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Fragmented Proteins as Food Emulsion Stabilisers: A Theoretical Study

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ABSTRACT

Using a model based on the primary structure of α_{s1}-casein, the colloid stabilising ability of fragmented protein and that of the intact chains are compared. We perform Self Consistent Field (SCF) calculations to obtain the induced interaction potentials between the oil droplets, resulting from the overlap of adsorbed protein layers in each case. For the intact α_{s1}-casein, we confirm the known result, that the mediated inter-particle interaction potential develops a deep attractive energy minimum at high salt concentrations and pH values close to the isoelectric point of the protein. The same does not occur for the appropriately fragmented systems, with improved emulsion stability predicted as a result, even at pH values close to pI. It is shown that this superior performance, for the case considered, is due to the diblock-type behaviour of one of the fragments. On the other hand, it is well known that α_{s1}-casein more closely resembles the less favourable triblock structure. However, it is also demonstrated that the presence of a "diblock" like fragment by itself may not always be enough to produce a better emulsion stabiliser. It is seen that the hydrolysis of some peptide bonds may indeed lead to a suitable polypeptide, but that this is displaced from the interface by the structurally less desirable ones, also generated by the fragmentation process. The displacement occurs due to the competitive adsorption between different fragments. The removal of the undesirable fragments from the solution is found to greatly enhance the predicted colloid stabilising ability of the remaining polypeptide.

INTRODUCTION

The formulation of the texture and structure of food systems is undergoing a number of major changes. On one hand, nowdays the texture of food is not only required to provide an appealing mouthfeel sensation, but it also has to serve as a vehicle for the design and delivery of healthier foods. Coupled with this, the general philosophy for the formulation of new food products is also gradually shifting from one largely based on the available processing operations, and the relatively limited textures that they provide, to one involving a more bottom up approach utilizing the principles emerging from the field of soft matter.[1,2] Thus, such phenomenon as arrested phase separation, self assembly[3] and controlled aggregation are all increasingly seen as design tools to be explored by food scientist, rather than as unavoidable aspects of a particular manufacturing process.

In foods colloids much of the structuring emerges from the arrangement of mesoscopically sized entities, namely emulsion droplets, fat crystals or protein particulates. A good degree of control over the interactions between such entities is essential if the soft matter approach to food structure design is realistically to be achieved. Food scientists have traditionally relied on the use of different proteins as the colloidal stabilisers of choice. These in food colloids fulfil the same role as that of dispersants in many other fields of technology, ensuring the long term stability of the colloidal particles or emulsion droplets.[4,5] This is achieved through the manipulation of surface forces and the provision of steric and electrostatic repulsion between the particles. Unfortunately nature had not intended for such food proteins to be deployed as general purpose dispersants. The structure and the sequence of hydrophilic and hydrophobic amino acid residues from which these proteins comprise, are usually far from the ideal ones for a colloidal stabiliser. As a result, the degree of colloidal and emulsion stability achieved is very sensitive to environmental conditions, such as temperature cycles, pH changes and the addition of background electrolyte, all of which are likely to alter during the course of processing of a particular food product. The effects of these changes on the stabilisation properties of the biopolymers can be dramatic. For example, quite commonly small drifts in process parameters, or minor changes in the specification of raw materials, can alter the protein mediated colloidal interactions from repulsive to attractive, or via versa. In other fields of technology, such as paints and coatings, inks, explosives and agrochemicals, where also the use of colloidal formulations is ubiquitous, suitable dispersants having appropriate size and architecture (e.g. linear, comb, branched), with desirable sequence of

hydrophobic and hydrophilic segments (homopolymers, diblocks, random co-polymers, block co-polymers, etc) can often be synthesised. The major factor limiting the use of the most optimal dispersants in these applications is normally then the cost. The same is of course not true of food colloids, where one is limited to the use of edible macromolecules, which increasingly also have to come from natural sources, rather than being synthetic. Even relatively minor chemical alterations to these natural biopolymers, though acceptable in some cases, are not preferred.

In obtaining superior food colloid stabilisers, while also attempting to maintain the degree of chemical modification to a minimum, several strategies have been proposed and examined in the past few years. The first of these has focused on the so called the "Pickering" route,[6] where small particles rather than individual molecules, adsorbed at the droplet interfaces, are responsible for the stability of the emulsion. The high desorption energies associated with displacing the particles from the surface, makes these types of Pickering emulsions and foams very stable against coalescence, as well as Ostwald ripening.[7-9] However, particles are much larger than molecules and therefore much slower at getting to the freshly created interfaces during the emulsification process. Furthermore, to allow for the formation of small submicron emulsions, one requires rather small nanoparticles. Obtaining such small, edible grade nanoparticles, with the right surface chemistries appropriate to their use as Pickering stabilisers, has been a challenge. One possibility explores the use of small amphiphilic calcium alginate derivatives in the form of nanogels.[10,11] An even more promising approach considers the filamentous fungi protein hydrophobin[12-14] as the possible stabilising Pickering nanoparticles. Hydrophobin is a highly surface active protein, which nevertheless seems to maintain its globular structure even upon adsorption onto a hydrophobic surface, or indeed at high temperatures. Theoretical support for this view has quite recently been provided by the molecular dynamic simulation work of Euston.[15] The high surface activity, coupled with the particle like nature of hydrophobin, makes it an excellent potential candidate as a superior emulsion and particularly foam stabiliser.

The second strategy for developing higher grade food colloid stabilisers explores the useful synergic effects[16] occurring between various biopolymers.[17,18] An interesting example of this approach, investigated in particular by McClements and his co-workers,[18-23] involves the formation of protein + polysaccharide multilayers on the surface of the emulsion droplets. Polysaccharides are by and large hydrophilic. Thus in water, a good solvent, there will be

strong excluded volume repulsion between the individual chains. Typically, polysaccharides are also much larger biopolymers than proteins. As such they are capable of forming much thicker interfacial layers, if made to adsorb at the interfaces. Both of these characteristics are highly desirable attributes of a good steric stabiliser.[4,24] The main difficulty of course is in enticing the otherwise hydrophilic polyelectrolyte to accumulate at a hydrophobic interface. On their own, these hydrophilic biopolymers will simply deplete from the interfacial regions. The trick is achieved using the mutual electrostatic attraction between the polysaccharide and an already adsorbed film of protein, with an opposite electrical charge.[23,25] The thick layer of polysaccharide, covering the surface of the droplets, is shown to greatly enhance the stability of the emulsion against environmental changes, such as increase in salt concentration[22] or thermal treatment.[21] In a series of recent theoretical studies, Ettelaie et al[26-29] applied the Self Consistent Field method to study the film structure and the strength and nature of colloidal interactions that arise when interfacial protein + polysaccharide mixed layers overlap. The main conclusion of this work was that much stronger repulsion and thicker interfacial films could result if the charge of the polysaccharide was non-uniformly distributed along its backbone. More specifically, for the best results the polyelectrolyte should have one or a few short, highly charged sections, with the rest of the chain consisting mainly of uncharged moieties.[26-28]

One major issue in preparation of emulsions stabilised by electrostatically formed protein + polysaccharides mixed layers is the tendency of emulsion to destabilise at intermediate stages of production. This is mainly driven by bridging flocculation induced by the polyelectrolyte during its initial addition to the solution.[20,25,30] Furthermore, it is difficult to deposit multilayers at pH conditions much higher than pI of the proteins (assuming an anionic polyelectrolyte is being used). Indeed in some cases the desorption of the polysaccharide from the interface is observed at such pH values,[31] not surprisingly at a stage when the charge of the protein layer also becomes strongly negative.[32] One technique in overcoming these difficulties is to link the protein and polysaccharide chains permanently through a covalent bond between them.[33,34] Such conjugate biopolymers occur naturally, as for example with gum Arabic,[35] and are routinely used in soft drink beverages for their emulsifying properties.[36,37] They can also be deliberately synthesised, in a more controlled and optimised manner, through Maillard reactions between protein and polysaccharide.[38] Emulsification properties of a number of such conjugate biopolymers, including ovalbumin-dextran,[39]

β-casein-dextran,[40,41] bovine serum albumin-dextran,[41] β-lactoglobulin-propylene glycol alginate[42] and whey protein-maltodextrin[43] have been studied by a number of researchers and shown to produce both finer and more stable emulsions than those possible with unmodified proteins. Nevertheless, there still remain many interesting questions regarding the optimal size of polysaccharide relative to protein, number of attached chains per molecule and the most suitable location for the formation of the bonds along the protein backbone. A notable early theoretical attempt to answer some of these questions is the Monte Carlo simulation work of Dickinson and Euston.[44] In their study the complexes consisted of a soft particle, modelling the globular protein part, which was joined irreversibly to a ramified, more open fractal structure, representing the polysaccharide section of the complex. More recently Akinshina et al,[45] using a similar calculation methodology to the one adopted here showed that for relatively short polysaccharide chains the attachment position begins to become rather critical. If such short chains are linked to the middle (i.e. more hydrophilic) part of α_{s1}-casein, they have a detrimental effect on the colloid stabilising properties of this protein. On the other hand, if the covalent bonds are formed towards the more hydrophobic ends of α_{s1}-casein, then the provision of steric repulsion by the protein is improved.[45] The linkage of protein to polysaccharide essentially makes the latter amphiphilic through the presence of hydrophobic residues of the protein. Where a marginally higher level of chemical modification is tolerated, the same objective can also be realised by the attachment of small hydrophobic fatty acid chains to the polysaccharide or through its esterification with dicarboxylic acids, as for example with the approved food grade hydrophobically modified octenyl-succinate starch (OSA) starch.[46,47] The high stability of emulsions stabilised by modified starch against pH changes or at high salt concentrations has been clearly demonstrated by Chanamai and McClements.[48]

While both of the methods above, whether using composite biopolymers or Pickering nanoparticles, involve stabilising agents that are larger than the protein molecules, a third possibility takes the opposite approach. The basic idea is to use smaller fragments of protein as emulsifiers and emulsion stabilisers. One hopes that by hydrolysing a protein to a smaller set of polypeptide fragments, some of these will have more desirable structures, boosting their emulsion stabilising functionality. Smaller molecules also have the added advantage of faster adsorption kinetics, making it in principle easier to produce very fine stable droplets. This is not only due to their higher diffusion coefficient, resulting from their smaller size, but

also the fact that such fragments are more likely to be in a coil like disordered conformation. Thus they require no major configurational rearrangements or unfolding before adsorption.

Emulsifying capacity and emulsion stability of droplets stabilised by polypeptide fragments have been studied for a wide variety of proteins, with different hydrolysing enzymes at various levels of hydrolysis.[49-64] Results have at best been somewhat mixed, with as many studies reporting improvements in various interfacial related functionalities of fragments relative to the intact protein as those detecting a deterioration or no change. However, there are a few general trends that clearly emerge from these studies when taken together. For example, Qi et al found that the emulsion activity of soy protein increased at first, reaching a maximum at 15% degree of hydrolysis (DH), but then decreased with a further level of modification.[52] The same trend has also been reported by Chen et al, using extruded and power ultrasound treated soy protein isolate, albeit with the optimum DH at a much lower value of 1.25%.[49,50] Using similar results for bovine milk proteins, Lee et al[51,65] have concluded that the polypeptide fragments should be at least 20 residues long before they can display any significant emulsifying activity. For imparting good emulsion stability, the chains are likely to have to be even longer than this, which implies a rather low value of DH for optimum modification. The nature of the enzyme used for fragmenting the protein is also expected to be important in determining the extent of improvement in the interfacial functionality of the protein. Papain hydrolysed pea protein was found to have a superior emulsifying and foaming capacity than the untreated protein, at least at some pH values. Yet, the opposite has been observed for protein fragments produced using the commercial protease.[60] Similarly, in a study involving whey protein, Turgeon et al noted that chymotryptic hydrolysed fractions had a lower emulsifying capacity than tryptic ones.[55] This was again attributed to a slightly higher portion of lower sized products in the former case, as well as the more hydrophobic nature of fragments resulting from treatment by trypsin. Indeed, the highly soluble fragments, lacking a sufficient number of hydrophobic residues are expected to be poor emulsifiers or stabilisers, as they will have little affinity for adsorption. Experiments confirming this view were conducted by Caessens et al,[63] where ultra filtration was used to isolate and examine fragments arising from different sections of β-casein. Fractions obtained from the more hydrophobic end of β-casein had better foaming ability, while those from the middle parts displayed superior emulsifying capacity, when they were compared to the intact protein. However, the fragments from the hydrophilic N-terminus side

of the molecule displayed distinctly inferior foam, emulsion and surface activity.[63] In another interesting recent study, McCarthy et al removed a section from β-casein that included most of the phosphoserine residues of this protein. Emulsions stabilised by the remaining fraction, although on average of a larger droplet size, were found to be considerably more stable against the addition of calcium salts,[56] as expected. Situations involving the production of more complex fragments can also arise under certain circumstances. A particular case is that involving simultaneous denaturation and hydrolysis of β-lactoglobulin, where disulfide covalent bonds form between the polypeptides as these are generated.[62] Another is the hydrolysis of complexes formed between different proteins[66] or between protein and polysaccharides.[67] There are also systems for which the reported synergic effects between several different fragment types is thought to be key to the superior surface functionality of the broken up protein.[53]

As can be inferred from the above discussion, many of the experimental studies have been conducted on a somewhat trial and error basis. In contrast not a great deal of attention has been paid to theoretical identification of the most suitable cleavage points along the protein backbone, by examining the primary and secondary structure of the resulting polypeptide chains. This is understandable, since in practice it is quite difficult to have enzymes which only target a single or a very small number of desired peptide bonds. For example take trypsin, which is considered to have a relatively narrow well defined specificity. This enzyme only hydrolysis the peptide bonds involving the carboxyl side of lysine or arginine amino acids residues.[68] Even so, there are around 21 such bonds along the backbone of the α_{s1}-casein (the protein considered in our study), on which trypsin can act. Thus, at any time there can potentially be 253 different types of fragments, including the intact chain itself, which to a greater or lesser extent will all be competing with each other for adsorption onto the surface of the droplets. Furthermore, the relative abundance of each fragment type changes as the degree of hydrolysis is altered. At present such a theoretical investigation or computer simulations, involving the presence of all the fragments produced by the action of enzyme, in correct concentrations as dictated by a certain value of DH, remain infeasible. Nevertheless, some progress in this direction has been made and will be reported elsewhere in future. For now then, we shall focus on the cleavage of certain specific bonds one at a time. Despite its simplicity, even for this relatively limited model of fragmentation, some

interesting result and insights emerge. These clearly demonstrate both the potential and pitfalls of using fragmented polypeptides as colloidal stabilisers in foods.

In the next section we give a brief account of the theory behind our SCF calculations. We then highlight our model of α_{s1}-casein and its fragments. The results of the calculations are provided next, showing the density profiles of fragments at the interfacial regions and the colloidal interactions that these mediate between the emulsion droplets. These are compared and discussed in relation to the position of hydrolysed bonds on the backbone of α_{s1}-casein.

METHODOLOGY

Self Consistent Field Calculations Applied to Adsorbed Interfacial Layers

The application of self consistent field theory to ensembles of polymer chains is almost as old as the first studies of these macromolecules using statistical mechanical techniques. The earliest numerically based SCF calculation, applied to the study of colloidal forces mediated between two surfaces, covered with polymer chains, is the one due to Dolan and Edwards.[69] However, it was the reformulation of the theory by Scheutjens and Fleer[70-72]and its subsequent extension to more complex co-polymers[73-75], that has allowed the method to be more conveniently applied to wide range of situations, including some classes of food biopolymers. Exploring certain similarities between disordered proteins, such as α_{s1}-casein and β-casein, and synthetic co-polymers, Leermakers et al[76] and Dickinson et al[77,78] considered the configuration of dense interfacial layers produced by these proteins. Not only the structure of layers thus predicted were in good agreement with the results of neutron reflectometry experiments,[79] but the calculations also led to a clear explanation for the observed differences in the stability behaviour of emulsions covered by α_{s1}-casein and β-casein.[77,78]

The first and possibly the most important step in applying the self consistent field theory to layers of macromolecules on surfaces, is to average out the molecular degrees of freedom using the appropriate statistical mechanics methods. The procedure is quite involved but can be applied to problems concerning polymers in bulk as well as those at interfaces.[80-84] Once carried out, this leads to a coarse grained free energy functional which is no longer expressed

in terms of the position or configuration of individual molecules, but instead is now a function of a set of density profiles for each of the monomer residue types that form the chains. The set also includes the density profile variation of the solvent molecules, as well as any free ions that may exist in the solution. For two parallel flat surfaces, a distance L apart, immersed in a polymer solution, the derived free energy functional per unit area is[81,82]

$$\frac{\Delta F}{k_B T} = -\int_0^L \left[\sum_i \frac{1}{N_i} \sum_\alpha \left(\phi_i^\alpha(r) - \Phi_i^\alpha \right) \right] dr \; - \int_0^L \left[\sum_\alpha \psi_\alpha(r) \sum_\iota \phi_i^\alpha(r) \right] dr$$

$$+ \frac{1}{2} \int_0^L \left[\sum_{i \neq j} \sum_{\alpha \neq \beta} \chi_{\alpha\beta} \phi_i^\alpha(r) \phi_j^\beta(r) \right] dr \; + \; \frac{1}{2} \int_0^L \left[\psi^{el}(r) \sum_\alpha q_\alpha \sum_i \phi_i^\alpha(r) \right] dr$$

$$+ \sum_\alpha \chi_{\alpha s} \sum_i \left[\phi_i^\alpha(0) + \phi_i^\alpha(L) \right]$$

, (1)

where the variation of the density of monomer residues of kind α belonging to chains of type i, with distance r, is indicated by $\phi_i^\alpha(r)$ and their bulk volume fraction far from the plates by Φ_i^α. The distance r is measured relative to one of the plates, with the other surface located at r = L. The symbols k_B and T denote the Boltzmann constant and temperature as usual. In the original Scheutjens-Fleer approach, the above equation is derived using a lattice based model and as such is given in its discretised form.[70,72,85]

The last three terms in the above equation are easy to understand and capture the enthalpic terms associated with any given set of density profile variations. These arise due to a multitude of molecular level interactions amongst different monomers, as well as those between the monomers and the solvent molecules and with the two surfaces. In the current model we divide these into short range (nearest neighbour type) interactions and a longer ranged electrostatic one operating only between the charged species. The strength of the net nearest neighbour interaction between two different monomer species of type α and β, are specified by the usual Flory-Huggins interaction parameter, $\chi_{\alpha\beta}$. When $\chi_{\alpha\beta}$ is large and positive, the contact between monomer species α and β is not favoured, whereas negative values indicate a favourable interaction. It should be noted that as is customary, the $\chi_{\alpha\beta}$

parameter measures the net difference between the α-β interactions relative to those between the α-α and β-β ones. As such, the positive value of $\chi_{\alpha\beta}$ may arise either as a result of direct repulsive interactions between the α and β type residues, or as is more likely, because of a strong affinity of one of these species for itself. Of course, for hydrophobic interactions in an aqueous environment, the origins of a positive value for $\chi_{\alpha\beta}$ are mainly entropic themselves. A set of parameters, $\chi_{\alpha s}$, appearing in the last term of Eq. (1), similarly indicate the affinity of each species type for adsorption onto the surface.[85] Once again a higher affinity implies a negative value of $\chi_{\alpha s}$, with monomers having a positive value of $\chi_{\alpha s}$ being the ones that avoid contacting the surface. The longer ranged electrostatic interactions between the charged species are taken care of by the fourth term in Eq. (1). This involves the electrostatic potential $\psi^{el}(r)$. Clearly this potential influences the spatial distribution of charged groups in the gap between the two plates. However, in turn $\psi^{el}(r)$ itself is determined by such a distribution as given by the Poisson equation:

$$-\varepsilon_r \varepsilon_0 \frac{\partial^2 \psi^{el}(r)}{\partial r^2} = \sum_\alpha q_\alpha \sum_i \phi_i^\alpha(r) \quad . \qquad (2)$$

We have denoted the charge of species α in Eqs. (1) and (2) as q_α. We have assumed that the plates are homogenous. Therefore, the potential ψ^{el} only various with the perpendicular distance in the gap between the surfaces, but not in a parallel direction to the plates.

Given a particular set of density profile variations, $\{\phi_i^\alpha(r)\}$, one can find many different possible spatial and conformational arrangements of biopolymers, ions and solvent molecules that will lead to this, the same density profile. The entropy contribution for each density profile arises from the number of such microscopic states associated with that profile. This is given by the combination of the first two terms in Eq. (1), with the first of these simply being the excess number of molecules of each kind i in the gap between the two surfaces. The quantity N_i is the size of chains of type i, given by the number of residues that these macromolecules contain. For the solvent and free ions we take $N_i = 1$. Finally, a set of auxiliary fields $\{\psi_a(r)\}$ appear in the second term of equation (1). These are fields that project out a particular density profile in the gap between the surfaces. They emerge naturally as part of the statistical mechanics averaging procedure used in deriving the coarse grained free energy functional. Each field $\psi_a(r)$ acts on its corresponding monomer species α

throughout the gap. These fields can be given a somewhat physical interpretation as follows. The set $\{\psi_a(r)\}$ are fields that when applied to an equivalent, but non-interacting set of chains, would result in the establishment of density profiles $\{\phi_i^{\alpha}(r)\}$ for which Eq. (1) is being calculated. By the term "non-interacting" we mean here chains that do not influence the conformation or spatial distribution of their neighbouring molecules and by "equivalent" we refer to chains that have exactly the same primary sequence of monomers, the same number of residues and the same bulk concentrations as the original system. In general it is easier to begin with fields $\{\psi_a(r)\}$ and then compute the resulting density profile $\{\phi_i^{\alpha}(r)\}$, as oppose to doing the calculations the other way round. Such calculations require the evaluation of the so called segment distribution functions for each type of biopolymer chain present in the system. For the purpose of the numerical calculations it is also necessary to discretize all the equations, including Eq. (2). In the Scheutjens-Fleer method,[70,72,85] this is achieved by dividing the gap between the planes into (L/a_0) parallel layers of thickness a_0 each, where a_0 is commonly taken to be the nominal monomer size (~ 0.3 nm here). The segment distribution functions, $G_i^f(n,z)$ and $G_i^b(n,z)$ are defined as the probability that a chain, consisting of the first n residues of the biopolymer i, will end up having its n^{th} monomer in the layer z, where z = 1 to (L/a_0). The suffix "b" or "f" differentiate the two ends of the polymer chain from which the n monomers are chosen. The procedure for calculating the segment distribution functions for a given set of fields $\{\psi_a(r)\}$ is well documented in the literature,[73,76,85] as well as described in our own previous work.[28,86,87] Therefore this will not be reproduced here. It suffices to say that once these distribution functions become available for all molecules and every value of n =1 to N_i, then the density of each monomer species α, forming a part of molecules of type i, can easily be determined for any desired layer z in the gap between the plates, through the composition law[73,85,87]

$$\phi_i^{\alpha}(z) = \frac{\left(\sum_{\alpha} \Phi_i^{\alpha}\right)}{N_i} \sum_{n=1}^{N_i} \left(\frac{G^f(n,z) G^b(N_i - n, z) \delta_{\alpha, t_i(n)}}{\exp(-\psi_{\alpha}(z))} \right) \quad . \qquad (3)$$

The function $\delta_{\alpha\beta}$ appearing in the above equation is the usual Kronecker delta function, which is equal to 1 if $\alpha=\beta$ and zero otherwise. We also define the function $t_i(n)$ such that it evaluates to the monomer species type number of the n^{th} monomer, on the backbone of biopolymer chains of kind i in our system.

The density profile variation of different monomer residues in the gap between the two surfaces are subject to fluctuations. Thus, in principle, any set of density profile variation, $\{\phi_i^{\alpha}(r)\}$ can arise in the gap between the two surfaces. The probability of this occurring at any time is proportional to ~ $\exp(-F(\{\phi_i^{\alpha}(r)\})/k_BT)$, where $F(\{\phi_i^{\alpha}(r)\})$ is the free energy for that profile, as given by Eq. (1). Hence, strictly speaking, all thermodynamic quantities of interest have to be averaged over all possible sets of density profiles, each one weighed with its own appropriate Boltzmann constant. Mathematically this is a very difficult task to carry out. Instead, in SCF theory the following important approximation is invoked. One assumes that the density profile with the lowest free energy (i.e. the highest Boltzmann weight) is the one that dominates the behaviour of the system. That is to say that the probability of any other profiles witch significantly deviations from this, the most probable $\{\phi_i^{\alpha}(r)\}$, is considered to be negligible. It is known that for concentrated polymer solutions or dense interfacial layers, like those occurring during the adsorption of protein at hydrophobic surfaces, the approximation is a valid one.[81,82,85] The condition for $F(\{\phi_i^{\alpha}(r)\})$, as given by Eq. (1), to take its minimum value is often considered in conjunction with an additional incompressibility restriction. This ensures that the sum of the concentrations of all species, at any point in the solution, is always the same. Therefore

$$\sum_i \sum_{\alpha} \phi_i^{\alpha}(r) = \sum_i \sum_{\alpha} \Phi_i^{\alpha} \quad . \qquad (4)$$

Note that the sum over i includes the solvent and ions, too. Taken together with the condition in Eq. (4), the minimum of $F(\{\phi_i^{\alpha}(r)\})$ can be shown to occur when

$$\psi_{\alpha}(r) = \psi_h(r) + \left(\sum_{\beta} \chi_{\alpha\beta} \sum_i \phi_i^{\beta} \right) + q_{\alpha}\psi^{el}(r) + \chi_{\alpha s}\left[(\delta(r) + \delta(r - L)\right] \quad . \qquad (5)$$

where $\delta(r)$ represents the Dirac's delta function and $\psi_h(r)$ is a hard core potential that ensures the incompressibility condition, Eq. (4). This hard core potential is the same for all monomeric species, be they part of a biopolymer chain, an ion or a solvent molecule. Equation (5) allows one to employ an iterative procedure in order to obtain the set of density profiles that minimise the free energy. One begins with an initial starting guess for $\{\phi_i^{\alpha}(r)\}$. These values are used in Eq. (5), in conjunction with (2) and (4), to obtain a set of fields $\{\psi_a(r)\}$. A new set of density profile variations is now calculated from these fields using

equation (3) and compared with the previous set. The process is repeated until the difference between $\{\phi_1^{\alpha}(r)\}$ values in two successive iterations, for all layers in the gap is found to be less than a pre-specified small tolerance limit. At this stage the convergence is said to be obtained, with the computed values of $\{\phi_1^{\alpha}(r)\}$ being those for which the interfacial free energy attains its minimum. Finally, the colloidal interaction potential per unit area between the two surfaces arising from the overlap of the adsorbed layers is obtained, by monitoring the changes in the free energy of the system, as the gap size between the plates is varied:

$$V(L) = \Delta F(L) - \Delta F(\infty) \quad . \qquad (6)$$

For $F(\infty)$ we use the value of the free energy evaluated when the two surfaces are sufficiently far apart, such that the presence of one surface does not influence the adsorption behaviour taking place at the other interface. Equation (6) gives the interactions between two flat surfaces. These can further be manipulated, using the well know Derjaguin approximation[4,5]

$$V_{par}(L) = \pi R \int_{\infty}^{L} V(x)dx \quad , \qquad (7)$$

to yield the mediated interactions between two spherical colloidal particles or emulsion droplets of radius R.

Model

In this study we shall consider the resulting interactions between emulsions stabilised by α_{s1}-casein or its fragments. Our reason for choosing this protein is two fold. Firstly α_{s1}-casein, like other caseins, is a disordered protein with no tertiary structure and a minimal amount of secondary structure, thought not to be of huge consequence in dictating its adsorption behaviour. This makes α_{s1}-casein much more amenable to calculations based on SCF theory, described above. Secondly, emulsions stabilised by this protein are considerably more prone to colloidal instability at high salt concentrations or close to pI of the protein, say in comparison to β-casein.[77,78] This makes it worthwhile then to study how the fragments of α_{s1}-casein fair in this respect, relative to the intact protein itself.

In adopting the model used in this work, the main objective was to maintain a reasonable representation of the hydrophobic, hydrophilic and charge sections of α_{s1}-casein, based on its primary structure. It has been shown by the pioneering work of Leermakers et al[76] that this can be achieved to a good degree of approximation, by having the amino acid residues divided into six distinct categories. These are the hydrophobic residues, the polar but non-charged amino acids and the positive and the negatively charged monomers. The other two groups consist of negatively charged phosphoserine, which has two pK_a values, and histidine, with a rather different pK_a compared to other positively charged residues. For this reason these two amino acids are placed in their own separate groups, distinct from other charged monomers. In this model, the chemical nature of the residues assigned to each group, manifest themselves through the interactions they have with monomers in other groups and with the surface, free ions and solvent molecules. Thus for example, the hydrophobic residues will have a positive, unfavourable Flory-Huggins χ interaction parameter with the solvent, as well as any other polar or charged residues and free ions. On the other hand, these hydrophobic monomers will also have a negative χ of a few k_BT for their interaction with the surface. This is typical of the magnitude of hydrophobic interactions between such residues and a hydrophobic interface. The negative value of χ entices the hydrophobic residues of our model biopolymers to adsorb and remain in contact with the plates. A full list of different χ parameters for the interaction between various groups of monomers is given in Table I. These are largely taken from the work of Leermakers et al[76] and were also found to be appropriate ones to use in our own previous work.[26,27,45] In addition, the ionisable groups also possess electric charges which vary with the pH of the solution. For a desired pH, these are calculated as normal using the corresponding pK_a values for each group as provided in Table I. The primary structure of α_{s1}-casein is that taken from Swaisgood,[88] as illustrated by the model of Akinshina et al.[45] Similarly, for any fragmented section of α_{s1}-casein, we take the sequence of amino acid residues to remain identical as that of the same part of the intact molecule. However, we do add the C-terminus and the N-terminus charges at the two sides of any hydrolyzed peptide bonds.

Apart from the biopolymers and solvent, there are also free positive and negative ions in the solution. By alternating the volume fraction of these in the calculations, the concentration of background electrolyte can be specified. We shall assume that these ions are monovalent, e.g. Na^+ and Cl^-. By making the concentration of one of these marginally higher than the

other as necessary, we can account for the presence of counter-ions arising from the charged biopolymers. In this way the charge neutrality of the entire solution is guaranteed. The preference of the ions for hydration by the solvent molecules is represented here by having the ion-solvent interaction parameter set to -1 (k_BT), thus promoting contact between the ions and the solvent molecules. Finally, in relation to Eq. (2), we take the relative permeability of water as 79 in our calculations.

RESULTS AND DISCUSSIONS

Intact α_{s1}-casein

It is useful to begin our study by first reproducing some of the known results, showing the expected conformation and the colloidal interactions induced by intact α_{s1}-casein layers. An α_{s1}-casein molecule adsorbed on the surface, is subject to Brownian fluctuations, constantly altering its configuration. However, using our SCF calculations, it is possible to predict the average distance of each monomer residue away from the surface. This is presented in the graphs of Figure 1, where the average distance from the interface is plotted against the monomer sequence number. The first residue, counting from the N-terminus side, is labeled as 1, the second as 2, and so on. The calculations were performed at neutral pH for the data presented in Figure 1a, and at pH=4.5, close to pI of our model protein, for those in Figure 1b. The background electrolyte volume fraction was set at a moderately high value of 0.01 (roughly equating to 0.3 mol/l), in both cases. The separation distance between the plates was 180 monomer units apart. At such a gap size, the two surfaces are essentially isolated and the adsorption taking place on either interface is not affected by the presence of the other surface. The protein bulk volume fraction was taken to be 10^{-11} in the calculations. It is important to note that this small value does not imply that the total amount of protein in the system is also small. The choice of a tiny number for the volume fraction is a reflection of the fact that, in the majority of practical formulations, almost all of the protein is adsorbed. Only a very small fraction of the molecules tend to remain in the solution. We choose the concentration of this remaining protein in the solution such that an adsorbed layer, in equilibrium with bulk, has the predicted protein coverage of roughly 1 mg/m^2, according to the calculations. This is in line with the experimental values often quoted for the coverage of emulsion droplets by such proteins.

The most obvious feature of both graphs in Figure 1 is the "triblock like" behaviour of α_{s1}-casein, where the two end sections of the protein form trains of adsorbed residues, remaining close to the interface. In contrast, the more hydrophilic middle part of α_{s1}-casein forms a loop, protruding some distance away from the surface into the bulk solution. The presence of this loop is crucial for the colloidal stabilising ability of α_{s1}-casein. It is the overlap of the loops on the surface of two approaching particles that induces the required strong steric repulsion between the particles, thus keeping the droplets apart. This is particularly significant at higher salt concentrations, where the electrostatic component of the inter-particle repulsive force is largely screened. Of course, it is important that the overlap should start at a reasonably far separation distance where the van der Waals attractive forces between the particles are still negligible. Comparing the graph of Figure 1b to that in Figure 1a, it is evident that the extension of the loop formed by the middle part of α_{s1}-casein is less at pH of 4.5 than at neutral pH. This leads to shorter ranged steric repulsion at pH=4.5. The triblock-type behaviour of α_{s1}-casein can itself lead to problems, causing bridging flocculation. The presence of another adjacent nearby surface means that the end sections of some of the chains on one droplet can desorb from it and become attached to the opposite interface, and vice versa. In other words, as well as forming loops, the chains can now also take up link conformations, extending from one surface to the other. This constitutes a rapid increase in the configurational entropy of the chains and therefore is favoured. The result is an attractive contribution to the mediated interactions between the droplets at short separation distances. In particular, at distances where the adsorbed layers on two adjacent droplets (or particles) just begin to overlap, the steric repulsion is as yet not strong. At these distances it is this entropically driven component that thus dominates, further enhancing the attraction between the droplets already in operation due to van der Waals forces. As droplets move even closer, the excluded volume interaction, arising from the increased overlap of the layers, starts to become larger and the steric forces become repulsive. A lucid theoretical demonstration of this effect was first given by Milner and Witten.[89] The effect is also shown to exist in multi-block chains,[87] and hence for the majority of proteins. At low salt concentrations, or pH values far from pI, the charge of the protein is normally adequate to provide a sufficient amount of electrostatic repulsion to largely mask this effect. However, at pH values close to the isoelectric point of the protein and high electrolyte concentrations, such bridging phenomenon gives rise to an attractive energy potential well in the inter-particle interaction potential. This is clearly demonstrated by our SCF calculated results,

presented in Figure 2. The graphs in this figure show the α_{s1}-casein induced particle-particle interaction potentials, obtained at several different pH values and for oil droplets of size 1 μm. All the other parameters, including background electrolyte concentration, are the same as those in Figure 1. The interactions also include the ubiquitous van der Waals attraction, which is present independent of the behaviour of biopolymers. For droplets of radius R, with a surface separation of r, this is given to be[4,5]

$$V_{vw} = -\frac{AR}{12r} \quad , \qquad (8)$$

where A is the composite Hamaker constant, around 1 k_BT for most oil in water emulsions. In most cases, we find that once the protein layers begin to overlap, the induced forces quickly overwhelm the van der Waals interaction. Nevertheless, the van der Waals attraction can be more significant at separations larger than the overlap distance. This leads to a small energy minimum in the V(r) graphs at these particle surface separations. This is evident in the results shown in Figure 2, obtained at pH=7. The depth of this energy well is only a few k_BT and therefore it can easily be overcome by the Brownian motion of the droplets. As the pH of the solution is lowered down to 5.0 the magnitude of the energy minimum increases. At pH=4.5, close to pI of our model protein, the depth of the minimum is now ~ 180 k_BT, as can be seen from the corresponding graph in Figure 2. This arises from a combination of a lower extension of the loop formed by α_{s1}-casein as already shown in Figure 1b, the bridging effect discussed above and the absence of any contribution from the electrostatic repulsion at this pH. The presence of an energy well of this magnitude, in the inter-particle interaction potential, leads to the strong aggregation of droplets. This in turn is likely to cause the coalescence and the eventual breakdown of the emulsion dispersion. At even lower pH of 3 the net charge of protein becomes positive. This leads to a significant decrease in the depth of the energy minimum to 26 k_BT, which nevertheless is still sufficient to lead to the formation of flocs of oil droplets in these dispersion systems. While for an energy minimum of -26 k_BT, the flocs are now weak enough to be broken by rigorous shaking or stirring of the emulsion, their presence will still manifest itself in the shear thinning and other such non-Newtonian rheological behaviour of the dispersions.

Fragments of α_{s1}-casein as Colloidal Stabilisers

The preceding discussions highlighted a major shortcoming in the use of α_{s1}-casein as an emulsion stabiliser. This feature is common to many other proteins too. In this section we will examine and compare the stabilising ability of fragments of α_{s1}-casein with those of intact protein. For this purpose, we shall take the hydrolysed peptide bond as one involving the carboxyl side of either lysine or arginine residues. There is no real reason for this apart from the fact that these are the bonds that the enzyme trypsin attacks, so there is a precedent for their specific hydrolysis. However, it should be noted that the enzyme breaks such bonds indiscriminately, whereas here we assume that only one chosen bond, out of the 21 or so possible ones on α_{s1}-casein backbone, is cleaved. We shall not address the separate and considerable practical difficulties in realising such a system. Rather, our aim is simply to see whether there are theoretical grounds for expecting a section of a protein to have superior emulsion stabilising properties, when it is compared to the whole protein itself.

In deciding upon the specific bond for the cleavage, it is useful to examine the known differences between the interfacial behaviour of α_{s1}-casein and β-casein.[76-78] Unlike α_{s1}-casein, the behaviour of β-casein more closely resembles those of diblock polymers.[76] For instance, the bridging effect, discussed in relation to α_{s1}-casein in the previous section, does not occur with diblock chains. Examining the conformation of α_{s1}-casein in Figure 1a, it is evident that such diblock segments can be produced by the breakage of the bonds close to either ends of the central hydrophilic loop of α_{s1}-casein. The locations of two such bonds, involving lysine or arginine, are indicated in Figure 1a by arrows (A) and (B). These occur between the residues 42-43 and 103-104 of α_{s1}-casein, respectively, counting from the N-terminus side of the molecule. For the first of these, the fragmentation separates the relatively hydrophobic N-terminus side of the protein from the rest of the chain. The remaining fragment should now have a more diblock like distribution of hydrophobic and hydrophilic amino acids, similar to that seen for β-casein. Our SCF predicted interaction potential, V(r), between two droplets of size 1 μm, in a solution of fragmented protein resulting from the breakage of bond (A), is displayed in Figure 3. Both fragments are simultaneously present and none are removed from the solution. The graphs for various pH conditions are plotted on the same scale as those in Figure 2 for comparison, where the overall volume fractions of fragmented and the full protein have also been kept the same. It is

markedly obvious that in the graphs of Figure 3, the presence of any energy minima is barely detectable. The difference between the induced inter-particle potentials by the intact and the fragmented chains is most striking at pH=4.5, where the depth of the energy well is 180 k_BT for the former system, but only 3.5 k_BT for the latter case. Thus, even at the isoelectric point of our model α_{s1}-casein, the emulsion droplets stabilised by fragments are predicted to remain stable. These results clearly demonstrate the potential for the appropriately chosen polypeptide fragments to outperform the colloid stabilisation ability of unmodified protein. The reason for the improved performance in this case is best understood from the results presented in Figure 4. This shows the average distance of each residue away from the surface for the fragmented chains obtained from the C-terminus side of α_{s1}-casein, adsorbed at the interface. The data was obtained at pH=4.5, for the same system as those in Figure 3. This is to be compared to the graph of Figure 1b, calculated under the same condition as those for the intact protein. Not only the more desirable diblock type behaviour of the fragment is evident, but one can also notice that the dangling end of the polypeptide extends somewhat further away from the interface than the loop of the unmodified α_{s1}-casein, at the same pH.

Similar type of behaviour should also be possible to achieve by breaking the bond on the other side of the loop, at the position labelled (B) in Figure 1a. This time it is the more hydrophobic C-terminus side that is separated away to leave a diblock type structure, involving the N-terminus end of α_{s1}-casein. The interaction potential mediated between the droplets in a solution of such fragmented protein, is displayed in Figure 5. Once again the graphs are at pH values of 7, 5, 4.5 and 3. While at pH=7 and pH=3, i.e. a few pH units on either side of isoelectric point, the interaction potential graphs indicate a reasonable degree of colloidal stability for the droplets, this is not the case closer to pI, in this instance. As with the unmodified α_{s1}-casein, but unlike the previous fragmented protein involving the broken bond (A), there are deep energy wells present in V(r) induced by these polypeptides. The graph of Figure 6 shows the conformation of the N-terminus side fragment at the interface. This seems to suggest that this fragment adopts a configuration not too dissimilar to a diblock polymer. The polypeptide also extends to a reasonable distance of ~ $7a_0$ which again is quite comparable to that for the C-terminus segment for the case of previous broken bond (see Figure 4 and Figure 6). The question arises then as to why in the previous system a good level of stabilisation was predicted, but yet this does not seem to be true for the fragments produced by the cleavage of bond (B). A significant clue to answering this question is

provided by considering the variation of the volume fraction of each of the two fragments that result from the breakage of bond (B), plotted as a function of distance away from the interface. Such graphs are shown in Figure 7. In this figure, the dashed line represents the results for the (undesirable) C-terminus fragment and the solid line those for the "diblock like", N-terminus side. While there is a reasonably large amount of C-terminus fragment on the surface, the amount of the more desirable N-terminus fragment is almost zero. That there is only a minuscule amount of the latter polypeptide adsorbed on the interface can be better seen in the inset graph of Figure 7, where we have magnified the y-axis by a factor of 10^5. Interestingly, the variation of the volume fraction, as seen in the inset graph, has a maximum occurring some distance away from the surface. This is a well known type of behaviour displayed by synthetic diblock polymers, consisting of a strong anchoring block and another that prefers to be in the solvent.[85] Thus, while the desired N-terminus side fragment possesses all the right structural attributes and will adopt the required conformation on the surface, it is not adsorbed in merely sufficient levels so as to have any influence on the particle-particle interaction potential. In other words, the desired polypeptide is displaced through the competitive adsorption by the other fragment. Indeed, in the absence of this other C-terminus side segment, the "diblock-like" fragment acts as a reasonably good stabiliser. The induced potential plotted in Figure 8, demonstrate this quite clearly. The calculations were carried out under the same conditions as those in Figure 6, for pH= 4.5. However, this time we had removed all C-terminus side polypeptide from the system. It can be seen that the depth of the energy minimum well in the inter-particle potential, is reduced from a value of ~ 70 k_BT in the presence of undesirable C-terminus fragments (see Figure 6), to a much lower value of ~ 7 k_BT in their absence (Figure 8).

The conclusion from the above discussion is that it is simply not enough to focus on producing fragments with promising structural attributes needed for good stabilisers. In as much as this is important, one should also consider other fragments being produced and the resulting possible competitive adsorption that will occur between these different polypeptides. In the examples we have considered, the fragmentation of α_{s1}-casein through the breakage of bond (A) in Figure 1a, produced a polypeptide with a more optimal primary sequence of amino acids, compared to the intact protein. This fragment also strongly dominated the adsorption at the interface. In contrast, for the case involving the cleavage of bond (B), a polypeptide with a suitable structure was achieved but could not compete for

adsorption with the other resulting fragment. We should finally add that in the preliminary calculations we presented above, the hydrolysis of the chosen bond was assumed to be complete. In practical cases of course, the degree of hydrolysis (DH) is often kept at a much smaller value. This means that some residual unbroken protein may also be present. Hence, in addition to the competitive adsorption amongst the fragments themselves, there is also competition for adsorption with the unmodified protein. Even a small concentration of the intact protein may turn out to be enough to dominate the interfacial properties, displacing all the fragments. In such a case, there are little expected gains to be made by fragmenting the protein.

CONCLUSIONS

By the virtue of a structure more conducive to the provision of inter-particle interactions, necessary to insure colloidal stability, one can argue that some fragments of protein may provide better emulsion stabilising properties than the protein itself. However, the experimental results involving fragmented proteins remain somewhat inconclusive on this issue, showing a diversity of behaviour dependent on the degree of hydrolysis, pH value, types of protein involved and the bond selectivity of the enzyme used in fragmentation. In the current work, we have addressed the above question from a theoretical point of view by using calculations based on the self consistent Field theory of Scheutjens and Fleer.[70,72] We have considered a model of α_{s1}-casein, and its fragments, in which the amino acid residues are divided into six separate categories.[76] The interactions that are mediated between the emulsion droplets by adsorbed layers of such polypeptides are then calculated. We have confirmed that the adsorbed α_{s1}-casein chains adopt a triblock-type conformation at the interfaces. At high salt concentrations and pH values close to the isoelectric point of the protein, the limited extent of the hydrophilic loop of α_{s1}-casein, leading to thinner interfacial layers, coupled with the possibility for bridging, causes the induced interactions between the emulsion droplets to become attractive. By focusing on the cleavage of bonds which produce polypeptide structures more closely resembling a diblock-type structure, it is shown that the

fragments can in principle outperform the colloid stabilising properties of α_{s1}-casein. This is particularly evident close to pI of the protein. Nevertheless, choosing part of the protein with the correct structure is only one of the requirements in achieving superior properties. The hydrolysis of the protein chains also produces other polypeptides. These compete with the desired fragment and can indeed displace it from the interface. We have again demonstrated this effect using our SCF calculations, by considering a system where a peptide bond towards the C-terminus side of the hydrophilic section of α_{s1}-casein was broken.

While our results have been for α_{s1}-casein, it is achieving good emulsions stabilisers, derived from globular vegetable proteins such as soya bean or peas, that would be of the greatest commercial interest to the food industry. Also, here we have considered a situation where only one specific peptide bond is cleaved, and then at 100% hydrolysis. At present, such a situation cannot be engineered using enzymatic fragmentation of the proteins. However, there is no reason why polypeptides involving a selected section of a given protein cannot be synthesized using chemical means.[90] Though clearly not suitable for any large scale production purpose, this nevertheless will allow many of the theoretical predictions of the current work to be verified experimentally. Should a more selective cleavage of bonds become commercially feasible, some of the undesired fragments, having higher surface affinities, can be filtered out by first passing the solution through a porous hydrophobic matrix. However, for now it is useful to extend the SCF calculations to situations involving many broken bonds and a larger set of different fragments, as is presently more realistically achievable in practice. Such theoretical work is currently underway and will be reported elsewhere.

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Table Caption

Table I - Set of values of χ Flory–Huggins interaction parameters (in units of k_BT) for different monomer types, making up the model α_{s1}-casein and its fragments, the salt ions and the solvent molecules. Also shown are the interaction parameters for each monomer group with the hydrophobic interface and the assumed pK_a values for the charge-carrying amino acid residues of the protein.

Figure Captions

FIGURE 1 – Average distance of each monomer residue, making up an adsorbed α_{s1}-casein molecule, away from a planner interface, plotted against the monomer sequence number. The numbering starts from the N-terminus side of the protein chain. Results are at a background electrolyte volume fraction of 0.01 and at pH=7 (a) and pH=4.5 (b). The arrows in (a) indicate the location of possible bonds, on either side of the central loop, susceptible to hydrolysis by trypsin.

FIGURE 2 – The interaction potential, V(r), induced between the oil droplets of size 1 μm, by the intact α_{s1}-casein, plotted against the surface separation distance. All the results are at a salt volume fraction of 0.01 and pH values of 7 (dash-dotted line), 5 (short dashed line), 4.5 (long dashed line) and 3 (solid line).

FIGURE 3 – The same results as those in Figure 2, but now involving fragmented α_{s1}-casein. The protein is cleaved at the peptide bond 42-43 (bond (A) in Figure 1a). Both resulting fragments are present in the system.

FIGURE 4 – Graph showing the conformation of the C-terminus side fragment of α_{s1}-casein, adsorbed on a hydrophobic surface at salt volume fraction of 0.01 and pH=4.5. The fragment is produced by the breakage of the bond 42-43 on α_{s1}-casein backbone. The sequence number on abscissa commences from the N-terminus side of the polypeptide fragment.

FIGURE 5 – The same results as those in Figure 2, but now involving fragmented α_{s1}-casein. The protein is cleaved at the peptide bond 103-104 (bond (B) in Figure 1a). Both resulting fragments are present in the system.

FIGURE 6 – Average distance from the interface for each residue of the N-terminus side fragment, adsorbed on the surface, at an electrolyte volume fraction of 0.01 and pH=4.5. The fragment is produced by the cleavage of bond 103-104, bond (B) in Figure 1a.

FIGURE 7 – Variation of the volume fraction of both polypeptides produced by the breakage of bond (B) in Figure 1a, away from the planner interface. The dashed line represents the C-terminus side fragment and the solid line the N-terminus one. The volume fraction of the latter is so small that it can only be observed in the inset graph, where the ordinate has been magnified by a factor of 10^5. The results are calculated at a pH=4.5 and salt volume fraction of 0.01.

FIGURE 8 – The same results as those in Figure 5, showing the variation of the induced inter-particle interaction. However, this time the C-terminus side fragment has been removed from the system.