Chitosan Gels for the Vaginal Delivery of Lactic Acid: Relevance of Formulation Parameters to Mucoadhesion and Release Mechanisms

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ABSTRACT

The aim of this work was to assess the effect of formulation parameters of a mucoadhesive vaginal gel based on chitosan and lactic acid, and to highlight its release mechanisms. Two molecular weight chitosans were used to prepare gels with 2 lactic acid concentrations. Both chitosan molecular weight and lactic acid concentration had a significant and mutually dependent influence on mucoadhesion, measured on pig vaginal mucosa. Similarly, the lactate release profiles were found to be dependent on lactic acid content and polymer molecular weight.

One gel formulation based on the stoichiometric lactate to chitosan ratio was subjected to release test in media with 2 different counterions and increasing ionic strength. This test demonstrated that the lactate release is mainly due to ionic displacement.

KEYWORDS: Vaginal delivery, mucoadhesion, chitosan, lactic acid.

INTRODUCTION

The human vagina has several effective mechanisms of defense against exogenous microbes. Among these mechanisms, the presence of nonpathogenic commensal microorganisms, mainly *Lactobacillus acidophilus,* which produces antimicrobial bacteriocins, peroxides, and lactic acid from glycogen, is of primary importance. Thanks to the presence of lactobacilli, the pH of the vaginal fluid in healthy women is maintained between 4 and 5. The natural resistance to the colonization of pyogenic organisms is therefore mediated by lactic acid, low pH, and antimicrobial polypeptides.[1,2] A strict coincidence has been observed between an increase in vaginal pH and a higher incidence of bacterial vaginosis and of trichomoniasis also resulting in the destruction of the natural flora of lactobacilli.[3] The increase of the vaginal pH (to as much as pH 7) may also occur in postmenopausal women with consequent possible colonization of the vaginal mucosa by pathogenic microorganisms and, therefore, increased risk of local infections. Irritation of the mucosa, with consequent itching, tenderness, and unpleasant smell are the main consequences of these pathological situations.[4,5]

Among vaginal formulations, gels are easy to manufacture, comfortable, and have the ability to spread onto the surface of mucus and to achieve an intimate contact with vaginal mucosa.[6-8] Moreover, because of their high water content and their rheological properties, they present the further advantage of a hydrating and lubricating action, which is particularly useful in pathological situations characterized by dryness of the vaginal mucosa. The employment of mucoadhesive polymers can improve the time of contact with the mucosa, delaying the loss of the formulation and prolonging the effect. Chitosan is a widely used biocompatible polymer known for its peculiar properties of mucoadhesion,[9] antimicrobial activity, and wound healing.[10] Chitosan-based formulations have been described in literature as drug vehicles,[11] and also for vaginal delivery of some antiinfective drugs.[12,13]Different semisolid preparations have been also proposed for the release of lactic acid.[14] Moreover, some lactate gels based on methylcellulose mixed with either Eudragit or chitosan, are described in the literature,[15,16] although the effect of formulation parameters on mucoadhesion and the influence of the ionic interaction between polymer and lactic acid still need to be investigated.

In this work, some mucoadhesive chitosan lactate gels were developed, which were specifically intended for the controlled release of lactic acid onto vaginal mucosa. Drug-polyelectrolyte complexes have been previously used in pharmaceutical systems, especially in matrix tablets.[17,18] They are often based on the combination of an anionic polyelectrolyte (carrageenan, carbomer, methacrylate derivatives), and sometimes on cationic polyelectrolytes.[19,20] In the present study, chitosan-lactate gels have been assessed not only as a possible vehicle for the maintenance of vaginal physiological pH conditions, but also as a semisolid polyelectrolyte-drug system. The aim of this work was to assess the relevance of some parameters, such as polymer molecular weight and lactic acid/polymer ratio, to the performance of the

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formulation. Two different viscosity grades of chitosan were combined in aqueous environment with lactic acid in 2 lactic acid/polymer ratios. Rheological and mucoadhesive properties and lactic acid release profiles were evaluated as a function of the molecular weight of the polymer and of the amount of lactic acid in the formulation, by means of a full factorial design, to put in evidence not only the main effects but also their interactions. Finally, the relevance of an ion-exchange release mechanism was evaluated by checking the influence of the ionic strength of the release medium on lactate release profiles. In particular, the release induced by different kinds of counterions was assessed.

Materials and Methods

Materials

Two chitosan viscosity grades were compared, hereafter indicated as AM and FH: (1) AM chitosan (medium molecular weight, Aldrich Chemical Co, Milwaukee, WI); the viscosity of a 1% (wt/vol) solution in 1% (wt/vol) acetic acid was ~280 mPa.s (at 37°C, 20 seconds^{-1}) and (2) FH chitosan (high molecular weight, Fluka BioChemika, Fluka Chemie AG, Buchs, Switzerland); the viscosity of a 1% (wt/vol) solution in 1% (wt/vol) acetic acid was ~770 mPa.s (at 37°C, 20 seconds^{-1}).

Lactic acid, United States Pharmacopeia (USP) grade, was used (ACEF Fiorenzuola d'Arda, Piacenza, Italy).

Two commercial preparations, Lactal (Trimedic AB, Kungsbacka, Sweden) and Replens (Jannsen-Cilag SpA, Milan, Italy), were also considered for comparison purposes. The first one contains lactic acid and hydroxypropylmethylcellulose. The second one is a mucoadhesive formulation intended for rehydration of vaginal environment, based on polycarbophil: as it does not contain lactic acid it was considered as a reference in the mucoadhesion test only.

Preparation of the Gels

The gels were prepared by hydrating the polymer (always 3.0% wt/wt final concentration) in distilled water and lactic acid. Final concentration of lactic acid was either 1.65% wt/wt or 3.0% wt/wt. The 1.65% wt/wt lactic acid concentration is close to a stoichiometric ratio between lactic acid and aminoglucosidic units in the chitosan chain. The pH of each formulation was checked after 24 hours hydration (Mettler TL 21 Titrator, Mettler, Novate Milanese, Italy).

Rheological Characterization

The rheological characterization of the gels was performed by means of a Bohlin CS rheometer (Bohlin Instrument Ltd, Cirencester, UK) equipped with a cone-plate 4/20 system. All analyses were performed at 37°C, after a 3-minute rest time. Viscosity curves were obtained at between 10 and 100 seconds^{-1} shear rates. Viscoelastic parameters G' (storage modulus) and G" (loss modulus) were measured in an oscillation test at frequencies ranging between 0.1 and 5.0 Hz, in the linearity range of viscoelasticity. The rheological characterization was effected, for comparison purposes, also on gels prepared with acetic acid, as this is quite commonly used to dissolve chitosan.

Mucoadhesion Test

Mucoadhesion was evaluated with an ex vivo detachment test, by means of a tensile stress tester.[21] A 100-mg quantity of gel was layered onto a filter paper support (30-mm diameter) glued to a mobile probe. This device was put in contact with a vaginal mucosa layer obtained from pigs weighing ~130 to 140 kg, and ranging from 1 to 2 years old. Porcine vaginal mucosa was deprived of connective tissue and glued, using cyanoacrylate glue, to a holder facing the formulation. Time of contact was 3 minutes, with a preload of 2500 milliNewtons (mN), to allow the formation of mucoadhesive joints. The mobile probe was then moved at constant speed (4 mm/min) up to complete separation of the 2 surfaces. The maximum force of detachment (Fmax) was measured and recorded on a personal computer. Fmax was used as response in a full factorial design evaluation of the effect of polymer molecular weight and of lactic acid amount on mucoadhesion. The statistical analysis was performed with a Statgraphics 5.0 package (Manugistics, Rockville, MD).

Lactic Acid Release Test

The release of lactic acid was measured by means of Franz cells with a 20-mm diameter orifice. The temperature was kept at 37°C by means of a water jacket. The release test was performed in 0.05 M KH_2PO_4/Na_2HPO_4 phosphate buffer pH 5.0. The sample (100 mg) was layered on the top of a dialysis membrane (12 000-14 000 d cut off). At predetermined intervals, 0.5 mL of receptor fluid was taken, and the lactic acid concentration was detected by high-performance liquid chromatography (HPLC) method. The receptor fluid was replaced and correction was made for dilution. The release profiles obtained were fitted according to the equation that describes diffusive phenomena:

$$\frac{Dt}{D\infty} = k\sqrt{t}, \qquad (1)$$

where Dt and $D\infty$ represent drug delivered at time = t and time = ∞, respectively.

The release rate constant K, obtained from the slope of the curves so obtained, was then used as response in a full

factorial design to assess the effect of polymer molecular weight and lactic acid amount on the release properties of the gels. The statistical analysis was performed with a Statgraphics 5.0 package.

Lactic Acid Detection

Lactic acid was detected by means of an HPLC method[22] using a Jasco apparatus (model 980-PU pump and model 975-UV detector, Jasco, Tokyo, Japan), with an Ultrasphere ODS column (5 μm, 150 × 4.6 mm) (Beckman Coulter, Milan, Italy). Mobile phase was 2% (vol/vol) methanol in phosphate buffer (0.025 M, pH 7.4) containing 0.002 M tetrabutylammonium iodide. Flow rate was 0.6 mL/min, UV detection was performed at 210-nm wavelength. In Figure 1, an example of a chromatogram showing the peak of lactic acid at 3.35 minutes retention time is given. The lactic acid amount was calculated by referring to a calibration curve that was repeated for each of the media where release studies were performed.

Assessment of the Counterion Influence on Lactic Acid Release

The influence of the presence of counterions in the medium on the maximum lactate release was assessed by filling dialysis bags (cutoff 12-14 kd) (Emanuele Mires, Milan, Italy) with accurately weighed samples (~4 g) of the gel prepared with the chitosan AM 3.0% wt/wt and lactic acid 1.65% wt/wt. The bags were maintained under agitation in 200 mL of either phosphate buffer or NaCl solution at different concentrations. Each medium was tested in triplicate. In the case of phosphate buffers, the following concentrations were used: 6.25 mM, 12.5 mM, 25 mM, and 50 mM. In the case of NaCl solutions, the following concentrations were used: 2.5 mM, 5 mM, 10 mM, 25 mM, and also 154 mM corresponding to the 0.9% (wt/vol) physiological solution.

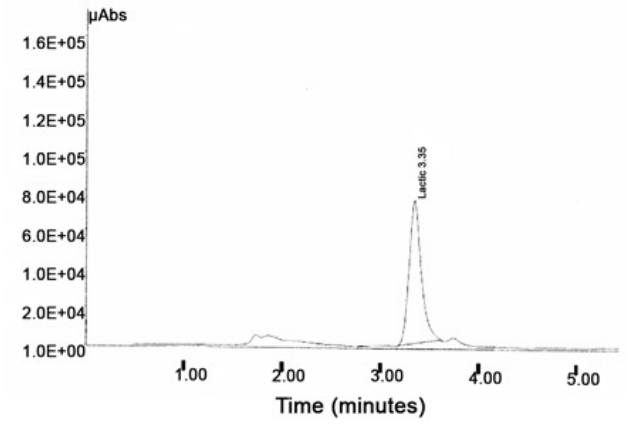

Figure 1. Example of a chromatogram of a lactic acid sample.

Table 1. pH Values of the Prepared Chitosan-Lactate Gels*

Chitosan	% Lactic Acid	pH
AM	1.65	4.1
AM	3.00	3.3
FH	1.65	3.8
FH	3.00	3.3

*AM indicates chitosan with a viscosity of a 1% (wt/vol) solution in 1% (wt/vol) acetic acid and ~280 mPa.s (at 37°C, 20 seconds^{-1}); FH, chitosan with a viscosity of a 1% (wt/vol) solution in 1% (wt/vol) acetic acid and ~770 mPa.s (at 37°C, 20 seconds^{-1}).

For comparison, lactate release was also tested in distilled water. Samples of 2 mL of the receiving medium outside the bags were taken at different times (at 1, 2, 3, 4, 5, and 6 hours, and after 24 hours), and the concentration of lactic acid was detected by HPLC method.

Results and Discussion

Preparation of the Gels and Rheological Characterization

The 1.65% (wt/wt) lactic acid concentration was found to be the minimum amount of lactic acid necessary to obtain solubility of the 3.0% (wt/wt) chitosan samples and clear gels. This finding is in line with data previously given[16] in which the lactic acid/chitosan ratio necessary to reach stoichiometric neutralization was found to be 0.6:1.0 and was, therefore, assumed to be the equimolar ratio. In Table 1 the pH values of all the prepared chitosan gels are given. Even the lowest lactic acid concentration (1.65% wt/wt) gave a pH value around 4.0 and therefore close to the physiological one. As expected, when 3.0% wt/wt lactic acid concentration was used, the pH values were slightly lower, although still compatible with physiological values.

In Figure 2, the apparent viscosity measured at 20 seconds^{-1} is given for the reference formulations, Lactal and Replens, and for the prepared chitosan formulations. All the tested formulations were also prepared using acetic acid instead of lactic acid. This formulation was made for the purpose of comparison, as acetic acid is quite often used to obtain chitosan solutions. No relevant differences could be observed between the samples with lactic acid and those with acetic acid. In almost all cases, the increase in acid concentration (from 1.65% to 3.0% wt/wt) corresponded to a slight decrease in viscosity, which however did not result as significant; the only exception being the FH sample in acetic acid ($P < .05$). The comparison, performed by means of t test, assumes that the variances of the 2 samples are equal: in this case this assumption seems to be reasonable, as an F test was performed to compare the standard deviations. In Figure 3, the viscoelastic parameters for the samples in lactic acid and in acetic acid are illustrated. The values found for the 2 reference formulations were in the case of Replens 590 (± 6.3) Pa for G' and 63 (± 0.9) Pa for G" and in the case

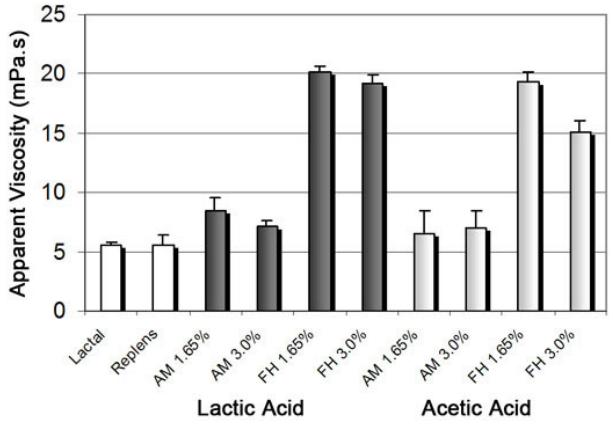

Figure 2. Apparent viscosity values (measured at 20 seconds^{-1} shear rate) of the 2 commercial formulations and of the chitosan gels prepared with lactic acid and with acetic acid. AM indicates medium molecular weight chitosan; and FH, high molecular weight chitosan.

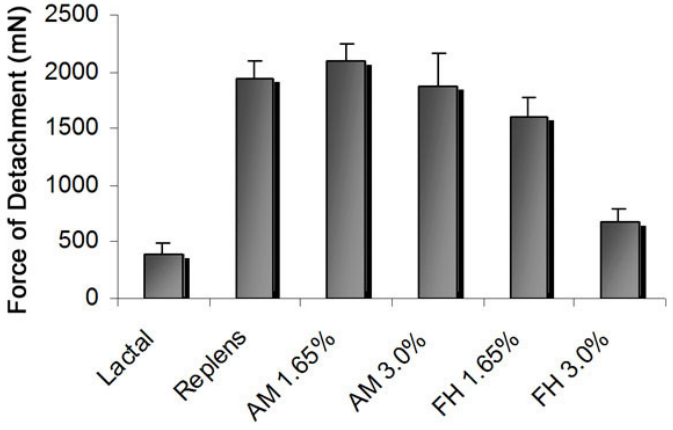

Figure 4. Mucoadhesion of the gels, measured as maximum force of detachment (mean values ± SD; n = 6), of the chitosan gels and of the 2 commercial formulations. AM indicates medium molecular weight chitosan; and FH, high molecular weight chitosan.

of Lactal 9.8 (± 6.3) Pa for G' and 30 (± 1.3) Pa for G". For Replens, based on polycarbophil, the viscoelastic parameters are quite high, in line with the cross-linked structure of the polymer that is responsible for the strong elastic character of the sample. Such strong elastic properties were not observed in any of the chitosan lactate gels, indicating that the gel structure is, as expected, mainly owing to physical entanglement of polymeric chains. Especially in the case of FH samples, both in lactic and in acetic acid, the viscoelastic parameters seem more strongly influenced by the amount of the acid in the formulation than by viscosity. The increase of the acid level results in the increase of both the loss modulus and the storage modulus, probably because a more complete ionization of the polyelectrolyte is accomplished, resulting in repulsion of similar charges and higher gel rigidity.

Mucoadhesion

Mucoadhesion of the gels, expressed as force of detachment from the excided mucosa, is illustrated in Figure 4. The difference between the 2 reference formulations is in line with the literature data, which describe hydroxypropyl methylcellulose (HPMC) as a quite poor mucoadhesive polymer, while polycarbophil is well known for its excellent mucoadhesion, especially at the quite low pH values as that in the healthy vaginal mucosa. Most of the considered formulations are comparable to Replens. In spite of the experimental variability, a clear trend can be observed between the different samples. As previously described,[23] the lower molecular weights correspond to better mucoadhesion. This finding can be explained by the high mobility (and interpenetration capability) of the low molecular weight polymer. Moreover, this result confirms that the adopted test

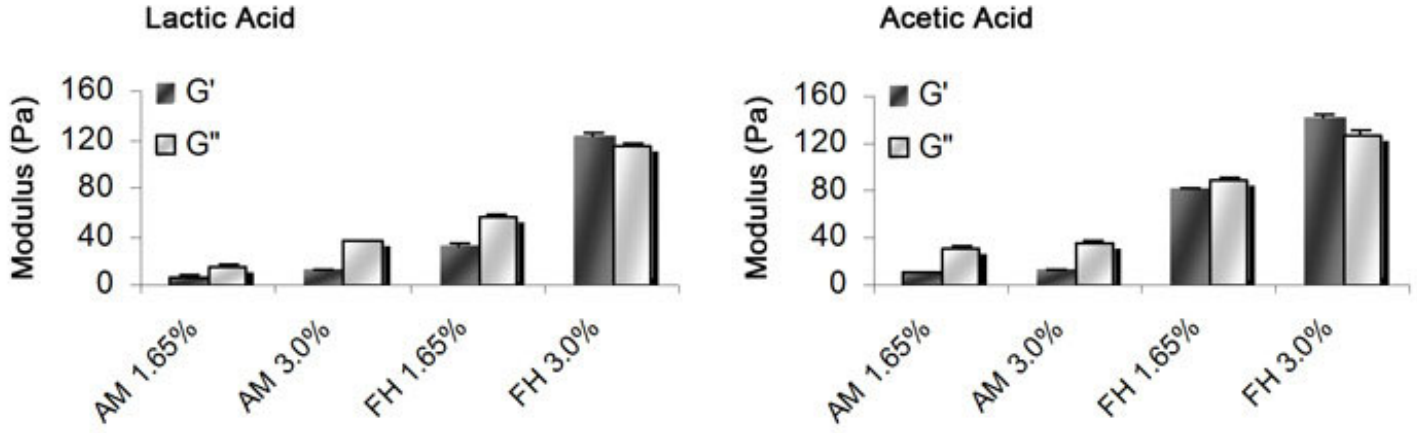

Figure 3. Viscoelastic parameters (elastic modulus G′ and viscous modulus G″) of the chitosan gels prepared with lactic acid and with acetic acid. AM indicates medium molecular weight chitosan; and FH, high molecular weight chitosan.

Table 2. Analysis of Variance for the Effects of the Formulation Parameters Polymer Molecular Weight and Lactic Acid Percentage and of Their Interaction on the Mucoadhesion Performance Measured as Maximum Force of Detachment*

Source	Sum of Squares	*df*	Mean Square	F Ratio	*P* Value
A: MW	4.51 E6	1	4.51 E6	30.68	.000
B: lactic acid	2.06 E6	1	2.06 E6	14.02	.001
AB	8.66 E5	1	8.66 E5	5.89	.025
Total error	2.94 E6	20	1.47 E5	—	—
Total	1.07 E7	23	—	—	—

*MW indicates polymer molecular weight; AB, interaction of molecular weight and lactic acid percentage; Fmax, maximum force of detachment.

was able to measure the strength of the mucus-polymer interface, and it was not affected by the polymer consistency: if the rupture had occurred within the gel layer, the Fmax would have been lower for the low molecular weight samples (characterized by lower viscosity and elasticity). Furthermore, it can be seen that the higher lactic acid contents correspond to poor mucoadhesion behavior, particularly evident in the case of high molecular weight FH samples. This finding can be explained by a reduced ionization of the acidic moieties of the sialic acids of the mucin chains, which are usually known to contribute to strengthening the mucoadhesive bonds with cationic polymers such as chitosan. To better understand the influence of both molecular weight and lactic acid amount, the mucoadhesion results of the chitosan formulations were analyzed according to a full factorial design: 2 factors (molecular weight and lactic acid content) and 2 levels. An advantage of full factorial designs is their ability to put in evidence not only the statistical significance of main factors, but also of their interactions. In this case, in fact, the results of the analysis of variance (ANOVA) test, given in Table 2, show that polymer molecular weight, acid amount, and their interaction had a significant effect on the response maximum force of detachment (Fmax) ($P < .05$).

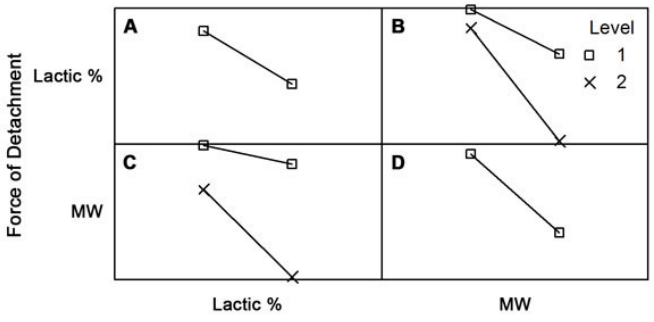

Figure 5. Effects of lactic acid concentration (lactic %) and chitosan molecular weight (MW) on maximum force of detachment (Fmax). (A) mean effect of lactic acid percentage, (B) interaction plot: effect of MW when lactic acid percentage is at the low[1] and at the high level,[2] (C) interaction plots: effect of lactic acid percentage when MW is at the low[1] and at the high level, (D) mean effect of MW.[2] AM indicates medium molecular weight chitosan; and FH, high molecular weight chitosan.

In Figure 5, in particular, the mean effects of the main factors and their mutual interactions (shown in the B and C sections) are illustrated: it is possible to see that between molecular weight and lactic acid amount there is a synergism; at the highest level of lactic acid factor, the negative effect of polymer molecular weight is more pronounced.

Lactate Release Studies

Figure 6 shows the comparison of the release curves of lactic acid from the prepared chitosan gels, and from Lactal gel used as reference. The 2 formulations containing the lactic acid at 3.0% wt/wt level show faster release, superimposable to that of HPMC-based formulation (Lactal). On the assumption of prevalent diffusive release mechanism, the percentage released vs time experimental points were fitted according to the square root equation, in the linearized form. For all the formulations, a quite good agreement was found between the curves and the chosen model (R2 of the regression ranging between 0.956 and 0.992). The linearized curves are illustrated in Figure 7, where, in the insert, the mean (± SD) values of the slope K are given. This parameter, which describes the release rate, was therefore used as response in a full factorial design aimed to investigate the effect of polymer molecular weight and of lactic acid

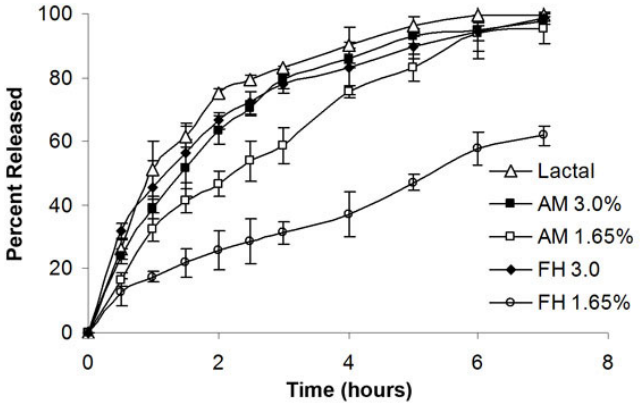

Figure 6. Lactate release profiles from the prepared chitosan gels and from the commercial formulation Lactal (mean values ± SD; n = 3).

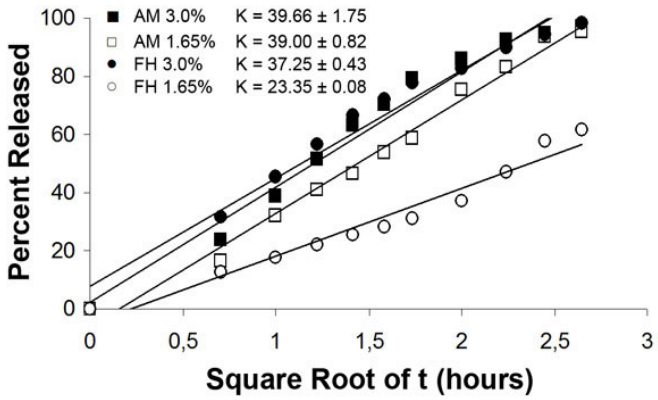

Figure 7. Linearization of lactate release profiles from the prepared chitosan gels according to square root equation (mean values ± SD; n = 3). The slope values K ($\%t^{-1/2}$) for each formulation are given (mean ± SD, n = 3). AM indicates medium molecular weight chitosan; and FH, high molecular weight chitosan.

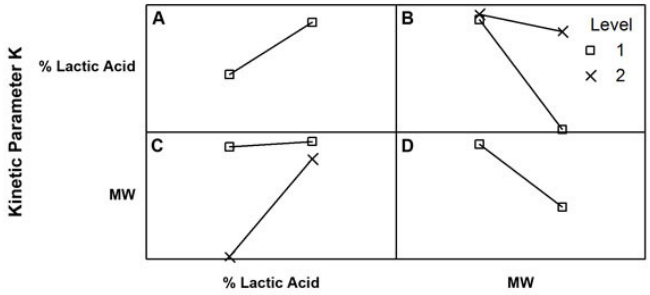

Figure 8. Effects of lactic acid concentration (lactic %) and chitosan molecular weight (MW) on lactate release rate K (%√t). (A) mean effect of lactic acid percentage, (B) interaction plot: effect of MW when lactic acid percentage is at the low[1] and at the high level,[2] (C) interaction plot: effect of lactic acid percentage when MW is at the low[1] and at the high level,[2] (D) mean effect of MW.

amount in the formulation on drug release. The results of the ANOVA test are given in Table 3, while the mean effects and their interactions are illustrated in Figure 8. Both the main factors and their interaction had results that were significant ($P < .05$). Chitosan molecular weight has a negative effect on lactate release, probably because of the slower diffusion through the more entangled and more rigid polymer network of FH gels. On the contrary, lactic acid amount has a positive effect on drug release, as could be expected on the basis of diffusive behavior, where lactate concentration acts as a driving force for diffusion. Diffusive behavior is conceivably the main release mechanism in the case of gels based on the nonionic polymer HPMC, such as the reference formulation Lactal. However, in the case of lactic acid (RCOOH) and chitosan (Ch), an ionic pair $RCOO^-$ ChH^+ is likely to form in the range of pH values between the pK_a of lactic acid (3.8) and that of chitosan (~6). Equilibrium is therefore likely to be involved in the lactate release according to the scheme illustrated in Figure 9.

This equilibrium can occur only after the ions of the medium have determined the lactate displacement from the complex with the polymer by ionic exchange. In particular, Figure 6 shows that all the chitosan-lactate samples, with the only exception being FH 1.65%, release the lactate to a quantitative extent. This finding suggests that the exchange step by the counterions of the medium is complete, under the experimental conditions, making the complete diffusion of lactate species possible.

To better investigate this aspect, the effect of medium counterions on the lactate release was further investigated.

Assessment of the Counterion Influence on Lactic Acid Release

The sample based on low molecular weight chitosan, containing a close to stoichiometric ratio of chitosan/lactic acid (AM 1.65%), was chosen to assess the effect that the counterions of the medium exert on lactate release. In Figure 10, the lactate release in media based on NaCl and on pH 5.0 phosphate buffer, respectively, is illustrated. An acetate buffer, more commonly used to mimic the vaginal environment could not be used because the acetic acid interferes with the chromatographic detection of lactate. The 2 buffers used were therefore chosen because the phosphate buffer made it possible to maintain the environmental pH at acidic values (pH 5.0) where chitosan is well soluble. NaCl solution seems more physiologically relevant than the phosphate one, as the chloride ions are the most represented in the vaginal

Table 3. Analysis of Variance for the Effects of the Formulation Parameters Polymer Molecular Weight and Lactic Acid Percentage and of Their Interaction on the Lactate Release Rate, Expressed by the Kinetic Parameter K*

Source	Sum of Squares	*df*	Mean Square	F Ratio	*P* Value
A: MW	244.35	1	244.35	247.3	.000
B: lactic acid	159.06	1	159.07	161.0	.000
AB	131.40	1	131.41	133.0	.000
Total error	7.90	8	0.99	—	—
Total	542.73	11	—	—	—

*MW indicates polymer molecular weight; AB, interaction of molecular weight and lactic acid percentage.

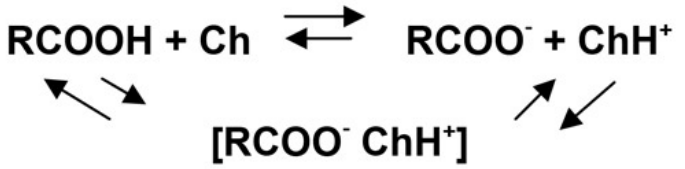

Figure 9. Scheme of the equilibrium possibly involved in the gel between the following species: lactic acid (RCOOH), chitosan (Ch), and the ionic pair $RCOO^{-}$ ChH^{+}.

physiological environment. However, in this case, no buffering effect was present, and the pH values were higher, ranging between 6.5 and 6.9.

The release profile in distilled water (given for reference in both the figures) stops after releasing ~10% of the lactate present in the formulation. This result was expected, as in the considered formulation there was no excess of unbound lactic acid. As the buffer concentration was increased, the maximum amount released also increased, to reach plateau levels dependent on the total amount of salts in the medium; this finding is clearly owing to the displacement mechanism governing the release of lactate. To better compare the effects

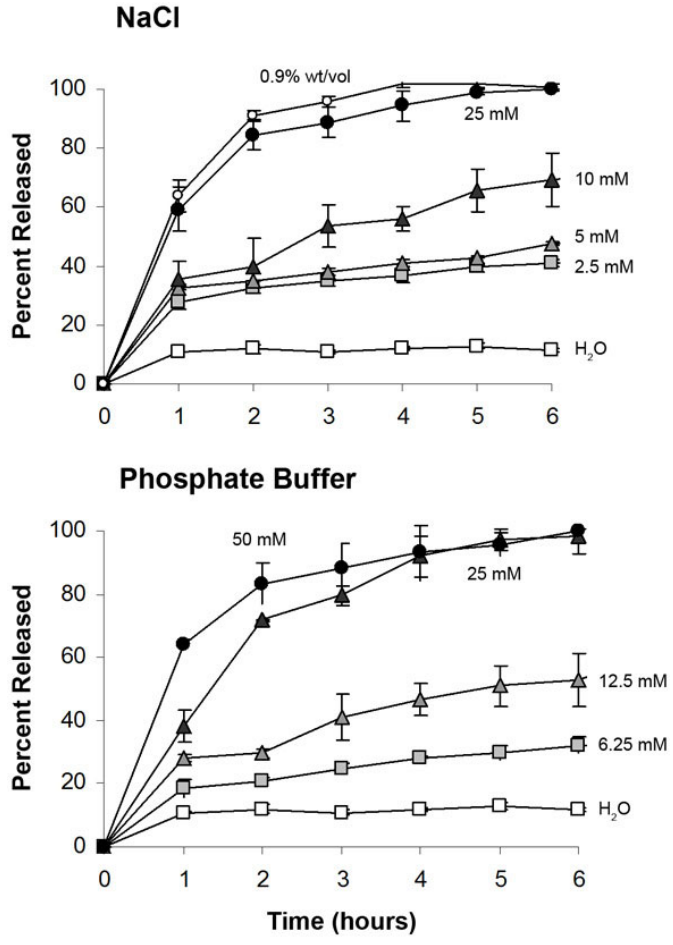

Figure 10. Lactate release profiles in media based on NaCl and phosphate buffer, at different concentrations.

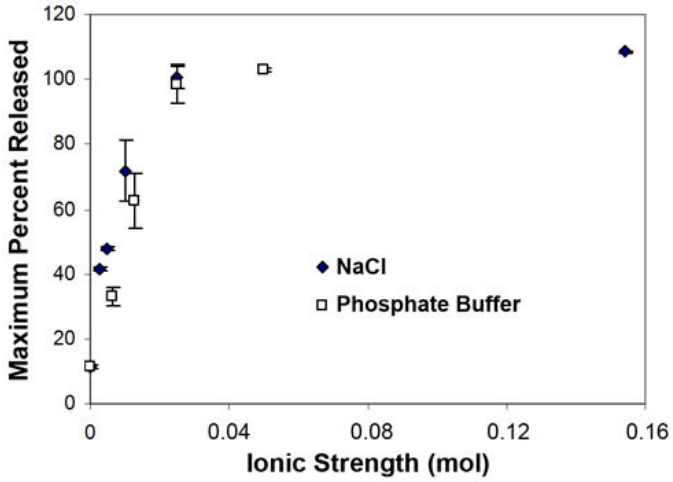

Figure 11. Maximum percentage of lactate released after 24 hours as a function of the medium ionic strength, in the different NaCl solutions and phosphate buffers.

of the 2 media, and therefore of the different counterions involved in lactate displacement, the maximum percentage released (after 24 hours) is plotted toward the ionic strength for each medium considered: the results are given in Figure 11. In both the case of NaCl and of phosphate buffer, the effect of the ionic strength on lactate displacement is quite similar. No relevance of the anionic counterion on the lactate release mechanism could therefore be appreciated.

CONCLUSIONS

The data obtained confirmed the good mucoadhesive behavior of chitosan, also in the studied lactate gels, although the effect of both polymer molecular weight and lactic acid concentration to optimize the mucoadhesive performance of the formulations and release profiles must be taken into account.

The observed influence of the ionic strength of the medium on the maximum amount of lactic acid released from the formulation AM 1.65%, based on a stoichiometric ratio between chitosan and lactic acid, confirms that not only diffusion but also ionic displacement plays a role in the release mechanism.

Moreover, the lactate release is completed at ionic strength values well below those characteristic of the physiological fluids (0.9% wt/vol NaCl solution). This finding makes it reasonable to envisage a complete release of lactate from the tested formulations in vaginal environment.

ACKNOWLEDGMENTS

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