Interfacial Mechanics of β-Casein and Albumin Mixed Protein Assemblies at Liquid-Liquid Interfaces

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Abstract

Protein emulsifiers play an important role in formulation science, from food product development to emerging applications in biotechnologies. The impact of mixed protein assemblies on surface composition and interfacial shear mechanics remains broadly unexplored, in comparison to the impact that formulation has on dilatational mechanics and surface tension or pressure. In this report, we use interfacial shear rheology to quantify the evolution of interfacial shear moduli as a function of composition in bovine serum albumin (BSA)/β-casein mixed assemblies. We present the striking difference in mechanics of these two protein and observe the dominance of β-casein in regulating interfacial shear mechanics. This observation correlates well with the strong asymmetry of adsorption of these two proteins, characterised by fluorescence microscopy. Using neutron reflectometry and fluorescence recovery after photobleaching, we examine the architecture of corresponding protein assemblies and their surface diffusion, providing evidence for distinct morphologies, but surprisingly comparable diffusion profiles. Finally, we explore the impact of crosslinking and sequential protein adsorption on the interfacial shear mechanics of corresponding assemblies. Overall, this work indicates that, despite comparable surface densities, BSA and β-casein assemblies at liquid-liquid interfaces display almost 2 orders of magnitude difference in interfacial shear storage modulus and strikingly different viscoelastic profiles. In addition, co-adsorption and sequential adsorption processes are found to further modulate interfacial shear mechanics. Beyond formulation science, the understanding of complex mixed protein assemblies and mechanics may have implications for the stability of emulsions and may underpin changes in the mechanical strength of corresponding interfaces, for example in tissue culture or physiological conditions.

Introduction

Protein emulsifiers play an important role in the formulation of many food products and their processing, as well as for the engineering of materials for consumer healthcare applications [1–5], although the latter field is largely dominated by small molecules, polymers and particulate emulsifiers. A range of different proteins are typically involved, from animal-derived proteins such as albumins, caseins, lysozyme and lactoferrin, proteoglycans such as mannoproteins, to plant-based proteins such as soy or pea proteins, allowing the stabilisation of a broad range of oils, mineral or plant-based [1,2].

In the biotechnology space, microdroplet microfluidic platforms have recently been proposed for a range of applications, from the monitoring of reaction kinetics, screening and biosensing to single cell sequencing [6–8]. Most surfactants used in these applications are synthetic, small molecules or macromolecular, for example based on various types of perfluoropolyethers and associated copolymers for the stabilisation of fluorinated oil droplets, or Span 80, Tween derivatives and other lipids for the stabilisation of mineral or silicone oil droplets [9,10]. Although tensioactive proteins have played a minor role for such technologies [11–14], they present interesting potential, for example to provide cytocompatibility and replace materials harmful to our environment.

Recently, the design of bioactive microdroplets enabling the adhesion and culture of adherent cells has been proposed [15–18]. Indeed, it was discovered that the self-assembly of proteins at liquid-liquid interfaces could enable the formation of protein nanosheets displaying strong mechanical properties, able to resist cell-mediated contractile forces [19–26]. In these systems, the protein nanosheets formed play a triple role, stabilising corresponding interfaces and associated emulsions (tensioactive), forming a percolated network with strong interfacial viscoelastic properties (scaffolding) and presenting ligands able to engage with cell membrane receptors (bioactivity). In turn, the resulting bioactive liquid-liquid interfaces and bioactive emulsions (or bioemulsions), enable the regulation of cell adhesion and maintenance of stem cell phenotype [15,19,20,22,25–27]. The role of interfacial shear mechanics, to enable the mechanical engagement of integrin receptors and downstream mechanosensing cascades was found to be essential to allow the maintenance of stem cell phenotype upon culture at liquid-liquid interfaces or on bioemulsions. The impact of a number of parameters such as the chemistry of the oil used [25], the presence of co-surfactants [20–23], pH of assembly [22], functionalisation with charged moieties [18] and physical or chemical crosslinking of proteins [17,19,20] on interfacial mechanics and cell culture

have been explored in the context of single protein solutions, from albumins and β-lactoglobulin to fibronectin. In particular, interfacial shear mechanics and viscoelasticity, rather than dilatational or indentation moduli, were found to correlate well with cell adhesion and proliferation [20,22,28]. However, how protein mixtures, which have been found to impact on dilatational mechanics, can also regulate the interfacial shear moduli of corresponding assemblies has not been explored systematically yet.

Competitive adsorption of protein at air-water or oil-water interfaces has received increasing attention, to improve the stability of foams and emulsions. In particular, systems combining globular and disordered flexible proteins have been studied broadly [29,30]. Globular proteins are typically described as more rigid and require denaturation to adsorb at hydrophobic interfaces [17,30,31], or at least conformational changes underpinning surface adsorption and self-association [32–34]. In turn, disordered proteins such as casein can adsorb faster at interfaces, owing to their softer, less structured architecture [31,35]. This reflects the required energy of activation associated with denaturation and protein conformation typical of globular protein assembly, therefore modulating protein adsorption kinetics and regulating competitive adsorption profiles [36–40]. In most cases, studies have focused on the interfacial composition and to some extent dilatational rheological properties of corresponding mixed assemblies, and few studies have investigated their interfacial shear mechanics.

In this manuscript, we explore the adsorption of mixed bovine serum albumin (BSA) and β-casein at the interface between the fluorinated oil Novec 7500 (relevant to microdroplet platforms for biotechnologies) and phosphate buffer saline. We characterise the impact such assemblies have on interfacial shear mechanics and correlate these changes with surface composition. We then characterise the nanostructure and diffusion of assemblies of both isolated proteins via neutron reflectometry and fluorescence recovery after photobleaching (FRAP), respectively. Finally, we explore the impact of chemical crosslinking and protein exchange on interfacial mechanics.

Materials and Methods

Materials and Chemicals. β-Casein (> 98%), BSA (> 98%), 1H,1H,2H,2H-perfluorodecanethiol (97%) and sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-l-carboxylate (sulfo-SMCC) were obtained from Sigma. The Alexa Fluor™ 488 NHS ester and the Alexa Fluor™ 488 NHS ester were purchased from Thermofischer Scientific. The fluorinated oil (Novec 7500; 2-(trifluoromethyl)-3-ethoxydodecafluorohexane) was from ACOTA. SPR gold coated sensor chips were obtained from Ssens.

Preparation of emulsions. For the generation of emulsions, 1 mL of fluorinated oil (Novec 7500) and 2 mL protein solution (1 mg/mL in PBS) were added into a glass vial. The vial was shaken using a vortex until the emulsion was created (10 s) and further incubated for 1 h at room temperature. The upper liquid phase was aspirated and replaced with PBS 6 times to wash off excess protein solution.

Interfacial shear rheology measurements. Interfacial shear rheology was selected for the evaluation of interfacial shear mechanics. Interfacial rheological measurements were carried out on a Discovery Hydrid-Rheometer (DHR-3) from TA Instruments, using a Du Nouy Ring geometry and a Delrin trough with a circular channel. The Du Nouy ring has a diamond-shaped cross section that improves positioning at the interface between two liquids to measure interfacial rheological properties whilst minimizing viscous drag from upper and sub-phases. The ring has a radius of 10 mm and is made of a platinum-iridium wire of 400 μm thickness. The Derlin trough was filled with 4 mL of fluorinated oil (with or without surfactant). Using axial force monitoring, the ring was positioned at the surface of the fluorinated oil, and was then lowered by a further 200 μm to position the medial plane of the ring at the fluorinated phase interface. 4 mL of the PBS solution were then gently introduced to fully cover the fluorinated sub-phase. Time sweeps were performed at a frequency of 0.1 Hz and temperature of 25°C, with a displacement of 1.0 10^{-3} rad (strain of 1 %) to follow the self-assembly of the protein nanosheets at corresponding interfaces. In each case, the protein solution (1 mg/mL) was added after 15 min of incubation and continuous acquisition of interfacial rheology data for the naked liquid-liquid interface. Before and after each time sweep, a frequency sweep (with displacements of 1.0 10^{-3} rad) and amplitude sweeps (at a frequency of 0.1 Hz) were carried out to examine the frequency-dependent behaviour of corresponding interfaces and to ensure that the selected displacement and frequency selected were within the linear viscoelastic region. For the sequential adsorption experiment, one hour after the

formation of the protein layer, replacement of the aqueous phase with PBS (no protein) was achieved using a high resolution flow control system (Elveflow, 30 min, 1 mL/min; without disrupting the formed nanosheet) in the rheology trough. This washing step ensures that non-adsorbed protein molecules are removed from the aqueous phase. Subsequent injection of a second protein solutions (final concentration of 1 mg/mL) or treatment with sulfo-SMCC (at a concentration of 2 mg/mL) was then monitored for 1 h, followed by 30 min of further washing with PBS and characterisation via frequency sweep (at an oscillatory displacement of 1.0 10^{-3} rad), stress relaxation experiment (0.5 % strain) and amplitude sweep (at a frequency of 0.1 Hz).

Surface Plasmon Resonance (SPR). SPR measurements were carried out on a BIACORE X from Biacore. SPR chips (SPR-Au 10 x 12 mm, Ssens) were plasma oxidized for five minutes and then incubated in a 5 mM ethanolic solution of 1H,1H,2H,2H-perfluorodecanethiol, overnight at room temperature. This created a model fluorinated monolayer mimicking the fluorophilic properties of Novec 7500. The chips were washed once with water, dried in an air stream and kept dry at room temperature prior to mounting (within a few minutes). Thereafter, the sensor chip was mounted on a plastic support frame and placed in a Biacore protective cassette. The maintenance sensor chip cassette was first placed into the sensor chip port and docked onto the Integrated μ-Fluidic Cartridge (IFC) flow block, prior to priming the system with ethanol. The sample sensor chip cassette was then docked and primed once with PBS. Once the sensor chip had been primed, the baseline was allowed to stabilize, and the protein solution (1 mg/ mL in PBS) was loaded into the IFC sample loop with a micropipette (volume of 50 μL). The sample and buffer flow rates were kept at 10 μL/min throughout. After the injection finished, washing of the surface was carried out in running buffer (PBS) for 10 min.

Protein labelling. Corresponding Alexa Fluor™ NHS ester dyes were conjugated to the primary amines of BSA (594 nm) and β-casein (488 nm) following protocols recommended by the supplier. Briefly, the dyes were dissolved in DMSO and mixed with the protein solution (10 mg/mL in 0.1M sodium bicarbonate buffer) and kept under vortex for 1 h at room temperature.

Epifluorescence microscopy, fluorescence recovery after photobleaching (FRAP) and data analysis. Fluorescence microscopy images were acquired with a Leica DMi8 fluorescence microscopy and images were analysed in Fiji ImageJ. Emulsions were prepared according to the protocol described above, using the labelled proteins (fluorophores: 594 nm for BSA and 488 nm for β-casein). An emulsion volume of 10 μL was placed in a microwell plate filled with 200 μL of

PBS. Images were acquired with a Nikon CSU-W1 SoRa Spinning Disk Confocal with a 63x oil objective.

Neutron reflectometry (NR). Reflectivity measurements were carried out using the INTER reflectometer at the ISIS Spallation Neutron Source, Rutherford Appleton Laboratory, Didcot, UK. Neutron reflectivity is sensitive to the average neutron refractive index, n, can be written as[41]:

$$n \approx 1 - \frac{Nb}{2\pi}\lambda^2\lambda$$

Where *N* is the atomic number density, *b* the coherent scattering length. The *Nb* of a medium can be directly calculated from its composition. To carry out neutron reflectometry at oil-water (PBS) interfaces, we applied the "spin-freeze-thaw" method [42]. Note that as the melting temperature of Novec 7500 was too low, it was not possible to carry out this procedure with this fluorinated oil, and hexadecane (~18.0 °C) was utilized as an alternative. The oil layer (hexadecane) was contrast matched to a hydrophobic Si substrate by spin coating. The oil layer was then frozen and sandwiched between the substrate and the aqueous phase. After ensuring the absence of trapped air within the system, the temperature of the cell was raised to 25 ± 1°C, causing the oil layer to melt. For a relatively thin oil film, the reflections from both the Solid substrate–oil (R_1) and oil–water (R_2) interfaces contribute to the measured reflectivity. The reflectivity can be calculated using the thick-film approximation [43]:

$$R_{Total} = \frac{R_1 + R_2A - 2R_1R_2A}{1 - R_1R_2A}$$

A represents the attenuation of the neutron beam upon travelling through the oil phase:

$$A = exp\left(\frac{-2\chi d}{sin\theta}\right)$$

χ is the attenuation coefficient of hexadecane which was experimentally measured. R_1 is determined through supplementary measurements, and the contributions from the substrates-oil interfaces are subsequently subtracted from the total reflectivity (R_{total}). The data is then normalized, and the reflectivity (R_2) originating exclusively from the oil-water interface is plotted as a function of the scattering wave vector Q. Q is defined by the beam incident angle θ and the neutron wavelength λ:

$$Q = \frac{4\pi\ \sin\theta}{\lambda}$$

A minimum of a two-layer model was found to provide a good fit to both datasets recorded for BSA and β-casein.

Surface pressure measurements. A trough designed for liquid-liquid interfaces (dense oil phase) for Langmuir-Blodgett (LB) deposition was used to evaluate changes in surface pressure/surface tension of protein layers formed at oil-water interfaces. The trough was a Kibron Langmuir Blodgett Trough (Kibron MicrotroughX Gn series) with Kibron's Layer X dip coater and a Wilhelmy plate coated with PTFE film. The trough has dimensions of 40 mm (width) by 151 mm (maximum length), while the single barrier is of 16 mm width. The Wilhelmy plate was placed at the sensor and calibrated first in air and then at the oil interface (16.2 mN/m surface tension, at room temperature). The trough was filled with 20 mL of fluorinated oil and the Wilhelmy plate was immersed completely in the oil phase. The PBS phase (60 mL) was added onto the oil phase by pipetting towards the barrier and not directly to the oil phase. The Wilhelmy plate was lifted using the exclusive sensor mechanism to ensure the plate was placed at the oil-water interface level. Changes in the surface pressure occurring during protein adsorption were recorded after zeroing the surface pressure, at a protein concentration of 1 mg/ mL (injected in the PBS phase drop by drop uniformly throughout the trough).

Statistical analysis. Statistical analysis was carried out using OriginPro 9, through one-way ANOVA with Tukey test for posthoc analysis. Significance was determined by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s., non-significant. A full summary of statistical analysis is provided in the supplementary information.

Results and Discussion

To investigate the assembly of β-casein at liquid-liquid interfaces, interfacial shear rheology was selected. Indeed, this technique enables shearing of liquid-liquid and air-liquid interfaces without inducing significant changes in surface area, and therefore enables to dissociate contributions from interfacial shear mechanics from surface tension effects [29,44]. Oscillatory dilatational rheology typically reflects both changes in surface tension and in plane shear mechanical properties of the interfaces characterised. Similarly, force probe microscopy allows the quantification of interfacial mechanics, but the indentation moduli obtained reflect a combination of interfacial mechanics and disjoining pressure (combining contributions from surface tension, as well as coulombic and van der Waals surface-probe interactions). As a result, force probe microscopy is relatively weakly sensitive to interfacial shear mechanics, with surface tension/disjoining pressure dominating indentation profiles at interfacial shear moduli near 10-100 mN/m, and only becoming significant in the range of 100 mN/m and above [28,45,46].

Upon adsorption of bovine serum albumin (BSA), the interfacial shear modulus dramatically increased within seconds of the injection, reaching a plateau near 13 mN/m (Figure 1A), from a baseline in the range of 10^{-5}-10^{-4} N/m, in good agreement with previous observations [21,23]. In contrast, upon adsorption of β-casein, only a small step almost undistinguishable from the baseline could be seen (Figure 1 A and Supplementary Figure S1), followed by a minor but steady increase in the interfacial shear modulus, overall remaining below 1 mN/m. β-casein was able to form stable emulsions (stable for several weeks to months) and led to very rapid changes in surface pressure (Supplementary Figure S2), in agreement with observations reported in the literature for various oils [47,48]. This behaviour indicates that although both BSA and β-casein rapidly adsorb at the surface of the fluorinated oil Novec 7500, BSA forms a networks displaying significant increase in interfacial shear mechanics, whereas β-casein does not, perhaps forming instead self-assembled monolayers closer in structure to those formed by low molecular weight surfactants.

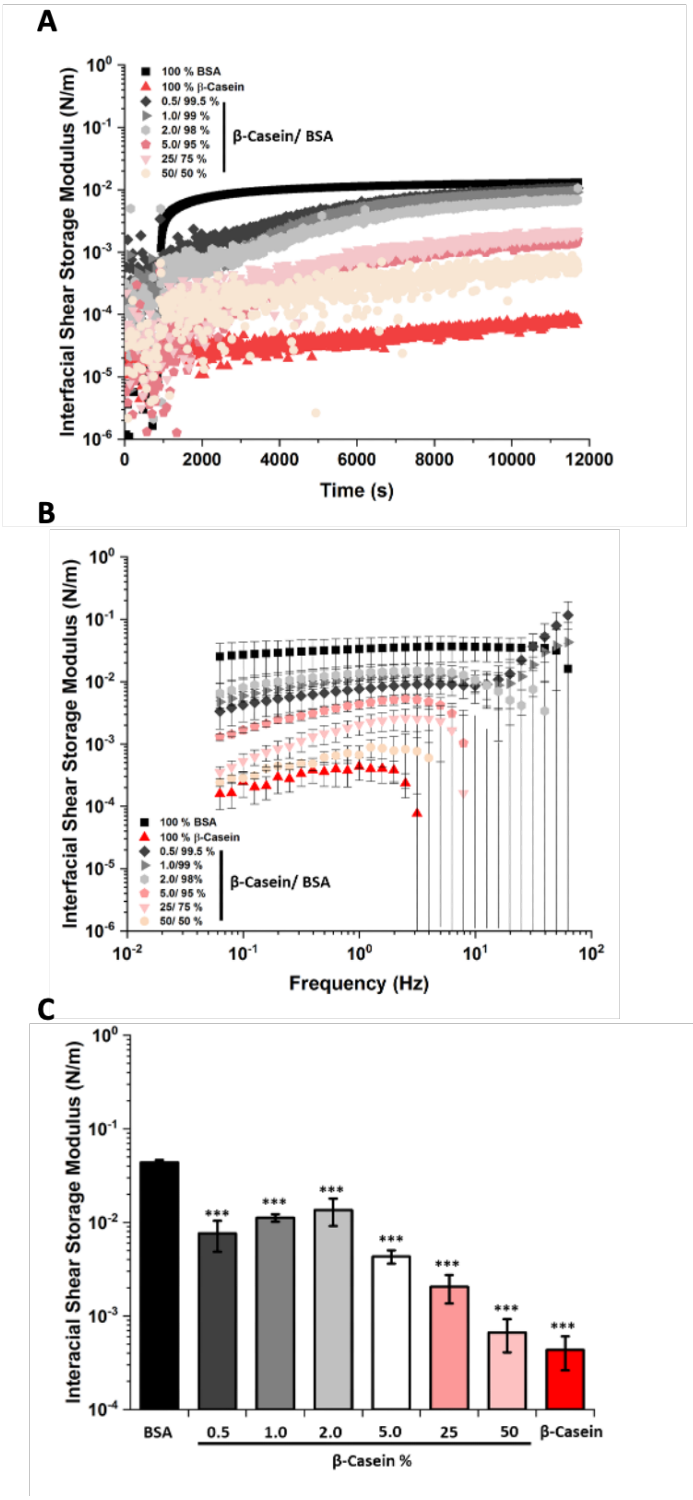

Figure 1. A. Evolution of interfacial storage moduli during the adsorption of BSA, β-Casein and their mixtures (0.5/99.5, 1.0/99, 2.0/98, 5.0/95, 25/75 and 50/50 wt % if β-Casein/BSA; total concentration of 1 mg/mL) at Novec 7500 oil-PBS interfaces. B and C. Frequency sweeps of the protein assemblies and their corresponding interfacial storage moduli measured at an oscillating amplitude of 10^{-4} rad and frequency of 1 Hz. One-way ANOVA with Tukey test; * $P < 0.05$; Error bars are s.e.m; n=3.

Interestingly, co-adsorption of β-casein and BSA led to a gradual increase in interfacial shear modulus (Figure 1), although this was not linearly correlated with the protein feed composition. β-casein introduction, even at moderate concentrations (0.5-2% in weight), had a significant impact on the ultimate interfacial shear modulus measured and significantly delayed the kinetics of the rise in moduli. Analysis of the frequency sweeps (Figures 1B and C) obtained after adsorption of corresponding mixed protein compositions clearly confirmed the trends observed from time sweeps (Figure 1A). The relatively broad linear viscoelastic region observed for BSA assemblies was significantly reduced upon the introduction of β-casein, above 5% by weight, with an increase in the slope of the frequency profiles and a reduction of the frequency at which breakdown of the waveform did not allow further quantification of associated moduli.

The rheological properties of other mixed protein films formed at the air-water interface (and in some cases oil-water interfaces) were also shown to be dependent on the composition, protein type and concentration of the constituents in the bulk phase. Indeed, a number of studies examined the co-adsorption of globular/globular or globular/random-coil protein mixtures at air-water or oil-water interfaces, and quantified the interfacial mechanics of resulting protein assemblies using dilatational rheology [49,50]. Proteins with different structures were proposed to have different abilities to undergo conformational reorganisation at interfaces. The compatibility of two proteins and the mechanical properties of the resulting complex are mainly determined by the molecular weight of the proteins, the conformation state of the proteins at the interface, protein-protein interactions and proteins-solvent interactions[31,37,40]. Studies showed that the composition of proteins in a mixed film is determined by the incompatibility of mixing of the adsorbing proteins with those in the interfacial film. The presence of the protein that arrives first at the interface was proposed to affect the binding affinity of the second protein. Although the changes in interfacial rheology observed for single protein assemblies (β-casein and BSA separately) are in good agreement with interfacial shear rheology data previously reported, they also point to significant differences compared to dilatational moduli observed [30,35,47,50]. Specifically, whereas β-casein is associated with a significant reduction in surface pressure and dilatational moduli, the interfacial shear moduli of assemblies measured at Novec 7500 interfaces remained mainly unchanged. This points to the significant contribution of surface tension and pressures to the measured dilatational moduli, which are not relevant to shear moduli measured in the absence of changes in surface area. Although the interfacial shear mechanics of mixed β-casein/BSA

interfaces has not been reported yet at other oil or air interfaces, it is however interesting to note that β-casein was found to dominate the dilatational mechanics of β-casein/β-lactoglobulin assemblies [50].

The non-linearity of the evolution of interfacial shear storage moduli as a function of feed solution composition suggested that protein adsorption at corresponding liquid-liquid interfaces also varied non-linearly with feed concentrations. To test this hypothesis, we labelled BSA and β-casein with complementary Alexa Fluor^{TM} dyes, and quantified their relative surface concentrations at the surface of oil-in-water emulsion droplets, after replacement of the aqueous phase with successive dilution/aspirations (Figures 2A-B). As the fluorescence intensities measured from corresponding microscopy images do not allow quantitative surface densities to be extracted, we quantified the surface densities of monolayers of BSA and β-casein at fluorinated interfaces (monolayers of perfluorinated alkanes) using surface plasmon resonance (SPR; Figures 2C-D). Although this corresponds to an approximation due to the differences in chemistry of the linear perfluorinated alkyl thiol selected and that of the branched Novec 7500, SPR allows precise quantification of surface densities. The surface coverage of β-casein was found to be approximately double that of BSA, near 200 ng/cm^2. Although values found in the literature vary significantly depending on the oil or air interface characterised, the surface densities quantified from our SPR data are in good agreement with those reported for both proteins at air-liquid interfaces (based on radiolabelling and radioactivity monitoring) [38–40] and comparable, although slightly below, values reported from neutron reflectometry measurements [51]. Therefore our work confirms that denser layers of β-casein form at Novec 7500 interfaces compared to assemblies formed with BSA. This suggests that the less structured β-casein proteins are able to pack more densely, perhaps similarly to polymer surfactants displaying a short hydrophobic head group with a long hydrophilic tail chain.

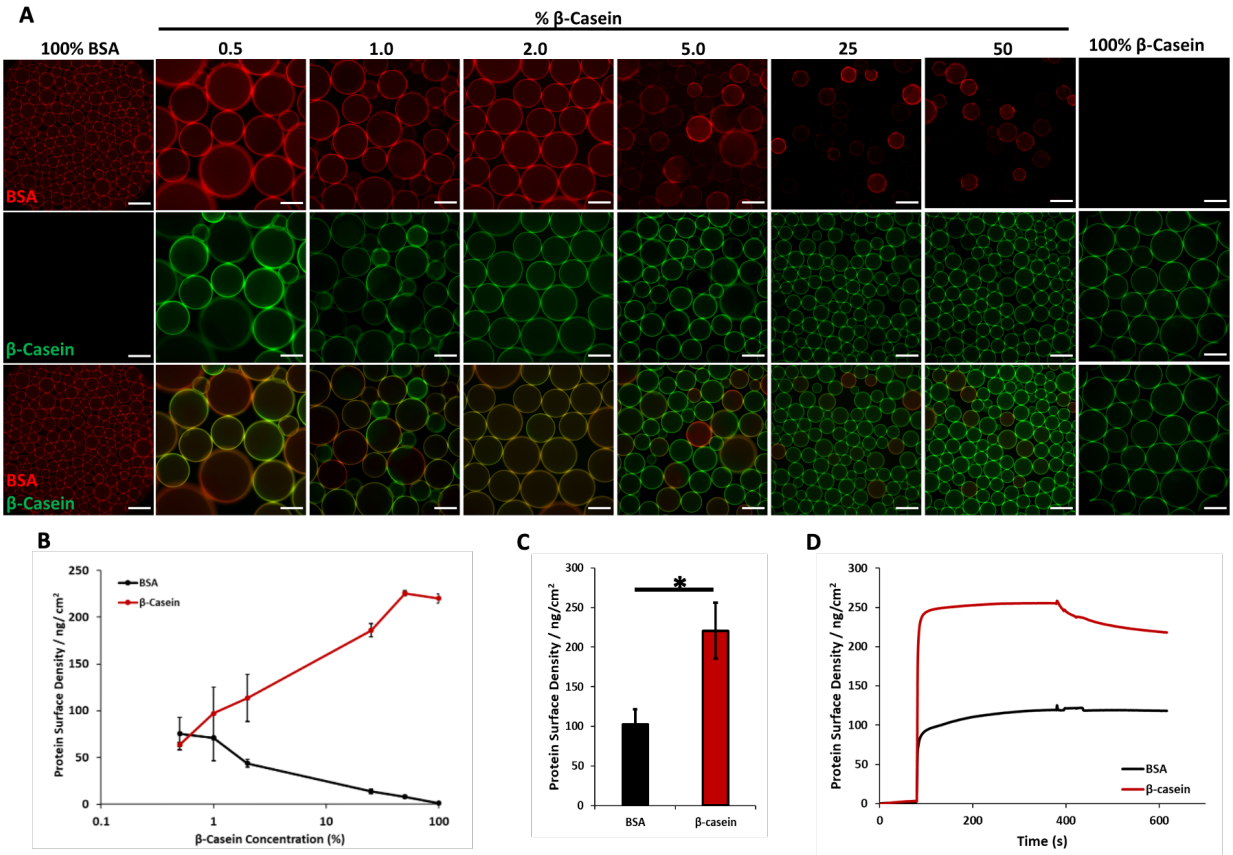

Figure 2. A. Epifluorescence microscopy images of labelled BSA (red) and β-Casein (green) stabilised emulsions. Scale bars, 200 μm. Emulsions were formed with mixtures of β-Casein and BSA (0.5/99.5, 1.0/ 99, 2.0/ 98, 5.0/ 95, 25/ 75, 50/ 50; β-Casein/ BSA wt %; total concentration of 1 mg/mL). B. Extrapolated protein surface densities of the corresponding BSA/β-casein assemblies (based on the single protein surface adsorption densities measured via SPR). C. Protein surface densities measured by SPR for BSA and β-casein alone, to model self-assembled monolayers. D. Corresponding representative SPR traces of the adsorption of BSA and β-casein to perfluorodecanethiol monolayers modelling fluorinated oil interfaces. One-way ANOVA with Tukey test; * $P < 0.05$; Error bars are s.e.m; n=3. Scale bars, 50 μm.

Analysis of the evolution of the protein composition at the interface evidenced a clear non-linearity, with significant levels of adsorption of β-casein even at low feed concentrations (e.g. 5% β-casein in the feed solution led to 50% assembly at liquid-liquid interfaces; Figure 2B). Such behaviour is well documented for co-assembly of proteins at liquid interfaces, in particular in the case of β-casein, although the dominance of β-casein adsorption to Novec 7500 interfaces was more pronounced compared to reports at air interfaces[38–40]. This was despite the excess protein presented in solutions (total concentrations of 1 mg/mL were used in these experiments). Therefore this behaviour is unlikely to arise from limited protein availability alone. In addition, our data suggested that assemblies formed at the surface of droplets were relatively homogenous at the single droplet level, although droplet-to-droplet adsorption levels and fluorescence intensities

varied more significantly (Figure 2A). This behaviour could be resulting from a tendency of these two proteins to self-associate and assemble at hydrophobic interfaces with proteins of the same type, as was reported at air interfaces, for which self-segregated domains of β-casein and BSA were observed[38]. The chemical and thermodynamic grounds for such segregation are not clear, but we note that this is unlikely to be associated with significant level of order in corresponding protein layers, as we had previously not observed any evidence for such order in BSA assemblies formed at Novec 7500 interfaces and transferred to TEM grids for imaging [20]. We also note that most droplets imaged featured mixed intensities of both proteins and therefore self-segregation was not found to be dominating protein adsorption at Novec 7500 interfaces.

To better understand the co-assemblies observed, we investigated the kinetics of adsorption of the two proteins alone and the architecture of the resulting interfaces via tensiometry and neutron reflectometry. The interfacial pressure of corresponding liquid-liquid interfaces increased rapidly upon adsorption of both β-casein and BSA, further confirming that both proteins adsorb rapidly in the system studied (Supplementary Figure S2). In addition, although increases in interfacial shear storage moduli were slow and overall remained very modest in the case of β-casein (Figure 1), the interfacial pressure rapidly and very significantly increased for this protein. This is in good agreement with the evolution of surface pressures previously reported for β-casein at other oil or air interfaces [35,47,48].

To investigate the structure of the protein assemblies formed in situ, we carried out neutron reflectometry experiments (Figure 3). We used a previously reported protocol in which a thin liquid film was formed at the surface of a silicon substrate (hydrophobised) and frozen prior to positioning at an aqueous (PBS) interface pre-cooled to preserve the thin film morphology, followed by warming up of the aqueous and oil phases and melting to form the liquid-liquid interface [42,43,51]. Note that as the melting temperature of Novec 7500 was too low, it was not possible to carry out this procedure with this fluorinated oil, and hexadecane had to be used instead. Both datasets recorded for BSA and β-casein assemblies fitted well with thin bilayers (Figure 3), in agreement with previous reports obtained for albumin [20,51]. These two layers are proposed to correspond to the more hydrophobic residues interacting with the oil phase and the hydrophilic fragments, swelling into the aqueous phase, away from the interface. However, the dimension and composition of these two layers differed significantly in both proteins. Indeed, the hydrophobic

layer of the β-casein assembly was thinner and less solvated than that of BSA, whereas its hydrophilic compartment was comparable in thickness but more solvated (Table 1).

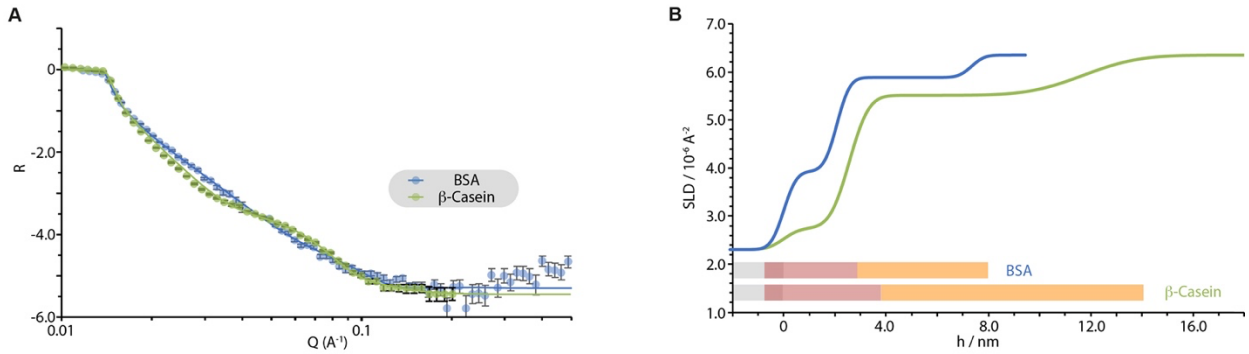

Figure 3. A. Neutron reflectometry data and corresponding fits recorded for BSA and β-casein assemblies at hexadecane (Si contrast matched oil, Nb = 2.1×10^{-6} Å^{-2})-PBS (D_2O, Nb = 6.35×10^{-6} Å^{-2}) interfaces. Fits shown correspond to two-layer models at hexadecane-water (PBS) interfaces. The Nb of BSA and β-casein in D_2O were calculated to be 3.05 and 2.6×10^{-6} Å^{-2}), respectively, based on their amino acid sequences (obtained from Uniprot), using the Biomolecular Scattering Length Density Calculator from the STFC/ISIS. B. Corresponding evolution of SLDs as a function of z-position (h) perpendicular to the interface towards the bulk water. The grey bars below the SLD profiles correspond to the position of the oil interfaces, whereas the red and orange bars correspond to the measured thicknesses of the hydrophobic and hydrophilic domains, respectively.

Table 1. Fitting parameters for BSA and β-casein (1 mg/mL) at hexadecane/water (PBS) interfaces. σ represents the layer roughness (the substrate roughness was 5 Å).

	d_1 (±1 Å)	σ_1 (±1 Å)	W_1 (±1 %)	d_2 (±1 Å)	σ_2 (±1 Å)	W_2 (±1 %)
BSA	21	4	27	53	4	86
β-casein	26	6	4	92	17	78

Therefore, overall our data clearly demonstrate the disconnection between the evolutions of the surface adsorption of the proteins studied at liquid-liquid interfaces and the mechanical maturation of corresponding assemblies. Both BSA and β-casein are found to rapidly assemble at fluorinated oil interfaces and form comparable film thicknesses at hexadecane interfaces, but whereas the interfacial shear storage modulus of β-casein layers does not significantly rise, it rapidly increased in the case of BSA. This difference in interfacial mechanics is proposed to result from the difference in structure of these two proteins. Whereas BSA is a globular protein presenting predominantly α-helical components (72% based on circular dichroism [17]), β-casein is a disordered protein displaying only one sizeable α-helix at its N-terminus, with a hydrophobic

terminal peptide sequence of VLILACLVALALA (based on data from Uniprot and AlphaFold structure predictions). This architecture presumably confers to β-casein amphiphilic properties comparable to other polymeric amphiphiles, and promotes the formation of dense layers in which macromolecules are not strongly interacting, with substantial associated fluidity and low interfacial shear moduli. In comparison, BSA presents a hydrophobic face that mediates the transport of lipid and other hydrophobic molecules and requires unfolding of α-helix bundles in order to assemble at hydrophobic interfaces [17]. This requirement presumably underpins a higher activation energy associated with BSA adsorption at oil and air interfaces and the kinetic control of β-casein adsorption in protein mixtures [36–38].

To further investigate molecular dynamics within protein assemblies at liquid interfaces, we carried out FRAP experiments on oil droplets stabilised by corresponding protein layers (Figure 4). Labelled proteins were assembled at the surface of droplets, followed by photobleaching of defined areas. Recovery of the fluorescence intensity was then monitored for at least 2 min. In contrast to quasi-2D protein assemblies that significantly recover intensities in biological systems, such as in focal adhesions within the same time frame [52,53], almost no recovery was observed for BSA and β-casein interfaces (Figure 4B). A very low level of recovery was initially observed in β-casein assemblies, but this reached a plateau at approximately 5% of the initial fluorescence (Figure 4C). BSA displayed negligible evidence of any recovery processes. This result was surprising, as we expected higher levels of fluidity and molecular diffusion at corresponding interfaces in the case of β-casein, based on the lack of changes in interfacial shear moduli observed (Figure 1). However, β-casein is known to form assemblies even in solution, upon association with $Ca^{2+}PO_4^{3-}$ species, with average diameters of 150 nm, corresponding to objects with molecular weights near 100 MDa [54]. Therefore, casein assembly at liquid-liquid interfaces, although forming relatively fluid interfaces with aggregates not coupled with each other from a mechanical point of view, does not display significant molecular diffusion at the timescales studied.

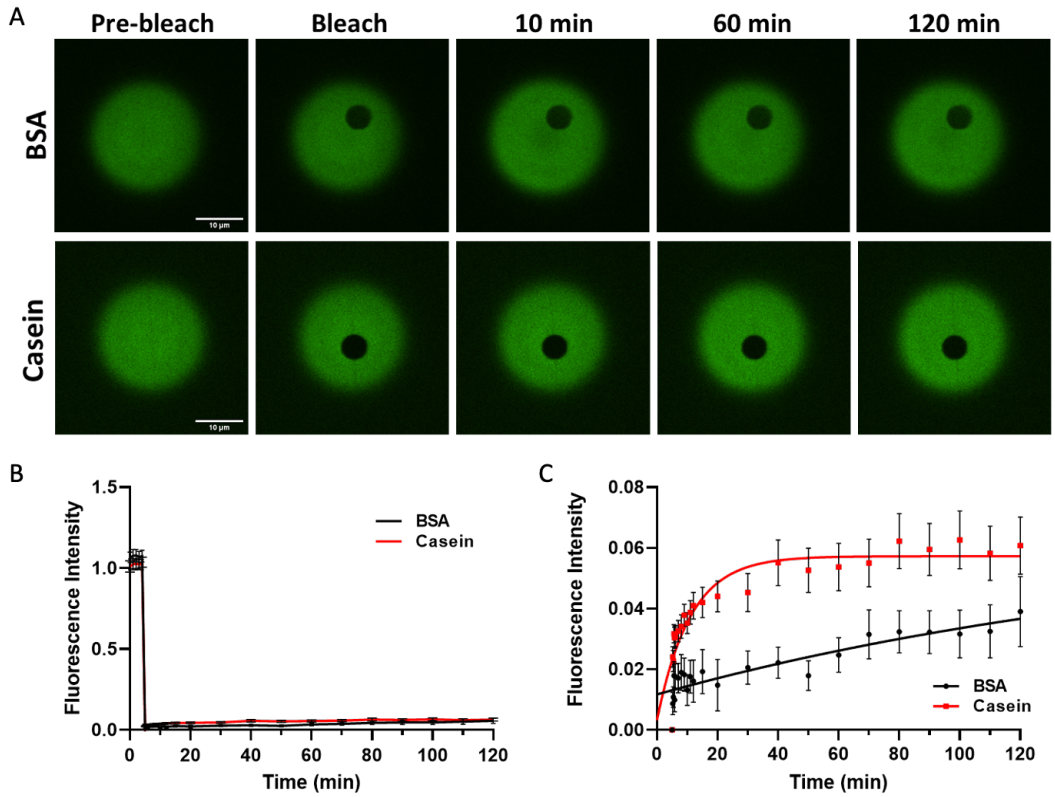

Figure 4. Fluorescence recovery after photobleaching (FRAP). A. Monitoring of fluorescently labelled BSA and β-casein pre- and post-bleaching and recovery for 120 minutes. Selected representative images. B. FRAP recovery profiles showing limited recovery for both BSA and β-casein assemblies. C. Recovery profiles of BSA and β-casein, zooming on the lower 8% of total fluorescence recovery. One-way ANOVA with Tukey test; * $P < 0.05$; Error bars are s.e.m; n=3.

To investigate whether crosslinking of β-casein interfaces could allow recovering the interfacial mechanics of BSA assemblies, time sweep experiments were followed by a complete removal of the free proteins from solutions, using a continuous flow of PBS, and injection of the hetero-bisfunctional crosslinker sulfo-SMCC (Figure 5). Throughout the washing step, we observed an excellent maintenance of the mechanical properties of the BSA interfaces generated (β-casein interfaces not displaying significant changes in interfacial shear moduli compared to pristine liquid-liquid interfaces, eventual changes occurring during washing could not be directly evidenced). Upon introduction of sulfo-SMCC, a steady increase in interfacial mechanics was observed, for both proteins, resulting in sulfo-SMCC crosslinked β-casein interfaces displaying comparable moduli to those based on BSA. In addition, interfacial stress relaxation profiles were also comparable for both crosslinked BSA and β-casein assemblies, further demonstrating that, at the protein densities achieved, effective crosslinking can be obtained in both cases. Similarly, BSA

and β-casein protein nanosheets formed at Novec 7500 interfaces in the presence of pentafluorobenzoyl chloride (previously shown to mediate physical crosslinking of albumin and β-lactoglobulin assemblies [17,18,20,21,23]) resulted in significantly stiffer interfaces with significant levels of elasticity (Supplementary Figure S3).

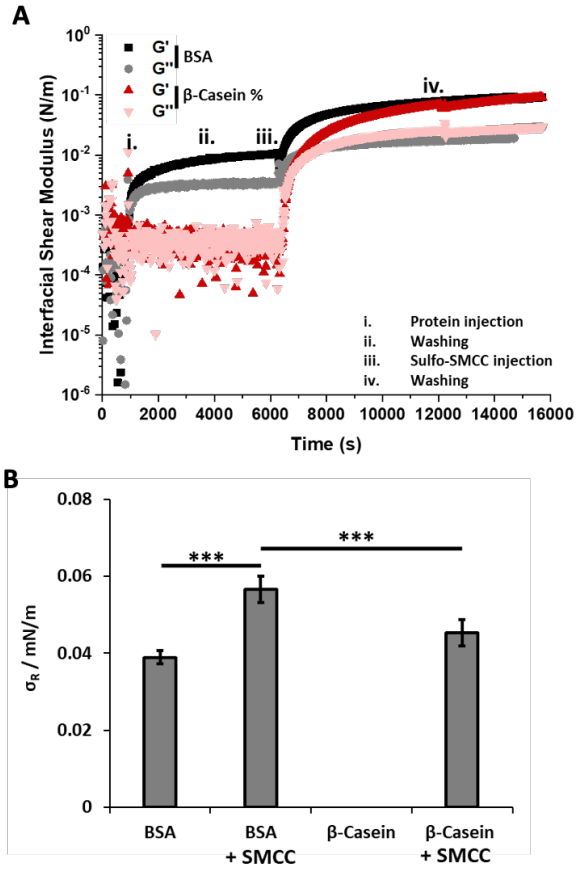

Figure 5. A. Adsorption of BSA and β-Casein, followed by washing with PBS for 30 minutes at 4000s. An injection of sulfo-SMCC (2 mg/mL) followed by another washing was carried out. B. The corresponding residual elasticities at a strain 0.5 % compared with the 2.0 and 5.0 % β-casein mixtures. One-way ANOVA with Tukey test; * $P < 0.05$; Error bars are s.e.m; n=3.

Having explored the impact of the composition of mixed protein assemblies on interfacial dynamics and mechanics, we then investigated the thermodynamic stability of protein layers in response to protein exchange. Indeed, such phenomena have important implications on emulsion stability and the stability and mechanical properties of protein-based interfaces. The evolution of interfacial mechanics during exchange experiments was first examined. After formation of mono-protein assemblies at oil-water interfaces, the excess free protein was washed as detailed above and the second protein was introduced in the aqueous (PBS) phase, at a concentration of 1 mg/mL.

Monitoring of changes in interfacial shear moduli again confirmed the excellent stability of interfaces throughout the wash step, but also evidenced a clear change in mechanics occurring upon introduction of the second protein (Figure 6A). In the case of BSA interfaces, introduction of β-casein led to a rapid decrease of the interfacial shear storage modulus from 11 to 2.5 mN/m. In contrast, in the case of β-casein interfaces, introduction of BSA led to an increase of the interfacial shear modulus from 0.18 to 2.8 mN/m, although at a much slower rate compared to the exchange observed with β-casein. Ultimately, after 1 h of exchange, the two resulting reconditioned protein interfaces displayed very comparable interfacial shear storage moduli, with frequency dependency profiles almost overlapping (Figures 6B and C).

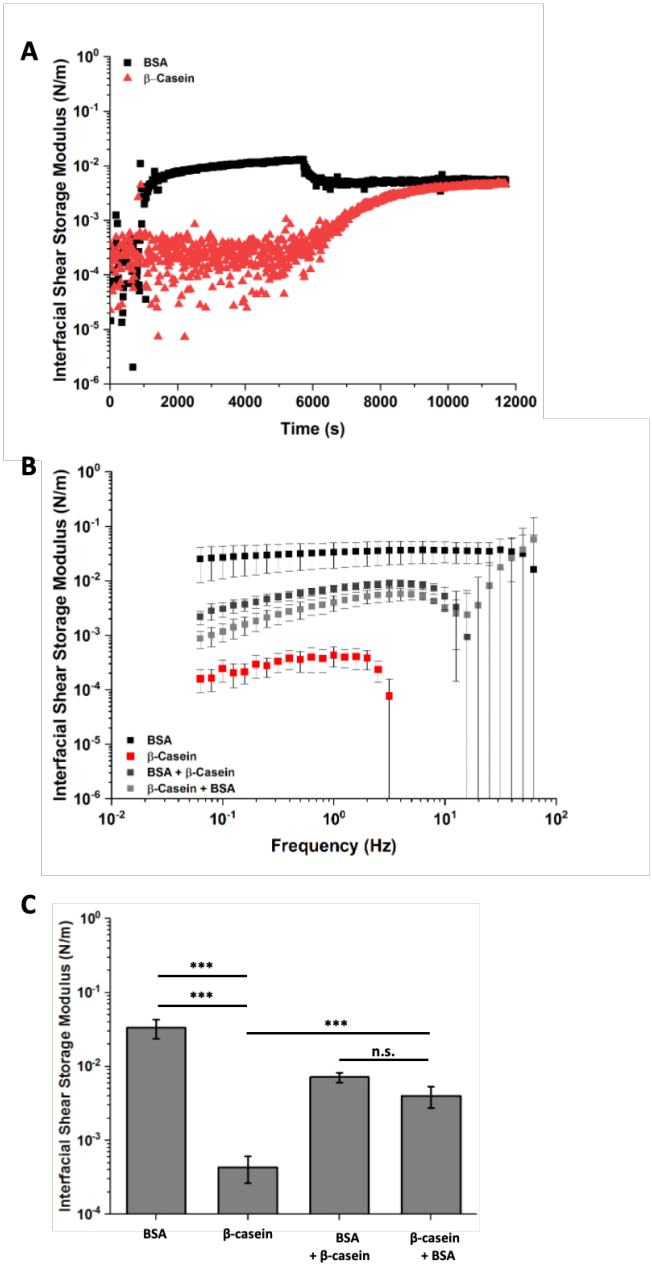

Figure 6. Sequential adsorption of BSA and β-Casein. A. Adsorption of BSA and β-Casein, followed by washing with PBS for 30 minutes at 4000 s and injection of β-Casein and BSA respectively. B. Frequency sweeps recorded at an amplitude of 10^{-4} rad. C. the corresponding interfacial storage modulus at oscillating amplitude 10^{-4} rad and frequency of 1 Hz.

The striking changes observed suggested significant changes in composition of the protein interfaces generated from single protein solutions. The comparable viscoelastic profiles of the resulting interfaces even suggested that comparable ultimate compositions of the exchanged protein interfaces. Literature reports had indicated that β-casein is not displaced by BSA at air-

liquid interfaces, whereas the opposite leads to partial displacement of adsorbed BSA [39]. To investigate such processes at Novec-PBS interfaces, we used our labelled proteins to form emulsions stabilised by single protein assemblies, followed by washing of the aqueous phase to remove excess free protein and injection of the second protein labelled with a different fluorophore. Microscopy images of the resulting emulsions, before and after injection of the second protein allowed the quantification of protein compositions at the corresponding interfaces (Figure 7). Upon introduction of β-casein to BSA-stabilised emulsions, a significant decrease in BSA density was observed, correlating with a significant increase in β-casein surface density. In contrast, upon introduction of BSA to β-casein-stabilised emulsions, no significant changes in protein densities were observed, suggesting that β-casein is not displaced and that very limited levels of BSA adsorption occurs.

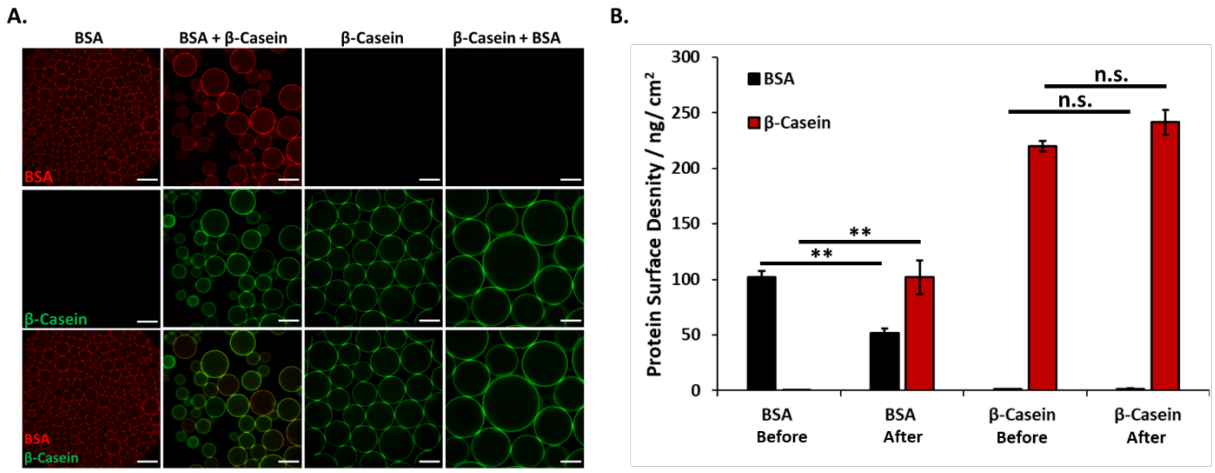

Figure 7. A. Epifluorescence microscopy images of labelled BSA (red) and β-Casein (green) stabilised emulsions. Scale bars, 200 µm. Emulsions formed with 100 % β-Casein and BSA followed by washing and exchange of the aqueous phase with 100 % BSA and β-Casein solutions, respectively. Protein concentrations: 1 mg/mL. B. Corresponding surface protein densities of adsorbed β-Casein and BSA. One-way ANOVA with Tukey test; * $P < 0.05$; Error bars are s.e.m.; n = 3.

These data suggest that, similarly to the behaviour of BSA and β-casein at air-water interfaces, exchange and displacement of these proteins at Novec 7500-PBS interfaces is asymmetrical. Whereas β-casein can displace approximately half of the BSA pool at the oil interface, leading to a mixed layer with protein densities corresponding to approximately half of the densities of the respective mono-protein layers, BSA was found to be unable to displace β-casein from this oil-water interface. Similar behaviour had been observed for exchange experiments at air-liquid

interfaces between other globular proteins (including BSA and soy 11s) and β-casein, whereas β-casein and lysozyme were not found to readily exchange [31,39].

Finally, considering the importance of interfacial mechanics and elasticity to enable processes such as cell adhesion, spreading and proliferation at liquid-liquid interfaces [15,16,20–24], we characterised the impact of the composition and processing of mixed protein layers on interfacial viscoelasticity. To do so, we applied an interfacial stress relaxation protocol previously reported [20,24], based on the application of a defined shear strain on protein interfaces using the du Nouy ring of our interfacial rheometer, followed by relaxation at this constant strain. In agreement with previously reported observations [20], BSA interfaces (alone) displayed moderate levels of stress retention σ_R (near 33 %), whereas β-casein interfaces (alone) displayed too fast relaxation profiles to be characterised using our assay. Similarly, at even moderate concentrations of β-casein in the feed solutions (e.g. 25%), interfaces were also found to be too fluid to allow interfacial stress relaxation experiments to be carried out. However, at intermediate β-casein concentrations of 2 and 5% (in the feed solutions), σ_R was found to increase to near 60%, indicating a significant increase in the elastic component of corresponding interfaces (Figures 8 and Supplementary Figure S4).

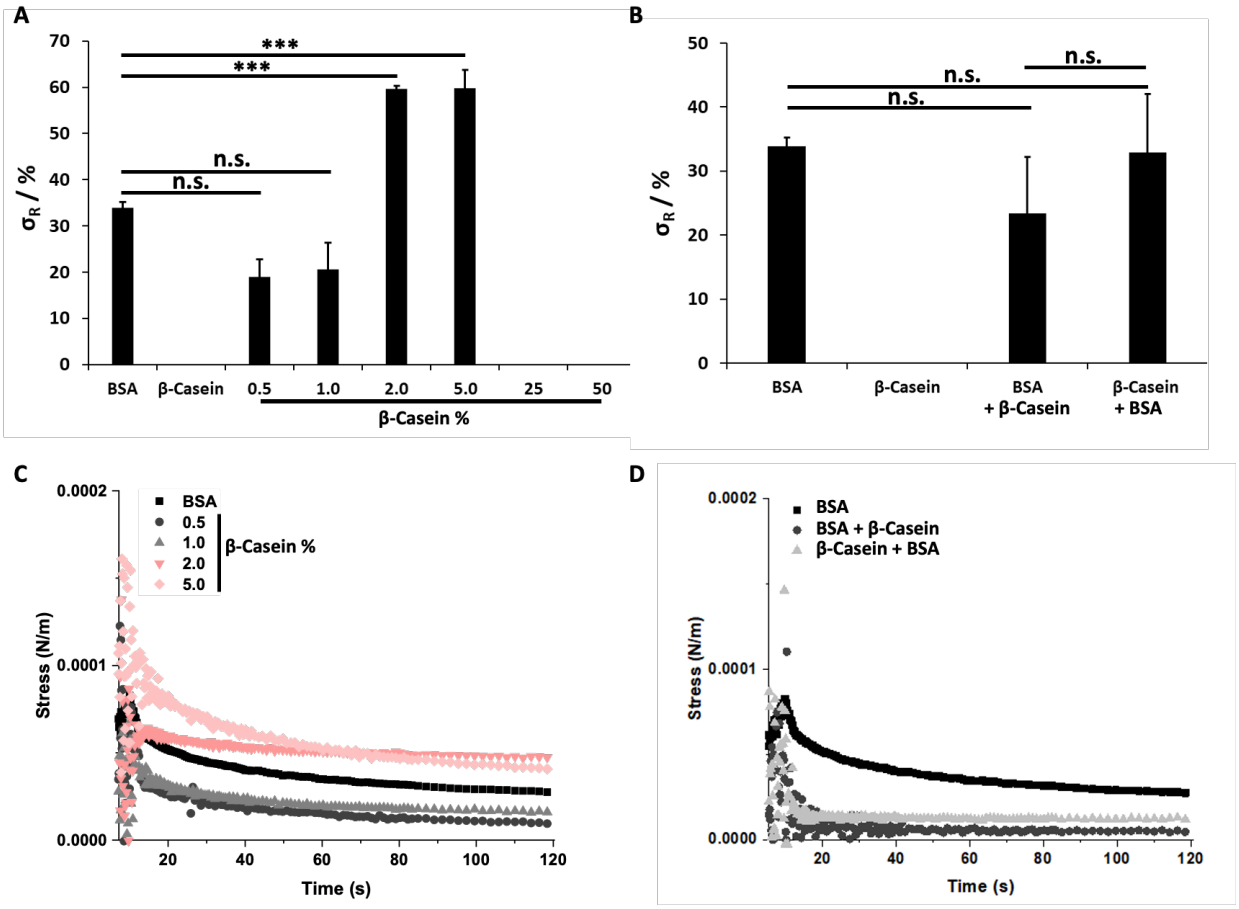

Figure 8. A. Residual elasticities σ_R (%) extracted from the fits of stress relaxation experiments at 0.5 % strain, for mixtures of BSA and β-casein (compositions in wt%, total protein concentration 1 mg/mL). B. Residual elasticities measured for assemblies after sequential adsorption of BSA + β-casein and β-casein + BSA. C and D. Corresponding stress relaxation profiles for the BSA/ β-casein mixtures and for the sequential adsorption of proteins, respectively. One-way ANOVA with Tukey test; * $P < 0.05$; Error bars are s.e.m; n=3.

These results are consistent with changes in interfacial shear storage moduli observed and the surface compositions quantified from microscopy of labelled mixed protein layers (Figures 1 and 2). Indeed, these data had indicated minor changes in interfacial shear moduli upon the adsorption of β-casein and β-casein/BSA binary layers at high β-casein concentrations. This correlated with the surface density of β-casein dominating the total protein surface density (>90%). However, at very low β-casein contents, high BSA surface densities were achieved at corresponding interfaces (>50%), therefore enabling the maintenance of entangled protein networks that displayed significantly higher elastic components. The enhancement of the elastic character of binary layers formed at 2 and 5% of β-casein (feed concentrations, corresponding to surface densities of β-casein

of 100 ng/cm^2) could be attributed to the retention of a percolated BSA network at the interface, whilst achieving sufficient packing of interstitial space with β-casein molecules. The combination of this network architecture and molecular crowding could be proposed to result in the enhancement in elasticity observed.

Analysis of stress relaxation profiles recorded for exchanged protein layers revealed further insight into their mechanical behaviour (Figure 8 and Supplementary Figure S4). Indeed, although addition of free β-casein at the surface of adsorbed BSA layers resulted in a significant reduction in interfacial shear storage moduli and BSA density (Figures 6 and 7), the stress retention of corresponding exchanged layers remained comparable to that of the pristine BSA layers (Figure 8C). In contrast, whereas pristine β-casein interfaces displayed negligible elastic components, exposure to free BSA solutions resulted in a significant enhancement of their elastic contribution. Therefore these results suggest that replacement of a pool of BSA adsorbed at oil-water interfaces and contributing modestly to the elastic component of corresponding layers was replaced by β-casein molecules, enhancing molecular packing and enhancing interfacial elasticity. However, in the case of β-casein to BSA exchange, very low levels of adsorption of BSA to interfaces (< 2 %) resulted in significant increase in interfacial shear moduli and elasticity, suggesting the crosslinking of β-casein layers and therefore interactions, through a mechanism not established yet, between β-casein adsorbates and BSA molecules.

Discussion and conclusions

Mixed protein layers adsorbed from binary solutions have been reported to display significant non-linearity in surface compositions, although this has been mainly studied at air-water interfaces [38,39]. Beyond BSA/β-casein couples, other mixed assemblies between globular proteins such as soy 11 globulin or egg lysozyme and β-casein have also been shown to display non-linear compositions, whereas mixed layers of α-lactalbumin and β-casein displayed more linear compositions [40]. These non-linear adsorption properties have been proposed to result from differences in activation energies, perhaps reflecting the need for globular proteins to partially denature and therefore change conformation prior to assembly. Therefore kinetically controlled mechanisms are proposed to regulate complex protein adsorption at liquid interfaces. However, some evidence of surprisingly large domain formation, at least in the case of BSA and β-casein suggest that incompatibility of adsorbed proteins (from a thermodynamic point of view and associated χ parameters), may also regulate adsorption profiles, at least at air-water interfaces [38]. Protein exchanges (e.g. between adsorbed proteins and free proteins) have also been shown to be asymmetrical and lead to partial displacement of some of the proteins, in particular in globular protein assemblies, whereas more disordered proteins such as β-casein are more stably adsorbed [31].

Although many of these observations were made at air-liquid interfaces, these processes and concepts are mirrored well by observations reported here for fluorinated oil-buffer interfaces. The retention and stability of protein layers may reflect a combination of the low activation energy associated with the adsorption of disordered proteins to interfaces, as well as the barrier to desorption that may result from unfolding and denaturation of globular proteins. However, the partial desorption of BSA observed in our results indicates the occurrence of a pool of more weakly adsorbed and potentially only partially denatured/unfolded proteins, which can be effectively displaced by β-casein proteins. Unlike the clear phase segregation reported at air-liquid interfaces [40], we did not observe such phenomenon at fluorinated oil-water interfaces (noting the large dimension of the segregated domains reported at air-liquid interfaces). Instead, we propose that a percolated network of denatured globular proteins occurs, with partly filled gaps that can enable the adsorption of substantial levels of β-casein (e.g. 100 ng/cm^2, from a 20-50 μg/mL solution, mixed with corresponding concentrations of BSA to make a total protein concentration of 1

mg/mL). Based on previous observations of BSA layers via TEM, these gaps are likely to be a few tens of nm at most [20].

Particularly insightful is the comparison between the interfacial shear mechanical data obtained for β-casein interfaces and the corresponding FRAP data. The lack of significant molecular diffusion at the time scales studied, without significant increase in interfacial shear moduli and elasticity, indicates the formation of β-casein aggregates, with molecular weights potentially 1-2 orders of magnitude higher than free proteins, and therefore significantly impacting surface diffusion. Such architecture is also in good agreement with the neutron reflectometry data obtained, indicating a very solvated hydrophilic shell with comparatively high roughness. Therefore, despite a high surface density and molecular crowding (200 ng/cm^2 corresponds to a surface area per β-casein molecule of 21 nm^2), β-casein assemblies and molecular aggregates (evidenced based on their low diffusion in FRAP experiments), remain isolated at corresponding fluorinated oil-water interfaces and can rapidly relax, resulting not only in weak changes in interfacial shear storage moduli, but also negligible elasticities. Observations that crosslinking, either through the covalent crosslinker SMCC or palmitoyl residues providing physical crosslinks, enables to form effective protein network that considerably enhances interfacial shear mechanics further suggest that the densities of β-casein achieved should sustain the formation of percolated networks, but are prevented to do so presumably through electrostatic repulsion between the highly hydrated β-casein assemblies.

Protein exchange experiments highlighted the impact that the first adsorbed protein layer has on further maturation of protein compositions and interfacial mechanics as adsorption of additional proteins takes place. Such processes are particularly relevant to the maturation of protein interfaces and were found to significantly impact interfacial shear mechanics and elasticity. Hence exposure of BSA adsorbed layers to free β-casein resulted in the displacement of a pool of BSA proteins and some level of softening of resulting assemblies. It is not clear why the elasticity of these systems did not increase as was observed in the case of binary adsorption from mixed BSA and β-casein solutions (at low concentration of β-casein, 2 and 5%), but possibly results from different architectures for the networks formed. Interestingly, this contrasts with the striking impact that adsorption of very low levels of BSA proteins onto a pre-adsorbed β-casein layer had on interfacial mechanics and elasticity.

Overall, these results provide insight into the chemical and structural parameters regulating interfacial mechanics in protein layers. Such properties not only have impact for the design and application of emulsions for food and consumer healthcare applications, for which such formulations are well-established, but also for the design of emulsions for emerging applications, for example in biotechnologies.

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Conflict of Interest

The authors declare no conflict of interest.

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