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On the encapsulation and viability of probiotic bacteria in edible carboxymethyl cellulose-gelatin water-in-water emulsions

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ABSTRACT

In this study, novel biobased dispersions to entrap probiotic bacteria were developed and characterized regarding their formation, microstructure and *in vitro* viability and culturability performance in model salivary, gastric and intestinal fluids. The systems are composed of type B pigskin gelatin and sodium carboxymethyl cellulose (NaCMC) which, depending on concentrations and temperature, can form water-in-water (W/W) emulsion droplets as observed by optical and fluorescence microscopy. Model probiotic bacteria, *Lactobacillus rhamnosus* GG (LGG), were successfully entrapped into the W/W emulsion droplets with surprisingly high viability. Moreover, the survival of the LGG cells, when exposed to the different model fluids, was improved after their entrapment in the W/W emulsions. Therefore, the developed dispersions display high potential for probiotic encapsulation and eventual delivery into the intestinal tract with acceptable viability.

1. Introduction

The development of efficient delivery systems for food and biomedical applications, able to encapsulate, protect, transport and deliver the active agent appropriately, remains unsolved in many aspects. One major challenge is that the carrier should deliver its content to the target area without any harmful side effect to cells and/or tissues. Probiotic bacteria arise as valuable cargo capable of treatment of different diseases or health problems (Holzapfel, Haberer, Snel, Schillinger, & Huis in't Veld, 1998; Iannitti & Palmieri, 2010; Passariello, Agricole, & Malfertheiner, 2014). These are living microorganisms, which are considered to provide beneficial health effects to the host by replenishing natural gastrointestinal microbiota. However, probiotic bacteria taken orally are very often deactivated by acidic stomach conditions, and consequently, the effectiveness of probiotics intake depends very much on the number of viable cells capable to reach the gastrointestinal tract (Shen & Cabasso, 1982).

Several physiological obstacles can delay or prevent the delivery of probiotic bacteria in a safe and effective fashion. Different strategies have been explored to protect probiotics from the harsh conditions of the gastrointestinal tract (i.e. low pH, bile salts and enzymes) such as microencapsulation (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). Various microencapsulation methods are being studied to address the problem of protecting and effectively delivering viable probiotic bacteria that preserve its metabolic functions. The microcapsules are often formed by mechanisms such as interfacial emulsion polymerization, ionic coacervation and sol-gel inmmobilization, using biocompatible macromolecules, such as polysaccharides and proteins, as encapsulating agents (Corona-Hernandez et al., 2013; Haffner, Diab, & Pasc, 2016). In this context, many different colloidal systems can be used for encapsulation in food formulation, including oil-in-water (O/W) emulsions, liposomes, coacervates, etc (Vemmer & Patel, 2013). Much less explored is the use of water-in-water (W/W) emulsions as templates for microencapsulation of probiotics in spite of these systems finding potential applications in food and drug delivery areas (Nicolai & Murray, 2017). These systems consist of droplets of an aqueous phase dispersed into another aqueous phase and are often obtained in different aqueous mixtures of hydrophilic polymers due to their

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thermodynamic incompatibility (Esquena, 2016; Frith, 2010; Grinberg & Tolstoguzov, 1997).

One of the main drawbacks of W/W emulsions is their poor stability; often fast droplet coalescence or flocculation occurs, resulting in irreversible phase separation (Frith, 2010). One strategy to increase colloidal stability is using gelifying species/reagents in the formulation which may form arrested gelled states upon temperature change, thus stabilizing the emulsion droplets (Esquena, 2016; Frith, 2010). The W/W emulsions are typically formed after segregative phase separation (repulsive interactions between the polymers), avoiding the conditions where associative phase separation (attractive interactions) may occur, and preventing the formation of coacervate particles instead of emulsion droplets (Doublier, Garnier, Renard, & Sanchez, 2000). Such segregative phase separation may eventually result in the formation of microgels, which are particularly relevant as efficient vehicles to entrap and deliver therapeutic agents due to their biocompatibility and biodegradability (Fernandez-Barbero et al., 2009; Oh, Drumright, Siegwart, & Matyjaszewski, 2008; Silva et al., 2013).

W/W can be an effective method for incorporating bacteria in fully biocompatible and reasonably mild conditions (moderate pH and temperature), without both surfactant and oil. Thus, W/W emulsions could become a platform for production of soft materials as carriers of living microorganisms. For example, probiotic bacteria could be directly introduced inside microgels. However, this process would require chemical crosslinking to prevent fast dissolution of microgels in aqueous solutions. Crosslinking might seriously affect or damage the bacteria and, in this context, alternative methods such as freeze-drying of emulsions might allow the production of novel soft biocompatible porous polymer materials with incorporated viable probiotic bacteria.

Mixtures of edible proteins and polysaccharides are particularly interesting candidates for food applications due to their biocompatibility profiles. A vast list of combinations of proteins and polysaccharides that result in segregative phase separation can be found elsewhere (Grinberg & Tolstoguzov, 1997), and it is known that stable W/W emulsions can be formulated in mixtures of such macromolecules (Esquena, 2016; Frith, 2010). Gelatin and carboxymethyl cellulose (NaCMC) were chosen for this work because of their biocompatibility, low-cost and different ionic character. NaCMC is an anionic derivative of cellulose with muco-adhesive properties, while gelatin has excellent membrane-forming ability, biocompatibility and non-toxicity features (Hanani, Roos, & Kerry, 2014; "Kamide, K., 1 - Introduction. In Cellulose and Cellulose Derivatives, Elsevier: Amsterdam, 2005; pp 1–23.,"). Moreover, due to its amphoteric nature, it is also a thermally reversible gelling agent for encapsulation, sharply increasing viscosity below ca.40 °C, which hypothetically may guarantee an enhanced colloidal stability (Esquena, 2016; Grinberg & Tolstoguzov, 1997). Depending on the concentration, temperature and pH, NaCMC and gelatin may change their protonation states and weakly attractive or segregative forces may induce the formation of complexes or separated phases.

The formation of colloidal dispersions in gelatin/NaCMC mixtures remains mostly unexplored, and in the present work, the formation of W/W emulsions is investigated and characterized. Although plate counting is a widely used method of choice for detection of live probiotics in the food industry, modern techniques such as flow cytometry provide much more information in terms of culture-independent viability assays, for example rapidity and the ability to detect dormant cells. Therefore, to improve the understanding the viability and culturability of probiotic cells the two different approaches (plate counting and flow cytometry) were used for the systems exposed to the different *in vitro* conditions. The main goal of this work is to determine if NaCMC/gelatin complexes and/or emulsions can be formed and used to entrap viable probiotic bacteria and eventually deliver them to the gut.

2. Experimental

2.1. Materials and methods

Sodium carboxymethyl cellulose, NaCMC, (Mw ca. 250 kDa with a degree of substitution of 0.7) and gelatin (bovine skin, Mw of ca. 50 kDa-100 kDa) were purchased from Sigma-Aldrich. *Lactobacillus rhamnosus* GG LMG 18243 (LGG) was bought from the Belgian Coordinated Collection of Microorganisms (BCCM). The MRS broth pH 6.4 and MRS agar pH 5.7 were obtained from VWR International. The PBS buffer was prepared in the lab with disodium hydrogen phosphate, sodium chloride, potassium chloride and potassium dihydrogen phosphate, all purchased from Sigma-Aldrich. Sodium bicarbonate, magnesium chloride hexahydrate, ammonium carbonate and calcium chloride (all analytical grade) were obtained from Sigma-Aldrich. The Live/Dead® *Bac*Light™ Bacterial Viability Kit L7012, containing the fluorescent probes Syto 9 and propidium iodine, PI, was purchased from Thermofisher Scientific, USA. Milli-Q water (18.2 MΩ.cm^{-1} at 25 °C, MQ) was used for the preparation of all samples. Porcine pepsin (EC 3.4.23.1), porcine trypsin (EC 3.4.21.4), bovine chymotrypsin (EC 3.4.21.1), porcine pancreatic α-amylase (EC 3.2.1.1), porcine pancreatic lipase (EC 3.1.1.3), porcine pancreatic colipase and bile extract B8631 (porcine) were all purchased from Sigma Aldrich.

2.2.1. Gelatin-NaCMC mixtures

Aqueous stock solutions of gelatin at different concentrations (from 5 to 64 wt%) were prepared by dissolving the appropriate amount of protein in MilliQ (MQ) water at 60 °C under continuous stirring. After achieving full dissolution, the solutions were kept at 4 °C before further use. On the other hand, aqueous stock solutions of NaCMC (from 0.5 to 7 wt%) were prepared by dissolving the polymer directly in MQ water at room temperature. Different ratios of the NaCMC and gelatin stock solutions were mixed by vortex and left to equilibrate at a constant temperature (60 °C) water bath. Samples were periodically inspected (i.e. naked eye and under polarized light microscopy).

2.2.2. Preparation of salivary, gastric and intestinal fluids

The Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were made up of the corresponding electrolyte stock solutions, enzymes and water as described in Minekus et al. (see Tables 1 and 2 therein for details) (Minekus et al., 2014). Briefly, the SSF was prepared using stock solutions of the following electrolytes: K^+ (18.8 mmol/L), Na^+ (13.6 mmol/L), Cl^- (19.5 mmol/L), $H_2PO_4^-$(3.7 mmol/L), HCO_3^- (13.7 mmol/L), Mg^{2+}(0.15 mmol/L), NH_4^+ (0.12 mmol/L), Ca^{2+}(1.5 mmol/L). In a typical example, five gelatin-CMC particles were mixed with 5 ml of SSF electrolyte stock solution. Human salivary α-amylase (EC 3.2.1.1) was further added to achieve 75 U/mL in the final mixture, followed by $CaCl_2$ (0.75 mM) and the required amount of water to dilute the stock solution of SSF. Note that the recommended contact time with the enzyme is 2 min at 37 °C (pH 7) and therefore all reagents were pre-heated before mixing.

For the SGF fluid, the electrolyte stock solution was prepared to achieve the following ion concentrations; K^+ (7.8 mmol/L), Na^+ (72.2 mmol/L), Cl^- (70.2 mmol/L), $H_2PO_4^-$ (0.9 mmol/L), HCO_3^- (25.5 mmol/L), Mg^{2+}(0.1 mmol/L), NH_4^+ (1 mmol/L), Ca^{2+}(0.15 mmol/L). Later, porcine pepsin (EC 3.4.23.1) was added to achieve 2000 U/mL in the SGF electrolyte mixture. 1 M HCl was added to adjust the pH to 3. Typically, the obtained gastric chyme

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