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Rapid Communication

Insights into the Stabilization of Interferon Alpha by Two Surfactants Revealed by STD-NMR Spectroscopy

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ABSTRACT

Surfactants are commonly used in biopharmaceutical formulations to stabilize proteins against aggregation. However, the choice of a suitable surfactant for a particular protein is decided mostly empirically, and their mechanism of action on molecular level is largely unknown. Here we show that a straightforward label-free method, saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy, can be used to detect protein-surfactant interactions in formulations of a model protein, interferon alpha. We find that polysorbate 20 binds with its fatty acid to interferon, and that the binding is stronger at pH closer to the isoelectric point of the protein. In contrast, we did not detect interactions between poloxamer 407 and interferon alpha. Neither of the two surfactants affected the tertiary structure and the thermal stability of the protein as evident from circular dichroism and nanoDSF measurements. Interestingly, both surfactants inhibited the formation of subvisible particles during long-term storage, but only polysorbate 20 reduced the amount of small soluble aggregates detected by size-exclusion chromatography. This proof-of-principle study demonstrates how STD-NMR can be employed to quickly assess surfactant-protein interactions and support the choice of surfactant in protein formulation.

Introduction

Therapeutic proteins are delicate molecules that fold into a marginally stable native state required for biological activity and are prone to degradation[1]; for example, perturbation of the native structure caused by interfaces or heat can lead to the formation of aggregates which in turn affects product quality and may lead to adverse side effects.[1–3] To develop a stable drug product, a careful selection of excipients and formulation conditions that minimize the aggregation of a therapeutic protein is essential.[4]

Surfactants are particularly important excipients due to their ability to stabilize proteins against aggregation and particle formation.[5,6] Nonionic surfactants like polysorbates and poloxamers are typically used in biopharmaceutical formulations.[5,7] Surfactants appear to stabilize proteins by different mechanisms, for example, by directly binding to a protein or by occupying hydrophobic interfaces like the air/liquid interface to prevent protein adsorption and subsequent degradation due to structural changes.[7–9] The question of whether a surfactant binds directly to a particular protein is important for understanding the mechanisms of stabilization.[7] However, protein-surfactant interactions are typically weak and transient (K_d > 1 mM), and therefore challenging to measure.[6]

There are different approaches to study protein-surfactant interactions.[5,10] Isothermal titration calorimetry (ITC) has been used to obtain thermodynamic parameters and the stoichiometry of binding between nonionic surfactants and proteins.[11–15] These ITC studies revealed that protein-surfactant interactions are highly dependent on the nature of the protein and the surfactant. For

Abbreviations: AF^{STD}, amplification factor; CD, circular dichroism; PEO, polyethylene oxide; IP, inflection point; ITC, isothermal titration calorimetry; nanoDSF, nanoscale differential scanning fluorimetry; NMR, nuclear magnetic resonance; PPO, polypropylene oxide; P407, Poloxamer 407; PS20, polysorbate 20; STD, saturation transfer difference.

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example, polysorbates typically bind to albumin and can also interact with some immunoglobulins.[11–13] However, ITC does not provide information on the binding site or chemical groups involved in the interaction.

Another approach to study surfactant-protein interactions is to use spectroscopic techniques like circular dichroism (CD) or fluorescence spectroscopy that indicate whether the structure of a protein changes in the presence of a surfactant.[5,12] Detecting an interaction in such case relies mostly on a binding mechanism that induces a conformational change in the protein. Further, surface tension measurement of protein-surfactant mixtures can provide insights into the mechanism of stabilization (e.g., surfactant-protein binding or surfactant-mediated steric hindrance at interfaces).[16,17]

Nuclear-magnetic resonance (NMR) is another spectroscopic technique with a broad potential to study biopharmaceutical proteins, including protein-excipient interactions.[10] In particular, both 1D and 2D NMR methods were recently applied to assess how interactions of surfactants with polypeptides,[18] human growth hormone,[19] and antibody fragments[12] affect the protein structure.

NMR offers a suite of experiments, including saturation transfer difference (STD), that are frequently used in fragment-based drug discovery to detect weak binding of small molecules to proteins, but have so far not been routinely applied to biopharmaceutical formulation. The STD experiment is a straightforward label-free method that requires only small amounts of unlabeled protein and has a relatively short experimental time compared to other NMR experiments.[20,21] It can be applied to any protein with rapid spin-diffusion (typically proteins >10 kDa) and does not impose any upper limit to the molecular weight of the protein. STD experiments rely on intermolecular magnetization transfer (also referred to as saturation but is in no way related to saturation in the sense of ligand binding) from protein target to its binding partner during selective saturation time. Ligand protons in closer proximity to the protein target receive higher degrees of saturation, which reflects in greater STD effects, and can be used to map the binding epitope of the ligand at atomic resolution,[20] and estimate the apparent affinity of binding.[22] The non-binding molecules either do not produce a signal in STD spectra or the intensity of the signal doesn't depend on the ligand concentration. We have previously applied STD-NMR to measure interactions between monoclonal antibodies and pharmaceutical excipients.[23]

Here we use STD-NMR to study the interactions of surfactants with a hydrophobic model therapeutic protein, interferon alpha. We investigate the impact of the measured interactions on the higher-order structure and key biophysical properties of the protein, as well as on the aggregation during long-term storage. The study demonstrates the feasibility of using STD-NMR to inform the selection of stable biopharmaceutical formulations.

Materials and Methods

Materials

Surfactant-free interferon alpha2a in bulk formulation was supplied by Roche Diagnostics GmbH, Penzberg, Germany. The formulation buffer was exchanged to 10 mM sodium acetate with pH 4 or 5 by extensive dialysis.[24] The surfactants were spiked into the dialyzed interferon from stock solutions. Unless stated otherwise, the concentrations of surfactant in the final formulations were 0.05 % (w/v) for polysorbate 20 and 0.2 % (w/v) for poloxamer 407. These concentrations were chosen as they reflect typical concentrations used in practice. The final interferon alpha concentration in the formulations was 1 mg/ml measured by ultraviolet spectroscopy NanoDrop 2000 (Thermo Fisher) and calculated with an $A280_{0.1\%}$ = 0.972. The storage stability studies were prepared with 2-mL fill volume in DIN2R glass type I vials as previously described.[24]

NMR

All NMR spectra were acquired at 25 °C on a 800 MHz Bruker Avance III spectrometer equipped with 5 mm triple resonance TCI cryoprobe and temperature control unit. Samples for NMR were prepared by addition of 5 % v/v ^2H_2O to 300 μL of each protein formulation and transferred to 3 mm NMR tubes (Wilmad). The spectra were acquired and processed using Bruker Topspin 3.5 (Bruker). Additional analysis was done in OriginPro9.1 (OriginLabs).

For each sample, a set of proton (1D ^{1}H) and saturation transfer difference (STD) spectra was acquired. ^{1}H spectra were acquired using a standard Bruker pulse sequence with excitation sculpting with gradients for water suppression (zgesgp) with 64 scans while stddiffesgp.3 pulse sequence with the interleaved acquisition of on- and off-resonance spectra with 32 scans was used for STD NMR experiments. On- and off-resonance saturation frequencies were 7.5 ppm and 20 ppm, respectively. Saturation time of 3 s was used, and 20 ms spin lock filter was applied to eliminate protein signals. STD spectra were obtained by subtracting on-resonance from off-resonance spectrum. Appropriate control experiments without addition of proteins were performed to confirm no direct irradiation of excipients. STD amplification factors were calculated using the equation:

$$AF^{STD} = \frac{I_{off} - I_{on}}{I_{off}} * \frac{[L]}{[P]} \quad (1)$$

where AF^{STD} is the STD amplification factor, I_{off} the intensity of excipient signal in the off-resonance spectrum, I_{on} the intensity of signal in the on-resonance spectrum, and *[L]* and *[P]* represent total concentrations of excipients and interferon alpha, respectively.

Apparent dissociation constants (K_D^{app}) were estimated by fitting the experimental AF^{STD} to the Langmuir isotherm[22] in which AF^{STD} is the STD amplification factor, AF_{max}^{STD} is the maximum STD-amplification factor, *[L]* is the total ligand concentration, and K_D^{app} is the apparent dissociation constant.

$$AF^{STD} = \frac{AF_{max}^{STD} * [L]}{K_D^{app} + [L]} \quad (2)$$

Near Ultraviolet Circular Dichroism (NUV-CD)

Near-UV circular dichroic measurements (250–340 nm) were performed with the Jasco J-810 spectropolarimeter (JASCO Deutschland). Therefore, 1.2 mL IFN α-2a solutions with a concentration of 1 mg/ml were filled into a 10 mm quartz cuvette. Spectra were collected in steps of 0.5 nm with 4 seconds data integration time per step and three accumulations per sample. Before each sample measurement, a blank of the respective buffer was performed. After buffer subtraction, the curves were smoothed using the Savitzky-Golay algorithm with seven smoothing points.

Nanoscale Differential Scanning Fluorimetry (nanoDSF)

A Prometheus NT.48 (Nanotemper Technologies) and standard glass capillaries were used. The temperature ramp was 1 °C/min. The excitation wavelength of the device is 280 nm and the fluorescence intensities at 330 and 350 nm are measured. In addition, a backscattering detector yields information on the aggregation of the sample. The inflection points (IPs) of the unfolding transitions from the fluorescence data and the aggregation onset temperatures (T_{agg}s) from the scattering signal were calculated with the PR.ThermControl 2.1 software (Nanotemper Technologies).

Size-Exclusion Chromatography (SEC)

The small soluble aggregates were measured on an HPLC (Dionex Summit 2) with a TSKgel G3000SWxl, 7.8 × 300 mm, 5 μm column (Tosoh Bioscience) using a running buffer composed of 50 mM sodium acetate pH 5 with 500 mM arginine hydrochloride. After injection of 25 μg interferon alpha, the elution of the protein was detected using a fluorescence detector (Dionex RF2000). The excitation and emission wavelengths were 280 and 343 nm, respectively. The chromatograms were evaluated with Chromeleon 7 (Thermo Fisher) to calculate the relative area of small soluble aggregates.

Flow Imaging Microscopy (FlowCAM)

A FlowCAM 8100 (Yokogawa Fluid Imaging Technologies) equipped with a 10x-magnification cell was used to analyze the subvisible particles in the formulations. A flow rate of 0.15 mL/min and a sample volume of 200 μL were used. The auto image frame rate was 29 frames per second with a sampling time of 74 s. The particles were identified using 3 μm distance to the nearest neighbour and segmentation thresholds of 13 for the dark and 10 for the light pixels. The images were processed with the Visual Spreadsheet software to determine the number of particles with a certain size (equivalent sphere diameter).

Results

Structural Groups in Surfactants Studied by NMR

We were interested in assaying the binding between surfactants and interferon alpha. We selected PS20 and P407 since these two surfactants belong to different structural classes used in biopharmaceutical formulations. PS20 is composed mainly of esters of lauric acid with polyethoxylated sorbitan, while P407 is a triblock co-polymer consisting of a polypropylene oxide (PPO) core flanked by polyethylene oxide (PEO) blocks (Fig 1a). The hydrophobic segments in the surfactants are the lauric acid and the polypropylene block for PS20 and P407, respectively. PS20 is widely used in biopharmaceutical formulations while the choice of P407 was motivated by earlier work showing that P407 stabilizes recombinant human growth hormone, a protein with a similar size and structure to IFN α-2a.[25] Additionally, P407 contains larger mass fraction of PPO (~30 %) compared to other, more commonly used, poloxamers such as poloxamer 188 (~20% PPO), thus offering a larger nonpolar region for interactions with a hydrophobic protein such as IFN α-2a.

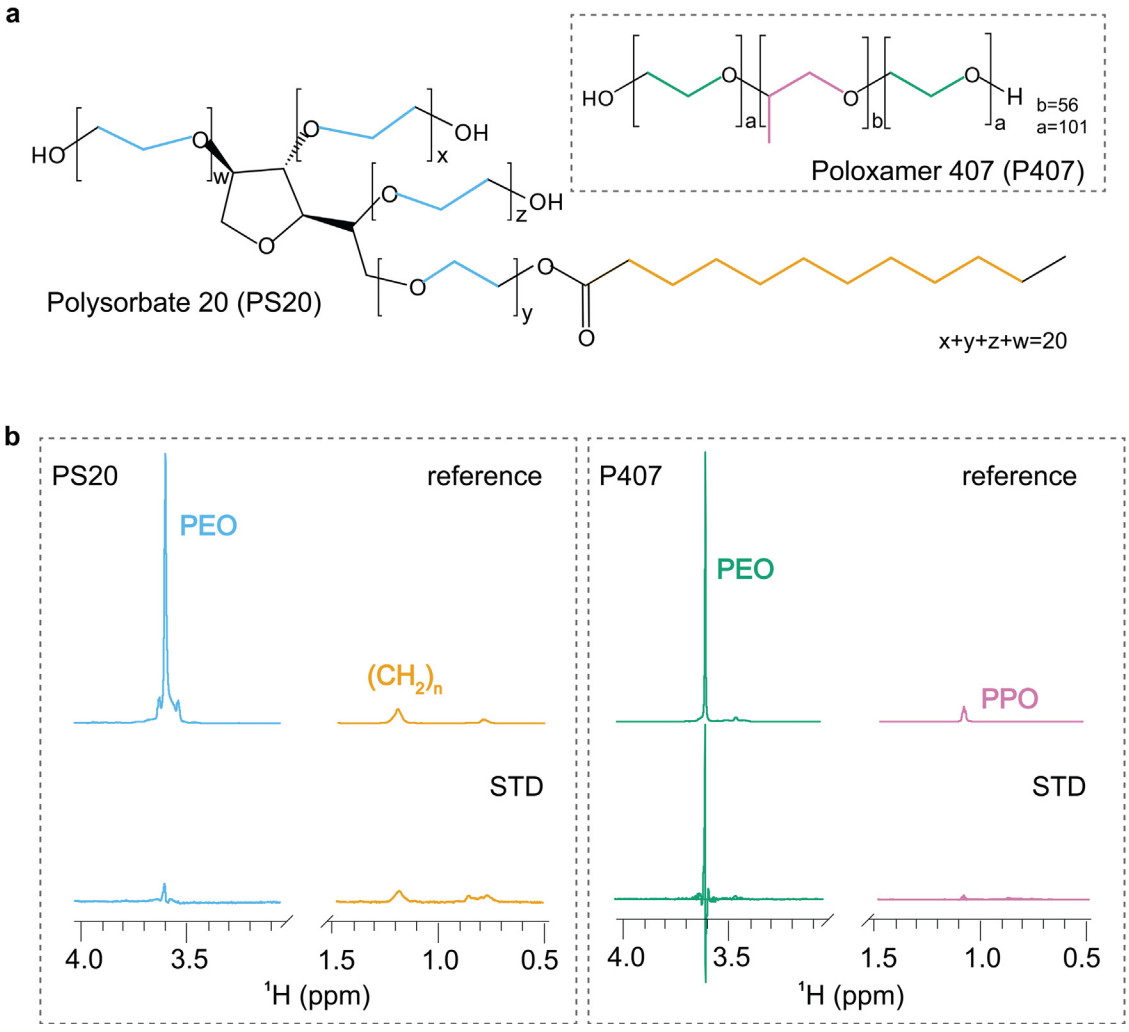

Figure 1. Surfactant structure and NMR analysis. (a) Chemical structures of PS20 and P407. (b) Reference and STD NMR spectra acquired at 1 mM surfactant showing the chemical shifts of specific groups in the surfactant structure. (CH2)n (yellow) – methylene groups in the fatty acid moiety of PS20. PEO (blue) – polyethylene oxide groups in the hydrophilic part of polysorbate 20. PEO (green) – polyethylene oxide of P407. PPO (magenta) – polypropylene oxide groups of P407.

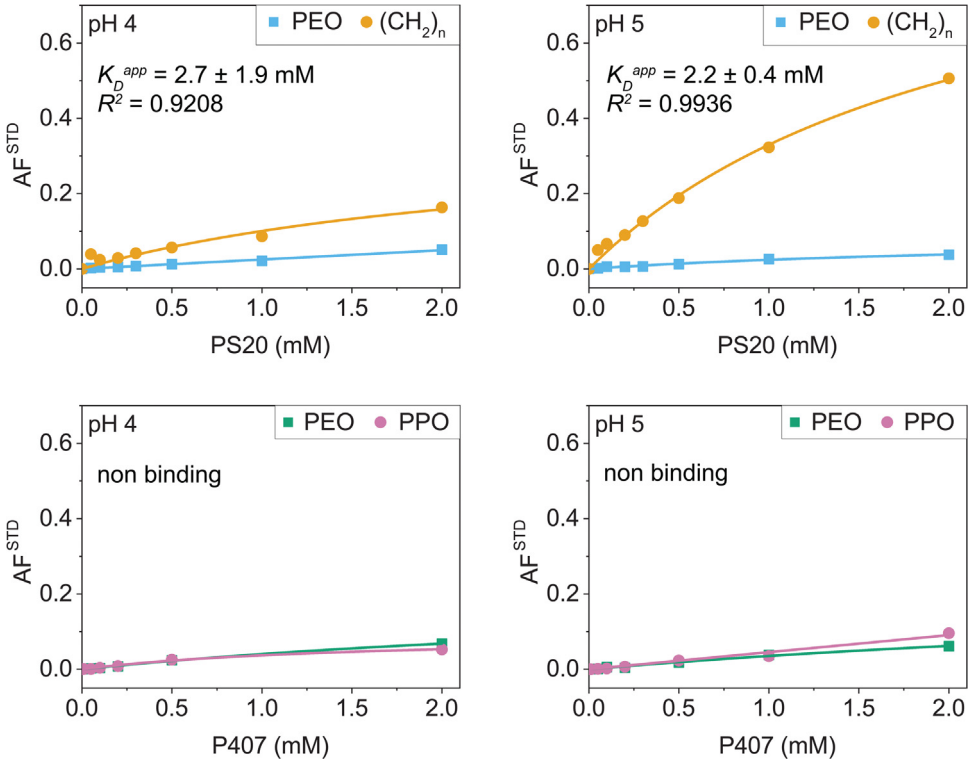

Figure 2. The interaction between interferon and surfactants can be assessed with STD-NMR. The $\mathbf{AF^{STD}}$ (amplification factor) of chemical moieties in the surfactants is depicted as a function of surfactant concentration. The lines are fits to the Langmuir isotherm that we use to determine the apparent dissociation constant ($\mathbf{K_D^{app}}$).

The chemical groups of the hydrophilic and hydrophobic parts of the surfactants have distinct chemical shifts in ^{1}H NMR spectra (Fig 1b). Accordingly, the STD-NMR spectra can be derived to assess whether certain parts of the surfactants interact with the interferon alpha (Fig 1b) which was further assessed in the subsequent section.

Binding of Surfactants to Interferon Alpha

To measure the binding between the two surfactants and interferon alpha in 10 mM acetate buffer at pH 4 and 5, we performed titrations and calculated the STD amplification factor (AF^{STD}) assigned to chemical groups in different parts of the surfactants (Fig 2). At surfactant concentrations used here, PS20 forms NMR-visible micelles that have the signals with the same chemical shift as monomeric PS20. Micelle formation in this case does not affect the interpretation of the results as the PS20 signal intensity increases linearly during titration (Fig S1). PS20 showed overall higher AF^{STD} compared to P407, indicating that PS20 binds more strongly to the protein than P407 (Fig 2). The AF^{STD} of the methylene groups in the fatty acid in PS20 was higher than the of the polyethylene oxide groups which reveals that PS20 binds to interferon alpha via this hydrophobic fatty acid moiety. The buffer pH also has an effect. Higher AF^{STD} of the methylene groups is observed at pH 5 compared to pH 4 (Fig 2). In contrast to PS20, P407 shows very low AF^{STD} for both the hydrophilic (PEO) and hydrophobic (PPO) moieties independent of the pH. This data suggests that P407 does not bind to interferon alpha or that the interaction is very weak.

Effect of Surfactant Binding on the Biophysical Properties of Interferon Alpha

We asked whether the binding of PS20 to interferon alpha will translate into effects on the biophysical properties of the protein. More specifically, we wanted to investigate whether the higher-order structure or thermal stability of interferon are affected by the surfactants, as previously reported for other proteins.[11,12] The near-UV CD spectra of the protein with or without surfactants were superimposable indicating that the interaction with the surfactant does not cause changes in the tertiary structure (Fig 3a).

We then used nanoDSF to investigate whether the surfactants have influence on the thermal unfolding profile and apparent melting temperatures of interferon. The unfolding traces of the protein were not affected by the presence of either PS20 or P407 (Fig 3b). At pH 4, the inflection point (IP) of the unfolding without surfactant was 71.7 ±0.3 °C whilst IPs were 71.5 ±0.4 °C and 71.7 ±0.3 °C in the presence of PS20 and P407, respectively. At pH 5, the surfactant-free sample had an IP = 66.0 ±0.1 °C, the addition of PS20 resulted in an IP = 65.8 ±0.1 °C, while the sample with P407 yielded an IP = 66.1 ±0.1 °C.

To assess whether the aggregation of interferon during heating is affected by the surfactants, we compared the light scattering traces from nanoDSF (Fig 3c). At pH 4, no aggregation onset was observed in any of the samples due to the small nature of the formed aggregates.[24] At pH 5, there was distinct onset of aggregation, and the aggregation profiles were identical for the surfactant-free and surfactant-containing samples (Fig 3c). The aggregation onset temperatures were 62.6 ±0.1 °C without surfactants, 62.3 ±0.1 °C in the presence of PS20, and 62.5 ±0.2 °C in the presence of P407.

Impact of Surfactant Binding on the Storage Stability of Interferon Alpha

An important question is whether the protein-surfactant interactions influence the long-term storage stability of interferon alpha. We have previously performed long-term storage stability studies with interferon alpha in surfactant-free formulations where we observed that pH and ionic strength have a strong effect on protein aggregation.[24] Complementing this data, we studied the long-term storage stability of interferon formulations containing either PS20 or P407 and compared them to the surfactant-free samples.

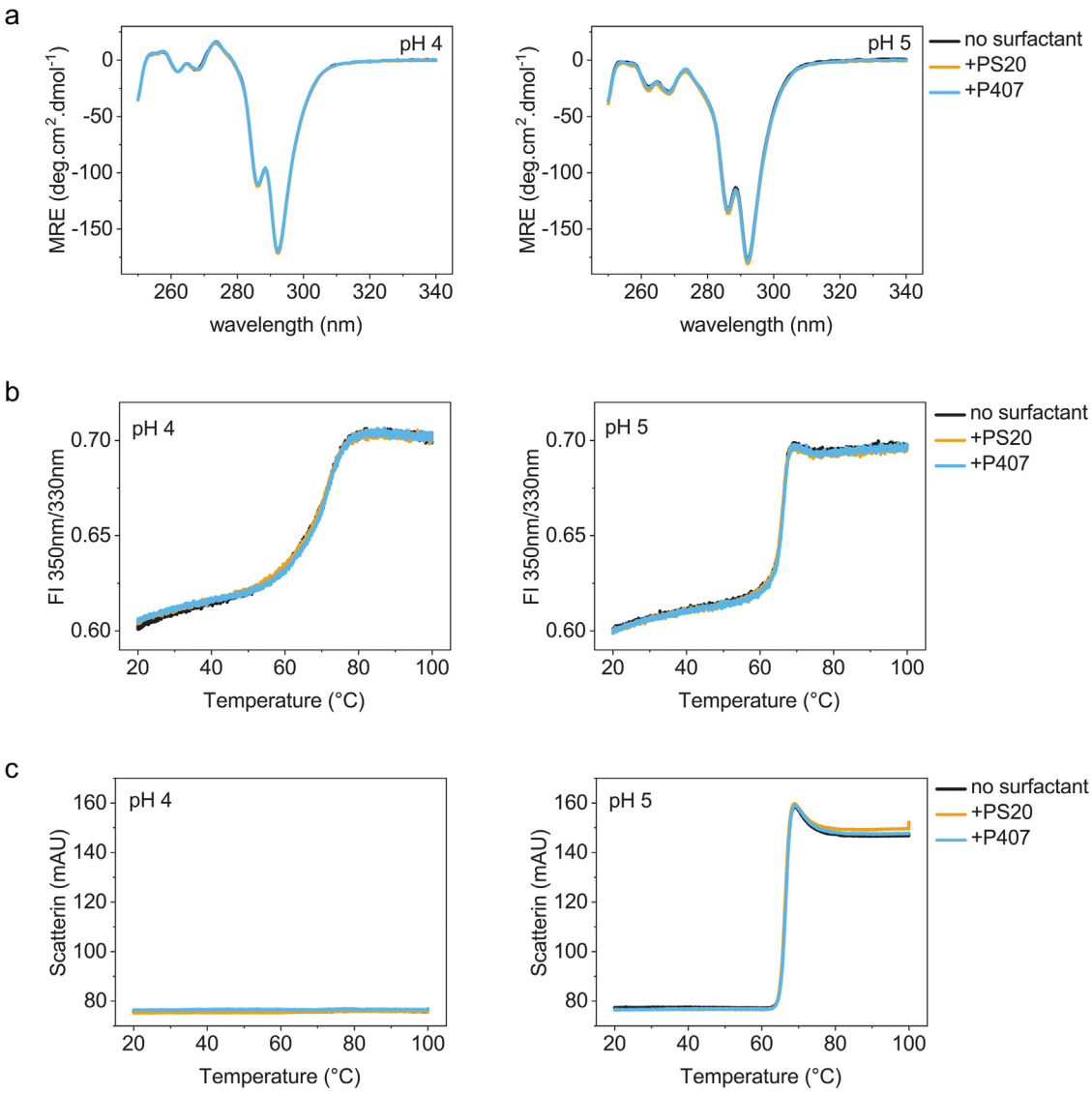

Figure 3. Effect of polysorbate 20 and poloxamer 407 on the structure and thermal stability of interferon alpha. (a) Near-UV CD spectra at pH 4 and 5. (b) Thermal unfolding profiles measured with nanoDSF (c) Aggregation during heating measured with the backscattering detector in nanoDSF. All curves are mean of triplicates.

Size-exclusion chromatography indicated that interferon alpha samples with pH 4 contained very low amounts of small soluble aggregates (≤ 0.2 %) during the entire span of the stability studies (12 m at 4°C) (Fig 4a). Strikingly, at pH 5, the samples with PS20 contained less (~0.1 %) small soluble aggregates compared to samples with P407 (~0.7 %) or to surfactant-free samples (~0.7 %) (Fig 4a).

Flow imaging microscopy analysis revealed that all samples at pH 4 also had very low particle counts during the entire stability study (Fig 4b). At pH 5, however, the surfactant-free sample contained more subvisible particles in all three size ranges (2-10 μm, 10-25 μm, and >25 μm) (Fig 4b). Notably, both PS20 and P407 greatly reduced the particle numbers at pH 5 (Fig 4b).

Discussion

Understanding protein-excipient interactions and their impact on the stability of a protein is critical for the rational development of biopharmaceutical formulations. Various techniques can be used to study protein-excipient interactions.[7,10] However, each of these techniques has strengths and limitations. Usually, no information about the interacting parts of the excipient with the protein can be obtained.[10]

Here we used STD-NMR to assay the interaction between surfactants and interferon alpha. The STD-NMR approach is particularly useful as it is a label-free technique that also provides information about the orientation of the ligand interacting with a macromolecule. In fact, a common application of STD-NMR is epitope mapping.[20] By using STD-NMR, we demonstrated that PS20 interacts mainly via its hydrophobic tail with interferon alpha while P407 showed no significant binding to the protein in tested conditions. The strength of the interaction was stronger at pH 5 compared to pH 4, which can be explained by the lower charge of the interferon near its isoelectric point (IEP = 6), leading to heightened non-polar and hydrophobic interactions. This agrees well with the notion that the aliphatic chains bind with hydrophobic regions on a protein.[26] In contrast, there was no detectable interaction between the PEO or PPO moieties in P407 and interferon alpha. Previous studies of protein adsorption of model proteins[27,28] and monoclonal antibodies[29] indicated that poloxamers, but not polysorbates associate with the proteins in solution. This further shows that protein-surfactant association very much depends on the chemical nature of both, surfactants and the protein surface,

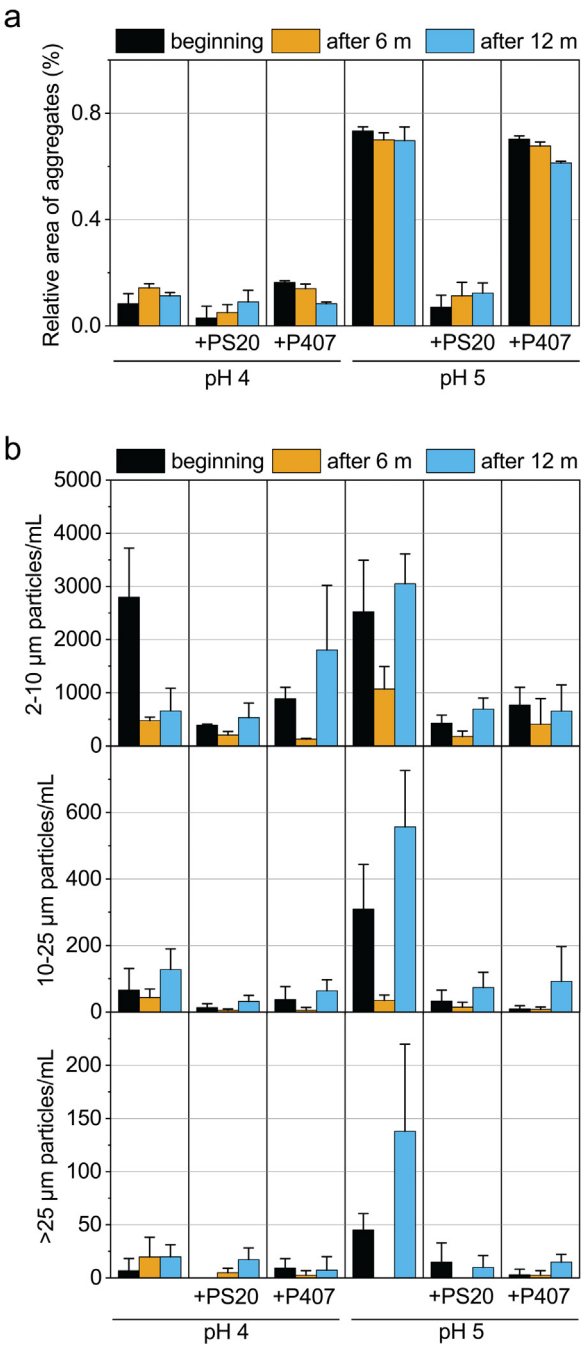

Figure 4. Long-term storage stability data of interferon alpha formulations incubated at 2-8 °C. (a) Relative amount of small soluble aggregates detected by size-exclusion chromatography. (b) Number of subvisible particles with size 2-10 μm, 10-25 μm, and >25 μm detected by flow imaging microscopy. The values are mean of triplicates with standard deviations.

therefore detailed assessment of this binding, as used in this study, can inform the choice of the surfactant used in final protein formulation.

In biopharmaceutical formulations, surfactants may be added above their CMC which could influence NMR signals with surfactants in micelles potentially being NMR-invisible due to their increased size which in turn affects their NMR properties, such as chemical shift, relaxation times and diffusion coefficients.[30,31] It is therefore crucial to assess how micelle formation influences NMR signals of surfactants on individual basis. We show that in the case of PS20, micelles do not have a significant impact on PS20 NMR signals at the tested concentrations, including their intensity and chemical shift perturbation. Here, P407 was used below CMC and no micelle formation was observed, however, poloxamers, are known to form NMR invisible micelles above CMC which results in line broadening and deviations from linearity of signal intensity upon increased concentrations.[32] Other surfactants, such as sodium dodecanoat[33] and sodium dodecyl sulphate[34,35] exhibit significant chemical shift perturbations as well as line broadening upon micelle formation. In case micelles exhibit a chemical shift different to that of monomeric surfactant, free concentration of the monomeric form can be estimated from the NMR spectra, and STD-NMR can be still used to detect the binding of the monomeric surfactant or the micelles to the protein, and to estimate the binding constant. However, if formation of micelles leads to line broadening, it is possible to detect surfactant-protein interactions using STD-NMR but binding constants can not be estimated.

Neither of the two surfactants affected the structure and thermal stability of interferon alpha. This is an interesting finding because the binding of PS20 can cause structural perturbations in a protein.[12] Markedly, the binding of PS20 to interferon alpha was manifested as a reduction in the amount of small soluble aggregates at pH 5 (Fig 4a). This reduction in aggregates was observed immediately after sample preparation and the trend was kept during the entire span of the stability study (12 m at 2-8 °C). This indicates that a surfactant-protein interaction could be required to disrupt interferon aggregates on a molecular level.

Interestingly, both PS20 and P407 reduced the numbers of subvisible particles formed at pH 5 (Fig 4b). This showed that the formation of large aggregates (in the subvisible range) was also suppressed by the non-interacting P407. We hypothesize that different mechanisms of surfactant-mediated stabilization are probably employed to inhibit the formation of small soluble aggregates or large insoluble aggregates.

Based on our results that demonstrate that we can distinguish between protein binding and non-binding surfactants, estimate binding constants and relate the protein-surfactant interaction with protein stability, we see three major applications of STD-NMR in biopharmaceutical formulation. Firstly, it can be applied in conjunction with other NMR methods to gain specific information on the interacting chemical moieties and establish binding stoichiometry as shown previously.[36] This contributes to a better understanding of the mechanism by which surfactants bind to proteins and can be used for troubleshooting when unusual results are obtained by other stability indicating methods. Secondly, STD-NMR is an established method routinely used in large scale screening for fragment-based drug design[37,38] and we expect that it can be automated and implemented for early screening of protein surfactant and other protein-excipient interactions in biopharmaceutical formulations as well. Because the analysis focuses on the NMR signals of chemical moieties in the surfactant molecule, the method can be quickly transferred to assay different non-labelled proteins. We envision establishing an NMR based platform for a range of surfactants that can be screened for interactions with therapeutic protein candidates during formulation development. Thirdly, our approach to combine STD-NMR with nanoDSF and CD could be particularly useful to quickly characterize biopharmaceutical formulations to detect protein-surfactant interactions and assess their impact on protein structure and stability. It will be particularly valuable to see if specific protein-surfactant interactions observed by STD-NMR can be correlated to higher stabilization of proteins against aggregation for a larger set of therapeutic protein candidates including peptides, antibodies, and fusion proteins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.xphs.2022.10.013.

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