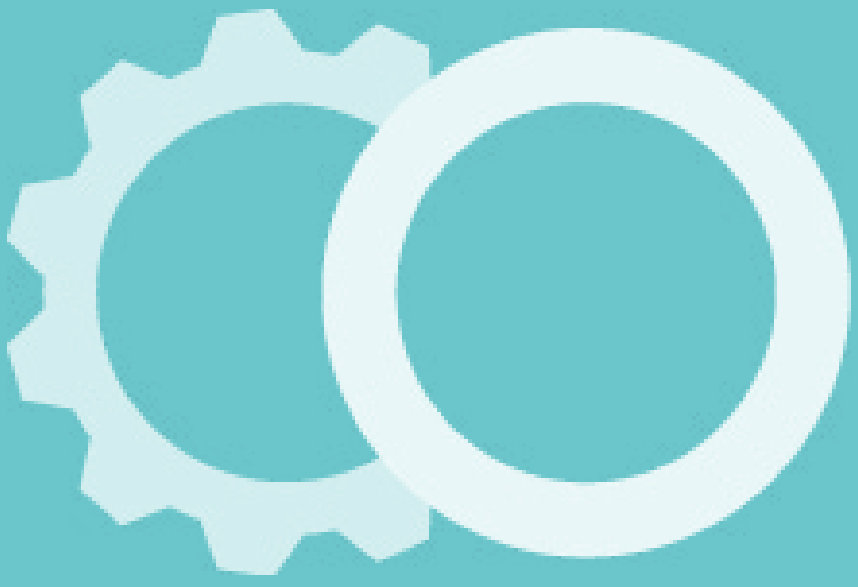
ALTHEA

THE POWER TO MAKE®

CRYSTALOMICS: A PATHWAY FORWARD FOR PROTEIN CRYSTALLIZATION

Protein-based therapeutics, specifically monoclonal antibodies (mAbs), have become the focus of pharmaceutical R&D over the last decade. While these biologics offer many possibilities when it comes to treating a variety of diseases, they also present considerable challenges during development. This is because, traditionally, protein-based therapies are delivered at high concentrations due to their size, biochemical complexity, and low bioavailability. These higher concentrations can increase solution viscosities, resulting in the possible formation of aggregates, which are associated with altered biological activity and increased immunogenicity. In addition, most protein-based biologics are administered via parenteral routes, making it necessary for patients to make long and expensive trips to a clinic to receive treatment. All of these factors combined make manufacturing and delivery of protein-based therapeutics very difficult. If these drugs could be produced at lower viscosities, then a lower volume could be used, making subcutaneous delivery possible; this would also significantly reduce the risk of aggregates. Now, a treatment that typically requires an hour in a clinic could potentially become a simple injection at home.



One way to lower the suspension viscosity of a drug is through the use of highly-concentrated crystalline suspensions, or protein crystals. Because protein crystals are packed in a repeating formation and held together by noncovalent interactions, the volume fraction, typically 5 to 30 microns, is considerably lower when compared to equivalent protein molecules in a solution. The stable form of crystallized proteins also protects against physical or conformational degradation to which proteins are often susceptible. The result is increased syringeability and injectability without adversely affecting a protein's biological activity.

The idea of crystallizing proteins for use in biologics is not new. In fact, insulin was the first crystalline protein to be approved for therapeutic use over 30 years ago[1]. Yet, it remains the only one. This is due to the specific expertise required to crystallize proteins for therapeutic use as well as the lack of clinical data available for crystalline formulations of larger, more complex molecules. By investing in the resources necessary to overcome these challenges, the industry would be able to offer alternate routes of drug delivery that are faster and more convenient for patients to administer.

THE CHALLENGES OF CRYSTALLIZATION OF PROTEINS FOR THERAPEUTIC USE

One of the biggest challenges of protein crystallization is finding a robust crystallization condition that produces crystals in a short period of time (sufficiently short for cost-effective GMP manufacturing). Each molecule used in drug development is unique, which means that crystallization conditions that work for one molecule may not work for others. For this reason, automation and imaging systems are used to screen various salts, excipients, pH levels, additives, etc., in order to find conditions that create crystals of the size that is needed. Initial experiments are typically done at around 2 to 5 microliters using a starting material of the highest purity. Supporting analytics also have to be available to show that the crystallization process does not adversely affect the molecule, in order to ensure the same efficacy pre- and post-crystallization. Once the appropriate conditions are identified, the process has to be scaled up to produce the required amount of crystals. Crystallization conditions tend to evolve during this part of the process; specifically the surface area to volume ratio changes as the size of the manufacturing vessels increases. Controlling the rate of reaction is imperative so that the size and amount of crystals remain as consistent as possible. This assures that the suspensions are relatively uniform and contain a narrow size distribution of the crystals.

Another major challenge of protein crystallization is, after finalizing an appropriate working volume, the crystals have to be re-formulated into generally recognized as safe (GRAS) excipients that are suitable for injection. Excipients used in the initial screening phase are typically not GRAS, even after a robust process to make small (10 milliliters) batches of uniform crystals has been developed. Excipients must also have the desired pH, osmolality range, and break loose energy, or BLE (the force needed to expel the material from a syringe). Downstream purification can also be a challenge, due to the various populations of crystal sizes. While centrifugation is an option, this is not preferred due to the potential contamination of particulates from the bottles as well as from the manual handling and potential errors of operators. Tangential flow filtration (TFF) is instead recommended, as it reduces these risks, particularly when it comes to particulates. Manual visual inspection is particularly challenging due to the "milk-like" appearance of crystalline suspensions. Specialized training is needed to detect particulates and the success of manual detection is dependent on

the experience, training, and skill of the operators.

PRESERVING EFFICACY DURING CRYSTALLIZATION

Development and manufacturing of protein crystals require specific expertise and experience, some of which are transferrable from the development of soluble protein formulations. In addition, specific equipment, such as robotics and process analytical technology (PAT) probes, is needed to monitor the entire process in real-time. Access to a platform and partner with experience is imperative, which is what makes Althea and its Crystalomics® Formulation Technology such a valuable resource. Concerns about crystallization affecting the biochemical characteristics and bioactivity of the soluble protein are addressed with extensive biophysical characterization and clinical data showing that there is no difference between the soluble API and its crystalline counterpart.

To demonstrate the potential of the Crystalomics® Formulation Technology, Althea shared the results after crystallization of the immunosuppressant Infliximab (Remicade®). Commercially available Infliximab is typically administered intravenously (IV) at a concentration of 10 milligrams per milliliter (mg/mL). In contrast, the crystalline suspension has a concentration of 250 mg/mL while reducing the volume approximately 96 percent, thereby showing potential to convert from IV administration to a subcutaneous route as shown in Table 1.

After 5 milligrams of Infliximab were desalted via a column, the crystallization solution was added dropwise to the desalted antibody and stirred at room temperature. Star-shaped crystals began appearing overnight, and crystallization was continued for 24 hours. Upon completion, the yield (percent conversion) of crystals was approximately 90 percent. The following graphs show the analytical, biological and biophysical characterization of crystallized Infliximab.

In Figure 1, a comparison of the viscosities between a soluble and crystalline Infliximab is shown. In contrast to the soluble form, viscosities of crystalline suspension remained relatively low even at concentrations above 150 mg/mL.

	Concentration (mg/mL)	Administration Volume (mL)
Infliximab		
Commercial	10	35
Crystalline	250	1.5
Change	+25x	-96%

TABLE 1. The results of crystallization increased protein concentration 240 mg/ml over the soluble dosage form while also reducing the volume approximately 96%.

COMPARISON OF VISCOSITIES — SOLUBLE VS. CRYSTALLINE

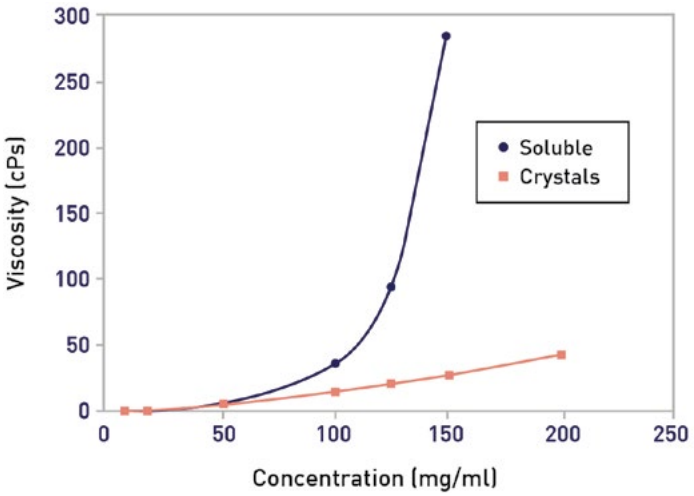


FIGURE 1. Viscosity of Infliximab. The viscosity of soluble and crystalline suspensions of Infliximab was measured by using a Cannon-Fenske viscometer according to the manufacturer's instructions.

Figure 2 shows the *in vitro* activity of soluble and crystalline Infliximab in a TNF-α neutralization assay. The efficacy of Infliximab is not affected by the crystallization process and final formulation.

In Figure 3, crystalline Infliximab administered subcutaneously displayed an improved pharmacokinetic profile over the soluble form.

The chart below (Figure 4) illustrates *in vivo* efficacy of Infliximab in a TNF-α mouse model. Crystalline Infliximab administered monthly has the same effect as the soluble form administered weekly for all histopathology clinical scores.

MALDI-TOF analysis and peptide mapping shown in Figure 5 on the next page shows that the physical structure of Infliximab is unchanged by the crystallization process.

Finally, in a structural analysis of Infliximab shown in Figure 6, circular dichroism (CD) and fourier transform infrared spectroscopy (FTIR) spectroscopy confirms that the secondary structure of Infliximab is unchanged after crystallization.

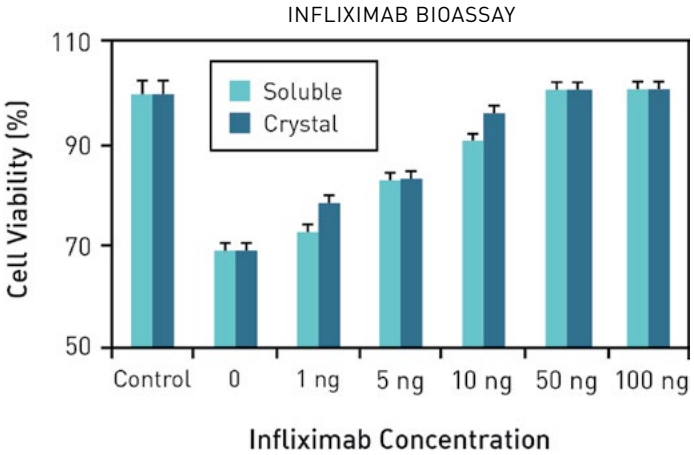


FIGURE 2. *In vitro* bioactivity of Infliximab. Cultured L-929 mouse fibroblast cells were detached, diluted to 2x105 cells per mL and added to 96-well plates (100 µL per well). TNF-α neutralization assays were performed by incubating mouse fibroblast cells overnight in the presence of 100 picogram/milliliters (pg/mL) TNF-α and various concentrations of Infliximab or dissolved crystals of Infliximab. The number of viable cells was determined by using a commercially available cell proliferation assay kit.

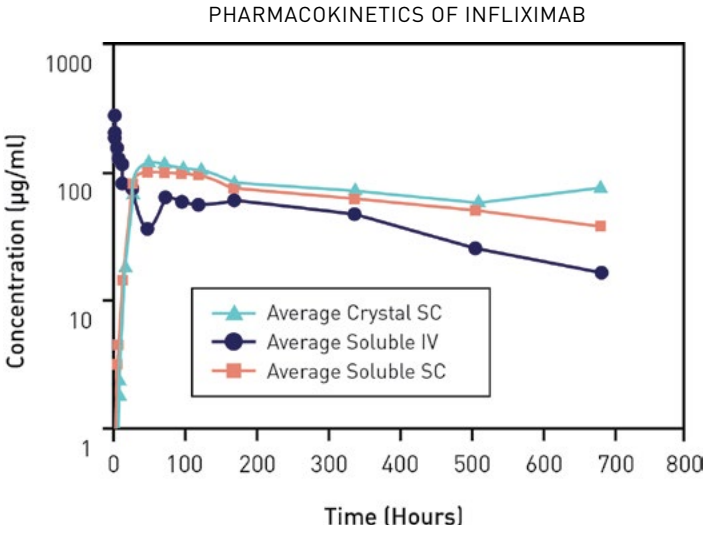


FIGURE 3. PK studies of Infliximab. Approximately 100 µL of Infliximab (20 mg/mL) was administered to BBDR/Wor rats having a body weight of 250g to provide a dose of 8 mg/kg. The mAb was administered in a soluble form either intravenously (IV) or subcutaneously (SC) or as a crystalline suspension SC.

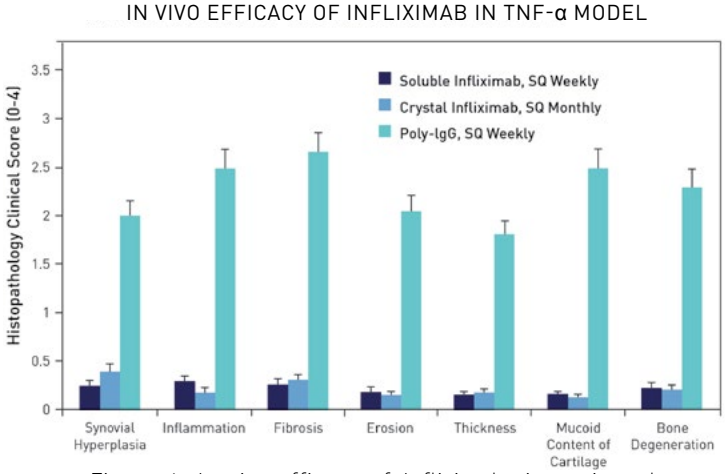


Figure 4. *In vivo* efficacy of Infliximab. Approximately 100 µL of Infliximab (20 mg/mL) was subcutaneously administered to TNF-α mice at a dose of 8 mg/kg in both soluble (weekly) and crystallized (monthly) forms. Non-specific IgG was used a control.

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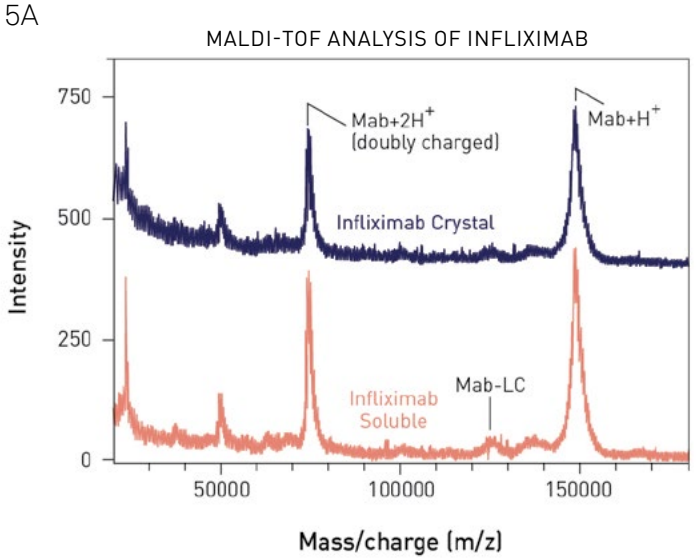

FIGURE 5A: MALDI-TOF analysis of Infliximab. ABI Voyager DE Pro and UV laser. High laser power was used to acquire spectra in one 100-scan experiment and caused the fragmentation into light chain (LC, 25 kDa) and heavy chain (HC, 50 kDa).

6A

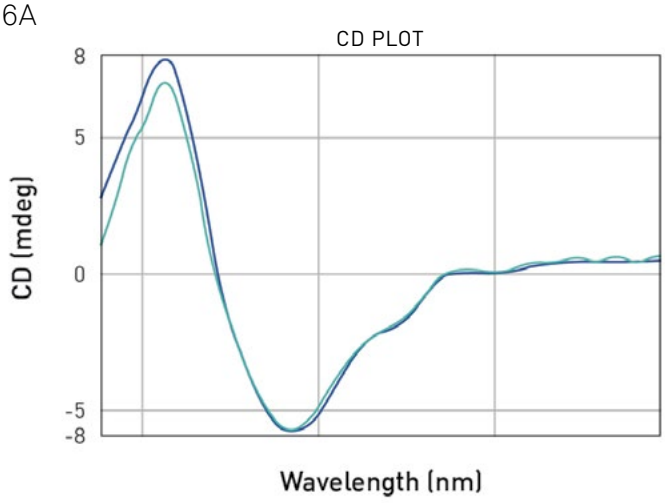

Figure 6A: CD Plot. Soluble Infliximab (Blue) and reconstituted Infliximab crystals (Green) spectra were collected using JASCO J-810 CD spectropolarimeter.

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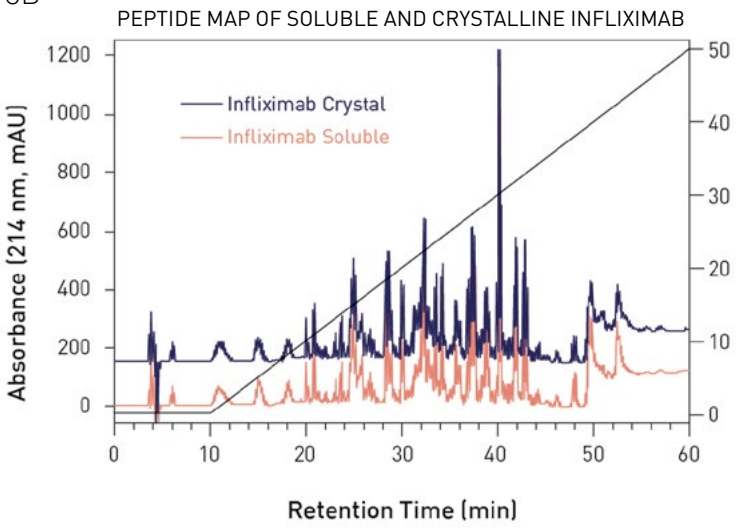

FIGURE 5B: Peptide map of soluble and crystalline Infliximab. Upper and lower chromatograms show crystalline and soluble Infliximab, respectively.

6B

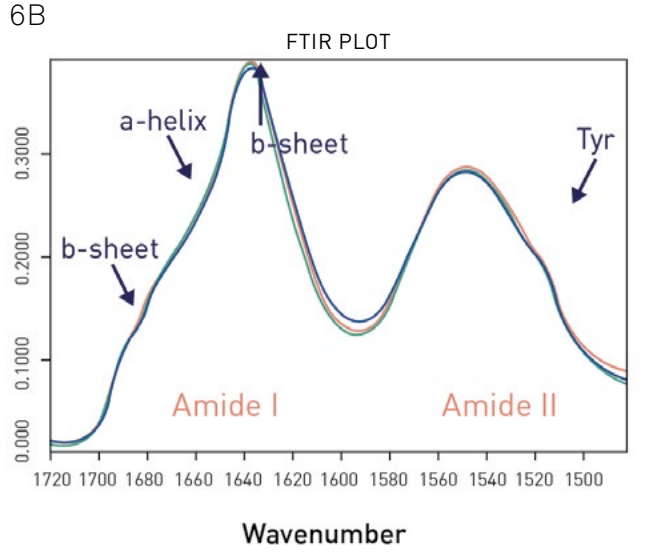

FIGURE 6B: FTIR Plot. Soluble Infliximab (Red) and reconstituted crystals (Blue; crystals were dissolved before the spectra were taken) and crystal suspension (Green) of Infliximab. The spectra were collected by using a Bruker FTIR instrument (Helios microanalysis) equipped with an ATR accessory.

A similar increase in protein concentration and reductions in dosage volume for both Rituximab and Trastuzumab were also achieved using Althea's Crystalomics® Formulation Technology.

SUMMARY

Protein crystals have shown the potential to address many of the issues associated with high concentration/ high viscosity therapeutic protein solutions. Crystallization, formulation, and manufacturing of these crystalline solutions require specific knowledge, equipment and experience, which is easily accessible through a partner like Althea. The result is a therapeutic product that increases patient compliance by enabling an easier and faster form of drug delivery.

References

1. Protein therapeutics: a summary and pharmacological classification — https://www.acsu.buffalo.edu/~abagati/dropbox/Newfolder/ProteinTherapeutics-Summaryand PharmacologicalClassification.pdf

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