



Potential of polymer stabilized nano-liposomes to enhance antimicrobial activity of nisin Z against foodborne pathogens

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ABSTRACT

The scientific and industrial interest in antimicrobial nano-carriers has significantly increased in recent years due to post-processing contamination. Liposomes are considered as promising food-grade carrier systems for bioactive-agents. However, due to relatively low stability of liposomes, surface modification or coating is required to improve its efficiency. The purpose of this study was to fabricate chitosan (CA) stabilized nano-liposomes (chitosomes) to enhance the controlled release and antimicrobial effect of nisin-Z against multidrug resistant (MDR) foodborne pathogens. Chitosome characteristics including encapsulation efficiency (EE %), SEM, thermal stability (TGA, DSC), FTIR analysis, antimicrobial potential and *in vitro* controlled release were investigated. Overall, chitosomes (CS) were thermally more stable, showed higher EE% ($86 \pm 1\%$) as compared to liposomes ($56 \pm 2\%$) and controlled *S. aureus*, *E. faecalis* and *L. monocytogenes* growth better than free or liposomal-nisin. SEM confirmed the size of liposomes from 54 to 108 nm, which increased after coating with chitosan. FTIR analysis revealed the interaction between CA and liposomes by merger of amide I and II peaks. Thus, CA-stabilized liposomes not only improve physico-chemical attributes while ensuring sustained release of nisin but also provide a potential approach to control bacterial contamination in food products for enhanced food safety.

1. Introduction

Pathogen contamination in food causes food spoilage, compromises food quality and ultimately leads to infection and illness. In spite of various food safety and food processing technologies to prevent transmission of these foodborne pathogens, they continue to implicate serious health and financial burden globally (Dinesh Kumar, Verma, & Singh, 2016). Emergence of antibiotic resistance in foodborne pathogens is now a global concern.

Pathogens including *Enterococcus faecalis*, *Listeria monocytogenes* and *Staphylococcus aureus* have been transmitted to consumers by fresh and minimally processed ready to eat dairy, meat and poultry food products (Yegin, Perez-Lewis, Zhang, Akbulut, & Taylor, 2016). These pathogens are well established opportunistic bacteria responsible for both food poisoning and food spoilage. In the past few years, in spite of increased antibacterial therapy, *S. aureus* related foodborne illness maintain high level of morbidity and mortality (Shi et al., 2017). Incidence of systemic listeriosis is much higher in immunocompromised

individuals and in pregnant females (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). Similarly *E. faecalis* is ranked as third major cause of bacteremia globally (Ammerlaan et al., 2013).

These Gram-positive foodborne pathogens have emerged as highly antibiotic resistant strains originating from food or by direct contact with animals (Bortolaia, Espinosa-Gongora, & Guardabassi, 2016). Due to unavoidable emergence of antibiotic resistance in pathogens and side effect of synthetic chemical food preservatives, it is necessary to develop alternative strategies for effective control of foodborne pathogens. Therefore growing need of safe antimicrobial agents from the natural origin seems to be vital (He et al., 2016). In this context nisin, 34 amino acids based natural antibacterial peptide derived from *Lactococcus lactis*, has been used widely in the food industry as a preservative. Nisin is the only bacteriocin which is categorized as Generally Recognized as Safe (GRAS) by Food and Drug Administration (FDA) since 1980 (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). In addition, nisin is highly active against a wide range of Gram-positive bacteria.

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However, application of nisin as a food preservative is limited due to its degradation by proteases enzymes present in food products. Moreover, it was observed that nisin losses its long term effectiveness as milk preservative due to its interaction with milk fat (Gharsallaoui, Oulahal, et al., 2016), which have different composition of lipids (triglycerides) than soy lecithin (phospholipids). Divalent cations associated with bacterial cell wall surfaces also reduce nisin's effectiveness due to electrostatic repulsion (Imran et al., 2015a). Therefore, to overcome the limitations associated with the use of free nisin as GRAS preservative, nanotechnology based novel encapsulation methods has been introduced. Encapsulation of nisin in nanocarrier systems extensively improves its delivery and controlled release (Khan & Oh, 2016).

Liposome is a promising delivery system and received significant interest from food industry in recent years, owing to its structural similarity to cell membrane (Hasan et al., 2016; Park, Jo, & Jeon, 2014). Nano-liposomes are formed by phospholipids obtained mostly from soy lecithin, which contains hydrophobic tail and amphiphilic polar head (Pinilla, Noreña, & Brandelli, 2017; Sadiq et al., 2016; Yamakami et al., 2016). Because of their anionic polar head group, liposomes prove to be a good candidate for the encapsulation of cationic nisin. However, liposomes' use is limited due to their poor stability, low encapsulation efficiency, fusion and aggregation which induce undesired or burst release (Gibis, Ruedt, & Weiss, 2016). Another limitation associated with encapsulation of nisin in liposomes is that nisin forms pores in the membrane after interaction with phospholipid's bilayer (Gharsallaoui Joly, et al., 2016; Gharsallaoui, Oulahal, et al., 2016), leading to a burst release of nisin eventually reducing its functional properties. Electrostatic interactions are considered as the initial step in the series of events leading to pore formation in lipid bilayer. Therefore, a promising way to improve stability of liposomes is coating these vesicles with biopolymer. This could be achieved by simply mixing liposome suspension with polymer without any chemical cross linking agent (Park et al., 2014). By polymer coating, mutual repulsion increases between nano-liposome, hence improving the stability of lipid bilayer nanoparticles. Previously, research work has been done on the stabilization of liposomes by coating with protein and carbohydrate based polymers e.g. polyethylene glycol, hyaluronic acid, poly(arginine) and chitosan to improve structural stability, cell penetration and mucoadhesion (Cosco et al., 2017; Fukui, Kameyama, & Fujimoto, 2017; Lane, Haller, Chavarroche, Almond, & DeAngelis, 2017).

Chitosan is one of the suitable candidates to be used as coating agent for liposomes due to its non-toxicity, bioavailability and biocompatibility. Chitosan is a polysaccharide based cationic polymer composed of 2-amino-2-deoxy- β -D-glucose units that are linked by 1–4 linkages (Niaz, Nasir, Shabbir, Rehman, & Imran, 2016). As liposomes contain negative charge (Abd-El-Azim, Ramadan, Nafee, & Khalafallah, 2017), chitosan adsorbs on their surface due to its cationic nature and forms a thin layer over liposomes. Chitosan coating increase absorption rate of encapsulated agent by improving stability of liposomes during food processing and storage. Coating with biopolymer also prevents aggregation and degradation by food components (Tan, Feng, Zhang, Xia, & Xia, 2016). Recently, studies have been published on chitosan coated liposomes for the improved drug delivery e.g. chitosan coated liposomes for enhanced pulmonary administration of curcumin (Manconi et al., 2017), Seong, J. S et al. reported enhanced skin permeation of quercetin by encapsulating it in liposomes coated with N-succinyl-chitosan (Seong, Yun, & Park, 2018). Lopes et al. explored pectin and polygalacturonic acid-coated liposomes as novel delivery system to check release behavior of nisin (Lopes, Pinilla, & Brandelli, 2017). However, controlled release and antimicrobial efficacy of chitosomes encapsulating nisin against MDR foodborne pathogens has not been explored yet. Chitosan is itself antibacterial against Gram-positive and Gram-negative pathogens, which can not only improve the interaction of chitosomes with the bacteria but can also exhibit synergistic antimicrobial activity along with nisin. Thus, the purpose of

the present study was to develop bacteriocin loaded nano-liposomes coated with chitosan (chitosomes) and to determine the physico-chemical characteristics of the nanostructures including their thermal stability, their potential for controlled release of nisin at different pH or temperature, and to evaluate the antimicrobial activity of nano-antimicrobials against resistant foodborne pathogens.

2. Materials and methods

2.1. Materials

Growth medium (Muller Hinton, Brain heart infusion and Nutrient agar) for strains' growth and preservation were purchased from Sigma-Aldrich. RapID ONE System kits for bacterial identification were procured from Thermo Fisher Scientific. Nisin Z was purchased from Honghao Chemical Co. (Shanghai, China). Nisin used in this study was more than 90% pure (according to the manufacturer, the formulation contains 38.4×10^6 I.U. per gram and 6.88% moisture content). Purified soy lecithin (phosphatidylcholine, $\geq 94\%$), was provided by Lipoid (Ludwigshafen, Germany). TPP (Tripolyphosphate-molecular formula: $\text{Na}_5\text{P}_3\text{O}_{10}$) and chitosan (medium molecular weight) with 85% degree of deacetylation was received from Sigma-Aldrich (USA). Acetic acid having molecular weight 60.05 g/mol was purchased from Riedel-de Haen chemicals.

2.2. Isolation, identification and characterization of foodborne pathogens

Different Gram-positive foodborne pathogenic bacteria (e.g. *Listeria*, *Enterococcus*, and *Staphylococcus*) were isolated from raw milk processed by dairy industries in Pakistan. Identification was carried out by using the API test kit (Thermo Scientific™ RapID™ ONE System). Prior to testing, isolates were cultured overnight at 37 °C on the respective agar medium (blood agar for *Listeria*, nutrient gar for *Staphylococcus* and *Enterococcus* species). Suspension was prepared from the colonies with the turbidity equivalent to 2 McFarland and injected to the wells, subsequently it was incubated for 4 h at 37 °C and reagents were added. Positive and negative results on the basis colour changes were determined according to RapID protocol (Akbari et al., 2015; Garedew et al., 2015). Tests were carried out according to manufacturers' instructions and results were interpreted using the appropriate laboratory computer software or reference indices recommended by the manufacturer. In addition, bacterial identification with conventional biochemical test e.g. coagulase, catalase, oxide, sugar fermentation acid etc., were also performed according to standard microbiological procedures (Karande, 2017). Bacteria can be identified to species level and sometimes to sub-species level. Multi drug resistant (MDR) profiles of the identified strains were carried out by disc diffusion method (Benmansour et al., 2016).

2.3. Quantification of bacteriocin by nano-photometer

Stock solution of nisin at 1 mg/mL (w/v) concentration was prepared by dissolving 0.01 g of nisin powder in 10 mL of distilled water. For the calibration curve, dilutions of 5, 10, 25, 50, 100, 250, 500, 750, and 1000 $\mu\text{g/mL}$ were prepared from the stock solution of nisin. After wave scan, the λ max selected was 591 nm by nano-spectrophotometer (Implen). Optical density (OD) values were recorded for each dilution in triplicate and then respective standard curve was prepared by Bradford assay (Lequeux, Ducasse, Jouenne, & Thebault, 2014).

2.4. Fabrication of nano-vesicles

Liposomes were prepared by following the procedure previously described by Imran et al. (Imran et al., 2015b) with slight modification. Accordingly, soy lecithin (1% w/v) was mixed in distilled water containing nisin (1 mg/mL). The mixture was stirred (1300 rpm) for 4 h.

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