Food Chemistry 230 (2017) 6–13

Contents lists available at ScienceDirect

Food Chemistry

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

Kinetic stability and membrane structure of liposomes during *in vitro* infant intestinal digestion: Effect of cholesterol and lactoferrin

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ARTICLE INFO

Article history:
Received 14 February 2016
Received in revised form 17 December 2016
Accepted 4 March 2017
Available online 6 March 2017

Keywords:
Liposomes
Digestion
Membrane structure
Stability
Microfluidity

ABSTRACT

The effects of cholesterol and lactoferrin on the kinetic stability and membrane structural integrity of negatively charged liposomes under *in vitro* infant intestinal digestion conditions were elucidated using dynamic light scattering, pH-stat titration, Fourier transform infrared spectroscopy, and pyrene steady state fluorescence probes. The liposomes had a smaller particle diameter, a wider size distribution, and a greater negative charge after digestion. The incorporation of cholesterol into the phospholipid bilayers resulted in a more ordered conformation in the aliphatic tail region and reduced micropolarity, indicating that cholesterol can improve the structural stability of liposomal membranes against intestinal environmental stress. Lactoferrin coverage facilitated the release of free fatty acids and increased the microfluidity of the bilayers, reducing the structural integrity of the liposomes. This study provides useful information on the design of liposomes and other microcapsules with improved and controlled release properties during digestion for particular groups of people.

1. Introduction

Liposomes, i.e. polar lipids that are dispersed in an aqueous medium, are self-assembling and cell-resembling colloidal delivery systems; they have been applied mainly in pharmaceutics, cosmetics, gene therapy and agriculture (Lasic & Papahadjopoulos, 1995; Liu, Ye, & Singh, 2015). Recent studies have considered the utilization of liposomes in the food and nutrition area to encapsulate and control the release of bioactive components, such as antioxidants, enzymes, proteins and vitamins (Kheadr, Vuillemard, & El-Deeb, 2003; Liu, Ye, Liu, Liu, & Singh, 2013; Rovoli, Gortzi, Lalas, & Kontopidis, 2014; Tan et al., 2014). However, environmental stresses, including pH, temperature, oxygen and enzymes, can cause the aggregation of liposomes, alteration to their surface properties and even damage to their structural integrity (Allen Zhang & Pawelchak, 2000; Liu, Ye, Liu, Liu, & Singh, 2012; Sułkowski, Pentak, Nowak, & Sułkowska, 2005). This instability of liposomes, against the environment and cellular uptake, leads to the loss of key molecules and, thus, limits their practical application (Chiou et al., 2009).

Recently, studies related to the digestion of food-based delivery systems in the human gastrointestinal tract (GIT) have attracted much attention (Ozturk, Argin, Ozilgen, & McClements, 2015; Teng et al., 2014). For liposomes, the emphasis has been on the release behaviour of the core ingredients and improved digestion stability by surface modification. Zou et al. (2014) prepared nanoliposomes using dynamic high pressure microfluidization and demonstrated that the stability of epigallocatechin gallate was significantly improved by liposomal encapsulation during *in vitro* digestion. Liu, Liu, Liu, Li, and Liu (2013) developed layer-by-layer self-assembly alginate–chitosan-coated nanoliposomes, which had improved stability of the lipid membrane and prevented leakage of medium chain fatty acids into the GIT. In our previous studies, we determined the detailed physicochemical characteristics and membrane integrity of liposomes during *in vitro* digestion; we showed that the pancreatic enzyme in simulated intestinal fluid (SIF) could modify the organized structure of assembled lipids or damage the liposomal membrane severely, indicating that the liposomes exhibited lower stability in SIF than in simulated gastric fluid (Liu, Liu et al., 2013). Previous studies have focused mainly on adults. However, there have been few studies on specific populations, such as infants. The sensitive and developing GIT of infants exhibits different conditions from the adult GIT, namely different pH gradients, enzyme levels and gastric emptying rates, which lead to differences in the response to food and carriers in infants

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http://dx.doi.org/10.1016/j.foodchem.2017.03.021
0308-8146/

(Bourlieu et al., 2014). Thus, more systematic knowledge about the structural properties of liposomes for these populations, especially during digestion in the small intestine, is required.

The kinetic stability of the liposomal bilayer can be influenced by its composition and surface properties. Cholesterol, a major constituent in many biological membranes, can increase bilayer thickness and the membrane ordering, and even reduce membrane permeability. The incorporation of cholesterol has been assumed to provide physicochemical stabilization to liposomes (Qin et al., 2011). Lactoferrin is a glycoprotein that is present in mammalian milk and exhibits a net positive charge at physiological pH (Levay & Viljoen, 1995). The surface modification of liposomes with lactoferrin improves cellular uptake efficiency and inhibits growth of cancer cells more efficiently compared with non-modified liposomes (Chen et al., 2011); the permeability of the neuron growth factor across the blood–brain barrier can also be increased by lactoferrin-surface-grafted liposomes (Kuo & Wang, 2014). However, with respect to a nutrient delivery system, the influences of cholesterol and lactoferrin on the membrane properties and dynamic digestion stability of liposomes have still to be clarified.

To extend our previous work and provide more information about the behaviour of liposomes during SIF digestion, we investigated the kinetic stability and structural properties of liposomes with and without cholesterol and lactoferrin (a positively charged protein). Negatively charged liposomes were prepared from food-grade phospholipids using a thin-layer dispersion method. Dynamic light scattering, pH-stat titration (lipolysis), Fourier transform infrared (FTIR) spectroscopy and fluorescence spectroscopy were used to determine the effects of cholesterol and lactoferrin on changes in the hydrodynamic radius, polydispersion index, zeta-potential, free fatty acid (FFA) release kinetics, membrane structure and membrane fluidity of liposomes under infant SIF conditions.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine from soybean containing ≥30% phosphatidylcholine, ≥73% polar lipids and ≥20 wt.% saturated fatty acids was purchased from Sigma-Aldrich Chemical Co (P3644, St. Louis, MO, USA), as was cholesterol from sheep wool (≥92.5%, C8503, Sigma-Aldrich). Lactoferrin (≥90%) was purchased from Tatua Co-operative Dairy Company Ltd. (Morrinsville, New Zealand). Pancreatin from porcine pancreas [P1750; 4 × United States Pharmacopeial (USP) specifications], bile extract porcine (B8631) and pyrene (82648) were purchased from Sigma-Aldrich. Absolute ethanol and all other reagents were analytical grade and were obtained from Sigma-Aldrich. Milli-Q water (water purified by treatment with a Milli-Q apparatus; D11971, Thermo Fisher Scientific, Waltham, MA, USA) was used for the preparation of all solutions.

2.2. Preparation of liposomes

Four different liposomal formulations were prepared using a thin-layer dispersion method, as described previously (Liu et al., 2012); details of the formulations are given in Table 1. Briefly, L-α-phosphatidylcholine, cholesterol, Tween-80 and vitamin E were mixed in a mass ratio of 6:1:1.8:0.12 and dissolved in absolute ethanol. The ethanol was evaporated under vacuum using a rotary evaporator at 55 °C (RE5298; Yarong Biochemical Instrument Factory, Shanghai, China) for at least 1 h to form a thin lipid film. This lipid film was then rehydrated with phosphate-buffered saline (pH 7.4, 0.05 mol/L) to obtain a liposomal suspension (PCLP), with a lipid (L-α-phosphatidylcholine and cholesterol) concentration of 8 mg/mL. Liposomes without cholesterol (PLPs) were also prepared using the same procedure. PCLFs (liposomes with cholesterol and lactoferrin) and PLFs (liposomes with lactoferrin but no cholesterol) were prepared by adding PCLPs and PLPs dropwise into a stirred lactoferrin solution (4 mg/mL, 1:1, v/v), respectively.

Table 1
Formulations of the four types of liposome.[a]

Liposomes	Composition	Mass ratio
PCLPs	L-α-Phosphatidylcholine:cholesterol	6:1
PLPs	L-α-Phosphatidylcholine:cholesterol	6:0
PCLFs	L-α-Phosphatidylcholine:cholesterol:lactoferrin	6:1:1.75
PLFs	L-α-Phosphatidylcholine:cholesterol:lactoferrin	6:0:1.75

[a] PCLPs, liposomes without lactoferrin but with cholesterol; PLPs, liposomes without lactoferrin and without cholesterol; PCLFs, liposomes with cholesterol and lactoferrin; PLFs, liposomes without cholesterol but with lactoferrin.

2.3. Preparation of simulated intestinal fluid (SIF)

SIF was prepared as described by Singh and Sarkar (2011). The SIF contained 6.8 g of K_2HPO_4, 190 mL of 0.1 M NaOH and 0.4 mg/mL of bile salts, and the pH was adjusted to 7.4 before the SIF was diluted to 1000 mL with Milli-Q water. Pancreatin was added into the system at the beginning of digestion, at a final concentration of 0.32 mg/mL.

2.4. Pancreatin-catalysed digestion

The *in vitro* pancreatin-catalysed digestion was carried out as described previously (Liu et al., 2015). Liposomes were mixed with SIF in a volume ratio of 1:3 and the mixture was adjusted to pH 7.4 before the *in vitro* digestion. The solution was preheated to 37 °C, with continuous shaking at 95 rev/min, for 30 min in a temperature-controlled water bath (Lab-Line shaker bath, Model LZ33070; Barnstead International, Dubuque, IA, USA). Samples were taken periodically for analysis (60 min).

2.5. Determination of Z-average diameter and zeta-potential of the liposomes

The *Z*-average diameter of the liposomes was determined by dynamic laser light scattering using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK) at a wavelength of 623 nm and 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. Samples were diluted 10-fold in Milli-Q water before determination. Mean particle diameters were calculated as the average of at least triplicate measurements.

The zeta-potential (ξ) of the liposomes was also measured using the same equipment and determined from electrophoretic mobilities (μ_E), using the Henry correction to the Smoluchowski equation:

$$\zeta = \frac{3\mu_E n}{2\varepsilon_0 \varepsilon_r f(k\alpha)} \quad (1)$$

where n is the viscosity of water, ε_0 is the permittivity of the vacuum, ε_r is the relative permittivity, k is the Debye length and α is the particle radius. Values of 1.5 and 1.0 are normally used for approximate determinations of $f(k\alpha)$. Generally, in an aqueous medium and at medium electrolyte concentrations, $f(k\alpha)$ is 1.5, namely the Smoluchowski approximation. For small particles in a medium