Article:
Mu, M, Karthik, P, Chen, J et al. (2 more authors) (2021) Effect of amylose and amylopectin content on the colloidal behaviour of emulsions stabilised by OSA-Modified starch. Food Hydrocolloids, 111. 106363. ISSN 0268-005X

https://doi.org/10.1016/j.foodhyd.2020.106363

Effect of Amylose and Amylopectin Content on the Colloidal Behaviour of Emulsions Stabilised by OSA-Modified Starch

Mingduo Mu[a], Pothiyappan Karthik[b], Jianshe Chen[b], Melvin Holmes[a], Rammile Ettelaie[a,*]

[a] Food Colloids Group, School of Food Science and Nutrition, University of Leeds, Woodhouse Lane, Leeds, LS2 9JT, UK
[b] School of Food Science and Bioengineering, Zhejiang Gongshang University, Hangzhou, Zhejiang, 310018, China

Submitted to Food Hydrocolloids

Keywords: Hydrophobically modified starch; Emulsion stability; Tailored controlled release; Amylose content

* Corresponding author. Tel: + 44 113 343 2981, Fax: +44 113 343 2982

E-mail: r.ettelaie@leeds.ac.uk (R.Ettelaie).

Abstract

The impact of the amylose content (AC) of hydrophobically modified starch on its emulsion stabilising behaviour has been examined in this study. Waxy maize (W) and normal corn starches (N) with different amylose contents, 5.48% and 28.37% respectively, were hydrophobically modified with Octenyl Succinic Anhydride (OSA) (3%) and used to fabricate oil-in-water emulsions. Emulsion samples were compared for their colloidal stability against pH change, increase of electrolyte concentration and enzymatic treatment by amylase. The amylose content of the OS-starch made a pronounced difference to emulsion stability in all cases. Increases in the electrolyte concentration, or decrease of pH to low values, left emulsions stabilised by OS-N (medium AC) strongly flocculated. However, little coalescence of droplets was detected, even after 21 days. In comparison, the OS-W (low AC) stabilised emulsion remained well dispersed throughout the entire storage period, following changes to pH or raised salt concentrations. The contrast between the behaviour of the samples is attributed to the provision of sufficient steric interactions by OS-W emulsifier but not OS-N. Destabilisation following α-amylase treatment revealed a different trend, with both *in vitro* and *in vivo* digestion experiments leaving the low AC (OS-W) stabilised droplets showing extensive coalescence immediately post treatment. Over the same short period, the OS-N stabilised emulsions became flocculated, but again without much droplet coalescence. The enzymatic degradation of OS-N interfacial layers seems to proceed more slowly than the OS-W samples. Varying destabilisation rates provide a means for realising tailored release profiles of flavours or active ingredients, achieved in principle by appropriate mixing of several emulsions stabilised by different AC modified starch.

1 Introduction

In the food industry, there has been an increasing trend in moving away from synthetic surfactants to more natural emulsifiers. In the past few decades, food biopolymers, i.e. proteins and polysaccharides have been studied extensively for their emulsifying and stabilising properties, with most surface active colloidal stabilisers currently in use being protein based (Dickinson, 2013; Wijaya, Patel, Setiowati, & Van der Meeren, 2017). Researchers continue to seek novel colloidal stabilisers derived from these two types of biopolymers, and progress has been made with fragmented proteins (Dai, Bergfreund, Reichert, Fischer, & Weiss, 2019; Ettelaie, Zengin, & Lee, 2014; Mokni Ghribi et al., 2015), conjugates of protein and polysaccharide through Maillard reaction (Akhtar & Dickinson, 2007; Benichou, Aserin, & Garti, 2002; Kato, Sasaki, Furuta, & Kobayashi, 1990), and hydrophobically modified starch (Arshad, Ali, & Hasnain, 2018; Chanamai & McClements, 2001, 2002; Ettelaie, Holmes, Chen, & Farshchi, 2016; Ettelaie, Zengin, & Lishchuk, 2017; Hu, Karthik, & Chen, 2019; Mu, Farshchi, Holmes, Chen, & Ettelaie, 2019; Nilsson & Bergenstahl, 2006).

Hydrophobically modified starch is also considered as an inexpensive and abundant substitute for Gum Arabic. Being of plant source and the “gold standard” of beverage emulsion industry, Gum Arabic is a composite polysaccharide containing a small amount of lower molecular weight protitious fraction (Dickinson, 2018; Randall, Phillips, & Williams, 1988), which is thought to be entirely responsible for its surface active properties. Due to its high heterogeneity and rather small portion of the glycoprotein fraction, usually large amounts are required in real formulations, resulting in higher cost. Upon hydrophobic modification, starch develops an amphiphilic nature and becomes surface active. Compared to proteins, this modified starch has been found to provide a higher degree of colloidal stability at low pH and high electrolyte concentrations (Chanamai & McClements, 2002). This is because the main stabilising mechanism provided by the hydrophobically modified starch is thought to be based on the provision of steric repulsion and is not electrostatic in origin (Tesch, Gerhards, & Schubert, 2002). Screening of any electrostatic interaction, as there might be in the case of modified starch esters, using high electrolyte concentrations or at low pH conditions, have provided support for this view (Chanamai & McClements, 2002; Lin, Liang, Zhong, Ye, & Singh, 2018b).

Currently, the only legally permitted material for hydrophobic modification of starch for use as food emulsifiers is octenyl succinic anhydride (OSA). Even then this is limited to less than 3% by weight (FDA, US) (Bai, Kaufman, Wilson, & Shi, 2014; Zhao et al., 2018). The modification reaction is most commonly carried out in an alkaline medium. OS-starch has many potential applications other than beverage emulsions, for example encapsulation of fragrances, essential oils and fish oils, and formulation of reconstitutable emulsions for easier transportation and storage (Cheuk et al., 2015; Garcia-Tejeda, Salinas-Moreno, Barrera-Figueroa, & Martinez-Bustos, 2018; Mu et al., 2019; Samakradhamrongthai, Thakeow, Kopermsub, & Utama-Ang, 2016). There is indeed a trend in using clean label ingredients in the food industry, which may lead to a reduction in the use and eventual phasing out of any chemically modified starch. Nonetheless, research on OS-starch is still likely to remain relevant in other industries such as nutraceutical and pharmaceutical, and any phasing out will be a gradual process.

In recent years, increasing attention has been paid to understanding the possible connection between the structure of starch molecules and the stabilising properties of the derived OS-starch. For example, these studies have involved examining starches of various botanical source or cultivar, and attempting to link their structural characteristics to their functional properties (Simsek, Ovando-Martinez, Marefati, Sjoo, & Rayner, 2015; Song, Zhao, Li, Fu, & Dong, 2013; Wang, He, Fu, Huang, & Zhang, 2016; Whitney, Reuhs, Ovando Martinez, & Simsek, 2016). Evidence indicates that starch granules have a layered structure, and modifying agents such as OSA gain access to the interior of the granule by the pores on the surface and channels leading to a central cavity (Huber & BeMiller, 2000; Liu, Li, Goff, Nsor-Atindana, & Zhong, 2018). The crystalline region of a starch granule consists of helices formed by the short branches (DP 10-15) of amylopectin, whereas amylose sits mostly in the amorphous region of the granule. Various studies on distribution of OSA in the starch granule have found that OSA mainly attaches to starch in the amorphous regions of the latter. If true this implies that the amylose molecules are the primary receptors for OSA in modified starch granules (Liu et al., 2018; Shogren, Viswanathan, Felker, & Gross, 2000; Wang et al., 2013; Whitney et al., 2016). One question that naturally arises from the above studies, and has caught the attention of several researchers in recent years, is whether the amylose content (AC) of a starch affects the substitution efficiency, and thus the emulsifying and stabilising properties of the modified starch. This is likely given the possible bias of OSA attachments to amylose chains. Importantly, this is not to say that amylose content is the only relevant factor

to the emulsification and stabilisation properties of OS-starch. Other structural factors such as the crystallinity of granule, crystal types, granule size, molecular chain length, degree of branching of amylopectin might also be responsible. Nevertheless the focus in the current work remains on the influence of the proportion of amylose in the modified starch on its emulsifying ability.

It has been suggested that the emulsification ability of OS-starch increases with higher degree of substitution (DS) in the range of interest here (modification <3%) (Bhosale & Singhal, 2006; Lin, Liang, Zhong, Ye, & Singh, 2018a; Ruan, Chen, Fu, Xu, & He, 2009). Studies by Lopez-Silva, Bello-Perez, Castillo-Rodriguez, Agama-Acevedo, and Alvarez-Ramirez (2020), Song, Pei, Zhu, Fu, and Ren (2014) and He, Song, Ruan, and Chen (2006) all supported this view. These researchers found that DS is positively correlated to amylose content of the native starch. However, other work conducted with starch from the same botanical source as the previous researches, as well as different ones, found either no correlation or a slightly negative correlation (Cruz-Benítez et al., 2019; Lopez-Silva, Bello-Perez, Agama-Acevedo, & Alvarez-Ramirez, 2019; Song et al., 2013; Sweedman, Hasjim, Schafer, & Gilbert, 2014). In terms of application oriented comparisons, encapsulation involving modified starch from different sources, has been addressed by Cruz-Benítez et al. (2019), even though colloidal stability was not of main interest and only the encapsulated dry powder was characterised.

Few studies focused on the impact of amylose content of OS-starch in liquid emulsions. Even in those systems that do involve the fabrication and characterisation of liquid emulsions, the OS-starch was sometimes kept in granular form to provide a Pickering type emulsifier (Lopez-Silva et al., 2019; Song et al., 2014). Pickering emulsion (Pickering, 1907) has a different stabilising mechanism in the sense that it maintains colloidal stability due to difficulty of displacing the adsorbed particles from the interface resulting from large desorption energies (> 1000 kT). A distinction is to be made with typical steric repulsion, as in molecularly adsorbed OS-starch ester stabilised emulsions, where the droplets are kept apart due to the excluded volume repulsion between overlapping interfacial layers. The typical size of starch granules ranges from 2 µm to 50 µm, so as a result, the oil droplets they stabilise through Pickering mechanism are usually no smaller than 10 µm (Matos et al., 2018; Simsek et al., 2015). However, once OS-starch granules are gelatinised, the solution is capable of producing sub-micron emulsions with amylose and amylopectin polymers (Chanamai & McClements, 2002; Sweedman, Schafer, & Gilbert, 2014).

Among the very few studies involving gelatinised OS-starch stabilised liquid emulsions and the impact of amylose content, there are both experimental and theoretical investigations. Experimentally, Song et al. (2013) produced stable emulsions with gelatinised modified rice starch, having droplet sizes ranging from 1 µm to 12 µm. Emulsion stability was reported to be enhanced with smaller AC values, whereas the initial droplet size seemed to change non monotonically with varying amylose content for the studied cultivars of *indica* rice. Sweedman, Hasjim, et al. (2014) used maize starch of various AC to emulsify an oil based solution of β-carotene (1 wt% oil content emulsion). They were able to fabricate emulsions with excellent colloidal stability at low amylose content, but also observed a faster destabilisation of droplets as AC was increased. High pressure homogenisation was employed in this work, and modified maize starch was able to confer a real sub-micron size for droplets (~300 nm). However, in both of these studies, emulsion stability was examined only in a rather limited range of electrolyte concentration and pH, relevant to the specific purpose of their investigation. For Song et al. (2013) this was to differentiate between the rice cultivars while Sweedman, Hasjim, et al. (2014) explored the possibility of achieving high oxidative protection for β-carotene. As a result, in these low stress systems, no additional electrolyte was added, and the pH of the systems were not even mentioned. The work of (Lopez-Silva et al., 2019) is potentially also a possible case of gelatinised OS-starch stabilised emulsion. They reported emulsion droplet sizes that are lower than their corresponding starch granule sizes, despite describing their emulsions as being of Pickering variety in their publication.

On the theoretical side, the work of Ettelaie et al. (2016), based on numerical Self Consistent Field (SCF) calculations, compared behaviour of amylose and amylopectin with equal degrees of hydrophobic modification. The study made two major assumptions. Firstly, it assumed that the background electrolyte concentration was sufficiently high so as to screen any electrostatic repulsion. Thus, any predicted emulsion stability can purely be attributed to steric interactions. Secondly, amylose and amylopectin were taken to have the same molecular weight, with the only remaining difference being the branched and linear nature of these two starch biopolymers. While the chosen molecular weight of chains was in a region where the size distribution of amylose and amylopectin just about overlap with each other, amylopectin tends to have a typical molecular weight 100x larger than average sized amylose. Nonetheless, under these limiting assumptions, the study suggested that amylopectin would form a more compact layer at the interface, and is therefore associated with a larger energy minimum, once van der Waals interactions are also included in the calculations. On the other

hand, amylose formed less dense but more extended films, resulting in longer ranged repulsion. However, this advantage was cancelled out by the prediction of an additional energy minimum at closer inter-droplet separation. This was shown to arise from larger tendency of modified amylose to cause bridging flocculation. It was also indicated that a mixture of amylopectin with a small amount of amylose would have better performance than pure amylopectin (Ettelaie et al., 2016).

Other than changes in electrolyte concentration and pH, colloidal systems stabilised with OS-starch can also be destabilised by enzymatic digestion of the starch. Amyolysis of OS-starch, in the absence of any oil droplets, has been studied and compared with that of native unmodified starch. It has generally been found that OSA modification reduces starch digestibility, regardless of starch source (Ai, Nelson, Birt, & Jane, 2013; Bajaj, Singh, & Kaur, 2019; Lopez-Silva et al., 2020; Simsek et al., 2015). Several recent studies have also examined the impact of the enzymatic digestion of OS-starch on the rate of emulsion degradation. The focus of these studies has largely been the sensory perception, substance release in the gastric-intestinal tract, and a comparison of the behaviour of starch stabilised droplets with protein stabilised ones upon the application of gastric-intestinal digestive enzymes (Dresselhuis, de Hoog, Cohen Stuart, Vingerhoeds, & van Aken, 2008; Hu et al., 2019; Lin et al., 2018a; Silletti, Vingerhoeds, Norde, & van Aken, 2007). One general conclusion from all such research is that emulsions stabilised with OS-starch can indeed be destabilised due to the break-down of adsorbed OS-starch surface layers by starch-digesting enzymes. It is natural to speculate that amylose content may have an impact on OS-starch digestibility of such interfacial films, much in the same way as native starch itself. If so, then AC should also influence the stability behaviour and the rate of break-down of emulsions stabilised with OS-starch during amylase treatment. Very recently, Lopez-Silva et al. (2020) reported tentative results supporting this view. They found that OS-starch digestibility is positively linked to amylose content. Although their work only focused on OS-starch digestion in bulk, without considering the implication for emulsion destabilisation, it nonetheless provides a foundation for the above idea. That is to say, different digestibility of OS-starch resulting from the variability in their amylose content, may have an impact on the enzymatically induced destabilisation behaviour of the emulsions fabricated using these emulsifiers. The work in this area is still rather sparse and, to the best of our knowledge, very little has been published in comparing the enzymatic degradation of emulsions that are stabilised by OS-starch of different AC.

In the research work presented here, we would firstly like to extend the previous studies in the literature by performing a more careful systematic experimental study, highlighting the differences in adsorption behaviour of starch polymers possessing varying amylose contents (AC). The colloidal stability of the emulsions produced from these, over a broad range of electrolyte concentrations and pH is investigated. Furthermore, preliminary studies involving enzymatic hydrolysis of stabilising OS-starch on droplet interface are performed. The work reported here includes both *in vitro* and *in vivo* experiments, mimicking starch digestion in the oral phase. The resultant degradation behaviour of the emulsions is examined, providing a base for more extensive future work. The prospect of different droplet destabilisation times, during enzymatic treatment of emulsions stabilised by hydrophobically modified starch of varying amylose content, opens up interesting possibilities. For example, they may lead to the tantalising prospect of achieving a broad range of desired tailored controlled release profiles, simply by an appropriate mixing of droplets stabilised by OS-starch of different AC.

2 Material and Methods

2.1 Materials

Waxy maize starch (W) and normal corn starch (N) were purchased from Shandong Fuyang Biotech Ltd. Co. (Dezhou, China) and Sigma-Aldrich (Shanghai, China) respectively. Octenyl succinic anhydride (OSA) was purchased from Sigma-Aldrich (Shanghai, China). Sunflower oil was purchased from a local supermarket. Our α-amylase (diluted with starch, from *Bacillus substilisa*) was purchased from Maya Reagent (Shanghai, China). All other chemicals, including mucin from porcine stomach type II, were purchased from Sigma-Aldrich (Shanghai, China). Deionised water was obtained from a Milli-Q apparatus (Millipore, Bedford, UK) and was used to prepare all solutions and samples.

2.2 Modification of starch with OSA

Starch was suspended in distilled water (33 wt%) with agitation. The pH of the slurry was adjusted to 8.2 with 0.5 M NaOH solution. OSA (3%) was added slowly over 2 h, and the reaction was carried out for 22 h in total. The weak acid produced during the reaction was neutralised continuously, ensuring that pH remained at 8.2 and allowing the reaction to proceed forward. In the final stage of the process, the reaction was terminated by adjusting pH to 6.0. This was achieved using 0.5 M HCl. After being centrifuged and washed twice

with ethanol and twice more with distilled water, removing any unreacted OSA, the sample was then oven dried at 40 °C for 48 hours.

2.3 Emulsification with OSA-starch

Oil-in-water emulsions containing 10 wt% sunflower oil and 1 wt% OS-starch were fabricated. OS-starch was suspended in Milli-Q water, and heated in hot water bath at 90 °C with intermittent stirring, for 60 min. This was to ensure a maximal degree of gelatinization. The resultant OS-starch solution was then cooled to room temperature and used for emulsion preparation. Primary emulsions were made at 18,000 rpm with high-speed homogeniser (IKA Ultra Turrax T25, UK). The final emulsions were prepared using either a Microfluidizer at 250 bar (Microfluidics M-110P, Westwood, Massachusetts, USA) or a University of Leeds in-house made Jet homogenizer operating at a constant pressure of 250 bar (Burgaud, Dickinson, & Nelson, 1990). When required, pH of the emulsion was adjusted by addition of 0.1 M HCl or NaOH, and electrolyte concentration by addition of NaCl (s). During such addition, the emulsions were kept under mild magnetic stirring for the entire process and 3 minutes after. Care was taken so that the solutions and solids were added very slowly into the meniscus to minimize the effect of high local concentration.

2.4 Recovering OSA-starch from the surface of emulsion droplets

The emulsion was centrifuged at 8500 rcf until a clear cream layer separated from the serum layer. The cream layer was carefully removed and the oil was extracted with 1:2 methanol and chloroform. The insoluble material was recovered starch. Solvents were evaporated and the recovered starch was oven dried at 40 °C after 3 washes by Milli-Q water. Perhaps an additional washing step for ultracentrifuged cream layer would help in getting rid of the last traces of unadsorbed starch chains, this step was not performed here.

2.5 Characterisation of starch

2.5.1 Amylose content

The amylose content of starch was determined using the iodine adsorption method (Hoover & Ratnayake, 2001), and the absorbance was measured at 600 nm with a UV-VIS spectrophotometer UV-2600 (Shimadzu, China).

2.5.2 NMR

NMR analysis was conducted to determine the degree of substitution (DS) of various starch samples. The procedure was conducted as described by Zhao et al. (2018). Native and OSA modified starches were dissolved in DMSO-d_6 using a previously described method (Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009). Recordings of ^{1}H spectra were made on a Bruker 400M System (Bruker, Fallanden, Switzerland) at 30 °C with a pulse angle of 30°, a delay time of 10 s and an acquisition time of 2 s. The degree of substitution was calculated according to the equation

$$DS = \frac{I_{0.89}}{3\left(I_{\alpha-1,6} + I_{\alpha-1,4} + I_{r-e}\right)}$$

where $I_{r\text{-}e}$ corresponds to the ^{1}H NMR integral of the α and β reducing-end signals at 4.91 and 4.28 ppm, respectively. Similarly, $I_{\alpha\text{-}1,4}$ and $I_{\alpha\text{-}1,6}$ are the ^{1}H NMR integrals of internal α-1,4 and α-1,6 glycosidic linkages.

2.5.3 Fourier Transform Infrared Spectroscopy (FT-IR)

The IR spectra of native and modified starches were obtained using a FTIR Nicolet iS5 (Thermo Fisher Scientific, WI, USA). A minute amount of sample was ground with FTIR grade potassium bromide (approximately 1:80) and pressed to form a pellet disc. The disc was placed in the sample compartment and the scanning range was kept at 400-4000 cm^{-1} to generate the spectra.

2.6 Characterisation of emulsion

2.6.1 Droplet size

The droplet size of emulsions was measured using a laser light scattering instrument Mastersizer 3000 (Malvern Panalytical, UK). Before droplet size measurement, emulsions were shaken by hand to ensure homogeneity. Sample was added to the dispersion unit connected to the laser light scattering instrument until an obscuration between 5% and 10% was obtained. The mean droplet size was reported as the volume-weighted mean diameter, $D_{4,3} = \sum_i n_i d_i^4 / \sum_i n_i d_i^3$, where n_i denotes the number of droplets with a diameter d_i.

2.6.2 ζ-potential

ζ-potential of the emulsions was measured with Zetasizer Nano ZS (Malvern Panalytical, UK). Emulsions were diluted by a factor of 2000, and then filled in a folded capillary cell DTS1070.

2.6.3 Apparent viscosity

The apparent viscosity of emulsions was measured using a Kinexus Ultra rheometer (Malvern Panalytical, UK) and a double gap concentric cylinder DG25 geometry (cup diameter 26.25 mm, bob internal diameter 24 mm, bob external diameter, 25 mm). The samples were gently mixed, poured into the temperature-controlled measurement cell, and allowed to equilibrate at 25 °C or 37 °C for 5 min prior to the measurement. Apparent viscosity of emulsions was measured for shear rates in the range 0.2-200 s^{-1} using application of continuous shear, at 25 °C or 37 °C.

2.6.4 Optical microscope

Emulsion droplets were observed under a Nikon optical microscope fitted with a Leica MC120 HD camera (Leica, Heidelberg, Germany). Objectives used were at magnifications of 40x or 100x. Images were captured with LAS v4.6 software.

2.6.5 Turbiscan

Emulsions or mixtures of emulsion and saliva were carefully added to a sample holder. The backscattering of light was measured using a TurbiscanLab instrument (Formulaction, Toulouse, France). A laser light source of 850 nm scanned across the height of the sample holder (42 mm), at 37 °C, for a period of 20 mins.

2.7 *In vitro* oral digestion

2.7.1 With amylase

Emulsions were mixed with amylase solution (2 g/L, pH 6.8) at 1:1 ratio, and immediately placed in a shaking water bath of 37 °C and 100 rpm. Droplet size measurements and microscopic images were taken of the samples at 10 s, 300 s, 600 s, 1200 s, using the methods described above.

2.7.2 With artificial saliva

Emulsions kept at various temperatures (4 °C, 25 °C or 50 °C) were mixed with artificial saliva (Supplementary S1) (Davis, Hartman, & Fincher, 1971; Karthik, Ettelaie, & Chen, 2019; Sarkar, Anwesha, Goh, & Singh, 2009) at 1:1 ratio, and immediately placed in a shaking water bath of 37 °C and 100 rpm. Droplet size measurements and microscopic images were taken of the samples at 10 s, 300 s, 600 s, and 1200 s using the methods described above.

2.8 *In vivo* oral digestion

The *in vivo* oral digestion was conducted with ten healthy panellists (5 females and 5 males, aged between 22 and 24, all non-smokers). It was ensured that no food was consumed by the panellists in the 2 h 30 min prior to the session. A 2 ml aliquot of emulsion was added into a 25 ml food-grade plastic sauce container with lid. The samples were coded with 3 digits and the order of presentation was randomised. For each sample, the panellists were asked to place the 2 ml emulsion in their mouth, gently stir it with their tongue to mix it with saliva, and spit it back into the sauce container after the designated time (3 s, 10 s, 30 s or 60 s). The collected digested samples were immediately characterised by droplet size measurement and, for a few of the samples, by optical microscopy. Before starting the session and after each sample, the panellists were asked to rinse their mouth and palate with bottled water at least for three times. Approximately, 5 min break was taken between the samples, for the panellists to rest and restore normal salivary secretion for the next trial. Consent was obtained in writing from each panellist and this study is covered by the ethical approval obtained from Zhejiang Gongshang University, China (2015111265).

2.9 Statistical analysis

All measurements were performed in triplicate, and the data presented were expressed as the mean values ± standard deviations. Two-tail paired t-tests were performed where applicable, and $p<0.05$ was set to determine the level of statistical significance.

3 Results and discussion

3.1 Physiochemical properties of native and OS starch

The FT-IR spectra of native and modified W (waxy maize) and N (normal corn) starch are shown in Fig 1. The broad peaks at approximately 3400 cm^{-1} indicate the hydroxyl groups, and those at 2930 cm^{-1} indicate C-H stretching. The peak at 1650 cm^{-1} represents bound water present in the starch. The characteristic peaks for starch materials are present between 800 cm^{-1} and 1200 cm^{-1} (Shingel, 2002). Compared to native W and N, the OSA modified starch showed two additional peaks at around 1740 and 1567 cm^{-1}. The peak at 1740 cm^{-1} is due to the characteristic IR stretching vibration of C = O, and an evidence for the formation of ester carbonyl groups. The peak at 1567 cm^{-1} is indicative of the asymmetric stretching vibration of carboxylate groups ($RCOO^-$) (Miao et al., 2014; Simsek et al., 2015). These

results confirmed the substitution of hydroxyl groups in starch by ester carbonyl and carboxyl groups in OSA, thus the formation of OS-W and OS-N.

The AC of native W and N were determined to be 5.48 ± 0.99% and 28.37 ± 0.10% respectively (Table 1), by a colorimetric method based on the iodine affinity of amylose (Hoover & Ratnayake, 2001). The amylose helices form dark blue complexes with iodine, and the colour change is used to determine the amylose content. Upon the attachment of OSA, the helical structures of amylose are disrupted, thus resulting in a decrease in the measured AC values for both types of starch (Lopez-Silva et al., 2020). The amylose content of starch and the degree of substitution (DS) upon modification, have been found to be positively correlated in some studies (He et al., 2006; Lopez-Silva et al., 2019; Song et al., 2014; Song et al., 2013), while negatively in others (Cruz-Benítez et al., 2019; Sweedman, Hasjim, et al., 2014). In our case, the native starch with lower AC (W) yielded a DS of 0.0160 ± 0.0021 when it was OSA modified. The same modification procedure for N, i.e. the starch with the higher AC, achieved a DS of 0.0229 ± 0.0012. This is in line with the preferential attachment of OSA to amylose molecules, which has been found in various previous research studies (Liu et al., 2018; Shogren et al., 2000; Wang et al., 2013; Whitney et al., 2016).

3.2 Emulsification and Stabilisation properties

3.2.1 Adsorption on the surface

OS-W and OS-N were then used in fabricating emulsions, and the adsorbed modified starch was recovered from the surface of oil droplets using the method described in section 2.4. The recovered OS-starch is referred to as OS-W_R and OS-N_R. As shown in Table 1, both adsorbed starch showed significantly (two-tailed paired t-test, $p<0.05$) higher DS than the bulk (OS-W and OS-N). This suggests that modification with OSA improves the surface affinity of the starch molecules, with the ones having a higher level of attached OS groups being preferentially adsorbed on the interface. For starch adsorbed on the surface of droplets, OS-W_R contained a significantly larger percentage of amylose than OS-W in bulk, whereas OS-N_R did not when compared to OS-N. The increase in AC from OS-W to OS-W_R indicates a stronger adsorption for amylose molecules than amylopectin, and thus once again seems to support the previous findings that OSA preferentially attaches to amylose (Liu et al., 2018; Shogren et al., 2000; Wang et al., 2013).

As the amount of amylose content of starch increases, one would expect smaller differences in DS between chains in bulk and those on the surface post adsorption. However, difficulties

in obtaining amylose molecules in their dissolved form with OS-N may also have played a role and cannot be ruled out. As AC rises, the gelatinization temperature of a starch is elevated, making it harder to achieve full gelatinization (Jeong & Lim, 2003). During the cooling down of starch solution before homogenization, retrogradation is more pronounced with higher AC (Dobosz et al., 2019), and thus possibly less amylose can remain in the form of free molecules available for adsorption.

3.2.2 Destabilising emulsions by lowering pH

One of the major advantages of OSA modified starch as a molecularly adsorbed colloidal stabiliser, is that it predominantly relies on the provision of steric repulsion for stabilizing oil droplets, rather than electrostatic forces. As a result, the emulsions formed by these are more resistant to changes in environmental factors such as variation in pH and electrolyte concentrations (Chanamai & McClements, 2002; Sweedman, Hasjim, et al., 2014). In order to verify and compare the steric stabilising effect of OS-W and OS-N, emulsions produced by these emulsifiers (referred to as W and N, respectively) using a Jet Homogeniser, were subjected to a series of pH changes and electrolyte additions. When adjusting pH of the emulsions, 0.5 M HCl or NaOH was added to the system. This of course can also affect the electrolyte concentration, aside from changing pH. In order to rule out this interference, NaCl was added to the emulsions to bring the background electrolyte concentration up to 0.003M. This ensures that any changes we caused by adjusting pH would remain negligible compared to this pre-existing background electrolyte level.

Fig 2 captures the changes in $D_{4,3}$ average droplet size for emulsions kept under different pH conditions, after various periods of storage. The sizes of droplets initially produced at pH 6.5 were 440 nm and 550 nm, for W and N samples, respectively. At day 0, emulsion W showed little variation in droplet size with pH adjustment throughout the range 3.5 to 9.5. However, with time, there was a slight increase in the droplet size of W when the sample was kept at more acidic pH values, increasing from 500 nm to 910 nm after 21 days at pH 3.5. On the other hand, almost immediately from day 0, emulsion N kept at acidic conditions (pH < 6.5) possessed markedly larger droplets than those stored at alkaline pH. For example, the average size at pH 7.5 was 549 nm, while at pH 3.5 it was found to be 1.91 μm. This difference in droplet sizes was maintained as the emulsions went through 21 days of storage, with the droplet size at pH 6.5 on day 21 growing to 1.71 μm, while at pH 3.5 it measured at 3.24 μm (see Fig 2). Due to the $–COO^-$ groups in the OS chain, the OS-starch is slightly negatively charged at neutral and alkaline pH. As a result, droplets with surfaces covered by OS-W and

OS-N had negative ζ-potentials (Fig 3). As pH is decreased to acidic values, the carboxylic groups become protonated (-COOH) and lose their negative charge. This is seen (Fig 3) in the smaller value of the measured ζ-potential. Nevertheless, it is worth pointing out that even at pH 7.5 to 9.5, the measured ζ-potential of approximately -6 mV to -11 mV was considered just short of sufficient (at 0.003M electrolyte) for the resulting electrostatic repulsion to act as the sole stabilising mechanism for emulsion droplets over a period of as long as 21 days. This was verified with calculation of DLVO interactions (not shown here) (Hunter, 2001).

These results indicate that emulsion W, based on OS-starch of lower amylose content, was reasonably stable across the pH range 3.5 to 9.5 for at least 21 days (Fig 2A). As pH changed from 9.5 to 3.5, the electrostatic force between droplets was almost completely removed, as is evident from the measured ζ-potential of merely -1.35 mV at pH 3.5 (Fig 3). The fact that emulsion W was still sufficiently stable under such a condition, even after 21 days, suggests that OS-W is capable of stabilising the emulsion almost entirely by the virtue of its induced steric repulsion forces between the oil droplets. On the other hand, our emulsion N, with its higher AC, exhibited lower emulsion stability as electrostatic interaction was removed by the decrease in pH. It was safe to conclude that any steric stabilising effect, as there might be due to OS-N starch adsorbed layers, was not strong enough to stabilise emulsion droplets on its own. It is most likely then that OS-N starch stabilised droplets, in contrast to OS-W ones, were at least partially dependent on electrostatic interactions for the provision of their stabilising mechanism.

3.2.3 Destabilising emulsions by increasing electrolyte concentration

Because OS-starches are slightly negatively charged molecules, both a lowering of pH and increasing the background electrolyte concentration of the continuous phase should reduce or screen out the electrostatic repulsion between the oil droplets. If our conclusion regarding the partial reliance of the stability of our N emulsions on electrostatic forces is true, then the same droplet behaviours should also present itself in systems with elevated electrolyte concentration.

In order to change the electrolyte concentration of emulsions without altering pH, various amounts of NaCl were introduced to emulsions W and N, and the resulting emulsions were kept and observed for 21 days. In Fig 4 we have plotted the average droplet size as a function of storage time for emulsions containing different concentrations of electrolyte, at pH 6.5. When there was no additional NaCl, both emulsions maintained their droplet sizes at around

500 nm throughout the 21 days. For W samples (i.e. ones stabilised by low AC modified starch) emulsions containing higher electrolytes retained their submicron size for the entire storage period. In comparison, the N emulsions (i.e. ones stabilised by higher amylose content modified starch) destabilised over time (Fig 4). At 0.1 M and 0.2 M NaCl, the droplet size of N samples exceeded 1 μm within hours of the NaCl addition.

ζ-potential values of W and N emulsions, with or without addition of NaCl are presented in Table 2. With no additional electrolyte, W and N droplets had measured ζ-potentials of -17.11 ± 1.02 mV and -15.13 ± 0.63 mV respectively. Upon the introduction of 0.2 M NaCl to the system, the charge on the droplet surface was screened and the ζ-potential dropped to -2.5 mV for both of our emulsions. At such electrolyte concentrations then, the electrostatic repulsion between droplets must be very small. The impact on the stability behaviour of the two sets of emulsions is seen to be identical to that of reducing pH, as discussed earlier. Thus, the same conclusions are reinforced with regards to the stabilising mechanism of OS-W and OS-N starch. In the case of emulsion W stored at 0.2 M NaCl concentration, one observes that the average droplet size remains at a submicron size, despite the lack of electrostatic repulsion between the droplets. Once again this shows that in these W emulsions, OS-W emulsifiers are able to provide strong steric forces that are sufficient to stabilise droplets on their own, even in the absence of electrostatic forces. However, for emulsion N, even though the steric forces may still be of some importance, the electrostatic repulsion between the droplets are necessary to ensure their colloidal stability. Once this latter is removed, e.g. by an increase in electrolyte screening or decrease of pH, destabilisation of the emulsion system is observed.

Rheological behaviours of the emulsions at various NaCl concentrations were characterised by measuring shear rates and corresponding shear stresses according to the method described in section 2.6.3, for each of the emulsion samples (see Supplementary material S2). A power law fluid equation (Barnes, Hutton, & Walters, 1989; Sopade & Filibus, 1995) was fitted to logarithmic plots of apparent viscosity, η, vs. shear rate $\dot{\gamma}$

$$\eta = K\dot{\gamma}^{n-1}$$

for the measured data. In the above equation, K is the flow consistency index and n the flow behaviour index. Newtonian behaviour of a fluid can be distinguished by an n value close to 1. Both emulsions W and N, in the absence of any added electrolyte exhibited Newtonian

behaviour during the observed time, with $n = 0.99$ and 0.98 at day 21, respectively (Supplementary S2). With 0.2 M of added NaCl, N emulsion became shear thinning, as indicated by the flow behaviour index changing to $n = 0.65 < 1$, as shown in Fig 5. This is likely due to the possible formation of a weak network of oil droplets (Chanamai & McClements, 2001), as both OS-W and OS-N solutions on their own exhibited Newtonian behaviour when subjected to the same measurement (Supplementary Material S6). The results in Fig 5 are after 21 days of storage, although the shear thinning behaviour was already evident even at earlier times. In contrast, W emulsion has $n \approx 1.04$ at day 21 and remains Newtonian even after such a long period of storage.

A weak network formed by emulsion droplets suggests aggregation rather than coalescence. For example, depletion flocculation in emulsions are reversible by dilution or shearing, whereas coalescence is an irreversible destabilisation process (Dickinson, 2019). Droplet aggregation in an emulsion stabilised by biopolymers can normally be distinguished from droplet coalescence by addition of sodium dodecyl sulphate (SDS). The dilution by SDS breaks many possible types of bonds between the droplets. So long as the droplets have not coalesced, this leads to a marked decrease in the measured particle size as the network of droplets falls apart (Demetriades & McClements, 2000). Upon the addition of 2 wt% SDS to emulsion N, at 0.2 M of added NaCl and kept for 7 days, the second peak in the droplet size distribution shifted significantly to the left. At the same time, the first peak at the lower sizes became visibly more pronounced (Fig 6). This large shift in the droplet size distribution to lower values suggests that the destabilisation in N was primarily due to droplet aggregation, with only a limited degree of coalescence having taken place. The formation of flocculated aggregates in N with 0.2 M salt can also be clearly observed in the microscopic images in Fig 7. In both W and N with no additional electrolyte, droplets remained uniformly dispersed, retaining their submicron sizes over the period of observation. With the introduction of 0.2 M NaCl, the average particle size of emulsion W increased slightly from 550 nm at day 0 and to 830 nm at day 4 but continued to remain below one micron even after 21 days. In contrast, in emulsion N the formation of large flocs (but without any extensive coalescence) was noticeable starting from day 11. This coincided with the time when shear-thinning behaviour began to also manifest itself in our rheological measurements of this system.

Both alterations of pH and changes in electrolyte concentration, demonstrate that OS-W is more efficient in provision of steric stabilisation. This low AC modified starch is less dependent on the availability of the electrostatic repulsion in order to provide a sufficient

degree of colloidal stabilisation of droplets, as compared to OS-N. The size and structure of starch granules to some degree depend on their botanic source and specific cultivar. However, if and once fully gelatinised, the granules of OS-W and OS-N can be considered to have disappeared and the starch then exists in the solution in the form of dissolved modified amylose and amylopectin molecules in our systems. Therefore, in such a state the fundamental difference between dissolved OS-W and OS-N starch has to be largely sought in their composition, including the ratio of their amylose and amylopectin content. Therefore, the conformation adopted by these two molecules adsorbed on the oil/water interface is likely the main contributor to the different steric stabilising properties of OS-W and OS-N, seen in our study here. Ettelaie et al. (2016) conducted numerically based calculations using Self-Consistent Field (SCF) theory in order to compare the interfacial behaviours of amylose and amylopectin. In their somewhat idealised models, all aspects of the structure of amylose and amylopectin were considered identical, including their molecular weights, degrees of hydrophobic modification and various interactions parameters associated with monomer comprising the chains. The only difference left was the level of branching, with amylose represented as a linear biopolymer and amylopectin as a highly dendritic branched macromolecule. The computations provided the interactions mediated by the overlap of adsorbed adjacent layers on neighbouring droplets. Also the direct attractive van der Waals forces were accounted for in the calculations. However, it was assumed that the system was under the conditions where electrostatic repulsion was negligible (e.g. low pH or high salt). It was found that for both modified amylose and amylopectin, when each considered on their own, there was a shallow energy minima ($< 5\ k_BT$) in particle interaction potentials, occurring at droplet separations where the adsorbed layers just began to overlap. This happened at shorter separations for amylopectin, which was attributed to its more compact but rather denser interfacial layers. The depth of such minima is too small to cause any appreciable droplet aggregation. However, the calculated interaction potentials between two droplets, as stabilised by modified amylose, also exhibited a second energy minimum at a closer droplet-droplet separation distance. This second energy minimum was much deeper. Its presence may well be important in accounting for the inferior relative steric stabilising ability of the hydrophobically modified amylose, as is observed in our current study. The theoretical models, attributed the second energy minimum to the less dense and more extended conformation of modified amylose layers, when compared to layers formed by amylopectin of the same hypothetical molecular weight. This allowed for various OSA hydrophobically modified sites on the amylose to become more easily associated with two

different nearby surfaces. In other words, Ettelaie et al. (2016) concluded that hydrophobically modified amylose has some affinity for inducing bridging flocculation, not otherwise present for amylopectin of a comparable M_w. Of course, in reality amylopectin is not of comparable molecular weight to amylose, but on average typically 100 times larger. Therefore, even with chains having a higher tendency to extend away from the surface, it is likely that the linear amylose molecules still form thinner interfacial layers, and thus weaker steric repulsion than amylopectin. Coupled with preferential attachment of OS groups to amylose as discussed previously, and with its higher tendency for bridging, the modified amylose when possessing little charge will have a higher potential to cause aggregation of the droplets.

Our experimental results with emulsion N are largely in line with the above theoretical predictions. We further verified that 23.59% amylose content on the interface was enough to induce flocculation of the droplets, when electrostatic repulsion was largely absent.

3.3 Destabilisation of emulsion through enzymatic digestion of OS-starch in the oral phase

3.3.1 Enzymatic destabilisation in oral phase: *in vitro*

To study the destabilisation of W and N emulsions induced by enzymatic digestion of OS-W and OS-N starch interfacial layers, emulsions were produced by Microfluidizer and the conditions were optimised so that the final emulsions W and N both had the same droplet size of $D_{4,3}$ = 0.55 μm (Supplementary S3). The final emulsions were stored and tested for their colloidal stability during a period of 16 days (Supplementary S4), and new batches were made under the same conditions to be used within 3 days for *in vitro* and *in vivo* experiments.

The changes in droplet size of W and N upon the action of 2 g/L α-amylase solution are shown in Fig 8A. In order to exclude any destabilisation of emulsion resulting from changes in the environmental factors, such as an elevated temperature (37 °C) and shaking (100 rpm), control samples were made by mixing emulsions with equal amounts of Milli-Q water. These were subjected to exactly the same temperature and shaking as samples containing the enzyme. The emulsion average size in both control samples (W control and N control) remained at $D_{4,3}$ = 0.55 μm within the 1200 seconds duration of the study. In contrast, both enzyme-treated samples W and N exhibited larger droplet sizes as a result of the action of α-amylase. In the presence of α-amylase, droplet size of W increased to 8.62 μm in the first 10 seconds, and 70.96 μm at 1200 s. On the other hand, the change in N was markedly more

gradual, with $D_{4,3}$ measured at 4.78 μm at the end of the 1200 s time period of the observation. During the oral stage, the enzyme α-amylase would cleave α-1,4 glycosidic bonds in the starch chains, leaving the branching points that are α-1,6 glycosidic linkage in amylopectin untouched (Smith & Morton, 2010a). Destabilisation of emulsion by the action of α-amylase was found to be more rapid for W system, which was stabilised by amylopectin rich OS-W, than in emulsion N with its higher AC. It is worth noting that the impact of the enzyme digestion on the stability of our two emulsion samples is completely opposite to that due to the addition of salt or lowering of pH discussed in the previous section.

After it was confirmed that α-amylase was able to digest OSA modified starch adsorbed at droplet interfaces, and therefore cause destabilisation of the emulsions as a result, artificial saliva (formulation given in Supplementary material S1) was used to better mimic the electrolyte and protein rich environment in the oral cavity. In order to exclude impact of environmental factors such as changes to electrolyte concentration and the presence of protein that may induce flocculation (Sarkar, A., Ye, & Singh, 2017; Silletti et al., 2007), the control samples in this experiment were mixed with empty artificial saliva, which is a solution containing all the inorganic salts and mucin as highlighted in supplementary material S1, except for α-amylase. Prior to mixing with artificial saliva, the emulsion systems were kept at various temperatures (4 °C, 25 °C, 50 °C) to mimic cold, room temperature and hot beverages. As seen in Fig 8B, 8C and 8D, in all three cases, both emulsions W and N were destabilised by the action of α-amylase. At the same time, the control samples maintained stable droplet sizes. Moreover, at all three temperatures, the enzyme treated W emulsions presented higher measured $D_{4,3}$ values than their N counterparts, when measured at 300 s, 600 s, and 1200 s following the introduction of the artificial saliva. In terms of the temperature of the emulsion prior to digestion, for the same emulsion type (emulsion W or N), temperature did not have any obvious influence on droplet size during destabilization.

When the digested emulsions were observed under optical microscope, it was very clear that significant coalescence of droplets was induced in emulsion W, while in emulsion N the formation of large clusters and extensive flocculation was more prominent (Fig 9). These observations are supported by our droplet size measurements, when the flocs in N were easily broken up into smaller clusters and even individual droplets, by the dilution and shearing within the dispersion unit of Mastersizer (Silletti et al., 2007). However, it is important to note that even though flocculation was also observed for emulsion N under high electrolyte conditions discussed in section 3.2.3, the situation here is somewhat different. It is tempting

595 at first to associate the flocculation with the raised electrolyte concentration in artificial saliva.
596 However, the calculated electrolyte concentration in the emulsion system following the
597 mixing with artificial saliva is estimated to be around 0.038M. Destabilisation induced by
598 such a relatively low salt concentration is rather slow and would take a few days to become
599 noticeable. This was confirmed by the N control sample, which showed no obvious changes
600 in size or the level of aggregation in the absence of α-amylase under this salt and protein
601 concentrations, during this short initial exposure time (20 mins maximum). Recall also that
602 our addition of salt experiments of section 3.2.3, displayed immediate flocculation only at
603 electrolyte concentrations at or above 0.1 M. Therefore, the OS-N interfacial layer must have
604 degraded partially at least by α-amylase to cause this degree of flocculation of the droplets.
605 But presumably, the degradation must have proceeded at a slower rate than that in the OS-W
606 case, with the extensive coalescence seen in the latter largely absent for N emulsion system.
607 The distinctions we observe in the destabilisation of the enzyme treated W and N emulsions
608 must have arose from their different AC contents, with a faster rate of enzymatic degradation
609 for low amylose content based emulsifier in system W.

610 Enzymatic digestion by α-amylase induced coalescence in emulsion stabilised by OS-W
611 which have 3.93% amylose content. Their impact on emulsions stabilised by OS-N with
612 21.86% amylose, over the same short period of 20 min, was to cause emulsion flocculation.
613 Once gelatinised and cooled down to room temperature, amylose and amylopectin molecules
614 start to recrystallize to some extent, and this retrogradation phenomenon is known to be more
615 prominent with amylose, making them less sensitive to enzymatic digestion (Fredriksson et
616 al., 2000; Patel et al., 2017; Sikora et al., 2019). As found by Zhou, Chung, Kim, and Lim
617 (2013). Therefore, post gelatinisation and retrogradation, normal corn starch contains more
618 resistant starch than waxy corn starch. It is speculated that for this reason, despite the fact that
619 droplets in emulsion W started with thicker interfacial layers of OS starch, a higher digestion
620 rate is associated with OS-W. This leads to a more rapid degradation of the protective surface
621 films in emulsion W. In addition, upon the digestion by α-amylase, amylose is cleaved into
622 shorter straight chains of glucose polymer, while amylopectin is chopped into straight chains
623 and small fragments around branch points (Smith & Morton, 2010b). Variations in the
624 surface activity of the fragments resulting from digestion would affect the surface coverage
625 of emulsions and hence their destabilisation behaviour.

626 Distinctions between the rates of α-amylase induced destabilisation behaviour of emulsions
627 fabricated with OS-starch, involving different AC, provides a possibility for achieving

628 tailored controlled release profiles for flavours or other food active ingredients. Our results
629 show that this in principle can be engineered by careful mixing of such emulsions at
630 appropriate ratios, to yield the desired release profile.

631 3.3.2 Enzymatic destabilisation in oral phase: *in vivo*

632 Emulsions W and N (both with initial droplet size of ~550 nm) were then subjected to *in vivo*
633 oral digestion with 10 panellists, and the *in vitro* results were successfully reproduced here.
634 Fig 10 shows the droplet size changes of emulsions with three representatives of the ten
635 panellists. In all ten cases, emulsion W had larger increases in $D_{4,3}$ values than N at the time
636 points 10 s, 30 s and 60 s. There were large variations in the final droplet sizes from different
637 panellists, and this is attributed to the various saliva secretion rate and α-amylase level in
638 saliva among individuals (Carpenter, 2013). The microscopic images in Fig 11 again revealed
639 similar results as the *in vitro* experiment. W was destabilised by coalescence of droplets,
640 whereas N sample displayed the formation of flocs from submicron, otherwise intact droplets,
641 with no extensive levels of coalescence.

642 To further characterise the behaviour of W and N when digested by α-amylase in the oral
643 cavity, the Turbiscan backscattering profile (20 min) was obtained. Emulsions were mixed
644 with either artificial saliva or freshly collected human saliva, before starting the measurement.
645 Same characteristic behaviours of digested emulsions were observed for artificial saliva and
646 human saliva. Here in Fig 12, only the profiles for samples treated with human saliva are
647 shown, as human saliva was more potent in destabilising droplets and thus differences in
648 behaviours of W and N can be more clearly observed. A rapid phase separation can be seen in
649 W, which is a result of the large coalesced droplets creaming out. Destabilisation in N was
650 significantly slower than that in W sample. Any visible serum layer at the bottom of the
651 sample took a much longer time to form. Once again, this preliminary *in vivo* experiment
652 confirmed the findings of our *in vitro* investigations. We hope that the results provided here
653 will stimulate more extensive *in vivo* based studies involving larger and border range of
654 panellists, as well as including sensory aspects. Such trials are beyond the scope of the
655 present work, but no doubt are necessary to establish the idea of mixing of emulsions,
656 stabilised by hydrophobically modified starch of different amylose content, as a feasible way
657 of realising highly tailored novel controlled release vehicle in future.

658 # 4 Conclusion

Upon hydrophobic modification with OSA, both waxy maize starch (denoted by W) and normal corn starch (referred to here as N) were able to produce modified starch with satisfactory emulsifying and stabilising abilities. Emulsion stabilised with OS-W was highly resistant to pH and electrolyte concentration variations. This indicated the likely provision of strong steric repulsion as the main stabilising mechanism as provided by the adsorbed layers of this biopolymer. On the other hand, OS-N stabilised emulsions exhibited a significant degree of flocculation and an increase in droplet size, at acidic pH conditions as well as at high electrolyte concentrations. The interfacial films formed by OS-N seem to provide weaker steric forces, and therefore are more dependent on the presence of some electrostatic interactions than the OS-W ones. We have attributed this to the higher amylose content in OS-N modified starch and the possibly thinner surface layers it forms. There is some theoretical justification for this provided by some recent self-consistent field type calculations. It has been shown that the linear nature of hydrophobically modified amylose makes it more prone to forming bridging contacts between two closely spaced neighbouring oil droplets, as compared to a more compact layer formed by highly branched polymers (Ettelaie et al., 2016). It was also noticed that while aggregation in OS-N fabricated systems occurred quickly upon addition of salt, the subsequent droplet coalescence proceeded much more slowly. This was demonstrated here through dilution with SDS, resulting in a significant downward shift in the droplet size distribution of the aggregated system, even when carried out after 11 days of storage.

As for enzyme-induced destabilisation, both *in vitro* and *in vivo* oral digestion revealed more rapid increase in the size of emulsions stabilised by OS-W starch (lower amylose content) relative to those fabricated with OS-N (higher AC). Digestion of W emulsion was associated with extensive droplet coalescence. In contrast, α-amylase digestion of N samples led to flocculation but without appreciable coalescence, at least over shorter time scales (~ 20 min) post enzymatic treatment. This interesting difference in the behaviour of the two emulsions under enzymatic digestion, suggests that interfacial adsorbed films of modified starch of various amylose content are enzymatically degraded at differing rates. Thus, while OS-W layers provided stronger steric forces and therefore were not as sensitive to changes in pH or electrolyte concentration, they were more prone to enzymatic degradation and droplet destabilisation due to α-amylase activity.

The result is significant in that it provides the potential to generate a gradient of flavour release. In principle, one can fabricate emulsions stabilised by several hydrophobically

modified starch of different amylose content. The droplets include a required flavour or an active ingredient within the dispersed phase. These emulsions can then be mixed together at an appropriate ratio. When treated with enzymes, as for example during the oral consumption, droplets stabilised by different starch layers in the system will become destabilise at different rates. A desired controlled release profile can be engineered and simply tailored by changing the mix ratio of different emulsion droplets.

In future research, it would be interesting to investigate in greater depths the interfacial arrangements of amylose and amylopectin, details of their digestion by α-amylase, and any conformational changes occurring on the interface that is caused by the digestion process. Once such a deeper understanding and a better control of the structure and the amylose content of the modified starch becomes available, formulations for combining emulsions fabricated with various starches can be produced to tailor specifically desired release profiles, in the manner outlined above.

Acknowledgements

We would like to thank Brent S. Murray for valuable comments and discussions, and express our gratitude to Sheng Fang for his help in acquiring the NMR spectra. One of us MM, also wishes to thank Zhejiang Gongshang University for their hospitality during her stay in Hangzhou, where part of this work was carried out.

Reference

Ai, Y., Nelson, B., Birt, D. F., & Jane, J. L. (2013). In vitro and in vivo digestion of octenyl succinic starch. *Carbohydr Polym, 98*(2), 1266-1271.

Akhtar, M., & Dickinson, E. (2007). Whey protein–maltodextrin conjugates as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids, 21*(4), 607-616.

Arshad, H., Ali, T. M., & Hasnain, A. (2018). Native and modified Sorghum starches as wall materials in microencapsulation of nutmeg oleoresin. *Int J Biol Macromol, 114*, 700-709.

Bai, Y., Kaufman, R. C., Wilson, J. D., & Shi, Y. C. (2014). Position of modifying groups on starch chains of octenylsuccinic anhydride-modified waxy maize starch. *Food Chem, 153*, 193-199.

Bajaj, R., Singh, N., & Kaur, A. (2019). Properties of octenyl succinic anhydride (OSA) modified starches and their application in low fat mayonnaise. *Int J Biol Macromol, 131*, 147-157.

Barnes, H. A., Hutton, J. F., & Walters, K. (1989). *An introduction to rheology* (Vol. 3): Elsevier.

Benichou, A., Aserin, A., & Garti, N. (2002). Protein-Polysaccharide Interactions for Stabilization of Food Emulsions. *Journal of Dispersion Science and Technology, 23*(1-3), 93-123.

Bhosale, R., & Singhal, R. (2006). Process optimization for the synthesis of octenyl succinyl derivative of waxy corn and amaranth starches. *Carbohydrate Polymers, 66*(4), 521-527.

Burgaud, I., Dickinson, E., & Nelson, P. V. (1990). An improved high-pressure homogenizer for making fine emulsions on a small scale. *International Journal of Food Science and Technology*(25), 39-46.

Carpenter, G. H. (2013). The secretion, components, and properties of saliva. *Annu Rev Food Sci Technol, 4*, 267-276.

Chanamai, R., & McClements, D. J. (2001). Depletion Flocculation of Beverage Emulsions by Gum Arabic and Modified Starch. *Journal of Food Science, 66*(3), 457-463.

Chanamai, R., & McClements, D. J. (2002). Comparison of Gum Arabic, Modified Starch, and Whey Protein Isolate as

746 Emulsifiers: Influence of pH, CaCl2 and Temperature. *Journal*
747 *of Food Science, 67*, 120-125.
748 Cheuk, S. Y., Shih, F. F., Champagne, E. T., Daigle, K. W., Patindol,
749 J. A., Mattison, C. P., & Boue, S. M. (2015). Nano-
750 encapsulation of coenzyme Q10 using octenyl succinic
751 anhydride modified starch. *Food Chem, 174*, 585-590.
752 Cruz-Benítez, M. M., Gómez-Aldapa, C. A., Castro-Rosas, J.,
753 Hernández-Hernández, E., Gómez-Hernández, E., & Fonseca-
754 Florido, H. A. (2019). Effect of amylose content and chemical
755 modification of cassava starch on the microencapsulation of
756 Lactobacillus pentosus. *Lwt, 105*, 110-117.
757 Dai, L., Bergfreund, J., Reichert, C. L., Fischer, P., & Weiss, J. (2019).
758 Shear rheological properties of acid hydrolyzed insoluble
759 proteins from Chlorella protothecoides at the oil-water interface.
760 *J Colloid Interface Sci, 551*, 297-304.
761 Davis, R. E., Hartman, C. W., & Fincher, J. H. (1971). Dialysis of
762 ephedrine and pentobarbital from whole human saliva and
763 simulated saliva. *J Pharm Sci, 60*(3), 429-432.
764 Demetriades, K., & McClements, D. J. (2000). Influence of sodium
765 dodecyl sulfate on the physicochemical properties of whey
766 protein-stabilized emulsions. *Colloids and Surfaces A:*
767 *Physicochemical and Engineering Aspects, 161*, 391-400.
768 Dickinson, E. (2013). Stabilising emulsion-based colloidal structures
769 with mixed food ingredients. *J Sci Food Agric, 93*(4), 710-721.
770 Dickinson, E. (2018). Hydrocolloids acting as emulsifying agents –
771 How do they do it? *Food Hydrocolloids, 78*, 2-14.
772 Dickinson, E. (2019). Strategies to control and inhibit the flocculation
773 of protein-stabilized oil-in-water emulsions. *Food Hydrocolloids,*
774 *96*, 209-223.
775 Dobosz, A., Sikora, M., Krystyjan, M., Tomasik, P., Lach, R.,
776 Borczak, B., Berski, W., & Lukasiewicz, M. (2019). Short- and
777 long-term retrogradation of potato starches with varying
778 amylose content. *J Sci Food Agric, 99*(5), 2393-2403.
779 Dresselhuis, D. M., de Hoog, E. H. A., Cohen Stuart, M. A.,
780 Vingerhoeds, M. H., & van Aken, G. A. (2008). The occurrence
781 of in-mouth coalescence of emulsion droplets in relation to
782 perception of fat. *Food Hydrocolloids, 22*(6), 1170-1183.

Ettelaie, R., Holmes, M., Chen, J., & Farshchi, A. (2016). Steric stabilising properties of hydrophobically modified starch: Amylose vs. amylopectin. *Food Hydrocolloids, 58*, 364-377.
Ettelaie, R., Zengin, A., & Lee, H. (2014). Fragmented proteins as food emulsion stabilizers: A theoretical study. *Biopolymers, 101*(9), 945-958.
Ettelaie, R., Zengin, A., & Lishchuk, S. V. (2017). Novel food grade dispersants: Review of recent progress. *Current Opinion in Colloid & Interface Science, 28*, 46-55.
Fredriksson, H., Björck, I., Andersson, R., Liljeberg, H., Silverio, J., Eliasson, A. C., & Åman, P. (2000). Studies on α-amylase degradation of retrograded starch gels from waxy maize and high-amylopectin potato. *Carbohydrate Polymers, 43*(1), 81-87.
Garcia-Tejeda, Y. V., Salinas-Moreno, Y., Barrera-Figueroa, V., & Martinez-Bustos, F. (2018). Preparation and characterization of octenyl succinylated normal and waxy starches of maize as encapsulating agents for anthocyanins by spray-drying. *J Food Sci Technol, 55*(6), 2279-2287.
He, G., Song, X., Ruan, H., & Chen, F. (2006). Octenyl Succinic Anhydride Modified Early Indica Rice Starches Differing in Amylose Content. *Journal of Agricultural and Food Chemistry, 54*, 2775-2779.
Hoover, R., & Ratnayake, W. S. (2001). Determination of Total Amylose Content of Starch. *Current Protocols in Food Analytical Chemistry*, E2.3.1-E2.3.5

Hu, X., Karthik, P., & Chen, J. (2019). Manipulating oral behaviour of food emulsions using different emulsifiers. *International Journal of Food Science & Technology, 54*(7), 2408-2415.
Huber, K. C., & BeMiller, J. N. (2000). Channels of maize and sorghum starch granules. *Carbohydr Polym, 41*, 269-276.
Hunter, R. J. (2001). *Foundations of colloid science*: Oxford university press.
Jeong, H.-Y., & Lim, S.-T. (2003). Crystallinity and Pasting Properties of Freeze-Thawed High Amylose Maize Starch. *Starch - Stärke, 55*(11), 511-517.

Karthik, P., Ettelaie, R., & Chen, J. (2019). Oral behaviour of emulsions stabilized by mixed monolayer. *Food Res Int, 125*, 108603.

Kato, A., Sasaki, Y., Furuta, R., & Kobayashi, K. (1990). Functional Protein polysaccharide conjugate prepared by controlled dry heating of ovalbumin dextran mixtures. *Agric. Biol. Chem., 54*, 107-112.

Lin, Q., Liang, R., Zhong, F., Ye, A., & Singh, H. (2018a). Effect of degree of octenyl succinic anhydride (OSA) substitution on the digestion of emulsions and the bioaccessibility of β-carotene in OSA-modified-starch-stabilized-emulsions. *Food Hydrocolloids, 84*, 303-312.

Lin, Q., Liang, R., Zhong, F., Ye, A., & Singh, H. (2018b). Physical properties and biological fate of OSA-modified-starch-stabilized emulsions containing β-carotene: Effect of calcium and pH. *Food Hydrocolloids, 77*, 549-556.

Liu, W., Li, Y., Goff, H. D., Nsor-Atindana, J., & Zhong, F. (2018). Distribution of octenylsuccinic groups in modified waxy maize starch: An analysis at granular level. *Food Hydrocolloids, 84*, 210-218.

Lopez-Silva, M., Bello-Perez, L. A., Agama-Acevedo, E., & Alvarez-Ramirez, J. (2019). Effect of amylose content in morphological, functional and emulsification properties of OSA modified corn starch. *Food Hydrocolloids, 97*.

Lopez-Silva, M., Bello-Perez, L. A., Castillo-Rodriguez, V. M., Agama-Acevedo, E., & Alvarez-Ramirez, J. (2020). In vitro digestibility characteristics of octenyl succinic acid (OSA) modified starch with different amylose content. *Food Chem, 304*, 125434.

Matos, M., Laca, A., Rea, F., Iglesias, O., Rayner, M., & Gutiérrez, G. (2018). O/W emulsions stabilized by OSA-modified starch granules versus non-ionic surfactant: Stability, rheological behaviour and resveratrol encapsulation. *Journal of Food Engineering, 222*, 207-217.

Miao, M., Li, R., Jiang, B., Cui, S. W., Zhang, T., & Jin, Z. (2014). Structure and physicochemical properties of octenyl succinic

esters of sugary maize soluble starch and waxy maize starch. *Food Chem, 151*, 154-160.
Mokni Ghribi, A., Maklouf Gafsi, I., Sila, A., Blecker, C., Danthine, S., Attia, H., Bougatef, A., & Besbes, S. (2015). Effects of enzymatic hydrolysis on conformational and functional properties of chickpea protein isolate. *Food Chem, 187*, 322-330.
Mu, M., Farshchi, A., Holmes, M., Chen, J., & Ettelaie, R. (2019). Effect of storage temperature and relative humidity on long-term colloidal stability of reconstitutable emulsions stabilised by hydrophobically modified starch. *Food Hydrocolloids, 95*, 62-75.
Nilsson, L., & Bergenstahl, B. (2006). Adsorption of Hydrophobically Modified Starch at Oil/Water Interfaces during Emulsification. *Langmuir, 22*, 8770-8776.
Patel, H., Royall, P. G., Gaisford, S., Williams, G. R., Edwards, C. H., Warren, F. J., Flanagan, B. M., Ellis, P. R., & Butterworth, P. J. (2017). Structural and enzyme kinetic studies of retrograded starch: Inhibition of alpha-amylase and consequences for intestinal digestion of starch. *Carbohydr Polym, 164*, 154-161.
Pickering, S. (1907). Cxcvi.—emulsions. *Journal of the Chemical Society, Transactions, 91*, 2001-2021.
Randall, R., Phillips, G., & Williams, P. (1988). The role of the proteinaceous component on the emulsifying properties of gum arabic. *Food Hydrocolloids, 2*(2), 131-140.
Ruan, H., Chen, Q.-h., Fu, M.-l., Xu, Q., & He, G.-q. (2009). Preparation and properties of octenyl succinic anhydride modified potato starch. *Food Chemistry, 114*(1), 81-86.
Samakradhamrongthai, R., Thakeow, P., Kopermsub, P., & Utama-Ang, N. (2016). Microencapsulation of white champaca (Michelia alba D.C.) extract using octenyl succinic anhydride (OSA) starch for controlled release aroma. *J Microencapsul, 33*(8), 773-784.
Sarkar, A., Goh, K. K. T., & Singh, H. (2009). Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva. *Food Hydrocolloids, 23*(5), 1270-1278.
Sarkar, A., Ye, A., & Singh, H. (2017). Oral processing of emulsion systems from a colloidal perspective. *Food Funct, 8*(2), 511-521.

Schmitz, S., Dona, A. C., Castignolles, P., Gilbert, R. G., & Gaborieau, M. (2009). Assessment of the extent of starch dissolution in dimethyl sulfoxide by 1H NMR spectroscopy. *Macromol Biosci, 9*(5), 506-514.

Shingel, K. I. (2002). Determination of structural peculiarities of dexran, pullulan and gamma-irradiated pullulan by Fourier-transform IR spectroscopy. *Carbohydrate Research, 337*, 1445-1451.

Shogren, R. L., Viswanathan, A., Felker, F., & Gross, R. A. (2000). Distribution of Octenyl Succinate Groups in Octenyl Succinic Anhydride Modified Waxy Maize Starch. *Starch/Stärke, 52*, 196-204.

Sikora, M., Krystyjan, M., Dobosz, A., Tomasik, P., Walkowiak, K., Masewicz, L., Kowalczewski, P. L., & Baranowska, H. M. (2019). Molecular Analysis of Retrogradation of Corn Starches. *Polymers (Basel), 11*(11).

Silletti, E., Vingerhoeds, M. H., Norde, W., & van Aken, G. A. (2007). The role of electrostatics in saliva-induced emulsion flocculation. *Food Hydrocolloids, 21*(4), 596-606.

Simsek, S., Ovando-Martinez, M., Marefati, A., Sjoo, M., & Rayner, M. (2015). Chemical composition, digestibility and emulsification properties of octenyl succinic esters of various starches. *Food Res Int, 75*, 41-49.

Smith, M. E., & Morton, D. G. (2010a). 2 - THE MOUTH, SALIVARY GLANDS AND OESOPHAGUS. In M. E. Smith & D. G. Morton (Eds.), *The Digestive System (Second Edition)* (pp. 19-38): Churchill Livingstone.

Smith, M. E., & Morton, D. G. (2010b). 8 - DIGESTION AND ABSORPTION. In M. E. Smith & D. G. Morton (Eds.), *The Digestive System (Second Edition)* (pp. 129-152): Churchill Livingstone.

Song, X., Pei, Y., Zhu, W., Fu, D., & Ren, H. (2014). Particle-stabilizers modified from indica rice starches differing in amylose content. *Food Chem, 153*, 74-80.

Song, X., Zhao, Q., Li, Z., Fu, D., & Dong, Z. (2013). Effects of amylose content on the paste properties and emulsification of octenyl succinic starch esters. *Starch - Stärke, 65*(1-2), 112-122.

Sopade, P., & Filibus, T. (1995). The influence of solid and sugar contents on rheological characteristics of akamu, a semi-liquid maize food. *Journal of Food Engineering, 24*(2), 197-211.
Sweedman, M. C., Hasjim, J., Schafer, C., & Gilbert, R. G. (2014). Structures of octenylsuccinylated starches: effects on emulsions containing beta-carotene. *Carbohydr Polym, 112*, 85-93.
Sweedman, M. C., Schafer, C., & Gilbert, R. G. (2014). Aggregate and emulsion properties of enzymatically-modified octenylsuccinylated waxy starches. *Carbohydr Polym, 111*, 918-927.
Tesch, S., Gerhards, C., & Schubert, H. (2002). Stabilization of emulsions by OSA starches. *Journal of Food Engineering, 54*, 167-174.
Wang, C., He, X., Fu, X., Huang, Q., & Zhang, B. (2016). Substituent distribution changes the pasting and emulsion properties of octenylsuccinate starch. *Carbohydr Polym, 135*, 64-71.
Wang, C., He, X., Huang, Q., Fu, X., Luo, F., & Li, L. (2013). Distribution of octenylsuccinic substituents in modified A and B polymorph starch granules. *J Agric Food Chem, 61*(51), 12492-12498.
Whitney, K., Reuhs, B. L., Ovando Martinez, M., & Simsek, S. (2016). Analysis of octenylsuccinate rice and tapioca starches: Distribution of octenylsuccinic anhydride groups in starch granules. *Food Chem, 211*, 608-615.
Wijaya, W., Patel, A. R., Setiowati, A. D., & Van der Meeren, P. (2017). Functional colloids from proteins and polysaccharides for food applications. *Trends in Food Science & Technology, 68*, 56-69.
Zhao, S., Tian, G., Zhao, C., Lu, C., Bao, Y., Liu, X., & Zheng, J. (2018). Emulsifying stability properties of octenyl succinic anhydride (OSA) modified waxy starches with different molecular structures. *Food Hydrocolloids, 85*, 248-256.
Zhou, X., Chung, H. J., Kim, J. Y., & Lim, S. T. (2013). In vitro analyses of resistant starch in retrograded waxy and normal corn starches. *Int J Biol Macromol, 55*, 113-117.