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Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting

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Abstract

Nanoencapsulation may improve activity of protein or polypeptide antimicrobials against a variety of microorganisms. In this study, nanoliposomes prepared from different lipids (Phospholipon 90H, Phospholipon 100H, dipalmitoylphosphatidylcholine (DPPC), stearylamine (SA), dicetyl phosphate (DCP) and cholesterol) by a new, non-toxic and scalable method, were tested for their capacity to encapsulate nisin Z and target bacteria (*Bacillus subtilis* and *Pseudomonas aeruginosa*). Factors affecting the entrapment efficiency (charge and cholesterol concentration in the vesicles) and stability of nanoliposomes were assessed. The nanoliposomes and their bacterial targeting were visualised, using different microscopes under air and liquid environments. Nisin was entrapped in different nanoliposomes with encapsulation efficiencies (EE) ranging from 12% to 54%. Anionic vesicles possessed the highest EE for nisin while increase in cholesterol content in lipid membranes up to 20% molar ratio resulted in a reduction in EE. Stability of nanoliposome-encapsulated nisin was demonstrated for at least 14 months at 4 °C (DPPC:DCP:CHOL vesicles) and for 12 months at 25 °C (DPPC:SA:CHOL vesicles).

Keywords: Nanotechnology; Nanotherapy; Mozafari method; Nisin; Nanoliposomes; Atomic force microscopy; Confocal laser microscopy; Transmission electron microscopy

1. Introduction

One of the applications of nanobiotechnology is the encapsulation and targeting the bactericidal and bacteriostatic agents such as nisin and other peptide-based antibiotics. Nisin is a cationic polypeptide composed of 34 amino acids produced by Lactococcus lactis strains (Gross and Morell, 1971; Verheul et al., 1997). Nisin has received much attention because of its broad inhibitory spectrum against a variety of gram-positive bacteria, including food pathogens and contaminants. Nisin is not inhibitory toward gram-negative pathogens if used without additives, but its spectrum of activity can be broadened by combining the polypeptide with chelators such as EDTA or polyphosphates (Boland et al., 2004; Branen and Davidson, 2004). There are two known natural variants of nisin (nisin A and nisin Z) that are equally distributed among nisin-producing strains (Mulders et al., 1991). These variants differ by a single substitution, at position 27, with histidine (nisin A) and asparagine (nisin Z) (Mulders et al., 1991). This structural modification gives nisin Z higher solubility and diffusion characteristics which are important for food and pharmaceutical applications (De Vos et al., 1993). However, the use of nisin in its free form (unpackaged or unencapsulated) is expensive and is associated with loss of activity due to degradation or deactivation and emergence of nisin-resistant bacterial strains (Benech et al., 2002; Laridi et al., 2003). To overcome these limitations, encapsulation of nisin in phospholipid vesicles may offer the following potential advantages: (i) reduce or prohibit nisin affinity to non-target components and eliminate undesired interactions, (ii) act as a long-term preservative in food and drug systems, (iii) protect nisin from inhibitors or unfavorable conditions – e.g. those that naturally occur in food matrix (Laridi et al., 2003), (iv) decrease (or preferably abolish) the risk of emergence of resistant strains, and (v) provide a mean for targeting the bacteria.

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Due to their several favourable properties, phospholipid vesicles are being considered for the encapsulation and controlled release of food material, nutraceuticals, cosmetics and pharmaceuticals (Barenholz and Lasic, 1996; Mozafari and Mortazavi, 2005). A distinctive advantage of phospholipid vesicles, compared to other encapsulation techniques, is that they can be made of natural ingredients that are beneficial for our health (Crook et al., 1991; Huwiler et al., 2000; Thompson et al., 2006). Recent research into the biological functions of phospholipids and sphingolipids has identified several health benefits, including liver protection, memory improvement and inhibition of cholesterol absorption (Crook et al., 1991; Crook et al., 1992; Koopman et al., 1985; Thompson and Singh, 2006; Thompson et al., 2006). Nanoscale phospholipid vesicles (nanoliposomes), and their micron-sized counterparts (liposomes), offer a potential means of delivery of bactericides to a wide range of bacteria in the treatment of infectious diseases (Bergers et al., 1995; Moribe and Maruyama, 2002). A number of studies have been carried out in which liposomes and nanoliposomes, with surface-bound lectins, have been targeted to bacteria (Jones, 2005; Sanderson and Jones, 1996). The ingredients (building blocks) of liposomes – i.e. liposome composition – is an important parameter determining shelf-life and targetability of the lipid vesicles. It has been found that vesicles incorporating phosphatidylinositol (PI) are effective at targeting to some oral and skin-associated bacteria possibly as a consequence of hydrogen bonding interactions (Catuogno and Jones, 2003; Jones and Kaszuba, 1994; Kaszuba et al., 1995). Cationic liposomes incorporating stearylamine (SA) also interact strongly with the skin-associated bacterium *S. epidermidis* (Sanderson and Jones, 1996). These studies were done using radiolabelling to assay the targeting of the liposomes to immobilised bacterial biofilms formed on microtitre plates. The phospholipid vesicles used in the above-mentioned studies were produced by several methods (sonication, reverse phase evaporation and extrusion), which generally involve application of toxic solvents or high-shear forces (for a review see Mozafari, 2005a). The apparent monolayer coverage of the bacteria has been estimated from the geometric area of the biofilm and the projected area of the liposomes, assuming that they are perfect spheres. While this method gives a useful apparent measure of targeting it does not take into account the surface roughness of the biofilm nor possible deformation of the liposomes on adsorption, effects which would lead to over- and under-estimation of adsorption, respectively (Kaszuba et al., 1997).

We have prepared nisin-loaded liposomes and nanoliposomes by a novel method without employing toxic solvents, high-shear forces or any other harmful procedure. Successful formation of nisin-loaded vesicles was confirmed by high-resolution imaging using various microscopical techniques. In order to gain further insight into the detailed nature of the interaction of nanoliposomes with both gram-positive and gram-negative bacterial surfaces we have attempted to observe the interaction by electron microscopy. In particular, the interactions of both anionic nanoliposomes and cationic SA-containing nanoliposomes with bacterial cells were investigated.

2. Experimentals

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Stearylamine (SA), dicetylphosphate (DCP), Triton X-100, glycerol and nisin were purchased from Sigma Chemical Co. (USA). Hydrogenated phosphatidylcholines (Phospholipon® 90H and Phospholipon® 100H) were kind gifts of Phospholipid GmbH (Cologne, Germany). All solvents (chloroform, methanol, Analar grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of commercial analytical grade. The liposomal ingredients were kept under nitrogen atmosphere at the recommended storage temperatures (0–4 °C) and thin layer chromatography (TLC) proved that all liposomal ingredients were of high purity with no sign of degradation as explained elsewhere (Mozafari et al., 2002).

2.2. Organisms and media

Stock cultures of *Bacillus subtilis* (a gram-positive bacteria) have been prepared by subculture from laboratory stocks of the microbiology laboratory of the Technology Department, Massey University. Each bacterium was inoculated in brain heart agar (BHA, Difco Laboratories, Detroit, MI, USA) at 30 °C for 24 h and then single colonies obtained by inoculating the nutrient broth (NB) bacterial culture onto BHA using the streak technique. A single colony was reintroduced back into fresh NB medium and incubated at 30 °C for 24 h. All stocks of bacterial cultures were stored at 4 °C after incubation.

Nonmucoid strains (PA-1 and PA-48912-2) and mucoid strains (PA-48912-1 and PA-48913) of *Pseudomonas aeruginosa* (a gram-negative bacteria) used in this study were isolated from sputum of CF patients with pulmonary infections at the Memorial Hospital (Sudbury, Ontario, Canada) and were maintained as described before (Omri et al., 2002).

2.3. Preparation of nanoliposomes

Nanoliposomes composed of different ingredients and molar ratios, as indicated in Table 1, were prepared by the Mozafari method, which is based on the heating method (Mozafari et al., 2002; Mozafari, 2005a,b), as explained below. The liposomal ingredients were added to a preheated (60 °C, 5 min) mixture of nisin (200 μg/ml) and glycerol (final concentration 3%, v/v). The mixture was further heated (60 °C) while stirring (approx. 1000 rpm) on a hotplate stirrer (RET basic IKAMAG® Safety Control, IKA, Malaysia) for a period of 45–60 min under nitrogen atmosphere. The reaction was performed in a home-made glass vessel specially designed by Mozafari (Fig. 1), typically in a total volume of 10 ml milli Q water (Millipore Corp., Bedford, MA, USA).