

Evaluation of chitosan based vaginal bioadhesive gel formulations for antifungal drugs

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The aim of the present study was to evaluate chitosan as a vaginal mucoadhesive gel base for econazole nitrate and miconazole nitrate. To this aim, different types of chitosan with different molecular masses and viscosity properties [low molecular mass chitosan (viscosity: 20,000 mPa s), medium molecular mass chitosan (viscosity: 200,000 mPa s), high molecular mass chitosan (viscosity: 800,000 mPa s)] have been used. First, rheological studies were conducted on chitosan gels. Mechanical, syringeability and mucoadhesive properties of chitosan gels were determined. Release profiles of econazole nitrate and miconazole nitrate from chitosan gels were obtained and evaluated kinetically. In addition, anticandidal activities of formulations were determined. Finally, vaginal retention of chitosan gels in rats was evaluated by *in vivo* distribution studies. Based on the results, it can be concluded that gels prepared with medium molecular mass chitosan might be effectively used for different antifungal agents in the treatment of vaginal candidiasis, since it has high mucoadhesiveness, suitable mechanical and release properties with good vaginal retention.

Keywords: chitosan, vaginal gel, mucoadhesion, econazole nitrate, miconazole nitrate, vaginal retention

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Vaginal yeast infections are among the most common reasons for women seeking medical care; it has been estimated that 70–75 % of women have an episode of *Candida vaginitis* at least once during their lifetime (1). Local administration of antimicrobial agents has been favored in the treatment of vaginitis because of the numerous side effects of systemically applied drugs. Vaginal dosage forms used for this purpose include solutions, gels, creams, ointments, foams, pessaries, tablets and vaginal inserts (1). To achieve a desirable therapeutic effect, vaginal delivery systems need to have the ability to spread onto the vaginal mucosa surface and guarantee an intimate and prolonged contact at the site of application (2). Therefore, localized mucoadhesive dosage forms

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may be regarded as a suitable formulation design to improve both the bioavailability of the drug and patient compliance while traditional vaginal preparations might not ensure satisfactory therapy.

Mucoadhesive gels have a high capability of adhering to mucosal surfaces, which cover various body cavities such as the vagina and prolong the residence time of the drug delivery system at the application site and decrease the number of applications required (3). Use of natural polymers is valuable for the preparation of mucoadhesive gel formulations owing to their proven biocompatibility and safety. In this respect, the polysaccharide chitosan has received particular attention over the past 20 years (4). Chitosan is a natural copolymer consisting of glucosamine and *N*-acetylglucosamine units. It has a cationic character based on its primary amino groups. These primary amino groups of chitosan are responsible for various properties (*i.e.*, controlled drug release, mucoadhesion, *in situ* gelation and permeation enhancement). In addition, chitosan and its derivatives have efflux pump inhibitory properties. They are able to reversibly inhibit efflux pumps and owing to this property the mucosal uptake of various efflux pump substrates (such as anticancer drugs, antimycotic drugs and antiinflammatory drugs) can be greatly improved (4). All of these properties make chitosan an ideal candidate for being used in drug delivery systems (4, 5).

Miconazole nitrate (MN) and econazole nitrate (EN) are imidazole derivative antifungal drugs used to treat topical fungal and yeast infections (6). The objective of this study was to comprehensively evaluate chitosan as a vaginal gel base for EN and MN. For this purpose, different types of chitosan with different molecular masses and viscosity properties have been used for the preparation of mucoadhesive gels. First of all, a comprehensive rheological study was conducted on prepared chitosan gels. Mechanical, syringeability and mucoadhesion properties of chitosan gels were determined with a TA-XT Texture Analyzer (7). Release profiles of MN and EN were obtained and evaluated kinetically. In addition, anticandidal activities of formulations were determined by the agar well diffusion method. Finally, the vaginal retention of chitosan gels in rats was evaluated by *in vivo* distribution studies.

EXPERIMENTAL

Materials

Low molecular mass chitosan [Brookfield viscosity (1 %, *m/V*, in 1 % acetic acid solution at 25 °C): 20,000 mPa s, 75–85 % deacetylated], medium molecular mass chitosan [Brookfield viscosity (1 %, *m/V*, in 1 % acetic acid solution at 25 °C): 200,000 mPa s, 75–85 % deacetylated], high molecular mass chitosan [Brookfield viscosity (1 %, *m/V*, in 1 % acetic acid solution at 25 °C): 800,000 mPa s, > 75 % deacetylated] and EN were purchased from Sigma-Aldrich (GERMANY). MN was obtained from İLSAN-İLTİŞ Pharmaceutical Company (TURKEY). All other materials were of analytical grade.

Preparation of formulations

Gel formulations were prepared using chitosan of three different molecular masses. Briefly, chitosan of 2 % (*m/m*) concentration was dissolved in diluted lactic acid solution (1 %, *V/V*). Percent chitosan concentration was chosen according to literature findings and preformulation studies (8). Chitosan concentration in gel formulations was kept constant to evaluate the difference between chitosan types. MN or EN was incorporated into formulations at 2 % (*m/m*) and 1 % (*m/m*) concentrations, respectively. MN and EN concentrations were chosen in accordance with their concentration in commercial products. Compositions of gels are given below:

F1: Formulations prepared with low molecular mass chitosan (2 %)

F2: Formulations prepared with medium molecular mass chitosan (2 %)

F3: Formulations prepared with high molecular mass chitosan (2 %).

Determination of pH

The pH values of gels were measured with a pH-meter (WTW series pH 720) at room temperature. The pH values of formulations were checked 24 hours upon preparation.

Rheological studies

Rheological analysis of chitosan gels was performed at 37 ± 0.1 °C with a Haake Mars rheometer (Thermo Fisher, Germany). Analysis was performed in flow mode and in conjunction with parallel steel plate geometry (40 mm diameter) with a gap of 0.3 mm, as described previously (9). In continuous shear analysis, the upward and downward flow curves for each formulation were measured over shear rates (10 – 2000 s^{-1}).

Oscillatory analysis of each formulation was performed after determination of its linear viscoelastic region at 37 ± 0.1 °C, where stress was directly proportional to strain and the storage modulus remained constant. Frequency sweep analysis was performed over the frequency range of 0.1–10.0 Hz following application of a constant stress. The standard gap size was 0.3 mm for each sample. Storage modulus (G') and loss modulus (G'') of the formulations were determined as previously described (10). In each case, rheological properties of the formulations were examined at least six times.

Mechanical properties of formulations

Textural analysis was performed to determine mechanical properties of the formulations using a software-controlled penetrometer (TA-TX Plus, Stable Micro System, UK) equipped with a 5 kg load cell. Formulations were initially transferred into glass vials (20 mL). An analytical probe was twice compressed into each formulation to a defined depth (15 mm) and at a defined rate (2 mm s^{-1}), allowing a delay period (15 s) between the end of the first and beginning of the second compression. Experiments were carried out at least six times. As a result of this study, a force-time curve was obtained (Fig. 1) and mechanical parameters (hardness, compressibility, adhesiveness, cohesiveness and elasticity) were calculated (11).

Hardness is the force required to attain a given deformation and the altitude of the first peak gives the hardness value. Compressibility is the work required to deform the product during the first compression of the probe and the first area under the curve (AUC) defines compressibility. Adhesiveness is the work necessary to overcome the attractive forces between the surface of the sample and the surface of the probe. The second AUC defines adhesiveness. Cohesiveness is the ratio of the area under the force-time curve produced in the second compression cycle to that in the first compression cycle. Elasticity is the rate at which the deformed sample returns to its undeformed condition after the removal of the deforming force. Cohesiveness and elasticity values were calculated according to the following equations:

$$\text{Cohesiveness} = AUC_3/AUC_1 \quad (1)$$

$$\text{Elasticity} = \text{Time difference}_{3-4}/\text{Time difference}_{1-2} \quad (2)$$

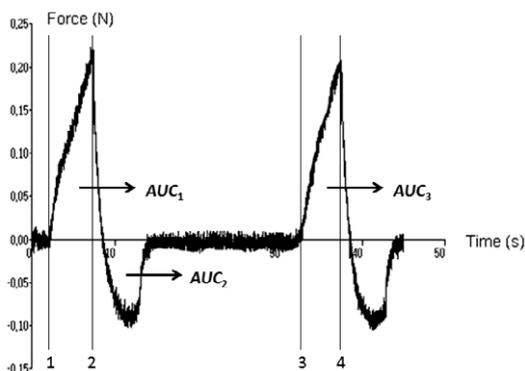


Fig. 1. Schematic representation of the force-time curve obtained from textural analysis.

Syringeability of formulations

The work required to expel formulations from a syringe without a needle was measured using a TA-XT Texture Analyzer (Stable Micro Systems) in compression mode, as previously described in literature (12). Shortly, formulations were packed into 2 mL plastic syringes without needles to a height of 25 mm, avoiding air bubbles. The syringe was then placed in the metallic support and immobilized with a plastic clamping ring. A force transducer was connected to the texture analyzer TA-XT to measure the force with which the arm of the texture analyzer moved and pushed the plunger of the syringe. The probe was lowered at a constant speed (2.0 mm s^{-1}) over a distance of 30 mm. The resistance to expression of syringe contents (work done) was determined from the area under the force-time curve recorded during plunger compression. All measurements were performed in at least six replicates (12).

Mucoadhesion studies

Mucoadhesion measurements were performed using a TA-XT Texture Analyzer (Stable Micro Systems) equipped with a load cell of 1 kg. A/MUC measuring system (mucoadhesion test ring) consisting of a ring in which the biological support can be fixed and a cylinder probe with a diameter of 1 cm were used. In this study, mucin dispersion (mucin from porcine stomach, Type II, Sigma-Aldrich, Germany) was used instead of a biological support. A filter paper disc was wetted with 10 µL of mucin dispersion in phosphate buffer (8 %, *m/m*, pH 6.4). 20 mg of each sample was applied to the cylinder probe. The sample and biologic substrate were put in contact with a preload of 6000 mN for 3 min. The cylinder probe was moved upwards at a predetermined speed of 2.5 mm min⁻¹ to separate the mucoadhesive interface (mucin-sample). The maximum detachment force, as a function of displacement, was recorded and the work of adhesion parameter (mN mm) was calculated as the area under the force *vs.* displacement curve. The test was conducted at 37 °C and each experiment was carried out six times (13).

Release studies

The *in vitro* release studies of MN and EN from different chitosan gel bases were carried out using Spectra/Por Regenerated Cellulose Dialysis Membrane Tubes (12.000–14.000 MWCO). Membranes were soaked in distilled water for 24 hours before the experiment. Phosphate buffer (pH 4.5) with dioxane (65:35, *V/V*) was used as receptor medium (14). Each formulation was packed in dialysis bags, which were placed in 150 mL of buffer at 37 °C in sealed glass vials. The receptor medium was continuously stirred. At certain time intervals, samples were withdrawn and the drug content of each sample was analyzed spectrophotometrically (Shimadzu UV-Vis 1208 spectrophotometer). Samples were measured at 273 nm and 270 nm for MN and EN contents, respectively. All the experiments were repeated at least three times.

Determination of release mechanism

The release mechanism from gels was investigated using the Ritger-Peppas equation (15, 16).

$$M_t/M_\infty = k t^n \quad (3)$$

In this equation; M_t is the drug released at time t , M_∞ is the quantity of drug released at infinite time, M_t/M_∞ is fractional release, k is the diffusional constant and n is the diffusional exponent that characterizes the drug release mechanism.

Determination of anticandidal activities of formulations

Anticandidal activities of formulations were determined by the agar well diffusion method. The standard *Candida albicans* ATCC 90028 strain was inoculated onto a Sabouraud Dextrose Agar (SDA, Oxoid) plate and incubated at 35 °C for 48 hours. A suspension equal to the 0.5 McFarland turbidity standard was prepared in a sterile sa-

line solution (8.5 g L^{-1}) from the candida colonies. $100 \mu\text{L}$ of the suspension containing approximately $1 \times 10^6 - 5 \times 10^6$ colony forming units (CFU)/mL was spread on the plates with a sterile swab and allowed to dry at room temperature for 5 minutes. Then, 20 mm diameter wells were made in the centers of SDA plates with a sterile scalpel. 0.5 mL of formulation with or without MN and EN was put into the wells aseptically. After incubation for 24–48 hours at $35 \text{ }^\circ\text{C}$, inhibition zone diameters formed around the formulations were measured. To control the possible contamination of formulations, they were placed on SDA plates that were not inoculated with *C. albicans* and incubated (17).

In vivo distribution studies

For *in vivo* distribution studies, the near-infrared dye IRDye 800RS Carboxylate (LI-COR Biosciences, NE) was loaded in chitosan gels ($10 \text{ mg}/10 \text{ g}$, *m/m*). Female Wistar albino rats (200–250 g) were anesthetized with ketamine. Vaginal and abdominal regions of the rats were shaved and 0.2 mL of gels was given intravaginally using a syringe without a needle. Rats were transferred to the MousePOD™ Adapter (model 9201-MP) scanning surface of an Odyssey Infrared Imaging System (model 9201-3; LI-COR Biosciences, USA). The MousePOD™ adapter was equipped with temperature regulators to maintain animal temperature during scanning. Vaginal regions of animals were scanned from tail to abdomen in two infrared channels simultaneously (700 and 800 nm), where one channel (700 nm channel) was used for normalization of the measured infrared intensities. The rats were scanned every hour for 8 h and 24 h after administration. The intensities were displayed in pseudo colors by the Odyssey software (LI-COR Biosciences) to isolate regions of interest (18). This *in vivo* study was performed following institutional approval from the Ethics Committee of Adnan Menderes University (Aydın, Turkey, No: B.30.2.ADU.0.00.00.00/050.04/2012/107). Experiments were carried out in accordance with the guidelines laid down by the Helsinki Declaration regarding the care and use of animals for experimental procedures and in accordance with local laws and regulations.

Statistical data analysis

Statistical data analysis was performed using the Student *t*-test with $p < 0.05$ as the minimal level of significance.

RESULTS AND DISCUSSION

Results of pH measurements

The vaginal pH of healthy women of reproductive age is acidic (4.5–5.5). It is maintained by *Lactobacilli* present in the healthy vagina that play an important role in the control of infection by common pathogens. These organisms also produce other bactericidal compounds such as hydrogen peroxide, bacteriocin-like substances and possibly biosurfactants (19). The pH of formulations is one of important parameters for vaginal compatibility. Therefore, the pH values of chitosan gels were determined first in this

Table I. pH values of formulations

Formulation code	pH \pm SD
F1	5.34 \pm 0.04
F2	5.44 \pm 0.06
F3	4.98 \pm 0.03
F1 + MN	4.82 \pm 0.07
F2 + MN	4.67 \pm 0.08
F3 + MN	4.53 \pm 0.04
F1 + EN	3.85 \pm 0.06
F2 + EN	3.92 \pm 0.09
F3 + EN	3.79 \pm 0.07

study. The results showed that all the formulations were suitable for vaginal application as they could maintain the acidic pH value of the vagina (Table I). It was also observed that the pH value of the gels did not change significantly when using different types of chitosan. Addition of EN and MN into gel formulations significantly decreased pH values in all formulations ($p < 0.05$).

Results of rheological studies

Rheological properties affect the *in vivo* behavior of formulations in the vagina such as application, spreadability and retention time. Spreadability of gels increased with a decrease in viscosity. Also, the retention time of chitosan gels was affected by their viscosity and rheological behavior. Therefore, were evaluated the rheological behavior of chitosan gels and determined if the flow types of the formulations were Newtonian or non-Newtonian.

It can be seen from Fig. 2 that the gels exhibited flow behavior in dependence on the chitosan type. F1 formulation showed Newtonian flow due to its low viscosity. This flow behavior is common in diluted gel and emulsion formulations. The reason for Newtonian flow behavior is that the molecules or micelles of this formulation did not get entangled. Further, the formulation did not undergo a phase change to turn into a gel and was still an easily flowing liquid, similar to pure water displaying typical Newtonian flow behavior (20, 21). Solutions began to flow after shear stress reached its yield point. Therefore, the flow curve for these solutions, under physiological conditions, exhibited pseudoplastic behavior with hysteresis. The hysteresis phenomenon indicated the presence of an elastic component. Thus, strong gels formed with these polymers can be characterized as viscoelastic materials. Viscoelastic materials normally show hysteresis under cyclic deformation. Viscoelastic materials are preferable to viscous materials because they flow more easily and are less susceptible to deformation due to stress (21). On the other hand, F2 and F3 formulations exhibited plastic flow. Plastics are shear thinning, their curve begins with a yield value and their viscosity decreases with an increase in shear rate (20, 21). It is clear that the type of chitosan affects the flow behavior of a formulation.

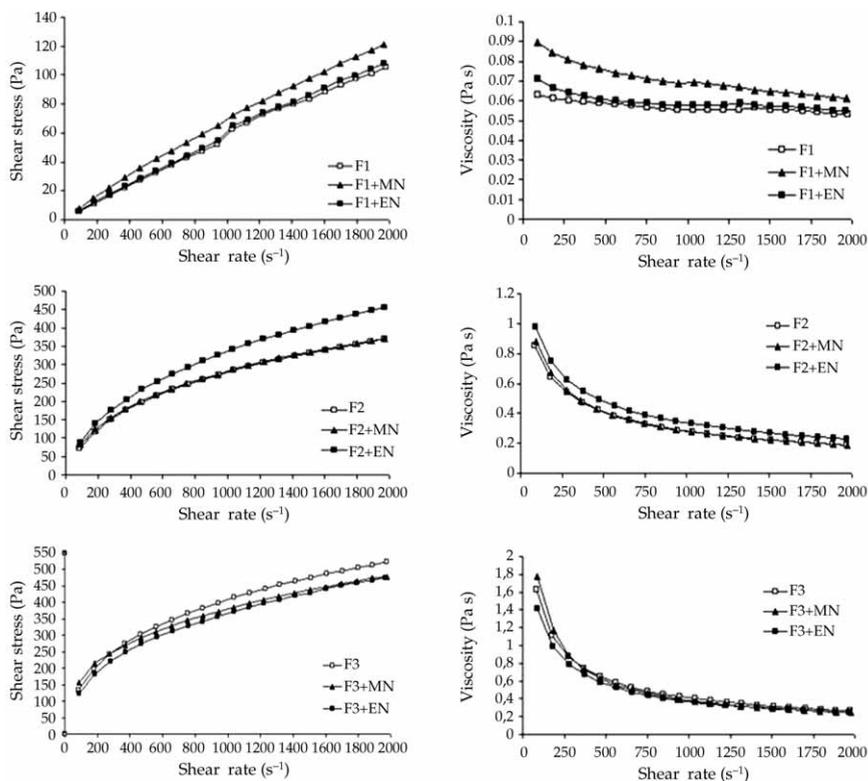


Fig. 2. Flow curves of chitosan gels.

Oscillatory studies determine the viscoelastic property of the formulation by subjecting the sample to sinusoidal shear stress. The effects of chitosan type and addition of an active substance on the viscoelastic properties, namely, storage modulus (G' , a measure of elasticity) and loss modulus (G'' , viscous components) of the formulations, are displayed in Fig. 3. As can be seen, these chitosan gels are characterized by lower G' and higher G'' values, pointing to a mainly viscous polymer solution containing a network established by physical entanglements. Also, at all frequency values a liquid-like behavior is observed, so the viscous properties are more pronounced than the elastic ones (22). Similar liquid type behavior has been reported for chitosan gels in the literature (22–24).

Results of our rheological studies also showed that the addition of MN or EN had no significant effect on the flow behavior or viscosity values for all formulations ($p > 0.05$).

Mechanical properties of formulations

Vaginal semi-solid formulations should have appropriate mechanical properties (*e.g.*, mucoadhesive properties, ease of application, low hardness, and good retention at the application site) to provide maximum benefit of the formulation to the patient (12).

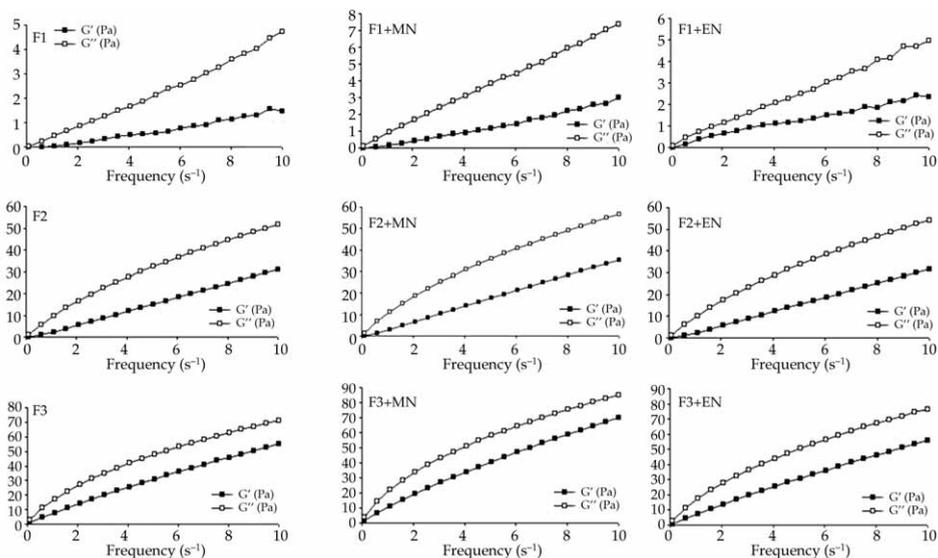


Fig. 3. Effects of chitosan type and addition of an active substance on storage modulus (G') and loss modulus (G'').

Therefore, vaginal bioadhesive gel formulations were evaluated according to their mechanical properties and the results are given in Table II.

Adhesiveness is the work required to detach the probe from the sample and is related to bioadhesion. Cohesiveness provides information on the effects of repeated shearing stresses on the structural properties of formulations. Hardness expresses the applicability of the gel to the desired site. Compressibility determines the extraction of the gel from the container and the simplicity of its spreadability on the application site. Elasticity represents the rate at which the deformed sample returns to its undeformed condition. Lower numerical values as determined by the Texture Profile Analysis (TPA) in elasticity mode indicate higher product elasticity (11). Formulations should have good adhesiveness properties to remain on the vaginal mucosa for a long period of time and thereby decrease application frequency. On the other hand, compressibility and hardness values of the formulations should be low to facilitate product removal from the container and provide product comfort within the vaginal cavity.

As can be seen from Table II, increase in chitosan molecular mass and viscosity altered the mechanical properties of all formulations. Formulations prepared with high molecular mass chitosan exhibited the highest adhesiveness values. On the other hand, the hardness and compressibility values of these formulations were, as expected, higher than the others, which might be disadvantageous for their spreadability on the vagina and patient compliance. Hardness and compressibility of formulations prepared with low molecular mass chitosan are low compared to other formulations. Nevertheless, their adhesiveness is comparatively low, which might cause leakage during the use of the formulation and restrict patient compliance.

Briefly, the compressibility and hardness values of formulations should be low while adhesiveness and cohesiveness values should be high. Also, formulations should show lower numerical values of elasticity, which indicate higher product elasticity. Based on the results of mechanical properties, gels prepared with medium molecular mass chitosan appeared to offer more suitable mechanical properties than gels for vaginal application prepared with low and high molecular mass chitosan.

In addition, the results showed that the addition of active substances in 1 or 2 % concentrations did not significantly affect the adhesiveness, cohesiveness, hardness, compressibility and elasticity of the formulations.

Results of syringeability studies

The work required to expel samples from a syringe is another important parameter for practical administration of gels with a syringe. This parameter shows the force required for the application of a formulation and affects clinical practice. Previously, Jones *et al.*, reported that increased product viscoelasticity (particularly elasticity) offered increased resistance to product deformation in TPA. In syringeability analysis, this situation causes an increase in work required for compression/expulsion of the formulation (25). Our results on elasticity, hardness and syringeability were in accord with this study and the ranking was: F3 > F2 > F1 (Table II). In addition, significant increases in syringing work were observed as the molecular weight and viscosity of chitosan increased ($p < 0.05$). F3 required most work to expel the liquid formulation from the syringe (or applicator), which might be disadvantageous for the ease of administering formulations with a syringe or applicator. In addition, the results showed that incorporation of 2 % MN or 1 % EN into chitosan gels had no significant effect on work required to expel formulations from the syringe.

Results of mucoadhesion studies

Mucoadhesive properties of formulations play an important role in prolonging the residence time and mucoadhesion should be determined sensitively. A TA-XTplus texture analyzer can be used to measure mucoadhesion of formulations under simulated vaginal conditions, based on the principle of applying tensile strength and shear stress to break the adhesive bond between the test sample and a model membrane (26). Therefore, the mucoadhesive properties of chitosan gels were determined with a TA-XTplus texture analyzer equipped with the A/MUC measuring system.

The results of mucoadhesion studies are presented in Table II. Chitosan is a well-known bioadhesive polysaccharide and its mucoadhesive properties are likely to be based on its cationic character. The mucus layer exhibits anionic structures in sialic acid and sulfonic acid form. Based on ionic interactions between the cationic primary amino groups of chitosan and these anionic substructures of the mucus, mucoadhesion can be achieved. In addition, hydrophobic interactions might contribute to chitosan mucoadhesive properties (4).

Table II. Results of mechanical properties, work of syringing and work of mucoadhesion of chitosan gels^a

Formulation code	Adhesiveness (N mm)	Cohesiveness	Hardness (N)	Compressibility (N mm)	Elasticity	Work of syringing (N s)	Work of mucoadhesion (N mm/cm ²)
F1	0.008 ± 0.001	0.974 ± 0.023	0.009 ± 0.000	0.025 ± 0.001	0.869 ± 0.062	6.893 ± 0.652	222.329 ± 5.199
F2	0.016 ± 0.001	0.912 ± 0.034	0.009 ± 0.001	0.019 ± 0.002	1.005 ± 0.139	21.908 ± 3.385	253.510 ± 38.041
F3	0.062 ± 0.005	1.048 ± 0.072	0.017 ± 0.002	0.032 ± 0.005	1.065 ± 0.043	39.727 ± 2.177	426.538 ± 63.285
F1 + MN	0.008 ± 0.001	0.925 ± 0.072	0.007 ± 0.001	0.012 ± 0.002	0.985 ± 0.131	7.509 ± 2.537	212.078 ± 24.193
F2 + MN	0.022 ± 0.003	0.965 ± 0.031	0.010 ± 0.002	0.016 ± 0.006	0.942 ± 0.099	20.055 ± 0.433	252.004 ± 55.222
F3 + MN	0.075 ± 0.017	1.165 ± 0.030	0.013 ± 0.001	0.016 ± 0.003	1.119 ± 0.104	35.858 ± 2.857	454.886 ± 22.704
F1 + EN	0.006 ± 0.000	0.990 ± 0.052	0.007 ± 0.000	0.012 ± 0.001	1.004 ± 0.052	6.450 ± 1.106	209.510 ± 32.027
F2 + EN	0.018 ± 0.002	0.887 ± 0.010	0.011 ± 0.000	0.033 ± 0.002	0.904 ± 0.076	18.377 ± 2.376	277.913 ± 25.010
F3 + EN	0.120 ± 0.010	0.978 ± 0.046	0.036 ± 0.012	0.036 ± 0.009	1.154 ± 0.013	38.620 ± 6.681	440.472 ± 36.946

^a Mean ± SD.

Results of mucoadhesion studies were in accord with adhesiveness results obtained from the texture profile analysis of formulations. Significant increases in the work of mucoadhesion were observed as the molecular weight and viscosity of chitosan increased ($p < 0.05$) and F3 showed stronger mucoadhesiveness than other formulations.

Results of release studies

The *in vitro* release profiles of MN and EN were evaluated in pH 4.5 phosphate buffer at 37 °C and the results are displayed in Fig. 4. As can be seen, the release of EN and MN were in range of 50–70 % over 8 hours. In 24 hours, the release rates of MN from F1, F2 and F3 reached 89, 91 and 96 %, respectively. In addition, at the end of 24 hours, the release rates of EN from F1, F2 and F3 were 71, 67, 78 %, respectively (data not shown).

Previous studies had shown that drug release might be influenced by gel viscosity. As the viscosity of the gel increased, the release of the drug was expected to be slower. (27). On the other hand, our results showed that the release profiles of MN and EN were not significantly affected by the viscosity of chitosan ($p > 0.05$). When we compared the

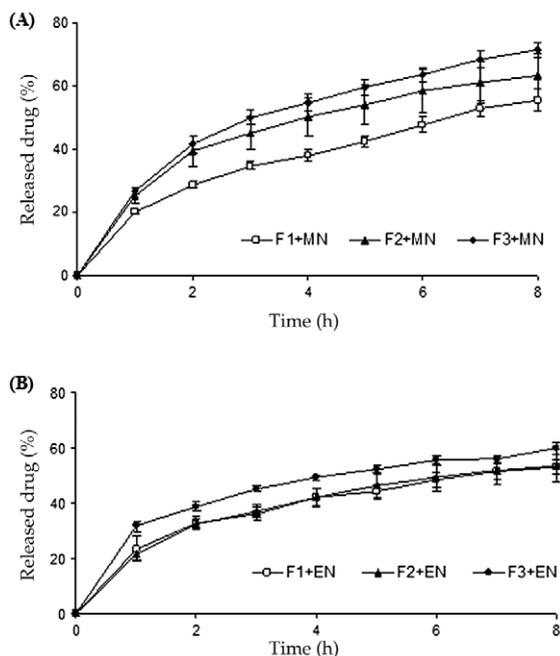


Fig. 4. Dissolution profiles of: a) MN and b) EN from chitosan gels in pH 4.5 phosphate buffer.

release rates of EN and MN from the same chitosan formulations, we found that the release rates of MN from F2 and F3 formulations were significantly higher than the release rates of EN from F2 and F3 ($p < 0.05$). On the basis of release studies, it can be concluded that F1, F2 and F3 formulations offer the advantage of controlling both MN and EN release over 8 hours.

Determination of the release mechanism

An empirical equation (Eq. 3), proposed by Ritger and Peppas, was used for the analysis of release data in gel formulations (16). Application of the general release equation enabled calculation of n and hence identification of the mechanism of release from the formulations. To this aim, the release data were fitted to Eq. 1, and best-fit parameters were calculated. According to this equation, $n = 0.5$ indicates case I (Fickian) diffusion or square root of time kinetics, $0.5 < n < 1.0$ indicates anomalous (non-Fickian) diffusion, $n = 1.0$ indicates case II transport, and $n > 1.0$ indicates super case II transport for thin film formulations (28).

The values of diffusional exponents n , release rate constants k and correlation coefficients R^2 are listed in Table III. The release exponents n of all formulations ranged from 0.288 to 0.470, which indicates case I (Fickian) drug release. In this case, diffusion of drug molecules is the controlling mechanism of drug release.

Table III. Drug release parameters of formulations

Formulation code	Release exponent (<i>n</i>)	Release rate constant (<i>k</i>)	Correlation coefficient (<i>R</i> ²)
F1 + MN	0.470	1.310	0.996
F2 + MN	0.412	1.440	0.985
F3 + MN	0.406	1.479	0.973
F1 + EN	0.349	1.400	0.982
F2 + EN	0.365	1.388	0.958
F3 + EN	0.288	1.510	0.991

Results of determination of anticandidal activities of formulations

Chitosan and its derivatives have a spectrum of antimicrobial activity against filamentous fungi, yeasts, Gram-positive and Gram-negative bacteria. Although chitosan is not primarily used as an antimicrobial agent, its utility as an ingredient in pharmaceutical formulations and food has lately gained more interest (29). Therefore, the anticandidal activity of chitosan gels against the standard *Candida albicans* ATCC 90028 strain was evaluated and the results are given in Table IV.

Although chitosan was known to have anticandidal action against *Candida albicans*, our results showed that F1, F2 and F3 formulations without MN or EN did not exhibit any antimicrobial activity. Chitosan's *in vitro* antimicrobial activity is influenced by various intrinsic and extrinsic factors, such as chitosan itself (type, molecular mass, deacetylation degree, solvent and concentration) and the environmental conditions (test strain, its physiological state and the bacterial culture medium, pH, temperature, ionic strength, metal ions, EDTA, organic matter) (30). Therefore, we might conclude that our formulations did not show antimicrobial activity due to some of these intrinsic or extrinsic parameters. It was stated in recent years that the antimicrobial properties of chitosan might have a negative impact on healthy vaginal microflora and that its vaginal use for treat-

Table VI. Inhibition zone diameters of formulations

Formulation	Diameter of inhibition zone (mm)
F1	0
F2	0
F3	0
F1 + MN	33.6 ± 1.5
F2 + MN	26.3 ± 0.5
F3 + MN	28.3 ± 0.5
F1 + EN	31 ± 1
F2 + EN	30.3 ± 1.5
F3 + EN	30.0 ± 2

ment of chronic diseases requires caution (4). From this point of view, we might say that the absence of antimicrobial effect in our formulations without active substances might be an advantageous outcome.

Chitosan gels with active substances showed anticandidal activity with inhibition diameters between 26–34 mm. There were no significant differences in this anticandidal activity among the formulations and active substances.

Results of in vivo distribution studies

Vaginal retention of gels is an important parameter to be evaluated in order to achieve efficacy. Optimally, a mucoadhesive vaginal gel must be distributed over the mucosa, maintaining an epithelial coating layer (31). To date, several *in vitro* and *in vivo* methods have been developed and used to evaluate the distribution and retention of formulations in the vagina (31, 32). Imaging studies such as magnetic resonance imaging and gamma scintigraphy still remain the most reliable methods for *in vivo* assessment of vag-

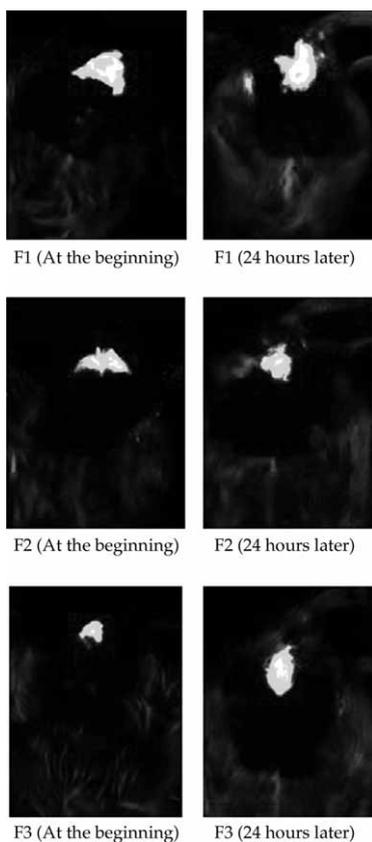


Fig. 5. Vaginal retention of formulations.

inal distribution and retention (33). In our study, we evaluated vaginal retention using a new imaging method: Odyssey Infrared Imaging System with MousePOD™ adapter (LI-COR Biosciences). The benefit of this method is that live animals are used and that they are not exposed to radiation like in radio-imaging studies (34). Near-infrared images of rats administered RDye 800RS Carboxylate dye (peak absorption at 786 nm) loaded chitosan gels are shown in Fig. 5.

The *in vivo* distribution studies showed that F1, F2 and F3 formulations remained in the vaginal mucosa 24 h after application. It was stated in previous studies that the rheology of vaginal gel could be considered as one of the most important factors affecting gel distribution and retention time in the vagina (35). Selection of correct viscosity of a gel can be decisive for its adequate retention and distribution in the vagina. On the other hand, our results showed that F1, F2 and F3 formulations with different chitosan viscosity values remained on the vaginal mucosa up to 24 hours and no significant difference was found among the formulations.

Previously, Di Fabio *et al.* evaluated the potential effectiveness of microbicides on vaginal HIV-1 transmission formulated with two different polymers (carbopol and hydroxyethylcellulose). The results showed that it was essential to achieve a uniform distribution on vaginal mucosa in order to form an effective barrier. However, the gel with a high viscosity value was difficult to apply and distribute on the vagina of mice. Then, polymers with lower viscosity were examined to achieve a uniform distribution over the entire vagina/cervix and the results showed that vaginal transmission of HIV was successfully prevented by the application of this formulation (36). On the other hand, gels with low viscosity values may result in leakage of the administered formulation from the vagina and restrict the efficacy of the formulation or patient compliance. Therefore, although no significant difference in vaginal retention time was noticed between the formulations, F2 seems to be more suitable than F1 and F3 considering the mechanical, mucoadhesive and syringeability properties. Anyway, in further *in vivo* studies, the difference between the formulations should be evaluated in more detail.

CONCLUSIONS

The aim of this study was the evaluation of chitosan (different molecular mass and viscosity values) for the preparation of mucoadhesive vaginal gels. For this purpose, miconazole nitrate and econazole nitrate were chosen as imidazole derivative active substances. The gels were first comprehensively evaluated in *in vitro* characterization studies (rheological evaluation, texture profile analysis, mucoadhesion, syringeability and release studies). In addition, vaginal retention of the gels was investigated *in vivo* through a distribution study on rats. Based on the results obtained from these studies, it can be concluded that vaginal gels prepared with medium molecular mass chitosan might be effectively used for different antifungal agents for the treatment of vaginal candidiasis, since they have high mucoadhesiveness, suitable mechanical and release properties with good retention in the vagina.

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