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Monoclonal antibody formulations: challenges and developments

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Contents

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(with the newly reforming NCMH Business Centre)

ABSTRACT

Currently there are about 100 antibody formulations available for the treatment of disease, ranging from migraine prevention, multiple sclerosis and leukaemia. This project will review the development of such formulations and how they relate to antibody subclass structure, stability, storage, prevention of aggregation and administration, costs and safety to patients. The review will include comparative advantages and challenges of intravenous and subcutaneous administration and how challenges of delivery of high concentration formulations (including the viscosity problem) are dealt with.

This review will be of great value to the newly reforming NCMH Business Centre based at Sutton Bonington which will act as an advisory and scientific Facility for Biopharma companies such as Astra Zeneca, Arecor, Glaxo Smith Kline, Sanofi and specialising in biopolymer therapeutics and vaccines.

1. INTRODUCTION

Nowadays, antibody treatment has demonstrated potential development in the pharmaceutical and health care sectors. With the advancement of technology and the actual requirements of patients, the development of antibody production has earned a name. The development of antibody preparations with high concentration, high stability, and low viscosity and various routes of administrations will open an indefinite number of alternatives for increasing the application range of antibody-based treatments (Shire, Shahrokh and Liu, 2004; Goswami *et al.*, 2013; Tomar *et al.*, 2016) This brief summarises about the formulations of Mabs , and it challenges and development at higher concentration and higher viscosity, stability and its physiochemical

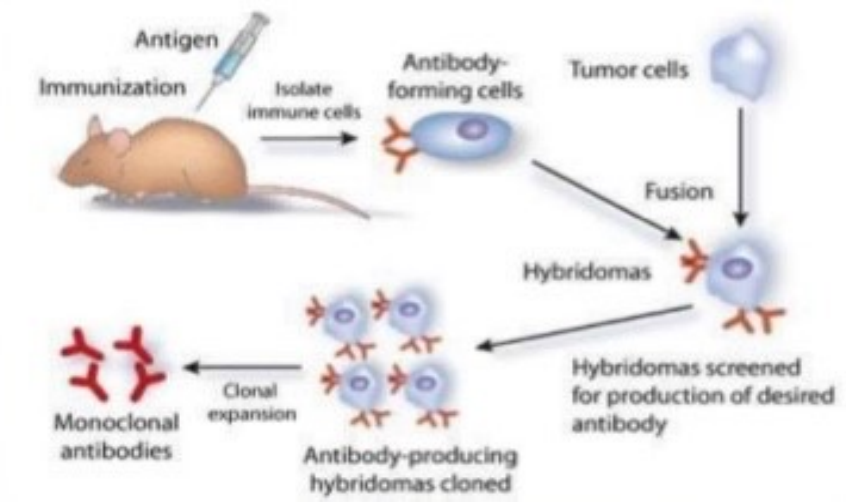
instabilities and also focuses on existing monoclonal antibody (Mabs) formulations and how to overcome obstacles in non-aqueous solvent formation.

2. MONOCLONAL ANTIBODIES (Mabs)

Monoclonal antibodies are made up of immunoglobulins which are indistinguishable and derived from a one B-cell clone . These antibodies are designed to recognize particular epitopes, or binding sites, on a single antigen. Monoclonal antibodies vary from polyclonal antibodies in that they are derived from a single B-cell clone and then target a single epitope. The development of genetic engineering techniques that allowed the generation of chimeric mouse/human mAbs largely made up of the variable regions of a mouse antibody combined with the constant regions of human IgG1 to yield a molecule that is approximately 30% mouse and 70% human in structure may define the mAb therapeutic era , for example Remicade, Rituxan (Jefferis, 2019)). Scientists have also developed the human gene antibody "HUMIRA" by researching the structural elements of mouse antibody which formed the paratope and transplanted the chimeric antibody sequences to human variable regions by developing new protocols. Because of the high efficacy and low adverse effects of antibody therapy, the number of such biopharmaceutical products has increased significantly since the first therapeutic monoclonal antibody product (Muromonab-CD3) was successfully introduced into the market and commercialized on a large scale in 1986 (Smith, 1996a, 1996b)

2.1 PREAPARATION OF MONOCLONAL ANTIBODY (Mabs)

Monoclonal Antibody production or Mab is produced by cell lines or clones obtained from the immunized animals with the substances to be studied (Figure 1).Cell lines are produced by fusing B cells from the immunized animal with myeloma cells. To produce the desired Mab, the cells must be grown in either of two ways: by injection into the peritoneal cavity of a suitably prepared mouse (in vivo method) or by in vitro tissue culture. The vitro tissue culture is the method used when the cells are placed in culture outside the mouse the mouse's body in flask



Source: www.googleimages.com Figure 1 Preparation of Mabs in vitro

2.2 MONOCLONAL ANTIBODY THERAPY

It is specialised form of immunotherapy which uses monoclonal antibodies to bind to specify target cells or proteins. In general, Mabs can achieve targeted therapy goals by inhibiting the activity of target receptors, stimulating the patient's immune system to attack the target cells, or delivering radioisotopes, cytotoxic chemicals, or therapeutic nanoparticles . Mabs having special properties like its therapeutic activity and time of acting towards the disease so it achieves a high market values and cures diseases ranging from migraine to cancer.

3. FORMULATION OF ANTIBODY BIOTHERAPEUTICS

Due to the innovations of technology, the formulation and production of biotherapeutics products has rapidly increased in biotechnology industry. Antibody formulation are usually produced in lyophilised or liquid forms. This formulations are available in liquids but they have higher molecular weight and its structure so they are not administered orally, they are administered through intravenous (IV), Sub-Cutaneous (SC), Intra-Muscular (IM), where IM route is rarely used. Intra-venous route is majorly use according to clinical specialists, among all the routes of administration IV route is more preferred for high dose drug administration though there are some complexities

and difficult to venepuncture and this route is uncomfortable for patients too. Recently, the clinical use and observation of subcutaneous injection of antibodies has been proved to be safe and effective, and many patients believe that subcutaneous injection at home is more convenient and friendly than IV in medical institutions (Berger, 2008) Clinical studies also show that SC delivery is quicker than intravenous treatment and has less systemic side effects for immunoglobulin therapy (Bittner, Richter and Schmidt, 2018)

Around 200 monoclonal antibodies got approved till 2021 and it indicates that mAbs are in demand for treatment and prevention of diseases such as multiple sclerosis, arthritis, plaque psoriasis, leukaemia, etc. "Since the first therapeutic monoclonal antibody product "(Muromonab-CD3)(Smith, 1996b)" was successfully introduced into the market and commercialised on a large scale in 1986 the number of such biopharmaceutical products has increased significantly due to the high efficacy and low adverse effects of antibody therapy. As of 7 June 2021, antibody therapeutics for the treatment of various indications have been approved by the Food and Drug Administration (FDA) of United States or the European Medicines Agency (EMA) **Table 1** and "many of them have also been extended for use in other global markets". (Kaplon *et al.*, 2020)

Table 1 *:* **Approved antibodies therapeutics by US and EU as of June 7 2021**

Generic name	Brand name	Target	Format	Indication first approved or reviewed	First EU approval year	First US approval year
Muromonab CD3	Orthoclone Okt3	CD3	Murine IgG2a	Reversal of kidney transplant rejection	1986*	1986#
Efalizumab	Raptiva	CD11a	Humanized IgG1	Psoriasis	2004#	2003#
Tositumomab-I131	Bexxar	CD20	Murine IgG2a	Non-Hodgkin lymphoma	NA	2003#
Nebacumab	Centoxin	Endotoxin	Human IgM	Gram-negative sepsis	1991*#	NA
Edrecolomab	Panorex	EpCAM	Murine IgG2a	Colon cancer	1995*#	NA
Catumaxomab	Removab	EPCAM/CD3	Rat/mouse bispecific mAb	Malignant ascites	2009#	NA
Daclizumab	Zinbryta	IL-2R	Humanized			

			IgG1			
Abciximab	Reopro	GPIIb/IIIa	Chimeric IgG1 Fab	Prevention of blood clots in angioplasty	1995*	1994
Rituximab	Rituxan	CD20	Chimeric IgG1	Non-Hodgkin lymphoma	1998	1997
Basiliximab	Simulect	IL-2R	Chimeric IgG1	Prevention of kidney transplant rejection	1998	1998
Palivizumab	Synagis	RSV	Humanized IgG1	Prevention of respiratory syncytial virus infection	1999	1998
Infliximab	Remicade	TNF	Chimeric IgG1	Crohn disease	1999	1998
Trastuzumab	Herceptin	HER2	Humanized IgG1	Breast cancer	2000	1998
Adalimumab	Humira	TNF	Human IgG1	Rheumatoid arthritis	2003	2002
Ibritumomab tiuxetan	Zevalin	CD20	Murine IgG1	Non-Hodgkin lymphoma	2004	2002
Omalizumab	Xolair	IgE	Humanized IgG1	Asthma	2005	2003
Cetuximab	Erbitux	EGFR	Chimeric IgG1	Colorectal cancer	2004	2004
Bevacizumab	Avastin	VEGF	Humanized IgG1	Colorectal cancer	2005	2004
Natalizumab	Tysabri	a4 integrin	Humanized IgG4	Multiple sclerosis	2006	2004
Panitumumab	Vectibix	EGFR	Human IgG2	Colorectal cancer	2007	2006
Ranibizumab	Lucentis	VEGF	Humanized IgG1 Fab	Macular degeneration	2007	2006
Eculizumab	Soliris	C5	Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2007	2007
Certolizumab	Cimzia	TNF	Humanized Fab, pegylated	Crohn disease	2009	2008
Ustekinumab	Stelara	IL-12/23	Human IgG1	Psoriasis	2009	2009
Canakinumab	Ilaris	IL-1β	Human IgG1	Muckle-Wells syndrome	2009	2009
Multiple sclerosis		2016#	2016#; 1997#			

Golimumab	Simponi	TNF	Human IgG1	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009	2009
Ofatumumab	Arzerra	CD20	Human IgG1	Chronic lymphocytic leukemia	2010	2009
Tocilizumab	RoActemra, Actemra	IL-6R	Humanized IgG1	Rheumatoid arthritis	2009	2010
Denosumab	Prolia	RANK-L	Human IgG2	Bone Loss	2010	2010
Belimumab	Benlysta	BLyS	Human IgG1	Systemic lupus erythematosus	2011	2011
Ipilimumab	Yervoy	CTLA-4	Human IgG1	Metastatic melanoma	2011	2011
Brentuximab Vedotin	Adcetris	CD30	Chimeric IgG1; ADC	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012	2011
Pertuzumab	Perjeta	HER2	Humanized IgG1	Breast Cancer	2013	2012
Adotrastuzumab emtansine	Kadcyla	HER2	humanized IgG1; ADC	Breast cancer	2013	2012
Raxibacumab	-	*B.anthrasis* PA	Human IgG1	Anthrax infection	NA	2012
Obinutuzumab	Gazyvaro	CD20	Humanized IgG1	Chronic lymphocytic leukemia	2014	2013
Siltuximab	Sylvant	IL-6	Chimeric IgG1	Castleman disease	2014	2014
Ramucirumab	Cyramza	VEGFR2	Human IgG1	Gastric cancer	2014	2014
Vedolizumab	Entyvio	α4β7 integrin	Humanized IgG1	Ulcerative colitis, Crohn disease	2014	2014
Nivolumab	Opdivo	PD1	Human IgG4	Melanoma, non-small cell lung cancer	2015	2014
Pembrolizumab	Keytruda	PD1	Humanized IgG4	Melanoma	2015	2014

Blinatumomab	Blincyto	CD19, CD3	Murine bispecific tandem scFv	Acute lymphoblastic leukemia	2015	2014
Alemtuzumab	Lemtrada	CD52	Humanized IgG1	Multiple sclerosis; chronic myeloid leukemia#	2013; 2001#	2014; 2001#
Evolocumab	Repatha	PCSK9	Human IgG2	High cholesterol	2015	2015
Idarucizumab	Praxbind	Dabigatran	Humanized Fab	Reversal of dabigatraninduced anticoagulation	2015	2015
Necitumumab	Portrazza	EGFR	Human IgG1	Non-small cell lung cancer	2015	2015
Dinutuximab	Unituxin	GD2	Chimeric IgG1	Neuroblastoma	2015	2015
Secukinumab	Cosentyx	IL-17a	Human IgG1	Psoriasis	2015	2015
Mepolizumab	Nucala	IL-5	Humanized IgG1	Severe eosinophilic asthma	2015	2015
Alirocumab	Praluent	PCSK9	Human IgG1	High cholesterol	2015	2015
Daratumumab	Darzalex	CD38	Human IgG1	Multiple myeloma	2016	2015
Elotuzumab	Empliciti	SLAMF7	Humanized IgG1	Multiple myeloma	2016	2015
Ixekizumab	Taltz	IL-17a	Humanized IgG4	Psoriasis	2016	2016
Reslizumab	Cinqaero	IL-5	Humanized IgG4	Asthma	2016	2016
Olaratumab	Lartruvo	PDGFRα	Human IgG1	Soft tissue sarcoma	2016	2016
Bezlotoxumab	Zinplava	*Clostridium difficile* enterotoxin B	Human IgG1	Prevention of Clostridium difficile infection recurrence	2017	2016
Atezolizumab	Tecentriq	PD-L1	Humanized IgG1	Bladder cancer	2017	2016
Obiltoxaximab	Anthim	*B.anthrasis* PA	Chimeric IgG1	Prevention of inhalational anthrax	In review	2016

Brodalumab	Siliq, LUMICEF	IL-17R	Human IgG2	Plaque psoriasis	2017	2017
Dupilumab	Dupixent	IL-4R α	Human IgG4	Atopic dermatitis	2017	2017
Inotuzumab ozogamicin	BESPONSA	CD22	Humanized IgG4; ADC	Acute lymphoblastic leukemia	2017	2017
Guselkumab	TREMFYA	IL-23 p19	Human IgG1	Plaque psoriasis	2017	2017
Sarilumab	Kevzara	IL-6R	Human IgG1	Rheumatoid arthritis	2017	2017
Avelumab	Bavencio	PD-L1	Human IgG1	Merkel cell carcinoma	2017	2017
Emicizumab	Hemlibra	Factor Ixa, X	Humanized IgG4, bispecific	Hemophilia A	2018	2017
Ocrelizumab	OCREVUS	CD20	Humanized IgG1	Multiple sclerosis	2018	2017
Benralizumab	Fasenra	IL-5R α	Humanized IgG1	Asthma	2018	2017
Durvalumab	IMFINZI	PD-L1	Human IgG1	Bladder cancer	2018	2017
Gemtuzumab ozogamicin	Mylotarg	CD33	Humanized IgG4; ADC	Acute myeloid leukemia	2018	2017; 2000#
Erenumab	Aimovig	CGRP receptor	Human IgG2	Migraine prevention	2018	2018
Galcanezumab	Emgality	CGRP	Humanized IgG4	Migraine prevention	2018	2018
Burosumab	Crysvita	FGF23	Human IgG1	X-linked hypophosphatemia	2018	2018
Lanadelumab	Takhzyro	Plasma kallikrelin	Human IgG1	Hereditary angioedema attacks	2018	2018
Mogamulizumab	Poteligeo	CCR4	Humanized IgG1	Mycosis fungoides or Sézary syndrome	2018	2018
Tildrakizumab	Ilumya	IL-23 p19	Humanized IgG1	Plaque psoriasis	2018	2018
Fremanezumab	Ajovy	CGRP	Humanized IgG2	Migraine prevention	2019	2018
Ravulizumab	Ultomiris	C5	Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2019	2018

Cemiplimab	Libtayo	PD-1	Human mAb	Cutaneous squamous cell carcinoma	2019	2018
Ibalizumab	Trogarzo	CD4	Humanized IgG4	HIV infection	2019	2018
Emapalumab	Gamifant	IFNg	Human IgG1	Primary hemophagocytic lymphohistiocytosis	Negative opinion from EMA	2018
Moxetumomab pasudotox	Lumoxiti	CD22	Murine IgG1 dsFv immunotoxin	Hairy cell leukemia	In review	2018
Caplacizumab	Cablivi	von Willebrand factor	Humanized Nanobody	Acquired thrombotic thrombocytopenic purpura	2018	2019
Risankizumab	Skyrizi	IL-23 p19	Humanized IgG1	Plaque psoriasis	2019	2019
Polatuzumab vedotin	Polivy	CD79b	Humanized IgG1; ADC	Diffuse large B-cell lymphoma	2020	2019
Romosozumab	Evenity	Sclerostin	Humanized IgG2	Osteoporosis in postmenopausal women at increased risk of fracture	2019	2019
Brolucizumab	Beovu	VEGF-A	Humanized scFv	Neovascular agerelated macular degener ation	2020	2019
Crizanlizumab	Adakveo	CD62	Humanized IgG2	Sickle cell disease	EC decision pending	2019
Enfortumab vedotin	Padcev	Nectin-4	Human IgG1; ADC	Urothelial cancer	NA	2019
[fam-] trastuzumab deruxtecan	Enhertu	HER2	Humanized IgG1; ADC	HER2+ metastatic breast cancer	NA	2019

Teprotumumab	Tepezza	IGF-1R	Human IgG1	Thyroid eye disease	NA	2020
Eptinezumab	VYEPTI	CGRP	Humanized IgG1	Migraine prevention	NA	2020
Isatuximab	Sarclisa	CD38	Chimeric IgG1	Multiple myeloma	2020	2020
Sacituzumab govitecan	TRODELVY	TROP-2	Humanized IgG1; ADC	Triple-neg. breast cancer	NA	2020
Inebilizumab	Uplizna	CD19	Humanized IgG1	Neuromyelitis optica spectrum disorders	NA	2020
Tafasitamab	Monjuvi	CD19	Humanized IgG1	Diffuse large B-cell lymphoma	In review	2020
Belantamab mafodotin	BLENREP	BCMA	Humanized IgG11; ADC	Multiple myeloma	In review	2020
Satralizumab	Enspryng	IL-6R	Humanized IgG2	Neuromyelitis optica spectrum disorder	EC decision pending	2020
Atoltivimab, maftivimab, and odesivima	Inmazeb	IgG1	Ebola virus; mixture of 3 human IgG1	Ebola virus infection	NA	2020
Naxitamab	Danyela	GD2	Humazied IgG1	High-risk neuroblastoma and refractory osteomedullary disease	NA	2020
Margetuximab	Margenza	HER2	Chimeric IgG1	Metastatic breast cancer	NA	2020
Ansuvimab	Ebanga	Ebolavirus glyco-protein	Human IgG1	Ebolavirus infection	NA	2020

Evinacumab	Evkeeza	Angiopoietein like 3	Human IgG4	Homozygous familial hypercholesterolemia	EC decision pending 2021	2021
Dostarlimab	Jemperli	PD-1	Humanized IgG4	Endometrial Cancer	NA	2021
Loncastuximab tesirine	Zynlonta	CD-19	Humanized IgG1 ADC	Diffuse large B-cell lymphoma	In Review	2021
Amivantamab	Rybrevant	EGFR, cMET	Human bispecific IgG1	NSCLC w/ EGFR exon 20 insertion mutations	In Review	2021
Aducanumab	Aduhelm	Amyloid Beta	Human IgG1	Alzheimer's disease	NA	2021
Toripalimab	Tuoyi	PD-1	Humanized IgG4	Nasopharyngeal carcinoma	NA	2021
Tanezumab	-	Nerve growth factor	Humanized IgG2	Pain due to osteoarthritis of knee or hip	In review	In Review
Tralokinumab	-	IL-13	Human IgG4	Atopic Dermatitis	NA	In Review
Teplizumab	-	CD-3	Humanized IgG1	Type 1 Diabetes	In review	In Review (Q2 2021)

Retifanlimab	-	PD -1	Humanized IgG4	Carcinoma of the anal canal (Squamous cell)	In review	In Review 02/07/2021
Oportuzumab monatox	-	EpCAM	Humanized scFv immunotoxin	Bladder cancer	In review	In Review 25/07/21
Anifrolumab	-	IFNAR1	Human IgG1	Systemic lupus erythematosus	In review	In Review 8/18/2021; Rolling BLA
Bimekizumab	-	IL-17A	Humanized IgG1	Psoriasis	NA	In Review 30/09/2021
Narsoplimab	-	MASP-2	Human IgG4	Hematopoietic stem cell transplant-associated thrombotic microangiopathies	NA	In Review 15/10/2021
Inolimomb	-	CD-25	Muine IgG1	Graft vs. host disease	NA	In Review 17/10/2021
Balstilimab	-	PD-1	Human IgG4	Cervical cancer	NA	In Review
Sutimlimab	-	C1s	Humanized IgG4	Cold agglutinin disease	NA	In Review
Ublituximab	-	CD20	Chimeric IgG1	Chronic lymphocytic leukemia	NA	In Review

Tisotumab vedotin	-	Tissue Factor	Human IgG1 ADC	Cervical cancer	NA	In Review
Omburtamab	-	B7-H3	Murine IgG1	CNS/leptomeningeal metastasis from neuroblastoma	In Review	In Review
Tezepelumab	-	Thymic stromal lymphopoietin	Human IgG2	Severe asthma	NA	NA
Sintilimab	-	PD-1	Human IgG4	Non-small cell lung cancer	NA	In Review
Penpulimab	-	PD-1	Humanized IgG1	Metastatic nasopharyngeal carcinoma	In Review	In Review (March 2022) NA; Oncology review

This Table has been adapted from (Kaplon *et al.*, 2020) Antibody therapeutics approved or in regulatory review in the EU or US. *The Antibody Society , June 7, 2021*. Products that were granted approvals but subsequently withdrawn from the market are included in the table. Biosimilar products are excluded .

“Table notes: *, country-specific approval. #, withdrawn or marketing discontinued. NA, not approved or in review in the EC, or not approved or information on review status not available in USA.”
(Dates written under review column of table data was found out by me . Yet to be approved)

Above mentioned monoclonal antibodies are used in treatment of diseases these days and recommended by healthcare professionals for betterment of patients as soon as possible . In the table most of antibodies are made from IgG class and its subclasses (IgA, IgG, IgM, IgD, IgE).
The choice of IgG subclass is critical for creating therapeutic mAbs. The choice of IgG subclass is critical for creating therapeutic mAbs, particularly in cancer . In this situation, IgG1 has the greatest potential for antibody-dependent cell-mediated cytotoxicity and is hence excellent for killing cancer cells . In contrast, IgG3 is rarely utilised for therapeutic mAbs because the lengthy hinge region is susceptible to proteolysis, resulting in a shorter half-life. Glycosylation of the Fc region of IgG mAbs is required for various effector functions to be activated, and cellular engineering can be utilised to create specific glycoforms of antibodies. Interestingly, IgG4 has the ability to trigger inflammatory responses via FcRs21, and IgG4 can display dynamic dissociation and Fab arm exchange.
Glycosylation of the Fc region of IgG mAbs is required for various effector functions to be activated, and cellular engineering can be utilised to create specific glycoforms of antibodies20. Interestingly, IgG4 has the ability to trigger inflammatory responses via FcRs21, and IgG4 can display dynamic dissociation and Fab arm exchange (Cymer *et al.*, 2018)

Table 2 : UK licensed monoclonal antibodies

Name	Type of antibody	Target	Licensed indication
Infliximab (Remicade)	Human–mouse chimaera IgG1	TNF-α	Refractory Crohn's, Crohn's fistulas, refractory rheumatoid arthritis
Palivizumab (Synagis)	Humanised IgG1	F protein on RSV	Prophylaxis, RSV in premature infants or brochopulmonary dysplasia
Abciximab (ReoPro)	Human–mouse chimaera	Platelet glycoprotein IIb/IIIa	High risk coronary intervention
Rituximab (MabThera)	Human–mouse chimaera IgG1	CD20	Refractory low grade or follicular B cell lymphoma
Basiliximab (Simulect)	Human–mouse chimaera IgG1K	IL-2 receptor α chain	Prophylaxis of acute rejection in allogeneic renal transplantation
Daclizumab (Zenapax)	Humanised IgG1	IL-2 receptor α	As Basiliximab
Trastuzumab (Herceptin)	Humanised IgG1	HER 2 growth receptor	Relapsed HER2 (high) breast malignancy

IL-2, interleukin 2; TNF-α, tumour necrosis factor α; RSV, respiratory syncitial virus. (Drewe and Powell, 2002)

In the above mentioned, **Table *2***, some well-known UK licensed monoclonal antibodies and which are useful in treatment of diseases. This monoclonal antibodies treatments are available in market and which also preferred by health care professionals for treatment of cancer.

4. ANTIBODY INSTABILITY STUDIES

Antibodies, like other proteins , can be broken down by a range of physical and chemical processes; however, antibodies appear to last longer on average than other proteins. Antibody instability can be detected in the liquid, frozen and lyophilized phases. The status of glycosylation of the antibody can have a significant effect on how quickly it degrades. In most cases, multiple degradation pathways may occur simultaneously and the degradation mechanism may vary depending on stressful conditions. These degradation pathways fall into two main categories: physical and chemical instability are listed below:

4.1 PHYSICAL INSTABILITY

DENATURATION: It denatures the conformation of protein order which is secondary and tertiary structure from 2 fAb fragments linked to Fc domain via hinge region and leads to disruption of disulphide bonds in the original or native structure of the protein which reduces therapeutic property (Kaur, 2021). Denaturation occurs due to extreme changes in pH or temperature of the environment. Direct disruption of the mAb's function, such as a loss in hinge flexibility, or encouragement of aggregation, can be the result of unfolding (le Basle *et al.*, 2020)

AGGREGATION: *"Aggregate formation is influenced by multiple aspects of the bioproduction process but can be mitigated by good process design and control."*

Aggregation is most common and severe issue observed in monoclonal antibodies as a one of the physical degradation pathway. It involves the self-association of unfolded proteins or its fragments which have high molecular weight such as dimers, oligomers, tetramers, etc. It causes the generation of particles which are too large in size and not able to detect with kind of analytical methods and exposes to multiple epitopes which increases the immunological reaction in patients and reduces its therapeutic activity which is a concern of safety of patients.(Ma, Ó'Fágáin and O'Kennedy, 2020)

Antibody aggregation can be induced by freeze-drying to varying degrees. In the absence of sugars, lyophilization of a recombinant humanised monoclonal antibody (rhuMAb HER2 resulted in a modest increase in aggregate size (less than 1.2 percent). A human monoclonal antibody aggregation was detected in PBS after lyophilization (Wang *et al.*, 2007; le Basle *et al.*, 2020; Kaur, 2021). This process is mostly triggered by unfavourable conditions or major changes in pH, temperature, ionic strength, concentration, etc. which is exposed to it during storage of mAbs.

4.2 CHEMICAL INSTABILITY

There are different mechanisms of chemical instabilities which has been observed in several research disulphide bond. It can happen with or without the presence of oxidants (such as peroxides, light, or metals), and is also known as self-oxidation. Some residues, such as methionine, histidine, and cysteine, are particularly vulnerable to oxidation. The synthesis of disulphide bond between two oxidised free species, with a thiolate anion intermediate is one of the outcomes of cysteine oxidation. In a basic environment the formation of bonding between intramolecular or intermolecular is increased (le Basle *et al.*, 2020; Kaur, 2021)). The second major chemical degradation pathway which reduces the generation of proteins is **deamidation**, where asparagine and glutamine residues are affected. The development of a cyclic imide which acts as intermediate and results in structural change of polypeptide structure, which is produces by an acid-base reaction between the unique residues are present nearby and it acts as proton donors for example, threonine and serine. Instant hydrolyzation takes place of an intermediate called succinimide in asparagine converted into aspartic acid or iso-aspartic acid (Shire, 2015; Kingsbury *et al.*, 2020)

Fragmentation in monoclonal antibodies (mAbs) can occur on disulphide bonds or peptides. Peptides or proteins bond cleavage produces low molecular weight species of varying type and size, which can be produced by enzymatic or non-enzymatic processes. This condition occurs

only in situation like where environmental conditions are likely to be highly acidic or high temperature (Shire, 2015; Strickley and Lambert, 2021).

Other chemical instabilities are like isomerization, cross-linking reactions, glycation, etc. also occurs due to the inappropriate storage conditions.

5. Formulation of Monoclonal antibody in higher concentration: challenges and development

Highly concentrated monoclonal antibodies are unique type of class in biopharmaceutical industry.

The current study examines the effect of manufacturing parameters on an ultra-high concentration IgG1 antibody subclass formulation that may be given as a single subcutaneous injection. Monoclonal antibodies, which require greater dosages for therapeutic efficacy but have poorer stability, are given as dilute infusions or two (low concentration) injections, both of which result in decreased therapeutic efficacy. It's worth noting that a high protein content (50 mg/mL) might be difficult to work with in terms of manufacturing of drug at high concentration of protein. During preparation, primary packaging, and manufacturing process development, and optimization of other dosage form-related factors like colloidal properties, physical, and chemical protein stability should be considered (Kollár et al., 2020a).

The objective of this type of research is to develop a pharmaceutical product that can preserve normal protein structure during its prolonged storage while also providing accurate and exact dose when administered. Its is necessary to inject adequate number of large dosages of mAbs which is related to the patient's bodyweight and several other factors but since mAbs are low potency drugs which needs to administered intra-venously which requires patient to admit in hospital. So, the recent studies are focused on administration of monoclonal antibodies via subcutaneous route and its comparison mentioned below in

'Table 3. It is observed that there is limitation in dose volume (< 2ml) via sub- cutaneous injection because the sub-cutaneous tissue back pressure the drug if high volume is injected and it results in pain at injection site. So, higher concentration of mAb results in formation

reversible non-covalent aggregation which affects protein conformation, pharmacokinetics and safety. (Shire, 2015; Deokar *et al.*, 2020)

Additionally, due to reversible non-covalent aggregation results in chemical instabilities like isomerization, elimination, and breaking of peptide or protein bonds which leads to irreversible form of covalent aggregates.

So, higher concentration mabs formulations should proper stability and appropriate viscosity of antibody solutions to prevent the discomfort in patients while administrating it via SC route.

Table 3 : Comparison between IV route and SC route of administration

Route of administration	Advantages	Disadvantages
Intra-venous route (IV)	• Rate of delivery and dose are precisely controlled • Rapid exposure • Its reduces the irritation of drugs at injecting site. • Large amount of doses can be administer. (1 to 100+ ml)	• It requires professional healthcare assistance. • Requires frequent hospitalization . • Requirement of observation after administration • Higher risk of systemic infections. • Patient's incompliance due to venepuncture.

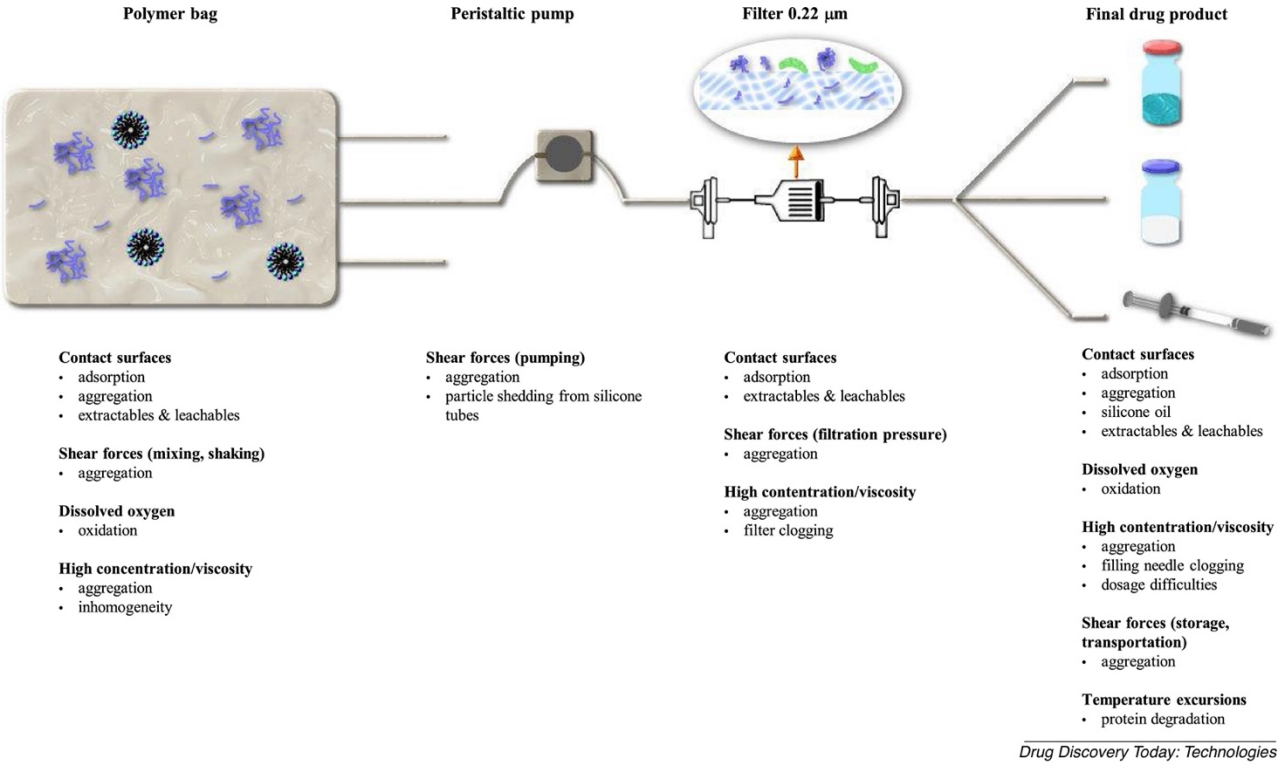
Sub – cutaneous route (SC)	• Self – administration of dose is done easily by patient • Decreased cost compared to IV. • Less pain and better comfort of patient than IV route administration	• Dosage volume limitations (< 2ml) because sub-cutaneous tissue back pressure the injected drug and causes pain at injection site. Less effective at injection site. • Ascertain adequate absorption and bioavailability.

(Modified by myself from reference (Kollár *et al.*, 2020a)

5.1 Challenges in formulation of Monoclonal antibodies with higher concentration

In recent studies, it mentioned that common issues related to manufacturing, stability, viscosity, and drug delivery are frequently linked with the development of monoclonal antibodies with high concentration.

The intense protein-protein interactions (PPIs) occurs due to increased viscosity, opalescence, and aggregation ((Tomar *et al.*, 2016; Garidel *et al.*, 2017). During formulation, primary packaging, and manufacturing process development, as well as the optimization of other dosage form-related factors, colloidal characteristics, physical, and chemical protein stability should be taken into account. The purpose of this type of research is to create a pharmaceutical drug that can preserve optimal protein structure during its shelf life while also ensuring proper and exact dosage when administered . Monoclonal antibodies (Mabs) are a constantly expanding special group of biopharmaceuticals which target a broad range of diseases, including several chronic and life-threatening diseases. Mabs are excellent for commercial pharmaceutical product development due to their high specificity, low non-mechanism toxicity, and tolerable immunogenicity. (Kollár *et al.*, 2020)



Source: https://ars.els-cdn.com/content/image/1-s2.0-S1740674920300202-gr1_lrg.jpg (Kollár *et al.*, 2020a)

6. VISCOSITY

The viscosity of a fluid (liquid or gas) is its resistance to a change in form or movement of neighbouring parts relative to one another. Viscosity implies resistance to flow . The interprotein structures which are depend on protein–protein interactions (PPIs) help to determine the viscosity of concentrated protein solutions. Measurement of viscosity is done by viscometers. By conducting studies in a controlled environmental condition such as, pH, temperature, etc, PPIs can be altered and results in lower viscosity (Zhang and Liu, 2017)). Large amount of samples are required to evaluate the viscosity of a particular solution because it shows difficulty in characterising of behaviour of solution. Additionally, diffusion interaction parameter (kD) measurement is a major predictor of solution behaviour. The opalescence and viscosity tendency of mAb solution may be consistently predicted by measuring kD (weak self

interactions in dilute solution when the average distance between individual mAb molecules is high relative to the molecular size). While a low kD in dilute solution indicates the presence of viscous or opalescent solutions at greater concentrations, a high kD (> +20 mL/g) indicates the presence of wet solutions (Kingsbury *et al.*, 2020)

Mab solutions having high viscosity will have unfavourable effect on the higher concentration and it will be difficult to eject the solution from syringe having needle size of 25G (gauge). Patients may have more discomfort because of the dispersion of back pressure caused by high viscosity solutions, which may necessitate the use of bigger needles. Syringeaibility studies assessed that higher concentration solution of mab may require larger needle for dispatch the fluid from syringe and its flowability of fluid.

The viscosity of monoclonal antibodies usually increases gradually at a concentration of > 50 to 100 mg/mL ((Shire, Shahrokh and Liu, 2004) (Badkar *et al.*, 2011). To assess injectability, a viscosity-glide force relationship based on the Hagen-Poiseuille equation was created. As per their measurements, the liquid viscosity should be kept below 20 cP when employing a No. 27 thinwalled needle (inner diameter: 0.241mm) to avoid a sliding force exceeding 20 N.

So to overcome all this challenges, (Kastelic *et al.*, 2018) suggests that rather than, changing the viscosity formulation of mab solution and it may loses it therapeutic properties can be controlled by changing it binding site.

Other two important issues regarding manufacturing of higher concentration Mabs solution are solubility and stability.

The mAb concentration at which the chemical potentials of the aqueous and solid phases are identical is termed as solubility. For an approved solution, solubility limit is to be transparent, and

should be nearer to supersaturation i.e. turbidity, precipitation, crystallisation, opalescence. This may be difficult to do with some insoluble antibodies, such as ACPs or ACNPs.

Solubility are divided into two categories are:

Kinetic and Thermodynamic solubility which are mostly studied this days.

There are several techniques for manufacturing protein of higher concentration , the most frequent and appropriate of which is UF / DF (ultra-filtration / dia-filtration) in aspects of GMP (good manufacturing practice) and manufacturing cost at commercial level ((Kollár *et al.*, 2020b); (Neergaard *et al.*, 2013)) .

Stability is one of the most crucial issue of higher concentration mab with leads to accumulation of proteins in it which effects the pharmacokinetics and therapeutic properties of monoclonal antibodies Primary degradation of protein formulations is irreversible protein aggregation while, reversible protein self-association is a secondary. In high concentration protein preparations, there are mainly two strategies for reducing irreversible protein aggregation. The technique relies on circumstances that keep proteins in their normal shape, while the other inhibits aggregation-causing molecular collisions (Garidel *et al.*, 2017); (Ma, Ó'Fágáin and O'Kennedy, 2020)). To maintain the quality of higher concentration mab subcutaneous formulations and eliminate the aqueous solutions instabilities so the commonly utilised technique is knows as Freeze-drying . The hydrolytic processes may be attenuated, the mobility and conformational flexibility of the molecules can be decreased, and the aggregation tendency of protein molecules can be minimised in the presence of suitable excipients. (Garidel, Pevestorf and Bahrenburg, 2015)

7. NON-AQUEOUS FORMAULATIONS OF MONOCLONAL ANTIBODIES

Many novel techniques, such as adding hydrophobic salts and plasticizers to concentrated protein solutions, have been developed to enhance the concentration and lower the viscosity of

antibody solutions as much as feasible. Furthermore, the researchers are attempting to alter the dose form.

Following Figure 2: Basic protocol for formulation of non-aqueous protein suspensions Figure 2, shows the basic manufacturing process of protein powder suspensions in non-aqueous vehicles. The main techniques are freeze-drying with subsequent micronization and spray drying used in development of non-aqueous protein formulations in biopharmaceutical industries. Some of potential non-aqueous vehicles which are used for research and development of protein powder suspensions are mentioned in ***Table 4***.

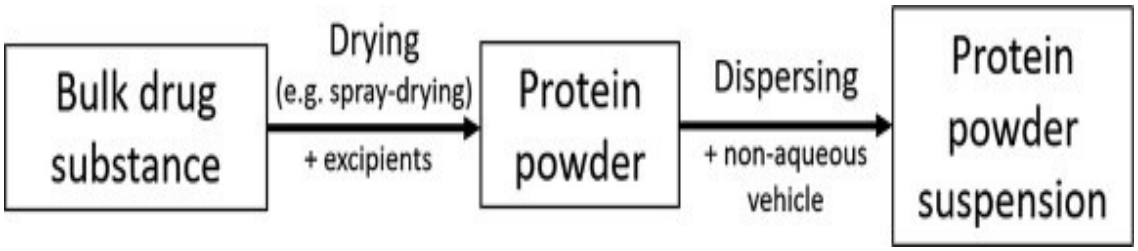


Figure 2: Basic protocol for formulation of non-aqueous protein suspensions

Other formulants used for development of non-aqueous protein powder suspensions are buffering agents, surfactants and antioxidants. Buffering agents are divided into amino-acid containing buffering agents and non-amino acid buffering agents. Protein stability also requires careful selection of the appropriate pH and buffering agent. Mostly histidine and arginine are present in amino acid containing buffer system and sodium phosphate, sodium citrate and tris buffer are non - amino acid containing buffer system. Development of proteins in non-aqueous solutions may have hydrophilic surfactants such as polysorbate or poloxamer are added to protein powder to prevent the degradation of proteins from aggregation and unfolding. Methionine acts an anti-oxidants

and prevents the protein suspension from oxidation while manufacturing and also at during drug release processes. (Srinivasan *et al.*, 2013)

The process of creating a finished medicinal product from protein powder suspensions in nonaqueous carriers is extremely difficult.

Table 4 : POTENTIAL NON-AQUEOUS VEHICES IN PROTEIN POWDER FORMULATIONS

Group	**Example**	**Viscosity [mPa·s] (Temp.)**
Plant oils	•Sesame oil	51–61 (25 °C)
	•Safflower oil	52 (26 °C)
	•Soybean oil	56 (25 °C)
Medium chain triglycerides	•Triglycerides of Caprylic and Capric acid	23–27 (25 °C)
Propylene glycol diesters of medium chain fatty acids	•Propylene glycol diesters of caprylic and capric acids	9 (20 °C)
Fatty acid esters	•Ethyl oleate	6 (25 °C)
	•Isopropyl myristate	5 (25 °C)
Polyethylene glycol	•PEG 200	48 (25 °C)

Esters	•Benzyl benzoate	8–9 (25 °C)
	•Ethyl lactate	2 (20 °C)
Alcohols	•Benzyl alcohol	5 (25 °C)
	•Isopropyl alcohol	2.4 (25 °C)
Group	**Example**	**Viscosity [mPa·s] (Temp.)**
	•Ethyl alcohol	1.2 (25 °C)
	•Propylene glycol	39 (25 °C)
Perfluorinated carbons	•Perfluorodecalin	6 (25 °C)
Semifluorinated alkanes	•Perfluorohexyloctane	3.44 (25 °C)
	•Perfluorobutylpentane	1.05 (25 °C)

This overview of potential vehicles use in protein powder suspensions are yet to be reviewed for administration of protein powders via Sub-cutaneous route. This table is adapted from several literatures and combined in form of table (Marschall *et al.*, 2021)

7.1 DELIVERY CHALLENGES

The desired application for protein powder suspensions to patients for treatment is via subcutaneous administration for diseases ranging from migraine prevention, multiple sclerosis and leukaemia. The problem arises when the suspension is not able flow easily through the

prefilled syringes and auto injectors due to its high concentration and high viscosity. So to minimizes the challenges it is better to change formulations by using low viscosity buffers which helps to glide properly in syringe. So It necessary to make appropriate choice of needle (Mathaes *et al.*, 2016)

8. CONCLUSION

Antibody treatment has already demonstrated its enormous potential for advancement. With the advancement of technology and the changing needs of patients, more demands are being placed on the creation of antibody formulations. Because not every antibody can be formed in any method to produce expected outcomes, it is essential to address antibody formulation concerns early in the research cycle. Most of the treated with mab treatment but there is major issue will solubility, stability, and have low concentration during the storage of it . Many studies have been conducted with the goal of elucidating the principles underlying these occurrences from diverse perspectives and offering practical remedies. Non-aqueous suspensions research is still in its infancy compared to aqueous solutions, but it has a lot of promise and economic value since it promises to offer high concentration, low viscosity formulations. The solid state of the proteins reduces physical and chemical instabilities, resulting in great protein stability. Although numerous studies have reported good protein stability in non-aqueous protein powder suspensions, little is known regarding the suspensions' physical stability, such as resuspend-ability, particle size stability, and injectability after storage.

To get to this stage, efforts and obstacles in improving current manufacturing methods and stable products with appropriate efficacy and with improved patient safety must be addressed in order to produce non-aqueous protein powder suspensions with improved characteristics compared to standard protein formulations.

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Characterization of an IgG fab fragment in non -aqueous solvent: Isopropyl Alcohol (IPA).

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CONTENTS

Report on experimental work.

Characterization of an IgG fab fragment in non -aqueous solvent: Isopropyl Alcohol (IPA).

Abstract : The aim of the study was to understand the protein-protein interactions in a non-aqueous solvents allow higher concentration, focusing on the stability of an antibody fragment, using the ultracentrifuge and dynamic light scattering with software to analyse and understand the protein-protein interactions with Iso-propyl alcohol (IPA) as the solvent. The comparison is done between with buffer and IPA to better understand the characterization. Instabilities and aggregation were also observed for IPA and showed it to be non – suitable for higher concentration formulations of antibodies.

Introduction

Antibody therapy is currently showing great promise in the pharmaceutical and health-care industries. The growth of the technology and the practical needs of patients have given rise to the development of antibody manufacturing. The development of antibody preparations with high concentrations, stability, and low viscosity, as well as a variety of administration routes, will expand the application range of antibody-based treatments indefinitely. While developing it at higher concentration there are number of factors to be considered such as instabilities like physio-chemical issues. Mostly the common instability phenomenon is aggregation which occurs due to the removal of hydrophobic residues from contact with the solvent resulting in a reduction in free surface energy, which drives protein aggregation. This experiments were conducted with the antibody fragment i.e. IgG Fab A'33 supplied by UCB Celltech company (Slough,UK) Characterization of Fab fragment was done using perkins.

Materials and Method

Instruments used : Analytical ultracentrifugation (Beckman, USA), Dynamic Light scattering zetasizer photometer (Malvern, UK) ,Viscometer (Schott- Gerate, Germany)

Sample : IgG A'33 Fab fragment (UCB cell tech).

Perkins software was used to study sequence of amino acid.

Preparation of Phosphate buffer saline : Di-sodium hydrogen orthophosphate dodecahydrate, potassium dihydrogen orthophosphate, sodium chloride and measured the concentration using spectrophotometer.

Preparation of IgG A'33 Fab fragment from the stock solution using previously made PBS buffer nd diluted by PBS buffer to make the appropriate concentration to carry out hydrodynamics experiments.

Preparation of sample for dialysis of protein : the fab sample was taken from the stock solution .i.e. 1ml and 5ml of PBS buffer. After dialysis the stock solution concentration was measured with a sprectrophotometer.

SDS GEL sample preparation : **Mini-PROTEAN ® TGX precast gels -** 12%, 10- well comb, 30 µl/well cat, **Laemmli sample buffer including -** 5µl of the 2-mercaptoethanol + 95 µl of the laemmeli = 100 µl , **Laemmli sample buffer: fab sample -** 2x of laemmli sample buffer (100 µl) : fab sample (50 µl). In general the

percentage should be 50%:50% of both but the concentration is concentrated twice. **10x Tris/Glycine/ SDS Buffer -** 80 ml of the SDS buffer + 720 ml of the RO/milliQ water= 800 ml. This used as a running buffer with a pH around 8.3. **Heating -** 5 minutes at 95.5 °C to denature the fab into a linear-like shape.

Preparation of sample for AUC-SV experiment : PBS buffer was used as reference and sample was from the stock solution, 7 cells named as XL/I 1 – 7 and it was serially dilute from highest concentration to lowest concentration. So, the amount of the sample in sample tubes was 750µg PBS buffer & 750µg sample whereas, in cell only 400µg of sample and buffer was analysed.

Preparation of sample for AUC-SE experiment : PBS buffer used as reference and antibody solution from stock solution and diluted it with PBS buffer and serial dilution from highest to lowest concentration.

Preparation of DLS Sample : 1mg/ml of Fab in cuvette and analysed it through zetasizer using the manufacturer's software.

Results and discussions :
So we performed both AUC experiments which are SV and SE experiments, but PBS buffer was used as reference so study the sedimentation coefficient as a function of radial distance during centrifugation and SE experiment was also performed.

SDS-PAGE results : SDS PAGE is an analytical techniques which is useful for the purification of proteins accordingly to their molecular weight and separate proteins according to the size . We had to perform SDS PAGE to find out the molecular weight of the antibody fragment as shown below
Figure [1]*:*

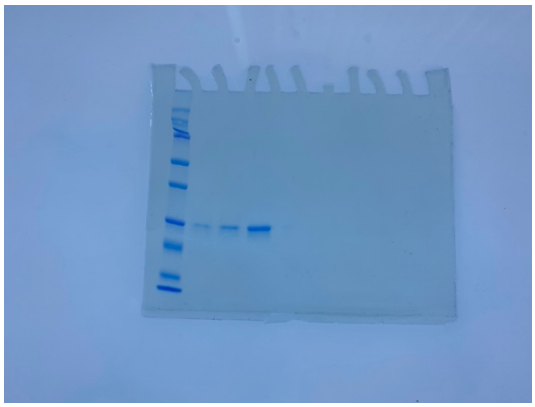


Figure 1. SDS PAGE ANALYSIS of IgG Fab. The protein standards are shown in the left hand column

SDS is detergent with a strong protein-denaturing effect and which binds to the backbone of the protein. In the presence of SDS and denaturing agent i.e. 2 – mercaptoethonol that cleaves disulphide bonds and unfolds the protein into linear chain with negative charge proportional to the polypeptide chain length.

We only investigated IgG Fab fragment, it is the antigen-binding site (Kresge et al., 2006; Wang et al., 2007).

Here we observed that SDS detergent breaks non-covalent bonds in the IgG Fab fragment and while measuring its weight through SDS Page technique resulted as approx. 45Kda.

Results of AUC-SV experiment in PBS buffer :

Sedimentation velocity is an analytical ultracentrifugation (AUC) method that measures the rate at which molecules move in response to centrifugal force generated in a centrifuge. This sedimentation rate provides information about both the molecular mass and the shape of molecules. In some cases this technique can also measure diffusion coefficients and molecular mass. In the sedimentation velocity method a sample is spun at very high speed (usually 40-60 K rpm) in an analytical ultracentrifuge. The high centrifugal force rapidly depletes all the protein from the region nearest the centre of the rotor (the meniscus region at the air/solution interface), forming a boundary which moves toward the outside of the rotor with time, until finally all the protein forms a pellet at the outside of the cell. The concentration distribution across the cell at various times during the experiment is measured while the sample is spinning, using either absorbance or refractive index detection in our Beckman ProteomeLab XL-I. SV analysis is very not time taking procedure compared to SE (Cole et al., 2008; Harding et al., 2015).

I had to perform at speed of 45000 rpm where we used the IgG Fab sample and PBS buffer and also measured the absorbance. The sample was serially diluted in the sample tubes i.e. 7 tubes and from the lowest to highest concentration and we observed leaks in 3 cells and repeated for those concentration later and analysed using software SEDFIT.

Following graph are for SV-analysis :

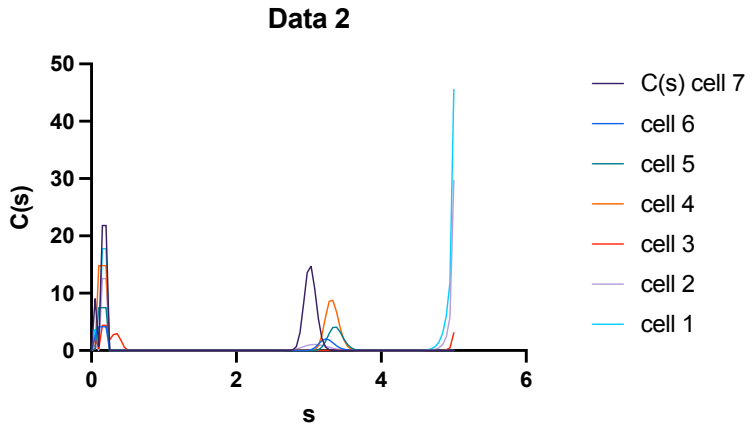


Figure 2Figure 2. Sedimentation velocity analysis of IgG Fab using interference optics in PBS buffer. The different colours correspond to different loading concentrations. The peaks at very low s value (<0.4S are artefacts of the analysis)

Results of AUC-SE experiment in PBS buffer : In sedimentation equilibrium, the sample is spun at a high enough speed in an analytical ultracentrifuge to push the protein toward the exterior of the rotor, but not high enough to induce the sample to form a pellet . Because the centrifugal force creates a concentration

differential in the centrifuge cell, diffusion works to counteract this gradient. The concentration distribution eventually achieves equilibrium once a precise balance between sedimentation and diffusion is achieved. In our Beckman XL-I, we assess the equilibrium concentration distribution across the cell while the sample is spinning, utilising either absorbance or refractive index detection.

The key point about sedimentation equilibrium is that the concentration distribution at equilibrium depends only on molecular mass, and is entirely independent of the shape of the molecule. The precision of the molecular masses determined by this technique is usually 1-2%.

Following graph Figure 3 is the result for my experiment which is IgG fab fragment in buffer .

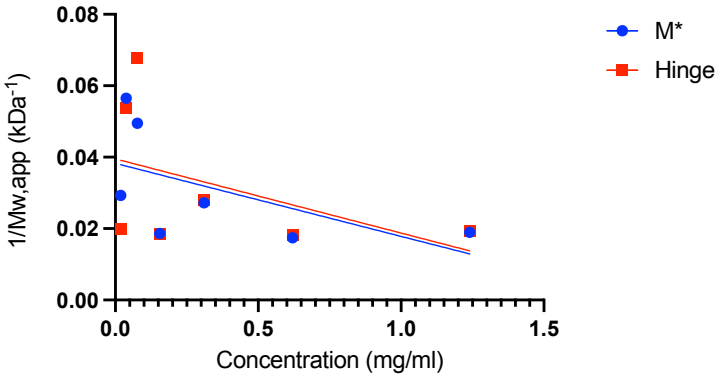


Figure 3 SE Analysis Graph. Measurement data points <0.5mg/ml are unreliable.

Sedimentation equilibrium was analysed by using MSTAR SEDFIT software.

Aggregation in isopropyl alcohol (IPA) and use of Dynamic Light Scattering

In IPA sedimentation velocity was not possible because of very large aggregate phenomena. However in Biology laboratories, dynamic light scattering (DLS) analyses are commonly used to detect aggregates in macromolecular solutions, quantify the size of proteins, nucleic acids, and complexes, and monitor ligand binding.

Aggregation is a phenomena which reduces the stability of the product and also involves the self-assembly of natural, unfolded protein or protein fragments into high molecular weight structures such as dimers, tetramers, oligomers, and even sub-micron or micron-sized particles(Kaur, 2021). Moreover, the aggregation in protein have increased the immunogenicity due to presence of multiple epitopes and reduced its therapeutic properties. As per the level of safety and its therapeutic concerns regarding the formation of aggregates, the guidelines have mentioned that limits the presence of the number of sub- visible particles (particulates) of size 10μm to 6000 particles/container and 25μm to 600 particles/container in the finished drug product (Kaur, 2021).

But using IPA as a solvent which has higher concentration and due to presence of alcohol group in IPA and amino acids in proteins it resulted in acid hydrolysis and formation of aggregates *Figure 4*. Aggregation leads to the degradation of therapeutic property of the protein.

When proteins are expressed at quantities higher than those seen in nature, aggregation is almost unavoidable(Zapadka et al., 2017).

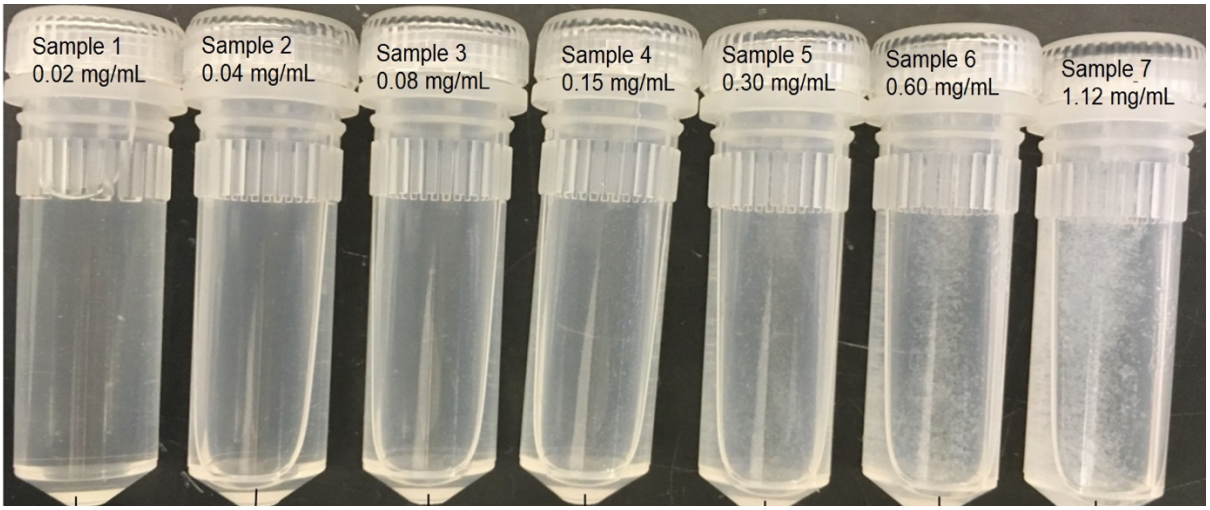
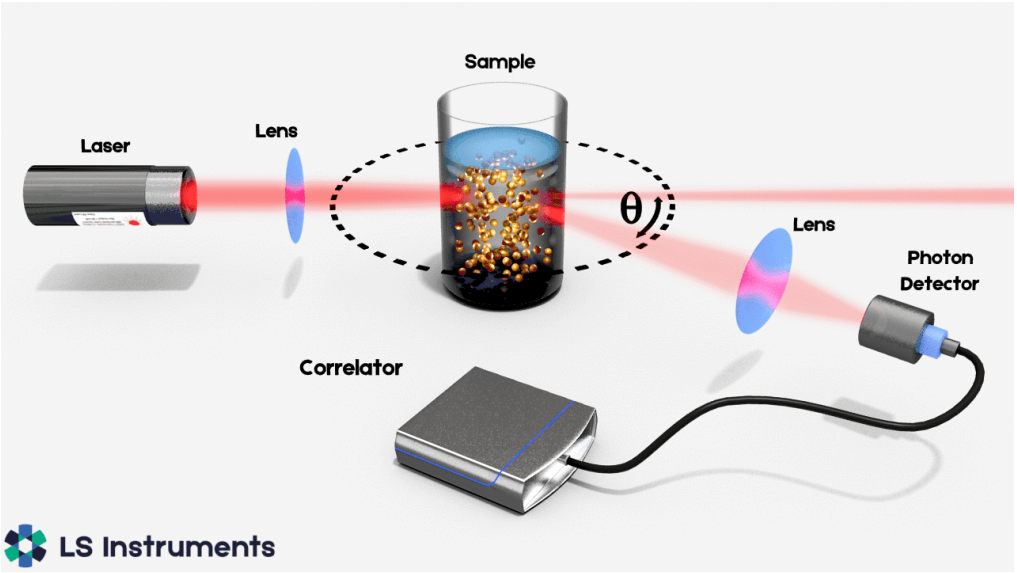


Figure 4 : Sample tubes for analysing the DLS .

Above, Figure 4 shows the clear aggregates in sample tubes numbered 5,6,7 having higher concentration of Iso-propyl alcohol.

Reversible aggregation is intrinsic to the native form of the protein and, being self-complementary in nature, the native protein surface self- associates to form reversible oligomers via weak non covalent interactions.(Bianco, 2018).

Since the experiment was mainly designed for the use of Iso-propyl alcohol as a non-aqueous solvent. IPA is volatile, colourless liquid with a sharp musty odour like rubbing alcohol. At higher concentration of it, denatures the protein and occurs in aggregation.
Analysing the sample with IPA resulted in aggregation and suitable analytical method was DYNAMIC – LIGHT Scattering (DLS) (Hoffmann et al., 2018)
DLS is an analytical method to analyse the aggregates in sample because it permits particle size down to 1 nm diameter, is one of the most common light scattering methods.
Emulsions, micelles, polymers, proteins, nanoparticles, and colloids are only a few examples of typical uses. The main idea is straightforward: A laser beam illuminates the sample, and a fast photon detector detects the fluctuations in scattered light at a specified scattering angle.



source : https://lsinstruments.ch/gallery/preview/431/dls-gif@2x.gif.

The diffusion coefficient *D* is then related to the radius *R* of the particles by means of the Stokes-Einstein Equation :

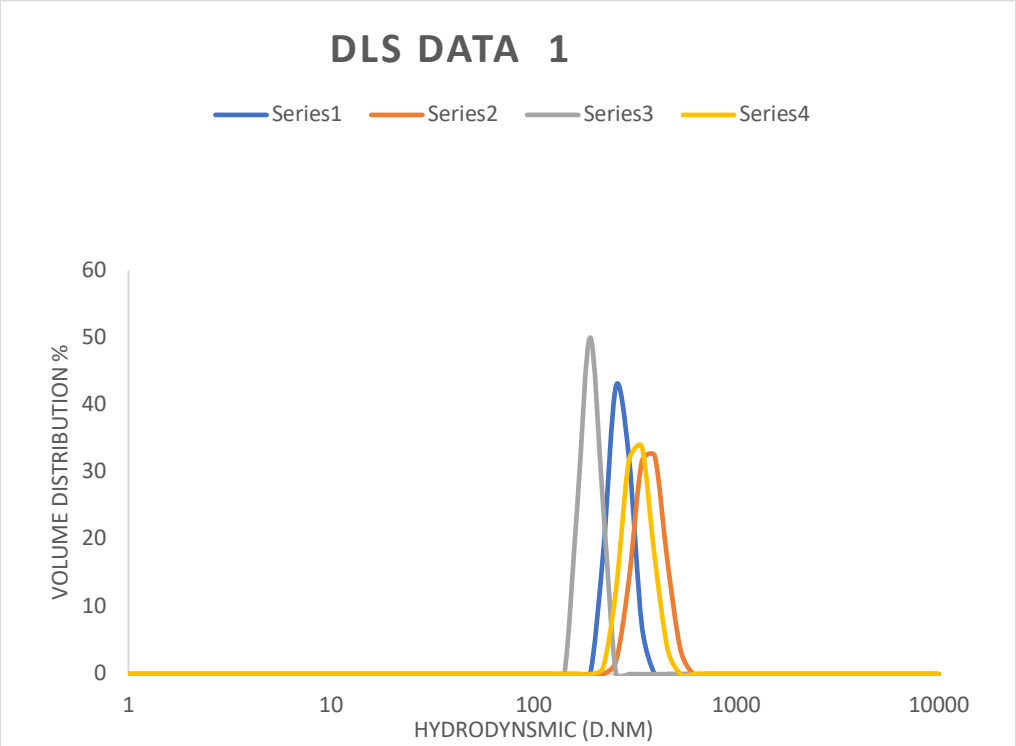
$$D = \frac{k_B T}{6\pi\eta R}$$

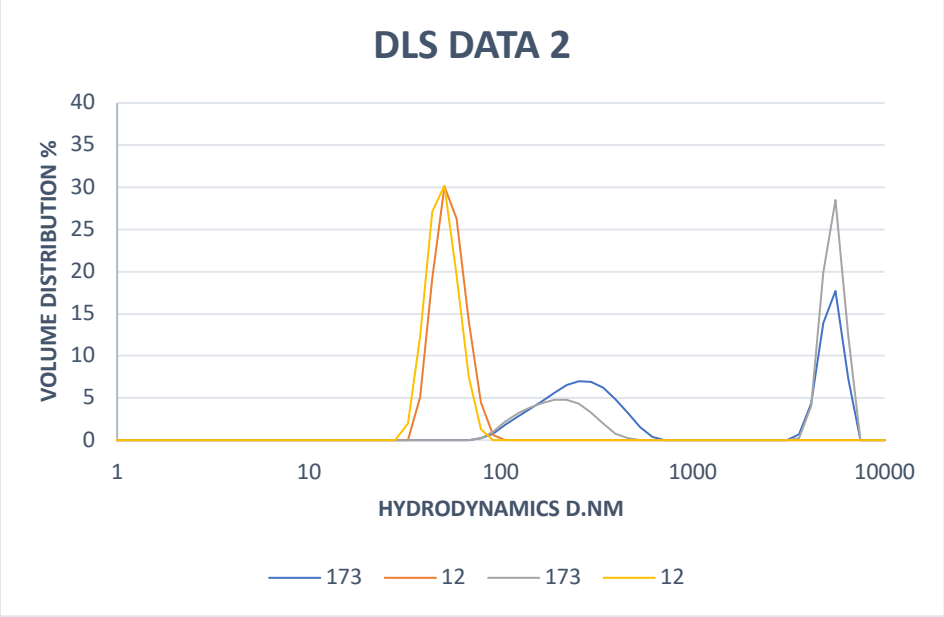
Where k_B is the Boltzmann-constant, *T* the temperature, and η the viscosity.

Dynamic Light Scattering (DLS) refers to an optical technique used for analysing dynamic properties and size distribution of a broad variety of physical, chemical, and biological systems composed of several suspended constituents (Postnov et al., 2020).

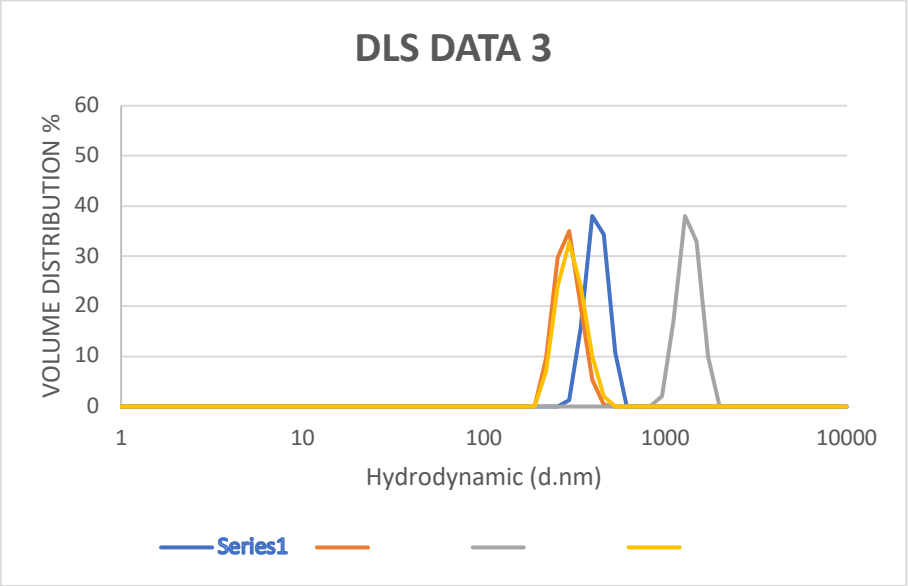
Results of DLS with IPA as:

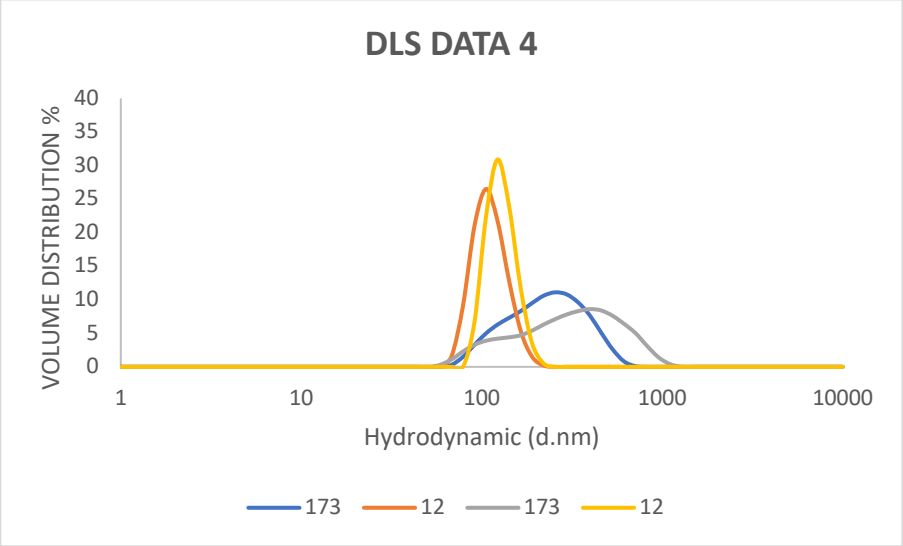
Aggregates were easily visible at higher concentration of IPA in sample tube and following graphs shows the aggregates of antibody fragment in IPA. Measuring angles for DLS were 173 and 13 degrees angle and choose multiple angle scattering.


DLS DATA 1
Series1
Series2
Series3
Series4
VOLUME DISTRIBUTION %
60
50
40
30
20
10
0
1
10
100
1000
10000
HYDRODYNSMIC (D.NM)


DLS DATA 2
VOLUME DISTRIBUTION %
40
35
30
25
20
15
10
5
0
1
10
100
1000
10000
HYDRODYNAMICS D.NM
173
12
173
12

”





Description of graphs :
Graph 1 and Graph 3 are unfiltered data set points where sample was not filtered with miniserts.
Graph 2 and Graph 4 are filtered data set points where sample was filtered using miniserts.

These graphs indicates the degree of aggregation and shows the size and volume distribution percentage in higher concentration sample tubes which sample tube -6 & 7.

How does aggregation occurs ?
In order to act efficiently as therapeutic molecules, proteins must generally be folded. Van der Waals and hydrophobic attractions between side-chain and backbone atoms; maximising hydrogen bonding; minimising steric clashes and energetically unfavourable bond torsional angles; maximising chain entropy; minimising (maximising) electrostatic repulsions (attractions); and minimising unfavourable interactions between amino acids and the solvent (water) and its co-solutes (Roberts, 2014; Wang, 2015)

How to minimise the aggregation ?

While it is presently impossible to anticipate when a protein will aggregate a priori, there are a variety of factors that impact whether and/or how soon protein solutions assemble. The following are the most important factors: solution conditions (pH, salt concentration and type, number and type of osmolytes present, and amphiphilic molecules such as surfactants); temperature; pressure; air-water contacts and other bulk water interfaces such as stainless steel (Roberts, 2014; Timasheff, 1998; Wang, 2015).

These are all parameters for a specific protein, and each protein reacts to changes in these "environmental" variables in a unique way. If the protein is generated recombinantly, it is also possible to change the molecular structure of the protein — a process known as protein engineering.

Viscosity was not able to perform for the IgG Fab in IPA because of high aggregation in IPA (Deokar et al., 2020; Hoffmann et al., 2018). When a non-organic solvent is added to an aqueous protein solution, it promotes protein unfolding, making the protein unstable. Changing the solvent property, on the other hand, may result in a decrease in protein solution viscosity. Due to high concentration of IPA resulted in aggregates formation which is difficult to measure which viscometer.

CONCLUSION :

Since Iso-propyl alcohol is a polar solvent. The hydroxyl group of the IPA molecule has a significant separation of electrical charge, in effect giving it both positive and negative end. Hence the protein interaction with IPA is a highly not suitable because of the formation of aggregates which is a form of physical instability. Aggregation occurs due to number of reasons such as during the thawing of antibody from freezer, change in buffer concentration, etc. Therefore, IPA is not suitable as a non- aqueous solvent. Because of the drawback of time constraints we could only analyse an IgG fab fragment which has 45kDa weight, which might be the one reason for the aggregation in IPA: low protein molecular weight, possessing less amino acids. We did not have time to analyse whole antibody molecules of larger molecular weight. We were also limited to using only one non- aqueous solvent due to time permitting. As stated above we couldn't perform AUC for IPA samples due to the formation of aggregates.

For future perspectives, in *Table 1 : POTENTIAL NON-AQUEOUS VEHICES IN PROTEIN POWDER FORMULATIONS.* a list of other non-aqueous solvents are given which could be used to analyse the stability of antibodies at higher concentration:

Table 1 : POTENTIAL NON-AQUEOUS VEHICES IN PROTEIN POWDER FORMULATIONS.

Group	Example	Viscosity

		[mPa·s] (Temp.)
Plant oils	•Sesame oil	51–61 (25 °C)
	•Safflower oil	52 (26°C)
	•Soybean oil	56 (25°C)
Medium chain triglycerides	•Triglycerides of Caprylicand Capric acid	23–27 (25 °C)
Propyleneglycoldiestersof mediumchainfattyacids	•Propyleneglycoldiestersof caprylicandcapricacids	9 (20 °C)
Fattyacidesters	•Ethyloleate	6(25°C)
	•Isopropylmyristate	5(25°C)
Polyethyleneglycol	•PEG200	48 (25°C

Esters	•Benzyl benzoate	8–9 (25°C)
	•Ethyl lactate	2(20°C)
Alcohols	•Benzyl alcohol	5(25°C)
	•Isopropyl alcohol	2.4 (25 °C)
Group	**Example**	**Viscosity [mPa·s] (Temp.)**
	•Ethyl alcohol	1.2 (25 °C)
	•Propylene glycol	39 (25°C)
Perfluorinated carbons	•Perfluorodecalin	6 (25°C)
Semifluorinatedalkanes	•Perfluorohexyloctane	3.44 (25 °C)
	• Perfluorobutylpentane	1.05 (25 °C)

This overview of potential vehicles use in protein-powder suspensions are yet to be. reviewed for administration of protein-powders via. Sub-cutaneous route. This table is adapted from several literatures and combined in form of table (Marschall *et al.*, 2021)

Use of stabilizers : In a liquid condition, proteins usually require a formulation excipient(s) as a protein stabiliser. The conventional preferred interaction method and/or additional suggested mechanisms such as nonspecific contact with surface hydrophobic pockets or charged amino acids, selective ligand binding, and increase of solution viscosity can all be used to stabilise proteins.

Protein aggregation can affect biopharmaceutical quality in a variety of ways. There are a variety of different “types” of aggregates, and there is currently little knowledge of the link(s) between the physical and chemical properties of the various aggregate types and a given product attribute, so new technologies for experimental characterization of protein products would be extremely beneficial to the field.(Roberts, 2014; Zapadka et al., 2017). Stabilizing excipients are added to formulations to slow down or prevent protein aggregation through different mechanisms, including strengthening of protein-stabilizing forces, destabilization of the denatured state, and direct binding to the protein, which are applied during isolation and purification, drying

(lyophilization, spray-drying, spray-freeze drying, foam-drying), storage in solution or after drying, and reconstitution after drying, according to Kemter.

Six categories of excipients are commonly used to stabilize proteins against aggregation, according to Kang: buffers, salts, amino acids, polyols/disaccharides/polysaccharides, surfactants, and antioxidants. These excipients prevent aggregation through several mechanisms. “First, pH is critical to protein stability and must be controlled to an optimal value through the use of appropriate buffers. Salts and amino acids increase the ionic strength of solutions while minimizing electrostatic interactions between protein molecules . (Cole et al., 2008; Harding et al., 2015; Marschall et al., 2021)

In summary, further research can be done using different non-aqueous solvents and whole antibody instead of a fragment.

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