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Evaluating the behaviour of curcumin nanoemulsions and multilayer nanoemulsions during dynamic *in vitro* digestion

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ABSTRACT

Nanoemulsions can be used to improve the bioaccessibility of lipophilic bioactive compounds, such as curcumin, being their behaviour highly influenced by their interfacial properties. The effect of chitosan and alginate layers' deposition on curcumin nanoemulsions' behaviour during *in vitro* digestion was evaluated using a dynamic gastrointestinal system. Results showed that polyelectrolyte layers' deposition improved curcumin antioxidant capacity during *in vitro* digestion. In addition, multilayer nanoemulsions showed a better control of the rate and extent of lipid digestibility by decreasing free fatty acids release, compared to uncoated nanoemulsions. However, a lower curcumin bioaccessibility was observed for multilayer nanoemulsions. Although cytotoxicity assays revealed that both nanosystems are toxic due to the use of sodium dodecyl sulphate (SDS), nanosystems were 3.3-fold less toxic than SDS itself.

This study showed that multilayer nanoemulsions could be used to increase satiety by retarding lipid digestion, which can be important for functional foods development for combating obesity.

1. Introduction

Consumers' demands for new and healthier foods are encouraging the food industry to seek for new strategies to fortify food products with bioactive compounds, turning foods into products that promote health and wellness (Cerqueira et al., 2013; Silva, Cerqueira, & Vicente, 2012, 2015b). Some of these bioactive compounds are lipophilic often resulting in low bioavailability, whereas some are pH- and temperature-sensitive, being prone to oxidative and chemical degradation (Guttoff, Saberi, & McClements, 2015; Mayer, Weiss, & McClements, 2013; McClements, 2015; Zou et al., 2015). Lipid-based delivery systems can be designed to encapsulate, protect and control/trigger the release of bioactive compounds at specific locations within the gastrointestinal (GI) tract. Also, they can control the digestion of lipophilic bioactive compounds in the GI tract, while improving their bioavailability (Li et al., 2010; McClements & Li, 2010; Sun et al., 2015). In particular, nanoemulsions can inhibit bioactive compounds' chemical and oxidative degradation, their large surface area can enhance lipid digestibility rates, improve the release of bioactive compounds, promote the faster formation of mixed micelles, while enhancing the bioactive compounds' permeability across the mucus layer and epithelium cells (Cerqueira et al., 2014; Sun et al., 2015; Ting, Jiang, Ho, & Huang, 2014). Nonetheless, nanoemulsions present some drawbacks and their stability can be influenced by their behavior towards dehydration, temperature changes (e.g. heating, chilling and freezing-thawing) and passage through the GI tract (Cerqueira et al., 2014; Silva, Cerqueira, & Vicente, 2015a). Multilayer nanoemulsions can be used as a strategy to improve the physical stability of nanoemulsions to environmental conditions

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; DLS, dynamic light scattering; FFA, free fatty acids; GI, gastrointestinal; H_d, hydrodynamic diameter; LbL, layer-by-layer; MCTs, medium chain triglycerides; O/W, oil-in-water; PBS, phosphate buffered saline; PdI, polydispersity index; SDS, sodium dodecyl sulphate; TEM, transmission electron microscopy; Zp, zeta potential

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such as pH, ionic strength, heating, chilling or freeze-drying cycles, while controlling lipids' digestibility and release of bioactive compounds in response to specific environmental triggers (Acevedo-Fani, Soliva-Fortuny, & Martín-Belloso, 2017). They can be produced using the layer-by-layer (LbL) technique, based on the deposition of charged polyelectrolytes onto oppositely charged lipid droplets (Acevedo-Fani, Silva, Soliva-Fortuny, Martín-Belloso, & Vicente, 2017; Pinheiro, Coimbra, & Vicente, 2016). Multilayer nanoemulsions' characteristics are known for being largely influenced by the properties of the outer polyelectrolyte layer, such as molecular weight, charge density, ionic composition and pK_a. However, the functional properties can be designed according to the type of polyelectrolyte, sequence of the polyelectrolyte layers, number of layers and the conditions to which the solutions are subjected during the construction of the system (Li et al., 2010).

The proper selection of the lipid nanosystem should be based on the final application. It is known that the large surface area of nanoemulsions allows the acceleration of the chemical reactions occurring at the oil-water interface such hydrolysis by lipases. Therefore, nanoemulsions' capacity to increase the rate and extent of lipids digestion, make them interesting for addressing human disorders that inhibit lipid digestion or absorption (Troncoso, Aguilera, & McClements, 2012). On the contrary, changing the interfacial properties through the deposition of polyelectrolyte layers may help controlling lipids' digestibility by enhancing the coating integrity, preventing lipase and other enzymes from reaching the encapsulated lipids (McClements & Li, 2010; Troncoso et al., 2012). By slowing down the digestibility rate of lipids, it is possible to stimulate ileal brake mechanism responsible for regulating hunger, satiety and satiation, thereby reducing the caloric intake of food products (Li et al., 2010; Maljaars, Peters, Haddeman, & Masclee, 2009).

Therefore, understanding of the behavior of lipid-based nanosystems within the GI tract is of the highest importance for the development of lipid nanosystems with tailored physiological attributes, such as satiety enhancement, improved bioavailability or reduced fat absorption (Liu, Ma, Zhang, Gao, & Julian McClements, 2017). With this view, the main purpose of this study was to evaluate the influence of the interfacial composition on the behavior of lipid-based nanosystems under *in vitro* GI conditions. To perform this evaluation, curcumin, a lipophilic bioactive compound with numerous beneficial effects on human health but a very low bioavailability, have been encapsulated in nanoemulsions. Nanoemulsions were produced using medium chain triglycerides (MCTs) as oil phase and SDS, as a anionic emulsifier. These nanoemulsions have been coated with oppositely charged biopolymers: alginate and chitosan, forming multilayer nanoemulsions. A dynamic GI model, comprising the simulation of stomach, duodenum, jejunum and ileum, was used in this study once it allows a more realistic simulation of the complex physicochemical and physiological processes that occur within the human GI tract. Also, the cytotoxicity of the nanosystems was evaluated using CaCo-2 cell line.

2. Materials and methods

2.1. Materials

Neobee 1053 MCTs, composed by caprylic/capric triglyceride oil with a fatty acid distribution of 55% of C8:0 and 44% of C10:0, was kindly provided by Stepan (The Netherlands) and was used without further purification. SDS, curcumin (*Mw* = 368.38 Da), 1,1-diphenyl-2-picrylhydrazyl (DPPH), pepsin from porcine gastric mucosa (600 $U{\cdot}mL^{-1}$), lipase from porcine pancreas (40 $U{\cdot}mL^{-1}$), pancreatin from porcine pancreas (8 × USP), bile extract porcine and the salts used for preparing the gastric and small intestinal electrolyte solutions, hydrochloric acid, sodium bicarbonate, Nile Red 9-diethylamino-5H-benzo [α]phenoxazine-5-one and dimethyl sulfoxide were purchased from Sigma-Aldrich (St Louis, MO, USA). Chitosan (deacetylation degree ≥95%) was purchased from Golden-Shell Biochemical CO., LTD (Zhejiang, China) and sodium alginate with *Mw* ≈ 15,900 Da and viscosity ≈ 200 cp (1% aqueous solution with Brookfield Model $LV^{-1}\ s^{-1}$ rpm at 25 °C) from Manutex RSX, Kelco 104 International, Ltd. (Portugal). Lactic acid (90%) was purchased from Acros Organics (Geel, Belgium). Sodium hydroxide and phenolphthalein were obtained from Panreac (Barcelona, Spain). Chloroform was obtained from Fisher Scientific (NJ, USA) and acetone from Fisher Chemical (Loughborough, UK). All cell culture media and supplements, namely RPMI 1640, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (PS) and trypsin/EDTA, were obtained from Invitrogen (Paisley, UK). For cytotoxicity experiments phosphate buffered saline (PBS) powder was purchased from Sigma-Aldrich (St. Louis, USA) and CellTiter 96® AQueous One Solution Cell Proliferation Assay was obtained from Promega (Wisconsin, USA). Human colon carcinoma Caco-2 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Distilled water (Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) was used to prepare all solutions.

2.2. Experimental procedures

2.2.1. Preparation of curcumin nanosystems

2.2.1.1. Curcumin nanoemulsions. Oil-in-water (O/W) nanoemulsions were prepared according to a previous work (Silva et al., 2015b), with slight modifications. Briefly, 0.1% (w/w) of curcumin was solubilized at 90 °C in MCTs during 30 min. The oily phase was then added to an aqueous phase containing 1% (w/w) of SDS in distilled water. An oil-to-aqueous phase volume ratio of 1:9 was used. The nanoemulsions were pre-mixed during 2 min at 83.3 s^{-1} using an Ultra-Turrax homogenizer (T 25, Ika-Werke, Germany) followed by 20 cycles of homogenization through a high-pressure homogenizer equipped with a zirconia nozzle (Z4 nozzle) with 100 μm of orifice (Nano DeBEE, BEE International, USA) at 15,000 Psi (103 MPa).

2.2.1.2. Curcumin multilayer nanoemulsions. Multilayer nanoemulsions were formed through adsorption of consecutive deposition of layers of polyelectrolytes onto the curcumin nanoemulsions using the LbL electrostatic deposition technique. The saturation method was applied, i.e. the layers were constructed by subsequent adsorption of polyelectrolytes from their solutions without the intermediate rinsing step. Therefore, an optimization step was conducted, in which different polyelectrolytes' concentrations (ranging from 0.01 to 0.1% w/w) were tested. The concentration at which the nanoemulsions are completely coated by the polyelectrolyte layer and there is no significant excess of polyelectrolyte in solution (i.e. optimum polyelectrolytes' concentration) was assessed. These concentrations have been chosen based on particle size and zeta potential measurements, in accordance with other authors (Pinheiro et al., 2016). Briefly, constant values of zeta potential with increasing polyelectrolyte concentrations suggest that the nanoemulsions became saturated with the polyelectrolyte and also, polyelectrolyte concentrations at which low particle sizes are obtained suggest that nanoemulsions are not susceptible to droplet aggregation due to the strong electrostatic repulsion between the droplets and to the saturation of the droplets surfaces with the polyelectrolyte. Concentrations of 0.04%, 0.04% and 0.02% (w/w) for the build-up of the 1st (chitosan), 2nd (alginate) and 3rd (chitosan) layers were selected to construct the multilayer nanoemulsions. Briefly, anionic curcumin nanoemulsions were coated with alternating layers of positively charged chitosan solution (dissolved in 1% lactic acid) at pH 3, and negatively charged sodium alginate solution (dissolved in distilled water) at pH 7 (volume ratio of 1:1, respectively) until the desired number of biopolymer layers was achieved (i.e. until obtaining the chitosan/alginate/chitosan/SDS-stabilized multilayer nanoemulsions). Polyelectrolyte solutions were used at the pH values where the polyelectrolytes were strongly charged and added dropwise with a syringe pump (NE-1000, New Era Pump Systems, Inc., USA) to

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