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Mucus interactions with liposomes encapsulating bioactives: Interfacial tensiometry and cellular uptake on Caco-2 and cocultures of Caco-2/HT29-MTX

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ABSTRACT

Structuring of delivery matrices in foods aquires careful designing for optimal delivery and subsiquent absorption of the beneficial compounds in the gut. There has been quite improvement in mimicking digestion and absorption *in vitro* but as of yet little is understood on mucus interference in nutrient absorption Therefore in this study interactions of human intestinal mucus with milk and soy phospholipids liposomes carring hydrophilic (epigallocatechin-3-gallate) or hydrophobic (β-carotene) bioactive molecules were investigated. Liposomes of about 100 nm were obtained using microfluidization and their behaviour with the human intestinal mucus were evaluated using drop shape tensiometry. The chemistry of the liposomes (milk or soy) and the encapsulated bioactive structure can affect the viscoelastic behaviour of the complex itself. Empty or loaded liposomes were differently interacting with the mucus at the interface. Mucus-liposomes interactions were also studied using cell cultures, Caco-2 (without mucus) and cocultures Caco-2/HT29-MTX (mucus producing). The interaction of mucus layer with liposomes was at some extent aligned with rheological studies. This work demonstrated that delivery systems may interact with the mucosal surface of intestinal cells, and *in vitro* approaches allow for screening of such interactions. These highlights could help us in carefully designing the delivery systems and moreover choosing the right carrier and/or bioactive that does not jeopardize the optimal delivery of the bioactive structure.

1. Introduction

Functional foods have been focus of much development in the past decade driven by increased interest of consumers for health and wellbeing. However, the limited bioactivity and bioavailability of bioactive molecules incorporated in foods make development of the appropriate food matrix extremely challenging. The limited bioefficacy of health promoting compounds is largely attributed to the instability of the molecules during processing and storage, their interactions with other food molecules, their high instability after ingestion, their rapid degradation during digestion, and poor intestinal absorption. Furthermore, the biological properties of bioactives may also depend on their ability to form complexes with other components interaction with food matrices, intestinal absorption, and the extent of bioconversion in intestine (Acosta, 2009; Arranz, Corredig, & Guri, 2016; Parker, 1996; Zhang, Zheng, Chow, & Zuo, 2004).

Encapsulation offers an effective approach to protect the bioactives during processing and storage, and enhancing their bioefficacy. Liposomes are spherical bilayer or multilayer vesicles comprised of phospholipids, thus both hydrophilic and hydrophobic bioactives can be simultaneously encapsulated (Mozafari, 2005). Liposomes have been employed in the pharmaceutical and cosmetic industry for decades to protect bioactive compounds, and in foods, they have been shown to improve the stability and absorption of incorporated bioactives under processing, storage and digestive conditions (Liu, Ye, Liu, Liu, & Singh, 2013; Nacka, Cansell, & Entressangles, 2001; Rashidinejad, Birch, Sun-Waterhouse, & Everett, 2016a; Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007).

The inner intestinal wall is comprised of a single layer of epithelial cells that are covered by a mucosal layer, acting as a natural defense and a protective barrier to pathogens and most bacteria, while permitting the exchange of nutrients (Karlsson, Wikman, & Artursson, 1993). Mucin proteins, the major component of mucus, significantly affect and modulate the rheological behaviour of the mucus layer (Lai, Wang, Wirtz, & Hanes, 2009). Recent studies suggest that mucus may hinder the diffusion of food particles depending on the physico chemical properties of such particles (Behrens, Vila Pena, Alonso, & Kissel, 2002; Borel & Sabliov, 2014). The diffusivity of the particles may affect not only the gastrointestinal transit but also their absorption in the gut, and ultimately the bioavailability of the bioactive compounds. It has

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been proposed that the cellular uptake of bioactives may be tailored by modifying the physico chemical properties of the delivery systems (Macierzanka et al., 2011).

In the small intestine, various digestive enzymes, bile salts and phospholipids work simultaneously to digest food components and form mixed micelles for intestinal cell absorption. It is known that liposomes are naturally present in the gut, and in breast milk (Keller, 2001). Information is available on the stability of liposomes under conditions similar to those found in the digestive tract (Gülseren & Corredig, 2013; Hwang, Tsai, & Hsu, 2010; Liu, Ye, Liu, Liu, & Sing, 2012). Liposomes can be directly absorbed by enterocytes as the bilayer vesicle structures are similar to cell membranes (Li et al., 2011).

To better understand the mechanisms related to delivery of bioactives in the intestinal tract, more information is needed on the interactions between human intestinal mucus components and liposomes prepared from milk and soy phospholipids. Milk phospholipids have a distinct phospholipids composition, containing high levels of sphingomyelin (SM) and phosphatidylserine (PS) (Burling & Graverholt, 2008). These molecules and their metabolites have been associated with beneficial health effects, such as antiinflammatory, antiproliferative, memory improvement functions and stress control (Burling & Graverholt, 2008). Soy phospholipids, on the other hand, are mainly comprised of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) with very low concentrations of SM and PS (Burling & Graverholt, 2008).

Epigallocatechin-3-gallate (EGCG) and β-carotene were employed as model bioactive molecules. The physicochemical properties of liposomes, morphology, encapsulation efficiency, and stability were first investigated. Then, the interactions between liposomes and the human mucus layer were studied using drop shape tensiometry. This approach has been previously used to understand possible interactions between mucus components and bioactive molecules (Guri, Li, & Corredig, 2015; Rossetti, Ravera, & Liggieri, 2013). In addition, uptake was evaluated using two *in vitro* cell models, Caco-2 and cocultures of Caco-2/HT29-MTX cells. These two cell lines were employed as the Caco-2 line represents a mucus free cell model, it forms a polarized monolayer of absorptive, gastric-like cells on the apical surface with distinct tight junctions (Karlsson et al., 1993; Pontier, Pachot, Botham, Lenfant, & Arnaud, 2001); while the HT29-MTX line consists of differentiated goblet cells with mucus secreting properties (Pontier et al., 2001). Hence, cocultures of Caco-2 and HT29-MTX will represent a model that closely mimics the intestinal epithelium, including the presence of a mucus layer.

2. Materials and methods

2.1. Materials

Milk phospholipids (NZMP Phospholipid Concentrate 700 (PC-700)) were donated by Fonterra (Fonterra Co-operative Group, Palmerston North, New Zealand). PC-700 contained 85.1% lipids (3% PS, 31% PC, 8.7% PE, and 16% SM). The moisture content was 1.7% and the extract also contained 6.6% lactose and 8.3% ash. Soy phospholipids (Ultralec P lecithin) were donated by ADM (Decatur, IL, USA), and they were composed of 23% PC, 18% PE and 15% PI. A tea polyphenol extract (Teavigo®) was donated by DSM Nutritional Products (Ayr, Ontario, Canada) containing mostly (−)-epigallocatechin-3-gallate (EGCG) (min. 94%). Sodium chloride, imidazole, HPLC-grade water, acetonitrile, methanol, chloroform, and glacial acetic acid were obtained from Fisher Scientific (Mississagua, ON, Canada) and 95% β-carotene powder was purchased from Sigma Aldrich (St. Louis MO, USA). Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), HEPES and, Hanks Balanced Salt Solution (HBSS) buffer were purchased from Sigma-Aldrich Corporation (Oakville, ON, Canada), while fetal bovine serum (FBS) heat inactivated, nonessential aminoacids (NEAA), trypsin 1 mM EDTA, L-glutamine and penicillin–streptomycin (10,000 units of penicillin and 10,000 mg of streptomycin per mL) were purchased from Invitrogen (Canada Inc., Burlington, ON, Canada).

2.2. Liposome preparation

Liposomes were prepared as previously described with some modifications (Thompson & Singh, 2006). Briefly, empty liposomes were prepared by dispersing a 10 mg · mL^{-1} of milk or soy phospholipids in imidazole buffer (20 mM imidazole, 50 mM NaCl in MilliQ water, pH 7) for 2 h. All dispersions were pre-homogenized using a Polytron mixer (Brinkmann Inst. Corp., Mississauga, ON, Canada) at 10,000 rpm for 5 min and then cycled through a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA) for 5 passes with an input air pressure of 58 MPa (Thompson & Singh, 2006).

To encapsulate tea polyphenols in liposomal dispersions, EGCG (4 mg · mL^{-1}) was also added to the buffered phospholipid dispersion. The concentration of EGCG was chosen based on previous studies (Gülseren & Corredig, 2013).

To encapsulate β-carotene, a molar ratio of 0.004 (β-carotene to phospholipids) was employed (Lee & Tsai, 2010; Liu & Park, 2009; Thompson, Couchoud, & Singh, 2009). The low molar ratio was used to minimize the structuring effect of this hydrophobic compound on the bilayer. 13.8 mg β-carotene was added to 10 g of milk or soy phospholipids extract (with an average molar mass of 800 g · mol^{-1}). To better dissolve β-carotene, ethanol (20 mL) was added to the mixture with constant stirring until a uniform paste was obtained. The solvent was then evaporated under nitrogen to form a lipid thin film. 20 mM imidazole, 50 mM NaCl buffer at pH 7.0 (100 mL) was then added, and the dispersion was pre-homogenized and cycled for 5 passes through microfluidizer. In all cases, samples were carefully covered to avoid light degradation.

2.3. Liposome morphology

Cryogenic transmission electron microscopy (Cryo-TEM) was employed to provide information on the microstructure of the liposome particles. The sample (4 μL) was pipetted onto a quantifoil (Quantifoil Micro, Jena, Germany) grid with 2 μm holes. The excess sample was blotted off in a vitrobot (FEI), (Eindhoven, Holland) and immediately plunged into liquid ethane held at liquid nitrogen temperature. The specimen was transferred under liquid nitrogen to a Gatan 626 Cryo holder (Warrendale, PA, USA) and viewed at −176 °C in the Tecnai G2 F20 TEM (Eindhoven, Holland). Images are recorded with a Gatan 4K bottom mount CCD camera.

2.4. Physical and chemical characterization of liposomes

Prior to storage, the liposome dispersions were adjusted to different pH values (3, 5, 7 & 9with either HCl 1 N or NaOH 1 N). The dispersions were stored under refrigeration temperature (4 °C) or room temperature (25 °C), and the stability was evaluated for mean particle size after one-week period. The average apparent diameter and ζ-potential of milk or soy liposomes were determined using dynamic light scattering (DLS) technique (Zetasizer Nano, Malvern Instruments, Worcestershire, UK). The latex reference samples used for size and ζ-potential measurements were purchased from Duke Scientific, Palo Alto, CA, USA. The liposomal dispersions were extensively appropriately diluted using 0.22 μm prefiltered imidazole buffer. A medium viscosity of 1.054 mPa · s was used for the calculation of the size from the diffusion coefficient data.

2.5. Encapsulation efficiency determination

Immediately following liposome preparation, the un-encapsulated EGCG or β-carotene was separated using gel permeation chromatography, for further analysis. Aliquots (1.5 mL) of the liposome dispersion