Colloids and Surfaces B: Biointerfaces 115 (2014) 86–92

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Ascorbyl palmitate/DSPE-PEG nanocarriers for oral iron delivery: Preparation, characterisation and in vitro evaluation

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ARTICLE INFO

Article history:
Received 30 May 2013
Received in revised form 3 November 2013
Accepted 17 November 2013
Available online 23 November 2013

Keywords:
Ascorbyl palmitate
Ferrous sulphate
Caco-2
Chitosan
Nanocarriers
Fortification

ABSTRACT

The objective of this study was to encapsulate iron in nanocarriers formulated with ascorbyl palmitate and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine polyethylene glycol (DSPE-PEG) for oral delivery. Blank and iron (Fe) loaded nanocarriers were prepared by a modified thin film method using ascorbyl palmitate and DSPE-PEG. Surface charge of the nanocarriers was modified by the inclusion of chitosan (CHI) during the formulation process. Blank and iron loaded ascorbyl palmitate/DSPE nanocarriers were visualised by transmission electron microscopy (TEM) and physiochemical characterisations of the nanocarriers carried out to determine the mean particle size and zeta potential. Inclusion of chitosan imparted a net positive charge on the nanocarrier surface and also led to an increase in mean particle size. Iron entrapment in ascorbyl palmitate-Fe and ascorbyl palmitate-CHI-Fe nanocarriers was 67% and 76% respectively, suggesting a beneficial effect of chitosan on nanocarrier Fe entrapment. Iron absorption was estimated by measuring Caco-2 cell ferritin formation using ferrous sulphate as a reference standard. Iron absorption from ascorbyl palmitate-Fe (592.17 ± 21.12 ng/mg cell protein) and ascorbyl palmitate-CHI-Fe (800.12 ± 47.6 ng/mg, cell protein) nanocarriers was 1.35-fold and 1.5-fold higher than that from free ferrous sulphate, respectively (505.74 ± 23.73 ng/mg cell protein) ($n = 6$, $p < 0.05$). This study demonstrates for the first time preparation and characterisation of iron loaded ascorbyl palmitate/DSPE PEG nanocarriers, and that engineering of the nanocarriers with chitosan leads to a significant augmentation of iron absorption.

1. Introduction

The world health organisation ranks iron deficiency as the most pervasive nutritional disorder, affecting as much as 20% of the global population [1]. It is prevalent in developed as well as developing countries, although the incidence is greater in the latter, primarily due to socio-economic factors [1].

Food fortification is generally recognised as a cost-effective and convenient approach to counter iron deficiency, and there is a substantial body of evidence that demonstrates the benefits of this approach [2]. The main requirements for an iron source to be used effectively as a fortificant is to have sufficiently high bioavailability without causing any undesirable sensory changes in the food vehicles such as flours, breakfast cereals, cereal-based complementary foods, salt, milk, and milk based products. This has proved particularly challenging, as iron salts such as ferrous sulphate are highly reactive to the food vehicle, whereas iron compounds that have a better compatibility profile such as electrolytic iron have been reported to have low bioavailability [3,4].

Ascorbic acid is often included in iron fortified foods on account of its role as a promoter of non-haem iron absorption [5,6] and has been shown to increase the absorption of all current iron fortification compounds [7]. It is thought that this enhancing action is due to the ability of ascorbic acid to reduce ferric iron to the bioavailable ferrous form and/or its capacity to chelate ferrous iron forming a soluble ferrous ascorbate complex that is resistant to the effect of iron inhibitors. Indeed a 2:1 molar ratio of ascorbic acid to iron increases iron absorption by at least two-fold in adult women as well as infants fed fortified foods. In case of phytate rich foods a minimum molar ration of 4:1 has been recommended [8,9]. Scheers and Sandberg investigated the mechanism of ascorbic acid on iron absorption in Caco-2 cells [10]. A short term increase in protein expression of the iron transporter divalent metal transporter 1 (DMT-1) and the ferrireductase duodenal cytochrome b (Dcytb) were reported in the presence of ascorbic acid,

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http://dx.doi.org/10.1016/j.colsurfb.2013.11.028

suggesting a possible explanation for the enhancement in iron absorption observed in the presence of ascorbic acid in single meals. However, conventional ascorbic acid is highly unstable and rapidly undergoes deterioration upon exposure to air, water, light or heat [11,12]. These properties significantly limit its application as an absorption enhancer for iron fortification products, particularly in case of flours and cereal products that are most commonly fortified.

To counter these drawbacks various ascorbic acid derivatives such as ascorbic acid-2-glucoside and ascorbyl palmitate have been synthesised, aiming at retaining its antioxidant effect whilst having an improved thermal and oxidative stability profile. Ascorbyl palmitate is a palmitic acid ester of ascorbic acid that is lipophilic in nature and has been used as an excipient in the cosmetic industry as a stable form of ascorbic acid [13]. Recently, Pizarro and co-workers investigated ascorbyl palmitate as an enhancer of iron absorption from iron fortified bread [14]. Wheat flour was fortified with ferrous sulphate with and without the addition of ascorbyl palmitate and then used to bake bread that was administered to human subjects. Inclusion of ascorbyl palmitate at a molar ratio of 2:1 and 4:1 significantly enhanced iron absorption from the fortified flour. These results demonstrate its activity as an enhancer of iron absorption in food vehicles, although the high organoleptic reactivity of free ferrous iron would remain an issue that might limit the utility of this approach.

Due to its hydrophobic nature ascorbyl palmitate requires the ampiphilic derivative polyethylene glycol grafted 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE-PEG) to spontaneously form vesicle structures in an aqueous medium [15]. The nanocarriers thus formed provide a suitable platform for incorporation of active ingredients, and previous studies have successfully demonstrated the use of such vesicles as carriers for a hydrophobic drugs including Amphotericin B and azidothymidine [16]. An additionl motivation to use this specific nanocarrier system was to employ ascorbyl palmitate prinicipally as an enhancer of iron absorption. We hypothesised that the further inclusion of chitosan, a known mucoadhesive, in these nanocarriers would lead to a greater enhancement of iron absorption. This would create a unique delivery system wherein the material used for nanocarrier formulation would also act as an absorption enhancer for iron.

Lo Nostro et al. first demonstrated the ability of ampiphilic ascorbic acid esters to self-aggregate and form micellar structures in an aqueous media [17]. The nanocarriers retained the antioxidant activity due to the presence of ascorbyl moiety in the polar head group, while the inner micellar provides a hydrophobic core. The ascorbic acid ester ascorbyl palmitate has been investigated for similar activity; however, vesicles were reported to form only in the presence of cholesterol. PEG has been in use for several decades as a surfactant and for steric stabilisation and conjugation of ligands to drug nanocarriers [18]. DSPE-PEG is a PEGylated phospholipid frequently used to develop drug nanocarrier systems due to its ability to form micellar rather than bilayered structures by self-assembly in a suitable aqueous environment [19].

Microencapsulation technology has been used widely in the pharmaceutical industry for coating and delivery of oral and parenteral drugs. This approach has also been successfully utilised in the food industry to protect a core active ingredient that is entrapped within an outer layer of lipidic or polymeric material thus preventing it from interacting with other food components as well as the surrounding environment [20]. A wide range of approaches including lipid based systems have been explored previously for iron delivery [21–23]. The main limitation of these formulations is the thermodynamic instability due to the high lipid content, and drug leakage from the vesicles due to chemical instability [24]. Hermida et al. recently prepared chitosan containing liposomes that demonstrated high iron loading and iron absorption in Caco-2 cells. Chitosan is a naturally occurring polysacchride that has been well characterised and studied extensively for drug delivery applications [26,27]. Chitosan is widely used in dietary supplement preparations, has a well established safety profile, and has Food & Drug Administration (FDA) approval for use in food applications [28–30].

Based on these rationales, this study aimed to formulate ascorbyl palmitate/DSPE nanocarriers for encapsulation of a hydrophilic molecule, ferrous sulphate, for oral iron delivery. Here we demonstrate for the first time formulation, characterisation and evaluation of in vitro iron absorption from ascorbyl palmitate and ascorbyl palmitate-chitosan (ascorbyl palmitate-CHI) loaded ferrous sulphate nanocarriers.

2. Materials and methods

2.1. Materials

Ascorbyl palmitate was purchased from Sigma–Aldrich (Dorset, UK) and DSPE-PEG was from Lipoid (Steinhausen, Switzerland). All other chemicals and reagents were either analytical or cell culture grade, and were purchased from Sigma–Aldrich (Dorset, UK) unless otherwise stated. Chitosan hydrochloride (HCL) was from Heppe Medical (Halle, Germany). Caco-2 cells were purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). Ferritin ELISA kit was from Ramco (ATI Atlas, Chichester, UK) and BCA protein assay kit was from Thermo Fisher Scientific (Northumberland, UK). Cell culture media, foetal calf serum (FCS) and reagents were from either Invitrogen (Loughborough, UK) or Lonza (Slough, UK). Cell culture plates (6-well and 96-well) and flasks were from Nunc (Roskilde, Denmark) and all other cell culture plasticware used was from Corning (Amsterdam, The Netherlands). All reagents used were prepared using ultrapure water (Milli-Q; resistivity of 18.2 MΩ cm). Prior to use all glassware and utensils was soaked in 10% HCl and rinsed with ultrapure water to remove any potential traces of residual minerals.

2.2. Preparation of iron loaded ascorbyl palmitate nanocarriers

Ascorbyl palmitate nanocarriers were prepared by thin film hydration method as described previously, with minor modifications [31]. Briefly, ascorbyl palmitate and DSPE-PEG (1:1 molar ratio) were dissolved in chloroform in a round bottom flask. Dry lipid film was formed by removing the solvent under reduced pressure for 10 min at 60 °C using a rotary evaporator (Hei-VAP Advantage Rotary Evaporator, Heidolph, Schwabach, Germany). Any residual solvent was further removed by purging the lipid film with nitrogen gas. The film was hydrated with a ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$) solution (1 mg/ml, pH 7.4) or Milli-Q water (pH 7.4) for blank nanocarriers. For chitosan coated nanocarriers chitosan-HCl was added to the hydration solution. Hydration was carried out by hand shaking the flask vigorously in circular motion for 1 min while maintaining at a constant temperature (60 °C) by keeping the flask immersed in a water bath. Ascorbyl palmitate nanocarriers were then stored in liquid nitrogen purged 10 ml glass vials at 4 °C. The preparations were coded ascorbyl palmitate, ascorbyl palmitate-Fe, ascorbyl palmitate-CHI and ascorbyl palmitate-CHI-Fe (Fe denoting iron loaded nanocarriers).

2.3. Iron entrapment

Iron entrapment in ascorbyl palmitate nanocarriers was determined by centrifugation followed by quantification analysis [32]. Aliquots of nanocarrier dispersions were subjected to ultracentrifugation (11,336 × *g*, 60 min, 4 °C) in a refrigerated laboratory centrifuge (Heraeus Fresco 70, Thermo Fisher, UK). The supernatant was collected and iron concentrations determined using the