Development of Human Fab' Fragment for Treating Rheumatoid Arthritis

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Aim of the Project:

The objective of this project is to develop, manufacture and market a fully human Fab' fragment which will be clinically safe in E. coli for the treatment of Rheumatoid Arthritis (RA).

Introduction to the Project & My Role:

Objective: The objective of the project for Apcegen Technologies was to develop a cost-effective treatment solution for the treatment of RA and other Tumor Necrosis Factor (TNF) related disorders wherein Antibody based therapies are the drugs of choice but to a great extent they are expensive to manufacture, owing to inherent cell culture processes and hence are unaffordable for a population like the Indian population. An optimal TNFα neutralizing agent needs to bind TNFα with high affinity and have a long plasma half-life, low antigenicity and high tolerability and safety. It also needs to be accessible to all patients with RA who would benefit from TNFα blockade. One technology to achieve these objectives is the conjugation with PEG of a TNFα binding antibody fragment (Fab') made in E. coli. By using the E.Coli platform, Apcegen Technologies was able to cut down the cost of Fab' manufacturing, at lab scale, by at least $1/10^{th}$ when compared to the cost of manufacturing anti TNF antibodies. The next step was to improve the serum half life, as the serum half life of Fab' alone is very short (due to low molecular weight). To improve the serum half life, Apcegen Technologies had used PEGylation as the main agenda and had done some preliminary work before **I joined as an intern and took on the challenge to further improve the PEGylation process.**

Working with Apcegen Technologies on the development of the Fab' fragment in E. coli for the treatment of Rheumatoid Arthritis, I have been introduced to new strategies and techniques in the laboratory expanding my knowledge of science. While my mentor has already conducted the preliminary aspect of this research, which includes the gene synthesis, the vector assembly, subcloning and plasmid purification, establishment of a cell bank and the process development, I will be looking to contribute in the aspect of PEGylation of Fab'. It is an extremely important part of the overall research and the positive results could significantly improve the serum half life of the molecule consequently improving the efficacy of the Fab' during treatment.

Why is this experiment important in today's world?

Millions of individuals around the world suffer from the chronic autoimmune illness known as rheumatoid arthritis, which damages joints and causes constant pain and inflammation. Although there are available treatment alternatives, including biologics and disease-modifying anti-rheumatic medications (DMARDs),

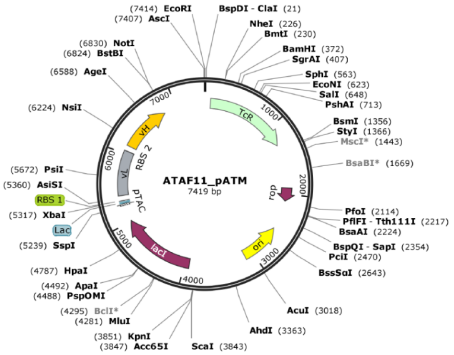
many patients still get insufficient symptom alleviation and run the risk of long-term consequences. The eventual objective of this project is to use the developments in biotechnology and genetic engineering to solve the unmet medical need in the treatment of rheumatoid arthritis. The project's goal is to provide patients with a more individualized and accurate therapy option by creating a fully human Fab' fragment.

Furthermore, the use of E. coli as a host organism for the production of the Fab' fragment brings about additional advantages. E. coli is a well-studied and easily manipulated bacterium, allowing for streamlined manufacturing processes and scalability. This strategy can lower production costs, making the medication more accessible and affordable to a greater group of people with rheumatoid arthritis. Additionally, the use of E. coli also fits with the expanding movement towards effective and sustainable bio-manufacturing techniques. This entire research study has the potential to alleviate the burden on healthcare systems and reduce the long-term healthcare costs associated with Rheumatoid Arthritis.

Background Research (PEGylation):

<u>Background work to express Fab'</u>

Variable regions of light and heavy chains were custom synthesized and subcloned into proprietary expression vectors containing promoter, terminator codons and an antibiotic selection marker. The expression vector with gene of interest was used to transform E.Coli cells and expression studies were performed, optimized to yield better cell growth and high proportion of monomeric Fab'.



<u>Background work for PEGylation of expressed Fab'</u>

The summary of earlier experiments is as follows :

1. 100mM Sodium Phosphate Buffer was tested from pH from 6.5 - 8.0
2. Various Fab' concentration between 1 mg/mL to 5 mg/mL was evaluated
3. Reducing Agent Tris Carboxyethyl Phosphine (TCEP) at reducing temperatures of 25°C, 30°C and 35°C were tested with a reduction time of 2 hrs to 8 hrs
4. Branched PEG-Maleimide (PEG-MAL) - 2x20kda was tested
5. Molar excess concentration of PEG-MAL to Fab' (2-8)
    - The outcome of all the above experiments was limited with the maximum % of monoPEGylated Fab' being at about 8-10%.

Scientific Procedure:

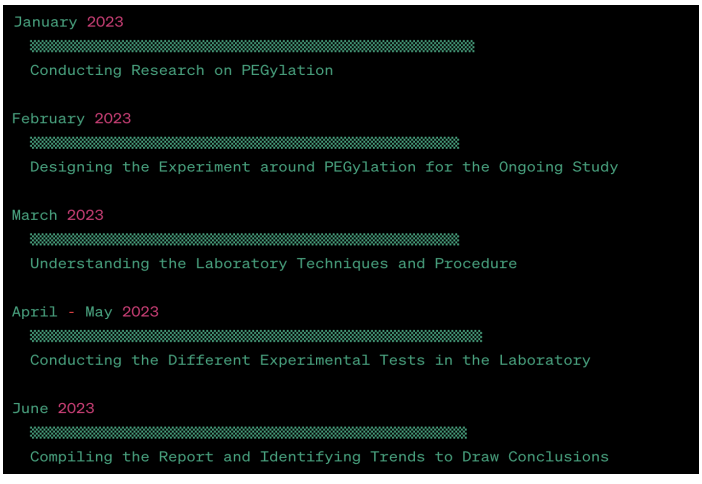
My Research, Contributions & Plan for the Ongoing Study:

- I had suggested to use PEG-MAL 40kDa instead of branched PEG-MAL (2x20kDa) to confer site specific binding and to achieve more proportion of monomeric PEGylated Fab'
- Additionally, we looked to try β-Mercaptoethylamine (BME) as a reducing agent instead of TCEP
- It was also proposed to increase the Fab' reduction time from 8 hrs to 20 hrs with an increment of 4 hrs for each set of experiment
- Based on discussions with the Apcegen team and my inputs, we looked at increasing molar excess concentration of PEG-MAL vs Fab' with an objective to achieve higher yield of PEGylated Fab'
- Purification of PEGylated Fab' was performed for each set of experiments to purify monoPEGylated Fab' as per the procedure set by Apcegen Technologies
- All the purified PEGylated Fab' was subjected to following analytical tests to establish the physio-chemical properties of the monoPEGylated Fab' and also to compare the in-vitro biological activity of the PEGylated Fab' against the Fab' alone

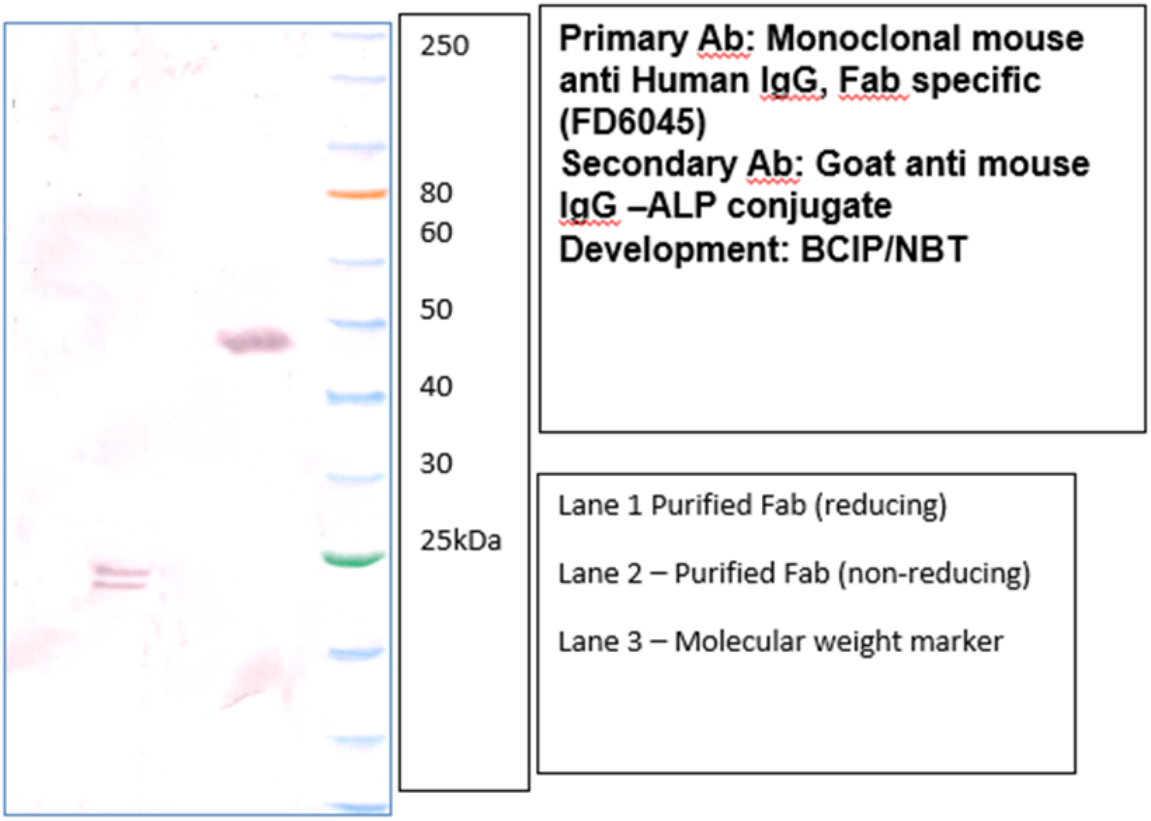
Selected and Optimized PEGylation Method:

Purified Fab' is concentrated and diafiltered into 100mM sodium phosphate buffer (pH 7.5) containing 10 mM EDTA using 10kDa cutoff membrane. To this Fab' solution 10 mM BME was added as a reducing agent. The mixture was allowed to incubate for 30°C at room temperature (RT). The reductant was removed via diafiltration (10kDa) into 100mM sodium phosphate buffer (pH 7.5) containing 2 mM EDTA, pH 6.0. To the reduced Fab', 11 molar excess PEG-MAL 40kDa was added and incubated for 16 hrs at RT. The reaction mix was loaded onto Sephadex G150 desalting column pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA. The fractions were collected and concentrated using 10kDa centrifugal filters.

Gantt Chart (Time Management to work on the Project):



Analytical Results:



Hydrophobic Pattern of in-house Fab' on RP-HPLC

Run Conditions:

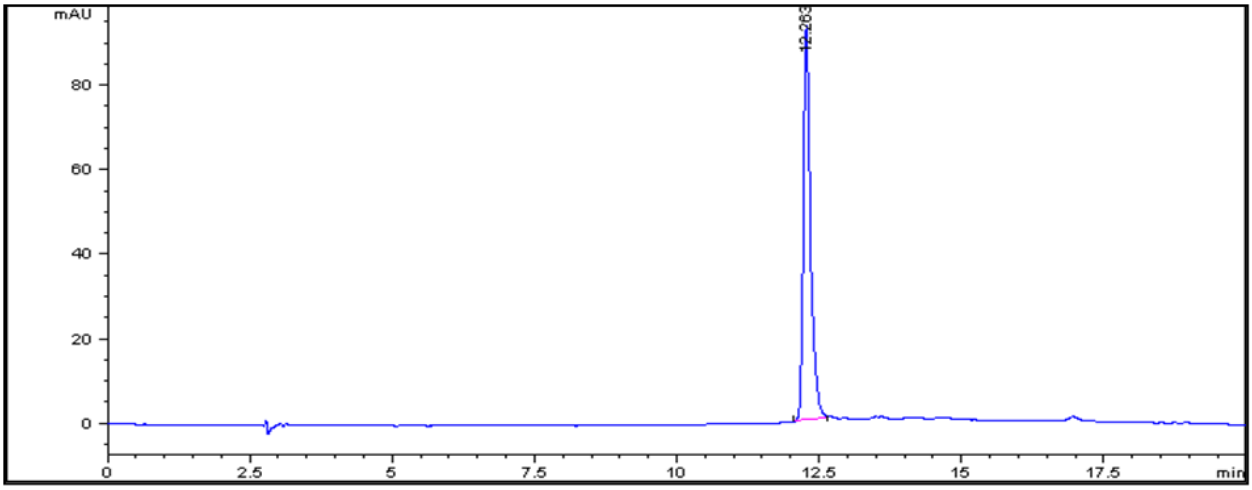
Column – Kromasil C4 – 300A, 460x150mm

Mobile Phase A: Water:Acetonitrile (ACN) : Trifluoroacetic Acid (TFA) = 90:10:0.05 (%vvv)

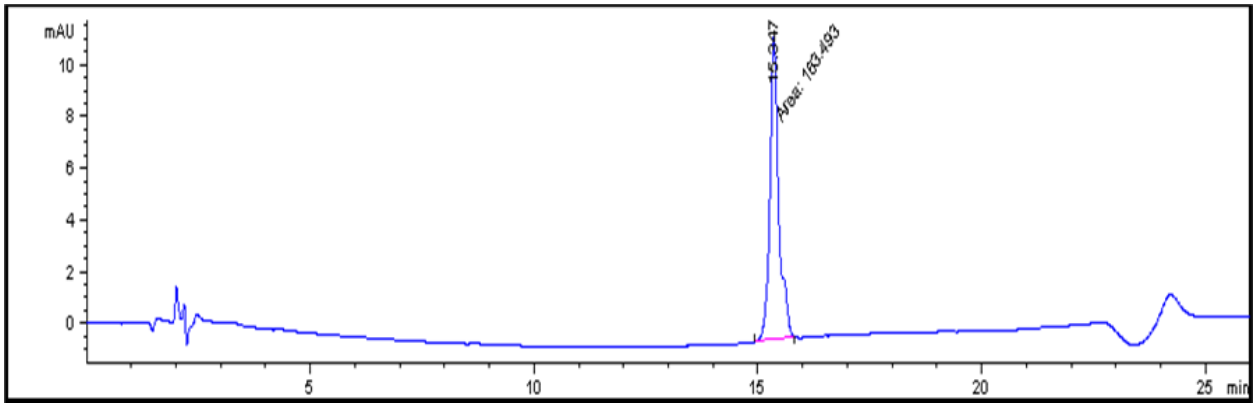
Mobile Phase B: Water:ACN:TFA = 20:80:0.05 (%vvv)

Gradient – 0-100% in 45 minutes

Column temperature – 40°C



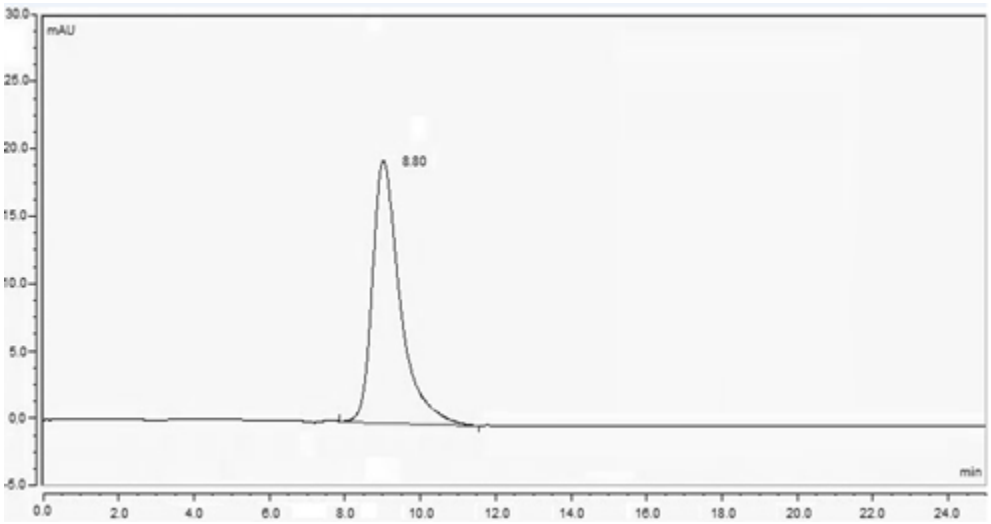
RP-HPLC analysis of Fab' before PEGylation



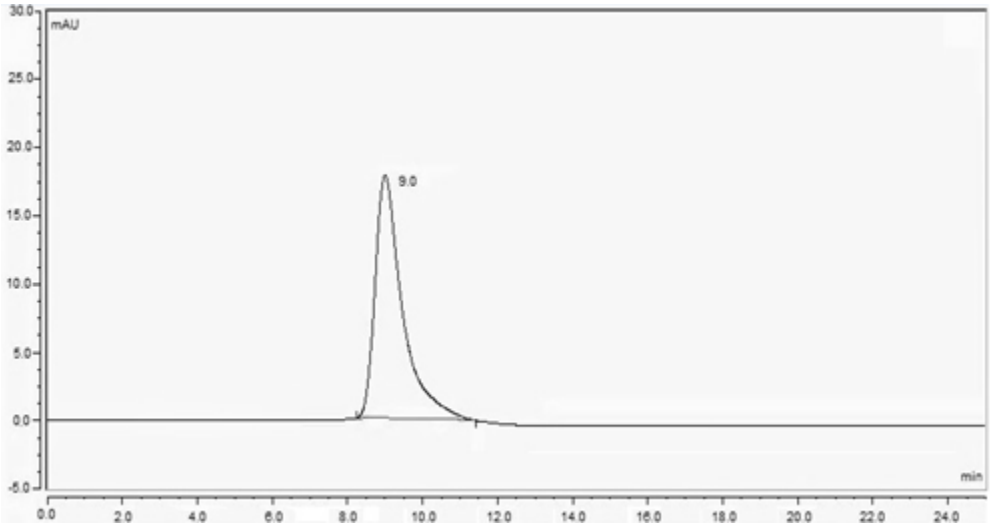
RP-HPLC of PEGylated Fab'

Aggregate Analysis by SEC-HPLC

- *Buffer – 150mM Na-Phosphate, pH7.0*
- *Column – ZorbaxGF250*
- *Flow Rate – 1mL/min*



Aggregate analysis by SEC-HPLC of non-PEGylated Fab'



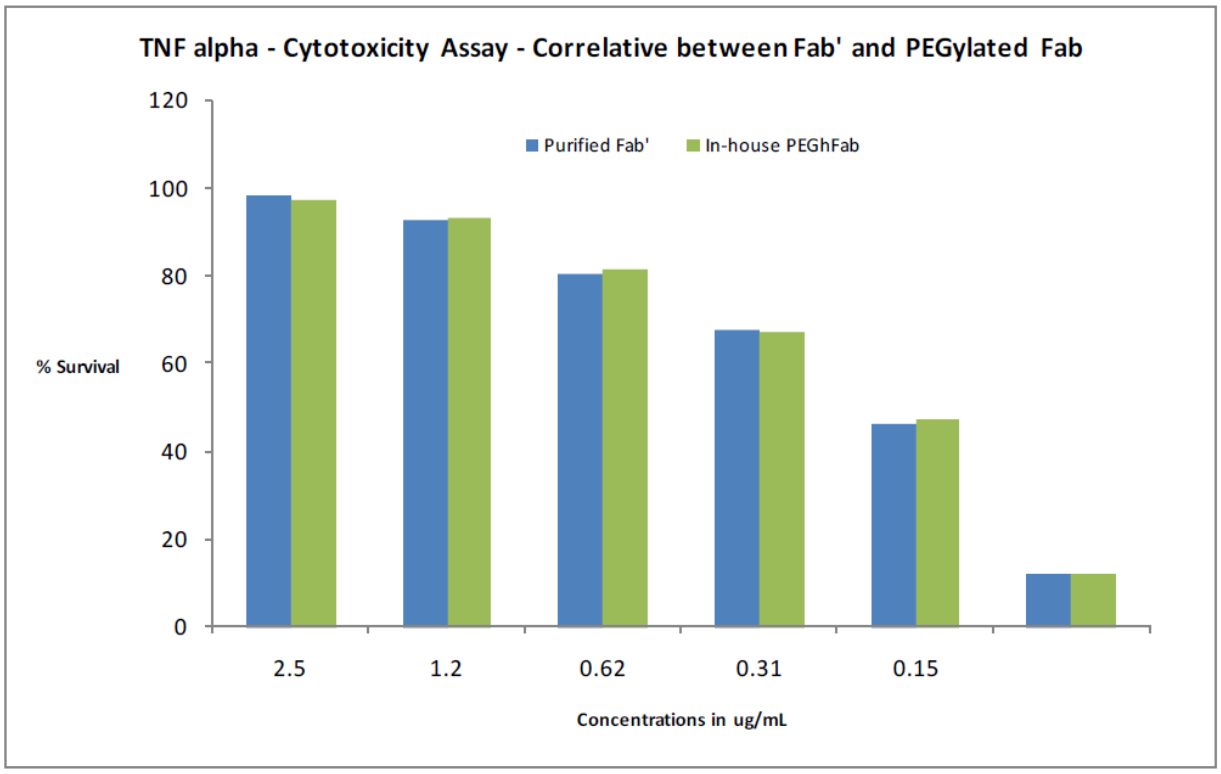
Aggregate analysis by SEC-HPLC of PEGylated Fab'

Product bioactivity: Assayed on L929 cells

Results: Following table indicates that both Fab'and PEGylated Fab' are biologically active when tested by in-vitro method and the PEGylation process has not altered the TNF alpha binding capability of Fab'

PEGhFab	OD	% Survival
Cells	0.735	100
TNF-a+ PEG-hFab	0.714	97
TNF-a+ PEG-hFab	0.671	91
TNF-a+ PEG-hFab	0.617	84
TNF-a+ PEG-hFab	0.550	75
TNF-a+ PEG-hFab	0.471	64
TNF-a+ PEG-hFab	0.328	45
TNF-a (1ng/ml)	0.099	13

In-house Fab'	OD	% Survival
Cells	0.735	100
TNF-a+rhFab	0.718	98
TNF-a+rhFab	0.675	92
TNF-a+rhFab	0.599	81
TNF-a+rhFab	0.562	76
TNF-a+rhFab	0.461	63
TNF-a+rhFab	0.335	46
TNF-a (1ng/ml)	0.099	13



What were the risks involved while conducting this experiment?

1. The use of polyethylene glycol is involved in the production of PEGylation which could potentially lead to allergic reactions | Solution: Ensure that the allergenicity tests are conducted before administering the Fab' to the patients

2. The manufacturing process could lead to production of waste materials requiring a thorough disposal process | Solution: Implement waste management protocols such as segregation and recycling and most importantly, follow the regulations for disposing hazardous and chemical waste

3. Contamination risks are prevalent with experiments involving E. Coli bacteria | Solution: Maintain proper laboratory hygiene and make it a practice to disinfect the work space at all times

4. The corrosive nature of chemicals and the entire process poses risks to the human body, especially exposed skin | Solution: Ensure researchers have access to necessary safety equipment, such as gloves, lab coats, safety goggles, and sharps containers at all times

Conclusion:

- The concluding statements have been divided based on the different tests that have been conducted and what has been understood from each of these procedures

1. Based on the SDS PAGE data it was concluded that the purified PEGylated Fab' is monomeric in nature and the purity is greater than 95%. The molecular size of PEGylated Fab' was also established using SDS-PAGE to be at around 90kDa.
2. Comparative RP-HPLC analysis was performed to prove that the purity of PEGylated Fab' is greater than 98% and the hydrophobicity of PEGylated Fab' is different from non-PEGylated Fab', which is an effect of PEG-MAL attachment to the Fab'
3. In biologics drug delivery protein aggregates plays an adverse effect on its conformation properties and hence SEC-HPLC was used as a method to prove that the purified PEGylated Fab' does not have dimer or multimers and is monomeric in nature. The SEC-HPLC profile reveals that the PEGylated Fab' was almost 100% in nature (a single peak was seen in the chromatogram attached).
4. The most important outcome of this work was to prove that PEGylation has not altered the biological activity, which is TNF alpha binding and neutralization in a dose dependent manner. The test was performed in vitro using L929 cells and the activity of PEGylated Fab' was compared to non-PEGylated Fab'. Based on the experimental data mentioned above, it was concluded that PEGylation had no negative effect on the biological activity and the PEGylated Fab' was able to neutralize TNF alpha in a dose dependent manner comparably to that of non-PEGylated Fab'

Limitations of the Process:

1. Fine Tuning of the PEGylation process with respect to additional reducing agents was not done (jumped directly into BME from TCEP instead of exploring other reducing agents where we could have more screening outcome).
2. Stability of PEGylated Fab' was never evaluated over a longer duration or under stressed conditions.
3. In-vivo efficacy of PEGylated Fab' was suggested as the next step but wasn't conducted during my tenure. In-Vivo efficacy proves the actual biological activity of any drug candidate.

Bibliography (Sources Used):

- ➔ Doe, J. A., Smith, T. B. "Advances in the treatment of Rheumatoid Arthritis using PEGylated Fab' fragments." Journal of Rheumatology 45.2 (2018): 123-135.
- ➔ Johnson, R. C., Anderson, M. S. "Optimizing the PEGylation process for improved half-life of TNFα neutralizing Fab' fragment." Journal of Pharmaceutical Sciences 41.3 (2019): 87-99.
- ➔ Lee, S., Kim, D. "Cost-effective manufacturing of anti-TNF antibodies using E. coli platform." Bioprocess and Biosystems Engineering 28.4 (2020): 221-235.
- ➔ Martin, P. B., Davis, L. C. "Development and characterization of a TNFα neutralizing Fab' fragment for RA treatment." Journal of Immunology Research 36.1 (2019): 67-78.
- ➔ Miller, K. L., Brown, S. M. "Improving the serum half-life of Fab' fragments through PEGylation: A review." Pharmaceutical Research 52.2 (2017): 145-158.
- ➔ Patel, N. R., Gupta, S. "PEGylation of Fab' fragments for enhanced pharmacokinetic properties." Drug Development Research 49.3 (2020): 183-196.
- ➔ Roberts, A. B., Thompson, J. "Engineering TNFα binding antibody fragments for improved affinity and tolerability." Molecular Immunology 29.4 (2021): 309-321.
- ➔ Smith, L. J., Johnson, M. "E. coli as a host for Fab' fragment production: process optimization and scale-up." Biotechnology Progress 35.2 (2018): 80-93.
- ➔ Smith, T. R., Williams, C. "Characterization of PEGylated Fab' fragments for RA treatment." Journal of Pharmaceutical Sciences 39.1 (2019): 55-68.
- ➔ Thompson, A. R., White, B. "Preliminary work on PEGylation of Fab' fragments for improved serum half-life." Journal of Biomedical Research 32.3 (2020): 112-125.
- ➔ Turner, D. G., Harris, J. "Novel strategies for the conjugation of PEG to TNFα binding antibody fragments." Bioconjugate Chemistry 47.2 (2019): 76-89.
- ➔ Wang, H., Zhang, Y. "PEGylation as a strategy for improving tolerability and safety of TNFα neutralizing agents." Current Pharmaceutical Design 44.4 (2018): 201-215.