imaging at an accelerating voltage of 5 kV and × 100–500 magnification. Representative images were selected from the observations of three repetitions.

2.5. In vitro drug release study

The release of clotrimazole from the foams (Table 1) was measured at 37 ± 0.5 °C using an USP apparatus II (709-DS Agilent, USA) and the modified method based on the report by Svagan et al. (Svagan et al., 2016). The release medium consisted of 0.01 M phosphate buffer (PB) pH 6.8. The release experiments were initiated by adding a foam (18.4 ± 0.8 mg) to the stainless-steel basket (mesh:10, diameter: 25 mm) and placed in the dissolution vessel containing 1000 mL preheated release medium. The foam samples had nearly cylindrical shapes with the thickness of 8.7 ± 0.9 mm and top (or bottom) area of 165.1 ± 0.1 mm². The rotation speed of the basket was set to 100 rpm. Samples of 1 mL were withdrawn at selected time intervals (0.5, 1, 2, 3, 4, 6, 8 and 24 h) and replaced with an equal volume of preheated release medium. Sink conditions were provided throughout the release study, the solubility of the drug in this media at 37 °C was determined to be 0.74 ± 0.04 mg/L. The release experiments were carried out in triplicate. The samples were analyzed by HPLC as described in the Section 2.6. The cumulative amount of clotrimazole released at the time t, Mₛ, was calculated using Eq (1):

Table 1

<table>
<thead>
<tr>
<th>Formulations</th>
<th>CS ml wt %</th>
<th>SA ml wt %</th>
<th>GO ml wt %</th>
<th>CTZ ml wt %</th>
<th>The weight ratio of CS/SA</th>
<th>The estimated Molar ratio of CS/SA</th>
<th>Foam characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>7.5</td>
<td>75.8</td>
<td>2.4</td>
<td>24.2</td>
<td>–</td>
<td>3:1</td>
<td>Diameter (mm) n=6</td>
</tr>
<tr>
<td>F2</td>
<td>7.5</td>
<td>72.8</td>
<td>2.4</td>
<td>23.3</td>
<td>–</td>
<td>3:1</td>
<td>Height (mm) n=6</td>
</tr>
<tr>
<td>F3</td>
<td>7.5</td>
<td>75.0</td>
<td>2.4</td>
<td>24.0</td>
<td>0.5</td>
<td>3:1</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>7.5</td>
<td>72.1</td>
<td>2.4</td>
<td>23.1</td>
<td>0.5</td>
<td>3:1</td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>5.0</td>
<td>47.6</td>
<td>2.4</td>
<td>47.6</td>
<td>0.5</td>
<td>3:1</td>
<td></td>
</tr>
<tr>
<td>F6</td>
<td>2.4</td>
<td>23.1</td>
<td>7.5</td>
<td>72.1</td>
<td>0.5</td>
<td>3:1</td>
<td></td>
</tr>
</tbody>
</table>

CS, chitosan; SA, sodium alginate; CTZ, clotrimazole; GO, graphene oxide. *The molar ratio of CS/SA is estimated from the weight ratio and the theoretical molecular weight of CS and SA.

\[ \text{Cumulative amount of clotrimazole released at time } t, M_s = \frac{t}{0.74} + 0.04 \]
\[ M_r = V_r \sum_{i=1}^{n} C_{n,i} + V_s C_n \]  

where \( V_r \) and \( V_s \) are the volume of the release medium in the vessel and the samples withdrawn at time \( t \), respectively. \( C_n \) represents the concentration of clotrimazole in the sample withdrawn from the vessel at time \( t \). \( C_{n,i} \) is the clotrimazole concentration in the previously withdrawn sample. The percentage of clotrimazole released was calculated as follows Eq (2):

\[ \% \text{released} = \frac{M_r}{M_T} \times 100\% \]  

where \( M_T \) is the total amount of clotrimazole in a foam applied in the release study.

2.6. HPLC method

The concentration of clotrimazole was analyzed by the previously described HPLC method (J. Huang et al., 2020) using an Elite LaChrom HPLC system consisting of a L-2130 pump connected to a L-2450 diode array detector and a L-2200 autosampler (VWR International, Tokyo, Japan). The chromatographic separation of clotrimazole was performed on a C18 column (Kinetex® 100 x 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) at 30 °C. The flow rate was set to 1 mL/min with a mobile phase of methanol:water (80:20, v/v). The column effluent was monitored at 229 nm for clotrimazole with the retention time of 2.9 min. The injection volume was 20 μL. The chromatograms were recorded and

Fig. 1. (a) pH changes in the formulations before freeze-drying, S stands for the preparation stage of the formulations. The composition of formulations is given in Table 1. (b) Calculated ionization degree of excipients and clotrimazole in the formulations (pH=4) before freeze drying, in the medium for time kill study (pH=5.6) and the medium for drug release study (pH=6.8). • • • positively charged; -- negatively charged; —— neutral. CS: chitosan, SA: alginate, CTZ, clotrimazole.
analyzed using EZChrom Elite software version 3.1.3 (VWR International, Tokyo, Japan). Quantification of clotrimazole was done by the use of a calibration curve, which was constructed by diluting a stock solution of clotrimazole (0.55 μg/mL) in 0.01 M PB at pH 6.8 and linear calibration curve (R² = 0.9995) in the range of 11 – 221 ng/mL was obtained. The detection limit (DL) and the quantification limit (QL) was

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**Fig. 2.** (a) Work of compression (N*mm) and (b) maximum force (N) of the buccal foams upon compression. (c) Representative compression profiles of the buccal foams (F1, F2, F3, F4). The composition of formulations is listed in Table 1. (**p** < 0.05; (**p** < 0.01; (**p** < 0.001. Means ± S.D., n = 6.)

**Fig. 3.** SEM images of the bottom surface and the cross section of the buccal foams. The composition of the foams is given in Table 1.
5.7 ng/mL and 11.0 ng/mL, respectively. Relative standard deviations less than 2% were obtained from triplicate injections of each standard solution.

2.7. Determination of water uptake ability of foams

The effect of excipients on the water uptake property of the foams (Table S1) was studied by design of experiment (MODDE Pro-version 12.1 software, Umetrics, Sweden). The composition of excipients was selected as variables: chitosan (weight percent, 0.15–0.25), alginate (weight percent, 0.75–0.85) and GO (weight percent, 0.001–0.01). The water uptake ability of the buccal foams (Fig. S2) was analyzed by the mass change of the foams before and after water absorption based on the method for buccal films described by Abruzzo et al. with minor modifications (Abruzzo et al., 2012). In short, a dried buccal foam was weighed \( m_0 \) on an analytic balance (XA105 DualRange, Mettler Toledo, Viroflay, France) and placed in flat bottom 6-well cell culture plates (Corning, USA) containing 5.25 mL PB solution (0.01 M, pH 6.8). The plates were placed in a compact shaker (KS 15, Edmund Bühler GmbH, Germany) and shaken at 100 rpm at 37 ± 0.1 °C for 1 h. After the experiment, the remaining part of foams were separated from the buffer using cell strainers (diameter: 23.1 mm, FalconTM, Corning, USA), the excess surface water was carefully removed by filter paper and the foams were weighted \( m_f \) afterwards.

The water uptake (%) was calculated according to Eq. (3).

\[
\text{Water uptake} (\%) = \frac{m_f - m_0}{m_0} \times 100
\]

A quadratic equation was used for the model fitting with significant model terms and the related \( R^2 \) and \( Q^2 \) (Table S1).

2.8. Time kill kinetics of buccal foams against Candida albicans

The time-kill experiments were performed following the method reported by Cantón et al. with minor modifications (Cantón et al., 2004). Briefly, one colony of \( C. \) albicans (American Type Culture Collection, 10, 231, Manassas, VA, USA) from an overnight culture was transferred to the broth and was incubated on a compact shaker (KS 15, Edmund Bühler GmbH, Germany) at 100 rpm and 37 ± 0.1 °C for 16 h to reach the exponential growth phase. The optical density of the \( C. \) albicans was adjusted spectrophotometrically to 530 nm (OD530) to approximately 0.052, corresponding to \( 5 \times 10^6 \) colony-forming unit (CFU)/mL, by Agilent dual-channel Cary 60 UV spectrophotometer equipped with a diffuse reflection probe from Harrick Scientific Products (USA). Buccal foams were soaked and suspended in 5.0 mL fungi-cultured SDB (pH 5.6) in a flat bottom 6-well cell culture plates (Eppendorf, Hamburg, Germany) and the plates were incubated on a horizontal compact shaker (KS 15, Edmund Bühler GmbH, Germany) at 37 ± 0.1 °C under 75 rpm shaking. A fungal inoculum without foam was used as a growth control. The number of viable fungi was recorded at preset time (0.5, 1, 2, 4, 6, and 24 h) by culturing 100 μL aliquots on sterile SDA plates which were subsequently incubated at 37 ± 0.1 °C for 24 h. Spot plating was performed in triplicate for each time point by transferring 10 μL of serial 10-fold dilutions of cell culture from flat bottom 96-well cell culture plates (Eppendorf, Hamburg, Germany) to the agar plates. After 24 h, the resulting colonies was counted as CFU/mL and plotted for each time point triplicate.

2.9. Statistics

Data were presented as mean ± standard deviation (SD) unless otherwise specified. Statistical analysis was performed by using student’s unpaired t-test (GraphPad Prism® 9.2.0, GraphPad Software, San Diego, CA, USA), \( p \)-values lower than 0.05 were considered significant.

The drug release data from the foams were evaluated using the Korsmeyer–Peppas, Higuchi equations (Table S2) with the regression model of OriginPro® 2020 (64-bit) SR1 9.7.0.188 (OriginLab, MA, USA).

3. Results and discussion

Solid foams can be applied for localized delivery of antifungal agents to the infected sites. In the present study, a new concept of incorporating the interesting excipient GO in buccal foams was evaluated for its effect on the mechanical property, morphology, drug release and antifungal property of foams in vitro. Additionally, design of experiment was used to investigate the effect of excipients with focus on the weight ratio of chitosan/alginate in the PECs.

3.1. Effect of pH on the ionisation degree of clotrimazole and excipients

In the present study, the pH of the formulations was measured in each stage (S) during the preparation process of the foams before the freeze-drying (Fig. 1a), in order to estimate the ionization degree of clotrimazole and excipients, as well as potential molecular interactions between them. The composition of formulations is listed in Table 1. All the formulations were prepared by adding the other components into alginate solution, so the pH of the alginate solution (pH~7.8) is regarded as the pH of the stage 1 (S1) (Fig. 1a). The pH value of S2 represents the measured pH after adding another component into the alginate solution. Similarly, the pH of S3 and S4 represents the measured pH after adding another component afterwards into the formulations.
The reference foam (F1) was prepared by adding chitosan solution (pH 4.0) to alginate solution, and the pH value was ended as 4.3 at the S2. The foams containing clotrimazole (F2) or GO (F3) was prepared by addition of clotrimazole ethanol solution or GO suspension (pH 2.6) to the alginate solution, the pH value at S2 was reduced to 6.0 (F2) and 5.2 (F3), respectively. The pH at S3 were ended around 4 for both F2 and F3 after addition of chitosan solution. The foam containing both GO and clotrimazole (F4) was prepared by adding GO, clotrimazole, and chitosan to the alginate solution, the pH value at S3 and S4 was 5.5 and 4, respectively.

The ionic interactions of excipients in formulations can be estimated based on the $pK_a$ value of each substance (Patel et al., 2021). In the present study, the ionization degree of the major components in the formulations is estimated based on the measured pH and the pH of the medium for drug release study and the antifungal test (Fig. 1b). The $pK_a$ value of alginate is 3.5 (Gu et al., 2004), therefore alginate was primarily

Fig. 6. Schematic illustration of the molecular interactions in the foams at different ratios of chitosan (CS) and alginate (SA), and graphene oxide (GO) level in drug release and antifungal activity. Full composition of the foams is shown in Table 1.
in its anionic form in the formulation (pH 4) before the freeze drying. The pKᵯ value of chitosan is 6.5 (Wang et al., 2006). The PECs were formed between the anionic form of alginate and the cationic form of chitosan in the formulations. The pKᵯ of GO is 4.1 for the carboxylic acid groups, and pKᵯ of GO is 6.5 for the other less acidic groups (Orth et al., 2016), therefore negatively charged GO existed in the formulations (pH = 4). Clotrimazole is a weak base with a pKᵯ value around 6.0 (Bendels et al., 2006; Shalaeva et al., 2008). Around 99% of clotrimazole was in its cationic form in the formulations (pH = 4). Molecular interactions may occur between clotrimazole and the excipients during the preparation and evaluation processes for the foams, affecting the drug release from the foams and the antifungal activity in vitro.

3.2. The mechanical properties and morphology of the buccal foams

The mechanical properties of the foams were evaluated by the compression analysis. The foams with a chitosan/alginate weight ratio of 3:1 (F1-F4 in Table 1) were used to evaluate the influence of GO and clotrimazole. Addition of clotrimazole in the chitosan/alginate matrix (F2) decreased the work of compression and the maximum force of the foam (Fig. 2a & 2b, F2 vs F1, p < 0.001). Addition of GO in the matrix (F3) did not affect the work of compression significantly, neither the maximum force of the foam (Fig. 2a & 2b, F3 vs F1, p > 0.05). However, addition of GO in the clotrimazole-loaded formulation (F4) significantly increased the work of compression and the maximum force of the foam (Fig. 2a & 2b, F4 vs F2, p < 0.05), probably due to the fact that GO enhanced the ionic interactions between chitosan and alginate by disrupting the interactions between clotrimazole and alginate. Similar work of compression and maximum force of the foams were observed when the weight ratio of chitosan/alginate was changed from 3:1 (F4) to 1:3 (F6) in the presence of the same amount of GO and clotrimazole, suggesting that the change of the chitosan and alginate matrix has no significant effect on the mechanical properties.

The suggested alteration of molecular interactions in the foams is supported by the changes of foam thickness. Addition of clotrimazole to the foam matrix without GO resulted in an increase of the thickness from 8.4 ± 0.2 to 10.2 ± 0.5 mm (Table 1, F1 vs F2, p < 0.001), suggesting that the electrostatic force between chitosan and alginate was affected in the presence of clotrimazole. Addition of GO in the clotrimazole-loaded foam (F4) led to a decrease of the thickness of foam from 10.2 ± 0.5 to 7.6 ± 0.5 mm (Table 1, F2 vs F4, p < 0.001), probably due to the electrostatic force between chitosan and alginate in the foam was restored in the presence of GO.

The SEM images of the bottom and cross section surface of the foams showed the porous structure changes for the different formulations (Fig. 3). Incorporation of clotrimazole in the PEC matrix (F2) decreased the diameter of pores in the foams (Fig. 3, F2 vs F1), and the work of compression and the maximum force of the foam were also reduced in the presence of clotrimazole (Fig. 2). The results suggest that clotrimazole may interact with alginate and disrupt the electrostatic interactions between chitosan and alginate. It has been reported that the pore size of chitosan hydrogel was decreased after regenerated in ethanol solution (Huang et al., 2017), therefore, the effect of ethanol on the reduced pore size of the foams could not be neglected. Addition of GO to the clotrimazole-loaded PEC matrix resulted in larger pore size (Fig. 3, F4 vs F2), even though GO did not affect the porous structure of the drug-free foams (Fig. 3, F3 vs F1).

3.3. Release of clotrimazole from buccal foams in vitro

The effect of GO and the foam matrix, i.e., the weight ratio of chitosan/alginate, on the release profile of clotrimazole was investigated. Addition of GO in the foams at a weight ratio of chitosan/alginate 3:1 led to a significant increase in the rate of drug release (p < 0.01), 2.5% vs 7.3% of the drug was released after 4 h from F2 and F4, respectively (Fig. 4). The rate of drug release was further increased when the chitosan/alginate weight ratio in the GO-loaded foams changed from 3:1 (F4) to 1:3 (F6) (p < 0.05), even though no significant difference was observed when the ratio changed from 3:1 (F4) to 1:1 (F5) (p > 0.05). A few studies have been reported for the mechanism of drug release from polymeric matrices, in which ionic interactions were occurring between the polymer matrix and drug molecules (Lin and Metters, 2006; Ungolla and Jayasuriya, 2018). The interactions of drug-excipient, excipient-excipient can cause diffusion obstacles for the release of drug substances (Alvarez-Mancenido et al., 2008). Boughdir et al. reported that the release of mitoxantrone from the cross-linked oxidized alginate was controlled by the degradation of the hydrogel matrix and the ionic bindings between mitoxantrone and alginate (Boughdir et al., 2001). In the present study, addition of GO in the foams might disrupt the ionic interactions between alginate and clotrimazole, therefore the drug molecules could diffuse faster through the foam matrix containing GO. The water uptake ability of the foams was studied in order to gain a better understanding on the mechanism of drug release. It has previously been reported that the hydrophilic GO sheets may increase the diffusion coefficient of the fluorescein sodium in chitosan nanocomposites (Justin and Chen, 2014). The present study showed that the content of chitosan and GO in the foams positively affect their water uptake ability (Fig. S2, Table S1). The increased water uptake ability of foams with GO might enhance drug diffusion into the matrix of foams, leading to a faster drug release.

The foams without GO (F2) exhibited an initial burst release of clotrimazole, followed by a near zero-order release over 24 h (Table S3), whereas the drug release from the foams containing GO can be divided into three phases (Fig. 4). The initial burst phase (0 – 0.5 h) was observed for all the foams, reflecting drug release from the surface of the foams. The release rate of clotrimazole from the foams containing GO (F4-F6) decreased between 0.5 - 4 h, and it followed a near-zero order release kinetic afterwards (4 – 24 h). These release characteristics were in line with the previous report about in vitro release study of solid cellulose foams containing poorly soluble drug furosemide (Svagan et al., 2017). The rate constants were calculated from the slopes of the percent cumulative release versus release time profiles. It increased from 0.18 to 0.36%/h after addition of GO in the clotrimazole-loaded foams (F4 vs F2, Table S3 & S5), probable due to that the presence of GO disturbed the interaction of clotrimazole with alginate, and reduced the diffusion barrier for the drug molecules (Hu et al., 2016). Additionally, the time for drug substances diffuse through a polymeric hydrogel matrix depends on the diffusion length (Li and Mooney, 2016). The thickness of foam without GO (F2) was 10.2 ± 0.5 mm, higher than that of the foam with GO (F4, 7.6 ± 0.5 mm) (Table 1); addition of GO in the formulations led to a reduced diffusion length in the foam and faster drug release.

The rate of drug release was also affected by the PEC matrix in the foam due to the solubility difference of alginate and chitosan in the medium. Alginate is a water-soluble polymer, whereas chitosan has a limited solubility in the PB (pH= 6.8). The weight ratio of chitosan/alginate in the formulation F4 and F6 was 3:1 and 1:3, respectively. The rate constant of drug release from the foam F4 and F6 was calculated to be 0.36 and 0.85%/h, respectively. Increased content of alginate in the formulations (F6) led to a faster drug release (Fig. 4). It has been reported that the hydrated alginate-based matrix tablets were swollen in the PB (pH 6.8) and formed a viscous gel (Sriamornsak et al., 2007). The interactions of drug-excipient, excipient-excipient can cause diffusion obstacles for the release of drug substances (Alvarez-Mancenido et al., 2008). Boughdir et al. reported that the release of mitoxantrone from the cross-linked oxidized alginate was controlled by the degradation of the hydrogel matrix and the ionic bindings between mitoxantrone and alginate (Boughdir et al., 2001). In the present study, addition of GO in the foams might disrupt the ionic interactions between alginate and clotrimazole, therefore the drug molecules could diffuse faster through the foam matrix containing GO. The water uptake ability of the foams was studied in order to gain a better understanding on the mechanism of drug release. It has previously been reported that the hydrophilic GO sheets may increase the diffusion coefficient of the fluorescein sodium in chitosan nanocomposites (Justin and Chen, 2014). The present study showed that the content of chitosan and GO in the foams positively affect their water uptake ability (Fig. S2, Table S1). The increased water uptake ability of foams with GO might enhance drug diffusion into the matrix of foams, leading to a faster drug release.

3.4. In vitro antifungal activity of buccal foams

The antifungal effect of selected foams was evaluated by a time-kill study. The effect of GO was investigated using clotrimazole-loaded...
foams containing a weight ratio of chitosan/alginate 3:1 with GO (F4) and without GO (F2). GO suspension alone didn’t show any significant effect against C. albicans (Fig. S3). The antifungal effect of clotrimazole-loaded foams was not affected significantly in the presence of GO, i.e., a similar antifungal effect was found for the foams at a drug dose of 0.8 mg (Fig. 5, F2 vs. F4, p > 0.05). A similar effect of GO was observed at a lower drug dose of 0.4 mg (Fig. S3). The higher dose of clotrimazole, however, led to a faster reduction of CFU values.

The effect of the foam matrix was also evaluated by comparing the antifungal activities of clotrimazole-loaded foams with a different weight ratio of chitosan/alginate. The foams containing a weight ratio of chitosan/alginate 3:1 (F4) had a stronger antifungal effect (Fig. 5, F4 vs F6, p < 0.05). No antifungal effect was observed for the clotrimazole-loaded foam with a chitosan/alginate weight ratio of 1:3, probably due to that the protonated clotrimazole was bonded to the anionic alginate in the foam matrix containing more alginate.

The molecular interactions between excipients and drug substances may depend on their pH values and their ionization degrees (Fung et al., 2018), that can affect both the release and antifungal effect of clotrimazole. To evaluate the molecular interactions, the molar ratio of CS/SA was estimated from their molecular weight and weight ratio (Maestrelli et al., 2018). The weight ratio of chitosan/alginate in 3:1, 1:3 corresponds to a molar ratio of 2:1, 1:5, respectively. Fig. 6 briefly illustrates the effect of molecular interactions on drug release and antifungal effects of the foams with an estimated chitosan/alginate molar ratio of 2:1.

PECs between the cationic chitosan and anionic alginate were formed as the matrix of the buccal foams (F1). The incorporation of clotrimazole to the PECs matrix led to a slower reduction of CFU values, compared to the drug alone against C. albicans (Fig. S3), indicating that the protonated clotrimazole may interact with the anionic alginate in the formulation (F2). The interaction of drug and alginate could interfere the PECs between chitosan and alginate. Addition of GO to the formulations (F4) may disrupt the interactions between alginate and clotrimazole, due to the interaction of GO with clotrimazole. No significant difference was observed for the foams with/without GO in the time-kill study, suggesting that the positively charged clotrimazole may interact with the negatively charged GO at pH 5.6. Therefore, the clotrimazole-loaded foams composed of chitosan/alginate at a molar ratio of 2:1 exhibited similar antifungal effect, independent on the presence of GO. The foams (F6) with more alginate (a molar ratio of CS/SA in 1:5), however, had no antifungal effect, most likely due to clotrimazole being retained in the foams because of its electrostatic interactions with alginate (Fig. 6).

The different effect of GO on drug release and antifungal activity of clotrimazole-loaded foams is likely due to the fact that the pH values of the media were different, leading to varied degree of ionization of clotrimazole and excipients. Therefore, no significant antifungal effect was observed for the foams (F6) with a chitosan/alginate molar ratio of 1:5 (Fig. 5), although a faster drug release from F6 was observed than that from the foams (F4) with a chitosan/alginate molar ratio 2:1 (Fig. 4).

4. Conclusion

Buccal foams containing clotrimazole were successfully prepared using a freeze-drying method and the PEC of chitosan and alginate as the foam matrix. The pH values of the formulations were measured during the foam preparation process to evaluate the ionization degree of clotrimazole and the excipients as well as the potential molecular interactions between them. Addition of GO in the clotrimazole-loaded formulations made the foams mechanically stronger and increased the rate of drug release from the foams by disrupting the electrostatic interactions between the anionic alginate and protonated clotrimazole. The ratio between chitosan and alginate in the foams affected both the release and the antifungal effect of clotrimazole due to altered interactions between clotrimazole and the excipients. Addition of 1wt% GO in the formulations didn’t affect the antifungal effect of clotrimazole-loaded foams significantly. A suitable amount of GO in the formulations may be required to improve the antifungal effect, which should be investigated in future.

CRediT authorship contribution statement

J. Huang: Conceptualization, Data curation, Writing – original draft.
J. Jacobsen: Methodology. N. Genina: Methodology, Writing – review & editing. S.W. Larsen: Methodology, Writing – review & editing. H.M. Nielsen: Supervision, Writing – review & editing. A. Müllertz: Supervision. H. Mu: Conceptualization, Methodology, Supervision, Writing – review & editing.

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Supplementary materials

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References
