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Chitosan–EDTA Conjugate: A Novel Polymer for Topical Gels

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Abstract

A recently developed chitosan–EDTA conjugate, neutralized with sodium hydroxide (NaChito–EDTA), has been tested for possible topical use. The technical properties and microbial stability of NaChito–EDTA have been compared with those of carmellose sodium (NaCMC), hydroxypropylmethylcellulose (HPMC), sodium polycarbophil (NaPCP) and sodium carbopol 980 (NaC980), well established gelatinizing agents.

NaChito–EDTA forms stable, colourless, completely transparent hydrogels at a polymer concentration of 0·5%. Of the polymers tested the novel polymer had the lowest incompatibility with multivalent cations and with ethanol, and much the best swelling properties. After 28 days of incubation at room temperature the rates of growth of the complete bacterial spectrum occurring in demineralized water and of *Escherichia coli*, serving as model strain representative of Gram-negative bacteria, were at least 2 log and 5·7 log, respectively, lower in NaChito–EDTA gels than in the other hydrogels. This antimicrobial activity of NaChito–EDTA can be explained by its highest binding affinity towards magnesium, which stabilizes the outer membrane of Gram-negative bacteria. However, this antimicrobial effect is insufficient to guarantee microbial stability. Further results showed that the antimicrobially acting polypeptide nisin can be recommended as an alternative novel preservative for NaChito–EDTA gels, because its antimicrobial spectrum could also be increased towards Gram-negative bacteria in combination with chelating excipients.

NaChito–EDTA seems, therefore, to be a promising novel polymer for topically-used gels, with advantages over well established gelatinizing agents.

The topical use of gels based on polymers such as poly(acrylate) and cellulose ether derivatives is still increasing (Regdon & Eros 1985; Braun 1991; Boer 1993; Foldvari et al 1993; Rebelo et al 1993; Ruiz et al 1994; Nortier et al 1995). They are widely applied excipients for thickening topical lotions, creams and ointments. These types of polymer are also used to modify the rheology of water-based systems and to stabilize multi-phase systems such as emulsions and suspensions. However, poly(acrylate) and cellulose ether gels are highly sensitive to electrolytes and to multivalent cations (for example Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}) and are incompatible with high concentrations of ethanol when neutralized with inorganic bases. On the other hand, organic neutralizers such as triethanolamine (TEA) or diisopropanolamine (DIPA) guarantee compatibility with high concentrations of ethanol, but contain traces of cancerogenic nitrosamines (Douglass et al 1978; Anderson 1979), the presence of which in topical products might pose a risk if they are absorbed through the skin. A further drawback is the microbial instability of aqueous formulations based on these gelatinizing agents (Bernkop-Schnürch et al 1995; Valenta & Schmatzberger-Wagerer 1995). The search for alternatives to these well established polymers is therefore a desirable target. Recently our research group has generated a novel polymer (Bernkop-Schnürch et al 1997) by covalent attachment of EDTA to chitosan (poly-D-glucosamine). The aim of this study was to evaluate the technical properties and microbial stability of the chitosan–EDTA conjugate in comparison with those of gelatinizing agents already established for topical use.

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Materials and Methods

Materials

Nisin (ambicin N) was from Aplin and Barrett (UK), carbomer (carbopol 980) and polycarbophil

(noveon AA1) from Goodrich (UK), HPMC (hydroxypropylmethylcellulose; metolose 60 SH) from ShinEtsu (Japan) and NaCMC (carmellose sodium; cellulose gum) from Hercules (The Netherlands). All other substances were obtained from Sigma (St Louis, MO).

Synthesis of the chitosan–EDTA conjugate

Chitosan (1 g) was suspended in demineralized water (100 mL) and the pH of the suspension was maintained constant at 6 by continuous addition of HCl (1 M, 500 μL) until the polymer had completely dissolved. EDTA (3·63 g) was suspended in demineralized water (10 mL) and the pH was adjusted to 6 by addition of the sodium hydroxide solution (5 M); this was then mixed with the 1% pH 6·0 chitosan–HCl solution (10 mL). To induce the formation of amide bonds between the amino groups of the chitosan and the carboxyl groups of EDTA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) was added at a final concentration of 0·1 M. The reaction mixture was incubated at room temperature with permanent stirring for 12 h and the resulting conjugate was isolated by exhaustive dialysis against demineralized water, 0·05 M sodium hydroxide solution and once more against demineralized water. The purified product was neutralized with sodium hydroxide and the so-called NaChito–EDTA lyophilized and stored at room temperature until use.

Neutralization of poly(acrylate) derivatives

Carbomer and polycarbophil were neutralized by gradually adding the polymer (10 g) to methanolic sodium hydroxide solution (4% w/w; 100 mL) while stirring. The precipitate of derivatives was separated by filtration, washed with methanol until the pH of the filtrate became neutral, and dried in a desiccator. The neutralized polymers, NaC980 and NaPCP, were stored at room temperature until use.

Formulations

Demineralized water obtained by ion-exchange (Seradest, Vario) was used to prepare 0·5% hydrogels from NaC980, NaPCP and NaChito–EDTA and 5% hydrogels from NaCMC and HPMC. For microbial stability studies hydrogels were also prepared with water for injection (European Pharmacopoeia 1991) and sterilized for 15 min at 121°C by autoclaving (Melag, Germany). For one series of experiments nisin (100 $\mu g\,g^{-1}$) was added aseptically under laminar air flow. All preparations were stored at room temperature until evaluation.

Incompatibility studies with multivalent cations and ethanol

To evaluate the influence of calcium, magnesium, zinc, aluminium and iron ions on the stability of different gels, aqueous stock solutions (0·2 M) of $CaCl_2$, $MgCl_2$, $ZnCl_2$, $AlCl_3$ and $Fe(NO_3)_3$ were prepared. These stock solutions and demineralized water were added to each hydrogel (700 mg) to furnish final cation concentrations of 100 mM, 50 mM, 10 mM, 5 mM and 2 mM in a final volume of 1·4 mL.

For incompatibility studies with ethanol each formulation (250 mg) was mixed with aqueous ethanol solutions (750 mg) to furnish final ethanol concentrations of 25, 35, 45, 55 and 65% (w/w). After 30-min incubation at room temperature all samples were investigated for viscosity loss, turbidity and precipitation. To determine viscosity loss, flow curves (rheograms) of each formulation were plotted at 23 ± 0·1°C by use of a Rotivisco (Haake, Germany) RT20 rheometer with cone plate (C35°/2) geometry. The flow curves were obtained under controlled stress beginning with shear rates from 0–100 s^{-1} for 90 s and ending with shear rates from 100–0 s^{-1} for 90 s. The viscosities of the gels were compared at a shear rate of 10 s^{-1}. Rheograms of samples free from multivalent cations or ethanol served as reference.

Magnesium- and calcium-affinity studies

The binding affinity of the NaChito–EDTA conjugate towards Mg^{2+} and Ca^{2+} ions at fixed pH was compared with those of NaC980, NaPCP and NaCMC using a slight modification of a method previously described by Lueßen et al (1995). To remove traces of soluble NaChito–EDTA conjugate, which would disturb evaluation of the binding affinity, the polymer conjugate was hydrated in demineralized water, centrifuged (1 h; 40 000 *g*; 20°C) and the supernatant discarded. The purification step was repeated four times and the remaining insoluble polymer was then lyophilized. The polymer conjugates NaC980 and NaPCP at concentrations of 0·25% (w/v) were hydrated in magnesium or calcium solution (0·1 mg mL^{-1}; 5·0 mL); these concentrations of magnesium and calcium were below the binding capacity of tested polymers. After incubation for 12 h at 20°C the polymers were centrifuged (1 h; 9000 *g*; 20°C) and the supernatant was analysed by complexometric titration to determine the amount of unbound Mg^{2+} (0·001 M EDTA; indicator: Eriochrome Black T) and Ca^{2+} (0·01 M EDTA; indicator: calcein) using a method described elsewhere Bernkop-Schnürch & Krajicek (1998).

Water-absorbing capacity

The water-absorbing capacity of each polymer system was determined by a gravimetric method. Aqueous gels of each polymer (30 mg) were placed on a water-permeable membrane serving as the bottom of a glass cylinder with a diameter of 28 mm. The gels were desiccated at 50°C and the cylinder was fastened to a tripod so that the membrane was situated above a vessel of water placed on a precision balance (PC 4400; Mettler, Switzerland). Thereafter the cylinder was lowered so that the membrane was completely immersed, with the polymer just above the water surface. The water uptake of each polymer system was then determined by measuring the decrease in weight of the water supported by the balance.

Diffusion test

Agar diffusion tests were performed by the method of the US Pharmacopoeia (1990), slightly modified as previously described by Valenta et al (1996). Briefly, cultures (1 mL; OD_{600} 0·2–0·3) of *Escherichia coli* (ATCC 8739) were inoculated on to Petri dishes containing bacterial media B (European Pharmacopoeia 1991). After 30 min prediffusion at room temperature each formulation (NaPCP, NaC980; NaChito–EDTA, NaCMC, HPMC; 1 g containing 0 or 100 μg nisin g^{-1}) was added as a spot to a plate. The plate was incubated at 37°C for 48 h and then evaluated qualitatively to determine whether bacterial growth had been inhibited.

Bacterial growth in the gel formulations

Test 1. The inoculum contained the complete microbial spectrum occurring in demineralized water, determined to be $1{\cdot}5 \times 10^4$ colony-forming units (CFU) mL^{-1} according to the method of Porter & Feig (1980). All gels were prepared with this demineralized water. NaPCP, NaC980, NaChito–EDTA, NaCMC and HPMC gels were stored at room temperature in glass cups during the test period of 28 days.

Test 2. *E. coli* was grown in liquid medium B (European Pharmacopoeia 1991) to a preset optical density (OD_{600} 0·41–0·60) corresponding to an average bacterial count between 1·15 and $1{\cdot}9 \times 10^8$ CFU mL^{-1}. This bacterial suspension (1 mL) was diluted in five steps to 1 : 100 000 with liquid medium B, the final dilution serving as inoculum for the gels. Each 20 g of sterile hydrogel with and without 2 mg nisin were contaminated with 2 mL of the diluted bacterial suspension and homogenized with a sterile spatula for 10 min under laminar flow. This furnished an initial inoculum in the range 50–100 CFU (g hydrogel)$^{-1}$.

The number of colony-forming units in each sample (test 1 and test 2) was counted 2 h and 7, 14, 21 and 28 days after preparation. The gel (1 g) was vigorously mixed with sterile NaCl–peptone buffer (European Pharmacopoeia 1991; 9·0 mL) and the resulting solution (1 mL) was plated on to a Petri dish containing bacterial medium B. The number of colony-forming units was counted after incubation at 37°C, and if necessary, the samples were diluted until the number was suitable for counting. Three independent replicate counts were obtained for each sample.

Statistical data analysis

Data are the means of results from three experiments ± s.d.. Statistical data analysis was performed using the Student's *t*-test, with $P < 0{\cdot}05$ as minimum level of significance.

Results and Discussion

Technical properties

The technical properties of NaChito–EDTA were compared with those of well established gelatinizing agents. Properties investigated were its compatibility with certain multivalent cations and with ethanol; rheological testing and visual observation were also performed. Incompatibilities were characterized by indications of no effect, viscosity loss, turbidity, and precipitation, as listed in Table 1. Pseudoplastic flow behaviour was demonstrated for all formulations. In contrast with HPMC, a nonionogenic polymer, all the anionogenic polymers were incompatible with multivalent cations. Of these anionogenic polymers the incompatibility with multivalent cations was lowest for NaChito–EDTA.

The results of incompatibility studies with ethanol shown in Table 2 demonstrate that carbopol and polycarbophil neutralized with sodium hydroxide have limited compatibility with ethanol. Even relatively low concentrations of ethanol lead to viscosity loss or turbidity, or both. In contrast with sodium hydroxide, organic bases lead to fewer problems of processing, handling and stability. Although the use of triethanolamine (TEA) and diisopropanolamine (DIPA) is preferred, it is known that these amines can contain nitrosamines as technically unavoidable impurities and many efforts have been made to replace these bases. Less problematic alternatives have been suggested, for example tetrahydroxypropylethylenediamine, aminoethylpropanol and tromethamol (Bremecker 1989). NaChito–EDTA gels are, however, much more compatible with ethanol; NaChito–EDTA can be used to produce colourless, completely clear

Table 1. Incompatibilities of different gelatinizing agents with multivalent cations.

Anion and quantity (mmol (g gel)$^{-1}$)	NaChito–EDTA	Sodium polycarbophil	Sodium carbopol 980	Sodium carboxymethylcellulose	Hydroxypropylmethylcellulose
Ca^{2+}					
100	P	P	P	–	–
50	P	P	P	–	–
10	P	P	P	–	–
5	V	V	TV	–	–
2	–	V	V	–	–
Mg^{2+}					
100	TV	P	P	–	–
50	V	P	P	–	–
10	V	P	P	–	–
5	–	V	V	–	–
2	–	V	V	–	–
Zn^{2+}					
100	P	P	P	TV	
50	P	P	P	TV	–
10	P	P	P	–	–
5	TV	TV	P	–	–
2	–	V	V	–	–
Al^{3+}					
100	P	P	P	P	–
50	P	P	P	P	–
10	P	P	P	T	–
5	P	P	P	–	–
2	T	TV	TV	–	–
Fe^{3+}					
100	P	P	P	P	–
50	P	P	P	P	–
10	P	P	P	P	–
5	P	P	P	P	–
2	P	TV	TV	–	–

– = Unaffected; V = viscosity loss greater than 30% (shear rate 10 s^{-1}); T = turbidity; P = precipitation.

hydroalcoholic gels containing as much as 65% ethanol.

The water-absorbing capacity of polymers depends upon the nature of the polymer, extent of cross-linking, temperature, polymer–solvent interactions and extent of ionization (Khare et al 1992). The importance of quantifying swelling properties is obvious, not only with regard to design and development of controlled-release dosage forms, but also for topical formulations. As shown in Figure 1, NaChito–EDTA had much the best swelling properties. Within 20 min the water uptake of this novel polymer was approximately twice as high as that of NaPCP or NaC980. NaChito–EDTA, NaPCP and NaC980 form stable gels even when polymer concentrations are as low as 0·5%. Because of poor water absorbency, a tenfold higher concentration of HPMC and NaCMC is required for formation of stable gels.

Microbial stability

To investigate the growth of the complete bacterial spectrum occurring in demineralized water (test 1) in the five different hydrogels, the number of CFU g^{-1} were counted 2 h, and 7, 14, 21 and 28 days after preparation. With the exception of the data obtained after 2 h, which can be regarded as

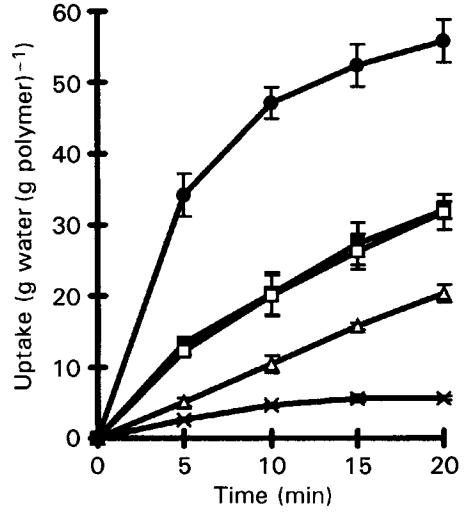

Figure 1. Water uptake of different gelatinizing agents: X HPMC; △ NaCMC; ■ NaC980; □ NaPCP; ● NaChito–EDTA. Data are means ± s.d. of results from three experiments.

Table 2. Ethanol-incompatibility of different gel preparations.

	Ethanol (%) w/w					
	20	25	35	45	55	65
Sodium carboxymethylcellulose	–	–	–	–	–	P
Hydroxypropylmethylcellulose	–	–	–	–	V	V
Sodium polycarbophil	–	–	–	TV	TV	TV
Sodium carbopol 980	–	–	–	TV	TV	TV
NaChito–EDTA	–	–	–	–	–	V

– = Unaffected; V = viscosity loss greater than 30% (shear rate $10\,s^{-1}$); T = turbidity; P = precipitation.

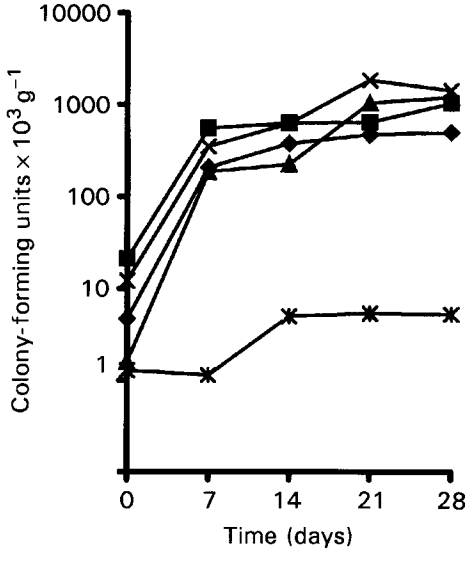

Figure 2. Viable bacterial counts of the complete spectrum occurring in demineralized water in hydrogels based on different gelatinizing agents: ◆ HPMC; ■ NaCMC; ▲ NaC980; X NaPCP; ∗ NaChito–EDTA. Data are means ± s.d. of results from three experiments.

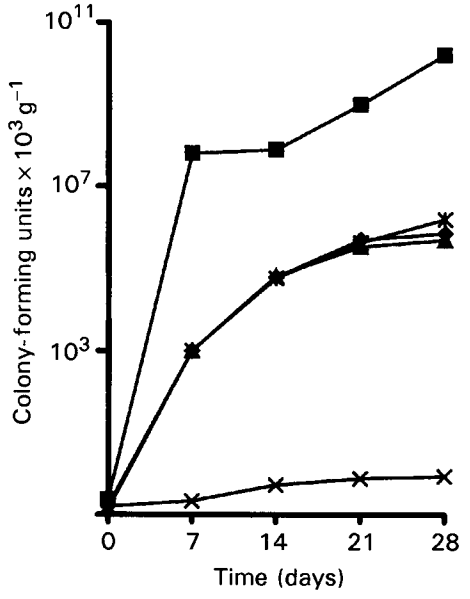

Figure 3. Viable counts of *Escherichia coli* (ATCC 8739) in hydrogels based on different gelatinizing agents: ◆ HPMC; ■ NaCMC; ▲ NaC980; X NaPCP; ∗ NaChito–EDTA. Data are means ± s.d. of results from three experiments.

the starting level, the standard deviations were less than 15%. As shown in Figure 2, the growth rate of bacteria in HPMC, NaCMC, NaPCP and NaC980 hydrogels remained consistently high. In NaChito–EDTA gels, however, after 28 days of incubation the bacterial growth was at least 100-times lower. In addition, the growth kinetics of *E. coli* (test 2), a model strain representative of Gram-negative bacteria, were monitored within the different types of hydrogel. As shown in Figure 3, even 7 days after inoculation a significantly greater number of colony-forming units could be detected in all the other hydrogels than in NaChito–EDTA, for which the bacterial count was only $866\,CFU\,g^{-1}$. These observations correlated with the results obtained from studies with the complete bacterial spectrum of demineralized water (test 1) and can be explained by the antibacterial activity of chitosan (Chen et al 1996) and of EDTA (McGregor & Elliker 1958; Repaske 1958; Marshall & Piddock 1994). It has been proposed that EDTA increases the permeability of the outer membrane of Gram-negative bacteria by chelating with the cross-bridging Mg^{2+} ions, which stabilize the lipopolysaccharides (Matsushita et al 1978). However, the inherent lack of broad-spectrum activity and the incompleteness of total bacterial kill strongly limits the use of EDTA as a sole preservative in topical systems. It has been reported that EDTA used in concentrations of 5 and 20 mM (Stevens et al 1991; Marshall & Piddock 1994), can increase the activity of preservatives, for example parabens, imidazolidinylurea, phenethyl alcohol and *t*-butylhydroxyanisol, towards Gram-negative bacteria (Hart 1984). The interpretation of the effect of EDTA is in agreement with observations of the role of the divalent cations magnesium and calcium in the inhibitory action of other complexing agents

Table 3. Results of effectiveness against *Escherichia coli* (ATCC 8739) of the different gel-preparations with and without nisin ($100\,\mu g\,g^{-1}$) and results from studies of the binding affinities to Mg^{2+} and Ca^{2+}.

Formulation	Effectiveness	Binding affinity (%) w/w	
		Mg^{2+}	Ca^{2+}
Hydroxypropylmethylcellulose	–	–	–
+ nisin	–	–	–
Sodium carboxymethylcellulose	–	–	–
+ nisin	–	–	–
Sodium polycarbophil	+	64·9 ± 5·2	77·63 ± 3·3
+ nisin	+		
Sodium carbopol 980	+	83·7 ± 1·5	85·3 ± 3·6
+ nisin	+		
NaChito–EDTA	+	100 ± 0	100 ± 0
+ nisin	+		

+ Inhibition; –no inhibition. Binding affinities are means ± s.d. of results from three experiments.

towards Gram-negative bacteria. For instance, Pelletier et al (1995) explained the antimicrobial activity of nitroxoline in terms of the disorganization of the bacterial outer membrane which resulted from chelation of the divalent cations magnesium and calcium. Further studies show that carbopol 940 promotes the microbial activity of preserving agents towards *Escherichia coli* and *Pseudomonas aeruginosa* (Scalco et al 1996). A possible reason for this might be the capability of this polymer to bind magnesium and calcium. Binding affinity studies towards magnesium and calcium of all the polymers investigated within this study could confirm this theory proposed for the antimicrobial activity of complexing agents. Whereas neither binding affinity nor antimicrobial activity was observed for HPMC and NaCMC hydrogels, all other polymers were able to bind these divalent cations and had antimicrobial activity in the diffusion test. With the exception of the binding affinity of NaPCP and NaC980 towards calcium, all other results of affinity studies were significant. Data from these studies and from the diffusion test are shown in Table 3. NaChito–EDTA had the highest binding affinity towards magnesium and calcium. As previously reported (Bernkop-Schnürch & Krajicek 1998) the coupling rate of the NaChito–EDTA conjugate is almost 100%, which corresponds to a concentration of approximately 11 mM EDTA in a 0·5% hydrogel. Because of the covalent attachment of EDTA the release of the complexing agent from the polymer can be excluded.

Preservation

Although growth–kinetic studies showed microbial stability to be highest for NaChito–EDTA gels, the antimicrobial activity of this novel polymer is still insufficient to conform with the requirements of the European Pharmacopoeia, showing the need for appropriate preservation. Progress in recombinant DNA technology and biotechnology has resulted in industrial capability to produce large numbers of antimicrobially active peptides and proteins, which represent potential candidates as preservatives (Valenta et al 1997) in commercial quantities. Because of their high molecular mass, which almost excludes percutaneous absorption (Bernkop-Schnürch et al 1996), side effects might be minimized. Because the antimicrobially active polypeptide nisin in combination with chelating agents has antibacterial effect not only against Gram-positive bacteria but also against Gram-negative (Blackburn et al 1989; Stevens et al 1991; Schved et al 1995), it has been chosen as a possible alternative to well established preservatives. Nisin is produced by *Lactobacillus lactis subs. lactis* (Jung & Sahl 1991) and has already been tested by our research group as a preservative for dermal products (Valenta et al 1995). Table 4 lists the effect on the growth of *E. coli* of different gelatinizing agents with and without addition of nisin. With the exception of the data obtained after 2 h, which can be regarded as the starting level for the evaluation of a preservative, the standard deviations were less than 15%. Addition of nisin to HPMC, NaCMC, NaPCP and NaC980 gels had no significant influence on the bacterial growth of *E. coli*. However, when used in combination with a NaChito–EDTA gel, nisin caused a significant 1·2 log reduction in viable counts of *E. coli* after 7 days and no bacteria survived after 21 and 28 days. These results show that nisin in combination with NaChito–EDTA has both bacteriostatic and bactericidal effect. Comparison of viable counts of *E. coli* in NaChito–EDTA with and without nisin, as shown in Figure 4, showed that even a concentra-

Table 4. Effect of nisin ($100\,\mu g\,g^{-1}$) on the growth of *Escherichia coli* (ATCC 8739) in different gel formulations.

Gelatinizing agent	Reduction of viable bacteria ($log\,CFU\,g^{-1}$)				
	2h	7 days	14 days	21 days	28 days
Hydroxypropylmethylcellulose	0·26	0·06	0·41	0·09	0·13
Sodium carboxymethylcellulose	0·56	0·08	0·11	0·24	0·15
Sodium carbopol 980	0·77	0·09	0·17	0·17	0·41
Sodium polycarbophil	0·78	0·11	0·16	0·18	0·26
NaChito–EDTA	0·49	1·20	1·60	2·88	2·93

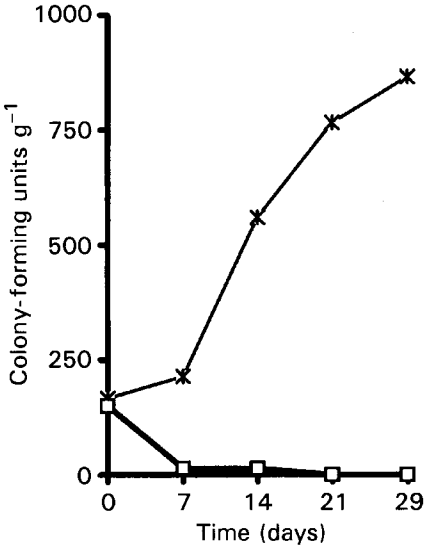

Figure 4. Viable counts of *Escherichia coli* (ATCC 8739) in NaChito–EDTA with (□) and without (*) nisin ($100\,\mu g\,(g\,gel)^{-1}$). Data are means ± s.d. of results from three experiments.

tion of 0·01% nisin suffices to reduce the number of colony-forming units to level $10^2\,CFU\,g^{-1}$ required for topical preparations. These results also correlate with binding affinity studies showing the significantly highest stability of the NaChito–EDTA complexes with magnesium and calcium. According to these results the polypeptide, at a concentration of at least 0·01%, can be suggested as preservative for NaChito–EDTA gels.

In summary, the novel NaChito–EDTA polymer can be recommended as an alternative gelatinizing agent for topically used hydro- and hydroalcoholic gels. NaChito–EDTA gels have the advantage of compatibility with higher concentrations of ethanol than NaPCP, NaC980, NaCMC and HPMC gels. The gels are also more microbially stable and have excellent swelling properties. The antimicrobially active polypeptide nisin seems to be a suitable preservative for NaChito–EDTA gels.

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