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Engineering of acidic O/W emulsions with pectin

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ABSTRACT

Pectins with distinct molecular design were isolated by aqueous extraction at pH 2.0 or 6.0 and were examined in terms of their formation and stabilisation capacity of model *n*-alkane–in–water emulsions at acidic pH (pH 2.0). The properties and stability of the resulting emulsions were examined by means of droplet size distribution analysis, Lifshitz-Slyozov-Wagner modelling, bulk rheology, interfacial composition analysis, large-amplitude oscillatory surface dilatational rheology, electrokinetic analysis and fluorescence microscopy. Both pectin preparations were able to emulsify alkanes in water but exhibited distinct ageing characteristics. Emulsions prepared using pectin isolated at pH 6.0 were remarkably stable with respect to droplet growth after thirty days of ageing, while those prepared with pectin isolated at pH 2.0 destabilised rapidly. Examination of chemical composition of interfacial layers indicated multi-layered adsorption of pectins at the oil-water interface. The higher long-term stability of emulsions prepared with pectin isolated at high pH is attributed to mechanically stronger interfaces, the highly branched nature and the low hydrodynamic volume of the chains that result in effective steric stabilisation whereas acetyl and methyl contents do not contribute to the long-term stability. The present work shows that it is possible by tailoring the fine structure of pectin to engineer emulsions that operate in acidic environments.

1. Introduction

Emulsions are increasingly being utilized for encapsulating and delivering bioactives at targeted locations in the gastrointestinal tract [1]. A wide variety of lipophilic bioactives, including vitamins (D, E), carotenoids, flavonoids, phytosrerols, polyunsaturated lipids or flavours have been encapsulated in colloidal systems [2–6]. Emulsions as delivery vehicles allow sustained release and protection from degradation during storage of lipophilic bioactive components that are incorporated into the hydrophobic core of the lipid droplets. Furthermore, protection of the bioactive from environmental conditions (e.g., gastric fluids) when it is loaded in the internal phase of the emulsions is another advantage that may result in more efficient delivery.

Emulsions are most commonly formed using proteins or low molecular weight surfactants. The problem with such molecules when used as emulsifiers is that they have limited resistance to the gastric environment (e.g., proteases or low pH). These factors, among others, alter the surface composition and change the properties of the colloidal system [7,8]. It is important, therefore, to control the physical stability within the stomach as a means to control the rate of release at the desired location (e.g., intestines or colon). Surface behaviour of emulsions can be tailored using surface-active polysaccharides with contrasting physical properties. Pectins from okra and sugar beet have unusual fine structures compared with other common pectin sources (e.g., citrus or apple), as they are highly acetylated and highly branched with variable amounts of arabinan side chains and ferulic acid residues that ultimately control their functional properties [9–11]. Using pectin to engineer the oil-water interface could be favourable, as it is resistant to enzymatic digestion in the upper gastrointestinal tract (e.g., mouth and stomach), nonetheless, is digested in the colon by pectinases. This functional characteristic makes pectin a suitable candidate to protect acid sensitive bioactives during gastric transit [12] or as a colon drug-delivery vehicle [13]. Other polysaccharide-based systems have been also tested as delivery methods due to biocompatibility and high potential to be modified and achieve the required functionality [14,15].

In our previous investigations, we have tuned the extraction protocols of pectin from okra pods and obtained polysaccharides with tailored structure (e.g., molecular weight, branching, methoxyl and acetyl content, etc.) [16]. In the present work, we build on our previous experimental findings with the aim to understand the behaviour of pectin at the oil-water interfaces in highly

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acidic environments. We have, thus, engineered and characterised pectin-stabilized oil-in-water emulsions at low pH values (pH 2.0), as a first step to understand the underlying fundamental mechanisms of emulsion coarsening at pH values in the vicinity of gastric pH.

2. Materials and methods

2.1. Materials

Pectins were isolated from okra pods [16], labeled as OP2 and OP6 and their major physicochemical characteristics are shown in Table S1. Sodium azide, citric acid monohydrate, sodium citrate dihydrate, phenol, *n*-hexadecane, *n*-dodecane, formaldehyde (37–40%), phosphate buffer saline (PBS) (all analytical grade reagents) were obtained from Sigma-Aldrich (St Louis, MO). Anti-homogalacturonan antibody LM19 and LM4 (non-pectin specific antibody) were supplied by PlantProbes (Leeds, UK). De-ionized water was used throughout the experiments.

2.2. Preparation of emulsions

Preliminary experiments on the optimum concentration of pectin towards emulsion stability showed that fine emulsions are produced at pectin concentration of 1.5% w/v with dispersed phase volume fraction of $\varphi = 0.1$ (*n*-dodecane or *n*-hexadecane) and under acidic conditions (pH 2.0). The aqueous phases of the emulsions were prepared by means of dissolving pectin at 1.67% w/v concentration in citric buffer (10 mM, pH 2.0) at room temperature. Emulsions were fabricated at room temperature in two stages: a) pre-emulsions were obtained with a high-speed (IKA T18 basic, Ultra-Turrax, Germany) homogenizer for 2 min and, b) the coarse emulsions were further emulsified using an ultrasound device (Hielscher Ultrasonics, Model UP 100H) equipped with 7 mm diameter MS7 tip immersed (two-thirds) in the coarse emulsion and operating at 30 kHz. Ultrasonic treatment of the emulsions was performed for 40 s with pulsed ultrasound (30% per second) at 100% amplitude (corresponding to ultrasonic waves of 125 μm). The sonication conditions were chosen in accordance to the preliminary data that showed the absence of "over-processing".

2.3. Determination of droplet size distribution

Droplet size distribution was measured immediately after the emulsion preparation and after 1 h followed by measurements at 1, 10 and 30 days of storage at room temperature using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) laser diffraction particle size analyzer equipped with the small volume sample dispersion unit Hydro 2000SM (Malvern Ltd, UK). Refractive indices of *n*-dodecane, *n*-hexadecane and dispersion medium (citric buffer, 10 mM, pH 2.0) were set to 1.421, 1.434 and 1.333, respectively. Consequently, droplet size was described using the surface-weighted mean diameter ($d_{3,2}$), volume-weighted mean diameter ($d_{4,3}$) and span.

The physical properties of *n*-alkanes required for the calculations of theoretical (ω_{th}) Ostwald ripening rates in the emulsions were taken from literature [17–19]. Solubility ($c_{r\to\infty}$) was 2.3×10^{-5} and 9.3×10^{-8} ($mol\,m^{-3}$), diffusion coefficient (D) was 5.4 and 4.6 ($10^{-10} \times m^2\,s^{-1}$), molar volume ($V_m$) was 2.27 and 2.92 ($10^{-4} \times m^3\,mol$) and molecular weight ($M_w$) was 0.170 and 0.226 ($kg\,mol^{-1}$) for *n*-dodecane and *n*-hexadecane, respectively. Interfacial tensions (γ) were 25.5 or 27.0 $mN\,m^{-1}$ for *n*-hexadecane-water interfaces stabilized by 0.1% w/v OP6 or OP2, and 34.4 or 30.9 $mN\,m^{-1}$ for *n*-dodecane-water interfaces stabilized by 0.1% w/v OP6 or OP2, respectively. Interfacial tension measurements were performed as described in Section 2.5.

2.4. Interfacial composition analysis

Interfacial composition analysis was performed by determining protein, pectin and acetyl contents at the of oil-water interface. Emulsions were ultracentrifuged at 60000*g* for 1 h (Optima L–100 K ultracentrifuge, rotor 50.2 Ti, Beckman Coulter, USA) until equilibrium phase separation conditions were achieved and serum was collected using a syringe. The interfacial composition was evaluated as the protein, pectin or acetyl concentration difference between the pectin solutions (*i.e.*, aqueous phase before emulsification) and serum solutions. Protein was measured with Bradford analysis using Quick Start™ Bradford Protein Assay kit. The quantification of adsorbed pectin was expressed as total carbohydrates in pectin solution and serum phase using the phenol-sulphuric method [20]. The acetyl content was determined with the hydroxamic acid method in the pectin solution and serum phases [21]. Interfacial protein and pectin concentrations (Γ, $mg\,m^{-2}$) were calculated as protein or pectin concentration difference between the biopolymer solution and serum phase divided by the specific surface area (SSA) of the oil droplets:

$$\Gamma = \frac{\text{mg of adsorbed protein or pectin}}{\text{SSA} \times \text{mL of alkane in emulsion}} \quad (1)$$

where specific surface area (SSA), $m^2\,mL^{-1}$ was obtained by the result analysis report of the instrument.

2.5. Interfacial rheology

The interfacial tension of the *n*-hexadecane- or *n*-dodecane-water interfaces stabilized by 0.1% w/v OP2 and OP6 was measured using a profile analysis tensiometer (PAT-1D, SINTERFACE Technologies, Berlin, Germany) at 20 °C. The *n*-alkane-water interfaces were equilibrated for 2 h and subjected to large-amplitude oscillatory dilatational deformations. The amplitude sweeps were performed stepwise from 2 to 50% strain at a frequency 0.1 Hz. Lissajous plots were constructed by plotting the surface pressure $\pi = \gamma - \gamma_o$, where γ_o was interfacial tension before the oscillation, versus deformation $(A - A_o)/A_o$, where $A_o = 20\,mm^2$ was the area at zero deformation.

2.6. Pectin immunolocalization at the o/w interface

Anti-homogalacturonan antibody LM19 [22] (PlantProbes, Leeds, United Kingdom) was used to localize pectin at the alkane-water interface and LM4 (non-pectin specific antibody) was used as a negative control.

Pectin aqueous phases with OP2 (1.67% w/v) were prepared in 10 mM PBS, pH 7.4. A drop of OP2 solution was placed on a microscopy slide and dried using Bunsen burner. Dried sample was fixed using 10% formalin solution buffered in 10 mM PBS. Following the washing step, samples were blocked with 5% BSA in 10 mM PBS. The immunolabeling of pectic epitopes started with incubation of the samples with the primary antibody (LM19 was used as 5-fold dilution of a hybridoma supernatant) overnight at 4 °C followed by a washing step in PBS (three times for 5 min). LM19 was visualized using secondary labelling with anti-rat IgG coupled to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, USA). The secondary antibody was diluted 1:5 in PBS and incubation was performed for 2 h at room temperature.

In order to use the probes for *in situ* immunolocalisation of pectin at the alkane-water interface, OP2-stabilized emulsions (1.5% w/v) were prepared using high-speed homogenizer (IKA T18 basic, Ultra-Turrax, Germany) for 2 min. Monoclonal antibody