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Abstract

There is a great deal of interest in the Food Industry in the use of polysaccharides and proteins to stabilise oil-in-water emulsions and there is particular interest nowadays in the use of polysaccharide–protein complexes. There are three classes of complexes namely; (a) naturally-occurring complexes in which protein residues are covalently attached to the polysaccharide chains as is the case, for example, with gum Arabic; (b) Maillard conjugates, which are formed by interaction of the reducing end of a polysaccharide with an amine group on a protein forming a covalent bond; and (c) electrostatic complexes formed between a polysaccharide and a protein with opposite net charge. This review sets out our current understanding of the nature of these different polysaccharide-protein complexes and their ability to stabilise oil-in-water emulsions.

Introduction

Proteins have amphiphilic characteristics and as such are able to adsorb strongly at the oil-water interface. They are commonly used in the Food Industry as emulsifiers in the stabilisation of oil-in-water emulsions [1-5]. The amount adsorbed and the conformation adopted at the oil-water interface will depend very much on the protein amino acid composition since adsorption occurs through hydrophobic groups present within their structure. Once adsorbed, the molecules can unfold in order to maximise the number of hydrophobic groups that are in contact with the surface enabling the hydrophilic groups to re-arrange and protrude away from the surface into the aqueous phase. Interaction can sometimes occur between adjacent adsorbed protein molecules through hydrophobic bonding or disulphide bond formation leading to the formation of a viscoelastic layer at the oil-water interface as has been demonstrated by surface rheology and atomic force microscopy [6].

The adsorbed protein molecules are able to stabilise emulsions by preventing droplet aggregation and coalescence through electrostatic and / or steric repulsive forces [1,2,4,5]. However, they have limitations in so much that, at the protein isoelectric point, the protein molecules will have a net zero charge and unless the adsorbed layer thickness is large enough, droplet aggregation will occur. A further cause of instability in oil-in-water emulsions is Ostwald ripening which becomes particularly important for emulsions containing water soluble oils, for example, flavour oils such as limonene which is used in beverages [3,5]. This phenomenon occurs as a consequence of that fact that the solubility of the oil increases as the size of the droplet decreases. The higher concentration of dissolved material surrounding the smaller droplets results in a concentration gradient and so the dissolved molecules move from the smaller droplets to the larger droplets giving rise to an overall

increase in droplet size. The most widely used emulsifier for the stabilisation of flavour oil-in-water emulsions is gum Arabic and its effectiveness is most likely due to the fact that it forms a thick adsorbed layer as will be discussed in more detail below. Gum arabic is a complex polysaccharide that contains certain fractions which are rich in protein and it has been shown that the protein is covalently attached to the polysaccharide moieties [7]. Currently, there is considerable interest in identifying other naturally-occurring polysaccharide–protein complexes and the material that has received most attention in recent years is pectin and in particular, sugar beet pectin [8-14]. The concept that polysaccharide–protein complexes can stabilise oil-in-water emulsions has led to the development of gum arabic look-alike molecules, that is, polysaccharide-protein conjugates formed through the Maillard reaction. This area of study was initiated by Kato et al (1990, 1992, 1993) and is still an active field of research today [15-17]. A further area that has received considerable attention in recent years is the use of polysaccharide-protein electrostatic complexes as emulsifiers particularly in the encapsulation of active compounds [18-21]. The complexes are formed between anionic polysaccharides and proteins at pH values below the protein isoelectric point where they carry a net positive charge. One approach, first reported by Bungeberg de Jong [22], is to use polysaccharide-protein coacervates as emulsifiers [23] but more recent interest has been given to the use of soluble electrostatic complexes [24, 25]. A second approach is to form an emulsion using a protein as the emulsifier and then add the polysaccharide which adsorbs onto the protein layer to form a secondary layer on top [26].

Naturally-occurring polysaccharide-protein complexes

Gum Arabic is commonly used as an emulsifier in the production of flavour oil-in-water emulsions for application in the Beverage Industry [27-30]. It is a tree gum exudate obtained from the stems and branches of acacia trees (*Acacia senegal* and *Acacia seyal*) which grow across the Sahelian belt in Africa, notably in Sudan, Chad and Nigeria. It is a complex, highly branched polysaccharide consisting of a core of $\beta-1,3$ linked galactose residues with branches consisting of galactose, arabinose, rhamnose and glucuronic acid. It has also been shown to contain a small amount of protein (~2.5% for gum from *Acacia senegal*) as an integral part of its structure. It is now recognised that the gum consists of three main fractions which differ mainly in their molecular size and protein contents. The bulk of the gum (90%) is referred to as the arabinogalactan fraction (AG). It has a molecular mass of 2-3 $x10^5$ g/mol and contains very little protein and has been reported to have a disk-like structure [31]. The second major component is referred to as the arabinogalactan-protein fraction (AGP) which represents ~10% of the gum. The AGP has a molecular mass of ~ 1-2 x 10^6 g/mol and contains about 10% protein. It consists of a polypeptide chain of ~250 amino acids with blocks of carbohydrate with molecular mass ~4 x 10^4 g/mol covalently attached

through serine and hydroxyproline residues [26]. The third minor fraction, referred to as the glycoprotein fraction (GP) represents ~1% of the total gum. It has a molecular mass of ~2 x 10^5 g/mol and contains 20-50% protein but little is known about its structure. The importance of the proteinaceous components within the gum to the emulsification properties was first recognised by Randall et al (1988) who demonstrated that protein-rich fractions adsorbed preferentially onto the surface of orange oil droplets and this has since been supported by the work of others [32,33]. Removal of the protein by treatment with proteolytic enzyme greatly reduces the emulsification properties [32, 34] which confirms the role of the protein. It is envisaged that the proteinaceous components adsorb onto the surface of the oil droplets and the covalently linked carbohydrate blocks protrude into the aqueous phase and prevent droplet aggregation through both electrostatic and steric repulsive forces (Figure 1).

Recently Yadav et al., 2007 reported that glycosylphosphatidylinositol lipids were present in the AGP fraction and that these also make a contribution to the emulsification properties [35]. Further work, however, is required to confirm this.

Zeta potential measurements have confirmed that gum Arabic – coated droplets have sufficient negative charge above pH 4 to prevent droplet aggregation through electrostatic repulsions due to the presence of the glucuronic acid groups in the gum Arabic structure [36-39]. However, the zeta potential decreases at lower pH values as the glucuronic acid groups become undissociated and steric repulsive forces predominate. It has been found that the amount of gum Arabic adsorbed onto limonene oil droplets is ~ 5mg/m^2 at pH 7.5 and ~6.5mg/m^2 at pH 3.5 [37]. These values are significantly higher than might be expected for monolayer coverage suggesting that multilayer adsorption occurs. This could arise through electrostatic interaction between adsorbed and non adsorbed protein and polysaccharide components of the gum Arabic molecules. It has been shown by rheological measurements that gum Arabic molecules have a tendency to self associate in solution [40] which supports this hypothesis. As noted above, the bulk of the gum (i.e. the AG component) has very little protein associated with it and hence does not play any significant role in the emulsification process.

There has been considerable interest in the Food Industry in recent years in the use of pectins for the stabilisation of oil-in-water emulsions. Pectin is a complex polysaccharide [41, 42] and consists of linear chains of 1,4 linked α- D- galacturonic acid residues, interrupted by 1,2 linked L- rhamnose residues. There are branches, consisting of neutral sugars linked to the rhamnose producing 'hairy regions' along the otherwise 'smooth' galacturonic acid main chain. Akhtar et al., 2002 [13] showed that depolymerised citrus pectin with a degree of esterification, DE of 70% and molecular mass of 70 kDa produced very stable rapeseed oil emulsions with comparable properties to emulsions prepared with gum Arabic. However, the pectin was not as effective at stabilising limonene oil-in-water emulsions. The supernatant

following emulsification was analysed and it was concluded that the material adsorbed was rich in protein. There has been particular interest in recent years in the use of sugar beet pectin as an emulsifier. Sugar beet pectin constitutes ~ 20% of sugar beet pulp which is obtained as a by-product during the extraction of sugar and in the EU amounts to ~400K tonnes p.a. A distinct feature of sugar beet pectin is that it contains ferulic acid which is not the case for citrus pectins. Leroux et al. investigated the interfacial and emulsification properties of citrus and sugar beet pectins and found that they both significantly reduced the interfacial tension [9]. Values for 2% pectin solutions at the paraffin oil / water interface were 31.3 mN/m for citrus pectin and 19.4 mN/m for sugar beet pectin. Emulsions were prepared using orange oil and rapeseed oil and the pectin remaining in the aqueous phase was recovered by precipitation and analysed. It was found that the fraction that adsorbed onto the oil contained a higher proportion of protein and had a higher acetyl content than the material in the aqueous phase. De-acetylation did not cause any significant loss in emulsifying capacity and it was concluded that the emulsifying properties were most probably due to the protein components present. Chee Kiong and Williams [10] determined adsorption isotherms for the adsorption of sugar beet pectin onto limonene oil droplets. It was found that the amount adsorbed was ~9.5 mg/m^2 which is significantly more than might be expected from monolayer coverage as is the case for gum Arabic as discussed above. Furthermore the adsorbed fraction contained 14.7% protein and 2.1% ferulic acid whilst the pectin sample as a whole contained 2.7% protein and 1.06% ferulic acid thus confirming that protein and ferulic acid – rich components adsorbed preferentially. In a further study Chee Kiong et al. reported that the thickness of the sugar beet pectin layer adsorbed onto a model substrate (polystyrene latices) was 140 nm at plateau coverage which was found to be roughly equivalent to the hydrodynamic diameter of the pectin molecules themselves [11]. The thickness was found to be sensitive to the pH and a value of 107 nm was obtained at pH 4 for a surface coverage of 20 mg/m^2 while a value of 70 nm was obtained at pH 8.8. At pH 4 the proteinaceous moieties are likely to carry a net positive charge and hence could form complexes through interaction with the glucuronic acid residues present and form multilayers. At pH 8.8 the proteinaceous moieties are likely to carry a net negative charge and hence are unlikely to form electrostatic complexes.

Nakauma et al., 2008 have compared the emulsification properties of sugar beet pectin, soybean soluble polysaccharide and gum Arabic using medium chain triglyceride, MCT as the oil [38]. They demonstrated that the polysaccharide concentration required to produce the minimum droplet size on emulsification of 15% MCT was in the order sugar beet pectin (~1.5%) < soybean soluble polysaccharide (~4%) < gum arabic (~10%). Whilst they found that the amount of gum Arabic adsorbed was ~ 6 mg/m^2 which is approximately the same as reported by Padala et al. [37], the amount of sugar beet pectin adsorbed was only ~ 2 mg/m^2

which was significantly less than that reported by Chee Kiong and Williams for limonene oil-in-water emulsions. The zeta potential of the fully coated droplets was found to be -55 mV, -48 mV and -40 mV for emulsions prepared with sugar beet pectin, soybean soluble polysaccharide and gum Arabic respectively.

Yadav et al. have recently reported on the emulsification properties of corn fibre gum, which is an arabinoxylan extracted from corn kernel pericarp and/or kernel [35]. They found that the emulsification properties were better for samples with a higher protein content and indicated that this supported the hypothesis of Randall et al. that the proteinaceaous components anchor the molecules to the surface of the droplet and the carbohydrate moieties provide an electrosteric barrier against droplet aggregation [32].

Recent attention has been given to crosslinking of adsorbed polymer layers to provide enhanced emulsion stability. Jung and Wicker [43], for example, employed lacasse to induce ferulic acid crosslinks between sugar beet pectin molecules. The crosslinked material was used to prepare emulsions with tetradecane as the oil phase and they found that it produced smaller sized droplets with a slightly more negative zeta potential. Littoz and McClements prepared corn oil emulsions using a combination of β-lactoglobulin and sugar beet pectin to produce a bilayer and then added lacasse to crosslink the pectin molecules [26]. The emulsions were found to have improved stability in the presence of electrolyte.

Polysaccharide-protein Maillard conjugates

The Maillard reaction was first reported by Maillard in 1912 [44]. It is a series of non-enzymatic browning reactions, which occur naturally between a reducing sugar and an amino acid and can be summarised as an initial condensation step between the reducing end of a carbohydrate and an amino acid. These products then isomerise to form Amadori (or Heyns if the carbonyl is a ketose) rearrangement compounds, including a Schiff base. This stage occurs spontaneously under mild conditions and can be seen in certain foodstuffs when stored. Following this, the protein-carbohydrate conjugate fragments creating reactive intermediates and can partially form cross-linked protein structures. The last step is the formation of melanoidins and other advanced glycation end products (AGEs), insoluble polymeric compounds resulting from further protein cross-linking and intermediate degradation. There are extensive surveys in the literature describing Maillard reaction pathways and potential products with a wide range of substrates [45,46] and a simplified schematic of the reaction is shown in Figure 2.

Work on the use of Maillard reaction products (MRPs) as emulsion stabilisers has been undertaken for many decades. Preliminary studies looking at the oxidation and stability of MRP containing emulsions was briefly mentioned in a paper concerned with the use of amino

acids to prevent oxidation of safflower oil emulsions by Riisom and co-workers [47]. Here the MRP of lysine and dextrose were used to show virtually no difference in oxidation rate and only a slight increase in emulsion stability when compared to a control containing no MRP [47]. However, work using polysaccharides rather than simple sugars was first initiated by Kato and colleagues [15-17, 48]. One of their early papers describes the induction of the Maillard reaction to create dextran-ovalbumin conjugates and then assesses the emulsifying properties, as an industrially suitable alternative to using cyanogen bromide activated polysaccharides to undertake the conjugation [15]. A powdered mixture of 1:5 weight ratio of ovalbumin to dextrose was dry heated at 60°C and relative humidity (RH) 65% for three weeks. Emulsions were formed using corn oil in a phosphate buffer, with stability measured by monitoring turbidity. Emulsion stability of the Maillard conjugate outperformed that of the cyanogen bromide ovalbumin-dextran prepared sample and was vastly greater in performance than the ovalbumin on its own. Emulsion stabilisation was effective in acidic environments (pH 3) and improved significantly at pH 10. Preheating the Maillard conjugate to 100°C also improved emulsion stability over the unheated control. They concluded that the qualities possessed by the Maillard conjugate offer emulsion stability over a wide pH and temperature range, preventing the need for unattractive chemical reagents.

Dickinson and Galazka used β-lactoglobulin and dextran to create Maillard conjugates which were found to bring about enhanced emulsion stability [49]. Conjugates were prepared by dry heating powders at 60°C at an RH of 35-40% for three weeks. Stable emulsions were formed using *n*-hexadecane, with changes in droplet size measured over time using laser diffraction and a visual assessment of creaming. Figure 3 illustrates the increased emulsion stability of the MRP over the dry heated native protein.

After three weeks of storage the emulsion made with dry heated native β-lactoglobulin showed an increase in the droplet diameter, with some increase starting to be noticed after a week whereas the MRP stabilised emulsion retained the same size distribution profile over the 4 week experiment. It was also found that serum and cream separation using the MRP was less than the native protein after 100 hours. β-lactoglobulin-dextran conjugates formed after four days had a poorer performance with respect to stability and emulsion formation than the three week reaction product, this was put down to a greater amount of unreacted dextran being present in the system. Overall it shows that protein-carbohydrate conjugation can enhance emulsion stability and performance; however, the reaction time would need to be reduced from three weeks to produce a commercially attractive product.

It is possible to follow the formation of Maillard conjugates using techniques such as the electrophoresis technique sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-

PAGE [16] and gel permeation chromatography (GPC). An example of the use of GPC is shown in Figure 4 for dextran and rice protein hydrolysate [50]. Chromatogram 1 shows two peaks, Peak A associated with the protein and Peak B for the dextran. Following heating, there is an increase in the amount of higher molecular weight material present (Peak A) and a decrease in low molecular weight material (Peak B). The appearance of Peak C in chromatogram 2, Figure 4, was attributed to the possible degradation products of early stage MRPs and the authors acknowledged further work was needed to verify this. Both of these methods used an indication of change in mass to provide evidence of a conjugation with the assumption being initial protein aggregation would lessen during the course of the reaction allowing conjugation to occur. This conjugation between protein and carbohydrate would then account for the increase in high molecular weight material.

Other analytical techniques to indicate Maillard reaction products include X-ray diffraction, where it has been suggested that structural changes resulting from conjugate formation between the amine groups of chitosan and xylan affect crystallisation [51]. Their results show changes in peak position and shape following the Maillard reaction relative to the unreacted material. It was also shown that longer reaction times were accompanied by increasing quantities of amorphous material showing that the Maillard reaction reduced crystallinity [51]. NMR has been used in conjunction with ion trap mass spectrometry operating in electrospray ionization mode, to identify Amadori and Heynes rearrangement products [52]. A tetrapeptide containing lysine was incubated with either fructose or glucose, and the rearrangement products isolated and characterised, with results showing the lysine group was the preferred reaction site. Matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry has also been used as a tool for detection of MRP in reactions between lysozyme and a range of sugars [53]. The resolution was not sufficient to identify low molecular weight additions to the full protein and so fragments of a digested protein were used. When using the digested protein fragment, addition of sugars following the reaction could be seen in the MALDI-TOF spectra by a mass peak increase equivalent to the carbohydrate mass. Further accounts of analytical techniques for proof of Maillard conjugation can be found in the work of Yaylayan et al [45].
There has been continued interest in the Food Industry in the use of Maillard conjugates as emulsion stabilisers over recent years and this is demonstrated in Table 1 which gives a summary of publications in the area over the last few years.

The preferred method for conjugate formation is by simple mixing of the dry solids and maintaining at a defined temperature and humidity for a given time since this is more viable as a commercial process compared to mixing solutions of the two components. However, the

mixing of solutions of the two components and then drying will lead to a more homogeneous mixture and the likelihood of enhanced reaction efficiency.

The molecular mass of the polysaccharide has been shown to be a key factor in determining the emulsification properties. For example, the emulsifying properties of complexes prepared with dextran of molecular mass 300,000 Da were reported to be far superior to complexes with dextran of molecular mass 10,000 Da and 20,000 Da [63].

More recently Wong et al. [54] used deamidated, soluble isolated wheat protein (IWP) and dextran with varying molecular mass to create Maillard conjugates. Emulsions were prepared using 20% canola oil with 80% aqueous wheat protein or 80% conjugate, all mixed using an Ultra-turrax. Zeta-potential measurements were performed and showed roughly similar values (~ 50 mV) for dextran (D10 [9-11 kDa] and D65 [64-76 kDa]) conjugates and the native wheat protein. However, across a pH range of 7 down to 4, the conjugate emulsions were shown to have a value below -30 mV over a much wider pH range while for the native wheat protein emulsions the zeta-potential dropped to zero below pH 4. The wheat protein-D65 conjugate emulsions had a constant droplet size of ~ 1.5 μm over the pH range, while the native wheat protein emulsions showed an increase ~ 1.7-142 μm as the surface charge of the protein was lost resulting in emulsion flocculation. The D10 conjugate emulsions showed a small increase, with droplet sizes between ~ 1.6-18 μm. The proposed mechanism of emulsion stabilisation is illustrated in the schematic below (Figure 5).
Figure 5A illustrates emulsions prepared using IWP alone. At pH 7, emulsions were found to be stable but at pH 4, due to the loss of protein charge and the collapse of protein steric layer, emulsions became strongly flocculated. Figure 5B illustrates the situation for IWP-D10 conjugates. At pH 7, emulsions were stabilised by the protein-dextran complex. At pH 4, the collapse of N-terminal protein diffuse layer was proposed to lead to the exposure of the conjugated D10 dextrans that provide a physical barrier against emulsion flocculation giving rise to some flocculation. Figure 5C shows the situation for IWP-D65 conjugates. At pH 7, D65 dextrans conjugated at the IWP N-terminus were reported to form an additional carbohydrate steric layer approximately 5.9 nm thick at the interface. At pH 4, the D65 dextrans were large enough to provide a steric barrier to prevent emulsion flocculation.

Most of the work on MRP has been in the area of non-ionic polysaccharides although some have involved charged polysaccharides. For example, Nakamura looked at the reaction of chitin and chitosan oligosaccharides, (together with galactomannan, xyloglucan and okara polysaccharides) with tofu whey protein [64], using 1:1 mixtures at 60°C and 65% RH for seven days. Emulsions were prepared from homogenised corn oil and MRP solutions then

analysed using ultraviolet-visible spectrophotometry at 500 nm to find emulsifying activity (EA). The half life of the absorbance after 10 min gave emulsion stability (ES).

The protein (>3 kDa)-polysaccharide conjugates showed improved EA compared to native protein. Chitosan oligosaccharide complexes also outperformed polysaccharide complexes in ES measurements and both showed improvement over the native protein. When using smaller proteins (<3 kDa), chitosan conjugates showed poorer EA and ES compared to those of chitin and xyloglucan This was attributed to the increased positive charge available to chitosan interfering with covalent linkages between negatively charged protein groups. Oil binding (OB) and water hydration (WH) were also measured. Chitosan conjugates with >3 kDa protein showed improved WH along with chitin and galactomannan conjugates, the OB test showed only chitin improved performance. Chitosan conjugates with acid precipitated protein showed better WH and OB and improved OB was also seen with conglycinin compared to with xylogucan and chitin. The whey protein <3 kDa, glycinin WH and β-conglycinin did not show any improvement with chitosan;chitosan's charged groups facilitate increased solubility of the smaller proteins and greater binding of water leading to higher WH values. Optimising the Maillard reaction requires an idea of the structure, including size and charge, of both the protein and carbohydrate starting material.

Kunzek and co-workers [65] investigated the reaction of whey protein isolate and sodium caseinate with high or low methoxylated (HM, LM) pectin. Conjugates were prepared at varying mixing ratios (1:1 – 1:5 protein:polysaccharide), at varying pHs (5.8 – 7), with the RH ranging from 65-80% and the temperature ranging from 50-60°C for 15 days. They concluded that the most important factor affecting emulsification properties was the choice of protein, rather than the degree of methoxylation of the pectin. With the whey protein both emulsion activity and stability improved following conjugation with either pectin species. However, for sodium caseinate performance was poorer following conjugation. This may be due to the fact that whey protein has a globular structure becoming more open at pH7 allowing the reaction to take place, whereas the micellar nature of sodium caseinate can result in a water in water emulsion during preparation thereby inhibiting the reaction [65].

It is evident that research on the development of Maillard conjugates as novel emulsifiers will continue to be an area of activity for some time to come. It is expected that the future direction of the work will make full use of plant proteins and polysaccharides which in many cases are considered as waste streams. A good example here is the development of conjugates using arabinoxylans produced as by-products in the biorefining of corn [58] thus helping to fulfil the Green Chemical potential and improved resource efficiency required.

Polysaccharide-protein electrostatic complexes

Bungenberg de Jong and Kruyt undertook the first systematic study of electrostatic interactions between polysaccharides and proteins using gum Arabic and gelatine as long ago as the 1920s and they referred to the complexes formed as complex coacervates as they were liquid – like rather than as solid precipitates [22]. Gum Arabic – gelatine coacervates are still used today to encapsulate flavour oils. The potential of using polysaccharide – protein complexes in a range of applications including protein entrapment and release, enzyme immobilisation and recovery, biosensors, protein separation processes and emulsification and encapsulation is now very much recognised [23,66,67]. Interest in this area of study may be gauged by the number of reviews dedicated to it, particularly in the last decade following the review by Benichou et al., 2002 [68]. The emulsifying properties of electrostatic polysaccharide-protein complexes were considered by Guzey and McClements, 2006 [69] and Dickinson, 2008 [70]. Such complexes are also included in numerous reviews of broader scope [2, 18, 71-78]. The review of Cooper et al. of polyelectrolyte-protein complexes generally is of particular merit in view its thorough treatment of experimental methods for studying the interaction [19].

A prerequisite for the formation of electrostatic complexes is naturally the existence of opposing charges on the reacting species. Conventionally this is achieved by utilisation of an anionic polysaccharide in combination with a protein bearing positive charge below its isoelectric point. For polysaccharides the negative charge most commonly arises out of the presence of carboxyl or sulphate moieties. The only naturally occurring cationic polysaccharide is chitosan and in this case electrostatic interaction occurs when the protein solution is at a pH above its isoelectric point. Zinoviadou et al. prepared (n-tetradecane) oil-in-water emulsions stabilised by a complex comprising chitosan and sodium caseinate at pHs above the protein isoelectric point [79]. Chitosan has similarly been used by Laplante et al. [80] and Yuan et al. [81]. The nature of polysaccharide – protein electrostatic complexes is dependent on the mixing ratio, pH and ionic strength and they may be soluble or in the form of an insoluble precipitate or coacervate. Vinayahan et al., for example, investigated the phase behaviour of gum Arabic - bovine serum albumin (BSA) mixtures and constructed a phase diagram for the system at varying mixing ratios and pH [21].This is shown in Figure 6. At low GA: BSA ratios (< ~1:2) the complexes formed were found to be soluble over a broad pH range. At pH values above ~pH 5.4 there was no complexation at any mixing ratio between the GA and BSA as expected since both carry an overall negative charge. However, soluble complexes were found to form between pH values of 4.8 and 5.4 which is above the isoelectric point of the BSA. It was assumed that interaction occurred between the GA carboxylate groups and positive patches on the BSA. At pH values between ~4.8 and ~ 3.0 (depending on the mixing ratio) the GA and BSA have opposite charges and insoluble

complexes were formed. At pH values below ~ 3 the GA carries only a very small negative charge and the BSA carries a significant positive charge and hence there may be soluble complexes formed or indeed no complexation at all.

Emulsions stabilised by electrostatic polysaccharide-protein complexes can be formed in different ways. Traditionally, emulsions have been prepared using premixed coacervates. Weinbreck et al., for example, used whey protein – gum Arabic complex coacervates to encapsulate flavour oils [66]. They reported that the best capsules are formed at the pH for maximum coacervation and maximum viscosity. In another example, Klein et al. used whey protein–gum Arabic blends to stabilise cloudy emulsions for application as flavour concentrates for soft drinks [82]. When using anionic soy polysaccharide with soy protein to stabilise (soybean) oil-in-water emulsions Yin et al. found it necessary to heat the emulsion (80 °C, 1 hour) to achieve a stable system [83].

Another approach is to form an emulsion using a protein as a primary emulsifier and then to add a polysaccharide to adsorb onto the protein layer forming a bilayer. A slight variation to this procedure is to add the protein and polysaccharide to the emulsion at the same time but at a pH at which there is no interaction [22]. Once the protein has adsorbed onto the oil droplets the pH can be adjusted and the polysaccharide will adsorb onto the protein layer thus forming the bilayer. The increase in adsorbed polymer layer thickness and likely increase in zeta potential will tend to stabilise the emulsion by inhibiting droplet aggregation through enhanced electrosteric repulsions. Multilayers can be formed by further sequential addition of protein and polysaccharide [69]. Emulsions produced using this procedure have been shown to have enhanced stability in respect of ionic strength [84] and pH [85]. A review of encapsulation techniques for lipid based drugs by Shchukina and Shchukin provides examples of 'multi' layer systems, although the scope of the review is not restricted to polysaccharide-protein systems [86].

In a further development, Jourdain et al. investigated the stability of *n*-tetradecane emulsions prepared using sodium caseinate and dextran sulphate [24]. They used two different methods to prepare the emulsions. In the first method a bilayer was produced by forming an emulsion with the caseinate and then adding the emulsion to a dextran sulphate solution at varying concentration. At low dextran sulphate concentrations bridging flocculation occurred but at higher concentrations stable emulsions were formed. In the second method, solutions containing a mixture of caseinate and dextran sulphate at varying mixing ratios were used. It is evident, although not explicitly stated, that the ratios chosen corresponded to the 1-phase region of the phase diagram, i.e. the complexes formed were soluble rather than coacervates. For these systems there was no evidence of bridging flocculation and the droplet size was smaller than for the bilayer emulsions. Mahendran et al. undertook similar studies using soluble gum Arabic – BSA complexes and found that they were more effective

than the gum Arabic and BSA alone [25]. Recent examples of studies employing electrostatic polysaccharide-protein complexes are summarised in Table 2 and Figure 7 presents a schematic of the various methods adopted to form stable emulsions using polysaccharide – protein complexes.

The future direction of research in this area will surely be aligned with the general trends in industrial sectors, particularly those in the food and beverage and pharma / nutraceutical sectors where these systems are already well established. In the latter there is certainly much need for carriers for hydrophobic active ingredients, AIs. Fish oil based O/W emulsions formulated using fish gelatine and whey protein isolate demonstrated potential for incorporation of beneficial oils rich in omega 3 fatty acids into acidic beverages [100]. For many delivery systems the goal is to be able to trigger release of the AI in response to an external trigger. Controlled release applications are important in a number of sectors, including agrochemicals etc and are another area where O/W emulsions have potential. A β-lactoglobulin / HM citrus pectin stabilised soy oil emulsion was employed as a model controlled release matrix for several volatile organic compounds [89]. Microencapsulation is an increasingly popular option for preservation of active ingredients. Rodea-Gonzalez et al. prepared W/O emulsions of an essential oil (chia oil from *Salvia hispanica L.)* utilising whey protein concentrate complexed with mesquite gum or gum Arabic which were then spray dried to produce microencapsulated oil [118]. It is also anticipated that further novel protein-polysaccharide combinations will arise out of the increased evaluation of biopolymers from waste streams, possibly generated as bio-refinery by-products. As highlighted in a recent review of the employment of vegetable proteins in microencapsulation there is a tendency to favour vegetable based proteins over animal based ones and an increase in the study of polysaccharide plant-protein electrostatic complexes is to be expected as a consequence [119].

Polysaccharide–protein complexes are not only employed to stabilise simple oil-in-water emulsions but can be employed in more complex systems. W/O/W emulsions have been stabilised by a whey protein isolate, WPI and enzyme modified pectin complex [97,98] and similarly complexes of whey protein with mesquite gum, gum Arabic or LM pectin [120]. Murillo-Martinez et al. demonstrated the use of sodium carboxymethyl cellulose or LM pectin in conjunction with WPI in stabilising W/O/W double emulsions, which were subsequently used to produce edible films [121]. It is of note that the preparation of these W/O/W emulsions relies upon conventional surfactants in order to prepare the initial W/O emulsion. This interesting area is also included in a recent review [122]. Studies on O/W/O double emulsions stabilised with WPI-xanthan gum or WPI-fenugreek gum have also been reported [123].

Concluding remarks

The ability of a number of polysaccharides including gum Arabic, pectin, soybean polysaccharide and corn fibre gum to stabilise oil-in-water emulsions is due to proteinaceous material present as an integral part of their structure. The protein component confers amphiphilic characteristics and enables the molecules to adsorb at the oil-water interface. The fact that these polysaccharides are particularly effective at stabilising limonene oil, which readily succumbs to Ostwald ripenening may be due to the fact that they form multilayers at the interface. Further work should be carried out to confirm this hypothesis.

In the case of sugar beet pectin, enzyme crosslinking of the adsorbed layers has been shown to lead to enhanced emulsification properties.

The formation of Maillard polysaccharide–protein conjugates has been an active area of study in recent years and provides an opportunity to develop a range of novel emulsifiers for application in food and related areas that have enhanced functionality compared to the protein itself. The molecular size and nature of the polysaccharide and protein have a significant influence on the overall properties. The protein component needs to be sufficiently large and hydrophobic to enable the conjugates to adsorb onto the surface of oil droplets while the polysaccharide needs to be above a certain critical size in order to form an adsorbed surface layer that is able to prevent droplet aggregation through steric repulsive forces. The use of charged polysaccharides is expected to lead to enhanced stability due to additional electrostatic repulsions and in addition could lead to the formation of multilayers at the interface due to protein – polysaccharide electrostatic interaction. This is an area that requires further study.

In the case of electrostatic complexes most of the work undertaken has involved either milk or soy proteins. A number of different preparative routes have been explored to form stabilised oil in water emulsions using these materials and 'order of addition' effects have been shown to be important. Whilst the use of coacervates may be most suited to encapsulation techniques where the emulsion is subsequently dried, the soluble complexes route may prove to be more appropriate for systems where the emulsion is the finished product. There is considerable potential for these systems to be employed in delivering sustainable solutions for emulsion formulators in a wide variety of industries.

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List of Figures

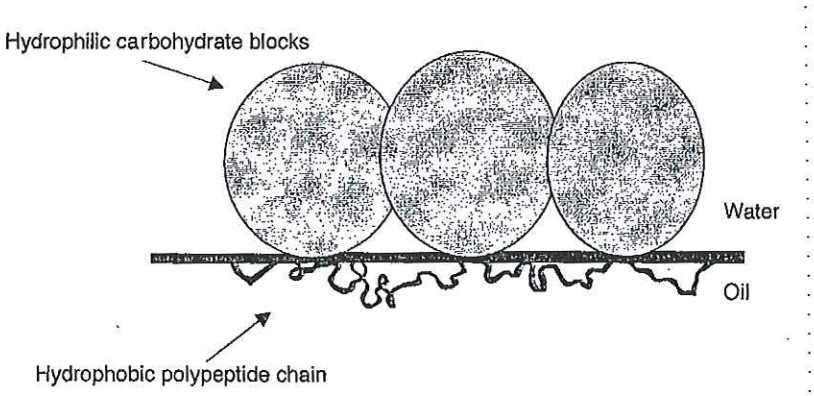

Figure 1 Schematic representation of gum Arabic adsorbed onto oil droplets

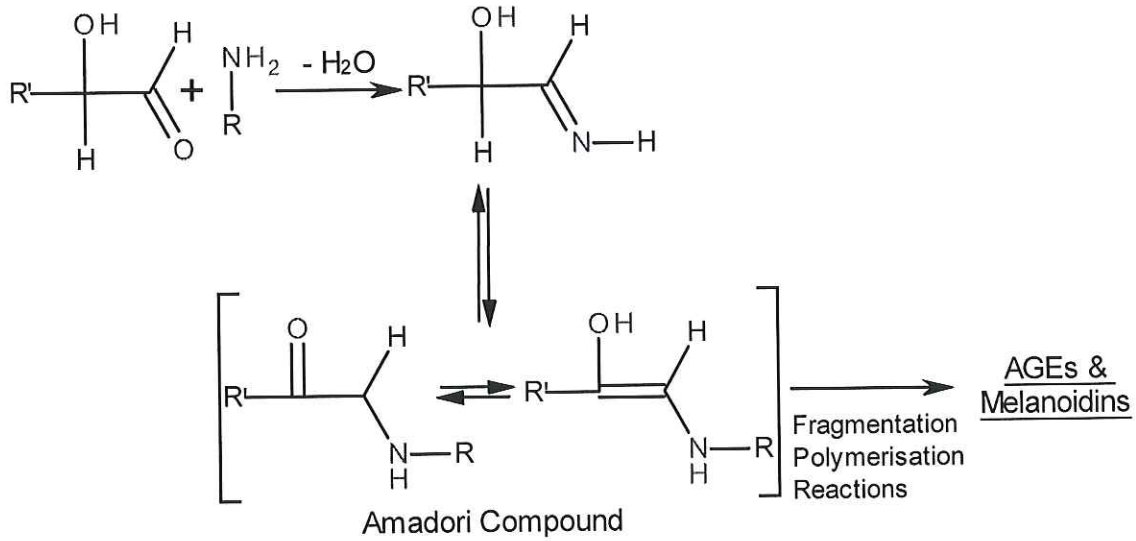

Figure 2: Schematic of the Maillard reaction, showing a reducing carbohydrate carbonyl reacting with a free amine group, leading to advanced glycation end-product (AGE) formation.

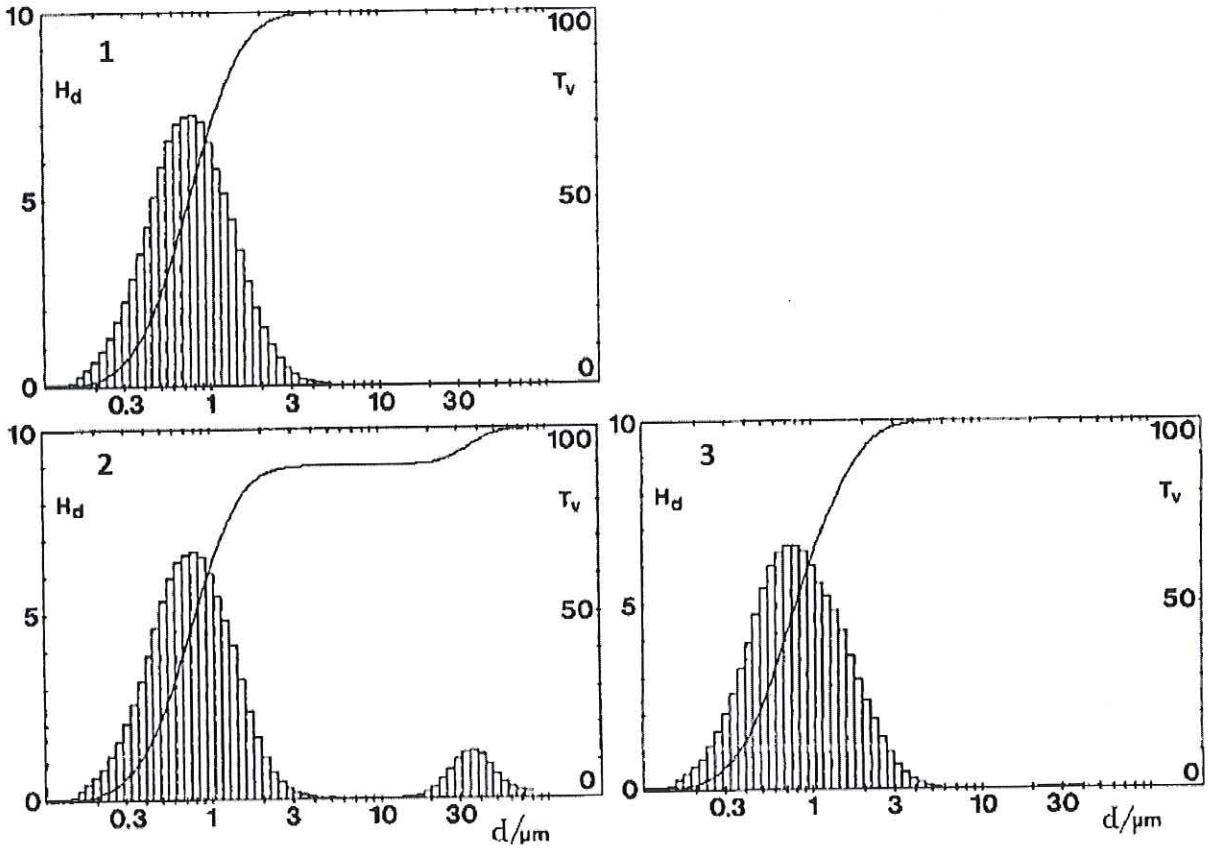

Figure 3: Emulsion droplet size distributions from [49]. 1: Dry heated β-lactoglobulin emulsion after preparation, 2: Dry heated β-lactoglobulin emulsion after 3 weeks, 3: 1:1 Maillard conjugate emulsion after 4 weeks – it is the same as the distribution immediately after preparation [49].

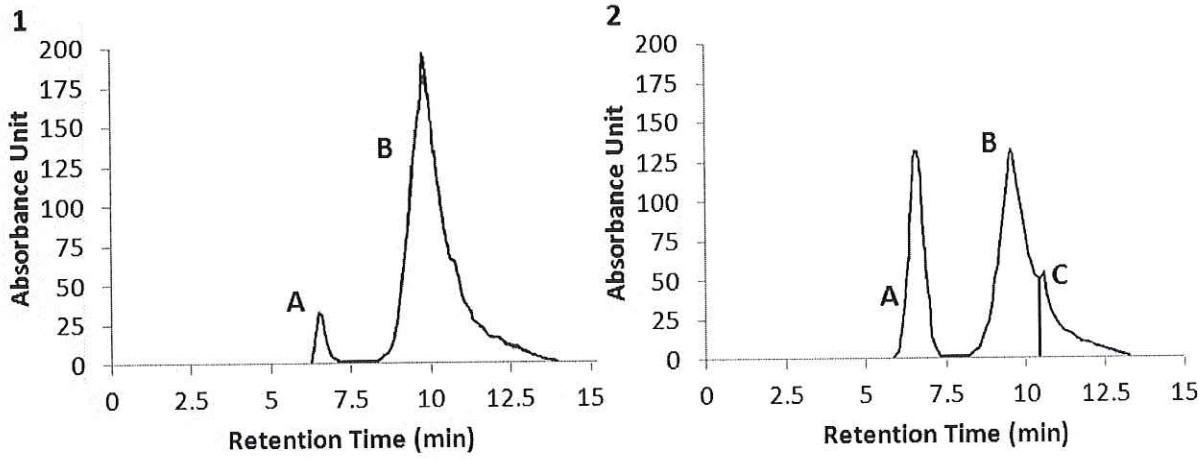

Figure 4: Example of GPC chromatogram from [50]. 1: Unheated dextran and rice protein hydrolysate, 2: After 40 min heating at 100°C.

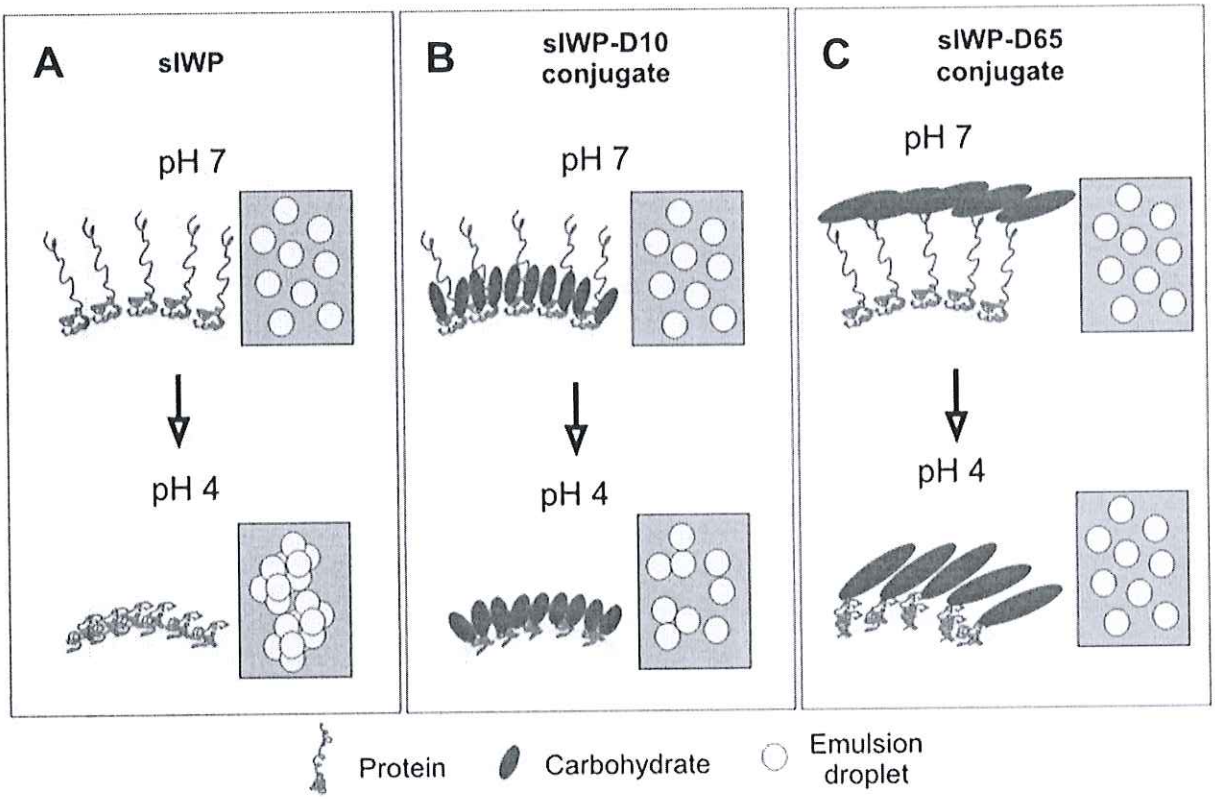

Figure 5: Illustration of the process of emulsion stabilisation taken from [54]. sIWP – soluble isolated wheat protein, D10 – dextran 6400 Da, D65 – dextran 41 kDa.

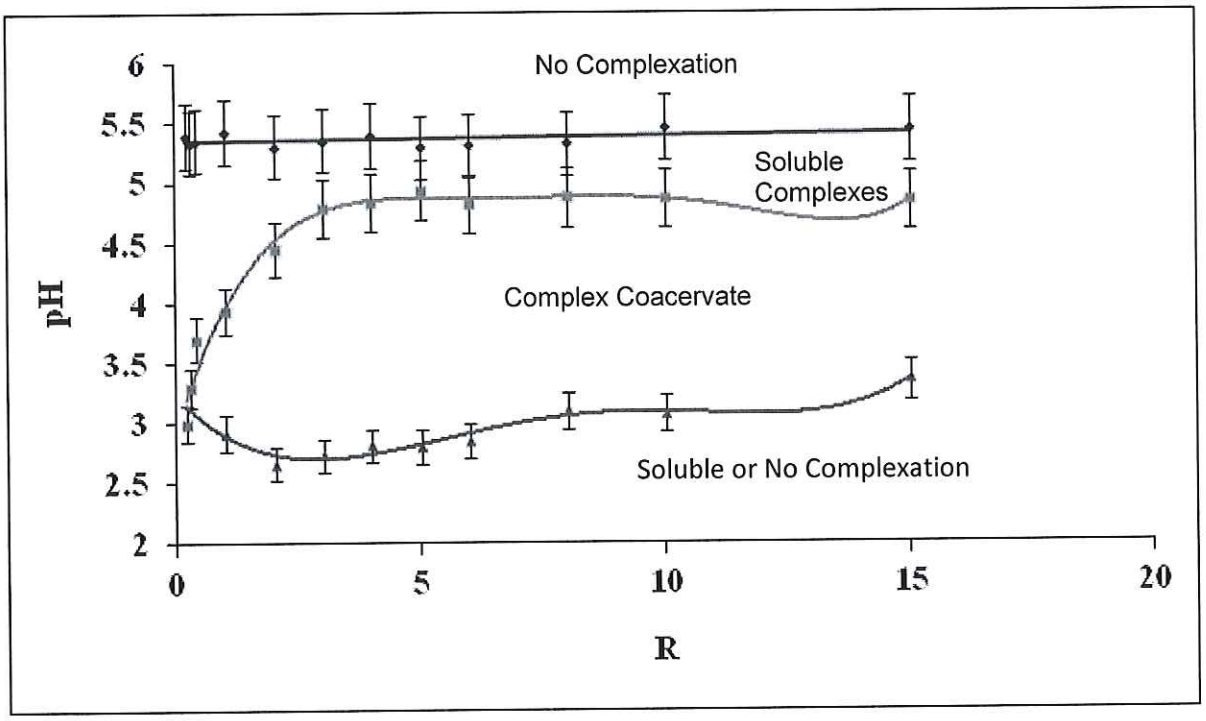

Figure 6: Phase diagram for mixtures of Gum Arabic and BSA as a function of pH and mixing ratio. (R = polysaccharide: protein), adapted from Figure 4 in Reference [21].

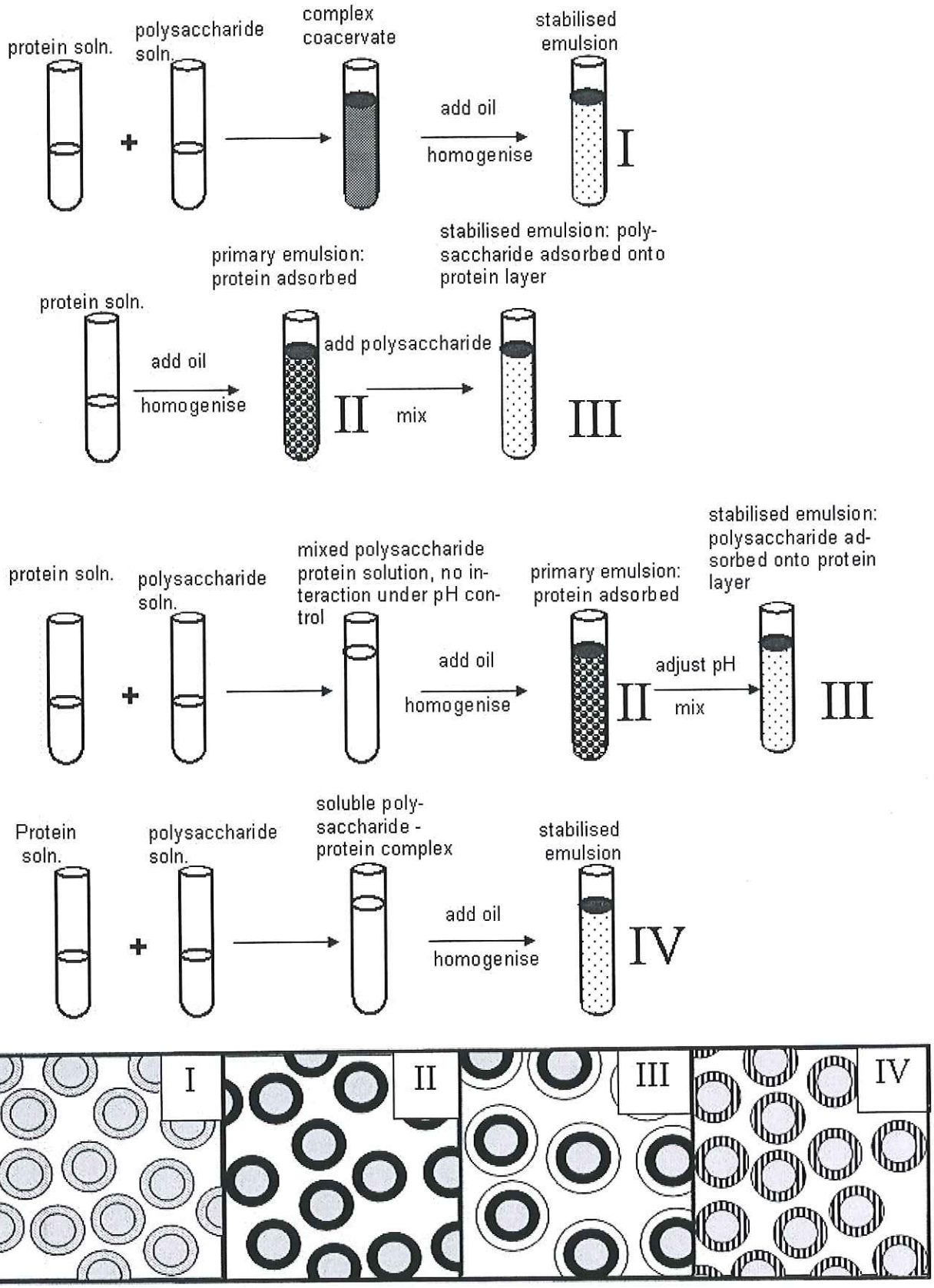

Figure 7. Schematic representation of four different preparation routes for emulsions stabilised by electrostatic polysaccharide-protein complexes. Inner oil droplet is protected by adsorption of (I) complex coacervate, (II) protein layer, (III) protein layer with outer polysaccharide layer (bilayer) or (IV) soluble complex.

System	Reaction Conditions	Reference
Rice protein hydrolysate + Glucose, Lactose, Maltodextrin & Dextran	1 :1 (%w/w) aqueous solution pH 11, 100°C heating for 0, 5, 10, 20, 30 and 40 min	[50]
Wheat protein + Dextran	1:1 dry heating, 60°C at 75% RH for 5 days	[54]
Egg white protein + Pectin	1:1 (%w/v) dry heating, 60°C at 79% RH for 6 – 48 hrs	[55]
Egg white protein + Fructose & inulin	2:1 dry heating, 60°C at 79% RH for 3 days	[56]
β-conglycin + Dextran	10:10 (%w/v) dry heating, 60°C at 75% RH for 6 days	[57]
β-Lactoglobulin, Whey protein isolate + Corn fibre gum	1:3 dry heating, 75°C at 79% for 2 days	[58]
β-Lactoglobulin + Galactose	1:1 dry heating, 40°C for 24 hrs and 50°C for 48 hrs, 44% RH	[59]
Bovine sodium caseinate + Galactose	1:0.2 dry heating, 60°C for 4 hrs and 50°C for 72 hrs, 67% RH	[60]
Soy whey protein + Fenugreek gum	1:1, 1:3, 1:5 dry heating, 60°C at 75% RH for 3 days	[61,62]

Table 1: Summary of recent publications producing Maillard conjugates.

(Key: Ratios are in the form Protein:Polysaccharide)

Protein	Polysaccharide	Reference
β-lactoglobulin	chitosan	[80]
β-lactoglobulin	gum Arabic	[87]
β-lactoglobulin	pectin – LM & HM	[88]
β-lactoglobulin	citrus pectin - HM	[89]
β-lactoglobulin	citrus pectin - HM	[90]
β-lactoglobulin	citrus pectin	[91]
β-lactoglobulin	chitosan plus citrus pectin or sodium alginate	[92]
β-lactoglobulin	sodium alginate	[93]
β-lactoglobulin	pectin	[84]
β-lactoglobulin	alginate, ι- carrageenan, gum Arabic	[94]
WPI	κ-, ι-, λ- carrageenan	[95]
WPI	CMC	[96]
WPI	gum Arabic	[82]
WPI	pectin – enzyme modified	[97, 98]
WPI	gum tragacanth	[99]
WPI	fish gelatine	[100]
WPI	chitosan	[81]
WPI	beet pectin	[101]
WPI	sugar beet pectin - HM	[102]
WPI	flaxseed gum	[103]
sodium caseinate	chitosan	[79]
sodium caseinate	CMC	[104]
sodium caseinate	dextran sulfate	[24]
sodium caseinate	κ-carrageenan	[85]
sodium caseinate	soy soluble polysaccharide, pectin – HM	[105]
soy protein	soy polysaccharide	[83]
soy protein isolate	gum tragacanth, carrageenan	[106]
soy protein isolate	soy soluble polysaccharide	[107]
soybean / flaxseed	gum Arabic	[108]
soy oleosin	κ-, ι-, λ- carrageenan	[109]
soy oleosin	beet pectin	[110]
casein	CMC	[111, 112]
non fat dry milk	ι- carrageenan, pectin- LM, HM	[113]
BSA	sugar beet pectin	[114]
egg white protein	gum Arabic	[37]
maize germ protein	xanthan gum	[115]
pea protein isolate	pectin–HM	[116]
lactoferrin	pectin- LM & HM, alginate	[117]

Table 2. Recent examples of emulsion stabilization studies employing electrostatic polysaccharide-protein conjugates. CMC refers to carboxymethyl cellulose; other acronyms are defined at first point of use in main text.