THE CHALLENGE OF FORMULATING HIGH CONCENTRATION THERAPEUTIC PEPTIDES

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Key Words: Therapeutic Peptides; High Concentration Formulation; Taylor Dispersion Analysis; Peptide-Peptide Interactions; Peptide Aggregation

Therapeutic peptides offer significant opportunities to treat serious and debilitating diseases, with several purported advantages over proteins, such as a simpler characterization process and a reduced regulatory burden. However, many peptides are known to be susceptible to aggregation, and surprisingly little work has been published on designing formulation principles for these therapeutic agents.

In this paper the focus will be on the challenges encountered in rational development of a stabilization strategy for two model peptides formulated at high concentration (>50 mg/ml). The first model peptide studied was carbetocin, a 9-amino acid long analogue of oxytocin with improved chemical and enzymatic stability. At high concentrations (70 mg/mL, ca 70 mM) carbetocin is sensitive to interface-induced stress, causing the formation of large amorphous aggregates. The addition of uncharged or charged surfactants surprisingly did not stabilize against this interface-induced stress; instead, positively and negatively charged surfactants steered the aggregation pathway towards the formation of amyloid-like fibrils [1]. The addition of very low concentrations of the uncharged surfactant Span 60 appeared to result in the formation of aggregates with a refractive index close to that of water, creating a false impression of stabilization. For carbetocin, only the removal of the air-water interface by fully filling the vials resulted in a significant stabilization against aggregation [1].

The second model peptide was a 33-amino acid negatively charged peptide which formed amyloid-like fibrils when exposed to heat stress (40 degrees C) at 50 mg/mL concentration. The limited impact of shaking indicated this fibrillation was primarily induced through peptide-peptide interactions in the bulk solution. Peptide-peptide interactions were therefore determined using Taylor Dispersion Analysis. The latter method suggested significant repulsive interactions for this peptide formulation in aqueous solution. Addition of salt (NaCl) or the amino acids Arg or Glu at 150 mM shielded these repulsive interactions. In contrast, addition of 250 mM of the amino acids Val or Ile had a very limited shielding effect. However, none of the added excipients appeared to stabilize the peptide against aggregation, although infrared spectroscopic analysis indicated that the aggregates in the NaCl, Arg and Glu containing solutions consisted of native-like peptides. The aggregates formed in the presence of Val and Ile, or in the absence of any excipients, contained a significant amount of intermolecular beta-sheet [2].

Our results illustrate the challenges in protecting high concentration peptides against aggregation and indicates a strong dependence of the aggregation pathway on the excipients added and their potential interaction with the peptide. A novel formulation toolbox may therefore be required for peptides. The high structural flexibility introduces a significant complicating factor in understanding their interactions with their local environment.

[1] Høgstedt UB, Østergaard J, Weiss T, Sjögren H, van de Weert M. Manipulating aggregation behavior of the uncharged peptide carbetocin. Journal of Pharmaceutical Sciences. 2018 Mar;107(3):838-847.
[2] Høgstedt UB. Formulation of concentrated peptide solutions – physical stability challenges and the impact of peptide-peptide interactions. PhD thesis University of Copenhagen, 2018.

TUNING PROTEIN BEHAVIOR BY MULTIVALENT CHARGES: AGGREGATION, DIFFUSION, AND ADSORPTION

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Key Words: phase diagram; dynamics; crystallization; charge effects; scattering

We discuss concepts for controlling and understanding protein aggregation pathways and the branching between them in aqueous solution by addition of multivalent ions. This route for the tailoring of the interaction potential is exploited for controlling a) crystallization, b) gelation and amorphous aggregation, as well as c) smaller aggregate formation including their dynamics and kinetics. Furthermore, we discuss the connection to and the role of interfaces in this context.

First, we present a real-time study of protein crystallization induced by multivalent ions using small-angle scattering and optical microscopy. Based on the crystallization kinetics, we propose a multistep mechanism. In the first step, an intermediate phase is formed, followed by the nucleation of crystals within the intermediate phase. During this period, the number of crystals increases with time, but the crystal growth is slowed down by the surrounding dense intermediate phase due to the low mobility. In the next step, the intermediate phase is consumed by nucleation and slow growth, and the crystals are exposed to the dilute phase. In this stage, the number of crystals becomes nearly constant, whereas the crystals grow rapidly due to access to the free protein molecules in the dilute phase. This real-time study not only provides evidence for a two-step nucleation process for protein crystallization but also sheds light on the role and the structural signature of the metastable intermediate phase in this process. Furthermore, the competing routes of aggregation are elucidated, as is the role of the general features of the phase diagram including liqiud-liquid phase separation (LLPS).

Second, we present complementary investigations of the dynamics of these systems using quasi-elastic neutron scattering, showing a remarkably universal behavior of the effective diffusion as a function of concentration and salt under suitable conditions.

Third, we show how these concepts can be transferred to protein-interface interations, and how adsorption behavior can be manipulated by multivalent charges. We try to connect the interface and the bulk behavior, including reentrant adsorption and anomalous interface behavior upon approaching bulk phase boundaries.

Finally, we comment on the role of specific ions and attempt a comprehensive discussion of models and theories suitable for a comprehensive explanation of these phenomena.

Invaluable contributions by F. Zhang, M. Fries, A. Sauter, F. Roosen-Runge, T. Seydel, M. Grimaldo, R. Jacobs, O. Matsarskaia, M. Braun, D. Stopper, R. Roth, and M. Oettel as well as numerous external collaborators are gratefully acknowledged.

References

A. Sauter et al., JACS (2015)
A. Sauter et al., Faraday Discussions (2015)
M. Grimaldo et al., J. Phys. Chem. Lett. (2015)
F. Roosen-Runge et al., Nature Scientific Reports (2014)
F. Roosen-Runge et al., PNAS (2011)
M. R. Fries et al., Phys. Rev. Lett. (2017)
O. Matsarskaia et al., Phys. Chem. Chem. Phys. (2018)
O. Matsarskaia et al., J. Phys. Chem. B (2019)

ION-SPECIFIC EFFECTS FOR TUNING THE PHASE BEHAVIOUR OF PROTEIN SOLUTIONS

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Key Words: phase behaviour; proteins; multivalent ions; scattering; thermodynamics

Protein phase behaviour is of importance in various areas of research such as structural biology, rational drug design and delivery, medicine (in particular protein condensation diseases), biotechnology, food science and cell biology. A particularly intriguing variety of phase behaviours can be induced in negatively charged, globular proteins in the presence of multivalent salts such as lanthanide (Ln) chlorides. These behaviours include reentrant condensation, crystallisation and cluster formation as well as liquid-liquid phase separation (LLPS) into a protein-rich and a protein-poor phase [1-3]. LLPS can occur upon a temperature decrease or increase, which is referred to as an upper or a lower critical solution temperature (UCST- and LCST-LLPS), respectively. Here, we present a challenging set of experiments investigating the complex phenomenon of LCST-LLPS in systems of bovine serum albumin (BSA) and multivalent salts from different perspectives including thermodynamic, (non-)equilibrium and spectroscopic studies.

First, the rather unusual phenomenon of LCST-LLPS in aqueous systems consisting of BSA and yttrium chloride (YCl_3) is characterised thermodynamically. Surface charge (zeta potential) and isothermal titration calorimetry (ITC) measurements show LCST-LLPS to be a hydration entropy-driven condensation [2]. This mechanistic explanation is corroborated by results obtained using extended X-ray absorption fine structure (EXAFS) spectroscopy. Based on the Y^{3+}-induced LCST-LLPS described above, the aspect investigated subsequently is the influence that the nature of the multivalent cations used has on this phase behaviour. The experiments focus on the three multivalent salts $HoCl_3$, YCl_3 and $LaCl_3$. A multi-technique approach including temperature-controlled UV-Vis absorbance and synchrotron small-angle X-ray scattering (SAXS) measurements shows that Ho^{3+} cations induce the strongest protein-protein attractions, while the interactions are weakest in the case of La^{3+}. The overall protein-protein and protein-cation interaction strengths can therefore be ranked according to the order $Ho^{3+} > Y^{3+} > La^{3+}$ [3]. Finally, the kinetics of LCST-LLPS of BSA in the presence of varying ratios of $HoCl_3$ and $LaCl_3$ is investigated using synchrotron ultra-small-angle X-ray scattering (USAXS). The growth of the characteristic length scale of the respective experimental systems as a function of time and temperature is found to be strongly influenced by the $HoCl_3/LaCl_3$ ratio. Notably, a higher volume fraction of $HoCl_3$ preferentially drives the samples into an arrested state even at low temperatures [4].

The present study thus shows how a careful choice of multivalent ions can be used to fine-tune protein-protein interactions and the resulting phase behaviour in solution. The results are of importance not only for a fundamental understanding of soft matter thermodynamics, but also for the design of so-called “smart” materials with implications for, e.g., drug delivery or water purification.

[1] Zhang et al., Phys. Rev. Lett. (101), 14810 (2008)
[2] Zhang et al., J. Appl. Cryst. (44), 755-62 (2011)
[3] Zhang et al., Pure Appl. Chem. (86), 191-202 (2014)
[4] Matsarskaia et al., J. Phys. Chem. B (120), 7731-6 (2016)
[5] Matsarskaia et al., Phys. Chem. Chem. Phys. (20), 27214-25 (2018)
[6] Matsarskaia et al., J. Phys. Chem. B Article ASAP, DOI: 10.1021/acs.jpcb.8b10725 (2019)

A COLLOID APPROACH TO SELF-ASSEMBLING ANTIBODIES

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Key Words: antibody self-assembly, protein interactions, protein solution viscosity

Concentrated solutions of monoclonal antibodies have attracted considerable attention due to their importance in pharmaceutical formulations, yet their tendency to aggregate and the resulting high viscosity pose considerable problems. Here we tackle this problem by a soft condensed matter physics approach which combines a variety of experimental measurements with a patchy colloid model, amenable of analytical solution. We thus report results of antibodies structural and dynamic properties obtained through scattering methods and microrheological experiments. [1] We model the data using a colloid-inspired approach, explicitly taking into account both the anisotropic shape of the molecule and their charge distribution. Our simple patchy model is able to disentangle self-assembly and intermolecular interactions, and to quantitatively describe the concentration dependence of the osmotic compressibility, collective diffusion coefficient and zero shear viscosity. Our results offer new insights on the key problem of antibody formulations providing a theoretical and experimental framework for a quantitative assessment of the effects of additional excipients or chemical modifications and a prediction of the resulting viscosity.

1) Nicholas Skar-Gislinge, Michela Ronti, Tommy Garting, Christian Rischel, Peter Schurtenberger, Emanuela Zaccarelli, and Anna Stradner, Molecular Pharm. (2019, submitted)

CONCEPT ON SELF-ASSEMBLY AND STRUCTURE OF GLOBULAR PROTEIN FLUIDS

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Key words: Protein, interaction, self-assembly, aggregate, flow.

Globular proteins are ubiquitous in our daily life. Not only they are naturally present in the biological matter, they also offer many possibilities to adjust the nutritional and flow properties of fluids or to design drug vehicles [1]. Globular protein systems interact through short range attractive forces; and the interaction between them may lead the system to form aggregates through self-assembling process. Since such biological monomers are complex systems, their aggregation process is most of the time out of control. The current main conceptual framework to describe that process is based on the idea that the monomers may self-assemble through a diffusion and reaction mechanism known as DLA for diffusion limited aggregation, and RLA for reaction limited aggregation respectively [2]. Beta-lactoglobulin (blg) solution gives, after heat-induced denaturation, a suspension of polydisperse aggregates as predicted by the random aggregation concept. Therefore, the transition from native blg to denatured blg aggregate suspension leads to complex correlation with the flow behavior [3]. Although the dependency of the aggregation process to physicochemical factors like, ionic strength, pH, temperature and concentration has been intensively investigated, it still remains much to do to control the aggregate polydispersity via self-assembling process. The composition of the raw product, thermal processing, pH and entropy instability during the aggregation process, are some of the factors influencing the polydispersity of the aggregates. We use different techniques such as SAXS/USAXS, LS, SEM, CSLM and image analysis methods to characterize thoroughly the structure of globular protein aggregates formed after heat-induced denaturation at different experimental conditions [4]. Whether these aggregates are in solution or entrapped by gelation, we do think that investigating their structure will provide us with relevant information to solve the issue related to their formation.

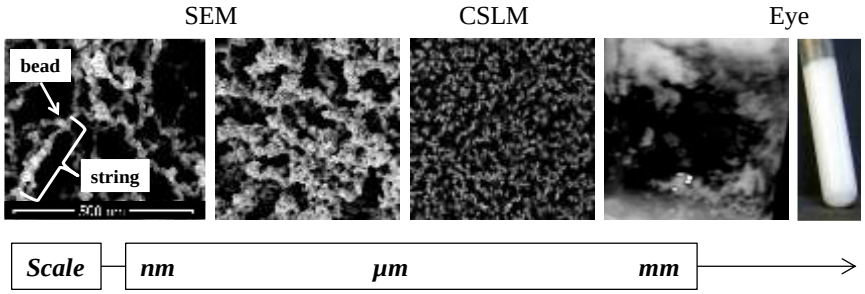

Figure 1- Pictures of globular protein solutions after heat-induced aggregation and gelation in different scales.

References:

[1] T. Nicolai, M. Britten, C. Schmitt, b-Lactoglobulin and WPI aggregates: Formation, structure and applications, Food Hydrocolloids 25 (2011) 1945-1962.
[2] H. Wu, J. Xie, Morbidelli, Massimo, Kinetics of Cold-Set Diffusion-Limited Aggregations of Denatured Whey Protein Isolate Colloids, Biomacromolecules 6 (2005) 3189-3197.
[3] A.C. Alting, R.J. Hamer, C.G. de Kruif, M. Paques, R.W. Visschers, Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels, Food Hydrocolloids 17 (2003) 469-479.
[4] C.D. Munialo, E. van der Linden, K. Ako, M. Nieuwland, H. Van As, H.H.J. de Jongh, The effect of polysaccharides on the ability of whey protein gels to either store or dissipate energy upon mechanical deformation, Food Hydrocolloids 52 (2016) 707-720.

ARREST TRANSITIONS IN PROTEIN SOLUTIONS – INSIGHT FROM COMBINING SCATTERING, MICRORHEOLOGY, AND COMPUTER SIMULATIONS

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Key Words: concentrated protein solutions, liquid-solid transition, Dynamic Light Scattering-based tracer microrheology, protein-protein interactions, patchy interactions.

The static and dynamic properties of concentrated protein solutions are essential ingredients for our understanding of the cellular machinery or formulating biopharmaceuticals. Here a combination of advanced characterization techniques such as light and x-ray scattering, neutron spin echo measurements [1] and microrheology experiments [2], combined with the theoretical toolbox from colloid physics and state-of-the-art computer simulations [3], considerably enhances our understanding of the link between protein interactions and the stability, dynamics and flow properties of these solutions up to high concentrations. We will address the enormous influence of weak attractive interactions known to exist between many globular proteins, and demonstrate the dramatic effect of an interaction potential anisotropy [1] such as attractive patches and shape anisotropy [3] on the dynamic properties. We will also discuss how we can combine interparticle interaction effects and the formation of (transient) equilibrium clusters in an attempt to understand and predict properties such as the concentration dependence of the zero shear viscosity of dense protein solutions [4].

(1) Bucciarelli, S.; Myung, J. S.; Farago, B.; Das, S., Vliegenthart, G.; Holderer, O.; Winkler, R. G.; Schurtenberger, P.; Gompper, G.; Stradner, A. “Dramatic influence of patchy attractions on short-time protein diffusion under crowded conditions” *Sci. Adv.* 2016, 2:e1601432.
(2) Garting, T. and Stradner, A. “Optical Microrheology of Protein Solutions using Tailored Nanoparticles” *Small* 2018, 1801548.
(3) Myung, J. S.; Roosen-Runge, F.; Winkler, R. G.; Gompper, G.; Schurtenberger, P.; Stradner, A. “Weak shape anisotropy leads to non-monotonic crowding effects impacting protein dynamics under physiologically relevant conditions” *J. Phys. Chem. B* 2018, 122, 12396-12402.
(4) Bergman, M.; Garting, T.; Schurtenberger, P.; Stradner, A. “Experimental Evidence for a Cluster Glass Transition in Concentrated Lysozyme Solutions” submitted to *J. Phys. Chem. B,* 2019.

AGGREGATION CHALLENGES IN THE FORMULATION DEVELOPMENT OF MULTI-DOSE PEPTIDE PRODUCTS

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Key Words: Peptide Aggregation, Peptide Fibrillation, Aggregation Kinetics, Preservatives, Formulation Development

The formulation development of parenteral peptide therapeutics frequently encounters aggregation challenges. In-depth biophysical understanding of the molecule and formulation are required to achieve formulation robustness. Further, unique considerations need to be given for peptide products that require multi-dose as the use of preservatives can promote aggregation while preservative effectiveness can also be impacted by its interaction with the peptide. This presentation will focus on the *reversible and irreversible fibril aggregates* in peptide formulations. Biophysical characterization of aggregation and formulation will be discussed in detail. Formation of reversible aggregates and the impact of excipients especially preservatives will be discussed. For the development of fibril-prone peptides, analytical challenges, formulation strategies, as well as predictive test for kinetics will also be discussed. In particular, studies on the temperature-dependent fibril nucleation kinetics and its impact on formulation development will be presented.

Reference:
J. Zhang, X. Mao, W. Xu. *Mol. Pharmaceutics* 2018, 15, 5591−5601
D'Addio et al. *Journal of Pharmaceutical Sciences* 2016, 105, 2989-3006

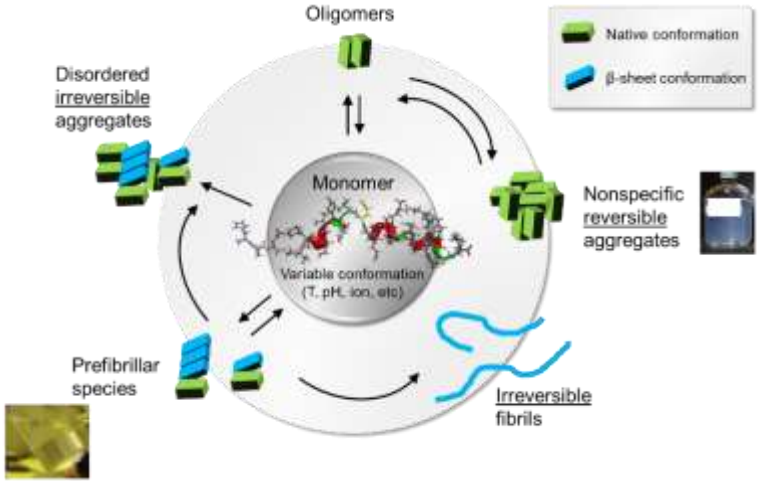

Figure 2 – Formation of reversible and irreversible aggregates by peptides

LINKING PROTEIN-PROTEIN INTERACTIONS TO THE DIVERSITY OF AMYLOID-LIKE AGGREGATES

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Key Words: Protein-Protein Interactions, Amyloid Aggregates, Sub-visible Particles, Polymorphism

Protein aggregation is often studied in the context of neurodegenerative diseases. Deposits of supramolecular aggregates, often appearing as ordered fibrils, are associated with the onset of devastating pathologies as Alzheimer´s and Parkinson´s diseases. Equally important is the impact that the formation of protein aggregates may have in the quality of a protein drug product. The presence of the so-called sub-visible particles (SVP) and visible particles in protein drug product is indeed considered one of the risk factors potentially inducing immune response in patients. Finally yet importantly, protein aggregates, and particularly amyloid fibrils, have unique structural, physico-chemical, mechanical and optical properties, making them appealing bio-inspired materials for several applications. Either one looks at protein aggregation in the context of diseases, drug development or biomaterials, understanding how protein-protein (PPIs) and protein-solvent interactions (PSI) determine aggregation kinetics and the morphology/structures of the final aggregates is a *conditio sine qua non* for unraveling the molecular mechanisms ruling the self-assembly reaction and for controlling it.

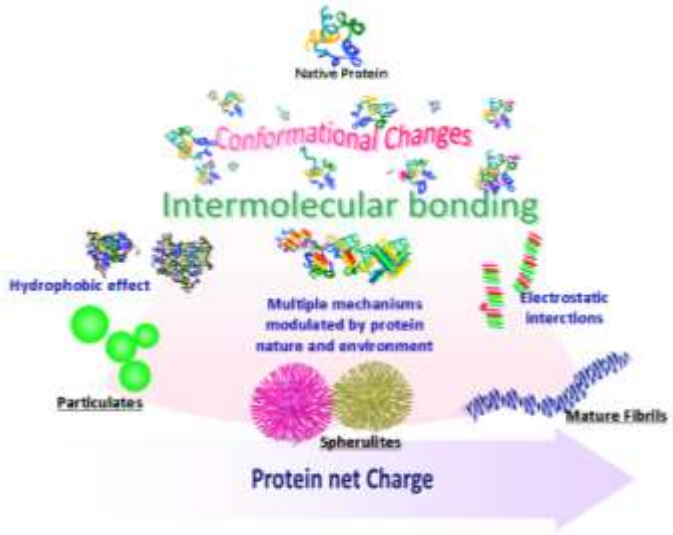

Figure 3 – Scheme representing the interplay between possible interactions and mechanisms during a protein aggregation process. In specific conditions, the formation of different morphologies and 3D arrangements of the final aggregates can occur [1].

In our group, we have reported the possibility for a large group of proteins under specific destabilizing conditions to form a variety of protein aggregates, being it not limited to the formation of amyloid fibrils (Figure 1) [1]. In line with the scope of the conference, I will present our unique approach based on advanced fluorescence microscopy, small angle X-ray scattering and spectroscopy and aimed at identifying the key PPIs and PSIs responsible for such variability in structures and morphologies [2-6]. We use surfactants, salts, alcohols in bulk and microfluidic setups to finely tune the interactions between proteins and, consequently, control the self-assembly process. Our results show that subtle changes in the PPIs and PSI do not only affect the kinetics, but they may also have a dramatic effect on the 3D arrangement, microscopic structures, mechanical properties and stability of the final self-assembled structures. Our findings provide a scenario in which a pool of highly heterogeneous structures can be generated as a result of interconnected aggregation pathways, being this aspect of key relevance especially for protein drug product development and optimization.

REFERENCES

[1] Vetri V. and Foderà V. 2015 *The Route to Protein Aggregate Superstructures: Particulates and Amyloid-like Spherulites*, FEBS Letters, 589, 2448–2463
[2] Vetri, V. Piccirili F., Krausser J., Buscarino G. , Lapinska U., Vestergaard B., Zaccone A., Foderà V. 2018 *Ethanol Controls the Self-Assembly and Mesoscopic Properties of Human Insulin Amyloid Spherulites*. Journal of Physical Chemistry B, 122, 3101-3112.
[3] Skamris Pedersen T., Marasini C. , Madsen K.L., Foderà V., and Vestergaard B. 2019 *Early Stage Alpha-Synuclein Amyloid Fibrils are Reservoirs of Membrane-Binding Species* Scientific Reports, 9, 1733
[4] Foderà V., Vetri V., Wind T.S., Noppe W., Cornett C., Donald A.M., Morozova-Roche L., Vestergaard B. 2014 *Observation of Early Structural Changes Leading to the Formation of Protein Superstructures ,* Journal Physical Chemistry Letters, 5, 3254–3258.
[5] Foderà V., Zaccone A., Lattuada M and Donald A.M. 2013 *Electrostatics controls the formation of amyloid superstructures in protein aggregation*, Physical Review Letters, 111, 108105.

[6] Foderà V., Pagliara S., Otto O., Keyser U.F. and Donald A.M. 2012 *Microfluidics Reveals a Flow-Induced Large Scale Polymorphism of Protein Aggregates* Journal Physical Chemistry Letters, 3, 2803-2807.

ACTIVATION ENERGIES DEFINE KINETIC (IN)STABILITIES OF THERAPEUTIC ANTIBODIES

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Key Words: Activation energy, Fluorescence, Formulation, Monoclonal antibody, Protein degradation.

Antibody degradation pathways are many-fold and can result in loss of function, efficacy and even to adverse effects in patients. Among others, aggregation and fragmentation is still the major challenge. The identification of the primary degradation pathway can be very complex. Monoclonal antibodies (mAbs) are large, multi-domain macromolecules. Due to their complex nature and inherent properties, temperature induced unfolding leads to rather complex unfolding kinetics. In this study, we determined activation energies (Ea) of thermal intrinsic fluorescence (IF) unfolding profiles unique for each individual antibody. The analyzed activation energies give insights both into kinetic (in)stabilities of single domains and the overall structure of the antibody. To realize this, we used a novel developed experimental setup to perform temperature dependent fluorescence unfolding profiles of various mAbs. In conclusion, the activation energies can be used as descriptors for kinetic (in)stabilities of therapeutic antibodies. Moreover, we show that lower activation energies correlate to monomer loss in long-term storage stabilities and can thus likely be used for shelf-life prediction.

BACTERIAL SURFACE LAYER PROTEINS: A NEW CLASS OF 'SMART' SWITCHABLE SURFACE-ACTIVE PROTEINS

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PROTEIN AGGREGATES AS BUILDING BLOCKS FOR OIL STRUCTURING

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Key Words: oleogels, protein interactions, capillary interactions

Over the recent years, structuring of organic solvents and oils into organogels (oleogels) has gained much attention from colloid, material and food scientists. Although many gelators are known, their use in applications is limited as they are often expensive, not effective, and have low stability. In this presentation, we will present a new category to the common oleogelators: proteins. As they are cheap, widely available, biocompatible and food grade, they have a large potential to be used in many different types of applications.

We have created heat-set protein building blocks of different dimensions (from nanometer to millimeter) that are used to create protein networks in oil. We have developed a method to transform the protein building blocks into the oil and change the interactions between the building blocks to control the network formation and the final properties of the protein oleogels. This presentation will give an overview of the different interactions between proteins that are of relevance in hydrophobic environments, and discuss how these interactions can be varied. To vary the interactions, different oils were used, water was added, and a heat treatment was applied. The oleogels show large versatility regarding rheological properties (strength, yield stress and plastic behavior), in a similar way as is known for very common protein hydrogels. Very firm protein oleogels can be formed that show fracture properties. But also protein oleogels with a spreadable behavior.

PROTEIN-STABILIZED EMULSIONS AND NANOEMULSIONS

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Key Words: emulsification, emulsion stability, protein adsorption, emulsion rheology, concentrated emulsions.

This presentation will summarize briefly our current understanding of the scientific and technological basis for the formation of emulsions containing micrometer and nanometer sized droplets, with applications in food, cosmetic and pharma industries [1-3]. The focus will be on protein-stabilized emulsions. Their specific features will be summarized in comparison to the surfactant- and particle-stabilized emulsions [3]. The subtle relations between the material characteristics of the emulsions (oil type, emulsifier, pH, etc.) and the optimal hydrodynamic conditions for emulsification will be discussed [3] in the context of obtaining emulsions with desired properties. Examples of appropriate mixtures of proteins with other emulsifiers (lipids, lysolipids, natural gums) will be given. The differences between oil-in-water and water-in-oil emulsions will be briefly discussed [4,5]. Special focus will be given on the recent advance in production of nanoemulsions using high pressure homogenizers, high viscosity of the continuous phase, and/or high oil volume fraction during emulsification [6,7]. Some new methods for self-emulsification will be briefly reviewed [8,9]. The basic physicochemical and hydrodynamic concepts will be illustrated by multiple examples with real systems.

References:

1. David Julian McClements, *Food Emulsions: Principles, Practices, and Techniques*, 3rd Edition, CRC Press, 2015.
2. Andreas Håkansson, Emulsion Formation by Homogenization: Current Understanding and Future Perspectives, *Annu. Rev. Food Sci. Technol.* 10 (2019) 239–258 (review article).
3. S. Tcholakova, N. D. Denkov, and A. Lips, Comparison of Solid Particles, Globular Proteins and Surfactants as Emulsifiers. *Phys. Chem. Chem. Phys.* 10 (2008) 1608-1627 (review article).
4. N. Politova, S. Tcholakova, N. D. Denkov, Factors Affecting the Stability of Water-oil-water Emulsion Films. *Colloids Surfaces A* 522 (2017) 608–620.
5. N. Politova, S. Tcholakova, S. Tsibranska, N. D. Denkov, K. Muelheims, Coalescence Stability of Water-in-Oil drops: Effects of Drop Size and Surfactant Concentration. *Colloids Surfaces A* 531 (2017) 32–39.
6. S. Tcholakova, I. Lesov, K. Golemanov, N. Denkov, S. Judat, R. Engel, T. Daner, Efficient Emulsification of Viscous Oils at High Drop Volume Fraction. *Langmuir* 27 (2011) 14783-14796.
7. D. Gazolu-Rusanova, I. Lesov, S. Tcholakova, N. Denkov, B. Ahtchi, Food grade nanoemulsion preparation by rotor-stator homogenization. *Food Hydrocolloids* (2019) under review.
8. S. Tcholakova, Z. Valkova, D. Cholakova, Z. Vinarov, I. Lesov, N. D. Denkov, K. Smoukov, Efficient Self-Emulsification via Cooling-Heating Cycles. *Nature Comm.* 8 (2017) 15012.
9. Zh. Valkova, D. Cholakova, S. Tcholakova, N. Denkov, S. K. Smoukov, Mechanisms and Control of Self-Emulsification upon Freezing and Melting of Dispersed Alkane Drops. *Langmuir* 33 (2017) 12155−12170.

INTERFACIAL RHEOLOGY OF MICROCAPSULES AND DYNAMICS IN FLOW

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Key Words: Interface, Rheology, Self-assembly, Microfluidics, Microcapsules

A capsule is a drop bounded by a thin solid membrane providing specific mechanical properties. It is used to control the spatio-temporal delivery of substances in numerous processes and also as a model system of cells. Its dynamics under flow depends on its membrane characteristics. Moreover, the delivery of encapsulated drugs is controlled by its deformation. The interfacial rheology of microcapsules can be tuned according to their formulation. We will focus on cross-linked membrane made with human serum albumin and chitosan assembled with a surfactant via electrostatic interactions. The interfacial rheological properties of these soft microparticles are deduced from their dynamics of deformation in elongation and shear flows. In elongation flow, the surface shear modulus of the membrane is measured and related to the kind of biopolymer used and to the main parameters of the process of fabrication. In the regime of large deformations, the microcapsules can present a non-linear elastic response or plastic deformations. Non-linear elastic constitutive law is deduced by comparison of the evolution of the shape of the microcapsule in the two main planes of deformation of the capsule with numerical simulations. In shear flow, the rotation of the membrane, i.e. the tank-treading, is visualised and quantified by decorating the membrane of microcapsules with particles. The tracking of the distance between two close microparticles showed membrane contraction at the tips and stretching on the sides. This dynamic of deformation induce viscous dissipation inside the membrane. The order of magnitude of membrane viscosity is determined by comparison with numerical simulations. Wrinkling instability is observed in extensional flow and studied by varying the interfacial properties of the microcapsules. In this way, the phase diagram of wrinkle instability for microcapsules has been deduced as the scaling law between the wrinkles wave-length and the membrane thickness. Finally, we have developed a set of tools to characterize the interfacial viscoelasticity of microcapsules, their bending modulus and their non-linear elastic properties. We conclude the talk with some results on break-up of microcapsules in flow.

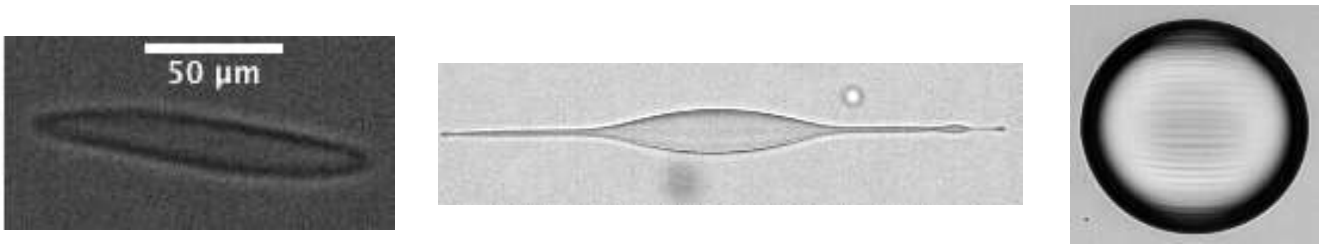

Figure 1: Microcapsules in flow. Left: A icrocapsule in shear flow. Middle: Break-up of a microcapsule in extensional flow. Right: Wrinkles on a microcapsule in flow.

References:

de Loubens, C., Deschamps, J., Georgelin, M., Charrier, A., Edwards-Levy, F., & Leonetti, M. (2014). Mechanical characterization of cross-linked serum albumin microcapsules. Soft matter, 10(25), 4561-4568.
de Loubens, C., Deschamps, J., Boedec, G., & Leonetti, M. (2015). Stretching of capsules in an elongation flow, a route to constitutive law. Journal of Fluid Mechanics, 767.
de Loubens, C., Deschamps, J., Edwards-Levy, F., & Leonetti, M. (2016). Tank-treading of microcapsules in shear flow. Journal of Fluid Mechanics, 789, 750-767.
Xie, K., De Loubens, C., Dubreuil, F., Gunes, D. Z., Jaeger, M., & Léonetti, M. (2017). Interfacial rheological properties of self-assembling biopolymer microcapsules. Soft matter, 13(36), 6208-6217.
Xie, K., de Loubens, C., Jaeger, M., & Leonetti, M. Wrinkling and folding on soft microcapsules. *Livret des résumés de la 22 e Rencontre du Non-Linéaire Paris 2019*, 105.

MICROFLUIDIC AND NANOTECHNOLOGY BASED ASSAYS FOR THE DEVELOPMENT OF SAFE BIOPHARMACEUTICALS

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Key Words: Protein aggregation, protein-protein interactions, formulation, interfaces, hydrodynamic flow.

Protein stability towards aggregation represents a potential challenge for the production and administration of pharmaceuticals. In particular, aggregation can compromise the developability and shelf-life of the products, with consequences for yield and safety, respectively. In this work, we discuss two novel approaches for the analysis of the stability of protein formulations:
(1) A microfluidic diffusion-sizing platform to analyze protein sizes and interactions at high protein concentration directly in the solution state with minimal perturbation of the sample. The limited dilution of the sample during the analysis and the possibility to characterize properties directly in the solution state make the technique suitable for the analysis of heterogeneous solutions of proteins under dynamic equilibrium. We show how the platform represents an attractive tool for the analysis of sizes and interactions of proteins in both diluted and high-concentration solutions during development, manufacturing, and formulation.
(2) A highly controlled assay of surface-induced protein aggregation based on nanoparticles. Protein aggregation is often due to heterogeneous nucleation events occurring at interfaces, including air/water interface, impurities and leachable particles. However, the development of screening tools against surface aggregation has been hindered by the difficulty in generating a controlled amount of surface stress in the formulation as well as in decoupling the surface effect from the contribution of hydrodynamic flows. In our assay, we leverage the flexibility of polymer chemistry to finely tune the properties and amount of surfaces provided by the nanoparticles, inducing aggregation of soluble peptides and proteins, including antibodies, in a time scale of a few hours. This platform represents i) an attractive tool for fundamental studies of heterogeneous nucleation events under stagnant and flow conditions, and ii) a high-throughput screening assay of the effect of intrinsic and extrinsic variables on protein stability towards interface-induced aggregation.

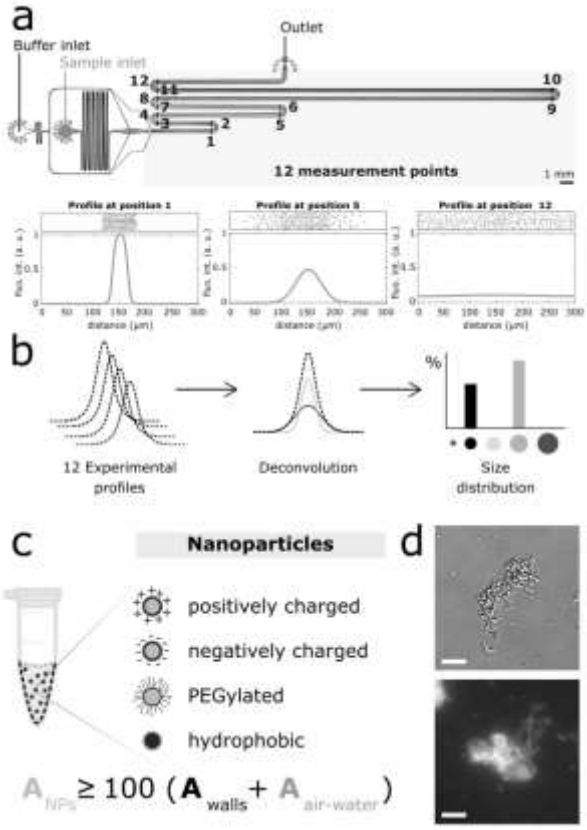

Figure 4 – a,b) Microfluidic diffusion sizing platform, allowing the measurement of size distributions. c,d) Polymeric nanoparticles library to screen for interface interactions in protein solutions.

References:

[1] Kopp M.R.G. Arosio P., Microfluidic Approaches for the Characterization of Therapeutic Proteins. J. Pharm. Sci., 2018, 107, 1228-1236.
[2] Kopp M.R.G., Villois A., Capasso Palmiero U., and Arosio P., Microfluidic Diffusion Analysis of the Size Distribution and Microrheological Properties of Antibody Solutions at High Concentrations. Ind. Eng. Chem. Res., 2018, 57 (21), 7112–7120.

BIOPHYSICAL CHARACTERIZATION APPROACHES TO AID THE SELECTION OF PROTEIN FORMULATIONS BY PREDICTING THEIR PHYSICAL STABILITY DURING LONG-TERM STORAGE

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The formulation of therapeutic proteins is a critical process which aims at finding the most suitable conditions that impede protein degradation during long-term storage. One degradation path of high interest is the non-native aggregation[1]. The latter can be greatly suppressed by the selection of suitable solution conditions[2]. Over the years, various biophysical techniques have been explored as tools to quickly select the most promising formulations for long-term storage.

In this talk, we share the experience in our lab how some of these techniques can be integrated into protein formulation studies. We discuss the application of differential scanning calorimetry and the usefulness of determining the apparent protein melting temperatures to select protein formulations with high physical stability[3]. In the next part, we show how state-of-the-art equipment that employs fluorimetric and light scattering measurements during heating allows the sample saving characterization of proteins to select stable formulations. We then discuss the limitations of the existing non-isothermal techniques and show how the results from formulation studies can be biased when some excipient properties change during heating. In this context, we explore whether isothermal chemical denaturation is a useful complementary tool to overcome the limitations of non-isothermal techniques[4].

Finally, we show how the assessment of the aggregation of partially folded species during refolding can provide additional information for the selection of protein formulations with high physical stability during storage[4]. The talk will be concluded with few general suggestions how to select solution conditions that impede aggregation during long-term storage of liquid protein formulations.

References

1. Roberts, C. J. *Non-Native Protein Aggregation Kinetics*. 98, (Springer New York, 2007).
2. Shire, S. J. Formulation and manufacturability of biologics. *Curr. Opin. Biotechnol.* 20, 708–714 (2009).
3. Youssef, A. M. K. & Winter, G. A critical evaluation of microcalorimetry as a predictive tool for long term stability of liquid protein formulations: Granulocyte Colony Stimulating Factor (GCSF). *Eur. J. Pharm. Biopharm.* 84, 145–155 (2013).
4. Svilenov, H., Markoja, U. & Winter, G. Isothermal chemical denaturation as a complementary tool to overcome limitations of thermal differential scanning fluorimetry in predicting physical stability of protein formulations. *Eur. J. Pharm. Biopharm.* 125, 106–113 (2018).

PREDICTING THE STABILITY OF BIOTHERAPEUTICS IN FORMULATION

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Key Words: Monoclonal antibodies, Chromatography, Formulation, Aggregation, Protein-Protein interactions

Biotherapeutics, especially monoclonal antibodies (mAbs), is one of the fastest growing groups of pharmaceuticals, corresponding to a treatment option to life-threatening conditions, such as cancer, autoimmune diseases and cardiovascular diseases. A key barrier in the production of these pharmaceuticals is the formation of protein aggregates, which can lead to increased production costs, loss of the biological function, and immunogenic responses from patients. To detect the presence of aggregates and ensure protein stability upon storage, efficient formulation screening methods are required.

Self-Interaction Chromatography (SIC) has previously been shown to be an accurate, automated and high-throughput technique to predict protein aggregation [1]. This study is adapting SIC into Interaction Chromatography (IC), which takes the interactions between two different proteins or species into account. The aim of the first case study was to analyse different formulations containing histidine buffer, phosphate-citrate buffer and sodium citrate buffer at 25 mM, and using a therapeutic mAb. Additionally, the stabilising effects of NaCl, polysorbate and L-arginine were examined in the range of 0-250 mM, 0-0.1% v/v and 0-200 mM, respectively. The protein-protein interactions for each formulation condition were quantified by calculating the osmotic second virial coefficient (B_{22}), a fundamental physicochemical property that describes protein-protein interactions in solution. These results were also compared with aggregation data and aggregation kinetics using size-exclusion chromatography and dynamic light scattering.

In a second case study bovine serum albumin (BSA) was mixed in formulation buffers in combination with sucrose and trehalose, where the protein-protein interactions were studied by determining B_{22} and the aggregation propensity measured. Later these conditions were lyophilised, which is a common way to formulate many biotherapeutics. The aim was to see if the results from the initial screenings also could predict the stability of the freeze-dried cakes.

The first case study showed that IC was a suitable technique to detect small changes in formulation conditions as it managed to predict the increase in stability of the mAb with the ionic strength and arginine concentration, but with no major effect with the addition of polysorbate. The second case study showed that BSA exhibited repulsive protein-protein interactions in all the range of concentrations analysed, as expected due to its high stability in solution, which could be increased with the addition of stabilisers, especially trehalose. Even in the freeze-dried cakes higher concentration of trehalose led to an increased Young's modulus, and therefore higher mechanical stability. Based on these results it could be seen that a protein-protein interaction technique such as IC could be used as an early predictor for formulation behaviour both in liquid and solid states.

References

1. Hedberg, S.H.M., Lee, D., Mishra, Y., Haigh, J.M., and Williams, D.R. (2018) Mapping the Mab Aggregation Propensity Using Self-Interaction Chromatography as a Screening Tool*. Analytical Chemistry*. *90*, 3878-3885.

NEUTRON SCATTERING: A VERSATILE TOOL FOR PROBING COMPLEX SOLUTIONS AND SURFACES

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Neutron scattering has significant benefits for examining the structure of complex samples. Cold (slow) neutrons are non-damaging and predominantly interact with the atomic nucleus, meaning that they can penetrate deeply into samples, which allows for flexibility in the design of samples studied. Most importantly, there is a strong difference in neutron scattering length (i.e., scattering power) between protium ((_1^1)H, 99.98% natural abundance) and deuterium ((_1^2)H, or D, 0.015%). Through the mixing of H2O and D2O in the samples and in some cases the deuterium labelling of the biomolecules, components within a complex can be hidden or enhanced in the scattering signal. This enables both the overall structure and the relative distribution of components within a complex to be resolved. In this talk I will discuss the technical premise behind performing neutron scattering experiments and highlight examples where neutron scattering has been used to examine complex bio-pharmaceutical related phenomena. Examples will include in situ chromatography measurements and complex and realistic bio-mimetics at buried interfaces.

KEY STABILITY ATTRIBUTES USED FROM TARGET PROTEIN DEVELOPMENT TO PROCESS OPTIMIZATION

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MULTI-LASER NANOPARTICLE TRACKING ANALYSIS (NTA): EVALUATING PROTEIN AGGREGATION

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THE DEVELOPMENT OF THERAPEUTIC PROTEINS CAN BE HINDERED BY POOR DECISION-MAKING STRATEGIES IN THE EARLY STAGE

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Key Words: Protein aggregation, Protein formulation(s), stability, Monoclonal antibody(s), Drug development

In this study we address two major issues related to the current development process of therapeutic proteins and their characterization. First, due to limited samples amounts, the selection of lead molecules in the early stages is often based on the results from a limited physicochemical characterization. The latter can be based on measurements of only 2-3 parameters, e.g. protein melting temperature, protein aggregation temperature, and is usually performed in only one buffer, e.g. PBS. The hypothesis we present is that such approach can lead to the rejection of lead candidates that can still be manufacturable and can move on to clinical trials. The second matter we address are the often-reported correlations between protein physicochemical parameters in the literature. We propose that such correlations can be found only in a small sample population, e.g. one protein in different solution conditions or different proteins from the same class. However, we expect that such correlations would not be valid in a large population, including various protein structures and solution conditions.
In order to address the above-mentioned issues, we created the PIPPI consortium (http://www.pippi.kemi.dtu.dk) and applied systematic approach to map the physicochemical properties of a wide range of proteins and extensively study their stability as a function of the solution conditions.
We show that promising therapeutic protein lead candidate can appear as non-manufacturable when only limited physicochemical characterization is performed, e.g. a few methods are used and only a few solution conditions are tested. Therefore, the rejection rate during early-stage development can be improved by more thorough physicochemical characterization. Moreover, only weak linear correlations between biophysical properties of proteins are observed in a large populations. This suggests that the often-reported correlations between parameters describing the protein stability are not representative of a global population. Understanding the connections between various physiochemical parameters would require a systematic database which is currently in development by the PIPPI consortium.

A NOVEL TECHNIQUE TO CHARACTERISE THE SURFACE HYDROPHOBICITY OF PROTEINS USING INVERSE LIQUID CHROMATOGRAPHY

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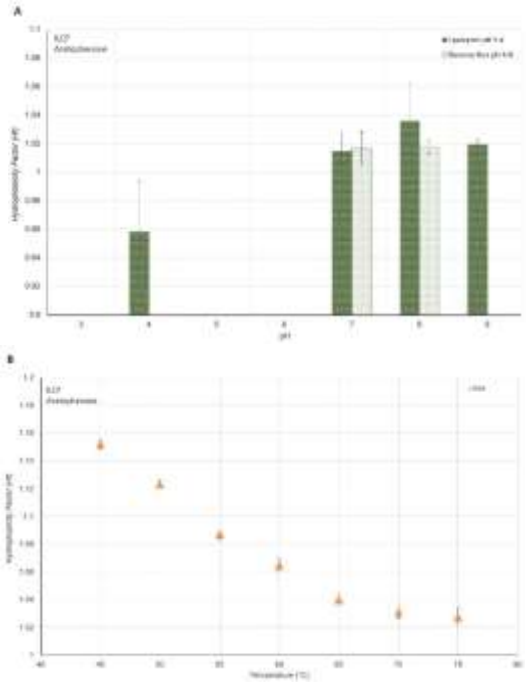
Key Words: Protein Hydrophobicity, Characterisation, HPLC, Affinity Chromatography

The surface properties of industrial and therapeutic proteins are key to understanding their behaviour in-situ, in the laboratory and in processes. Protein surface hydrophobicity is a marker for three dimensional structure, stability and function. Inverse Liquid Chromatography of Proteins (ILCP) with small molecule hydrophobic probes can be used to obtain direct thermodynamic measurements of the surface hydrophobicity of column resin-bound proteins using changes in probe retention behaviour. The dimensionless hydrophobicity factor (Hf) for Lysozyme and BSA was obtained with changing temperature and pH. The two sets of results are similar to published data from fluorescence and dielectric spectroscopy. The effects of pH appear to be reversible. The effects of temperature are reversible if the thermodynamic load is mild. This novel technique could provide reliable and quick hydrophobicity and other surface parameter data sets using standard equipment and methods for any conceivable protein of interest.



Hedberg, S. H. M., Heng, J. Y. Y., Williams, D. R., & Liddell, J. M. (2015). Self-interaction chromatography of mAbs: accurate measurement of dead volumes. Pharmaceutical research, 32(12), 3975-3985.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochimica et biophysica acta (BBA)-Protein structure, 624(1), 13-20.

Tessier, P. M., Lenhoff, A. M., & Sandler, S. I. (2002). Rapid measurement of protein osmotic second virial coefficients by self-interaction chromatography. Biophysical journal, 82(3), 1620-1631.

Figure 5 – A. Surface Hydrophobicity Factor Hf for lysozyme with pH. B. Hf for BSA with Temperature.

POSSIBLE OBSTACLES AND PITFALLS DURING BIOPHARMACEUTICAL PROCESSING OF HIGH CONCENTRATED LIQUID FORMULATIONS (HCLF)

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Key Words: high concentrated liquid formulation, biopharmaceutical processing, fill and finish

The need of high concentrated liquid formulations in the field of biologicals has evolved over the last years. The increase in protein concentration comes along with the advantage of a decreased volume of drug product which needs to be administered to obtain the desired therapeutic dose preferably within a single injection. Thus, the usage of high concentrated liquid formulations is associated with a higher degree of freedom with regard to e.g. primary packaging material such as syringes, small volume applicators as well as route of administration both leading to a higher patient convenience ideally to self-administration and thus hospital independency. An increase in protein concentration can result in higher viscosities, which results in challenges with regard to administration to the patient or biopharmaceutical processing steps (e.g. purification, fill and finish (1)). In literature, key interactions of high concentrated liquid formulations are described, which are of importance for the purification (e.g. donnan effect for diafiltration (2)) or the fill and finish step (e.g. differences in densities). The oral presentation will give an overview of obstacles and pitfalls within certain biopharmaceutical processing steps and approaches with which they could be tackled.

1. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004;93(6):1390-402.
2. Stoner MR, Fischer N, Nixon L, Buckel S, Benke M, Austin F, et al. Protein-solute interactions affect the outcome of ultrafiltration/diafiltration operations. J Pharm Sci. 2004;93(9):2332-42.

PREDICTING VISCOSITY OF HIGH CONCENTRATION MAB FORMULATIONS BASED ON DATA FROM HIGH THROUGHPUT CHARACTERIZATION METHODS

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Key Words: High conc. protein formulations, viscosity prediction, protein-protein-interaction

Development of high concentration liquid formulations is especially attractive for biopharmaceuticals due to their generally low bioavailability and amenability to self-administration as sub-cutaneous injections. Elevated protein concentration can be associated with increased viscosity, which becomes an important factor for protein drug product development, manufacturing and dose administration. High viscosity can be detrimental to manufacturing setups causing increased process times or failures. Dose delivery to patients are also impacted, as high viscosities, associated with high injection force can cause difficulties in self-administration or even pain. *In vitro* viscosity assessments are thus key to early developability efforts. However, these studies can be resource intensive due to multiple candidates, high material requirements and complex methods. Problems of high viscosity are thus often detected very late, putting pressure on project time lines and development teams. The development of reliable tools for early prediction of viscosity at high protein concentration is essential for selection of optimal candidates in a time and cost efficient way. We present here approaches to this end leveraging on data from high throughput measurements including dynamic light scattering and associated measurements/calculations.

MULTIPLE INTERACTIONS AND COMPLEX VISCOSITY: THE IMPACT OF HIGH FREQUENCY RHEOLOGY FOR THE DEVELOPMENT OF HIGH CONCENTRATION PROTEIN FORMULATIONS

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Key Words: Viscosity, Protein-Protein Interaction, Formulation Development, High protein concentration

Clinical doses of therapeutic proteins range up to 2 mg/kg bodyweigth per patient and even higher. For patient convenience and competitiveness, subcutaneous (s.c.) applications are required. Therefore, liquid formulations for s.c. applications can reach concentrations of up to 200 mg/ml. One key parameter for the development of biotherapeutics as high concentrated liquid formulations (HCLF) is viscosity. Consequently, high solution viscosity is challenging due to e.g. impeded syringeablitiy and injectability that directly link to patient inconvenience, and high shear stress that potentially impair protein inherent stability. Following Jezek et al. 2011, we consider protein concentrations of >100 mg/ml as “highly concentrated”.

During early phases in development of biopharmaceutics only limited material is available. Therefore, prediction of the solution viscosity at higher concentrations (e.g. for HCLF conditions), if required, will be of great benefit. In this study, we applied different approaches comprehensively investigating parameters describing protein-protein interaction, protein hydration, protein conformation at different concentrations, and the volume fraction of the protein molecule in solution. At a molecular level, Protein-Protein Interaction (PPI) are a result of electrostatic-interaction, van-der-Waal (vdW)-forces and hydrophobic forces of bi- or multimodal interaction as well as protein-excipients interaction. At the macroscopic level, these parameters describe a crucial influence on the protein-stability and its rheological behavior in solution. However, during formulation development commonly evaluated PPI parameter such as the second virial coefficient (B_{22}), and/or the concentration- dependent diffusion coefficient (k_D). These parameters only describe interactions in dilute conditions, which poses limitations in predicting interactions at high protein concentrations. At dilute conditions, mostly electrostatic double layer repulsion and charge-shielding effects of buffer and excipients components dominate. In contrast, at high concentration, distances between individual molecules are narrowed, and thus attractive forces such as vdW interactions are predominantly present. Therefore, a direct correlation of PPI parameter obtained from dilute to crowded conditions is only a shaky compromise. The mechanisms and principles driving the formation of highly viscose systems are not fully understood, especially at the molecular level. As a consequence, the attempts to reduce viscosity are often left to chance. In a case study, we evaluated the behaviour of concentrated protein formulations under high-frequency shear excitation in the MHz range. As a result, we propose an explanation for interaction potentials between individual protein molecules linked to high solution viscosity by extending the complex colloid theory. Multiple attraction forces result in a complex viscous behavior, the formation of a transient micro-rheological network of multiple interacting protein molecules, and the formation of an elastic modulus. In order to lower the viscosity, such multiple interactions have to be disrupted and disordered by different excipients.

However, the interaction potential is correlated to the characteristics of each mAb molecule and can be altered by excipients in a defined way. By relating low concentration PPI measurements and wet-lab determined molecular characteristics (e.g. effective surface charge, dipole moment) it is possible to predicts the potency for describing a high viscosity for each mAb. Knowing the effects of pH and different buffer and excipients, a guided development for decreasing the viscosity by different excipients and formulation conditions is possible. High frequency rheology allows a rapid and early evaluation of the viscosity properties of early candidates and thus support subsequent formulation development.

COMPRESSION AND PROTEIN-PROTEIN INTERACTIONS AS TRIGGERS FOR AGGREGATION OF MONOCLONAL ANTIBODIES AT INTERFACES

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Key Words: Aggregation, Shaking, Compression, Microscopy, B2

Aggregation of biopharmaceuticals triggered by interfaces is a challenge at various levels from upstream processing to patient application. We specifically investigated the air-liquid interface. A combination of Langmuir-Blodgett Trough experiments, Infrared Reflection-Absorption Spectroscopy, Brewster Angle Microscopy, Atomic Force Microscopy and Profile Analysis Tensiometry could demonstrate that the film formed by monoclonal antibodies (mabs) at the interface can be substantially condensed upon compression due to interface movement. Protein-protein interactions subsequently are a key element which determines whether large aggregates result from this phase upon decompression.

Thus, not only addition of surface active molecules is a remedy to solve the problem of surface induced aggregation. Additionally, factors which strongly affect the protein-protein interactions, specifically pH and ionic strength are starting points.

Furthermore, the protein film formation itself depends on the monoclonal antibody properties. In a series of mabs the drug candidate which was most stable against shaking stress showing the most repulsive A2 values and the least hydrophocity was the one which adsorbed the slowest at the air-liquid-interface.

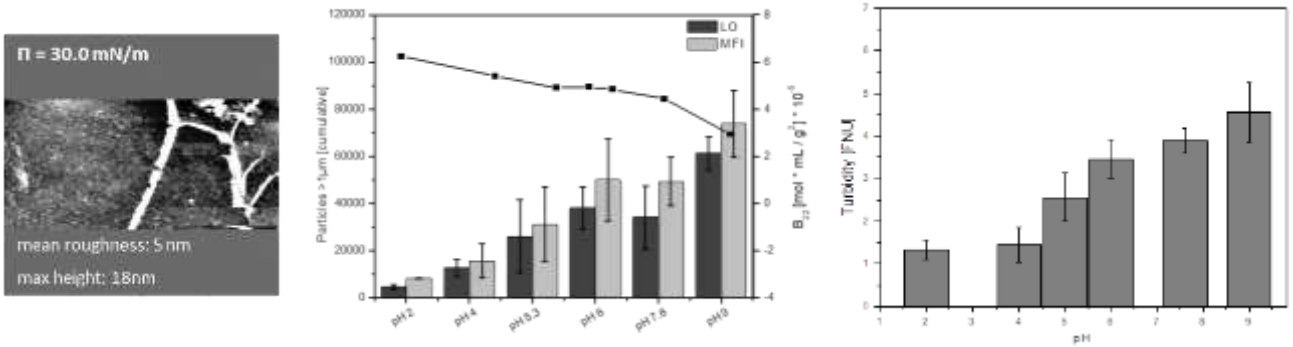

Figure 6 – Submerse AFM of blotted, compressed mab film; subvisible particle formation and turbidity upon stressing as well as B22 as a function of pH

References:
(1) Koepf E., Schroeder R., Brezesinski G., Friess W., The missing piece in the puzzle: Prediction of aggregation via the protein-protein interaction parameter A*2, Eur. J. Pharm. Biopharm. 128, 2018, 200-209.
(2) Koepf E., Eisele S., Schroeder R., Brezesinski G., Friess W., Notorious but not understood: How liquid-air interfacial stress triggers protein aggregation. Int. J. Pharm. 537, 2018, 202-212

PREDICTING, CHARACTERIZING, AND CONTROLLING INTERFACIAL ANTIBODY ADSORPTION AND AGGREGATION

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Key Words: interfacial, aggregation, surface tension, formulation

Exposure to interfaces can accelerate aggregation of antibody therapeutics, particularly under stresses encountered during manufacture, transportation, and administration. Controlling interfacial antibody adsorption is critical to limiting aggregation. A panel of monoclonal antibody therapeutics was characterized by multiple surface-sensitive techniques to predict their risk of interfacially mediated aggregation. The antibodies with the highest interfacial aggregation propensity exhibited more than a five-fold faster rate of surface pressure increase and rapidly formed hydrophobic films upon adsorption, which indicated these molecules unfolded rapidly at the interface. Strong, essentially irreversible protein-protein interactions, likely dominated by hydrophobic interactions, resulted in high levels of aggregation for these antibodies. In contrast, the antibodies with a low propensity to aggregate at the interface required many minutes of interfacial exposure to form hydrophobic films and did not develop strong, irreversible interactions. Overall, characterization of the interfacial properties of the antibodies allowed us to predict their extent of interfacial aggregation.

The risk of interfacial aggregation measured using surface-sensitive techniques was then related to the level of surfactant required in a formulation to stabilize against aggregation. Specifically, a small-scale IV bag agitation model was used to assess aggregation after simulated handling and transportation in the clinic. The level of surfactant required to stabilize against the agitation stress correlated directly with the risk of interfacial aggregation of the antibody, suggesting that surface-sensitive techniques can be used to enable rapid surfactant screening during formulation development.

UNDERSTANDING THE STABILIZATION OF PROTEINS IN HETEROGENEOUS SURFACE ENVIRONMENTS

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Key Our group is broadly focused on understanding and controlling the intersection of biology and materials at the molecular level. This intersection is critical in many areas of biotechnology where proteins and enzymes are integrated into or in constant contact with materials, including biocatalysis, tissue engineering, drug delivery, biosening, and therapeutic protein formulation. In line with this interest, we have developed a novel approach to elucidate the structure and transient behavior of protein molecules at the solution-solid interface based on dynamic single-molecule tracking. This approach, which is uniquely sensitive to structural and interfacial dynamics, includes the use of high-throughput tracking of protein molecules by means of internal reflection fluorescence (TIRF) microscopy in combination with intramolecular as well as intermolecular Forster resonance energy transfer (FRET). Notably, in this approach, as many as 106 protein molecules are tracked as they adsorb, desorb, diffuse, and simultaneously undergo conformational changes and/or intermolecular associations, permitting the statistical identification of dynamic, spatial, and population heterogeneity. The subsequent correlation of these dynamic behaviors on a molecule-by-molecule basis via large-scale multi-variate analyses, moreover, provides new insights into the connection between interfacial dynamics and protein structure. In this talk, I will discuss our recent results applying this approach to elucidate the molecular mechanisms involved in the stabilization of proteins through intentionally creating chemical heterogeneity in polymer brush and lipid bilayer surfaces. Additionally, I discuss how this understanding can be extended to design biomaterials that have improved biocompatibility.

INSIGHTS INTO FREEZE-THAW PROCESSES FOR THERAPEUTIC PROTEIN FORMULATIONS

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Key words: freeze, thaw, cryo-concentration, downscale model

For transport logistical reasons and for the decoupling of DS and DP shelf-life, most Biologics Bulk Drug Substances (BDS) are frozen and thawed before further processing. There are contradictory reports and publications on the influence of freezing processes - sometimes ultra-fast freezing is worse in terms of increased aggregate formation, sometimes slow freezing is inferior. Effects may only be visible upon subsequent long time storage (1, 2). The influence of the thawing process is usually neglected in the considerations and investigations, although here also protein can be damaged.

Case studies reported in the presentation on various therapeutic proteins show that slow and apparently mild thawing at low temperatures can lead to gelation or precipitation due to protein-protein interactions. Following CFD simulations, we have changed the design of the existing blast freezers and also added fast blast thawing (fig.1). The presentation will introduce experiences with this new blast-freezer-thawer as well as with representative freeze-thaw downscale models applied to formulations of therapeutic proteins. It could be nicely demonstrated that cryo-concentration of a monoclonal antibody and of the buffer agent had the same pattern in the downscale model and in the 2 L at scale bottle (fig. 2). Techniques such as micro CT for studying ice structures and the distribution of formulation components in ice are also presented.

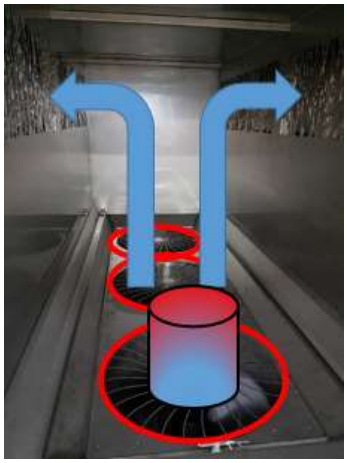

Figure 1: Novel Blast Freezer Thawer

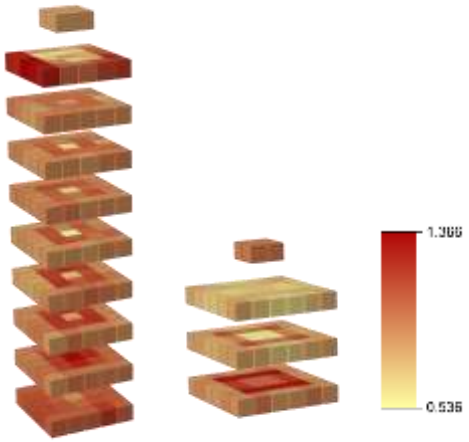

Figure 2: Cryo-concentration factor of mAb in a 2 L bottle (left) and in the downscale model (right)

References:
(1) B. Connolly et al., J. Pharm. Sci. 104:4170 – 4184, 2015
(2) Satish K. Singh et al, Pharm. Res. 28:873 – 885, 2011
(3) Oliver Blümel et al., Comparison of cryoconcentration in PET bottles and an innovative scale-down device, Poster at Conference “Freeze Drying of Pharmaceuticals & Biologicals”, September 19 – 21, 2018, in Garmisch, Germany

A PERSPECTIVE ON COLD PROCESSING OF PROTEINS

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Key Words: Cold denaturation, protein aggregation, freezing stresses, scale-down

Temperature is one of the most critical variables influencing the stability of biological systems and therefore a major concern for the production and commercialization of biopharmaceutical products. The general observation and practical rule is that refrigeration (including freezing) extends significantly the preservation of biological products.

Decreasing temperature is generally expected to decrease degradation kinetics, however, the complexity of proteins and the multiple phenomena that are triggered by cooling, often brings unanticipated results. For example, when a solution is freezing, the growth of the ice crystals causes all the solutes concentrate, typically by one or two orders of magnitude, potentiating interactions such as crystallization or protein aggregation. Buffer salts may precipitate, causing pH shifts, osmotic pressure and ionic force increase, protein molecules unfold (by cold), ice interfacial area extends, oxygen and other gases saturate forming air interfaces (bubbles) and dehydration is generalized. The mechanistic contribution of each of these variables cannot be clearly deconvoluted and it is also not easily correlated with product quality because of essentially three factors: (1) the stochastic nature of phase nucleation (either ice or dissolved substances), (2) the spatial anisotropy that is generated by freezing (on local composition, interfacial area, etc.), (3) and the amplification of the previous by limited (or deficient) process control (e.g. heat transfer). In general this setting contributes to inconsistent thermal history from container to container (which can be bags, vials, bottles or large steel vessels). This is somewhat tolerable when molecules are robust, but can trigger frustrating hurdles for sensitive proteins. In this work we approach the “cold” challenge by identifying cornerstones related to the preservation of protein molecules and by selecting some examples that can provide a mechanistic interpretation for the contribution of important process variables.

IMPLICATIONS OF POROUS-FLOW IN THE DESIGN OF FREEZE-THAW SYSTEMS FOR BIOPHARMACEUTICALS AND RATIONAL SCALE-DOWN METHODOLOGY.

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Key Words: freezing, thawing, scale-down, unidirectional, biopharmaceutical solutions.

Porous-flow through an ice matrix is a ubiquitous phenomenon during both freezing and thawing of biopharmaceutical formulations. During the freezing process, when the ice dendrites are formed, the proteins and excipients are excluded from the ice front. A concentrated aqueous solution is entrapped by the ice dendrites, creating what is called a mushy layer. By the action of gravity or pressure forces, the concentrated solution may flow through the ice matrix, disrupting the homogeneity of the mixture at the macro-scale. The concentrated solution is transported away from the mushy layer, creating spots of more concentrated solution in some regions of the container. During the thawing process, the same phenomenon occurs and is responsible for the strong stratification of the biomixture observed in the final liquid solution. Macro-concentration in the ice matrix and final stratification of the liquid solution are adverse phenomena that may trigger protein aggregation. Therefore, well-designed scale-down methods should mimic these adverse conditions that happen at large scales.

In this presentation, the impact of porous-flow on the macro-cryoconcentration and solution stratification after thawing will be analyzed inside bags, for vertical and horizontal heat transfer directions, using both freeze-thawing experiments and CFD simulations. In particular, the impact of the temperature and solution composition on porous-flow magnitude during thawing is examined based on the observed stratification during bottom-up unidirectional thawing (Fig. 1). Based on these experimental and theoretical insights, new rational designs for freeze-thawing scale-down systems are proposed for bags. This methodology is illustrated for the particular case of bottom-up freeze and thawing of bags, using a scale-down system that works with a sample of 27 mL to mimic a system of 10 L.

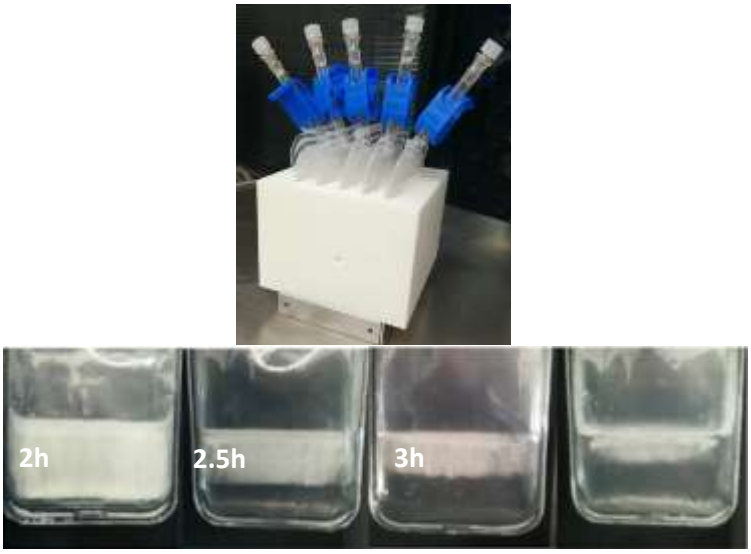

Figure 7 – Top) Bottom-up unidirectional scale-down device for bags. Bottom)Time-lapse of 27 mL bags initially at -30ºC thawed unidirectionally at 26 ºC (100mg/mL Sucrose solution, 5 mg/mL BSA, 3gpL NaCl.),

STABILITY OF PROTEIN FORMULATIONS AT SUB-ZERO TEMPERATURES BY ISOCHORIC COOLING

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Key words: isochoric cooling, protein formulations, osmolytes

Optimization of protein formulations at sub-zero temperatures is required for many applications such as storage, transport and lyophilization, particularly for high-value proteins used for clinical and diagnostic applications. Using isochoric cooling (constant volume) is possible to reach sub-zero temperatures without freezing aqueous solutions. This accelerates protein damage at sub-zero temperatures as protein unfolds by cold denaturation and diffusion in the liquid phase still exists, therefore speeding up optimization of protein formulations (Rosa et al., 2013). The proof of concept for the use of isochoric cooling to faster protein formulations was first demonstrated for the biomedical relevant protein disulfide isomerase (PDIA1). Three typical osmolytes, sucrose, glycerol and L-arginine, increased very significantly the long-term stability of PDIA1 at -20°C with all of them tested under isochoric cooling within the short time frame of around 700 h. The extremely stable redox green fluorescent protein 2 (roGFP2) was selected to evaluate the applicability of isochoric cooling on the optimization of highly stable proteins. This derivative of GFP is 2.6 fold more stable than the already very stable GFP β-barrel. Nevertheless, it was possible to denature to some extent roGFP2 at -20°C under isochoric cooling and to assign a stabilizing effect to sucrose. The isochoric method was also applied to insulin, a widely used therapeutic protein. Insulin damage was evaluated through the magnitude of a signalling event elicited by insulin on human hepatocyte carcinoma cells. Insulin at -20°C under isochoric cooling lost 22% of its function after 15 days and 0.6M sucrose prevented this deactivation.

Rosa, M., C. Lopes, E. P. Melo, S. K. Singh, V. Geraldes, M. A. Rodrigues (2013) Measuring and modelling hemoglobin aggregation below freezing temperature. J. Phys. Chem. B 117, 8939-8946.

EVALUATION OF ALTERNATIVE SURFACTANTS AS STABILIZERS FOR THERAPEUTICAL PROTEIN FORMULATIONS

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Key Words: surfactants, monoclonal antibody, formulation development, interface phenomena

Surfactants are potent stabilizers of proteins, preventing protein unfolding and aggregation presumably by competitive adsorption to interfaces. Due to limited data on e.g. performance and safety, only three surfactants are routinely found within marketed formulations of parenteral protein products: polysorbate 20 (PS20), polysorbate 80 (PS80), or poloxamer 188 (Px188).

These molecules are well-established and safe for parenteral administration but possess liabilities such as degradation of the surfactant itself and/or chemical inhomogeneity (PS) or show decreased stabilizing effects at silicone oil-water interfaces (Px188). Thus, there is a need to evaluate alternative surfactants to expand the toolbox for product development and to ensure optimal drug product stability and quality.
In our studies, potential alternative surfactants were evaluated and compared to PS20 and Px188 (e.g. during real-time and stress stability studies using a model mAb). Data from these efforts will be presented.

STRUCTURAL INTEGRITY OF PROTEIN NANOCAGE AT LIQUID-LIQUID INTERFACE

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Key Words: Protein nanocage, supramolecular assembly, oil-water interface, structural integrity, molecular dynamics simulations

Globular proteins adsorb at the interface of two immiscible liquids by maintaining thermodynamically favorable state which often results in a denatured structure and compromised functionalities. However, the behavior of highly structural proteins at the interface of two immiscible liquids is still unexplored. In this study, we focused on the structural behavior of supramolecular protein at the interface. Our previous studies show that highly structural protein adsorbs at the interface and act as a Pickering emulsifier. Theoretical analyses by Molecular Dynamic Simulation proved that the supramolecular protein E2, a highly structured protein nanocage, has retained structural integrity at the liquid-liquid interface. Further, experimental analyses by Small angle X-ray scattering (SAXS) and quartz crystal microbalance and dissipation (QCM-D) confirm the adsorption of E2 on the liquid-liquid interface with zero penetration depth. Moreover, molecular structural analyses using Circular Dichroism (CD) and tryptophan fluorescence for secondary and tertiary structures respectively, also suggest the structural integrity of the cage structure of E2 at the oil-water interface. This study brings new insights into the behavior of highly symmetrical supramolecular protein assembly at the liquid-liquid interface.