International Journal of Pharmaceutics xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

# Evaluation of antibacterial activity of nitric oxide-releasing polymeric particles against *Staphylococcus aureus* and *Escherichia coli* from bovine mastitis

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ARTICLE INFO

*Article history:*
Received 13 October 2013
Received in revised form 28 March 2014
Accepted 25 June 2014
Available online xxx

*Keywords:*
Antibacterial activity
Bovine mastitis
*E. coli*
Nitric oxide
Polymeric particles
*S. aureus*

ABSTRACT

Bovine mastitis is a serious veterinary disease that causes great loss to the dairy industry worldwide. It is a major infectious disease and is difficult to manage and control. Furthermore, emerging multidrug resistant bacteria that cause mastitis have complicated such management. The free radical nitric oxide (NO) is a potent antimicrobial agent. Thus, the aims of this study were to prepare and evaluate the antibacterial activity of nitric oxide-releasing polymeric particles against *Staphylococcus aureus* (MBSA) and *Escherichia coli* (MBEC), which were isolated from bovine mastitis. Fifteen MBSA isolates and fifteen MBEC were collected from subclinical and clinical bovine mastitis. Biocompatible polymeric particles composed of alginate/chitosan or chitosan/sodium tripolyphosphate (TPP) were prepared and used to encapsulate mercaptosuccinic acid (MSA), which is a thiol-containing molecule. Nitrosation of thiol groups of MSA-containing particles formed S-nitroso-MSA particles, which are NO donors. The NO release kinetics from the S-nitroso-MSA particles showed sustained and controlled NO release over several hours. The antibacterial activity of NO-releasing particles was evaluated by incubating the particles with an MBSA multi-resistant strain, which is responsible for bovine mastitis. The minimum inhibitory concentration for S-nitroso-MSA-alginate/chitosan particles against MBSA ranged from 125 μg/mL to 250 μg/mL. The results indicate that NO-releasing polymeric particles are an interesting approach to combating bacteria resistance in bovine mastitis treatment and prevention.



## 1. Introduction

Bovine mastitis is an infection in the mammary gland and is the major illness for dairy ruminants, which has reduced milk production and it is often associated with cattle disorders such as fever as well as altered heart and/or respiratory rates and rumen motility (Soto et al., 2003; Lohuis et al., 1998). Bacteria such as *Escherichia coli* or *Staphylococcus aureus* are the major causes of bovine mastitis (Rainard and Rioleet, 2006). Dairy cow mastitis is a disease with considerable economic importance. Throughout the course of a lactation, the incidence rate for mastitis may reach 100% in dairy herds with averages at 30–50% in many countries. In many countries and continents, including Europe, North and South America and New Zealand, *S. aureus* is among the most common causes of clinical and subclinical mastitis in dairy cattle (Holmes and Zadoks, 2011). The economic loss associated with clinical mastitis in the United States has been estimated at approximately $179 per mastitis case. Such loss is calculated from the reduced milk production, discarded milk, increased cull rates, pharmacologic costs, and increased labor costs (Ballou, 2012).

Further, emerging multidrug resistant bacteria that cause mastitis have complicated its management, and such resistance has complicated its prevention and treatment (Bhasme et al., 2013). Thus, using new antibacterials is desirable for controlling bovine mastitis. Certain compounds, such as violacein, have been tested against resistant bacteria with bactericidal effects; however, this antibiotic showed host toxicity (Cazoto et al., 2011).

Recent studies have indicated that nitric oxide (NO) is a key mediator for inflammatory responses caused by bovine mastitis (Piotrowska-Tomala et al., 2012). Indeed, nitrite ($NO_2^-$), nitrate ($NO_3^-$) and staphylococcal enterotoxin C were observed in

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http://dx.doi.org/10.1016/j.ijpharm.2014.06.051
0378-5173/

mammary gland secretions infected with *S. aureus*, which indicates that NO-related species (NOx) are important for animal immune responses against bacteria (Komine et al., 2004). The free radical NO is a key molecule in the immunological system, which is important for the natural host defense against invading pathogens such as bacteria (Martínez-Ruiz et al., 2011; Seabra et al., 2010). NO is enzymatically synthesized by several types of cells such as macrophage and neutrophils. Furthermore, given its small size and lipophilicity, when it is secreted by immune cells, NO readily diffuses across cells membranes and causes oxidative and nitrosative damage to invading pathogens (Schairer et al., 2012; Han et al., 2009). NO toxicity is based on its concentration. At low concentrations (nanomolar range), NO is a signaling molecule; in contrast, at high concentrations (micromolar-millimolar range), NO is a potent toxic agent, which can bind vital biomolecules in a pathogen, such as DNA, proteins and lipids (Schairer et al., 2012). Animal cells produce low concentrations of NO for regulatory functions, and NO acts as an intracellular signal. However, animal cells also synthesize high concentrations of NO (>1 μmol/L) through the action of the enzyme inducible NO synthase (iNOS). Indeed, host iNOS is a key component of the innate immune system. Therefore, NO is a mean of animal defense against microbes, and the use of exogenous NO donors for antimicrobial therapies is similar to the action of endogenous iNOS, i.e. production of high amounts of NO for longer periods of time to combat pathogens (Schairer et al., 2012). In addition, several papers described that the topical application of NO donors has been effective in inducing a local immune host response with minimal side effects (Mowbray et al., 2009).

As a free radical, certain molecules in biological media, such as hemoglobin, readily react with NO inactivate this molecule (Cooper, 1999). Thus, S-nitrosothiols (RSNOs) are important molecules that act as spontaneous NO carriers and donors to preserve NO bioavailability (Seabra et al., 2004, 2007, 2010). RSNOs are molecules with a thiol (SH) group covalently bound to NO; thus, they are NO donors that act through homolytic S—N bond cleavage and free NO release, which is indicated in Eq. (1) (De Oliveira et al., 2002; Shishido et al., 2003; Seabra and de Oliveira, 2004; Schairer et al., 2012).

$$2\,\mathrm{RSNO} \rightarrow 2\,\mathrm{NO} + \mathrm{RS} - \mathrm{SR} \quad (1)$$

In addition, RSNOs can transfer NO to other thiols in biomolecules, such as cysteine-containing proteins, through an S-transnitrosation reaction (Schairer et al., 2012). Important RSNOs, such as S-nitrosoglutatione (GSNO) and S-nitrosopolyesters, have antibacterial activities against gram-negative and gram-positive pathogens (Schairer et al., 2012; Seabra et al., 2010).

NO is an important player in natural host defenses against microbes; however, as a free radical, the therapeutic applications for NO are limited by a lack of effective transport and delivery vehicles. Thus, there has been increasing interest in developing NO-releasing materials in a safe, low-cost, controlled and sustained manner for antimicrobial applications (Cabrales, 2011; Carpenter and Schoenfisch, 2012; Seabra and Duran 2010; Seabra et al., 2010). Polymeric nanoparticles for biomedical applications, particularly delivery vehicles for bactericidal agents, have been the center of intense investigations over the last few years (Seabra and Durán, 2012; Seabra et al., 2012; Mihu et al., 2010).

We have already reported a preparation technique for NO-releasing polymeric nanoparticles composed of alginate/chitosan (Marcato et al., 2011, 2013). Indeed, alginate/chitosan systems have been widely used at micro and macro scales for biomedical applications because they have low toxicity and are biocompatible and biodegradable (Marcato et al., 2011, 2013; Douglas et al., 2006). In particular, chitosan is a versatility material that can be used to form fibers, films, gels, sponges, beads or nano/micro particles (Dutta et al., 2011). Chitosan is a cationically charged polymer derived from crustacean exoskeletons and can disrupt cellular membranes and damage cell walls (Mihu et al., 2010). Therefore, chitosan has antimicrobial activities against many pathogenic microorganisms such as fungi as well as gram-positive and gram-negative bacteria (Dutta et al., 2011; Berezin et al., 2012). Classically, chitosan/sodium tripolyphosphate (TPP) nanoparticles are the most studied system for drug delivery. This system was first introduced by Calvo et al. (1997). More recently, hybrid systems comprised by alginate and chitosan were reported (Goycoolea et al., 2009; Marcato et al., 2013). This hybrid system has been reported to improve some physical properties, such as the particle stability in biological media, pharmacological performance in comparison with conventional particles (i.e. comprised solely by chitosan/TPP) (Goycoolea et al., 2009).

In this work, biodegradable and biocompatible polymeric particles composed of alginate/chitosan or chitosan/sodium tripolyphosphate (TPP) were prepared and used to encapsulate mercaptosuccinic acid (MSA), which is a thiol-containing molecule. Nitrosation of thiol groups in MSA-containing particles led yielded S-nitroso MSA particles, which act as NO carriers and donors. The kinetics of NO release from S-nitroso MSA particles were monitored for 12 h at a physiological temperature. The antibacterial activity of NO-releasing particles was evaluated by incubating the particles with a *S. aureus* multi-resistant strain responsible for bovine mastitis. The results indicate that NO-releasing polymeric particles could be used to combat bacterial resistance as well as treat and prevent bovine mastitis.

## 2. Materials and methods

### 2.1. Materials

The chitosan (105 kDa/∼81% acetylation) was from Polymar, Ciência e Nutrição S/A, Fortaleza, CE, Brazil. The alginate (∼250 cps), mercaptosuccinic acid (MSA), sodium nitrite, sodium tripolyphosphate (TPP), acetic acid, 5′5-dithiobis(2-nitrobenzoic acid) (DTNB), and phosphate buffer saline (PBS), pH 7.4 were from Sigma, St. Louis, MO, USA and used as received. The culture media were from Difco®. The disks used for the agar diffusion technique were from Laborclin®. The aqueous solutions were prepared using analytical grade water from a Millipore Milli-Q Gradient filtration system.

### 2.2. Bacterial strains

The *S. aureus* (15) (MBSA) and *E. coli* (15) (MBEC) isolates were from subclinical and clinical bovine mastitis, respectively. The field isolates were from farms in Central São Paulo State with a history of chronic subclinical and clinical bovine mastitis problems (Cabral et al., 2004; Ribeiro et al., 2006). An antimicrobial resistance profile was determined using a disk-diffusion test (Cazoto et al., 2011) in accordance with the Clinical Laboratory Standards Institute guidelines (CLSI, 2012). The strains were stored at −80 °C in a 2.5 mol/L glycerol solution.

### 2.3. Synthesis of MSA-containing polymeric particles

In this work, four types of polymeric particles were prepared: (i) alginate/chitosan (0.75 ratio) without MSA (control), (ii) alginate/chitosan (0.75 ratio) with 4.0 mg/mL of MSA (26.5 mmol/L), (iii) chitosan-TPP particles without MSA (control 2), and (iv) chitosan-TPP particles with 80 mg/mL MSA (0.53 mol/L).



Please cite this article in press as: Cardozo, V.F., et al., Evaluation of antibacterial activity of nitric oxide-releasing polymeric particles against *Staphylococcus aureus* and *Escherichia coli* from bovine mastitis, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.051