Violacein/poly(ε-caprolactone)/chitosan nanoparticles against bovine mastitis: Antibacterial and ecotoxicity evaluation

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Abstract: The nanocarrier was synthesized by nanoprecipitation, using poly(ε-caprolactone) (PCL) as polymer, Tween 80 as surfactant and the biopolymer chitosan (CS) as a charge modification agent. Charge, size and morphology were analyzed by zeta potential, photo correlation spectroscopy (PCS), scanning electron microscopy (SEM) and differential scanning calorimetry (DSC). Bactericidal assays were carried out using a resistant strain of Staphylococcus aureus, and the acute ecotoxicity tests were performed with Daphnia similis. The nanoparticle without CS (PCLnp) exhibited an average size of 200 nm and zeta potential of -4.28 mV, while the nanoparticle with 0.04% (w/v) of CS (CS_PCLnp) had 250 nm and +21.3 mV. Both were stables for at least 30 days. 200 µg mL⁻¹ violacein was encapsulated in CS_PCLnp, which was dissolved in the polymer matrix, a shown by DSC analysis. The minimal inhibitory concentration against S. aureus of CS_PCLnp-vio was 25 µmol L⁻¹, while for free violacein it was > 25 µmol L⁻¹. Nanoparticles exhibited an EC50 between 0.3 – 1.1 µmol L⁻¹ with Daphnia, while free violacein was around 3.3 – 5.0 µmol L⁻¹. Thus, it was possible to control the charge of the nanoparticles, without extreme changes in size and that it is possible also to encapsulate a powerful antibactericidal compound such as violacein in nanoparticle.

1. Introduction

Mastitis occurs when the udders become inflamed due a microbial infection that invades the teat canal, multiplies and produces toxins that are harmful to the mammalian gland. This disease can be classified in two ways: subclinical, without visible signs, and clinical, with visible signs such as flakes or clots in the milk, slight swelling, secretion, fever, loss of appetite, dehydration and depression. Bovine mastitis is a complex problem because there are several causative agents. Over 100 microorganisms can cause mastitis, the most common being Staphylococcus aureus [1, 2]. Bovine mastitis is the main economic loss in milk production, estimated at $ 185/cow [2] and reach $ 1.8 billion/year in the United States. In addition, this kind of infection of the mammary glands also affects humans. Therefore new treatment approaches should be attempted. Among several possibilities, drug delivery has been promising approach against several diseases in recent years.

The extensive research in drug delivery is due to several advantages that these systems present, compared with conventional forms of drug administration. Drug delivery presents such
interesting features as the possibility to introduce poorly soluble drugs in formulations, increase diffusion through the skin [3], and prolong residence time of the drug and its action in specific cells [4], besides allowing decreases in toxicity. The carriers employed in drug delivery can be in micro or nano size ranges, including liposomes [5], emulsions [6], and lipid nanoparticles [7], dendrimers [8], oxides [9] and polymer nanoparticles [10]. Concerning polymeric nanoparticles, poly(ε-caprolactone) (PCL) is widely used, being compatible with a wide range of drugs [11]. Its slow degradation rates allow a long releasing period, up to several months [11] and, as PCL is lipophilic, it allows a good interaction with lipophilic compounds. Usually the lipophilic drugs are uniformly distributed in the matrix while hydrophobic drugs generally go to the polymer-solvent interface [11]. Regarding a potential candidate to encapsulate in PCL nanoparticles, violacein is a lipophilic compound and acts against bovine mastitis, since one of its characteristic is effectiveness against *S. aureus* [12].

Violacein is a violet pigment produced by *Chromobacterium violaceum*, which exhibits antiviral, antiparasitic, antibiotic and antibacterial activities [13-15]. However, violacein shows low bioavailability due its poor solubility. Therefore, violacein usually needs an organic solvent in order to be dissolved; in many cases toxic ones such as dimethyl-sulfoxide (DMSO) [14].

Since, the drug delivery method is an interesting option to increase the bioavailability of violacein, the aim of this work was the nanoencapsulation of violacein in a PCL nanoparticle and in order to control the charge of the nanoparticles the mucoadhesive chitosan (CS) [4] was also used.

2. Methods

2.1 Preparation of polymeric nanoparticles

The PCL nanoparticles coated with chitosan were prepared by the nanoprecipitation technique – solvent displacement method, modified from Govender *et al.* [16]. In a typical synthesis of unloaded nanoparticles, PCL was dissolved in acetone. The organic phase was poured into an aqueous phase containing 1% acetic acid (v/v), 1 mg mL\(^{-1}\) Tween 80 and 0 to 0.08% of chitosan (w/v). After mixing, the acetone was eliminated by evaporation under reduced pressure. To produce nanoparticles containing violacein, 8 mg of the active compound was dissolved in the organic phase and the same synthesis procedure used for unloaded nanoparticles was followed. The unloaded nanoparticles were called CS_PCLnp and the loaded nanoparticles as CS_PCLnp_vio.

2.2 Physical characterization

Particle size distribution (z-average), polydispersity index and zeta potential were studied by dynamic light scattering, using a Zetasizer Nano instrument, ZEN3600 (Malvern Instruments). All samples were diluted in 1 mmol L\(^{-1}\) solution of KCl, ratio 1:19 of sample:KCl solution, then placed in capillary polystyrene cuvettes. Measurements were performed at 25°C.

The morphology of the nanosystem was evaluated using scanning electron microscopy, with a JSM-6360LV (Jeol) instrument. A drop of sample diluted in MilliQ\(^{\circledR}\) water (1:19, for sample:water) was placed on the carbon ribbon.

2.3 Stability of nanoparticles

The stability of the system was analyzed with respect to size distribution and zeta potential, varying the temperature and pH. The pH was modified by an external module, MPT – 2 (Malvern Instrument). The modification of size and zeta potential were monitored using DLS, using a Zetasizer Nano instrument, ZEN3600 (Malvern Instrument). To correct the pH, acidic (HCl 0.25 mol L\(^{-1}\)) and basic (KOH, 0.5 mol L\(^{-1}\) and 0.25 mol L\(^{-1}\)) solutions were used.

2.4 Differential scanning calorimetry

The interaction between compounds in the nanostructure and their physical state were studied by a differential scanning calorimetry DSC-Q10 (TA Instruments). The samples were lyophilized, placed in aluminum pans and sealed. Samples were heated from 30°C up to 180°C, at 10°C min\(^{-1}\) and held isothermally for 1 min, in a nitrogen atmosphere (50 mL min\(^{-1}\)).
2.5 Loaded efficiency

The entrapment efficiency and total amount of violacein were analyzed by UV-vis spectrometry, Hitachi U-2000, using Beer’s law ($\varepsilon$ at 575 nm in ethanol $3.13 \times 10^2 \text{ mL mg}^{-1} \text{ cm}^{-1}$) [14]. To obtain the total amount of encapsulated violacein, 100 µL of suspension was dissolved in ethanol (1:15) and centrifuged at 10,000 rcf for 5 min. The supernatant was recovered and the UV-vis spectra measured. To determine the percentage of loaded violacein, 1000 µL from the sample was centrifuged at 10,000 rcf for 5 min, the supernatant was recovered and analyzed in UV-vis spectrophotometer.

2.6 Antibacterial assay

The isolates of S. aureus (MBSA) were obtained from farms located in the central region of the State of São Paulo, Brazil, with histories of chronic subclinical and clinical Bovine mastitis problems [17]. The diagnosis of clinical mastitis was performed using the strip cup test [18]. Subclinical mastitis was diagnosed based on the California Mastitis Test-CMT [19]. The S. aureus used were: MBSA 4 if it was subclinical mastitis and resistant to penicillin and MBSA19 for subclinical mastitis and resistant to penicillin/erythromycin. The determination of MIC was performed using the micro-dilution assay in 96-well plates, according to CLSI [20]. Single colonies were diluted in saline solution and adjusted to 0.5 index on the MacFarland scale ($2 \times 10^8 \text{ cfu mL}^{-1}$).

2.7 Ecotoxicity assay

Free violacein, PCL and violacein nanoparticles (CS_PCLnp_vio and PCLnp_vio) were tested for acute toxicity with Daphnia similis according to OECD 202 guidelines [21]. Twenty neonates (<24 h old) were placed in each concentration of the test solutions. Experiments were performed in four replicates at 21 ± 0.3°C in the dark. DMSO was used to dissolve free violacein and negative control was performed accordingly. After 48 h, the number of immobile organisms was recorded. The results were statistically analyzed using the Trimmed Spearman–Karber method for estimating the effective concentration EC50 [22].

3 Results and discussion

Before encapsulating the violacein, the nanoparticle system produced by the nanoprecipitation method was optimized by varying the amount of CS, in order to obtain a positively charged surface, with a satisfactory nanoparticle size. The effect of CS addition on mean diameter size, polydispersity and zeta potential of the nanoparticles were evaluated using Zetasizer Nano. Studies carried out by Quemeneur et al. [23] showed that CS with molar masses between 50,000 and 500,000 g mol$^{-1}$ present the same adsorption mechanism onto polymer, thus the same zeta potential behavior. In this way, we used only one molar mass of CS, changing just the amount of CS in the synthesis.

| Chitosan concentration (% w/v) | Zeta potential (mV) | Particle sizes (nm) | PdI   |
|-------------------------------|---------------------|---------------------|-------|
| 0.00                          | -5 ± 2              | 210 ± 10            | 0.24 ±0.08 |
| 0.04                          | +40 ± 3             | 280 ± 30            | 0.25 ±0.03 |
| 0.08                          | +44 ± 5             | 320 ± 50            | 0.21 ±0.06 |

In this first part of the study, each system containing a specific amount of CS (from 0.00 to 0.04 and 0.08% w/v) was synthesized at pH 3.5. Table 1 shows the effect of chitosan on particle size, polydispersity and zeta potential of the systems. As expected, the addition of chitosan increased the mean diameter and zeta potential of the particles, due to the increase of molecule adsorption on the nanoparticle surfaces.
The sizes and zeta potentials of the nanoparticles were evaluated by varying the pH since, due to the pKa of CS (pKa=6.5), it was expected that variation of pH could cause modification in the mean diameter size and zeta potential. Figure 1 (a) shows that higher amounts of CS cause agglomeration above pH 8. However, the zeta potential behavior was the same for different amounts of CS, Figure 1 (b). The nanoparticles without CS presented a negative zeta potential, which increased with basic pH. Regarding the DLVO (Derjaguin, Verwey, Landau and Overbeek) theory [24] the systems containing chitosan (0.04% and 0.08%) were stable up to pH 6, while the systems containing only Tween 80 was stable above pH 7.5, Figure 1 (b). Comparing the systems with 0.04% and 0.08% of CS, it is possible to observe a more stable behavior of the system containing 0.04% of CS.

![Graph 1](image1)

**Figure 1:** Mean diameter size (a) and zeta potential (b) changes with pH, from 3 up to 9 of three systems with different amounts of chitosan.

After these results, the system containing 0.04% of chitosan was selected for further studies. The system having only nanoparticle was named CS_PCLnp and the system containing violacein was named CS_PCLnp-vio. In this latter system 180 µg mL⁻¹ of violacein were encapsulated with a yield of 91 ± 1%, having violacein at 11% in mass of PCL (w/w). The same study of mean diameter size and zeta potential was carried out. Figure 2 shows the effect of loading violacein on the size and zeta potential with pH variation. Probably the observed changes are due the presence of violacein in the nanoparticle surfaces. SEM images of CS_PCLnp_vio showed spherical shape without agglomeration, Figure 2(c). Moreover, the presence of violacein did not affect the mean diameter in relation to the temperature, Figure 3.

![Graph 2](image2)

**Figure 2:** Mean diameter (a) and zeta potential (b) of unloaded and loaded nanoparticles with violacein in relation to the pH, (c) SEM image of CS_PCLnp_vio.
Figure 3: Mean diameter in relation to the temperature variation of unloaded and loaded nanoparticles with violacein.

A differential scanning calorimetric measurement was carried out to determine the state of violacein in polymer nanoparticle matrix and the results are presented in Figure 4. Violacein exists in one polymorphic form (melting point of 135°C), which was absent in the CS_PCLnp-vio systems. The absence of an observable melting point of violacein in the nanostructure can be attributed to complete drug dissolution in the polymer matrix [25].

Figure 4: DSC curves for CS_PCLnp, CS_PCLnp_vio and free violacein, obtained at 10°C min⁻¹ in a nitrogen atmosphere.

The free violacein and the violacein nanosystems, presented antibacterial activity. The CS_PCLnp_vio system presented a minimal inhibitory concentration of 25 µmol L⁻¹ against two resistant S. aureus strains, MBSA4 and MBSA19, while free violacein was less effective (minimal inhibitory concentration greater than 25 µmol L⁻¹).

Toxicity tests with Daphnia revealed that CS_PCLnp did not present acute toxicity to Daphnia, Figure 5 (a). In order to facilitate the dissolution of free violacein, 1% of DMSO was used. Controls performed only with 1% of DMSO did not show any toxicity to the test organism. EC50 for CS_PCLnp_vio was in the range of 0.3 – 1.1 µmol L⁻¹; and EC50 for free violacein was 4 µmol L⁻¹, with a confidence interval of 3.3 – 5.0 µmol L⁻¹, Figure 5(a). The higher toxicity of CS_PCLnp_vio in relation to the free violacein could be explained by the mucoadhesive property of CS [4] that would enhance the retention time of the nanoparticles in the organism’s gut. A purple color was observed in the gut of the organisms exposed to the nanosystem, Figure 5 (b), in contrast to no color for the free violacein exposed organisms. Both systems, free violacein and CS_PCLnp-vio, were more toxic to Daphnia in comparison with S. aureus strains, but at the same order of magnitude (µmol L⁻¹).
4. Conclusions

The control of charge was successfully achieved using chitosan, changing the negative zeta potential to positive. The nanoparticle was increased due chitosan addition. The violacein was loaded with a high efficiency in the CS_PCLnp_vio nanosystem and showing a better result against Bovine mastitis infection with *S. aureus*, compared with free violacein. Related to the ecotoxicity test, CS_PCLnp did not present acute toxicity to Daphnia. Both free violacein and CS_PCLnp_vio were toxic to Daphnia being the violacein nanosystem the most toxic one. Therefore, attention should be given when environmental exposures to those systems are expected.

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5. References

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