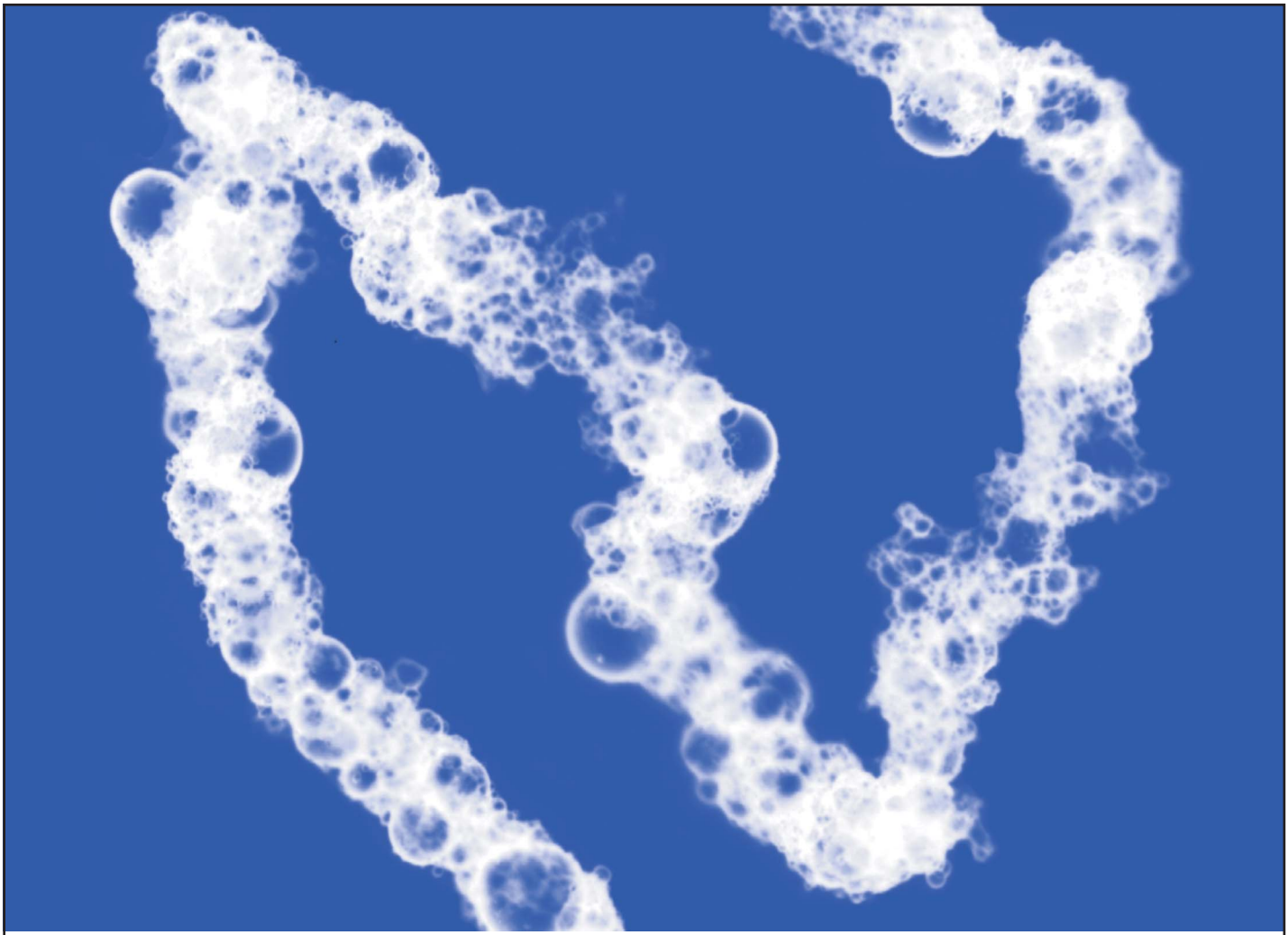

Showcasing research by A.R. Patel and K.P. Velikov *et al.* under the Molecular Structure of Food program in NanoNextNL at Unilever R&D Vlaardingen, The Netherlands.

Title: Fabrication and characterization of emulsions with pH responsive switchable behaviour

We report the fabrication of pH responsive switchable emulsions that quickly destabilize at acidic pH, but switch back to a stable state when pH is neutralized. The pH triggered flocculation is further used to influence the assembly of droplets into predetermined shapes. Such systems, stabilised using natural non-toxic components, can find important applications in the field of green colloidal structuring.

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Fabrication and characterization of emulsions with pH responsive switchable behavior†

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We report the fabrication of pH responsive switchable emulsions that quickly destabilise at acidic pH, but switch back to the stable state when the pH is neutralized. The pH triggered flocculation is further used to influence the assembly of droplets into predetermined shapes. Such systems, stabilised using natural and non-toxic components, can find important applications in the field of green colloidal structuring.

Colloidal systems such as emulsions with an on–off property have attracted a lot of interest recently because of their potential applications ranging from oil recovery[1] and template for emulsion polymerization[2] to heavy oil transportation.[3] At the same time, controlled stabilization and destabilization of emulsions could also find interesting applications in bio-related fields to release large payloads of functional bioactives on-demand for desired biological purposes.[4]

Due to the anticipated potential of such reversible emulsions, a great deal of effort has been recently put into the development of pH responsive particulate-based emulsifiers such as pH-responsive latexes,[5] stimulus-responsive microgel particles,[6] nanocomposite particles,[7] hydroxyapatite nanoparticles,[8] cross-linked copolymer micelles,[9] layered double hydroxide particles[10] and surface modified alumina coated silica nanoparticles.[11] However, these emulsifiers require a significant synthesis effort and the universal applications of some of these particles (such as in foods and pharmaceuticals) are severely limited due to the hazardous and toxic nature of the materials used.

Coacervates and complexes of natural biopolymers such as proteins and polysaccharides have been used for a long time in food and related fields for oil–water interfacial stabilization where usually the primary emulsion stabilised by an emulsifier is coated with coacervates *via* electrostatic deposition.[12,13] Xanthan gum (XG) – a natural, food grade biopolymer[14,15] is one material which is widely used in conjunction with proteins and water soluble celluloses to generate functional coacervates.[16–21] As previously reported by us, XG also interacts non-covalently with another natural material, shellac (SL)[22] leading to the formation of colloidal coacervates.[23]

In the current paper, we exploited the colloidal interaction of XG with SL at the oil–water interface to generate stable oil-in-water (o/w) emulsions with oil content as high as 60 wt%. Furthermore, due to the pH dependent solubility profile of SL (which is soluble in aqueous alkali but resistant to acid),[24] the emulsions demonstrated pH responsive switching from stable to flocculated and back to the stable state based on a simple alteration of the pH between neutral and acidic.

XG is a hydrocolloid which is used for a variety of applications involving structuring and thickening of the aqueous based products.[25,26] XG is not an emulsifier but it has been reported to stabilize o/w emulsions (at a low oil volume fraction) by modifying the rheology of the continuous phase.[27,28] The emulsion stabilizing property of XG has also been demonstrated in combination with one or more surface active proteins.[29–31] However, on its own, XG does not offer enough emulsion stabilization, as also observed from our experiments. Fig. 1a shows the optical microscopy image of an o/w emulsion prepared at 10 wt% oil and stabilized using 0.25 wt% XG. The emulsion showed coalescence within 2 hours of its preparation suggesting the ineffectiveness of XG in stabilising the emulsion. Similarly, SL in the dissolved state, has also not been shown to have surface activity at oil–water interfaces and there are no reports on the use of shellac in emulsion stabilisation. Accordingly, we observed that the emulsions prepared using SL as a stabilizer (at a concentration ranging from 0.25 to 2.5 wt%) showed quick coalescence within 30 minutes of the preparation (Fig. 1b).

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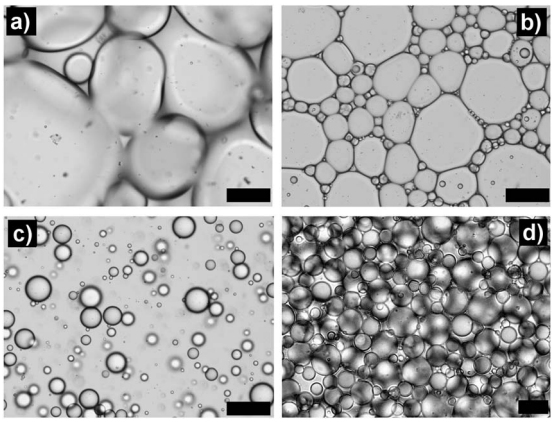

Fig. 1 Optical microscopy images of (a) emulsion prepared at 10 wt% oil and stabilized using 0.25 wt% XG. The emulsion showed coalescence within 2 hours of its preparation (scale bar = 50 μm); (b) emulsion prepared at 10 wt% oil and stabilized using 2.5 wt% SL. The emulsion showed coalescence within 30 minutes of its preparation (scale bar = 50 μm), (c) and (d) emulsion stabilized using XG : SL, 1 : 1 wt ratio at a concentration of XG equivalent to 0.25 wt% with the lowest and the highest oil contents (10 and 60 wt% respectively) (scale bar = 50 and 20 μm respectively).

As reported by us recently, colloidal interactions can be used to enhance the interfacial stabilization property of hydrocolloids.[32,33] On a similar line, the colloidal interaction between XG and SL was exploited in the current work to fabricate stable emulsions at varying levels of oil, 10–60 wt% (Fig. 1c and d). The average droplet size in all cases was in the range of 10–25 μm (Fig. 2a). The value of average droplet size at constant oil content (10 wt%) decreased with the increase in the SL proportion (Fig. 2b). This could be attributed to the relatively better surface active property of SL compared to XG. This was also confirmed from the fact that with the increase in the SL proportion, emulsion samples showed increased foaming during the preparation.

XG is an anionic polysaccharide and has a strong negative charge at neutral pH.[23,34–36] Accordingly, emulsions prepared with only XG as a stabiliser showed a zeta potential value of −80.7 mV. SL too is negatively charged at neutral pH due to the ionization of hydroxy acids.[22,37] Thus, the emulsions prepared with 10 wt% oil and stabilized at varying XG : SL proportions showed highly negative zeta potential values (in the range of −84 to −111 mV). Emulsions prepared at different oil contents (10–60 wt%) and varying XG : SL ratios were characterized over the storage period (number of days) for average droplet sizes. Based on the characterisation data over the storage period, the emulsions showed good stability against coalescence with no changes in the average droplet sizes and morphology, as viewed under the microscope. It can be assumed that the electrostatic repulsion of highly charged surfaces of oil droplets contributes to the enhanced stability of the emulsions. Fig. 3 shows the plot of average droplet sizes recorded for emulsions at 10 wt% oil (stabilized with varying XG : SL ratios) over the storage period of 18 days at 4 °C. Analysis beyond this period was not possible due to the observation of microbial growth in the samples.

The interactions of XG and SL were evaluated based on the binding parameters obtained from isothermal titration calorimetry (ITC). Raw data plot of heat flow against time and the corresponding plot after integration and normalisation are shown in Fig. 4. SL has an amphiphilic nature and the polar groups of the hydroxy acids are believed to be oriented towards the interface of the SL molecules.[38] The blank titration of SL in water resulted in endothermic peaks, probably indicating the dissociation of self-associated SL due to the dilution. In the

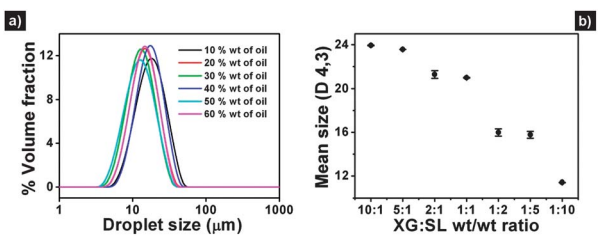

Fig. 2 (a) Droplet size distribution curves for emulsions stabilized by XG : SL = 1 : 1 wt ratio (XG concentration equivalent to 0.25 wt%) with an oil content ranging from 10–60 wt% and (b) volume weighted mean droplet size of emulsions prepared at 10 wt% oil and stabilized with varying XG : SL ratios (10 : 1 to 1 : 10 wt ratios).

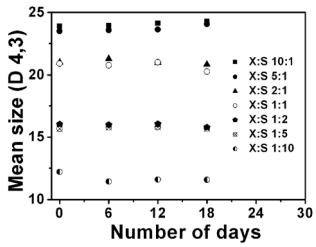

Fig. 3 Average droplet sizes of emulsions prepared at 10 wt% oil and stabilized at varying XG : SL ratios (10 : 1 to 1 : 10 wt/wt). The droplet sizes showed no apparent changes over 18 days of storage at 4 °C.

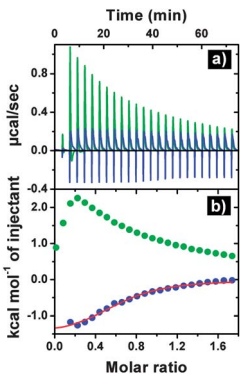

Fig. 4 (a) Raw data plot of heat flow against time for the titration of 6.25 mM SL into 0.033 mM XG. Each peak indicates the heat change associated with injection of 10 μL aliquots of SL solution into a calorimeter cell containing the XG solution. A negative heat flow represents an exothermic change and a positive peak represents an endothermic change; (b) corresponding plot after integration of peak areas and normalisation with respect to injectant concentration to yield a plot of molar enthalpy change (ΔH_{obs}) against the molar ratio of XG : SL. Green symbols represent the blank titration of SL in water.

presence of XG, the heat of binding recorded by ITC displayed a complex pattern with the successive appearance of an exothermic and endothermic signal. The occurrence of such complex signals has been previously reported to be induced by the aggregation of complexes[39,40] or coacervation.[41]

The integrated and normalized curve showed a sigmoidal shape (Fig. 4b) with saturation reached at a molar ratio of XG : SL > 1 : 6. Interactions with a binding constant (K_a > 10 000 M^{-1}), are considered to be of high affinity.[42] Thus, the high binding constant (8.4×10^4 M^{-1}) observed in the study suggested a strong interaction between XG and SL. The stoichiometry ($n = 0.6$) suggests that approximately two molecules of SL bind to one molecule of XG. Since both XG and SL are negatively charged, there is no ionic interaction between these two molecules. However, due to the presence of numerous hydroxyl groups in SL molecules, the interaction can be based on hydrogen bonding. XG has also been reported to interact with macromolecules *via* non-electrostatic, hydrophobic interactions.[16,43] The low and negative value of binding enthalpy ($\Delta H = -1.6$ kJ mol^{-1}) confirms that interaction between XG and SL was non-covalent in nature (probably hydrogen bonding and hydrophobic interactions). The free energy of binding ($\Delta G = -6.67$ kJ mol^{-1}) was negative, which is a requirement for spontaneous non-covalent interactions involving biopolymers.[44,45] The negative value of entropy ($\Delta S = -0.017$ kJ mol^{-1} K^{-1}) also suggests an increase in molecular order, which would occur upon aggregation and may imply a role of hydrogen bonding in the formation of the complex.[45,46] The property of shellac to participate in hydrogen bonding with hydrocolloids (such as pectin and cellulose derivatives) is attributed to the presence of its main component (hydroxy aliphatic fatty acid – aleuritic acid) which has a large number of carboxylic and hydroxyl groups.[47,48] Hence, we can assume that aleuritic acid is mainly responsible for the strong interactions between shellac and xanthan gum.

The XG : SL complexes formed due to the strong interactions between these two molecules could be considered responsible for the Pickering type stabilization of emulsion resulting in better stability than the emulsions prepared using individual compounds as stabilizers. Pickering type stabilization using natural materials is currently a topic of widespread interest among researchers from varied disciplines.[49–54]

SL has a pH dependent solubility which has been exploited extensively in the pharmaceutical industry to formulate enteric release systems.[22,55] Due to the presence of a large number of hydroxy acids, SL can be solubilised at alkaline pH whereas at acidic pH, it is practically insoluble.[24] As reported in our earlier study, SL colloidal particles prepared at neutral to alkaline pH (~7.2) show instant aggregation in acidic medium due to the acid resistant nature of SL.[23] This pH dependent behaviour of SL was used in the current study to impart pH responsiveness to the stabilized emulsions. The emulsions showed instant destabilization (flocculation) when the pH was changed from neutral to acidic (pH 1.2). On neutralizing the pH, flocculated emulsion could be switched back to the stabilized state. Fig. 5a shows the pH switching behaviour of emulsion (prepared at 10 wt% oil and stabilized by 1 : 1 wt/wt of XG : SL).

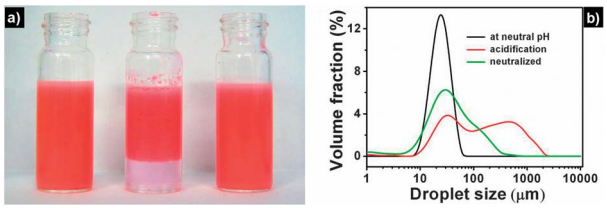

Fig. 5 (a) Photographs of emulsion prepared at 10 wt% oil and stabilized using a 1 : 1 wt/wt mixture of XG : SL. From left to right: stable emulsion prepared at neutral pH; flocculated emulsion after acidification (using 0.1 N HCl) and stable emulsion neutralized back to neutral pH (using 0.1 N NaOH), the emulsion was prepared with fluorescently labelled XG and the oil phase enriched with Nile red; (b) droplet size distribution curves for emulsion samples after acidification and neutralization.

For clear visualization, the emulsion was prepared with fluorescently labelled XG (synthesized in-house by covalently linking rhodamine B to XG) and the oil phase enriched with Nile red. The photograph clearly demonstrates the stabilization and destabilization of emulsion based on the pH changes. The stable emulsion prepared at neutral pH showed instant flocculation on changing the pH to acidic (~1.2), neutralizing the pH back to 7.2 resulted in switching the emulsion back to the stabilized state assisted by mild shaking. The droplet size distribution curves for the emulsion sample after acidification and neutralization are shown in Fig. 5b. The destabilization of emulsion at acidic pH is clearly represented by the appearance of double peaks; neutralization of the same sample shifted the curve back to normal with a single peak. After neutralization, the droplet size was recorded without use of any homogenization (only mild shaking), this explains the difference in the shape of curves for the emulsion prepared at neutral pH and the neutralized emulsion.

The pH dependent changes in the sample characterized using optical microscopy are shown in Fig. 6a. The discrete oil droplets showed flocculation at acidic pH resulting in phase separation with XG : SL covered oil droplets separating from the

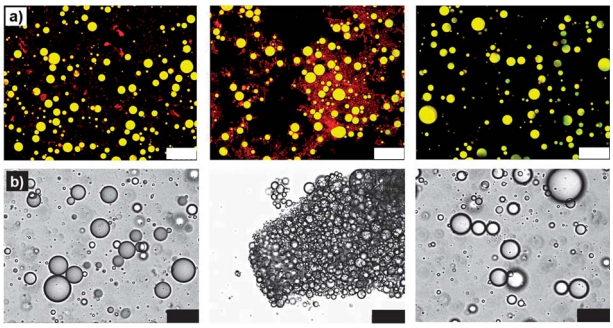

Fig. 6 (a) Confocal microscopy images of emulsion prepared at 10 wt% oil and stabilized using a 1 : 1 w/w mixture of XG : SL. From left to right: stable emulsion prepared at neutral pH; flocculated emulsion after acidification and stable emulsion neutralized back to neutral pH (scale bars = 50 μm); (b) optical microscopy images of emulsion prepared at 10 wt% oil and stabilized using a 1 : 1 w/w mixture of XG : SL. From left to right: stable emulsion prepared at neutral pH (scale bar 50 μm); flocculated emulsion after acidification (scale bar 100 μm) and stable emulsion neutralized back to neutral pH (scale bar 50 μm).

bulk. However, the oil droplets did not coalesce with each other. This observation can be attributed to the precipitation of the XG : SL complex from the solution due to the insoluble nature of SL at acidic pH. Because the oil droplets did not coalesce together, it was easier to obtain a stabilized emulsion by changing the pH back to neutral. To know exactly the location of XG during acidification, confocal microscopy images (Fig. 6b) were taken of emulsion prepared using rhodamine B labelled XG. From the images, it can be assumed that the oil droplets are stabilized by the network of XG : SL, as the signal from fluorescence of XG is seen in the bulk phase as well. On acidification, the XG : SL network stabilizing the oil droplets phase separates, resulting in the flocculation of the emulsion. On changing the pH back to neutral, the XG : SL gets re-dispersed in the bulk phase leading to the stabilization of emulsion with discrete oil droplets.

The pH dependent flocculation of these emulsions was further utilized to influence the assembly of oil droplets into predetermined shapes by simply extruding the emulsion using a syringe and a needle in an acidic medium. Fig. 7a shows the photograph of an extruded emulsion in the shape of alphabets, microscopy image provided as Fig. 7b shows the confined flocculation of emulsion droplets. In this way, we demonstrated a simple exploitation of the pH triggered flocculation of these emulsions to prepare soft structures with controlled shapes.

In conclusion, we successfully utilized the molecular interactions of XG and SL to stabilize emulsions with a high oil content (up to 60 wt%). The emulsions showed destabilization (flocculation without coalescence) at acidic pH and could be switched to stable emulsion merely by changing pH to neutral accompanied by mild shaking. This is an important aspect of these emulsions which could be used to deliver oil-soluble actives orally, where the release could be controlled based on the pH of the gastrointestinal tract. Moreover, the food grade status and naturalness of both these components extend its usage as surfactant-free, natural stabilisers for food products.

The components are non-hazardous and abundantly available at cheap rates, thus the pH switching behaviour of these emulsions can also find important applications in fields such as oil recovery where triggered stabilization and destabilization is required.

Fig. 7 (a) Photograph of concentrated emulsion (prepared at 60 wt% oil and stabilized using a 1 : 1 w/w mixture of XG : SL) extruded using a syringe and a needle in an acidic medium and (b) microscopy image of the extruded strand of emulsion droplets (scale bars = 400 μm).

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