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Research paper

A new lipid emulsion formulation with high antimicrobial efficacy using chitosan ☆

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Abstract

The antimicrobial activity of chitosan in lipid emulsions as well as in aqueous solutions was investigated. Two types of long-chained chitosan were used differing in the molecular weights, degree of the deacetylation and their viscosity: type I, mol. weight 8.7×10^4 g/mol, 92% degree of deacetylation and a viscosity of 14 mPa s, type II, mol. weight of 5.32×10^5 g/mol, 73% degree of deacetylation and a viscosity of 461 mPa s. In order to assess the pH optimum of the antimicrobial activity of the biopolymer, suspensions of the microorganisms *Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Aspergillus niger* were incubated at different pH-values in lactic acid solution (1% w/v) containing different concentrations of chitosan up to 1.5% (w/v). Emulsion formulations containing either 0.25%, 0.5% or no chitosan, respectively, were inoculated with the same microorganisms and were incubated at 25°C. The aqueous solutions as well as the emulsions were examined for microbial counts on agar plates after different periods of incubation. After 24 h of incubation in aqueous solutions only the cfu numbers of the bacteria were reduced. Both types of chitosan revealed a pH optimum of their antibacterial activity at pH 5.0–5.1 for *P. aeruginosa*, and at pH 5.3 for *S. aureus*. In addition, chitosan with a mol. weight of 8.7×10^4 g/mol, high degree of deacetylation and low viscosity showed a higher antimicrobial activity than the other chitosan type of this study. It was found that lipid emulsions containing 0.5% chitosan (type I) conformed to the requirements of the preservation efficacy test for topical formulations according to the European Pharmacopoeia while the emulsion without chitosan and a lactic acid solution with and without the biopolymer did not conform. In hemolysis studies on human erythrocytes, the hemolytic activity of the lipid emulsions with chitosan was assessed. These emulsions showed a negligible hemolytic behavior. The results indicate a use of chitosan as antimicrobial preservative in emulsion formulations for mucosal as well for parenteral applications. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Chitosan; Lipid emulsion; Aqueous solution; Antimicrobial efficacy; Hemolytic activity

1. Introduction

In the literature lipid emulsions have been described as a drug delivery system with novel properties [1]. It has been reported that lipid emulsions increase the solubility and the stability of drugs with poor water solubility [2,3]. At the same time the drugs incorporated in these systems showed an improved activity and minimized side effects [4–6]. Recently, positively-charged emulsions have been suggested as a new type of lipid emulsion with interesting properties [7,8], especially for ocular application and drug targeting purposes [9,10].

In a previous study the possibility of producing positively-charged emulsions using chitosan was shown. These emulsions also displayed sufficient stability to pass the autoclaving process without changes in their physicochemical properties [11].

Chitosan is a deacetylated product of chitin obtained from crab or krill and possesses a wide safety margin [12,13] compared to the other positively-charged emulsifying agents which were used to produce positively-charged systems [14,15]. In addition to the positive charge, a number of suggestions have been made regarding many interesting properties of chitosan, which may be utilized in many pharmaceutical applications [16,17]. One of these properties is the antimicrobial activity of chitosan which is suggested for use in a variety of different formulations, e.g. tapes for wound dressing, tooth paste or artificial tears [18–20]. The overall objective of the current study was to investigate the antimicrobial efficacy of chitosan in lipid emulsion formulations as well as in aqueous solutions in order to produce a formulation with improved activity in reducing the numbers

☆ Dedicated to Professor Dr B.C. Lippold, University of Düsseldorf/Germany on occasion of his 60th birthday.

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of microorganisms. In addition, while the in vitro hemolytic test provided a highly significant rank correlation to the in vivo results [21], the measurements of the hemolytic activity were intended to assess the toxicity potential of the prepared formulations.

2. Experimental section

2.1. Materials

Purified castor oil was purchased from Henry Lamotte (Bremen, Germany) and medium chain triglyceride MCT (Miglyol 812) was obtained from Hüls (Witten/Ruhr, Germany). Poloxamer 188 was provided by ICI (Atlas Chemie, Germany). Two types of chitosan were furnished by Fish Contract Bremerhaven (Bremerhaven, Germany) [type I] Chitopure with 92% degree of deacetylation and viscosity of 14 mPa s; [type II] Chitopure 026 with 73% degree of deacetylation and viscosity of 461 mPa s. The molar masses of the two chitosans were indicated by the supplier as being 4.1×10^5 g/mol, thus suggesting they were identical. The method as to how the molecular weights were determined was not given. The determination of the molar masses of the chitosans was carried out by the laser light-scattering technique. For the chitosan type I a molecular weight of 8.7×10^4 g/mol and for the chitosan type II a molecular weight of 5.32×10^5 g/mol was found. These values were regarded as relevant because this method represents an absolute molecular-weight determination technique for polymers [22].

Soybean lecithin (SL) was purchased from Lipoid (Ludwigshafen, Germany). Sorbitol, lactic acid, peptone from meat, sodium chloride peptone buffer (pH 7.0), agar medium with casein-peptone soymeal-peptone (CSA) and Sabouraud 4% dextrose agar medium were supplied by Merck (Darmstadt, Germany). Double distilled water was used for all preparations. All other chemicals were of reagent grade or higher. All materials were used as received. The culture media were mixed with water according to the prescription of the supplier and were sterilized at 121°C for 15 min before use.

The pH measurements were performed using a pH-meter type 540 GLP from WTW (Weilheim, Germany) connected to a pH-electrode Blue Line 16 pH by Schott (Mainz, Germany).

Pseudomonas aeruginosa (ATCC 9027) *Staphylococcus aureus*(ATCC 6538) *Candida albicans* (ATCC 10231), and *Aspergillus niger* (ATCC 16404) used for the microbiological assay were obtained from the German Collection for Microorganisms (Braunschweig, Germany).

2.2. Preparation and characterization of emulsions

The method used to prepare and characterize the chitosan submicron emulsions was reported elsewhere [11]. Briefly, chitosan was dispersed in a 5% aqueous solution of sorbitol to enable isotonicity adjustment and an equal amount of a 2% (w/v) solution of lactic or acetic acid was added since chitosan is only soluble in an acidic medium. The resulting mixture was stirred vigorously without heating for 60 min until chitosan was dissolved. The pH of the resulting solution was 3.6–3.8 and this was adjusted to 5.0 using sodium hydroxide (0.1 N) to avoid any flocculation of chitosan. The solution was filtered using a 0.45 mm filter (cellulose acetate filter, diameter 25 mm) to separate the non-soluble accompanying fibres. The first 5 ml of the filtrate were rejected to minimize problems arising from adsorption on the filter.

Phospholipids and poloxamer 188 were dissolved in the oil phase by heating. A mixture of castor oil with MCT (1:1) was the oil phase of choice as it showed low interfacial tension and relatively low viscosity [11]. The emulsions were produced by preparing a premixture of oil phase in aqueous solution using an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) at 8000 rpm for 3 min. The premixture was passed through a high-pressure homogenizer (Micron Lab 40, APV Gaulin, Lübeck, Germany) eight times successively at a pressure of 20 mPa and a temperature of 40°C. Each emulsion batch was prepared in triplicate.

The mean droplet size of the emulsions was determined by photon correlation spectroscopy (PCS) (Malvern spectrometer RR 102, Malvern, UK, with Helium–Neon laser $\lambda = 632.8$ nm, Siemens, Germany) covering the size range 5 nm to approximately 3 μm. Size analysis was studied by adding approximately 1 μl fat emulsion to 1 ml distilled water in order to obtain the optimum scattering intensity. The size of each fat emulsion was analyzed twice and the H_2O:fat samples were analyzed 10 times. The data were expressed in terms of mean diameter and polydispersity factor (deviation of the system from a monodispersed distribution). The mean droplet size of all emulsion systems prepared was found to be between 150 to 200 nm with the polydispersity factor ranging from 0.15 to 0.2, which indicates a monodispersed distribution [23].

Larger particles were detected using a laser diffraction analyser LDA (Helos, Sympatec, Clausthal-Zellerfeld, Germany) at a focal length of 20 mm which corresponds to a measurement range of 0.18–35 μm. The emulsions were characterized by the D_{max} and the D_{50} quantiles of the volumetric distribution. D50, mean diameter, is defined as the size in which 50% of the particles are smaller. D_{max} is defined as the size in which all particles are smaller. All systems displayed D_{50} values between 0.57 and 0.63 μm and D_{max} values were less than 1.8 μm.

The charge on emulsion droplets (ζ potential) was measured using a Zeta Sizer 3 (Malvern Instruments, Malvern, U.K.). The electrolyte solution used for the dilution consisted of double distilled water with a conductivity of 50 μS/cm adjusted by NaCl (0.5 mM). 500 μl of each emulsion formulation was diluted with 20 ml of the electrolyte solution. Phospholipid, poloxamer 188 and chitosan-poloxamer emulsions were found to have −55, −10 and

+24 mV ζ potential-values, respectively. These values of particle size and zeta potential are in agreement with previously reported data [11,24,25].

2.3. Microbiological assays

2.3.1. Determination of the antimicrobial efficacy of chitosan in aqueous solution

The test solutions contained chitosan in different concentrations (dissolved in 1% (w/v) lactic acid) mixed with meat peptone (0.5% (w/w)), dextrose (0.5% (w/w)), sodium chloride (0.5% (w/w)), and the suspension of the organisms tested. The concentration of the microorganisms in the test tubes was adjusted to be $1.5–3.5 \times 10^5$ cfu /ml. The content of microorganisms in the inocula was determined spectrophotometrically at a wavelength of 620 nm (spectrophotometer Nanocolor by Macherey & Nagel; Dueren, Germany) for the bacteria and for *C. albicans,* as well. The concentration of spores of *A. niger* was determined microscopically using a Thoma chamber (Microscope CH 30 by Olympus; Tokyo, Japan). In addition, the cfu numbers in the inocula were determined by plate counts on CSA. These numbers were considered as actual concentrations for the inocula.

Prior to each experiment the pH of the chitosan stock solution (in lactic acid) and the 1% lactic acid solution (without chitosan as a reference) was adjusted to the desired pH using NaOH (2N). Two millilitres of each inoculated solution were incubated for 24 h at 35°C.

Aliquots of 100 μl of the undiluted suspensions and of their 10^{-2} dilutions were plated on CSA for bacteria or on Sabouraud 4% dextrose agar medium for fungi. The plates were incubated for 2 up to 5 days at 35°C for bacteria and at 25°C for fungi and then they were examined for microbial growth and the colony forming units were counted. The effect of chitosan on the microorganism growth was determined in each test sample and, compared to the chitosan-free control tube, it was expressed as a 10-logarithmic reduction value (LRV).

2.3.2. Preservation efficacy testings

The antimicrobial efficacy of the emulsions as well as the aqueous formulations was investigated as a preservation efficiency test according to Ph. Eur. (26), which was considered as an appropriate test system particularly with the sampling frequency as it is required for formulations for topical application. Subcultures of *P. aeruginosa* and *S. aureus* were obtained on CSA after 24 h incubation at 35°C. Subcultures of *C. albicans* and *A. niger* were obtained on Sabouraud 4% Dextrose Agar at 25°C after incubation for 48 h and 7 days, respectively. The subcultures were washed with a sterile solution of 0.9% sodium chloride to prepare inocula with microbial numbers between 10^7 and 10^8 cfu/ml. The number of cfu in the inocula was counted as described above (2.3.1). Ten gramme emulsion samples were inoculated with a 0.1 g of the microrganism suspensions to obtain an initial number between 10^5 and 10^6 cfu/g. The inocula were homogeneously distributed in the samples by gentle stirring for 3 min and after this the inoculated containers were stored at 25°C. Samples of 1 g were taken from the bacteria containers for microbial counts after 2, 7 and 28 days, and from the fungi containers after 14 and 28 days. After the samples were diluted with sodium chloride peptone buffer (pH 7.0) to obtain 10^{-1}, 10^{-2} and 10^{-3} concentrations, microbial counts were carried out using the plate count method using 1 ml of each dilution on CSA medium for bacteria and on Sabouraud 4% Dextrose Agar for fungi.

2.4. Erythrocyte toxicity assay

The erythrocyte toxicity assay was conducted as described by Bock et al. [27]. Red blood cells (RBCs) were obtained from a healthy 30 year old, type A positive male with normal blood chemistry by centrifugation ($1000 \times g$ for 5 min). The RBCs were washed three times with isotonic saline buffer (0.15 M NaCl and pH 7.4) before diluting with buffer to prepare an erythrocyte stock dispersion with a fixed concentration of hemoglobin (3:11 centrifuged erythrocytes: buffer). The buffer consisted of $Na_2HPO_4 \cdot 12\ H_2O$ (7.95 g), KH_2PO_4 (0.76 g), NaCl (7.20 g) and distilled water (add 1000 ml). The washing step was repeated in order to remove debris and serum protein. The stock dispersion was stored in a refrigerator for a maximum of 24 h. The hemoglobin concentration in the stock dispersion was about 4 mM. A 100 μl aliquot of the erythrocyte stock dispersion was added per milliliter of sample. Incubation was usually carried out at 37°C for different periods of time. After incubation under shaking, debris and intact erythrocytes were removed by centrifugation ($750 \times g$ for 3 min) and 100 μl of the resulting supernatant was dissolved in 2 ml of an ethanol/HCl mixture (39:1 99% ethanol (v/v): 37% hydrochloric acid (w/v)). This mixture dissolved all components and avoided the precipitation of hemoglobin, with the exception of chitosan. Thus an additional centrifugation was necessary ($750 \times g$ for 3 min) to separate the non-soluble chitosan. The absorption of the resulting supernatant was determined at 398 nm by photometric monitoring against a blank sample. Control samples of 0% lysis (in buffer) and 100% lysis (in double-distilled water) were employed in all experiments. The trial was repeated and the mean value of 5 measurements using different samples was recorded.

3. Results and discussion

3.1. Effect of the pH-value on the antimicrobial activity of chitosan in aqueous solutions

Since there are no data available from the literature regarding the pH influence on the antimicrobial activity of chitosan, preliminary experiments were carried out at pH

values between 4.8 and 5.5. Flocculent fibres were observed in the chitosan solutions at pH values higher than 5.5 indicating a reprecipitation of the dissolved chitosan. Control (chitosan free) samples containing only lactic acid with a pH value below 5.0 caused a reduction in the microorganism number of at least four log-steps, whereas no effect was observed at pH values 5.0 to 5.5. In experiments with *P. aeruginosa, S. aureus, A. niger,*Wallhäußer also demonstrated that lactic acid has no antimicrobial effect at pH 5.0 and higher [28].

Based on these findings the influence of chitosan was investigated at pH values from 5.0 to 5.5. In Table 1A and 1B, the antimicrobial activities of two different chitosan types against *P. aeruginosa* and *S. aureus* are presented at different pH values (in the tested range). In preliminary experiments the chitosan concentration was varied between 0.15 and 1.5% (w/v). The tubes containing the chitosan solution and the test organisms were incubated for 24 h. The reduction in microorganism number was recorded for each concentration and the lowest test concentration is given where the maximum reduction in microorganism number was observed. When the maximum reduction was found at the concentration of 0.15% further measurements were carried out at the same pH-value using lower chitosan concentrations, in order to determine the maximum capacity of the antimicrobial activity of chitosan.

The highest efficacy of chitosan against *P. aeruginosa* was observed at pH 5.0. Chitosan type I (mol. weight 8.7×10^4 g/mol) caused a reduction in cfu numbers of more than 5 log-steps even at a concentration of 0.014%. In contrast, in the presence of the other chitosan type used in this study (type II, molecular weight 5.32×10^5 g/mol) the decrease in the number of the colony forming units was only one log-step.

The highest efficacy against *S. aureus* was observed at a pH value of 5.3. A difference between the antimicrobial activities of the two investigated types of chitosan against this microorganism was not observed.

In contrast to the results with bacteria, both types of chitosan did not show any activity against the yeast *C. albicans* and the mould *A. niger* in the short term experiments (24 h incubation). No reduction in the numbers of the colony forming units of both fungi was observed in the pH-range 5.0–5.5 up to a chitosan concentration of 1.5% (in concentrations above this value there was in complete dissolution of chitosan).

A difference between the short term effects of chitosan against bacteria and the yeast *C. albicans* was also reported by other authors. Olsen et al. examined the antimicrobial properties of a chitosan acetate salt against different bacteria species and against the yeast in incubations of 6 h [29]. At the concentration of 0.02% (w/v) a reduction for *P. aeruginosa* of 3 log-steps was found, for *S. aureus* of 1 log-step, while *C. albicans* investigated at the concentration of 0.05%, showed no reduction.

It was shown that the antibacterial activity of chitosan is affected by the pH value. The inhibiting mechanism of this polycationic polymer may be attributed to its binding to anionic molecules at the cell surface. The binding of chitosan to charged molecules in the bacterial cell wall is supported by the observations that a slight shift of the pH resulted in a sudden change in the active concentration of chitosan and in a noticeable difference in the antimicrobial activity of chitosan. From studies which have shown that chitin deriva-

Table 1
Effect of pH on the antimicrobial efficacy of two types of chitosan (in lactic acid solution)

(A) Type I: mol. weight of 8.7×10^4 g/mol, 92% deacetylation and viscosity of 14 mPa s

P. aeruginosa – logarithmic reduction value (LRV) after 24 h[a]

pH-value	5.0	5.1	5.2		5.3	5.4	5.5
Chit.%	0.014	0.45	1.05	0.15	0.15	1.05	0.6
LRV	> 5	> 5	> 3	> 2	> 2	> 2	> 1

S. aureus – logarithmic reduction value (LRV) after 24 h[a]

pH-value	5.0	5.1	5.2	5.3	5.4	5.5
Chit.%	0.3	0.15	0.45	0.068	0.3	0.3
LRV	> 2	> 2	> 4	> 5	> 5	> 3

(B) Type II: mol. weight 5.32×10^5 g/mol, 73% deacetylation and viscosity of 461 mPa s

P. aeruginosa – logarithmic reduction value (LRV) after 24 h[a]

pH-value	5.0	5.1	5.2	5.3	5.4	5.5
Chit.%	0.022	0.15	0.15	0.15	0.15	0.15
LRV	> 1	< 1	< 1	< 1	< 1	< 1

S. aureus – logarithmic reduction value (LRV) after 24 h[a]

pH-value	5.0	5.1	5.2	5.3	5.4	5.5
Chit.%	0.15	0.9	0.15	0.068	0.3	0.45
LRV	< 1	> 3	> 2	> 5	> 4	> 5

[a] Controls (chitosan 0%) pH 5.0–5.5: LRV <1.

Table 2
(A) Composition of the tested emulsion formulations and (B) antimicrobial efficacy of different emulsion formulations

(A) Formulation	Emulsifier (%)	Oil Phase (20%)
1	SL[a] (1.5%)	Castor oil–MCT[b] (1:1)
2	Pol.[c] 188 (2%)	Castor oil–MCT (1:1)
3	Pol.188 (2%) + 0.25% Chitosan	Castor oil–MCT (1:1)
4	Pol.188 (2%) +0.5% Chitosan	Castor oil–MCT (1:1)

(B) Type of emulsion	Antimicrobial efficacy[d,e]
SL (1.5%)	−
Pol.188 (2%)	−
Pol.188 2% + 0.25% Chitosan	−
Pol.188 2% + 0.5% Chitosan	+

[a] Soybean lecithin.
[b] Medium chained triglyceride.
[c] Poloxamer.
[d] +, Conformed to requirements of the preservation efficacy test according to Ph. Eur. 3, for topical formulations; −, did not conform to the requirements.
[e] Each formulation was tested in a single determination.

tives inhibit hemagglutination by bacteria and the microbial adherence to epithelial cells, it has been proposed that this inhibition is due to the selective binding of chitin derivatives to sugar receptors on the cell surfaces [29].

In addition, chitosan with a molecular weight of 8.7×10^4 g/mol is more active than chitosan with a molecular weight of 5.32×10^5 g/mol. Since the smaller chitosan used in this study has a greater degree of deacetylation than the other type, the increased activity of type I is not to be directly correlated to the degree of polymerization but may also be affected by the degree of deacetylation. This may mean that as greater numbers of the cationic groups in the chitosan molecule become available, the cell-binding activity increases.

3.2. Antimicrobial efficacy of chitosan in lipid emulsions

In a previous study a chitosan formulation with sufficient stability during autoclaving was developed [11]. It was shown that chitosan emulsions are stable at pH values of 5.0 to 5.1. This fact should be taken into consideration when preparing chitosan formulations.

In order to utilize the antimicrobial efficacy of chitosan in emulsions, different lipid emulsion formulations with and without chitosan were investigated to determine the most effective formulation. The composition of the tested emulsions is listed in Table 2A. It is shown in Table 2B that the emulsion formulation containing 0.5% chitosan (type I) revealed an antimicrobial activity which conformed to the requirements of the preservation efficacy test for topical formulations according to Ph. Eur [26]. The bacteria should be diminished at least by about 2 log-steps after two days, by about 3 log-steps after 7 days and on day 28 their number must not be increased. In the case of fungi, the cfu should be reduced at least about 2 log-steps after 14 days and on day 28 there should be no increase of the cell numbers. Emulsions with 0.25% chitosan and those without chitosan did not conform to these requirements. In fact, in chitosan-free formulations an increase in the number of the microorganisms was observed.

In contrast to the 24-h results with the aqueous solution in which chitosan only reduced the numbers of bacteria cells, after one day of incubation emulsions containing chitosan (0.5%) of type I revealed an antimycotic activity which resulted in a reduction of more than 2 log-steps for *C. albicans* and more then 1 log-step for *A. niger* (Table 3). Chitosan type II had no significant effect against both fungi species (less than 1 log-step reduction). Although the correlation between the antimicrobial activity and the degree of polymerization is not evident because of the difference in the degree of deacetylation of the two chitosans (see also section 2.1), nevertheless there may be the possibility of the influence of the polymerization degree. Cuero [30] reported that pentamer and heptamer chitosan units seem to have better antifungal action than larger units.

By comparing the preservation efficacy of the emulsion formulation containing 0.5% chitosan (type I) with the

Table 3
Effect of two types of chitosan (0.5%) on *C. albicans* and *A. niger* (cfu: colony forming units)[a]

Chitosan type	*C. albicans* (cfu/ml)		*A. niger* (cfu/ml)	
	Before	After 24 h	Before	After 24 h
Type I	1.0×10^5	2.7×10^2	3.4×10^5	6.1×10^3
Type II	1.0×10^5	4.3×10^4	3.4×10^5	1.6×10^4

[a] Type I: mol. weight of 8.7×10^4 g/mol, 92% deacetylation and viscosity of 14 mPa s Type II: mol. weight 5.32×10^5 g/mol, 73% deacetylation and viscosity of 461 mPa s.

Table 4
Antimicrobial efficacy of an emulsion containing 0.5% chitosan (mol. weight of 8.7×10^4 g/mol, 92% deacetylation and viscosity of 14 mPa s) assayed in the preservation efficacy test for topical formulations (pH 5.0–5.1)[a]

(A) Bacteria Test organism	cfu on day			
	0	2	7	28
P. aeruginosa in chitosan emulsion	1.7×10^6	< 10	< 10	< 10
In control 1		< 10	< 10	< 10
In control 2		4.1×10^6	5.6×10^6	3.9×10^7
S. aureus in chitosan emulsion	5.7×10^5	5.1×10^3	< 10	< 10
In control 1		1.2×10^3	5.0×10^2	< 10
In control 2		7.1×10^5	4.3×10^5	1.7×10^4
(B) Fungi Test organism	cfu on day			
	0	14	28	
C. albicans in chitosan emulsion	2.4×10^5	< 10	< 10	
In control 1		< 10	< 10	
In control 2		1.4×10^4	3.9×10^4	
A. niger in chitosan emulsion	1.0×10^5	1.2×10^2	< 10	
In control 1		5.0×10^4	4.8×10^4	
In control 2		1.0×10^4	1.7×10^4	

[a] Control 1: 1% lactic acid solution, pH 5.1, containing chitosan; control 2: 1% lactic acid solution, pH 5.1.

controls (aqueous acidic solutions with and without chitosan) it can be seen that the antimycotic activity is increased in emulsions. The emulsion conforms to the requirements for a formulation for topical application whereas the aqueous control solutions do not conform because the mould *A. niger* did not show the required reduction of the colony numbers (Table 4).

These findings may be of practical use regarding the fact that topical formulations which contain water should be equipped with antimicrobial properties, and multi-dose formulations of ophthalmics as well as injectables must be preserved. The addition of a chemical agent in the concentration required for the conformity to the preservation efficacy test is not always to be recommended because of possible allergic reactions of the user. Chitosan may serve as an appropriate additive or, in topic formulations, as a complete replacement of a chemical preservative.

From the results of the preservation efficacy tests it can be concluded that the antimicrobial activity of chitosan especially against the mould *A. niger* is enhanced in emulsions.

In a study performed by Goldberg et al. [31] the effect of chitosan on the microbial hydrophobicity was tested as determined by the adhesion of microbial cells to hexadecane. The extent of the adhesion was measured turbidimetrically. The adhesion of all three tested strains (*Escherichia coli, Candida albicans and Acinetobacter calcoaceticus*) increased from around zero value to over 90% in the presence of low concentrations of chitosan (125–250 μg/ml). Cells of *E. coli* preincubated in the presence of chitosan were highly adherent to the hydrocarbon, whereas hexadecane droplets pretreated with chitosan were unable to bind to untreated cells. The result shows that the presence of chitosan in a non-polar liquid reduces the polarity of the surfaces of suspended microbial cells. In lipid emulsions this fact may lead to an enrichment of chitosan-bound cells in the oil phase or in the interface. The higher antimicrobial activity of chitosan in the emulsions might be due to the immobilization of the microbial cells by hydrophobic interactions among them or/and with lipophilic molecules.

In order to prove if there were differences in the hydrophobicity of the organisms used in this study, their adherence to hexadecane was measured. 1.2 ml aliquot of the cell suspension used for inoculation was mixed with 0.2 ml hexadecane for 60 s and the percent reduction in the optical density at 620 nm of the aqueous phase was determined. The cells of *C. albicans*, *P. aeruginosa* and *S. aureus* exhibited low adherence to hexadecane, whereas the amount of cells remaining in the non-polar phase was much higher in the case of spores of the mould *A. niger* (Table 5). Its higher hydrophobicity may strengthen the chitosan-induced hydrophobic interactions in the interface and in the lipid phase of the emulsion which lead to an inactivation of the cells. This effect may result in the difference between the antifungal activity of chitosan in emulsions and in aqueous solutions. However, further work should be carried out to investigate the possible mechanism of the interactions.

Table 5
Hydrophobicity of the test organisms[a]

Microorganism	Hydrophobicity (%)[b] (mean ± SD)
P. aeruginosa	4.8 ± 3.9 (5)
S. aureus	11.4 ± 5.7 (5)
C. albicans	1.1 ± 2.4 (4)
A. niger	37.0 ± 7.4 (4)

[a] Numbers of measurements are given in parentheses.
[b] Amount of cells which remained in the hexadecane phase after mixing the cell suspensions with aliquots of hexadecane.

The lipid-induced increase of the antimicrobial activity of chitosan is in good agreement with the statement that the bioavailability and/or effectiveness of many drugs with poor water solubility can sometimes be enhanced when the drugs are incorporated into lipid emulsions [3–5]. Moreover, in connection with the antimycotic action, Levy et al [32] reported that the incorporation of amphotericin B in the lipid emulsion extended the survival time of mice infected with *C. albicans* when compared to Fungizone®, the marketed corresponding product. Furthermore, Gruenberg and his workers [33] reported that positive, fluid vesicles undergo a fusion process with the plasma membrane of Trypanosoma brucei while solid negative vesicles are only adsorbed to the membrane. This is of importance regarding the application to microorganism therapy by the encapsulation of drugs in the positively-charged vesicles. More promisingly, this may open pioneering work in specific targeting of the encapsulated drugs using these positively-charged vesicles [34,35].

Chitosan emulsions were prepared with ABA block copolymer (poloxamer 188). For this purpose poloxamer 188 was added to chitosan lactic acid solutions in order to investigate whether the antimicrobial action of chitosan may be enhanced. The addition of the poloxamer to chitosan solutions resulted in a decrease of antimicrobial efficacy (Table 6). This behavior could be due to the formation of a complex between chitosan and poloxamer molecules resulting in a lack of free amount of chitosan. Consequently its antimicrobial efficacy was strongly reduced. It was shown elsewhere [11] that the addition of the positively-charged chitosan to poloxamer emulsions lead to a decrease in emulsion particle sizes and a change of the surface charge of the oil droplets from negative to positive (from −11 to +23 mV). This indicates that chitosan molecules are localized at the interface and intercalated between the non-ionic surfactants. Hence a mixed interfacial film consisting of the poloxamer 188 and chitosan molecules was formed at the oil/water interface which resulted in an overall positive surface charge. Such unfavourable interactions between preservatives and surfactants have also been described by other authors [36,37].

Table 6
Effect of the addition of poloxamer 188 on the antimicrobial efficacy of chitosan (mol. weight of 8.7×10^4 g/mol, 92% deacetylation and viscosity of 14 mPa s) (pH 5.1 with 2% poloxamer 188)

	LRV	
(A) *P. aeruginosa* – LRV[a] after 24 h		
Chitosan	0.25%	0.5%
With poloxamer188	3	4
Without poloxamer	5	5
(B) *S. aureus* – LRV after 24 h		
Chitosan	0.25%	0.5%
With poloxamer 188	2	3
Without poloxamer	2	4

[a] LRV, logarithmic reduction value.

3.3. Hemolytic activity

Erythrocyte-induced hemolysis in vitro can be considered to be a simple and reliable measure for estimating the membrane damage caused in vivo [21,38]. Therefore, the behavior of chitosan emulsions in vivo was predicted by investigating the degree of hemolysis in vitro.

Only the hemolysis induced by the emulsions prepared with chitosan type I was studied since they showed a higher antimicrobial activity and thus may cause higher irritation and/or toxicity.

Increasing the chitosan concentration and incubation time causes a slight increase in the hemolytic activity of the investigated emulsions (Fig. 1). However, this hemolytic activity was lower than 6% for acetic acid emulsions after 30 min incubation with 0.5% chitosan at 37°C, whereas only 2% hemolysis was observed for lactic acid emulsions under the same conditions (Fig. 2). Hemolysis in the lactic emulsions was mostly not affected by increasing the chitosan concentration (data not shown). Acetic acid was also used here, as it is generally used in the literature to dissolve chitosan [21]. In order to investigate whether chitosan really showed such hemolytic activity, the effect of the acid type (0.5% w/w) was studied using lactic acid and acetic acid in lipid emulsions (with chitosan) and in acidic aqueous solution (chitosan-free). It is well established from previous work that chitosan-free emulsions caused little, or no detectable hemolysis, respectively [39].

Hemolysis results demonstrated in Fig. 2 show that a negligible hemolysis (lower than 2%) was produced after 30 min. at 37°C in lactic acid solution and in the chitosan lipid emulsion with lactic acid. In contrast, an aqueous solution of acetic acid (chitosan-free) revealed relatively higher hemolysis activity (about 4%) in comparison with lactic acid, whereas the chitosan lipid emulsion with acetic acid showed about 6%. Hemolysis that is lower than 2% can be attributed to the presence of chitosan, which indicates a wide safety margin according to the hemolysis test. Moreover, it could be deduced from the above results that only the requisite amount of the acid to dissolve chitosan should be used, avoiding any excess amount, which can cause addi-

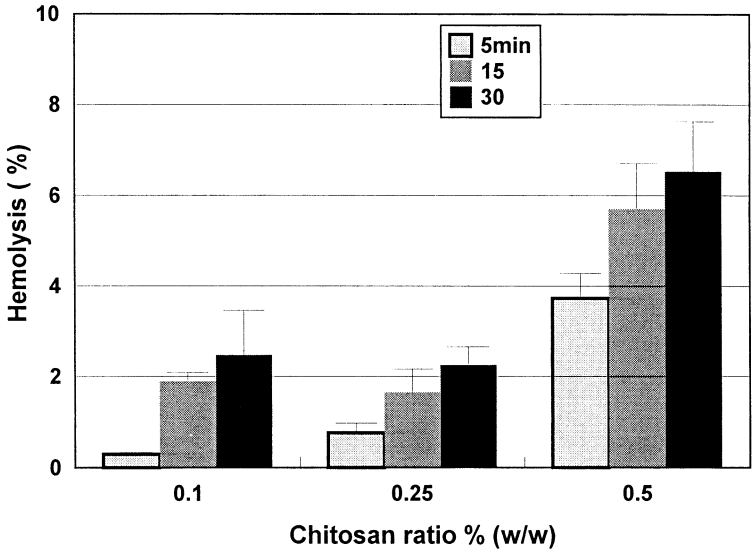

Fig. 1. Erythrocyte membrane damage induced by lipid emulsions prepared with various concentrations of chitosan using acetic acid after different incubation times at 37 °C ($n = 5$).

tional hemolysis (acetic acid). Lactic acid is more suitable and has better tolerability for use in forming chitosan emulsions for ophthalmic and parenteral administrations.

4. Conclusions

The antibacterial activity of chitosan was affected by changing the pH-values of the acidic aqueous solutions. Lipid emulsions prepared with chitosan displayed an antimycotic efficacy which conformed to the requirements of the preservation efficacy test for topical formulations of the European Pharmacopoea, whereas acidic solutions containing chitosan in the same concentration are not as active to conform these requirements because of the resistance of the mould *A. niger*.

Moreover, only the requisite amount of the acid should be used to avoid any excess amount which will result in an increase of hemolysis. It was shown that lactic acid is more suitable than acetic as it has a negligible hemolytic activity.

The above results demonstrate the importance of lipid emulsions as novel carriers and indicate that the role played by chitosan as an antimicrobial component in emulsion formulations needs further investigation.

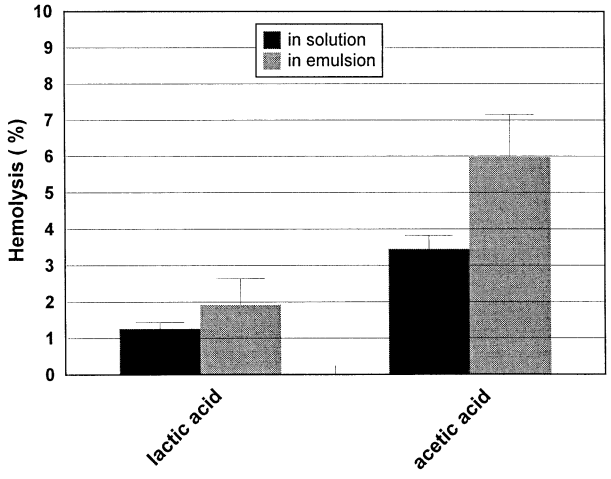

Fig. 2. Hemolytic activity of 0.5% chitosan in lipid emulsions and in acidic solutions (lactic acid and acetic acid) after 30 min incubation at 37 °C ($n = 5$).

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