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

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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, 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 addition 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 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. Material 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 (MilliQ; 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 minutes 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 ($FeSO_4.7H_2O$) solution (1 mg/ml, pH 7.4) or MilliQ 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 minute 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 (11336 *g*, 60 minutes, 4 ºC) in a refrigerated laboratory centrifuge (Heraeus Fresco 70, Thermo Fisher, UK). The supernatant was collected and iron concentrations determined using the iron

chelator ferrozine [33] as previously described , [34,35], and measuring the absorbance of the coloured complex spectrophotometrically at 562 using a microplate reader (VersaMax, Molecular Devices, USA) [33]. Measurements were repeated in triplicate, and results expressed as means ± standard deviations.

Nanocarrier iron entrapment was calculated as per the following formula:

Iron entrapment (%) = (Ti - Fi / Ti) x 100

Where T*i* is the total iron added during formulation and F*i* is the free (unentrapped) iron measured in supernatant.

2.4. Size analysis

Size analysis was carried out using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Three independent measurements were performed on each sample. The Fraunhofer model was used to analyze the results and the mean volume diameters (MVD) and standard deviations (SD) were calculated.

2.5. Zeta potential measurements

Zeta potential of ascorbyl palmitate nanocarriers was determined using an electrophoretic light-scattering technique (Zetasizer Nano ZS, Malvern Instruments, UK). The nanodispersion was added to the ZetaMaster electrophoresis cell and electrophoretic mobility was measured. All analyses were carried out in triplicate.

2.6. Transmission electron microscopy (TEM)

For visualisation of ascorbyl palmitate nanocarriers a droplet of the nanodispersion diluted in water was added to a copper grid as a thin film. Phosphotungstic acid solution (2%) was used as a staining agent and nanocarriers imaged by transmission electron microscopy (Philips Biotwin CM120, Philips Co, The Netherlands).

2.7. *Cell culture*

Caco-2 cells were obtained at passage 20 and used experimentally between passages 40 to 55. FCS supplemented Dulbeccos Modified Eagle Medium (DMEM) was used as culture medium. For iron absorption experiments cells were seeded onto 6-well plates at an initial seeding density of 1 x 10^4 cells/cm^2. Parallel 6-well plates were also seeded similarly to be used for assessment of cell viability prior to the commencement of the absorption experiment and following completion. Iron absorption experiments were carried after 24 h at day 13-15 post seeding by which time Caco-2 cells differentiate to a fully matured gastrointestinal (GI) tract phenotype. Cells were therefore cultured in media containing minimal amounts of iron 24 h prior to the experiment to ensure that cells form low levels of ferritin prior to the start of the experiments. Caco-2 cells were incubated with equivalent concentrations of iron from each of the formulations. Iron concentration of 20 μM was selected, based on previous studies that have shown this concentration to be optimum for iron studies, since at higher concentrations a progressive decline in the iron protein transporter DMT-1 takes place [36] . Under these conditions Caco-2 cells demonstrate maximal iron and therefore minute variations in iron between the various samples can be measured accurately in terms of the ferritin formed. Ferrous sulphate was used as a reference standard in our experiments.

2.8. In vitro cytotoxicity

Cytotoxicity of ascorbyl palmitate nanocarriers in Caco-2 cells was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay [37]. Briefly, Caco-2 cells were seeded onto 96-well culture plates at an initial density of 1 x 10^4 cells/cm^2. On day of experiment culture media was aspirated and replaced with phenol red free minimum essential media (MEM) containing ascorbyl palmitate nanocarriers (20μm, 50μM and 100 μM iron concentration). Following 48 h exposure 20 μl MTT reagent (5 mg/ml) was added to each well and the plates incubated for a further 4 h in a cell culture incubator (at 37 °C in a 5% CO_2). Media was then aspirated carefully and the formazan crystals formed were solubilised by adding Dimethyl sulfoxide (DMSO) and agitating the plates in an orbital shaker for 15 minutes. Absorbance was then measured spectroscopically at 550 nm (VersaMax, Molecular devices, USA). Viability of control cells was considered 100% and that of test cells expressed as a percentage of control.

2.9. Caco-2 iron absorption experiment

On day 13 post seeding cells were prepared for experiment by aspirating growth media and washing Caco-2 cell monolayers three times with wash solution (140 mM NaCl, 5 mM KCl, 10 mM PIPES buffer, pH 6.7, 37 ºC) and then incubated in serum-free MEM for 24 hours. On day 14 test media was prepared by titrating MEM with 0.1 M HCL or 0.1 M NaOH to pH 5.8, which represents the physiological pH in the duodenum. Test media was then sterile filtered using a 0.2 µm filter unit, buffered with 2-(N-Morpholino) ethanesulfonic acid (MES, 10 mM) and aliquoted into individual falcon tubes. Samples from the test preparations were added to the test media to achieve a final concentration of 20 µM elemental iron for each condition. Prior to commencing iron absorption experiments trypan blue exclusion assay was carried out in a parallel 6-well plate to assess Caco-2 cell viability (one well per condition). Caco-2 cells in test plates were washed three times with wash solution and then incubated with iron enriched test media (six wells per condition) for 2 hours at 37 ºC in a plate incubator rocking gently at 25 rpm. After incubation period test media was aspirated and cells washed twice with wash solution and finally with a removal solution (wash solution plus 5 µm Na hydrosulphite and 1 µm bathophenanthroline disulfonate) to remove any surface bound iron, as described previously [38]. Caco-2 cells were then incubated with fresh MEM for a further 24 hours in a cell culture incubator (37 ºC, 95% air and 5% CO_2). Parallel 6-well plates were also incubated with test media and subjected to similar conditions (one well per condition). Caco-2 cell viability was assessed at the completion of the experiment using trypan blue dye exclusion assay. Following 24 hours of incubation, media was aspirated and Caco-2 cells washed twice with wash buffer. Cells were harvested by addition of 350 µl lysis buffer (50 mM NaOH supplemented with 1 µg/ml protease inhibitor cocktail) per well for 40 minutes while rocking gently on a plate shaker (6 rpm). Cells were then scraped and the resultant lysate was pipetted into 0.5 ml microcentrifuge tubes and stored immediately at -20 ºC for further analysis.

2.10. Ferritin and total protein quantification

Total ferritin concentration of cell lysates was determined using a spectrophotometric ELISA kit following manufacturer's protocol with a few modifications. Briefly, frozen cell lysates were centrifuged for 10 minutes (13226 *g*, 4 ºC) and the resultant supernatant was used for the assay. A standard curve was generated using the standards provided (0, 6, 20, 60, 200, 2000 ng standard/ml). Samples and standards (30 µl each) were loaded in triplicate onto a 96-well plate and incubation steps carried

out as described in the protocol. Absorbance was determined at 490 and 630 nm using a microplate reader (VersaMax, Molecular devices, USA). Protein content of Caco-2 cells was determined using the Pierce BCA kit following manufacturer's protocol using the bovine serum albumin (BSA) stock (2 mg/ml) provided in the kit as standard. All samples were assayed in duplicate. Ferritin concentration was standardised against total protein concentration and ng ferritin/ mg protein was considered an indice of iron and absorption by Caco-2 cells.

2.11. Statistical analysis

Data is presented as mean ± standard deviation (SD) and difference between samples was analyzed by one-way analyses of variance (ANOVA) followed by Tukey's post-hoc test using the PRISM software package (Version 4, Graphpad Software Inc., San Diego, USA). Results were considered significantly different if $p \leq 0.05$.

3. Results and Discussion

3.1. Preparation and iron entrapment efficiency of ascorbyl palmitate nanocarriers

In this study ascorbyl palmitate was combined with DSPE-PEG to formulate a novel carrier system to deliver iron for oral absorption. The nanocarrier preparation methodology was similar to that of Moribe et al. using the thin film method, with the addition of chitosan to prepare ascorbyl palmitate-CHI nanocarriers. This protocol was adhered to as the authors have reported the formation of stable nanocarriers that efficiently entrapped model drugs. Drug loaded and blank nanocarriers were size maintained and formed a suspension that did not exhibit phase separation on storage at 20 °C for one month.

Iron entrapment in ascorbyl palmitate nanocarriers was determined by spectrophotometric analysis. The results are presented in Fig. 1. Prior to conducting these experiments optimisation experiments were carried out to determine the initial amount of iron to be added. It was observed that increasing the concentration of ferrous iron as a percentage (w/w) of the encapsulating material led to a gradual decrease in iron entrapment, with an initial iron loading at 10% concentration resulting in as little as 3% entrapment [35]. Chitosan concentration in the nanocarrier was also optimised in a previous work performed by the authors [35]. This is in agreement with previous studies that have reported a

correlation between decreased iron entrapment and high initial ferrous iron concentration. It was suggested that the low iron entrapment was due to the strong electrolytic behaviour of ferrous sulphate that has an unfavourable effect upon electrostatic stability of the nanocarriers [22,39].

Initial iron loading was therefore established to be 1% w/w of total encapsulating material. Iron entrapment efficiency in ascorbyl palmitate-Fe nanocarriers was 67%, whereas iron entrapment efficiency in ascorbyl palmitate-CHI-Fe nanocarriers it was 76%; a 13% increase. Although chitosan is incorporated in nano and microparticle formulations as an absorption enhancer on basis of its mucoadhesive properties, several studies have reported an increase in drug entrapment upon its inclusion [40,41]. Iron is known to bring about the hydrolysis of phosholipid ester bonds leading to poor vesicle wall formation [25]. Chitosan coating of the carrier membrane might lead to the formation of a rigid nanocarrier wall microstructure. Xia et al. observed that the encapsulation efficiency of iron containing liposome increases almost 3.7-fold when a suitable iron chelator such as citric acid is used during formulation [39]. This can be attributed to the ability of the chelator to form a complex with iron thereby having a favourable effect upon its entrapment in the carrier.

The interaction between iron and chitosan has been studied previously and the formation of a stable chitosan-iron complex has been reported [42]. The high iron entrapment observed in chitosan containing ascorbyl palmitate nanocarriers can therefore be attributed to the above phenomenon, either occurring in concert or independent of each other.

3.2. Zeta potential

Zeta potential is an essential parameter for characterisation of micro or nanoparticle delivery systems as it is a predictor of formulation stability as well as carrier interaction with cell membranes. Blank and iron loaded ascorbyl palmitate nanocarriers demonstrated a net negative charge of -34.26 ±1.45 mV and -3.49 ± 0.53 mV respectively (Table 1). As expected, inclusion of chitosan imparted a positive charge on blank and drug loaded nanocarriers (ascorbyl palmitate-CHI = 13.4 ± 0.42, ascorbyl palmitate-CHI-Fe = 2.42 ± 0.24), due the presence of positively charged amine groups in its structure. The net positive zeta potential values of ascorbyl palmitate-CHI nanocarriers suggests that chitosan

deposition takes place on the outer surface of the nanocarrier structures. Iron entrapment resulted in a shift in zeta potential of both ascorbyl palmitate-Fe and ascorbyl palmitate-CHI-Fe nanocarriers.

3.3. Size analysis

Particles size of all ascorbyl palmitate nanocarriers was found to be of submicron dimensions, ranging from 47 nm to 290 nm (Table 1). DSPE-PEG is a dispersing agent and its introduction in an aqueous media results in the formation of nanometre sized nanocarriers [43]. Our data is consistent with previous studies where ascorbyl palmitate-DSPE-PEG nanocarriers had a size range less than 300 nm [16]. Ascorbyl palmitate-CHI and Ascorbyl palmitate-CHI–Fe nanocarriers had the largest particle size, having a mean diameter of 290.83 ± 43 nm and 97.15 ± 5.7 nm respectively. Chitosan is known to adsorb on the surface of lipid carriers, and previous studies have reported a similar increase in particle size upon its incorporation in the formulation [25]. [18]. A reduction in ascorbyl palmitate-CHI nanocarrier size following iron incorporation can possibly be attributed to the formation of a chitosan-iron complex, as discussed earlier, that may result in complex deposition at the core of the carrier vesicle rather than the surface, and the consequent consolidation of nanocarrier size.

Size is an important parameter that can influence the bioactivity as well as stability of the formulation. Particles size of a formulation is dependent upon its ultimate application. For delivery of certain small drugs molecules sub-100 nm particles are desired to enhance the residence time of the drug in the circulation by avoiding phagocytic clearance and hepatic filtration [44]. In the context of iron delivery the objective was primarily to formulate an iron carrier system that was physically stable and composed of particles of nanometre size range.

3.4. Transmission electron microscopy (TEM)

TEM micrographs (Fig. 2.) illustrate that iron loaded ascorbyl palmitate nanocarriers were uniformly spherical and had a dense appearance. Ascorbyl palmitate-CHI nanocarriers are less spherical, possibly due to some adsorption of chitosan on the nanocarrier surface.

3.5. In vitro cytotoxicity

Caco-2 cells were incubated for 48 h with ascorbyl palmitate nanocarriers at varying iron concentrations. Results are shown in Fig. 3. Iron loaded ascorbyl palmitate as well as ascorbyl palmitate-Chi nanocarriers did not demonstrate any significant reduction in cell viability as compared to control. In fact Caco-2 cells incubated with ascorbyl palmitate nanocarriers nanocarriers at high iron concentration (Fe =100 μM) exhibited a significant increase in Caco-2 cell viability. It is likely that Caco-2 cells incubated with these nanocarriers absorbed greater amounts of iron over an extended duration; the increased cell viabilities observed may therefore be attributed to the promoter effect of iron on cell growth and differentiation [45].

3.6. Iron absorption from ascorbyl palmitate nanocarriers

Ferritin concentrations were measured as an estimation of iron absorption in Caco-2 cells. This method is very sensitive, has been well characterised, and shows good correlation with human absorption data at this time point [46,47]. Caco-2 cells synthesise ferritin in response to their iron status as well as iron levels in their surrounding environment [38].

Iron absorption from ascorbyl palmitate nanocarriers was higher than that from ferrous sulphate alone (Fig. 4.; $p < 0.05$). The highest level of iron absorption was from ascorbyl palmitate-CHI-Fe nanocarriers (800.12 ± 47.6 ng/mg cell protein); 1.35 fold/ 35.13 % ($p < 0.05$) higher than that from ascorbyl palmitate-Fe nanocarriers (592.17 ± 21.12 ng/mg cell protein), and 1.5 fold/ 58.4 % ($p < 0.05$) higher than that from free ferrous sulphate (505.74 ± 23.73 ng/mg cell protein). These results are in agreement with Pizarro et al., who showed ascorbyl palmitate to be a strong enhancer of ferrous iron in fortified bread [14]. Ascorbyl palmitate retains the ascorbyl moiety of ascorbic acid in an esterified form that protects it from the poor thermal and oxidative stability characteristic of ascorbic acid.

Chitosan is often used in drug delivery formulations as a permeation enhancer due to its mucoadhesive properties, which are attributed to its cationic polyelectrolyte structure allowing it to bind to negatively charged cell surfaces [48]. Chitosan coating or integration onto the surface of the drug carrier enhances its interaction with the cell membrane, thereby promoting cellular uptake [49] . Although the inclusion of chitosan has not been explored in DSPE-PEG based nanocarrier formulation, our results are in agreement with previous literature wherein a coating of chitosan on

liposome surfaces led to an increase in cellular uptake of the encapsulated material [50]. It has been suggested that positively charged chitosan exhibits strong electrostatic attraction with the negatively charged lipidic components of the vesicles resulting in a stable surface coating [51]. The presence of a chitosan on the surface of our ascorbyl palmitate nanocarriers was confirmed by zeta-potential studies (See Table 1).

Caco-2 cells were used to simulate duodenal absorption and therefore the pH was adjusted to 5.8 during the course of the experiment. Chitosan exerts maximal cell permeation effect in an acidic environment [52]. The pH in the duodenum region of the intestine is 5.8, and this provides an environment particularly suited for chitosan to act as a mucoadhesive, as the pH is close to its pKa value (5.5) [53]. Our results are in agreement with this; chitosan containing nanocarriers demonstrated high iron absorption, an effect that may be attributed to its mucoadhesive properties at the intestinal physiological pH. Chitosan inclusion in the nanocarriers therefore appears to exert an effect that complements and further augments the effects of ascorbyl palmitate on iron absorption in Caco-2 cells.

The peristaltic movement of the small intestine propel the contents at a flow rate of 0.188 ml/second [54]. This provides a very limited window for nutrients to undergo absorption, before they are washed off into the large intestine to be processed for excretion. In the context of actual physiological conditions of oral absorption it is intriguing to speculate that our ascorbyl palmitate-CHI nanocarriers might bind to the mucosal cell surface thereby prolonging the retention time in this region potentially leading to increased accumulation and absorption. However, the current study does not address this phenomenon, and further studies involving a dynamic absorption system or an in vivo model would be required to examine this possibility.

4. Conclusion

This study reports for the first time the preparation and characterisation of iron loaded ascorbyl palmitate-DSPE-PEG- nanocarriers. Relatively high iron entrapment was observed in the nanocarrier preparations, with zeta potential analysis confirming the presence of a net positive charge on the surface of nanocarriers containing chitosan. Significantly higher iron absorption from ascorbyl

palmitate nanocarriers than ferrous sulphate alone suggests that the ascorbyl moiety may have a role in the increased iron absorption from these nanocarriers. However, it is not possible to delineate from the present data whether this increase in absorption is due to individual components of the carrier system, or due to higher uptake of the nanocarrier vehicle itself. Engineering the nanocarriers with chitosan not only leads to an enhancement in iron entrapment, but also a significant increase in iron absorption in Caco-2 cells. These results demonstrate the potential of ascorbyl palmitate nanocarriers as novel iron delivery vehicles for nutritional applications.

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References

[1] M.B. Zimmermann and R.F. Hurrell, Nutritional iron deficiency, The Lancet, 370 (2011) 511-520.

[2] R.F. Hurrell, S. Lynch, T. Bothwell, H. Cori, R. Glahn, E. Hertrampf, Z. Kratky, D. Miller, M. Rodenstein, H. Streekstra, B. Teucher, E. Turner, C.K. Yeung, M.B. Zimmermann, and null, Enhancing the absorption of fortification iron. A SUSTAIN Task Force report, Int J Vitam Nutr Res, 74 (2004) 387-401.

[3] R.F. Hurrell, Preventing Iron Deficiency Through Food Fortification, Nutrition Reviews, 55 (1997) 210-222.

[4] J.H. Swain, L.K. Johnson, and J.R. Hunt, An Irradiated Electrolytic Iron Fortificant Is Poorly Absorbed by Humans and Is Less Responsive than FeSO4 to the Enhancing Effect of Ascorbic Acid, The Journal of Nutrition, 136 (2006) 2167-2174.

[5] R. Hurrell and I. Egli, Iron bioavailability and dietary reference values, The American Journal of Clinical Nutrition, 91 (2010) 1461S-1467S.

[6] A.L. Forbes, M.J. Arnaud, C.O. Chichester, J.D. Cook, B.N. Harrison, R.F. Hurrell, S.G. Kahn, E.R. Morris, J.T. Tanner, P. Whittaker, and ., Comparison of in vitro, animal, and clinical

determinations of iron bioavailability: International Nutritional Anemia Consultative Group Task Force report on iron bioavailability, Am. J. Clin. Nutr., 49 (1989) 225-238.

[7] R. Hurrell, How to ensure adequate iron absorption from iron-fortified food, Nutr. Rev., 60 (2002) S7-15.

[8] A. Stekel, M. Olivares, F. Pizarro, P. Chadud, I. Lopez, and M. Amar, Absorption of fortification iron from milk formulas in infants, Am. J. Clin. Nutr., 43 (1986) 917-922.

[9] D.P. Derman, T.H. Bothwell, A.P. MacPhail, J.D. Torrance, W.R. Bezwoda, R.W. Charlton, and F.G. Mayet, Importance of ascorbic acid in the absorption of iron from infant foods, Scand. J. Haematol., 25 (1980) 193-201.

[10] N.M. Scheers and A.S. Sandberg, Ascorbic acid uptake affects ferritin, Dcytb and Nramp2 expression in Caco-2 cells, Eur. J. Nutr., 47 (2008) 401-408.

[11] M.H. Sayers, S.R. Lynch, P. Jacobs, R.W. Charlton, T.H. Bothwell, R.B. Walker, and F. Mayet, The effects of ascorbic acid supplementation on the absorption of iron in maize, wheat and soya, Br. J. Haematol., 24 (1973) 209-218.

[12] L. Hallberg, L. Rossander, H. Persson, and E. Svahn, Deleterious effects of prolonged warming of meals on ascorbic acid content and iron absorption, Am. J. Clin. Nutr., 36 (1982) 846-850.

[13] R. Austria, A. Semenzato, and A. Bettero, Stability of vitamin C derivatives in solution and topical formulations, Journal of Pharmaceutical and Biomedical Analysis, 15 (1997) 795-801.

[14] F. Pizarro, M. Olivares, E. Hertrampf, S. Nuñez, M. Tapia, H. Cori, and D.L. de Romana, Ascorbyl palmitate enhances iron bioavailability in iron-fortified bread, The American Journal of Clinical Nutrition, 84 (2006) 830-834.

[15] D. Gopinath, D. Ravi, B.R. Rao, S.S. Apte, D. Renuka, and D. Rambhau, Ascorbyl palmitate vesicles (Aspasomes): formation, characterization and applications, International Journal of Pharmaceutics, 271 (2004) 95-113.

[16] K. Moribe, S. Maruyama, Y. Inoue, T. Suzuki, T. Fukami, K. Tomono, K. Higashi, Y. Tozuka, and K. Yamamoto, Ascorbyl dipalmitate/PEG-lipid nanoparticles as a novel carrier for hydrophobic drugs, International Journal of Pharmaceutics, 387 (2010) 236-243.

[17] P. Lo Nostro, G. Capuzzi, A. Romani, and N. Mulinacci, Self-Assembly and Antioxidant Properties of Octanoyl-6-O-ascorbic Acid, Langmuir, 16 (1999) 1744-1750.

[18] K. Knop, R. Hoogenboom, D. Fischer, and U. Schubert, Poly(ethylene glycol) in Drug Delivery: Pros and Cons as Well as Potential Alternatives, Angewandte Chemie International Edition, 49 (2010) 6288-6308.

[19] B. Ashok, L. Arleth, R.P. Hjelm, I. Rubinstein, and H. Onyüksel, In vitro characterization of PEGylated phospholipid micelles for improved drug solubilization: Effects of PEG chain length and PC incorporation, J. Pharm. Sci., 93 (2004) 2476-2487.

[20] V. Nedovic, A. Kalusevic, V. Manojlovic, S. Levic, and B. Bugarski, An overview of encapsulation technologies for food applications, Procedia Food Science, 1 (2011) 1806-1815.

[21] B. Ding, S. Xia, K. Hayat, and X. Zhang, Preparation and pH Stability of Ferrous Glycinate Liposomes, J. Agric. Food Chem., 57 (2009) 2938-2944.

[22] S.L. Kosaraju, C. Tran, and A. Lawrence, Liposomal Delivery Systems for Encapsulation of Ferrous Sulfate: Preparation and Characterization, Journal of Liposome Research, 16 (2006) 347-358.

[23] R. Wegmüller, M.B. Zimmermann, D. Moretti, M. Arnold, W. Langhans, and R.F. Hurrell, Particle Size Reduction and Encapsulation Affect the Bioavailability of Ferric Pyrophosphate in Rats, The Journal of Nutrition, 134 (2004) 3301-3304.

[24] A. Sharma and U.S. Sharma, Liposomes in drug delivery: Progress and limitations, International Journal of Pharmaceutics, 154 (1997) 123-140.

[25] L.G. Hermida, A. Roig, C. Bregni, M. Sabés-Xamaní, and R. Barnadas- Rodríguez, Preparation and characterization of iron-containing liposomes: their effect on soluble iron uptake by Caco-2 cells, Journal of Liposome Research, 21 (2010) 203-212.

[26] K.A. Janes, P. Calvo, and M.J. Alonso, Polysaccharide colloidal particles as delivery systems for macromolecules, Advanced Drug Delivery Reviews, 47 (2001) 83-97.

[27] M. Garcia-Fuentes and M.J. Alonso, Chitosan-based drug nanocarriers: Where do we stand?, Journal of Controlled Release, 161 (2012) 496-504.

[28] T. Kean and M. Thanou, Biodegradation, biodistribution and toxicity of chitosan, Advanced Drug Delivery Reviews, 62 (2010) 3-11.

[29] M. Rinaudo, Chitin and chitosan: Properties and applications, Progress in Polymer Science, 31 (2006) 603-632.

[30] B. Miralles, M. Mengíbar, R. Harris, and A. Heras, Suitability of a colorimetric method for the selective determination of chitosan in dietary supplements, Food Chemistry, 126 (2011) 1836-1839.

[31] E. Pupo, A. Padrón, E. Santana, J. Sotolongo, D.g. Quintana, S. Dueñas, C. Duarte, M.C. de la Rosa, and E. Hardy, Preparation of plasmid DNA-containing liposomes using a high-pressure homogenization-extrusion technique, Journal of Controlled Release, 104 (2005) 379-396.

[32] K. Ruckmani and V. Sankar, Formulation and optimization of Zidovudine niosomes, AAPS PharmSciTech, 11 (2010) 1119-1127.

[33] W.W. Fish, Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples, Methods Enzymol, 158 (1988) 357-364.

[34] M.G. Zariwala, S. Somavarapu, S. Farnaud and D. Renshaw, Comparison study of oral iron preparations using a human intestinal model, Sci Pharm (2013) in press doi:10.3797/scipharm.1304-03.

[35] M.G. Zariwala, N, Elsaid, T.L. Jackson, F. Corral López, S. Farnaud, S. Somavarapu, D. Renshaw. A novel approach to oral iron delivery using ferrous sulphate loaded solid lipid nanoparticles. Int J Pharm. 456 (2013) 400-407.

[36] P. Sharp, Methods and options for estimating iron and zinc bioavailability using Caco-2 cell models: benefits and limitations, Int. J. Vitam. Nutr. Res., 75 (2005) 413-421.

[37] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, Journal of Immunological Methods, 65 (1983) 55-63.

[38] L. Zhu, R.P. Glahn, C.K. Yeung, and D.D. Miller, Iron uptake by Caco-2 cells from NaFeEDTA and FeSO4: Effects of ascorbic acid, pH, and a Fe(II) chelating agent, J. Agric. Food Chem., 54 (2006) 7924-7928.

[39] S. Xia and S. Xu, Ferrous sulfate liposomes: preparation, stability and application in fluid milk, Food Research International, 38 (2005) 289-296.

[40] M. Phetdee, A. Polnok, and J. Viyoch, Development of chitosan-coated liposomes for sustained delivery of tamarind fruit pulp's extract to the skin, International Journal of Cosmetic Science, 30 (2008) 285-295.

[41] M. Zaru, M.L. Manca, A.M. Fadda, and S.G. Antimisiaris, Chitosan-coated liposomes for delivery to lungs by nebulisation, Colloids and Surfaces B: Biointerfaces, 71 (2009) 88-95.

[42] S.C. Bhatia and N. Ravi, A Mössbauer Study of the Interaction of Chitosan and d-Glucosamine with Iron and Its Relevance to Other Metalloenzymes, Biomacromolecules, 4 (2003) 723-727.

[43] A. Krishnadas, I. Rubinstein, and H. Onyuksel, Sterically stabilized phospholipid mixed micelles: in vitro evaluation as a novel carrier for water-insoluble drugs, Pharm. Res., 20 (2003) 297-302.

[44] F. Alexis, E. Pridgen, L.K. Molnar, and O.C. Farokhzad, Factors affecting the clearance and biodistribution of polymeric nanoparticles, Mol. Pharm., 5 (2008) 505-515.

[45] N.T.V. Le and D.R. Richardson, The role of iron in cell cycle progression and the proliferation of neoplastic cells, Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1603 (2002) 31-46.

[46] S. Yun, J.P. Habicht, D.D. Miller, and R.P. Glahn, An in vitro digestion/Caco-2 cell culture system accurately predicts the effects of ascorbic acid and polyphenolic compounds on iron bioavailability in humans, J. Nutr., 134 (2004) 2717-2721.

[47] S. Fairweather-Tait, I. Phillips, G. Wortley, L. Harvey, and R. Glahn, The use of solubility, dialyzability, and Caco-2 cell methods to predict iron bioavailability, Int. J. Vitam. Nutr. Res., 77 (2007) 158-165.

[48] I. Henriksen, K.L. Green, J.D. Smart, G. Smistad, and J. Karlsen, Bioadhesion of hydrated chitosans: An in vitro and in vivo study, International Journal of Pharmaceutics, 145 (1996) 231-240.

[49] J. Thongborisute, H. Takeuchi, H. Yamamoto, and Y. Kawashima, Properties of liposomes coated with hydrophobically modified chitosan in oral liposomal drug delivery, Pharmazie, 61 (2006) 106-111.

[50] H. Takeuchi, J. Thongborisute, Y. Matsui, H. Sugihara, H. Yamamoto, and Y. Kawashima, Novel mucoadhesion tests for polymers and polymer-coated particles to design optimal mucoadhesive drug delivery systems, Adv. Drug Deliv. Rev., 57 (2005) 1583-1594.

[51] J. Guo, Q. Ping, G. Jiang, L. Huang, and Y. Tong, Chitosan-coated liposomes: characterization and interaction with leuprolide, Int. J. Pharm., 260 (2003) 167-173.

[52] C.M. Lehr, J.A. Bouwstra, E.H. Schacht, and H.E. Junginger, In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers, International Journal of Pharmaceutics, 78 (1992) 43-48.

[53] A.M.M. Sadeghi, F.A. Dorkoosh, M.R. Avadi, M. Weinhold, A. Bayat, F. Delie, R. Gurny, B. Larijani, M. Rafiee-Tehrani, and H.E. Junginger, Permeation enhancer effect of chitosan and

chitosan derivatives: Comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells, European Journal of Pharmaceutics and Biopharmaceutics, 70 (2008) 270-278.

[54] A. Gutzeit, M.A. Patak, C. von Weymarn, N. Graf, A. Doert, E. Willemse, C.A. Binkert, and J.M. Froehlich, Feasibility of small bowel flow rate measurement with MRI, J. Magn. Reson. Imaging, 32 (2010) 345-351.