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Chitosan/alginate complexes for vaginal delivery of chlorhexidine digluconate

A. Abruzzo[a], F. Bigucci[a], T. Cerchiara[a], B. Saladini[b], M.C. Gallucci[c], F. Cruciani[a], B. Vitali[a], B. Luppi[a,*]

[a] Department of Pharmaceutical Sciences, Via San Donato 19/2, University of Bologna, 40127 Bologna, Italy
[b] PolyCrystalLine s.r.l., Via F.S. Fabri 127/1, 40059 Medicina, Bologna, Italy
[c] Department of Chemistry, Ponte P. Bucci, University of Calabria, 87036 Arcavacata di Rende, Cosenza, Italy

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ABSTRACT

Chitosan/alginate complexes were prepared at different polycation/polyanion molar ratios and freeze-dried vaginal inserts were obtained for chlorhexidine digluconate local delivery in genital infections. Complex yield, FT-IR spectra, and TGA thermograms were studied to confirm the interaction between the two polyions. The influence of different complexes on physical handling, morphology, and drug distribution in the samples were evaluated by friability test, scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDS), respectively. In vitro water-uptake, mucoadhesion and release tests were performed as well as microbiological tests toward pathogenic vaginal microorganisms. The results showed that the selection of suitable chitosan/alginate molar ratio and drug loading allowed modulate insert ability to hydrate, adhere to the mucosa, and release chlorhexidine digluconate. The insert containing an excess of alginate was found to be the best performing formulation and showed good antimicrobial activity toward the pathogens *Candida albicans* and *Escherichia coli*.

1. Introduction

Disturbances in the vaginal environment due to abnormal vaginal flora and vaginal infections are highly prevalent among reproductive-aged women. Vaginal candidiasis is ranked as one of the most common gynecological infections, and it has been estimated that about 75% of women experience an acute episode once in their lifetime. It has been reported that 30–35% of vaginitis episodes are due to *Candida albicans* (Das Neves et al., 2008; Nyririesy, Weitz, Grody, & Lorber, 2001; Sobel, 1988). Aerobic vaginitis is another frequent form of abnormal vaginal flora which has been considered an important cause of pregnancy complications, such as ascending chorioamnionitis, preterm rupture of the membranes, and preterm delivery. Aerobic vaginitis is defined as a disruption of the lactobacillary flora, accompanied by signs of inflammation and the presence of a predominantly aerobic microflora, composed of enteric commensals or pathogens, especially *Escherichia coli* and *Streptococcus agalactiae* (Donders, Bellen, & Rezeberga, 2011; Donders et al., 2002).

Topical imidazoles are considered standard treatments of candidiasis, while kanamycin or quinolones are a good choice for the therapy of aerobic vaginitis (Tempera & Furneri, 2010). In the case of mixed vaginitis, the use of a monotherapy becomes ineffective, whereas treatment with a wide-spectrum antibacterial and antifungal substance, such as chlorhexidine digluconate, may be promising for a more rapid healing (Molteni et al., 2004).

Several drug delivery systems are used for treatment of vaginal infections (Alamdar & Fakhrul, 2005). Indeed, conventional vaginal formulations (suspensions, pessaries, cream, and solutions) are characterized by short residence time at the site of administration, due to washing action of physiological secretions of vaginal fluids. Bioadhesive vaginal drug delivery systems, such as tablet, inserts, and gels, may adhere to vaginal mucosa in order to bring drug in contact with target tissues for sufficient period of time and prevent expulsion of formulation (Ceschel, Maffei, Borgia, Ronchi, & Rossi, 2001; Dobaria, Mashru, & Vadia, 2007; Dobaria, Badhan, & Mashru, 2009; Kast, Valenta, Leopold, & Bernkop-Schnürch, 2002; Valenta, 2005; Woodley, 2001). Tablets and some gel-based vaginal delivery systems are associated with problems like messiness and leakage of formulations causing inconvenience to users and leading to poor patient compliance (Dobaria et al., 2007). For this reason, in this study we focused the attention on the possibility to formulate a new suitable delivery system, able to overcome these limitations and characterized by a convenient application and easy handling. To achieve this goal, the vaginal insert was chosen as final dosage form, easily applicable and able to deliver a unique dose of drug in the vaginal cavity, while chitosan and sodium alginate were selected in order to obtain good insert mucoadhesion ability. Furthermore, different chitosan/alginate molar ratios were tested in

* Corresponding author at: Department of Pharmaceutical Sciences, Bologna University, Via San Donato 19/2, 40127 Bologna, Italy. Tel.: +39 0512095615; fax: +39 0512095615.
E-mail address: barbara.luppi@unibo.it (B. Luppi).

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order to obtain a system releasing the suitable chlorhexidine digluconate amount, accordingly to the therapeutic needs and providing the complete inhibition of pathogens, such as *C. albicans* and *E. coli*.

Chitosan, a N-deacetylated product of the polysaccharide chitin, shows interesting biological properties, including biocompatibility, non-toxicity, biodegradability, and mucoadhesivity (Dutta, Dutta, & Tripathi, 2004; Koga, 1998; Muzzarelli, 1997, 2010; Ravi Kumar, 2000). It was also widely used for different type drug delivery systems (Dodane & Vinod, 1998; Luppi, Bigucci, Cerchiara, & Zecchi, 2010) and largely employed to prepare vaginal mucoadhesive dosage forms (Bonferoni et al., 2008; Perioli et al., 2008; Rossi, Sandri, Ferrari, Bonferoni, & Caramella, 2003; Valenta, 2005). Chitosan can also interact with anionic polymers in order to prepare ionically crosslinked hydrogels (Berger, Reist, Mayer, Felt, Peppas, & Gurny, 2004; Hamman, 2010; Meshali & Gabr, 1993; Remuñán-López & Bodmeier, 1996). Sodium alginate, an anionic, biocompatible, hydrophilic, and biodegradable polymer, derived primarily from brown seaweed and bacteria, is a linear polysaccharide that consists of β-D-mannuronic acid and α-L-guluronic acid repeating units in various ratios (Hanne & Jan, 2002; Tønnesen & Karlsen, 2002).

Chitosan/alginate complexes were obtained by mixing polymeric solutions with different molar ratios of chitosan and alginate and then freeze-drying the precipitates. Complex yield, FT-IR analysis, TGA thermograms were studied to investigate the interaction between the two polyions. The complexes were used to prepare vaginal inserts loaded with chlorhexidine digluconate. Physical handling, morphology, and drug distribution in the samples were studied by friability test, scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDS) analysis. In vitro water-uptake, mucoadhesion, release and microbiological tests were performed in order to investigate the polyelectrolyte complexes ability to adhere to mucosa, to release chlorhexidine digluconate and to study the antimicrobial activity toward *C. albicans* and *E. coli.*

2. Materials and methods

2.1. Materials

Sodium alginate low viscosity ($M_w \approx 140{,}000$ Da, viscosity 100–300 cP, 2%), chitosan low molecular weight ($M_w \approx 150{,}000$ Da, viscosity 20–300 cP, $T = 20\,^{\circ}C$, 1% in 1% acetic acid; deacetylation degree 97%) and chlorhexidine digluconate used for this study were obtained commercially from Sigma–Aldrich (Milan, Italy). All other chemicals and solvents were of analytical grade and purchased from Carlo Erba (Milan, Italy). Complex preparation, water-uptake, mucoadhesion, and release studies were carried out in aqueous buffers with the following compositions per liter of distilled water: 8.99 ml CH_3COOH 2 N and 2.62 g CH_3COONa for acetate buffer at pH 5.0; 13.61 g KH_2PO_4, adjusted with hydrochloric acid to pH 4.5, for buffer simulating vaginal secretions.

2.2. Preparation of chitosan/alginate complex and solid complex weight measurement

Chitosan/alginate was prepared according to a method reported in a previous work (Bigucci et al., 2008) with some modifications. Briefly, chitosan (1.50 mmol of monomer in 200 ml) and alginate (1.50 mmol of monomer in 200 ml) were separately dissolved in acetate buffers at pH 5.0 at the same ionic strength (50 mM). Different volumes of chitosan solutions were added to alginate solutions and stirred at room temperature for 24 h, in order to obtain different chitosan/alginate molar ratios (1:9, 3:7, 1:1, 7:3, and 9:1).

The precipitate was separated by ultracentrifugation at 10,000 rpm for 10 min (ALC 4239R Centrifuge; Milan, Italy). Then it was washed with deionized water and homogenized at 17,500 rev min^{-1} for 5 min (Ultra-Turrax, T 25 basic homogenizer; IKA, Dresden, Germany) for three times in order to eliminate sodium acetate. Finally, the precipitate was suspended again in deionized water and freeze-dried (Christ Freeze Dryer ALPHA 1-2, Milan, Italy), obtaining five different chitosan/alginate complexes:

CH/ALG(1:9), CH/ALG(3:7), CH/ALG(1:1), CH/ALG(7:3), and CH/ALG(9:1).

Each precipitate was weighted for the determination of solid complex weight.

2.3. FT-IR spectroscopy and thermogravimetric analysis (TGA)

To verify interactions between chitosan and alginate, FT-IR spectroscopy (FT-IR-4100 spectrophotometer recorded with a Jasco, 650–4000 cm^{-1}) and TGA (STA 409 PC Luxx® Netzsch, temperature range: 5–1700 °C, heating and cooling rates: 0.01–50 K/min, inert atmospheres) of unloaded complex, chitosan and alginate powders and their physical mixture were performed. The IR spectra for the test samples were obtained using KBr disk method. Measurements were carried out at least in triplicate (relative standard deviation ±5%).

2.4. Preparation of chitosan/alginate complex vaginal inserts

The freeze-dried chitosan/alginate complexes were used to prepare vaginal inserts. For unloaded inserts (average diameter 0.6 cm, height 1.0 cm) 200 μl of phosphate buffer at pH 4.5 were added to 20 mg of different complex/mannitol mixtures (9:1; w/w). Mannitol was added, as a bulking agent in order to improve mechanical strength of lyophilized vaginal inserts when handled (Luppi et al., 2009; McInnes et al., 2005). Loaded inserts were prepared in the same way adding 200 μl of chlorhexidine digluconate solutions (in phosphate buffer at pH 4.5) at different concentration in order to obtain three different complex/drug weight ratios (2:0.5, 2:1, and 2:2) for every type of complex. The resultant suspensions, filled into polypropylene microcentrifuge tubes, were allowed to settle to swell and remove air and finally lyophilized, obtaining cone-like shaped solid inserts. The inserts were stored in a desiccator until use (Luppi, Bigucci, Abruzzo, et al., 2010).

Moreover, control formulations were prepared, without chitosan/alginate complexes, using 20 mg of mannitol and 200 μl of chlorhexidine digluconate solutions at different concentration (mannitol/drug weight ratio, 2:0.5, 2:1, and 2:2).

2.5. Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)

The morphology of vaginal inserts was studied by SEM analysis. Inserts were cut with a razor blade to expose the inner structure, fixed on supports and coated with gold–palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Samples were then observed with LEO 420 (LEO Electron Microscopy Ltd., England) using secondary electron imaging at 15 kV in order to examine the surface morphology and structure of the inserts.

Moreover, drug distribution in the samples was evaluated by energy dispersive X-ray spectroscopy (EDS).

2.6. Friability studies

Friability tests were conducted by subjecting at least 10 inserts to repeat revolutions using a friability tester. Inserts were weighted before and after the testing and % friability was measured as a percentage of weight lost during a standardized abrasion.

2.7. Water-uptake ability

Water-uptake studies were performed in phosphate buffer at pH 4.5 that simulate vaginal fluids and with the procedure reported in our previous work (Luppi, Bigucci, Abruzzo, et al., 2010).

The water-uptake behavior of loaded inserts with different complex/drug weight ratios: 2:0.5, 2:1, and 2:2 was also investigated.

2.8. Insert mucoadhesion properties

For these studies, vaginal mucosa obtained from freshly slaughtered pig was used. In fact, porcine vaginal mucosa was found to be very similar to human one in many characteristics, such as lipid compositions and histological properties (Kremer, Wertz, & Squier, 2001; Van Eyk & Van der Bijl, 2005). The in vitro mucoadhesion was measured in terms of the force needed to pull out a freshly excised porcine vaginal mucosa (surface area 1 mm^2) from the inserts with an adapted tensiometer (Krüss 132869; Hamburg, Germany) as reported in a previous work (Luppi, Bigucci, Baldini, et al., 2010).

The mucosa, suspended from the tensiometer spring, was lowered until it just contacted the surface of the insert, previously immersed in phosphate buffers at pH 4.5 for 15 min. A 500 μN force, measured by the torsion balance of the instrument as a negative force, was applied to the inserts for 30 s. Then, the vaginal mucosa was raised until it was separated from the formulations. This point represents the adhesive bond strength between these elements and is expressed as a positive force in dyne.

2.9. In vitro release studies

In vitro release studies were performed as reported by Luppi, Bigucci, Abruzzo, et al. (2010). Briefly, loaded inserts were placed on the sintered-glass filter plate of a Borosil glass filter crucible and the whole system was closed with Parafilm to avoid evaporation of release medium (filled with 10 ml of pH 4.5 phosphate buffer) and adjusted exactly to the height of the release medium surface so that the porous glass membrane was wetted but not submersed. The experiments were performed at 37 °C under magnetic stirring. Samples of 200 μl were taken at predetermined time points and replaced by fresh medium and analyzed using UV-spectrophotometer set at 254 nm.

2.10. Microbiological assays

The antimicrobial activity was evaluated against *E. coli* ATCC 11105 and *C. albicans* ATCC 10231. *E. coli* was grown aerobically in LB medium (Difco, Detroit, MI) at 37 °C for 24 h. *C. albicans* was grown aerobically in SD medium (Difco) at 30 °C for 48 h.

Viability of *E. coli* and *C. albicans* in phosphate buffer (pH 4.5) was compared with viability of the respective bacterium and yeast cultured in the presence of vaginal insert based on CH/ALG(1:9) complex and containing chlorhexidine digluconate (complex/drug weight ratio, 2:1). A microbial suspension, prepared from a broth culture in log phase growth of *E. coli* or *C. albicans*, was used to inoculate the Erlenmeyer flasks containing 120 ml of phosphate buffer. The initial concentration of *E. coli* and *C. albicans* was about 6 log of colony forming unit (CFU) per ml of experimental medium, corresponding to the physiological amounts in cases of infection. Counts of viable *E. coli* and *C. albicans* were carried on LB and SD agar plates, respectively, at the inoculum time (T0) and after 6 h (T6), and 24 h (T24) of incubation at 37 °C (physiological temperature). LB plates were incubated aerobically at 37 °C for 24 h. SD plates were incubated aerobically at 30 °C for 48 h. All plates were made in triplicate. Microbial concentration was expressed as a mean of log CFU/ml ± standard deviation (SD).

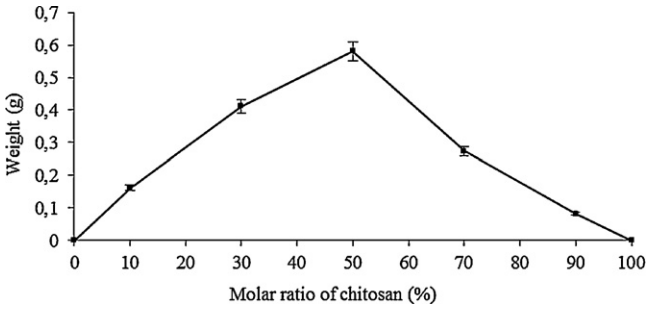

Fig. 1. Solid complex weights as a function of chitosan/alginate molar ratio at pH 5.0 obtained from 400 ml of final polymeric solutions.

2.11. Statistical analysis

All the experiments were done in triplicate. Results are expressed as mean ± SD. ANOVA tests were used to determine statistical significance of studies, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results and discussion

3.1. Chitosan/alginate polyelectrolyte complex weight measurement

Fig. 1 shows the effect of chitosan/alginate molar ratio on complex formation at pH 5.0. The molar ratio for maximum insoluble complex formation at pH 5.0 was 1:1. In fact, at pH 5.0 most of the chitosan amino groups and alginate carboxylic groups were charged (pK_b value of chitosan = 6.3 and pK_a value of alginate = 3.5), thus providing the greater interaction between the polymers. Moreover, the amount of precipitated complexes CH/ALG(7:3) and CH/ALG(9:1) was lower with respect to that of CH/ALG(3:7) and CH/ALG(1:9), respectively ($P < 0.05$); this suggest that greater moles of alginate were charged with respect to chitosan and that the presence of greater amount of alginate provided the formation of major amount of complex. For this reason, the amount of positively and negatively charges was evaluated. In particular, NH_3^+ and COO^- theoretical concentration (mM) was calculated considering a complexation reaction between chitosan (5 mM) and alginate (5 mM) as a function of pK_a values of the two polysaccharides and molar ratio chitosan/alginate at pH 5.0. NH_3^+ and COO^- theoretical concentration (mM) was 4.28 and 0.49 for CH/ALG(9:1), 3.33 and 1.45 for LG(7:3), 2.38 and 2.42 for CH/ALG(1:1), 1.43 and 3.39 for LG(3:7), 0.48 and 3.36 for CH/ALG(1:9), respectively, thus demonstrating the greater ionization of alginate with respect to chitosan.

3.2. FT-IR spectroscopy and thermogravimetric analysis (TGA)

Fig. 2 showed the FT-IR spectra of chitosan and alginate powders, physical mixture and CH/ALG(1:1) complex. The FT-IR spectra of chitosan showed bands at 1654 cm^{-1} relative to the vibration of the carbonyl group of acetylated amide and at 1580 cm^{-1} relative to stretching of the free amino group. Alginate showed the typical band at 1620 cm^{-1} relative to the vibration of C=O group. These characteristics bands were also in the FT-IR spectra of physical mixture of chitosan and alginate. The FT-IR of complex CH/ALG(1:1) showed the shift in amide carbonyl group to 1626 cm^{-1} and the shift in amino group of chitosan to 1554 cm^{-1}, confirming the interaction between chitosan and alginate, also reported by Muzzarelli, Tosi, Francescangeli, and Muzzarelli (2003).

Fig. 3 shows thermograms of chitosan, alginate, physical mixture, and CH/ALG(1:1) complex. Chitosan and alginate degraded at 293 and 238 °C, respectively. In the physical mixture there were

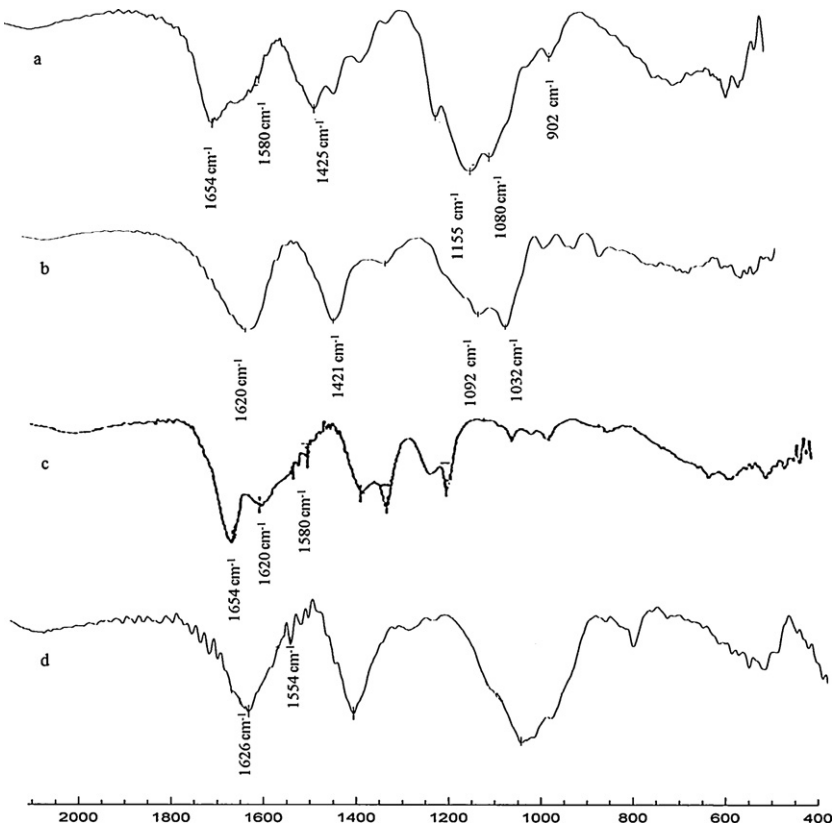

Fig. 2. FT-IR of chitosan (a), alginate (b), chitosan/alginate physical mixture (c), and complex CH/ALG(1:1) (d).

two peaks at 238 and 293 °C that could be related to the weight loss of alginate and chitosan, respectively. The complex CH/ALG(1:1) showed one event at 202 °C that can be considered as a proof of chitosan and alginate complexation. The shift in a lower temperature in the thermal degradation of the complex indicates that there was a loss of organization, due to the formation of ionic bonds between chitosan and alginate.

3.3. Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)

The structure of the inserts, observed by scanning electron microscopy (SEM), depends on the composition of chitosan/alginate complexes.

Fig. 4(A)–(E) shows the morphology of vaginal inserts based on the different complexes with 10 mg of chlorhexidine digluconate (complex/drug weight ratio, 2:1). The presence of drug in the complexes produced a rough and less porous surface rather than smooth as unloaded complexes (images of unloaded complexes are not reported).

Moreover, the complexes structure was more rough with the increase of the content of alginate in the complexes (Fig. 4D and E), probably due to the interaction between alginate and chlorhexidine digluconate. This interaction was studied measuring the turbidity (UV spectrophotometer set at 500 nm) of alginate solutions (2%, w/w) with increasing chlorhexidine digluconate content (Bertram & Bodmeier, 2006). The drug concentration, at which precipitation started, was determined by extrapolating the linear correlation of

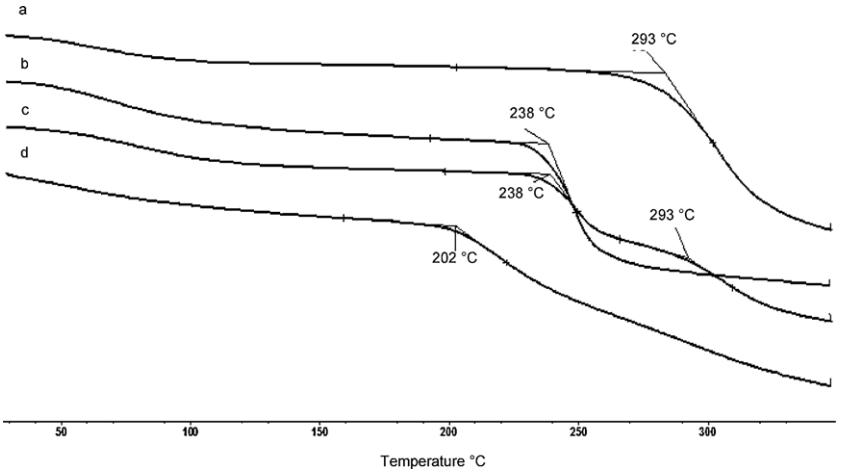

Fig. 3. Thermogravimetric analysis of chitosan (a), alginate (b), chitosan/alginate physical mixture (c), and CH/ALG(1:1) (d).

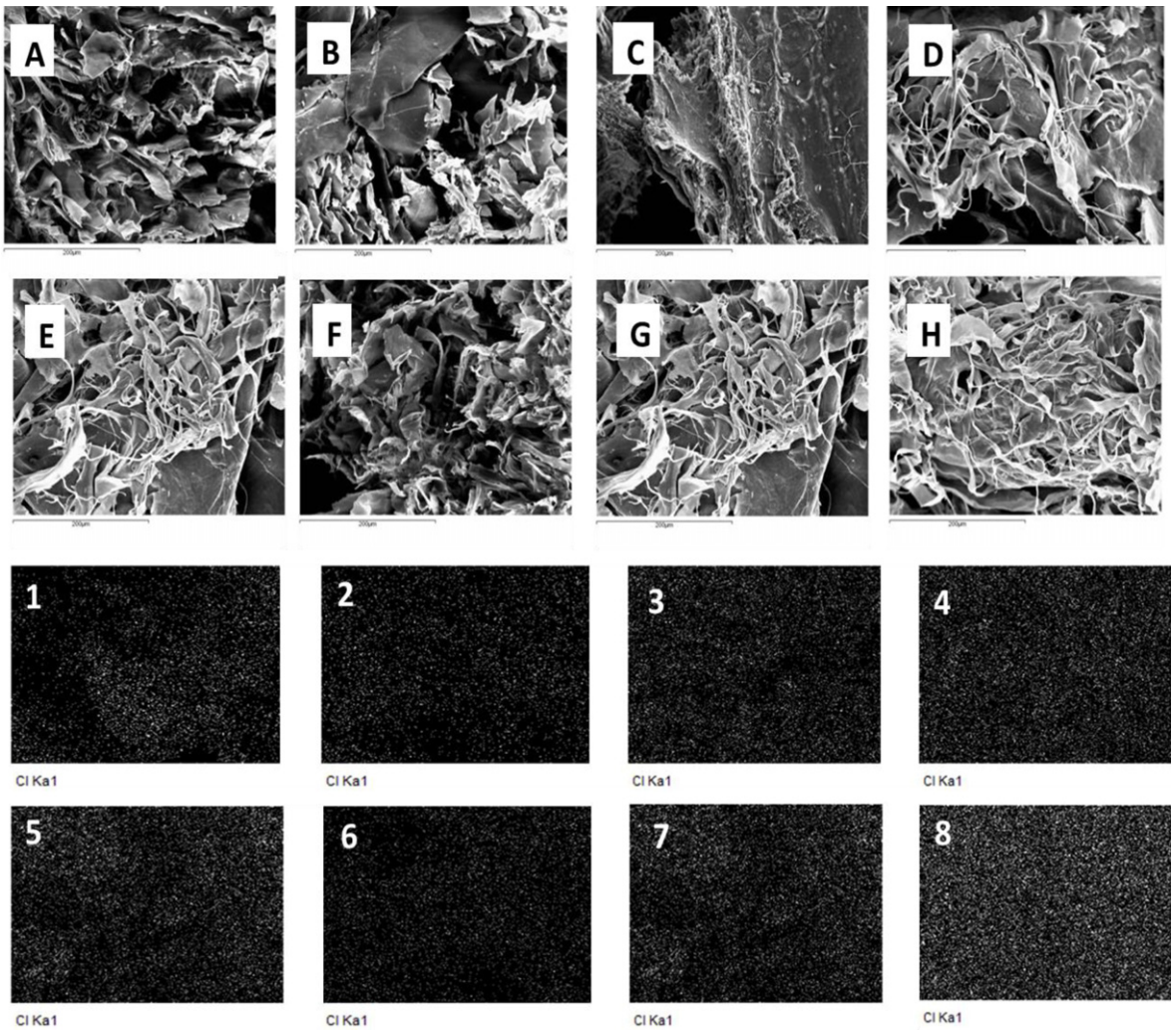

Fig. 4. Scanning electron micrographs/EDS of the different chitosan/alginate complexes: (A/1) CH/ALG(9:1), (B/2) CH/ALG(7:3), (C/3) CH/ALG(1:1), (D/4) CH/ALG(3:7), (E/5) CH/ALG(1:9) with complex/drug molar ratio 2:1. (F/6) CH/ALG(1:9) with complex/drug molar ratio 2:0.5, (G/7) CH/ALG(1:9) with complex/drug molar ratio 2:1, (H/8) CH/ALG(1:9) with complex/drug molar ratio 2:2.

the measured data points to an absorption of zero. Drug polymer interaction was observed as precipitation in polymeric solutions at concentration >0.06 mg/ml.

Fig. 4(F)–(H) shows the influence of different amount of drug on CH/ALG(1:9) complex. As can be seen, inserts based on complex/drug weight ratio 2:0.5 provided a more smooth surface with respect to inserts based on complex/drug weight ratio 2:1 and 2:2.

Fig. 4(1)–(8) shows the drug distribution in the inserts. As can be seen, the drug was homogeneously distributed in all the inserts based on the different complexes and also in the case of CH/ALG(1:9) inserts with complex/drug weight ratios 2:0.5, 2:1, and 2:2.

3.4. Friability studies

Inserts should be hard enough to be easily removed from their packaging and to be placed intact into the vaginal cavity. Friability is a function of the hardness of a solid form and was measured in order to assess insert tendency to chip, crack, or crumble due to friction and abrasion resulting from physical handling. The lower the friability, the more resistant the solid dosage form is to handling. In general, friability is affected by factors such as the size, shape, and weight of the dosage form, as well as the formulation. In particular, friability values of loaded inserts based on CH/ALG(1:9), CH/ALG(3:7), CH/ALG(7:3), and CH/ALG(9:1) were 9.3 ± 0.6, 10.1 ± 0.3, 9.5 ± 0.5, and 9.8 ± 0.4%, while complex CH/ALG(1:1) provided a friability of 80 ± 1.7%. All loaded inserts, except CH/ALG(1:1), were handled without damage and can be considered as promising formulations for vaginal application.

3.5. Water-uptake ability

Water-uptake was influenced by the medium (phosphate buffer pH 4.5) and by chitosan/alginate molar ratio. All the complexes showed the highest water-uptake ability at 120 min. In particular, the complex CH/ALG(1:1) showed the lower water-uptake ability among all the complexes (404.4 ± 11.2); while a large excess of chitosan and alginate allowed a greater water-uptake ability. In fact, water-uptake ability % of CH/ALG(9:1), CH/ALG(1:9), CH/ALG(7:3), and CH/ALG(3:7) were 686.2 ± 9.5, 767.9 ± 10.2, 484.9 ± 7.1, and 548.7 ± 7.6. This behavior is due to the presence of major charges in the complexes CH/ALG(9:1) and CH/ALG(1:9) that provided the entry of major amount of water in the systems. Moreover, in the complex CH/ALG(1:1), the ionization of the same amounts of polymers provided a system with a major interaction between chitosan and alginate and a minor amount of charges, thus limiting water-uptake ability. Furthermore, the complexes CH/ALG(1:9) and

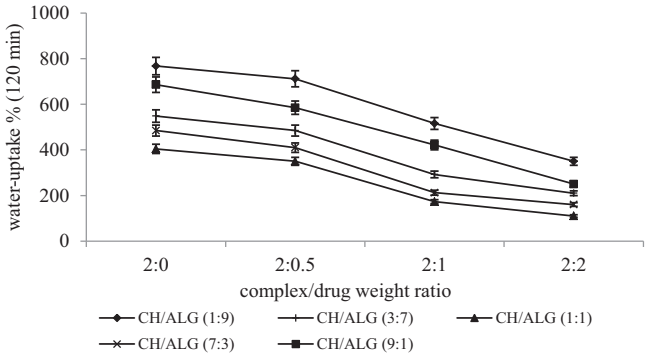

Fig. 5. Water uptake ability after 120 min of differently loaded (complex/drug weight ratios: 2:0.5, 2:1, 2:2, and 2:4) complexes at pH 4.5 ($n = 3$, the SD did not exceed 5%).

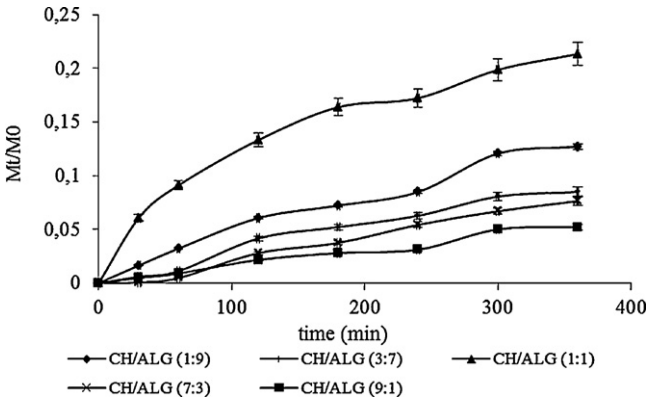

Fig. 6. Fractional amount of chlorhexidine digluconate released over time at pH 4.5 from the different chitosan/alginate complexes (Mt drug amount released over time, M0 drug amount in the formulation at $t = 0$). Each datum represents the average of three determinations ± SD.

CH/ALG(3:7) showed a major water-uptake ability ($P < 0.05$) with respect to CH/ALG(9:1) and CH/ALG(7:3), due to the major ionization of alginate at pH 4.5 (see Section 3.1).

The influence of chlorhexidine digluconate on the water-uptake ability of the insert was also investigated (Fig. 5). As can be seen, the presence of the drug in the vaginal inserts gradually reduced water-uptake. In fact, for the preparation of loaded inserts, the drug was dissolved in phosphate buffer at pH 4.5 and the amino group of chlorhexidine (pK_b 10.3) and the carboxylic groups of gluconic acid (pK_a 3.6) were positively and negatively charged, respectively. When drug solution was added to complex/mannitol mixture, these groups can interact with negative (alginate carboxylic groups) and positive (chitosan amino groups) charges, respectively, thus reducing the amount of free charges in the inserts.

3.6. Insert mucoadhesion properties

The presence of different amounts of chitosan or alginate in the formulations influenced significantly the inserts mucoadhesion properties. In particular, chitosan hydrochloride insert showed a higher detachment force value with respect to that of alginate insert (150 ± 7 μN and 55 ± 3 μN, respectively). This behavior can be due to the presence of chitosan amino groups that at pH 4.5, were positively charged and could interact with the negatively charge of sialic acid (pK_a 2.6) and sulfate residues of mucin glycoprotein (Peppas & Sahlin, 1996). For the same reason, a decrease in the mucoadhesion values was observed with reduction of chitosan amount in the inserts. In fact, the detachment force for CH/ALG(9:1) and CH/ALG(7:3) was higher with respect to that for CH/ALG(1:9) and CH/ALG(3:7) inserts (88 ± 4, 70 ± 6, 55 ± 3, 57 ± 4 μN, respectively). Moreover, in the complex CH/ALG(1:1) the high interaction between chitosan and alginate limited the presence of the positively charges, and, consequently, the detachment force (27 ± 2 μN). There was no significant difference in mucoadhesion results between loaded and unloaded inserts ($P > 0.05$).

3.7. In vitro release studies

Release profiles from loaded vaginal inserts (complex/drug weight ratio, 2:1) at pH 4.5 are shown in Fig. 6. In the case of the control formulation, the total amount of loaded drug was released after 30 min, due to the fast dissolution of the mannitol insert (data not reported in Fig. 6). On the other side, a sustained drug release can be observed for all the complex based formulations, due to the interaction of chlorhexidine digluconate with alginate and chitosan. Among all the inserts based on the different complexes, CH/ALG(1:1) showed the higher drug release due to the higher degree of interaction between chitosan and alginate in the complex and the lower free charges amount able to interact with chlorhexidine digluconate. Surprisingly, despite the comprovate interaction between alginate and chlorhexidine digluconate suggesting a major sustained release, CH/ALG(1:9) insert allowed higher drug release with respect to CH/ALG(9:1) insert.

As previously described, drug-complex interaction provided a decreased water-uptake. However the same interaction determined a decrease of density from unloaded to loaded inserts, indicating the capability of the hydrogel network to extend the polymeric chains in a greater way in presence of drug. In particular, the density values for unloaded and loaded (complex/drug weight ratio, 2:1) inserts based on CH/ALG(1:9) and CH/ALG(9:1) were 0.54 ± 0.02 g/cm^3, 0.65 ± 0.03 g/cm^3, 0.45 ± 0.02 g/cm^3, and 0.52± 0.03 g/cm^3, respectively. From these data we can also observe that the decrease in density was more evident for the insert with the excess of alginate, thus resulting in a major drug release from the polymer matrix, due to a greater drug diffusion ability. Finally in the case of the complex/drug ratio 2:2 all the inserts were unable to control drug release (Fig. 7).

3.8. Antimicrobial activity of the vaginal insert containing chlorhexidine digluconate

Viability of *E. coli* and *C. albicans* in phosphate buffer at pH 4.5 in the absence and presence of unloaded and loaded vaginal insert based on CH/ALG(1:9) complex (complex/drug weight ratio, 2:1) is shown in Table 1.

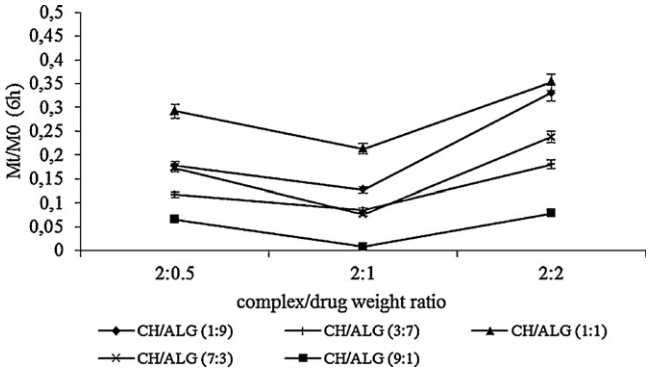

Fig. 7. Fractional amount of chlorhexidine digluconate released after 6 h from differently loaded (complex/drug weight ratios: 2:0.5, 2:1, and 2:2) chitosan/alginate complexes at pH 4.5. Each datum represents the average of three determinations ± SD.

Table 1
Viability of *Escherichia coli* and *Candida albicans* in phosphate buffer at pH 4.5 with and without the vaginal insert containing chlorhexidine digluconate.

Time point (h)[a]	Microbial concentration (log CFU/ml ± SD)[b]			
	Escherichia coli		*Candida albicans*	
	Unloaded insert	Loaded insert	Unloaded insert	Loaded insert
T0	6.15 ± 0.24	6.18 ± 0.15	5.90 ± 0.19	5.90 ± 0.23
T6	4.38 ± 0.28	<2	5.85 ± 0.20	5.00 ± 0.31
T24	3.15 ± 0.19	<2	4.86 ± 0.16	<2

[a] Counts of viable *E. coli* and *C. albicans* were carried at the inoculum time (T0) and after 6 h (T6) and 24 h (T24) of incubation at 37 °C.
[b] Microbial concentration was expressed as a mean of log CFU/ml ± SD.

Cell concentration of *E. coli* decreased after 6 h (T6: 4.38 log CFU/ml) and 24 h (T24: 3.15 log CFU/ml) of incubation at 37 °C, compared to the baseline value (T0: 6.15 log CFU/ml), with a survival of 71.2% and 51.2% at T6 and T24, respectively. Loaded insert exerted a strong antibacterial activity against *E. coli* as evidenced by the bacterial concentrations that were below the detection limit (<2 log CFU/ml) at the time points T6 and T24.

A good viability in phosphate buffer was demonstrated by *C. albicans*, as its concentration remained approximately constant after 6 h (T6: 5.85 log CFU/ml) and decreased about one logarithmic unit after 24 h (T24: 4.86 log CFU/ml) of incubation, compared to the baseline value (5.90 log CFU/ml). The survival of the yeast was found to be 99.2% and 82.4% at T6 and T24, respectively. The addition of the loaded vaginal insert to the experimental medium caused the loss of almost one logarithmic unit in the viability of *C. albicans* at T6 (5.00 log CFU/ml; survival: 84.7%) and a strong reduction of the yeast count at T24 (<2 log CFU/ml).

The present microbiological data demonstrated the inhibitory activity of the chlorhexidine digluconate formulated in vaginal insert against the principal pathogens which are responsible for aerobic vaginitis and candidiasis.

4. Conclusions

This investigation verified the formation of polyelectrolyte complexes between chitosan and sodium alginate in the vicinity of the pK_a interval of the two polymers and confirmed the potential of these complexes, able to hydrate and adhere to vaginal mucosa. Moreover, these complexes can be used to prepare new suitable carrier system capable to overcome limits of the conventional delivery formulations, such as messiness and leakage of formulations, thus increasing patient compliance. The selection of the appropriate chitosan/sodium alginate molar ratio as well as the drug amount allowed the modulation of insert water-uptake behavior and chlorhexidine digluconate release. In particular, inserts based on the complex CH/ALG(1:9) provided the higher amount of released drug and microbiological data demonstrated that chlorhexidine digluconate released from this insert can inhibit the principal pathogens responsible of aerobic vaginitis and candidiasis.

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