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Stability during in vitro digestion of lactoferrin-loaded liposomes prepared from milk fat globule membrane-derived phospholipids

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ABSTRACT

Liposomes loaded with positively charged lactoferrin (LF) were prepared from milk fat globule membrane-derived phospholipids using a thin-layer dispersion method. The entrapment efficiency of LF in the liposomes and the stability during in vitro gastrointestinal digestion were characterized and examined using dynamic light scattering, transmission electron microscopy, and PAGE. The entrapment efficiency of LF encapsulated in the liposomes was about 46%. The entrapped LF remained unchanged as a function of time and pepsin concentration when the liposome samples were digested in a simulated gastric environment, suggesting that the liposomes prepared from milk fat globule membrane-derived phospholipids were stable and protected the entrapped LF from pepsin hydrolysis. In simulated intestinal fluid, the entrapped LF was more susceptible to hydrolysis by the protease in pancreatin, as shown by changes in the diameter and membrane structure of the liposomes. The release of free fatty acids from the liposomes during digestion in simulated intestinal fluid revealed that the phospholipids in the liposomes were partly hydrolyzed by pancreatic lipase. It was suggested that liposomes may prevent the gastric degradation of LF and reduce the rate of hydrolysis of LF in intestinal conditions.

Key words: lactoferrin, liposome, milk fat globule membrane-derived phospholipid, digestion stability

INTRODUCTION

Lactoferrin (**LF**), a glycoprotein, is present in mammalian milk at an average concentration of 1.4 mg/mL and exhibits a net positive charge at physiological pH (Levay and Viljoen, 1995). It is able to bind ferric ions and has bacteriostatic, bactericidal, and fungistatic activities (Palmano et al., 2011). Because LF binds ferric ions with high affinity, it has been proposed as an antioxidant and has been used in infant formulas (Satué-Gracia et al., 2000). However, it is generally recognized that LF is easily hydrolyzed by the enzymes in the gastrointestinal (**GI**) tract, which may affect its biological activity (Kuwata et al., 2001). Therefore, the encapsulation of LF into a delivery system, such as an emulsion or liposomes, could be useful in preventing the degradation of LF and maintaining its structural integrity.

A liposome, in which a lipid bilayer encapsulates a fraction of the surrounding aqueous medium, is a self-assembling and cell-resembling colloidal delivery system (Lasic and Papahadjopoulos, 1995). Because of their ability to protect and control the release of bioactive components, the applications of liposomes have focused mainly on the pharmaceutical industry, the cosmetic industry, food science, and agriculture (Taylor et al., 2005; Min et al., 2011; Gabizon et al., 2012). Some reviews have specifically considered the utilization of liposomes in encapsulating bioactive proteins such as LF, which are susceptible to digestion and exhibit low permeability through the intestinal membrane. Trif et al. (2001) demonstrated that the entrapment of human LF in liposomes could prolong its retention at sites of local inflammation, such as the rheumatoid joint. Ishikado et al. (2005) encapsulated LF in multilamellar liposomes and found that liposomal LF might act more effectively than conventional LF in the immune system of rats. Roseanu et al. (2010) also stated that a liposomal formulation of LF could increase its uptake and accumulation into cells, and could probably protect it from degradation. To date, however, most studies have focused on whether the bioactivity of LF is improved by liposomal encapsulation. Little information is available to show whether or not liposomes and the entrapped ingredients maintain their structure during GI digestion (Hermida et al., 2009).

Traditionally, liposomes have been produced from high-cost phospholipids extracted from soybean oil or egg yolk, which may limit their applications. Milk fat globule membrane (**MFGM**)-derived phospholipids, which are isolated commercially from waste dairy streams such as buttermilk, can be used for liposome

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preparation. They contain approximately 25% sphingomyelin, and the FA in the chains attached to the phospholipid headgroups are primarily saturated and monounsaturated (Spitsberg, 2005; Singh, 2006). This unique composition of MFGM-derived phospholipids, which is very different from that of the commonly used phospholipids, can play a role in the transport and delivery of active agents in the GI tract (Corredig and Dalgleish, 1998; Roesch et al., 2004). When MFGM-derived phospholipids were used in our laboratory, the liposomes prepared had a higher phase-transition temperature, thicker membrane, and lower membrane permeability compared with liposomes prepared from soybean-derived phospholipids (Thompson et al., 2006b). The liposomes prepared from MFGM-derived phospholipids also had improved stability under a range of pH conditions, at a variety of storage and processing temperatures (Thompson et al., 2006a; Liu et al., 2012). Waninge et al. (2003) also reported that liposome-like vesicles could be produced from simulated MFGM-derived phospholipids. However, the stability to digestion and the structural integrity of liposomes prepared from MFGM-derived phospholipids and of their entrapped contents under GI conditions have not been explored.

In this study, we aimed to extend our previous studies (Thompson et al., 2006a; Liu et al., 2012) and to provide detailed information on the stability of an LF-loaded liposomal system under simulated GI tract conditions. Milk fat globule membrane-derived phospholipids were used to prepare multilayer liposomes by thin-layer dispersion methods, and their properties, including *z*-average diameter, surface charge, entrapment efficiency (**EE**), and morphology, were evaluated. Furthermore, to assess the stability of the LF-loaded liposomes, lipid hydrolysis and protein release during in vitro digestion were also investigated.

MATERIALS AND METHODS

Materials

A phospholipid-rich fraction derived from MFGM (Phospholac 600) was a gift from the Fonterra Co-operative Group Ltd. (Auckland, New Zealand). It contained ≥23% phosphatidylcholine, ≥73% polar lipids, and ≥37% (wt/wt) SFA. Lactoferrin (≥90%) was purchased from Tatua Co-operative Dairy Company Ltd. (Morrinsville, New Zealand). Pepsin from porcine gastric mucosa (P7000; enzymatic activity of 800 to 2,500 U/mg of protein), pancreatin from porcine pancreas [P1750; 4 × US Pharmacopeia (USP) specifications], and bile extract porcine (B8631) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade and were obtained from Sigma-Aldrich.

Preparation of Liposomes

A thin-layer dispersion method was used to prepare suspensions of liposomes. Briefly, MFGM-derived phospholipid, cholesterol, Tween-80, and vitamin E were mixed in a mass ratio of 6:1:1.8:0.12, as described in our previous study (Liu et al., 2011). The mixture was well dissolved in absolute ethanol and then evaporated to a thin film under vacuum in a rotary evaporator (R-215; Büchi Labortechnik AG, Flawil, Switzerland) at 60°C. The dried lipid film was rehydrated with PBS (pH 7.4, 0.05 *M*) containing LF at 2 mg/mL. Liposomes, with lipid (phospholipid and cholesterol) concentrations of 8 mg/mL and loaded with the LF suspension, were obtained after 10 min of sonication.

Characterization of LF-Loaded Liposomes

Determination of z-Average Diameter and ζ-Potential. A Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK) was used to determine the *z*-average diameter of the LF-loaded liposomes at 20°C. The relative refractive index [i.e., the ratio of the refractive index of the phospholipid (1.490) to that of the dispersion medium (1.330)] was 1.120. The absorption of the phospholipid was 0.001. Samples were diluted 10 fold in PBS. Mean particle diameters were calculated as the average of at least triplicate measurements. The ζ-potential values of the liposomes were measured using the same instrument and samples were diluted 10 fold with PBS before analysis. An individual ζ-potential measurement was calculated from the mean and the standard deviation of at least 10 readings from each sample.

Determination of EE. The percentage of entrapped LF was measured after the free protein had been separated from the liposomes using an ultracentrifuge (Sorvall WX Ultra 100; Thermo Scientific, Asheville, NC) at 396,420 × *g* for 1 h at 4°C. The supernatant was withdrawn and the remaining liposome pellet was washed 3 times with Milli-Q water (water purified by treatment with a Milli-Q apparatus; Millipore Corp., Bedford, MA) followed by resuspension in the same initial volume of PBS. The suspension was then used to measure the amount of LF encapsulated in liposomes by the Kjeldahl method (2100 distilling unit and 2006 digestor block; Tecator AB, Höganäs, Sweden). The total amount of LF in the liposome dispersion before centrifugation was determined using the same method. The EE was calculated using the following equation: