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ORIGINAL ARTICLE

Chitosan nanoparticles for daptomycin delivery in ocular treatment of bacterial endophthalmitis

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Abstract

Context: Chitosan nanoparticles were prepared to encapsulate daptomycin and proposed as a delivery system of this antibiotic to the eye for the treatment of bacterial endophthalmitis.
Objective: The aim of this study was to develop daptomycin-loaded nanoparticles to apply directly to the eye, as a possible non-invasive and less painful alternative for the treatment of endophthalmitis, increasing the effectiveness of treatment and reducing toxicity associated with systemic administration.
Materials and methods: Nanoparticles were obtained by ionotropic gelation between chitosan and sodium tripolyphosphate (TPP). Physicochemical and morphological characteristics of nanoparticles were evaluated, as well as determination of antimicrobial efficiency of encapsulated daptomycin and stability of the nanoparticles in the presence of lysozyme and mucin.
Results: Loaded nanoparticles presented mean particle sizes around 200 nm, low polydispersity index, and positive zeta potential. Morphological examination by scanning electron microscopy (SEM) confirmed their small size and round-shaped structure. Encapsulation efficiency ranged from 80 to 97%. Total *in vitro* release of daptomycin was obtained within 4 h. Determination of minimum inhibitory concentrations (MICs) showed that bacteria were still susceptible to daptomycin encapsulated into the nanoparticles. Incubation with lysozyme did not significantly affect the integrity of the nanoparticles, although mucin positively affected their mucoadhesive properties.
Discussion and conclusion: The obtained nanoparticles have suitable characteristics for ocular applications, arising as a promising solution for the topical administration of daptomycin to the eye.

Keywords

Antibacterial activity, chitosan, daptomycin, nanoparticles, ocular infection

History

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Introduction

The human eye, due to its position, is constantly exposed to the surrounding environment and to a wide range of pathogenic microorganisms. Serious eye infections can affect the ocular globe causing sight-threatening conditions, such as endophthalmitis. Bacterial endophthalmitis is an ocular inflammation resulting from the infection of the posterior segment of the eye by pathogenic microorganisms (Callegan et al., 2007). Endophthalmitis frequently culminates in severe visual disabilities causing irreversible damage to the photoreceptor cells of the retina, and it can result in partial or complete vision loss (Callegan et al., 2002).

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Gram-positive microorganisms are the most common bacterial pathogens causing endophthalmitis (Romero et al., 1999). Endogenous endophthalmitis is usually caused by *Staphylococcus aureus* and *Streptococcus* species (Bispo et al., 2008). Bacteria causing acute post-operative endophthalmitis have usually colonized ocular surface structures and include mainly coagulase-negative staphylococci, *S. aureus*, *Streptococcus viridans,* and other Gram-positive cocci and Gram-negative bacilli (Kresloff et al., 1998; Bispo et al., 2008). Enterococci are also relevant in post-operative cases, both for prevalence and severity of disease (Callegan et al., 2002).

Daptomycin is a natural lipopeptide antibiotic active against most Gram-positive microorganisms implicated in bacterial endophthalmitis (Jeu & Fung, 2004; Robbel & Marahiel, 2010). It is active against some antibiotic resistant bacteria, including methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis*, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant enterococci (Thorne & Alder, 2002).

Daptomycin has been approved for the nontopical treatment of skin structure infections caused by Gram-positive bacteria, as well as for the treatment of bacteremia and

right-sided endocarditis caused by *S. aureus* strains (Enoch et al., 2007; Robbel & Marahiel, 2010). It is available for intravenous route only, which means invasiveness and high hospital costs. Nonetheless, daptomycin may offer a novel antibacterial tool for the treatment of endophthalmitis, in particular if caused by MRSA.

Local drugs applied to the eye represent a non-invasive, safe, and less painful solution. However, treatment of bacterial infections in the eye is particularly difficult due to innate protective barriers in the precorneal area of the eye. Both corneal and conjunctival epithelia have tight junctions that limit the entrance of substances into the eye, including beneficial molecules such as antibiotics (Kinoshita et al., 2001; Nanjawade et al., 2007). Also, the mucus layer in the eye blocks the entrance of not only debris particles but also medicines, which are then removed through the lachrymal system (Corfield et al., 1997; de Campos et al., 2004).

Due to these limitations, drug delivery systems for topical ocular administration are interesting alternatives, especially due to the non-invasive way of releasing drugs in a controlled fashion directly to the eye (Diebold & Calonge, 2010). Among the possible strategies, the use of mucoadhesive polymeric carriers able to interact intimately with extraocular structures has been considered very promising (Ding, 1998; Motwani et al., 2008; Zarbin et al., 2010). In particular, chitosan has some favorable properties for controlled drug delivery to the eye. It is biocompatible, biodegradable, mucoadhesive, and non-toxic (de Campos et al., 2004; Severino et al., 2007). Mucoadhesive properties of chitosan contribute to enhance the residence time of the delivery system in the precorneal area, thus increasing drug penetration across intraocular structures and decreasing the frequency of administration. Biocompatibility of chitosan with ocular structures increases the contact time of drugs on the cornea, improving topical absorption and avoiding invasive treatments (Severino et al., 2007). Also, chitosan has penetration-enhancing properties, which results in reorganization and opening of tight junctions between epithelial cells, especially important in the transport of hydrophilic compounds such as therapeutic peptides (Mourya & Inamdar, 2008; de la Fuente et al., 2010; Subramanian et al., 2011). Until now, no study has demonstrated the potential of chitosan nanosystems for the protection of antimicrobial lipopeptides for efficient ocular administration.

Materials and methods

Materials

Daptomycin (degree of purity 94.9%) was a gift from Novartis Pharma AG (Basel, Switzerland). Low molecular weight chitosan with a degree of deacetylation of 85% and sodium tripolyphosphate (TPP) with a technical grade of 85% were obtained from Sigma-Aldrich® (*St. Louis, MO*). Acetic acid at 100% was purchased from Pronalab (Lisbon, Portugal). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were from Merck (Darmstadt, Germany). Acetonitrile, trifluoroacetic acid, and triethylamine were of analytical grade and purchased from Sigma-Aldrich® (USA), as well as lysozyme and mucin. Ultra-pure water was obtained in our laboratory using a Millipore™ water purification equipment (Billerica, MA).

Preparation of chitosan nanoparticles

Determination of the isoelectric point of daptomycin

For determination of the isoelectric point of daptomycin, 1 mg/mL of daptomycin solutions were prepared in ultra-pure water and adjusted to different pH values (pH = 2.25, 3.20, 4.30, 4.40, 5.33, and 5.47) with 1 M NaOH or 1 M HCl. The electric charge of daptomycin molecules was then determined by laser Doppler anemometry using ZetaPALS – Zeta Potential and Particle Size Analyzer (Brookhaven, NY).

Preparation of unloaded nanoparticles

In order to determine the optimal experimental conditions for the formation of nanoparticles, various concentrations of chitosan and TPP were tested. Chitosan was dissolved in acetic acid solutions in order to achieve final chitosan concentrations of 0.05, 0.5, 1, 2, 3, and 5 mg/mL. In all cases, concentration of acetic acid was 1.75 times higher than that of chitosan, as suggested by Calvo et al. (1997a). The pH of chitosan solutions was adjusted to 5.4–5.5 with 1 M NaOH. TPP solutions were prepared in ultra-pure water at final concentrations of 0.05, 0.1, 0.2, 0.5, 1, and 2 mg/mL.

To determine the influence of chitosan concentration on nanoparticle formation, 2 mL of a TPP solution (1 mg/mL) was added dropwise to 5 mL of the different chitosan solutions previously prepared, under magnetic stirring (1000 rpm) for 90 seconds, at room temperature. The same procedure was used to determine the influence of TPP concentration. Thus, 2 mL of the prepared TPP solutions at different concentrations was added to 5 mL of a chitosan solution (2 mg/mL), at the same temperature and stirring conditions stated above. The experiments were performed in triplicate.

Preparation of daptomycin-loaded nanoparticles

Daptomycin-loaded nanoparticles were formed spontaneously upon dropwise addition of 2 mL of TPP solution (1 mg/mL) to 5 mL of chitosan solution (2 mg/mL), under magnetic stirring (1000 rpm) for 90 seconds, at room temperature. Daptomycin was incorporated into nanoparticles by diluting daptomycin stock solution in pH-adjusted chitosan solution, in order to achieve final daptomycin:chitosan mass ratios of 0.05:1, 0.1:1, 0.15:1, 0.2:1, and 0.3:1. An alternative methodology was performed in which daptomycin was added to the TPP solution, instead of being incorporated into the chitosan solution. The experiments were performed in triplicate.

Nanoparticle suspensions were centrifuged at 20 000 × *g* for 20 min (Sigma-Laboratory centrifuges, Osterode am Harz, Germany). Supernatants were collected and stored at −20 °C for daptomycin assay by reverse-phase high-performance liquid chromatography (RP-HPLC), while nanoparticles were stored in the centrifugal tube at 4 °C for further analysis.

Physicochemical and morphological characterization

Particle size and polydispersity of freshly prepared unloaded and loaded nanoparticles were determined by

photon correlation spectroscopy using ZetaPALS (USA). Nanoparticle suspensions were analyzed at 25 °C with a detection angle of 90°. Zeta potential was determined by laser Doppler anemometry, at 25 °C, using the same equipment.

Scanning electron microscopy (SEM) was used to observe the morphology of the nanoparticles. Unloaded and daptomycin-loaded nanoparticles were dried at room temperature and placed on metal stubs with adhesive tape, sputter coated with gold (SC7620 Sputter Coater, Quorum Technologies, UK), and then observed under a scanning electron microscope (JSM-5600LV, JEOL, Japan) operating in high vacuum mode at an accelerating voltage of 20 kV.

Determination of encapsulation efficiency

Encapsulation efficiency of daptomycin-loaded nanoparticles was calculated as the difference between the total amount of daptomycin used to prepare the nanoparticles and the amount of daptomycin present in the supernatant after centrifugation of nanoparticle suspensions, as shown in Equation (1):

$$\text{Encapsulation efficiency } (\%) = \frac{\left\{\begin{array}{l}\text{Total amount of daptomycin} \\ -\text{Free amount of daptomycin}\end{array}\right\}}{\text{Total amount of daptomycin}} \quad (1)$$

Daptomycin content in the supernatant was determined by RP-HPLC using a method adapted from Martens-Lobenhoffer et al. (2008). The chromatographic equipment consisted of an Alliance® HPLC system (Waters Corporation, Massachusetts, USA) and the analytical column was a ZORBAX Eclipse XDB-C8 4.6×150 mm 5 µm particle size (Agilent Technologies, Santa Clara, CA). Briefly, the mobile phase for the chromatographic separation of daptomycin consisted of pure acetonitrile and a buffer solution prepared with 20 mM trifluoroacetic acid and 15 mM triethylamine in ultrapure water. For the chromatographic separation, a gradient was applied starting at 30% acetonitrile and rising to 40% acetonitrile in 5 min, then constant for 3 min, and finally decreasing to 30% acetonitrile for 3 min. The applied flow rate was 1 mL/min, the column temperature was set to 30 °C, and the injection volume was 50 µL. For quantification of daptomycin, peak areas at 224 nm were determined.

In vitro release profile of daptomycin

For daptomycin *in vitro* release studies, loaded nanoparticles were suspended in 10 mL of a phosphate-buffered saline (PBS) solution, pH 7.4, and placed in a water bath at 37 °C with gentle agitation. At 30 min, 1, 2, 3, 4, 5, and 24 h, the suspension was centrifuged at $20\,000 \times g$ for 5 min (Sigma-Laboratory centrifuges, Germany), and 5 mL were collected from the tube for RP-HPLC analysis. At each time, the tube was replenished with 5 mL of PBS solution.

Determination of minimum inhibitory concentration

Determination of minimum inhibitory concentration (MIC) was performed according to Clinical and Laboratory Standards Institute (CLSI, 2005a,b) guidelines for the broth microdilution method, described in M7-A6 (2005a) and M100-S15 (2005b) documents.

Assays were performed in 96-well microplates (Nunc, Roskilde, Denmark) in order to test free daptomycin, unloaded nanoparticles, and daptomycin-loaded nanoparticles. Tested daptomycin concentrations ranged from 0.06 to 16 mg/L. The Muller–Hinton (MH) broth (Biokar Diagnostics, Beauvais, France) was adjusted to a final calcium concentration of 50 mg/L and used to prepare the inocula and dilute the samples. Inocula were prepared by suspending bacteria colonies (grown in Nutrient agar media for 24 h) in calcium adjusted MH broth to a turbidity equivalent to that of a 0.5 McFarland standard (corresponds to 1×10^8 CFU/mL). The inocula were then diluted (1:100) and the final test concentration of bacteria in the wells was 5×10^5 CFU/mL.

Assays were performed in duplicate against the following organisms, obtained from a culture collection of our laboratory: MRSA ATCC 43300, methicillin-susceptible *S. aureus* (MSSA) ATCC 25923, *S. epidermidis* ATCC 14990, *Staphylococcus lugdunensis* ATCC 43809, *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus hominis* ATCC 27844, *Staphylococcus warneri* ATCC 27836, and *Enterococcus faecalis* ATCC 29212. The MIC was determined by observing the lowest concentration that inhibited bacterial growth.

Stability of nanoparticles in the presence of lysozyme and mucin

Particle size, polydispersity, and zeta potential of daptomycin-loaded nanoparticles were determined using ZetaPALS (USA), before and after incubation of nanoparticles in lysozyme (1 mg/mL, pH = 7.4) and mucin (0.4 mg/mL, pH = 7.4) aqueous solutions (incubation conditions: 37 °C, under moderate stirring, for 2 h). The conditions applied in this experiment were adapted from de Campos et al. (2004). Loaded nanoparticles before incubation were used as controls.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Scheffé post hoc test for comparison of groups with normal distribution, and the Mann–Whitney test for groups with non-normal distribution. Analysis was performed using IBM SPSS Statistics v 19.0.0 (Chicago, IL). Differences were considered to be significant at a level of $p < 0.05$.

Results and discussion

Preparation and characterization of unloaded nanoparticles

In this work we describe the preparation and characterization of a chitosan nanoparticulate system able to incorporate daptomycin, with appropriate mucoadhesiveness and antimicrobial characteristics for the treatment of ocular infections. Chitosan nanoparticles were produced by ionotropic gelation, a method based on the spontaneous formation of complexes between chitosan and TPP, under mild conditions. This method has enabled the formation of nanoparticles upon mixing of chitosan and TPP solutions, through the establishment of inter- and intramolecular bonds between

the phosphate groups of TPP and the amino groups of chitosan (de la Fuente et al., 2010). Usually, this method allows the formation of nanoparticles with small diameters (200-500 nm) and a spherical shape (Reis et al., 2006).

For the development of these nanoparticles, we first determined the conditions necessary to the production by testing different chitosan and TPP concentrations (Table 1). At first, chitosan concentration was set at 2 mg/mL while TPP solutions at different concentrations (0.05, 0.1, 0.2, 0.5, 1, and 2 mg/mL) were added dropwise to the chitosan solution. Secondly, the same protocol was repeated but this time TPP concentration was fixed at 1 mg/mL and added to chitosan solutions at different concentrations (0.05, 0.5, 1, 3, and 5 mg/mL).

Results of this experiment are presented in Table 1, in which it can be observed that nanoparticles are formed only when using a limited range of chitosan and TPP concentrations. In fact, it was possible to identify two distinct situations when mixing chitosan and TPP. For TPP concentrations ranging from 0.05 to 1 mg/mL, an opalescent suspension was formed, which should correspond to a suspension of very small particles, as suggested by Calvo et al. (1997a). The same occurred for chitosan concentrations of 0.05, 3, and 5 mg/mL. However, a TPP concentration of 2 mg/mL led to the formation of aggregates that would rapidly precipitate, as well as for chitosan concentrations of 0.5 mg/mL and 1 mg/mL, meaning that at these concentrations it is not possible to obtain nanoparticles.

Considering these results, the obtained opalescent suspensions were subjected to further studies and analyzed for determination of particle size, polydispersity, and zeta potential by photon correlation spectroscopy and laser Doppler anemometry, respectively.

It was observed that smaller particles were produced with increasing TPP concentrations up to 1 mg/mL. Chitosan concentration also seems to have some influence on particle size, as it seems to vary according to the concentration of the chitosan solutions used to prepare the nanoparticles. However, no statistical differences were observed among the formulations tested, and thus this variation in size is not considered significant ($p > 0.05$).

The obtained nanoparticles showed low polydispersity values ranging from 0.171 to 0.244 ($p > 0.05$), which suggests the presence of monodispersed particles with a narrow size distribution (Bihari et al., 2008; Mehravar et al., 2009). Zeta potential of the nanoparticles was also determined, since it represents the stability of the particulate system in the sense that the greater the absolute value of zeta potential, the greater the surface charge of the particles. Thus, as the absolute value of zeta potential increases, the electrostatic repulsion interactions between the particles will be greater, the stability of the particles will increase, and size distribution will be more homogeneous. Zeta potential values obtained in this study were all positive and relatively high, suggesting good physicochemical stability of the colloidal suspensions. Moreover, positive nanoparticles may interact with negative surface cells like ocular cells in a stronger and longer way, being an additional advantage of these nanoparticles. The only exception was for the formulation with chitosan:TPP mass ratio of 1:8, as expected from the excess TPP mass ratio and the predominance of TPP negative charges. No statistical differences were observed for the tested formulations concerning the variations in chitosan and TPP concentrations ($p > 0.05$).

From this experiment it can be concluded that nanoparticle formation is only possible for some chitosan and TPP concentrations, with the minimum nanoparticle size obtained for the formulations prepared with chitosan concentration of 2 mg/mL and TPP concentration of 1 mg/mL (Table 1). Further experiments were conducted using these concentrations, since they led to the production of nanoparticles with the smallest size. Regarding zeta potential, these nanoparticles were relatively stable and their positive charges are favorable for interactions with the negatively charged residues in mucus and cell membranes of ocular tissues.

Preparation and characterization of daptomycin-loaded nanoparticles

To prepare daptomycin-loaded chitosan nanoparticles, the pH of the chitosan solution was adjusted to 5.4–5.5, in order to assure that daptomycin molecules had a negative electric charge when daptomycin was added to chitosan solution. This way, daptomycin molecules would interact and bond with positively charged chitosan. In order to establish this pH value, daptomycin solutions were prepared at different pH values and zeta potential for these solutions was obtained. Zeta potential corresponds to the electric charge of the

Table 1. Particle size, polydispersity, and zeta potential of unloaded chitosan nanoparticles prepared at different chitosan and TPP concentrations.

Chitosan concentration (mg/mL)	TPP concentration (mg/mL)	Chitosan:TPP mass ratio	Particle size (nm)	Polydispersity	Zeta potential (mV)
2	0.05	1:0.01	508.95 ± 167.09	0.214 ± 0.211	$+23.82 \pm 4.84$
2	0.1	1:0.02	396.15 ± 120.99	0.244 ± 0.155	$+30.86 \pm 8.98$
2	0.2	1:0.04	371.60 ± 188.23	0.171 ± 0.052	$+27.42 \pm 15.46$
2	0.5	1:0.1	251.60 ± 64.11	0.209 ± 0.034	$+41.82 \pm 14.90$
2	1	1:0.2	214.73 ± 20.10	0.172 ± 0.022	$+21.79 \pm 8.17$
2	2	1:0.4	Aggregates	Aggregates	Aggregates
0.05	1	1:8	1568.60 ± 1181.15	0.197 ± 0.100	-2.10 ± 7.45
0.5	1	1:0.8	Aggregates	Aggregates	Aggregates
1	1	1:0.4	Aggregates	Aggregates	Aggregates
3	1	1:0.13	279.53 ± 57.80	0.209 ± 0.082	$+33.82 \pm 1.30$
5	1	1:0.08	351.60 ± 122.61	0.133 ± 0.039	$+32.07 \pm 1.27$

Concentrations of chitosan and TPP are initial concentrations, previous to nanoparticle formation.
TPP: sodium tripolyphosphate. Data shown are the mean ± standard deviation ($n = 3$).

molecules and thus the isoelectric point of daptomycin was found to be between 4.3 and 4.4.

It is known that peptides are generally better encapsulated into chitosan nanoparticles in a medium with a pH greater than the isoelectric point of the peptide, due to attractive electrostatic interactions between the negatively charged peptide molecules and the positively charged chitosan molecules (Gan & Wang, 2007). Thus, to prepare daptomycin-loaded nanoparticles, chitosan solution had to be adjusted to a pH value above 4.3–4.4 in order to assure that daptomycin molecules were negatively charged. Moreover, the pH of chitosan solution should be below 6.5, since above this value chitosan precipitates (Nagpal et al., 2010). For this reason, to prepare loaded nanoparticles, chitosan solution was adjusted to pH 5.4–5.5. The same pH value was used to prepare unloaded nanoparticles.

The incorporation of daptomycin into chitosan nanoparticles was obtained by diluting a daptomycin solution either in chitosan or TPP solution, in order to achieve final daptomycin:chitosan mass ratios of 0.05:1, 0.1:1, 0.15:1, 0.2:1, and 0.3:1. Results present in Table 2 show that mean particle size for these formulations ranged from 141.63 ± 32.69 nm to 204.20 ± 19.93 nm, which seems adequate for ocular delivery. Particle size of loaded nanoparticles seems to generally decrease as daptomycin:chitosan mass ratio increases. However, no statistical differences were found among the formulations tested ($p > 0.05$). Also, no significant differences were detected when daptomycin was added either to chitosan or TPP solution, and when comparing loaded and unloaded (2 mg/mL chitosan and 1 mg/mL TPP solutions) nanoparticles ($p > 0.05$). For the morphological examination of the nanoparticles, daptomycin-loaded nanoparticle suspensions were centrifuged and dried at room temperature. These samples were observed by scanning electron microscopy (SEM) that revealed the presence of small round-shaped particles with a solid structure (Figure 1). Although SEM images showed the presence of particles, it was not possible to confirm their actual size or surface morphology since the equipment used in this study did not allow higher magnifications in order to observe the samples in a more detailed way.

Zeta potential of loaded nanoparticle formulations was positive and above 30 mV ($p > 0.05$), indicating physically stable systems prone to interact with the positive ocular surface. Polydispersity values were low, ranging from 0.106 ± 0.039 to 0.203 ± 0.037, and it also seems to change independently of daptomycin concentration ($p > 0.05$).

Using a rapid and mild method such as ionotropic gelation, it was possible to obtain daptomycin-loaded nanoparticles around 200 nm. Similar results were obtained in other studies with chitosan nanoparticles prepared for encapsulation of different protein compounds. Pan et al. (2002) obtained chitosan nanoparticles for intestinal absorption of insulin with mean particle sizes of 265 nm. Gan & Wang (2007) used bovine serum albumin (BSA) as a model molecule for encapsulation into chitosan nanoparticles, which exhibited mean sizes around 300 nm and a high positive zeta potential. Considering zeta potential, results of this study were similar to those obtained by de Campos et al. (2001) and de Salamanca et al. (2006).

Encapsulation efficiency and release profile

Encapsulation efficiency was very high for all the formulations tested, ranging from $80.82 \pm 0.05\%$ to $97.93 \pm 0.01\%$ (Table 2). Generally, an increase in encapsulation efficiency was observed with increasing daptomycin concentration. However, statistical analysis revealed that for daptomycin concentrations above 142.8 μg/ml (daptomycin:chitosan mass ratio of 0.1:1), an increase in daptomycin concentration does not significantly affect encapsulation efficiency ($p > 0.05$). Also, no differences were found in the formulations in which daptomycin was added to chitosan or TPP solution, suggesting that daptomycin can be added to either solution with no influence on the final encapsulation ($p > 0.05$).

Contradictory results are sometimes observed regarding the effect of drug concentration on encapsulation efficiency. Gan & Wang (2007) reported an increase in encapsulation efficiency of bovine serum albumin (BSA) into chitosan nanoparticles from 38.7 to 72.5% with increasing protein concentration. However, many studies have reported opposite results, such as Xu & Du (2003) who showed that

Table 2. Particle size, polydispersity, and zeta potential of daptomycin-loaded chitosan nanoparticles prepared at different daptomycin:chitosan mass ratios.

	Daptomycin:Chitosan mass ratio	Particle size (nm)	Polydispersity	Zeta potential (mV)	Encapsulation efficiency (%)
Daptomycin added to chitosan solution	0.05:1	193.60 ± 44.29	0.185 ± 0.038	$+34.50 \pm 3.47$	80.82 ± 0.05*
	0.1:1	172.50 ± 44.90	0.203 ± 0.037	$+36.36 \pm 1.57$	93.80 ± 0.02
	0.15:1	154.60 ± 32.25	0.163 ± 0.056	$+34.75 \pm 5.91$	95.20 ± 0.00
	0.2:1	147.77 ± 17.86	0.106 ± 0.039	$+38.04 \pm 1.34$	95.03 ± 0.02
	0.3:1	141.63 ± 32.69	0.134 ± 0.036	$+33.99 \pm 2.33$	97.93 ± 0.01
Daptomycin added to TPP solution	0.05:1	204.20 ± 19.93	0.141 ± 0.049	$+31.72 \pm 11.57$	82.32 ± 0.02*
	0.1:1	177.77 ± 40.37	0.136 ± 0.050	$+33.17 \pm 8.70$	93.19 ± 0.03
	0.15:1	193.37 ± 27.42	0.155 ± 0.008	$+30.26 \pm 6.98$	96.25 ± 0.03
	0.2:1	146.87 ± 30.04	0.144 ± 0.052	$+35.23 \pm 8.11$	97.09 ± 0.01
	0.3:1	144.20 ± 28.99	0.108 ± 0.048	$+34.83 \pm 8.75$	96.86 ± 0.02

Daptomycin was added to chitosan or TPP solution during nanoparticle preparation. Chitosan concentration: 1.43 mg/mL; TPP concentration: 0.286 mg/mL; daptomycin concentrations: 71.4, 142.8, 214.3, 285.7, and 428.6 μg/ml (corresponding to daptomycin:chitosan mass ratios of 0.05:1, 0.1:1, 0.15:1, 0.2:1, and 0.3:1, respectively). These are final concentrations in nanoparticle suspensions. TPP: sodium tripolyphosphate. Data shown are the mean ± standard deviation ($n = 3$).

*Significant at the 0.05 level.

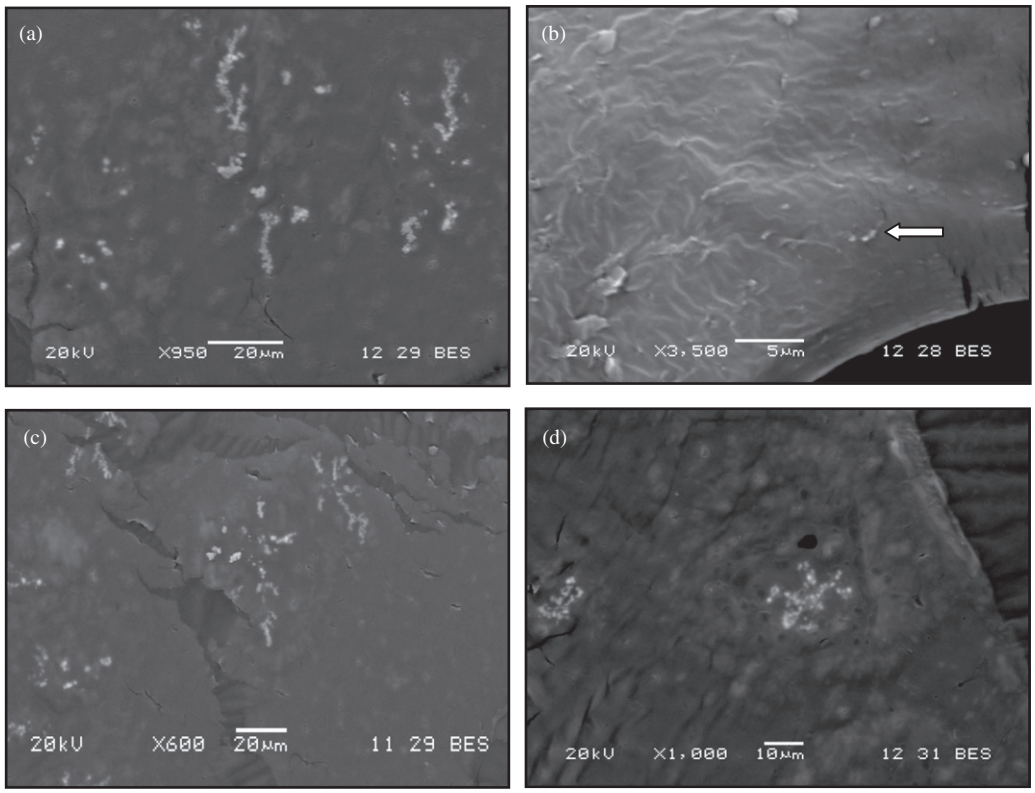

Figure 1. SEM images of unloaded nanoparticles (a) and daptomycin-loaded nanoparticles (b, c, and d). Samples were sputter coated with gold.

encapsulation efficiency was significantly affected by BSA initial concentration, with lower concentrations leading to higher encapsulation efficiency. Similar results were obtained by Pan et al. (2002), who reported a slight decrease in association efficiency with increasing insulin to chitosan ratio. Different and sometimes inconsistent results could be due to differences in the experimental conditions and type, molecular weight, and degree of deacetylation of chitosan, for example.

Encapsulation efficiency obtained in this study can be considered very high, with most formulations showing results over 90%. These results appear to be in agreement with some other studies with chitosan nanoparticles prepared by ionotropic gelation. For example, Huang et al. (2009) obtained insulin-loaded chitosan nanoparticles with average encapsulation efficiency up to 95.54%. Bayat et al. (2008) prepared chitosan nanoparticles for oral delivery of insulin with association efficiency over 84%. de Campos et al. (2001) obtained an encapsulation efficiency of 73.4% for cyclosporin A-loaded chitosan nanoparticles, considered particularly high due to the hydrophobic character of cyclosporin A. Fernández-Urrusuno et al. (1999) obtained high-encapsulation efficiency values ranging from 87.4 to 96.7% for chitosan nanoparticles loaded with insulin.

In vitro release profile of daptomycin (Figure 2) revealed a continuous release of daptomycin from the nanoparticles in a pH 7.4 medium during a time period of 4 h. Over 90% of the antibiotic was released during the first 3 h and after 4 h daptomycin had been completely released from the chitosan nanoparticles.

Release profiles can vary deeply depending on several factors, namely the morphology and size of the particulate

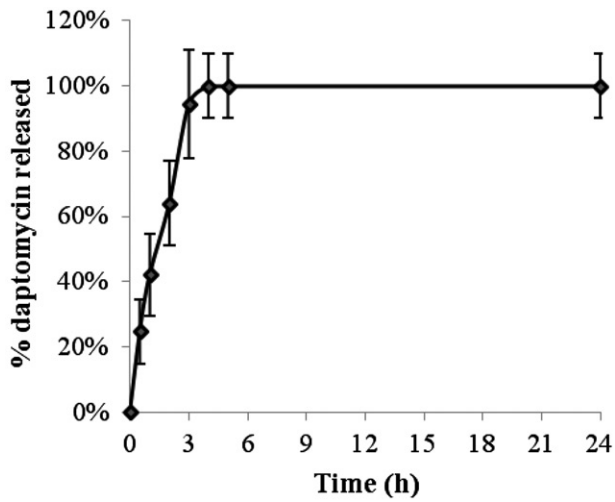

Figure 2. *In vitro* release profile of daptomycin from chitosan nanoparticles (daptomycin:chitosan mass ratio of 0.3:1). Release assay was performed in PBS solution, pH = 7.4, at 37 °C with agitation ($n = 3$).

system, physicochemical characteristics of the encapsulated drug, and pH of the release medium (Agnihotri et al., 2004). Boonsongrit et al. (2006) observed a high burst release of insulin from chitosan microparticles, with an almost complete release of the drug within just 10 min in a phosphate buffered solution (pH = 7.4). The authors concluded that the ionic interaction between insulin and chitosan molecules was too weak to control the drug release. Similar conclusions were obtained by Férnandez-Urrusuno et al. (1999), who observed a complete release of insulin within 2 h.

Concerning daptomycin, it is possible that an initial release occurred due to desorption of daptomycin molecules from

Table 3. Minimum inhibitory concentrations (MICs) for daptomycin solution, unloaded chitosan nanoparticles, and daptomycin-loaded chitosan nanoparticles against eight different Gram-positive bacteria.

MIC (μg/mL)							
			Daptomycin:Chitosan mass ratio				
Microorganism	Daptomycin solution	Unloaded nanoparticles	0.05:1	0.1:1	0.15:1	0.2:1	0.3:1
Staphylococcus aureus (MSSA) ATCC 25923	0.5	–	2	1	2	2	2
Staphylococcus aureus (MRSA) ATCC 43300	1	–	2	1	2	2	1
Staphylococcus epidermidis ATCC 14990	1	16	2	1	2	2	1
Staphylococcus lugdunensis ATCC 43809	1	16	1	1	1	2	1
Staphylococcus haemolyticus ATCC 29970	0.5	16	1	1	2	2	1
Staphylococcus hominis ATCC 27844	0.5	16	1	1	1	2	2
Staphylococcus warneri ATCC 27836	1	16	2	2	2	2	1
Enterococcus faecalis ATCC 29212	1	16	2	2	1	2	2

MRSA: methicillin-resistant *Staphylococcus aureus*; MSSA: methicillin-susceptible *Staphylococcus aureus*.

the surface of the particles, with 25% of the antibiotic being released within 30 min of the beginning of the experiment. During the final part of the experiment, daptomycin release could be due to instability of the nanoparticulate system and dissociation of the ionic matrix between chitosan and daptomycin, leading to a complete release of the antibiotic within 4 h. Also, at pH 7.4, both chitosan and daptomycin are negatively charged, reducing ionic interactions between the molecules. However, more studies should be performed to further understand the release behavior of daptomycin and observe if it can be modulated by altering the physicochemical characteristics of the nanoparticles, the molecular weight of chitosan, and the experimental conditions, for example.

Determination of antimicrobial efficiency

Determination of MIC for free daptomycin and unloaded and loaded nanoparticles was performed using a broth microdilution method against eight different microorganisms. MIC corresponds to the lowest concentration of the agent tested that inhibits visible bacterial growth (by absence of turbidity when compared to a control).

MIC values for the different formulations of loaded nanoparticles were equal or higher than those obtained for free daptomycin, for every microorganism tested (Table 3). MIC for free daptomycin (as a daptomycin aqueous solution) varied between 0.5 and 1 mg/L, and all microorganisms were susceptible, with MIC below the susceptibility breakpoints established for staphylococci and enterococci ($\leq$1 mg/L and $\leq$4 mg/L, respectively). MIC for daptomycin-loaded nanoparticles ranged from 1 to 2 mg/L. This decrease in susceptibility when daptomycin was encapsulated into the nanoparticles could be due to interactions of some free chitosan with bacterial cells. Chitosan may bind to the negatively charged bacterial cells and block the access of daptomycin to its binding sites, leading to a reduction in antibacterial effect. However, although there was a decrease in antimicrobial susceptibility of 2-to-4-fold when daptomycin was encapsulated, good results were still obtained for loaded nanoparticles. Antimicrobial activity of daptomycin seems to have been preserved when the antibiotic was encapsulated into chitosan nanoparticles.

Unloaded chitosan nanoparticles were also tested for their antimicrobial properties (Table 3), since antibacterial and antifungal activities have been ascribed to chitosan (Rhoades & Roller, 2000; Fernandes et al., 2008). MIC values of 16 mg/L were obtained for all the strains tested except for MSSA and MRSA. For these two strains, at the highest concentration (16 mg/L), turbidity was observed when compared to a control, confirming that at this concentration chitosan did not inhibit bacterial growth of MSSA and MRSA.

Previous studies have suggested that the antibacterial activity of chitosan may result from its polycationic structure. Positively charged chitosan may bind to negatively charged bacterial cells and destabilize the normal function of the membranes, causing leakage of intracellular components or inhibiting the transport of nutrients into the cells (Du et al., 2009). For example, Qi et al. (2004) obtained MIC values of 0.125 mg/L for a chitosan nanoparticle suspension against *S. aureus* ATCC 25923, compared to 8 mg/L for a chitosan solution against the same strain. The authors suggested that chitosan nanoparticles interact with bacterial cells in a greater degree than chitosan itself due to higher surface charge density of nanoparticles. Due to their larger surface area, nanoparticles can be tightly adsorbed onto the surface of bacterial cells, leading to disruption of the membrane and death of cells (Qi et al., 2004). These results are in accordance with those obtained in this study, suggesting that chitosan nanoparticles can have a high antibacterial effect.

Stability of nanoparticles in the presence of lysozyme and mucin

An important aspect of the characterization of nanoparticles is the evaluation of their stability in the presence of biological fluids containing proteins, enzymes, or other relevant compounds. In the case of ocular applications, it seems important to test the stability of nanoparticle formulations in the presence of two main components of the precorneal fluid: lysozyme and mucin (de Campos et al., 2004). Thus, the effect of lysozyme and mucin was tested by observing the physicochemical characteristics of nanoparticles before and after incubation with these compounds. Concentrations of lysozyme and mucin, as well as pH of the solutions, were chosen considering the physiological characteristics of the human eye (Calvo et al., 1997b).

Table 4. Particle size and zeta potential of daptomycin-loaded chitosan nanoparticles prepared at different daptomycin:chitosan mass ratios, before (B) and after (A) incubation with lysozyme and mucin.

	Lysozyme				Mucin			
	Particle size (nm)		Zeta potential (mV)		Particle size (nm)		Zeta potential (mV)	
Daptomycin:Chitosan mass ratio	B	A	B	A	B	A	B	A
0.05:1	214.33 ± 44.06	165.57 ± 10.78	24.75 ± 9.63	20.92 ± 0.27	199.57 ± 71.31	413.60 ± 35.92	23.62 ± 4.73	−31.56 ± 0.62
0.1:1	210.53 ± 29.29	143.47 ± 7.99	28.52 ± 1.79	21.02 ± 0.62	121.57 ± 5.25	339.70 ± 46.68	24.40 ± 1.61	−32.78 ± 1.32
0.15:1	117.60 ± 14.93	139.83 ± 4.11	23.24 ± 3.29	22.62 ± 2.83	208.57 ± 38.91	367.00 ± 62.23	24.36 ± 1.10	−26.99 ± 3.37
0.2:1	108.45 ± 3.75	164.77 ± 19.87	24.59 ± 0.88	24.28 ± 1.01	132.75 ± 73.61	362.43 ± 65.03	23.16 ± 1.10	−24.11 ± 1.54
0.3:1	112.70 ± 57.06	268.90 ± 43.04	31.37 ± 2.68	26.30 ± 2.34	117.30 ± 0.85	316.27 ± 67.83	24.07 ± 2.36	−32.77 ± 1.17

Incubation conditions: 1 mg/mL lysozyme solution or 0.4 mg/mL mucin solution, pH = 7.4, at 37 °C under moderate stirring for 2 h. Data shown are the mean ± standard deviation ($n = 3$).

Results present in Table 4 show an apparent reduction in nanoparticle size upon incubation with lysozyme; however, this only occurred for nanoparticles with daptomycin:chitosan mass ratios of 0.05:1 and 0.1:1. For the other formulations, particle size increased. However, statistical analysis revealed no significant differences in the size of nanoparticles before and after incubation with lysozyme ($p > 0.05$). Concerning zeta potential, no significant differences were found before and after incubation with this enzyme ($p > 0.05$). Since lysozyme is a cationic protein, interactions with the positively charged nanoparticles does not significantly affect their zeta potential due to electrostatic repulsion.

Incubation of loaded nanoparticles with mucin did no significantly affect their size ($p > 0.05$). Nonetheless, results of zeta potential suggest an interaction between mucin and chitosan nanoparticles. Before incubation with mucin, nanoparticles showed zeta potential values above +23 mV. However, after incubation, zeta potential decreased significantly ($p < 0.05$), with all formulations showing negative values. This reduction could be attributed to ionic interactions and consequent bonding of negatively charged mucin onto the surface of positively charged nanoparticles. This ionic interaction between mucin and chitosan nanoparticles is favorable to prolong the contact time between nanoparticles and the mucus layer of the eye surface, thus contributing to the absorption of the drug and reduction of the frequency of administration (Dudhani & Kosaraju, 2010).

Thus, this stability study suggests that lysozyme does not significantly affect the integrity of chitosan nanoparticles within 2 h of incubation. However, the presence of mucin significantly alters the surface charge of the nanoparticles, changing from positive to negative values, which is important for interaction and association of the nanoparticles to the mucosal tissues of the eye.

Conclusion

This is the first time, to our knowledge, that mucoadhesive chitosan nanoparticles were prepared for encapsulation of daptomycin, as an alternative therapy for the treatment of ocular infections caused by Gram-positive bacteria, particularly in endophthalmitis cases. The obtained nanoparticles have appropriate characteristics for topical ocular administration and antimicrobial activity of daptomycin was preserved after encapsulation. Loaded nanoparticles were able to interact with mucin, a component of ocular fluids important for the desirable mucoadhesiveness of the delivery system. This nanoparticulate system could arise as a possible way to deliver the antibiotic directly to the site of infection and enhance its residence time in the eye.

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