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Probing interfaces of pea protein-stabilized emulsions with a fluorescent molecular rotor

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Pea protein isolate (*Pisum sativum L.*, PPI) has been much studied in the last decade because of its potential as a bio-based alternative for surfactants to produce innovative and environmentally friendly emulsion products. PPI is ideal due to its favorable nutritional properties, low allergenicity and low environmental impact. Despite its growing popularity, understanding the stabilisation mechanism of emulsions stabilized with PPI remains a key question that requires further investigation. Here, we use fluorescence lifetime microscopy with molecular rotors as local probes for interfacial viscosity of PPI stabilized emulsions. The fluorescence lifetime correlates to the local viscosity at the oil-water interface allowing us to probe the proteins at the interfacial region. We find that the measured interfacial viscosity is strongly pH-dependent, an observation that can be directly related to PPI aggregation and PPI reconformation. By means of molecular rotor measurements we can link the local viscosity of the PPI particles at the interface to the Pickering-like stabilisation mechanism. Finally, this can be compared to the local viscosity of PPI solutions at different pH conditions, showing the importance of the PPI treatment prior to emulsification.

KEYWORDS

pea protein isolate, molecular rotor, interfaces, emulsions, local viscosity

1 Introduction

Emulsions are mixtures of two or more liquid immiscible phases, one dispersed in the other as droplets. Due to high interfacial tensions and droplet sizes greater than 0.1 μm, these systems are typically thermodynamically unstable Shahidzadeh et al. (1999); Aswathanarayan and Vittal (2019). Despite the existence of thermodynamically stable emulsions (e.g., microemulsions) or systems that display spontaneous emulsification, standard emulsion preparation includes other surface-active ingredients to guarantee kinetic stability, such as classic surfactants, proteins and other bio-based particles Shahidzadeh et al. (1997); McClements (2004); Yan et al. (2020). The use of proteins to stabilize emulsions has been extensively studied in recent decades, especially in the case of dairy proteins such as casein and whey Zhang et al. (2021); Hinderink et al. (2020); Burger and Zhang (2019); Lee et al. (2009); Tcholakova et al. (2006). Lately, the food industry has

developed a great interest in the development of products with plant-based proteins. Indeed, the global context has shown a need for a shift in dietary habits from synthetic or animal-based products to other alternatives Poore and Nemecek (2018). Thus, complex systems such as emulsions stabilized by soy or lentil proteins have become alternatives for surfactants for environmentally responsible product development Tang (2017); Liu and Tang (2013); Can Karaca et al. (2011).

Pea is one of the plant-based proteins that has attracted a lot of attention recently. Due to its nutritional and emulsifying properties as well as low allergenicity, research on PPI-stabilized emulsions has grown Kornet et al. (2022c); Li et al. (2022); Sridharan et al. (2020b); Lu et al. (2020); Lam et al. (2018). Pea protein isolates (PPI) are mainly composed by two globular proteins: Globulin and Albumin in a minor percentage. The former can be also separated into Legumin and Vicilin. In addition, there is also a minority presence of carbohydrates, lipids and moisture Burger and Zhang (2019). The relative amounts of these components can vary and how these different components interact with water-oil interfaces depending to the environment is responsible for the great complexity of the behavior of these proteins.

PPI as an emulsion stabilizer has been studied from different points of view. For instance, many parameters such as the globulin/albumin ratio, protein solution pH Kornet et al. (2022c),Kornet et al. (2022a); Liang and Tang (2013); Liu et al. (2009) or the agitation process Yang et al. (2022); Kornet et al., 2022b, Kornet et al., 2021 have been identified as key aspects in the emulsifying capacity, the stability of emulsions against coalescence, their rheological behavior Kornet et al. (2020) and interfacial activity Drusch et al. (2021); Chang et al. (2015); Gharsallaoui et al. (2009). Given its amphiphilic nature and interfacial activity, PPI has been considered to stabilize interfaces through a mechanism equivalent to classical surfactants (hereafter referred to as the "molecular mechanism"). This includes having a decrease in interfacial tension but also other mechanisms and forces of uttermost importance such as van der Waals interactions, electrostatic repulsions and depletion forces Burger and Zhang (2019); Tcholakova et al. (2008). However, it has been reported in several cases that these macromolecules can also behave as particles to stabilize interfaces through a Pickering mechanism Wang et al. (2022); Li et al. (2022); Sridharan et al. (2020a); Liang and Tang (2014). Pickering emulsions are emulsions whose interface stabilisation is mainly based on the existence of a steric barrier of particles around the interfaces, without a significant decrease in interfacial tension Velandia et al., 2021a; Velandia et al., 2021b); Dinkgreve et al. (2016). Such emulsions have attracted attention for food applications in recent years as they are believed to be highly resistant to coalescence due to an almost irreversible adsorption of the particles Schmitt et al. (2014); Sarkar and Dickinson (2020); Yan et al. (2020); Berton-Carabin and Schroën (2015). Nevertheless, whether particles decrease the interfacial tension is a matter of debate due to the many articles reporting both behaviors Manga et al. (2016); Kutuzov et al. (2007); Forth et al. (2019). Similarly, the high resistance to coalescence has been questioned, since it has been shown in several cases that Pickering emulsions can coalesce relatively easily Dinkgreve et al. (2016); French et al., 2016, French et al., 2015). For PPI-stabilized emulsions, front surface fluorescence and gravimetric techniques have been used to deduce the existence of this stabilization mechanism Hinderink et al. (2021); Sridharan et al. (2020b). Also, imaging methods including confocal laser scanning microscopy (CLSM) and cryo scanning electron microscopy (CryoSEM) have been employed to observe the location of PPI at the interfaces as Pickering particles Kornet et al. (2022a); Sridharan et al. (2020a). While all these methods have yielded valuable information, more work is needed to understand in detail how PPI films are built and stabilize interfaces.

Perhaps one of the most important features of protein-stabilized emulsions is the strong dependence on the behavior of their interfaces Berton-Carabin et al. (2018). In this aspect, interfacial rheology plays a key role in protein film characterization. Whether by means of dilational tests or interfacial shear, interfacial rheology techniques allow to obtain information on molecular interactions and network formation in the protein films Niu et al. (2023); Tseng et al. (2022). Also, it has been shown that interfacial rheology can be related to the bulk rheology of emulsions. This is notably the case under high dispersed phase volume fraction conditions in which the energy required to deform emulsions is strongly related to the energy to deform an oil droplet Fuhrmann et al. (2022); Kim and Mason (2017); Kim et al. (2016); Mason and Scheffold (2014). In other cases, these methods have also been related to emulsion stability. For example, it has been shown in protein films of emulsions stabilized with oxidized proteins that a decrease in the interfacial elastic modulus is characteristic of samples that are more prone to coalescence Berton-Carabin et al. (2018). On a smaller and local scale, microrheology techniques have also been used to study interfacial properties of food gels and emulsions Moschakis (2013); Lu and Corvalan (2016); Moschakis et al. (2006); Tisserand et al. (2012); Wu and Dai (2007); Yang et al. (2017). The latter are very attractive as they allow to obtain more information on the interface stabilization process at a nanometer scale. However, these still present challenges to be solved as the spatial resolution is limited to thermal fluctuations and the tracer particles are limited by their size and the nature of their surface Moschakis (2013); Wu and Dai (2007); Yang et al. (2017). In the context of the complexity and industrial interest in PPI as an emulsifier, the mechanical properties of PPI films have been characterized Gharsallaoui et al. (2009). Higher values of the interfacial elastic modulus were identified under pH conditions in which PPI is charged. Interestingly, this correlates with other researches in which PPI-stabilized emulsions show better emulsifying capacity in the same conditions Liang and Tang (2013); Gharsallaoui et al. (2012).

We set out here to obtain more information about this interfacial stabilisation mechanism using molecular rotors. Upon photo-excitation, these molecules can return to their ground energetic state through intramolecular twisting and fluorescence, with both pathways being dependent on their local environment, in particular on their local viscosity Mirzahossein et al. (2022); Caporaletti et al. (2022); Bittermann et al. (2021); Hosny et al. (2013); Uzhinov et al. (2011); Jee et al. (2010); Haidekker and Theodorakis (2010, 2007); Strehmel et al. (1997). The scale of sensitivity of the fluorescence probes to local structural changes and microviscosity is about a few nm. Therefore, this property has been used to measure the local viscosity of complex systems such as cells Kuimova (2012); Kuimova et al. (2008), lipid membranes Páez-Pérez et al. (2021), micro bubbles Hosny et al. (2013) and water-in-oil interfaces Kang et al. (2020). Additionally, they have been successfully used to assess the structural variation and aggregation process of proteins

such as lysozyme and insulin in solution Kubánková et al. (2017); Thompson et al. (2015); Hawe et al. (2010); Kuimova et al. (2008). Due to the scale which they are sensitive to, the use of molecular rotors at oil-water interfaces can potentially be viewed as a microrheology technique allowing to measure structural changes in the constituent parts of protein films.

In this research we probe the interfacial local viscosity of silicone oil-in-water emulsion droplets stabilized with PPI by means of the molecular rotor *trans*-4-[4-(dimethylamino)styryl]-1-methyl-pyridinium iodide (4-DASPI). We are particularly interested in getting a better understanding on how PPI, which is said to exhibit both molecular and Pickering stabilisation mechanisms, stabilizes water-oil interfaces. To this end, measurements of the fluorescence lifetime of the molecular rotor within the protein films are performed. To induce PPI structural modifications, pH variations prior to emulsification are done. We then compare these results to the fluorescence lifetime of 4-DASPI in PPI solutions and relate them to the protein structure. To our knowledge, this is the first time that the local viscosity of oil-water interfaces stabilized with proteins are probed with molecular rotors and our results pave the way to the understanding of the microscopic, molecular mechanisms responsible for protein-based stabilisation of emulsions.

2 Materials and methods

2.1 Materials

Yellow pea seed (*Pisum sativum L.*) were provided by Alimex Europe BV (Sint Kruis, Netherlands). Milli-Q filtrated and deionized water (18 MΩ cm resistivity) was used to prepare all the protein solutions. Silicone Oil (density 0.96 g/mL at 25°C, kinematic viscosity 50 cSt at 25°C), hydrochloric acid (HCl) 0.1 M and 4-DASPI (98% purity) were purchased from Sigma-Aldrich. All reagents were used as received unless specified.

2.2 Methods

2.2.1 Protein extraction

Pea protein isolate was obtained from a standard isolation process and based on previous studies Kornet et al. (2020, 2021, 2022a). Briefly, yellow pea flour was dispersed and stirred for 2 h in deionized water (1:10 ratio). Prior to stirring, the pH was adjusted to 8 with NaOH 1 M. The solution was then centrifuged at 20°C and 10000 g for 30 min to remove the starch fraction. The supernatant was then fractionated through a freeze-drying process by means of an Alpha 2–4 LD plus freeze dryer (Christ, Osterode am Harz, Germany). The resulting isolate was stocked at −18°C.

2.2.2 Protein solution preparation

Protein solutions were prepared by dispersing 1 wt% of the protein isolate in water (pH ≈ 7.3). To break up as many aggregates as possible resulting from the extraction process, the mixture was magnetically stirred at 500 rpm and 20°C for 3 h. The solution was stocked under refrigerated conditions overnight to ensure complete protein hydration. Subsequently, the solution was diluted to a final protein concentration of 0.5 wt% and the molecular rotor 4-DASPI was added to obtain a 10^{-5} M concentration. The pH was adjusted to acidic (pH = 3.0), isoelectric (pH = 4.6) or close to neutral conditions (pH = 6.3) with 0.1 M HCl and the resulting solution was magnetically stirred for 1 h.

2.2.3 Emulsion preparation

Silicone oil-in-water emulsions were stabilized using the PPI solution of 0.5 wt% as the continuous phase. The dispersed phase volume fraction remained constant at $\phi_O = 0.5$, all samples had a total emulsion volume of 40 mL and the emulsification temperature was held constant at 20°C. Silicone oil was progressively added to the PPI solution while using a Silverson L5 M-A emulsifier at 2000 rpm for approximately 2 min. Then, the samples were homogenized by stirring at 6000 rpm for 18 min. An ice bath was used to control the temperature. Emulsions were characterized after preparation and stored in a fridge at 4°C. The pH was verified before characterization with a pH-meter.

2.2.4 Confocal microscopy

An inverted confocal microscope (Leica TCS SP8) with a hybrid detector was used for the fluorescence lifetime imaging (FLIM) measurements of the emulsions. A 470 nm wavelength pulsed laser with 40 MHz repetition rate was used for all measurements. 4-DASPI presents an absorption maximum at 488 nm and an emission maximum at 600 nm Kim and Lee (1999). Therefore, an emission range between 500 nm and 700 nm was selected. A ×20 dry objective and a ×100 oil immersion objective were used for emulsion visualization (Figure 1), to follow the droplet size variation and to study the fluorescence lifetime at the interfaces (Figure 2A). It is worth noting that only the fluorescent signal immediately surrounding the drops, as shown in yellow in Figure 2A, is selected as the interface. This is done using the Leica Application Suite X software. For each drop of emulsion, a minimum of 10000 counts were taken to obtain a representative signal. To obtain an average lifetime value, a fluorescence decay curve is analysed as shown in Figure 2B. All fluorescence lifetime measurements (interfacial and proteins in solution) were fitted to a bi-exponential decay as hemicyanine dyes like 4-DASPI usually require for better fit Jee et al. (2010); Kim and Lee (1999):

$$I(t) = A_1 e^{-\frac{t}{\tau_1}} + A_2 e^{-\frac{t}{\tau_2}} \quad (1)$$

where $I(t)$ is the number of detected photons at time t, while A_i and τ_i are the amplitude and the lifetime of the ith decay component respectively. The average lifetime values reported here are amplitude-weighted average lifetimes $\langle\tau\rangle$:

$$\langle\tau\rangle = \frac{A_1\tau_1 + A_2\tau_2}{A_1 + A_2} \quad (2)$$

The error bars indicate the standard error obtained from 10 measurements (10 drops of emulsion) for each pH tested.

3 Results and discussion

Fluorescence intensity images along with the normalized line profiles $(I - I_{min})/(I_{max} - I_{min})$ of oil droplets stabilized with pea-protein isolate (PPI) at different pH values are shown in

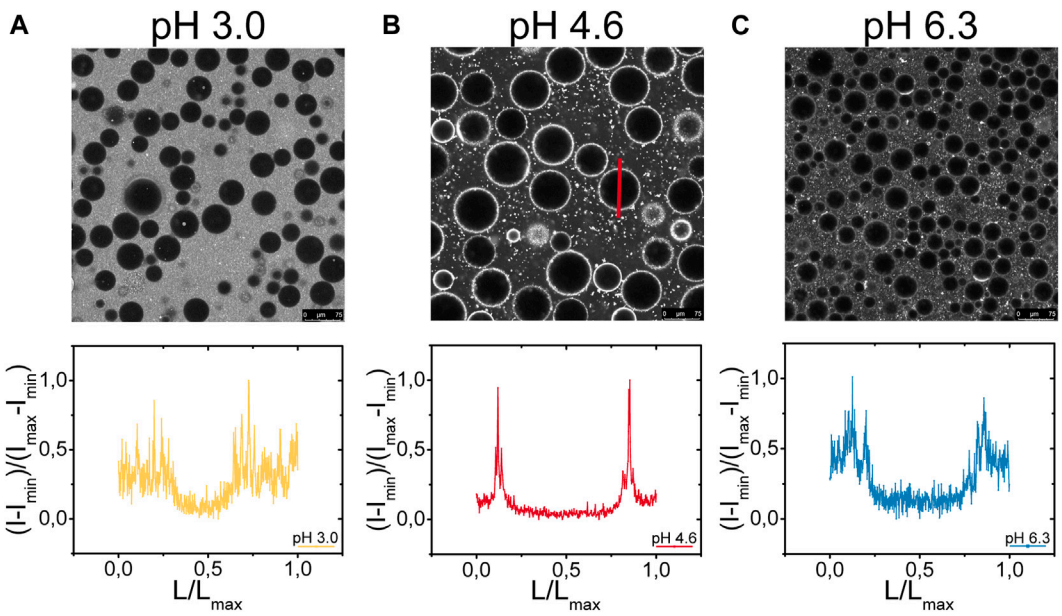

FIGURE 1
(Top) Fluorescence intensity images of 4-DASPI in PPI stabilized emulsions at pH 3.0 **(A)**, 4.6 **(B)** and 6.3 **(C)**. (Bottom) Line profiles of a selection of ≈10 oil droplets per sample. The data is based on the fluorescence intensity I measured in each pixel normalized with the minimum and the maximum value (I_{min} and I_{max}) along the normalized cross section of each drop L/L_{max} (red line illustrated in B-top).

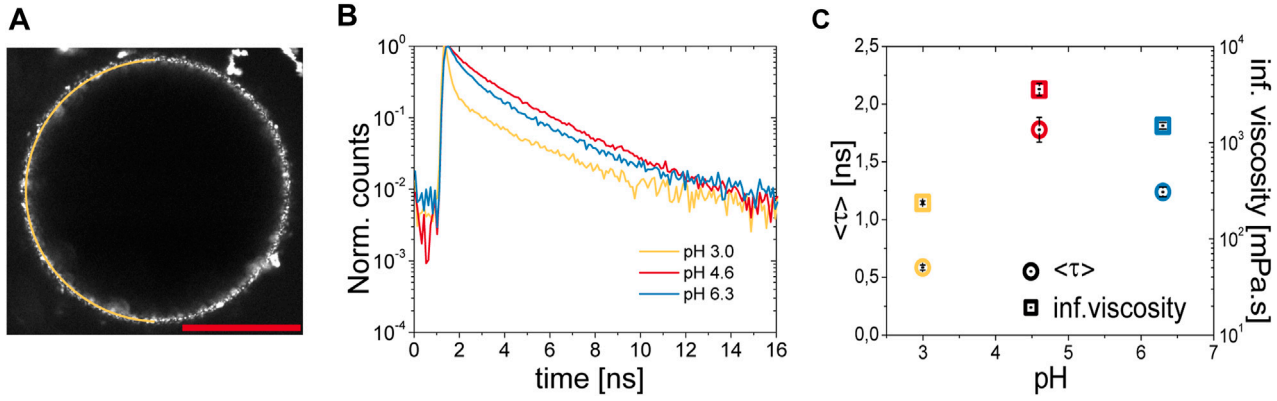

FIGURE 2
Fluorescence lifetime analysis of 4-DASPI at the interface of PPI-stabilized emulsions at different values of pH. **(A)** Snapshot of an interface (interface selection in yellow, pH = 4.6) investigated using fluorescence lifetime microscopy (magnification = ×100). Scale bar is 25 µm. **(B)** Fluorescence decay curves of droplet interfaces at different pH's. **(C)** 4-DASPI exhibits the highest value in $\langle\tau\rangle$ (circles) at the interface stabilized at pH 4.6. Using the calibration curve predicts a sharp increase in viscosity (squares) with respect to the emulsions stabilized at pH 3.0 and 6.3.

Figure 1. As a water-soluble dye 4-DASPI only stains the aqueous protein phase: the inside of the oil droplets remains non-fluorescent. Under pH 3.0 and pH 6.3 we find 4-DASPI to homogeneously stain the continuous phase (Figures 1A,C top). At the iso-electric point (IEP, pH 4.6), however, we observe thick crowns at the peripheries of the oil droplets, along with aggregates in the continuous phase (Figure 1B top). Thinner crowns are also observed at pH 6.3 while no clear crowns can be seen at pH 3.0 (See Supplementary Figure S1). The intensity profiles also show this (Figure 1 bottom). A closer look at the images (See Supplementary Figure S1) reveals that protein aggregates accumulate in large numbers at the interface in the IEP and pH 6.3 conditions. Additionally, PPI aggregates at the isoelectric point are shared between oil drops, in the same fashion as bridging particles in Pickering-stabilized emulsions French et al. (2015). Interestingly, the PPI particles at the interface are not evenly distributed around the film and thinner sections (or smaller aggregates of particles) can also be observed at the interfaces, suggesting the additional presence of a protein layer (See Supplementary Figure S1). These observations at the IEP and pH 6.3 allow to consider the presence of both the Pickering

and molecular stabilisation mechanisms coexisting in the PPI emulsions, as documented by other authors. Previously, Sridharan et al. observed that it is possible to stabilize emulsions by adsorption of self-assembled PPI particles in coexistence with PPI molecules in the bulk Sridharan et al. (2020a); French et al. (2015). The authors argued for the existence of these particles by claiming that hydrophobic and van der Waals forces overcome electrostatic interactions. Although from Figure 1A we did not observe a clear crown at the oil/solution interface at pH 3, there is an indication of difference between the lifetime measured at the interface compared to the one in the bulk (0.58 ± 0.01 ns vs. 0.51 ± 0.02 ns), suggesting that protein aggregation at the interface happens also at pH 3.0.

The fluorescence lifetime analysis at the interface of the PPI-stabilized emulsions is shown in Figure 2. Based on the known mechanism of interface stabilisation with proteins and the proven interfacial activity from interfacial tension studies at different pH's, PPI forms an interfacial film around the droplets Drusch et al. (2021); Gharsallaoui et al. (2009). We anticipate that both film structure and emulsification properties of proteins vary with pH as shown in other studies Chang et al. (2015); Liang and Tang (2013); Gharsallaoui et al. (2009). For instance, pH influences protein aggregation and therefore the local (nm) viscosity which is probed by molecular rotors (see, for example, Thompson et al. (2015)): a longer fluorescence lifetime will reflect a more viscous environment. The fluorescence decay curves measured directly at the oil-water interfaces (Figures 2A,B) allow us to confirm this effect. For the case of pH 3.0 an equivalent measurement was performed as for the other conditions, selecting the fluorescent area in the interfacial region. A maximum amplitude-weighted average lifetimes of $\langle\tau\rangle$ = 1.78 ± 0.11 ns is observed at the IEP followed by an intermediate ($\langle\tau\rangle$ = 1.23 ± 0.04 ns) and minimum value ($\langle\tau\rangle$ = 0.58 ± 0.01 ns) at pH 6.3 and pH 3.0 respectively (Figure 2C). It is important to mention that through molecular rotors we are probing the film structure once a state of equilibrium between proteins in the bulk and proteins at the interface is reached. Also, it has been shown that molecular rotor binding can influence the average lifetime in cellular systems Thompson et al. (2015). In our case we know that 4-DASPI does not generate covalent bonds with PPI. Possibly other interactions (electrostatic, hydrophobic, van der Waals) may be influencing our measurement but quantifying their effect separately in a complex system such as proteins at interfaces is beyond the scope of our study. The empirical Förster-Hoffmann equation $\tau_f = k \cdot \eta^x$ with τ_f the fluorescence lifetime and η the viscosity can be used to relate the local viscosity with the fluorescence lifetime of 4-DASPI at the PPI stabilized interfaces (See Supplementary Figure S4 for the calibration curve of 4-DASPI) Förster and Hoffmann (1971). From extrapolation of the obtained calibration curve, an inferred viscosity is calculated for each interfacial condition as shown in Figure 2C right *Y*-axis. Such values reflect states of higher (pH 4.6) or lower (pH 6.3 and pH 3.0) film density in the environment of 4-DASPI. Surprisingly, the values for the inferred viscosities are between two to three orders of magnitude above the viscosity of a PPI solution measured using rheometry (≈ 1 mPa.s). Understanding the meaning of such an increase in film density as a function of pH is a question that now arises.

A higher film density would intuitively be related to better stability properties of the emulsions or to an increase of the interfacial viscoelastic modulus. However, multiple reports indicate that emulsions stabilized with PPI (and any other protein) have the least optimal stability conditions against coalescence at the IEP Chang et al. (2015); Liang and Tang (2013); Gharsallaoui et al. (2009). Such behavior was also observed by us for the samples at pH 4.6 (data now shown). As mentioned before, confocal images of samples at the IEP (denser film condition) show that the protein films are not homogeneous (See Supplementary Figure S1). As there are areas of high PPI density, e.g., where proteins are shared between interfaces, there are also less denser regions similar to those observed at pH 6.3. The former are produced by PPI aggregation which, in turn, is mainly due to intraprotein electrostatic interactions and Van der Waals forces Zhang et al. (2022a). Indeed, at the IEP almost no electrostatic interactions occur which favours protein aggregation Lam et al. (2018). However, at pH 6.3 and pH 3.0 repulsive electrostatic interactions arise which also result in a decrease of the particle size compared to pH 4.6. The PPI aggregation was confirmed on the protein solutions and characterized with dynamic light scattering (DLS) and infrared spectroscopy (IR) (See Supplementary Figures S2, S3 for details about DLS and IR results). By comparing how the PPI hydrodynamic radius in solution varies with pH and the behavior of the local viscosity in the interface we identify that both follow the same trend. The denser environment probed with 4-DASPI at the IEP suggests that the observed behavior results from the nanometric scale (protein aggregates) and not the microscopic scale (protein film). This also agrees with the spatial resolution of the molecular rotor since its estimated size is 1 nm. Thus, it is to be expected that 4-DASPI shows greater sensitivity to structural changes occurring at a scale close to their spatial resolution (PPI particles and not protein films). Protein aggregation and the consequent increase in the density of the medium explain the different local viscosities measured at the interface. However, to go further, it is important to determine if what is observed in Figure 2C comes only from PPI aggregation or whether other mechanisms play a role. For this, we measured the amplitude-weighted average lifetimes for PPI solutions under different pH conditions in a separate experiment, as shown in Figure 3A. This provides a structural indicator on how pH impacts the building blocks in the bulk, before film formation. Indeed, being in solution, only the confinement associated with inter-protein and intra-protein interactions in the bulk is probed. Two aspects stand out about these results. First, the major effect of pH on the amplitude-weighted average lifetime is also seen for PPI solutions. The same trend is observed as for 4-DASPI behaviour at oil-water interfaces. $\langle\tau\rangle$ has a maximum under isoelectric conditions and two lower values at pH 6.3 and pH 3.0. This can be again correlated to protein aggregation (See Supplementary Figure S3). Higher hydrodynamic radius in solution suggest that the protein aggregates are more densely packed at pH 4.6. As a consequence this induces greater local confinement to the fluorescence probe and causes a larger fluorescence lifetime with $\tau_{solution}$ = 2 ns at the IEP. Since the fluorescence lifetime measures the local confinement of the protein aggregates and local crowding, it follows the same trend as the hydrodynamic radius of PPI measured by DLS. Secondly, $\tau_{interface}$ shows comparable values to $\tau_{solution}$, which indicates that PPI preserves its structure at the interface. This highlights that the

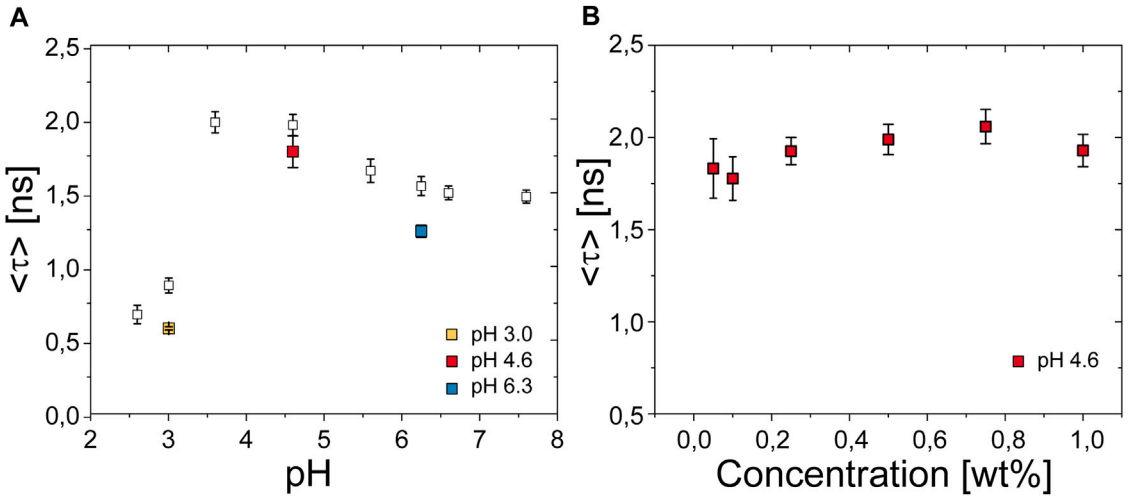

FIGURE 3
Fluorescence lifetime analysis of 4-DASPI in PPI solutions (empty squares) with varying pH (at constant protein concentration 0.5 wt%) **(A)** and varying protein concentration **(B)**. The amplitude-weighted average lifetime reaches a maximum at the isoelectric point, whilst remaining largely independent of protein concentration. The lifetimes measured at the oil-water interfaces (filled squares in A) are systematically lower than the ones observed in solutions at the same pH.

final film structure greatly depends on the protein structure and conformation before emulsification Zhang et al. (2022a); Kornet et al. (2022b), Kornet et al., 2021). Such a characteristic supports both the particle-like activity of proteins as emulsifiers and the presence of a Pickering stabilizing mechanism with PPI. These results can also be contrasted to the dependence on pH of the dilational elastic modulus of PPI films and the stability of emulsions with PPI as measured in other studies under comparable circumstances Amine et al. (2014); Liang and Tang (2013); Gharsallaoui et al. (2009) While we observe a maximum in the IEP and minima in electrostatically charged conditions, the opposite trend has been reported in literature for emulsion stability and interfacial elastic modulus Ladjal-Ettoumi et al. (2016); Gharsallaoui et al. (2009). This confirms, on the one hand, that we are not directly measuring the resistance of the film to deformation with 4-DASPI, but rather the molecular structure of the constituent parts. On the other hand, our findings are consistent with the theory that a more homogeneous film is produced under electrostatically charged conditions (i.e., pH 3.0), primarily as a result of a significantly smaller PPI size Gharsallaoui et al. (2009). It was previously proposed that the high elasticity of the interfacial films under non-isoelectric conditions could be due to the fact that the subunits of PPI (molecular mechanism) had enough time to adsorb and reorganise at the interface Gharsallaoui et al. (2009). By directly measuring the interfacial film region with molecular rotors and confocal microscopy observations, the results support this theory. This remains an interesting piece of information as it is also seen that the Pickering mechanism is more marked as a function of particle aggregation and favoring it implies less optimal stability properties for the emulsions Amine et al. (2014). This suggests, from a nanometre scale, that the most important mechanism for film elasticity and emulsion stability is also the molecular one as mentioned by other studies Sridharan et al. (2020a).

While the values for $\tau_{interface}$ and $\tau_{solution}$ are comparable in magnitude, it should be mentioned that a small difference between amplitude-weighted average lifetime in solution and at the interface is observed. The $\tau_{interface}$ is in all cases slightly smaller than $\tau_{solution}$. To identify whether this is caused by PPI interparticle interactions or is due to the oil-water interface, exploring a solution-based environment analogous to the protein film at the interface is a useful method. This can be accomplished by increasing the protein concentration in solution to look for a rise in fluorescence lifetime brought on by a change in confinement. This was carried out in the isoelectric case where higher PPI aggregation was found as shown in Figure 3B. Interestingly, no amplitude-weighted average lifetime variation was observed while increasing protein concentration in the studied range. This suggests that increasing the concentration of protein does not change the structure of aggregates, and only increases the number of aggregates. PPI structural modifications in the oil-water interfacial region is thus the last feature that could explain the observed lifetime of 4-DASPI. Indeed, it is well known that the way in which proteins interact with oil also impacts the interfacial reconformations Zhang et al. (2022b). Therefore, in presence of an interface, the hydrophobic regions tend to relocate to be in contact with the oil Beverung et al. (1999). This might explain the small but systematic decrease in the lifetime of 4-DASPI at the oil-water interface compared to the solutions. Additionally, this is in agreement with the molecular size of 4-DASPI given that such reconformation is occurring on the scale of protein particles. Molecular rotors therefore appear as a robust tool to identify multiple features of protein interfacial films, notably how these are built in a nanometric scale. In the future, we foresee that these tools are also used to probe multiscale properties so a more direct relationship between local viscosity of the protein films and properties such as film elasticity or resistance to coalescence can be obtained.

4 Conclusion

To conclude, the molecular rotor 4-DASPI was used to probe local interfacial viscosities of silicone-oil-in-water emulsions stabilized with PPI under pH modifications. A Pickering stabilisation mechanism was identified with PPI proteins, especially under isoelectric conditions. Bridging of PPI aggregates was observed between oil droplet interfaces. We successfully identified how protein film structures are modified as a function of pH. PPI aggregation and PPI reconformation in the interface characterise the stabilisation mechanism of these oil-water interfaces and can be correctly quantified with 4-DASPI. Amplitude-weighted average fluorescence lifetime in solutions showed an equivalent trend, but systematically higher, as the measurements in the interface with varying pH. Protein aggregates at the interface thus present lower local viscosity compared to simple solutions. We also observe that increasing the protein solution concentration did not impact the measured local viscosities, showing that 4-DASPI is mainly measuring a structural modification at the nanometer scale of PPI. From this study we identify that molecular rotors can be a robust tool to characterize oil-water interfaces with globular proteins that exhibit a Pickering stabilisation mechanism such as PPI.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

All authors contributed in the design of this research. SV, MB, and EM did the experiments with support from GG and FC. T-CR, PM, VS, MM, and DB guided the research, edited the manuscript and contributed in the analysis. The manuscript was written by SV with support from MB and EM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frsfm.2023.1093168/full#supplementary-material

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