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Behaviour of liposomes loaded with bovine serum albumin during *in vitro* digestion

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ABSTRACT

This study examined the stability of liposomes loaded with negatively charged protein (bovine serum albumin, BSA) during *in vitro* digestion. Zeta-potential and morphology measurements confirmed that BSA-loaded liposomes were successfully prepared, with an encapsulation efficiency of around 34%. The encapsulated BSA and the integrity of the liposomes remained unchanged with time when the liposomes were digested in a simulated gastric environment, suggesting that the liposomal membrane protected the entrapped BSA from pepsin hydrolysis. BSA-loaded liposomes exhibited lower stability in simulated intestinal fluid, as shown by damaged membranes and the release of free fatty acids. Also, lipolysis kinetics revealed that bile salts and ionic strength could facilitate a high level of free fatty acid release. This work further supplemented our knowledge about the effects of gastrointestinal digestion conditions on liposomal properties and provided valuable information for the design of liposome formulations for the food and health care industries.

1. Introduction

Despite dynamic growth in the food and nutritional supplement industry in recent decades, the stability and the ingestion efficiency of bioactive ingredients present major challenges. The most potent ingredients can be very unstable, can have low bioavailability and are often digested in the gastrointestinal (GI) tract, thus not reaching the desired organs and cells in appropriate concentrations (Qian, Decker, Xiao, & McClements, 2012). Delivery systems are able to alter the digestion kinetics and the biodistribution of encapsulated agents, which can reduce side effects and increase bioavailability (Kost & Langer, 2012). The liposome is considered to be a promising delivery system because of its amphiphilic encapsulation, protective ability, large carrying capacity and biocompatibility (Lasic, 1996). However, the instability of liposomes during their passage through the GI tract poses a major challenge. Typically, low pH conditions alter the angle of the headgroups and tails of lipids, leading to changes to the surface charge of liposomes (Lähdesmäki, Ollila, Koivuniemi, Kovanen, & Hyvönen, 2010). In addition, bile salts accelerate the hydrolysis of the bilayer membrane (Klinkesorn & McClements, 2010).

The surface properties of liposomes and the encapsulated ingredients are two crucial factors that influence the liposomal digestion behaviour. Surface modification, using charged substances conjugated with the liposomal membrane, is considered to be a strategy for stabilising liposomes (Torchilin, 2005). For example, pH-responsive polymers (Barea, Jenkins, Gaber, & Bridson, 2010), polysaccharides (Bai et al., 2011) and polyelectrolytes (Liu, Liu, Liu, Li, & Liu, 2013) can be coated on to the surface of liposomes and can protect against degradation and efficiently reduce the release of the liposomal contents during digestion in the GI tract. However, there is little published information on the effect of the encapsulated ingredients on liposomal behaviour during digestion. Stamp and Juliano (1979) demonstrated that the encapsulation efficiency (EE) of liposomes was governed largely by the type of their contents. Although Tsunoda et al. (2001) used positively charged lysozyme and negatively charged bovine serum albumin to examine the effect of the type of protein on the characteristics of liposomes, they provided no information on the effect of the type of liposomal content on the digestion stability.

Our previous studies have provided detailed structural and functional insights into the stability of liposomes during *in vitro* digestion (Liu, Ye, Liu, Liu, & Singh, 2012, 2013). We found, by encapsulating positively charged lactoferrin using a negatively charged liposome, that the EE was about 46% and that the membrane thickness increased because of the protein/phospholipid

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electrostatic interaction. In addition, the structure of the liposomes remained intact, with no release of protein after gastric digestion. However, as discussed by Cheng, Wu, Li, and Xu (2008), the electrostatic interaction between the encapsulated ingredient and the wall material of the carrier may influence the EE of liposomes. In addition, the electrokinetic charge of phospholipids can be altered by the nature of the encapsulated molecules, which will lead to modification of enzyme/lipid surface interactions and thus to changes in the digestion behaviour (Shayman, Kelly, Kollmeyer, He, & Abe, 2011). In our previous study (Liu, Ye et al., 2013), we attempted to clarify the digestion behaviour of negatively charged liposomes containing positively charged molecules; however, as of yet, there is no clear understanding of the effect of negatively charged contents on the structural properties and the release kinetics of anionic liposomes during digestion.

The present study was aimed at extending our previous work and to provide more information on the stability of negatively charged protein-loaded liposomes under simulated GI tract conditions. A model protein, bovine serum albumin (BSA), was encapsulated in anionic liposomes, and changes in the *Z*-average diameter, surface charge, morphology and protein release of the liposomes were evaluated. Moreover, to provide more insight into the factors influencing the stability of liposomes, the kinetics of the release of free fatty acids (FFAs) during *in vitro* digestion were also investigated.

2. Materials and methods

2.1. Materials

L-α-phosphatidylcholine from soybean (P3644, Sigma–Aldrich Chemical Co., St. Louis, MO, USA) contained ⩾ 30% phosphatidylcholine, ⩾73% polar lipids and ⩾20 wt% saturated fatty acids. BSA (30060-578) was purchased from Invitrogen Corporation. Pepsin from porcine gastric mucosa (P7000, enzymatic activity of 800–2500 units/mg protein), pancreatin from porcine pancreas (P1750, 4 × U.S. Pharmacopeial (USP) specifications) and bile extract porcine (B8631) were obtained from Sigma–Aldrich. All other chemicals were of analytical grade and were obtained from Sigma–Aldrich.

2.2. Preparation of BSA-loaded liposomes

BSA-loaded liposomes were prepared by a thin layer dispersion method, as described in a previous study (Liu, Ye, Liu, Liu, & Singh, 2012). Briefly, soybean phospholipid, cholesterol, Tween-80 and vitamin E were mixed in a mass ratio of 6:1:1.8:0.12. The mixture was well dissolved in absolute ethanol and then evaporated to a thin film under vacuum in a rotary evaporator (R-215, Buchi, Flawil, Switzerland) at 60 °C. The dried lipid film was rehydrated with phosphate-buffered saline (PBS; pH 7.4, 0.05 M) containing 2 mg BSA/ml. Liposomes loaded with BSA suspension were obtained after 10 min of sonication; the concentration of lipids (phospholipid and cholesterol) was 8 mg/ml.

2.3. Characterisation of BSA-loaded liposomes

2.3.1. Determination of Z-average diameter and zeta-potential

Before analysis, liposomal samples were diluted 10-fold in PBS. The *Z*-average diameter of the BSA-loaded liposomes was determined using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK) at 20 °C. The measurement parameters were as follows: 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. Mean particle diameters were calculated as the average of at least triplicate measurements. The zeta-potential values of the liposomes were measured using the same instrument. An individual zeta-potential measurement was calculated from the mean and the standard deviation of at least 10 readings from each sample.

2.3.2. EE measurement

The percentage of BSA entrapped in the liposomes was measured by the Kjeldahl method, as described in our previous study (Liu, Ye et al., 2013). The free protein that was not encapsulated was separated from the liposomes using an ultracentrifuge (Sorvall WX Ultra 100, Thermo Scientific) at 60,000 rev/min for 1 h at 4 °C. The supernatant was then withdrawn and the remaining liposome pellet was washed three times with Milli-Q water followed by resuspension in the same initial volume of PBS. The suspension was then used to measure the amount of BSA encapsulated in liposomes using a Kjeldahl apparatus (2100 Distilling Unit and 2006 Digestor Block, Tecator AB, Hoganas, Sweden). The total protein content of the liposome dispersion without centrifugation was determined using the same method. The EE was calculated using the following equation:

$$\text{EE } \% = \frac{W_{\text{in}}}{W_{\text{total}}} \times 100 \quad (1)$$

where W_{in} is the analysed weight of BSA encapsulated in the liposomes and W_{total} is the total weight of protein.

2.3.3. Morphology

The morphology of the BSA-loaded liposomes was observed using confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM).

For CLSM, a 500 μl sample was stained with 40 μl of Nile red solution (0.25 mg/ml) and 80 μl of fast green solution (1 mg/ml), which were used to stain the phospholipids and the protein, respectively. The mixed solution was placed on a concave confocal microscope slide and 50 μl of agarose (0.5%) was added to fix the sample. The sample was then covered with a cover slip and dried at room temperature under dark conditions. Images of the samples were acquired using a confocal laser scanning microscope (Leica DM6000 B, Heidelberg, Germany) with a 63× magnification lens.

A negative-staining TEM technique was used to provide visual confirmation of the formation of liposomes. A copper mesh grid was placed on to droplets of a nanoliposome suspension, which was diluted to a phospholipid concentration of 1 mg/ml with distilled water. After 4 min, the grid was stained with uranyl acetate solution (2%) for 4 min and air dried at room temperature after excess liquid had been removed with filter paper. The morphology of the nanoliposomes was recorded with a transmission electron microscope (CM10, Philips, Eindhoven, Netherlands) at 25,000× magnification.

2.4. In vitro digestion stability of BSA-loaded liposomes

2.4.1. In vitro digestion of liposomes

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in our previous study (Liu et al., 2012). The digestion stability of the BSA-loaded liposomes was measured both in SGF and in SIF. Liposomes were mixed with SGF containing pepsin (0.032 mg/ml) or with SIF containing pancreatin (0.032 mg/ml) at a volume ratio of 1:3. The pH of the mixture (60 ml) containing SGF was adjusted to 1.5 and the mixture containing SIF was adjusted to 7.4. These mixtures were then incubated in a shaking water bath (95 rev/min) at 37 °C and sub-samples (4 ml) were taken for analysis at different time intervals (0, 1, 5, 15, 30, 60 and 120 min).