Particle characterization and analysis in therapeutic protein formulation

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IN

BIOTECHNOLOGY

By

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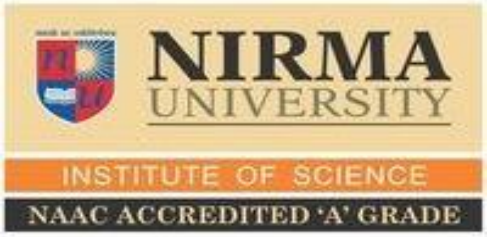

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List of Abbreviations

US - United states

EU - European

SbVP - Subvisible Particle

LO - Light Obscuration

USP- United States Pharmacopeia

MFI - Micro Flow Imaging

FDA - Food and Drug Administration

API - Active Pharmaceutical Ingredient

USP - United States Pharmacopeia

LPC - Liquid Particle Counter

Abstract

The aim of the study was characterization and analysis of particles in protein formulation. Characterization of particles was carried out by compendial method such as light obscuration (LO). But it has limitations for detecting various particles such as glass, silicone oil droplets, air bubble, proteinaceous and non-proteinaceous particles. The US and EU pharmacopeias require sub-visible particle (SbVP) analysis of parenteral drug products. hence there was a requirement of an orthogonal method which can analyze, quantify and characterize particles on parameters such as size, morphology, optical density etc.

Micro Flow Imaging (MFI) was the orthogonal method available which could characterize and quantify particles on the basis of their morphology. Therefore, the main aims of the study were 1) Development of a method which could differentiate between proteinaceous and non-proteinaceous particles and also transluscent and transparent particles. 2) Comparative evaluation of compendial method i.e. LO and orthogonal method i.e. MFI. The method development for MFI and comparative evaluation was carried out using polystyrene beads standards, which showed comparable results. After that placebo and real protein formulation were analyzed for the same. MFI was able to provide more information regarding particle morphology and the particle subpopulation based on their morphological filters. Considering advantages and limitations of both methods, MFI was proved to be more sensitive method with respect to counting accuracy and size accuracy. hence quantification and characterization of protein formulations with both LO and MFI had provided more information regarding the particle morphology and particle nature, which helps in the development of stable and potentially safe protein therapeutic products.

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Introduction
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4.1. Protein Formulations:

In recent years, the major drugs approved by the FDA are Biopharmaceutical drugs, also known as a Biologic medical products or Biologics (Sarah Zolls et al.,2011). The pharmaceuticals that are biological in nature, manufactured in extracted from, or semi synthesized from biological sources. Biopharmaceuticals have revolutionized the treatment for many diseases like diabetes, cancer, hepatitis and multiple sclerosis etc. and used for therapeutic purposes or in vivo diagnostics and it should be clinically effective, approvable by regulatory authorities and commercially viable (Rader et al., 2008).

These can be categorized into broad range of products such as:

1. Cytokines
2. Enzymes
3. Hormones
4. Clotting factors
5. Vaccines
6. Monoclonal antibodies
7. Therapeutic proteins-based drugs
8. Cell therapies and gene therapies

The main advantages of biologics are its potential target specificity, lower side effects and the potential to actually cure diseases rather than just treating the symptoms. Biologics are different in many aspects from conventional drugs, from manufacturing techniques, molecular size and complexity, stability of molecules to clinical properties (Sarah Zolls et al.,2011).

Though it has many advantages, it encounters major challenges, which mainly includes chemical and physical instability. Chemical degradation includes deamidation, isomerization, hydrolysis, racemization, oxidation, disulfide formation and β-elimination. While physical stability can be termed as the capability of a protein to maintain its tertiary structure which is essential for biological activity. So, the loss with this leads to physical degradation, which involves reversible or irreversible denaturation with loss of tertiary structure and unfolding with reactions like chemical degradation, aggregation and precipitation. This will develop several side effects, immunogenicity and allergic reactions, on administering in to the patients. To prevent such reactions, there is a strong need for the development of highly pure and stable biopharmaceutical products has arisen (Kuriakose et al.,2016; Ratanji et al.,2014, Derrick et al., 2014; Rosenberg et al.2006; Wang et al., 2015).

The morphology of protein particles in protein formulation is highly variable, from spherical aggregates to long, irregular fibers. These are surface-active agents and are drawn to hydrophobic interaction, which leads to unfolding and following aggregation (Ripple et al.,2014).

As proteins in formulations have dynamic structures, which are prone to aggregation and unfolding processes. To conserve the native structure and biological activity of the proteins, it requires some formulation excipients as a protein stabilizer in a liquid state to minimize the adverse effects of aggregation. This is the main challenge to formulate protein in such a way that it retains its clinical significance without losing biological activity (Ripple et al.,2014).

Besides this, protein formulation comprises of reversibly or irreversibly related protein molecules ranging in size from small oligomers to large aggregates of micrometers size. In which, protein particles signify a very low portion by mass of the total protein, which reduces safety concerns. But it limits method development and characterization and understanding about the origin and causes particles formation (Rosenberg et al., 2006; Wang et al., 2015).

Therefore, some main aspects that's need to be focused on such, the kinetics of formation of protein particle, the structure of protein particles and physicochemical characteristics of protein particles with respect to patient's immune system. Therefore, due to the various sources of introduction, products need to be screened at each stage of manufacturing, at different batches and delivery devices. Hence there is rising necessity to understand the morphology of particles in biopharmaceutical products, raised by significant advancements in particle analysis and concerns related to potential impact of particles on product quality and safety (Ratanji et al., 2014; Tovey et al., 2011).

4.2. Characterization of particles in protein formulation:

4.2.1. Types of Particles:

Particles in therapeutic proteins have different characteristics by count, size, morphology, chemical composition, and other physicochemical properties. particles can be categorized on the basis of size into: nanometer-sized aggregates, sub micrometer particles up to 1 µm, 1 µm to 100 µm termed as "subvisible", and greater than 100 µm termed as "visible". Particles are dynamic and can easily change (count, size, morphology, etc.) in response to minor changes in their environment**.**

Submicron particles	Subvisible particles	Visible particles
• < 1 µm	• 1- 100 µm	• > 100 µm

Figure 1: Represents range of particles in therapeutic protein formulation

Beside this, on the basis of chemical- composition particles can be classified as: homogeneous i.e. chemical entity and source for e.g. protein. or heterogeneous i.e. protein with a coating of non-protein entity which includes fiber, glass particle, silicone oil droplet (Ratanji et al., 2014). So, it is necessary during formulation development to differentiate the particles on the basis of size, shape or structure, depending on the various conditions the protein has been exposed, which lead towards the susceptibility of the protein to different stress conditions and the identification of the cause (Sarah Zolls et al., 2011).

Various formulation developmental studies, packaging material or excipients, manufacturing process or intrinsic particles from the container closure, or aggregation of protein API that develop during the product's life cycle and storage conditions are the major source for the formation of particles (Demeule et al., 2010). So, it is critical to monitor the presence of these particulates to ensure the safety and efficacy of drugs, therefore, development of formulation can be highly sensitive and of great importance (Ratanji et al., 2014).

Table 1: Typical particles found in therapeutic protein formulation

EXTRINSIC PARTICLES	INTRINSIC PARTICLES
Fibers – shed from clothing filters or packaging	Protein aggregates – formed during manufacturing or storage as a result of: • Protein-protein interactions • Protein-air/liquid interface interactions • Protein-container interactions • Protein-contaminant interactions
Dust – from the environment	Silicone oil – used to lubricate moving parts in devices, such as syringe barrels; introduced during drug administration
	Fragments of glass, plastic or rubber – shed during manufacturing or packaging, including shreds of rubber from stoppers and shards of plastic or glass from vials or devices

(Corvari et al., 2015)

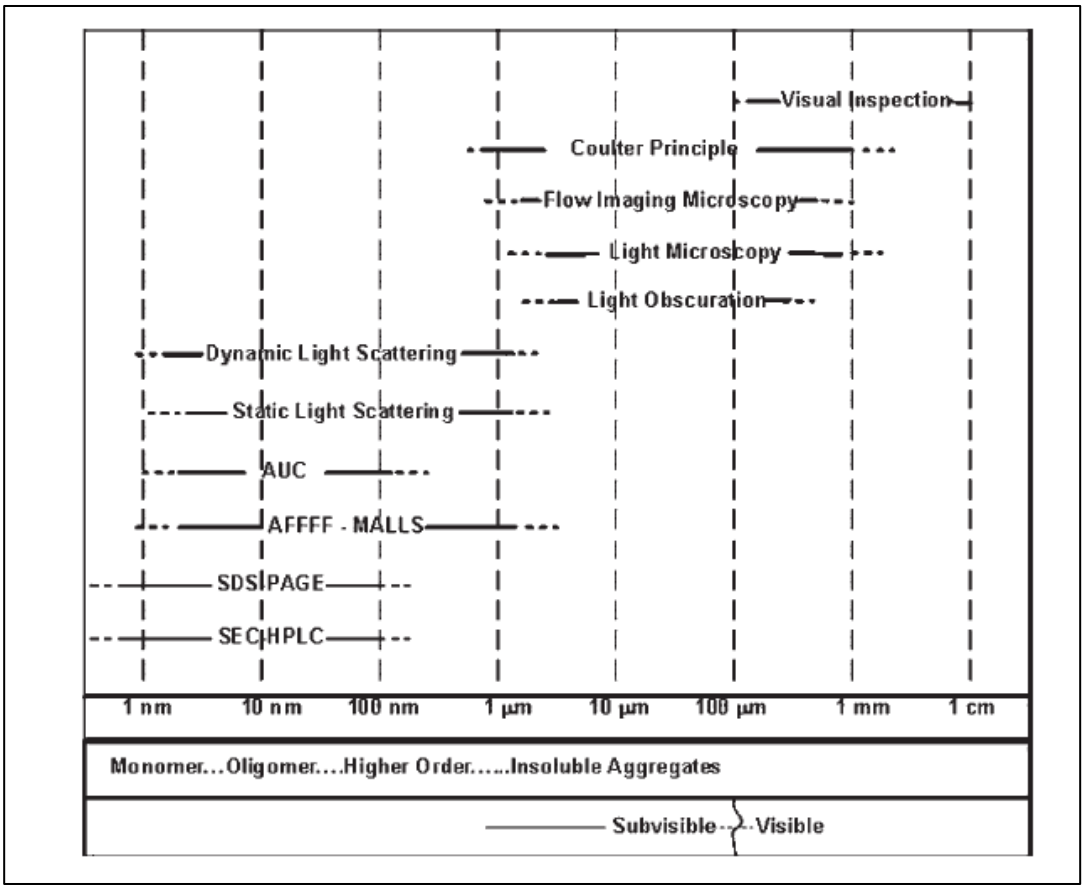
Along with these particles have properties such as count, size, morphology, optical density, chemical composition, and other physicochemical properties (Corvari et al., 2015). which are normally used as parameter for the analysis, quantification and characterization of particles.

Table 2: Overview of measurable particle properties.

Size	Hydrodynamic diameter Equivalent circular diameter (ECD) Equivalent spherical diameter (ESD) Ferret diameter Molecular weight
Concentration	Total particle concentration Size distribution
Shape	Aspect ratio Circularity
Optical Properties	Transparency Refractive index
Identity	Chemical identity (proteinaceous vs. non-proteinaceous) Further characterization of proteinaceous particles (secondary/tertiary structure)

(Sarah Zolls et al., 2011)

The accepted range for visible and sub visible particles in parenteral products are restricted by the pharmacopoeias, which makes quantification of protein particles very crucial. There cannot be single method which can give information about every aspect of particle size, count, morphology, origin and cause. The need for the method to characterize and quantify particles depends on the type of analysis being done. So, there are various methods with different purposes, principles and applications being used for the identification, quantification and characterization of particles in biopharmaceutical industry for intended range.



(Sarah Zolls et al., 2011)

Figure 2: Depiction of the approximate size range of analytical methods for size determination of subvisible and visible particles.

4.3. Role of particles in Protein aggregation:

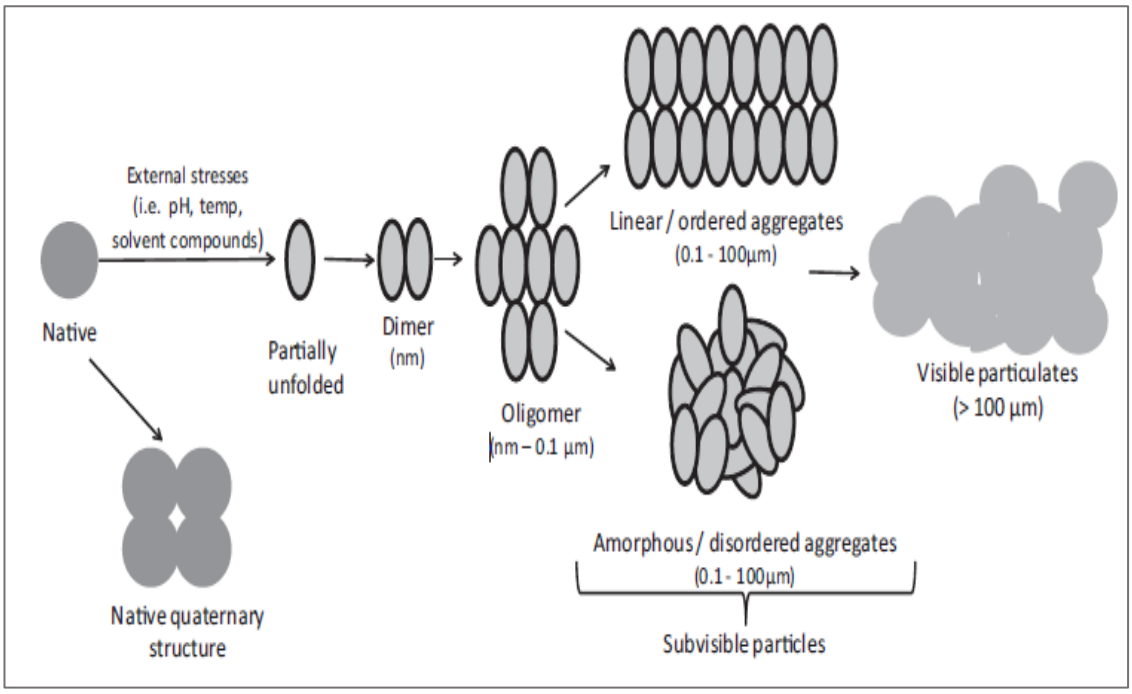
Protein aggregation is one of the main challenges in the development of protein biotherapeutics. The aggregates formed, are the assemblies of the protein monomers and their size ranges from dimers to large aggregates ranging from nanometer to hundred microns, which are visible to the human eye (Scherer et al., 2012). Formation of the Particles (visible and sub visible) in biotherapeutics are the major concern during development of drug. This leads to potential safety concern, critical quality issues and also impact drug activity (Sekhon et al., 2010). Hence these particles are to be analyzed before administration in to the patients, due to increase risk associated with immunogenicity it generates if it remains in the body for long time.

Aggregation comprises of interactions which causes self-association of protein molecules into assemblies except native quaternary structure. They can range from dimers to subvisible and visible particles, they include covalent or non-covalent linkages, ordered or disordered in structure, soluble or insoluble, and reversible or irreversible formation (Narhi et al., 2012).

The protein particles can be formed by adsorbing at interfaces such as air, solid, or liquid, environmental factors, temperature, most commonly by mechanical stress leads towards;

a) Partial or full unfolding of the protein leads to association with other proteins or particles
b) Adsorption of protein (unfolded) to a non-protein particle or interface

These demonstrates the need to characterize particles and to elucidate chemical composition of particles and the mechanisms behind. This will help to minimize and control particle formation. The exact mechanism of protein aggregation is not fully understood and main reason behind which is considered to be the role of partially folded entities. Hence quantification and Characterization of the protein is necessary at each stage of formulation development and quality of product. and it is essential for the regulatory submission (Ratanji et al., 2014; Ripple et al., 2014).



(Narhi et al., 2012).

Figure 3: Schematic model of protein aggregation

4.4. Implications of particles in protein formulation:

Protein therapeutics derived from endogenous human protein is capable of eliciting immunogenicity in the patients. Therefore, the main focus of the clinical studies is on the consequences related to sub visible and visible particles on parenteral formulation.

4.4.1. Immunogenicity of drug:

It is the ability to induce undesirable immune responses. The development of high affinity anti-therapeutics antibody response is the most common effect found during the administration of protein therapeutics. It may lead towards the reduced efficacy of the drug, occasionally life-threatening autoimmunity and adverse conditions like Anaphylaxis, Cytokine Release Syndrome, Infusion Reactions (Corvari et al., 2015; Jorgensen et al.,2009; Kamerzell et al., 2011; Kuriakose et al., 2016; Ratanji et al., 2014; Rosenberg et al., 2006; Subramanyam et al., 2006; Tovey et al., 2011)

A. Patient-Specific Factors That Affect Immunogenicity

1. Immunologic status and competence of the patient:

Patients who are immunologically suppressed may be at lower risk of eliciting immune responses to therapeutic protein products compared to healthy persons with normal immune responses.

2. Sensitization/History of allergy

Exposure to a therapeutic protein product or to a structurally similar protein may lead to pre-existing antibodies at baseline. Sensitization to the excipients or process/product-related impurities of a therapeutic protein product may also predispose a patient to an adverse clinical consequence.

3. Route of administration and dose

Route of administration can affect the risk of is foremost important. Basically, intradermal, subcutaneous, and inhalational routes are related to increased immunogenicity with respect to intramuscular and intravenous (IV) routes. The IV route is generally considered to generate least immune response following dose.

4. Genetic Status

Genetic factors may alter the immune response to protein therapeutics. human leukocyte antigen (HLA) haplotypes may predispose into patients to elicit undesirable antibody responses to specific products.

5. Status of Immune Tolerance to Endogenous Protein

Humans are not evenly immunologically tolerant to all endogenous proteins. Thus, the robustness of immune tolerance to an endogenous protein affects the ease with which a therapeutic protein product counterpart of that endogenous protein.

B. Product-Specific Factors That Affect Immunogenicity

Product-specific factors may increase or decrease the potential for and the risk associated with an immune response. Following factors are considered with respect to immunogenicity related to product (Corvari et al., 2015; Jorgensen et al.,2009; Kamerzell et al., 2011; Kuriakose et al., 2016; Ratanji et al., 2014; Rosenberg et al., 2006; Subramanyam et al., 2006; Tovey et al., 2011).

1. Product Origin (foreign or human)

Origin of product is an important factor to generate Immune responses against proteins derived from natural sources or foreign antibodies can develop to the desired therapeutic protein product and other foreign protein components potentially harmful which are present in the product.

2. Primary Molecular Structure/Posttranslational Modifications

Primary sequence, higher-order structure, species origin, and molecular weight of therapeutic protein products are all crucial factors that may provoke immunogenicity.

3. Quaternary Structure: Product Aggregates and Measurement of Aggregates

Protein aggregates are defined as self-associated protein species. Aggregates are categorized on characteristics: size, reversibility/dissociation, conformation, chemical modification, and morphology.

4. Glycosylation/Pegylation

Glycosylation may strongly modulate immunogenicity of therapeutic protein products. Glycosylation indirectly changes protein immunogenicity by minimizing aggregation and by shielding immunogenic protein epitopes from the immune system. Pegylation has lead to reduce their immunogenicity and causes loss of product efficacy and adverse safety concerns.

5. Impurities with adjuvant activity

Adjuvant activity can arise through multiple mechanisms, including the presence of microbial or host-cell-related impurities in therapeutic protein products.

6. Immunomodulatory Properties of the Therapeutic Protein Product

The immunomodulatory activity of any given therapeutic protein product critically influences immune response directed to itself and also to other co-administered therapeutic protein products, endogenous proteins, or even small drug molecules.

7. Formulation

Formulation components are principally chosen for their ability to preserve the native conformation of the therapeutic protein in storage by preventing denaturation due to hydrophobic interactions, as well as by preventing chemical degradation, including oxidation, and deamidation. Formulation may also affect immunogenicity of the product by altering the amount leachable from the container closure system.

8. Container Closure Considerations

Interactions of protein products with container closure leads towards decrease in product quality and causes immunogenicity. It is common in prefilled syringes of products. These syringes are composed of multiple surfaces and materials that interact with the therapeutic protein product over a prolonged time period and thus have the potential to alter product quality and immunogenicity.

4.4.2. Bioactivity:

It can be defined as the beneficial or adverse effects of a drug on living matter. For a drug to be an effective and to make it suitable for use, it should not be only target specific but also acquires the proper adsorption, distribution, metabolism, excretion. If the concentration of particles increases in the parenteral formulation, it will lead towards the immunogenicity and indirectly towards decrease in bioactivity of the protein-based products.

4.4.3. Shelf life of the product:

It can be described as the period of time from the data of manufacture, the drug product is expected to remain within its approved specification while stored under defined condition. Particle concentration has major impact on shelf life of protein formulation, which makes product less commercially viable.

Therefore, considering these implications of protein aggregates on protein formulations, measurement and characterization of these entities are found to be of great importance.

Hence quantification of sub visible particles larger than 10 μm and 25 μm in parenteral is commonly performed using LO. But for therapeutic protein products regulatory agencies increasingly ask for quantification and characterization of particles with a size below 10 μm. Furthermore, the availability of an increasing number of other orthogonal techniques, has raised the need for particle analysis tools and which enable the characterization of the particles to greater extent (Sharma et al., 2010). There cannot be one method which can characterize and quantify all the aspects of particle analysis; hence there is a growing need for the other methods which can fulfill the requirements raised by the regulatory authorities. Below is the table which describes the other orthogonal methods used during formulation development for the particle analysis in protein formulation based on the protein of interest and intended application.

Table 3: Overview of analytical methods for particle analysis, optical quantification methods

Principle	**Visual inspection**	**Visual inspection**	**Microscopic methods**	**Microscopic methods**	**Light absorption / blockage**	**Light scattering**	**Light scattering**
Method	Human or automated visual inspection	Flow Particle Image Analyzer	FlowCAM	Electron microscopy	Nephelometry / turbidimetry	Dynamic light scattering	Nanoparticle tracking analysis
Further information	Detects only presence of visible particles		-	-	Detects only presence of particles	-	-
Size	-	Different diameters (ECD, Feret diameter)	Different diameters (ECD Feret diameter)	Limited	-	Hydrodynamic diameter	Hydrodynamic diameter
Size distribution	-	Limited	Yes	-	-	Limited	Limited
Shape	-	Aspect ratio; circularity	Aspect ratio; circularity	Limited	-	-	-
Structure	-	-	Transparency related values	Surface morphology	-	-	-
Identity	-	-	Distinction of protein vs. non-protein material by selective fluorescent dyes	-	-	-	-

(Zölls et al., 2013)

4.5 Specification:

According to the United States Pharmacopeia (USP), there are requirements for testing and characterization of sub visible (>10 um) and visible (>25 um) particles of therapeutics. Until now particles sized above 10µm have gained attention in development of therapeutic protein product but detection and characterization of sub visible particles within the range 1-10µm is still developing (USP<788>, USP<787>).The Compendial methods used for the analysis of particles are light microscopy and Light obscuration but both the methods have certain limitations, such as unable to differentiate between translucent and transparent particles and proteinaceous or non-proteinaceous particles. Hence there is a need for the development of an orthogonal method i.e. Micro flow imaging (MFI), which may overcome the limitations and helps in the detection, quantification and characterization of particles in the protein formulation (Ripple et al.,2012).

Table 4: Specification given by USP General chapter <788>, <787> and <789>

	USP<788>	**USP<787>**	**USP<789>**
Product	Parenteral products	For protein-based therapeutics	For ophthalmic solution
Method	Quantitative LO and microscopic count test	LO and qualitative microscopic count test	LO and microscopic count test
Sample volume	≥ 5 Ml	0.2 to 5 mL	Not specified
General limits	_	(Small volume injectables) ≥ 10 µm→ NMT 6000 ≥ 25 µm → NMT 600	(for LO) ≥ 10 µm→ NMT 50 per mL ≥ 25 µm→ NMT 5 per mL
Specifications	(Large volume injectables) ≥ 10 µm → NMT 25 ≥ 25 µm → NMT 3	_	(for microscopic test) ≥10 µm→ NMT 50 per mL ≥ 25 µm→ NMT 5 per mL ≥ 50µm→ NMT 2 per mL

(USP General chapter <788>, <787> and <789>)

4.6 Compendial methods for analysis:

Compendial is related to compendium which termed as standard. With respect to analysis of protein formulation, compendial methods are referred to as standard methods for the analysis of protein formulation.

4.6.1. Light Microscopy:

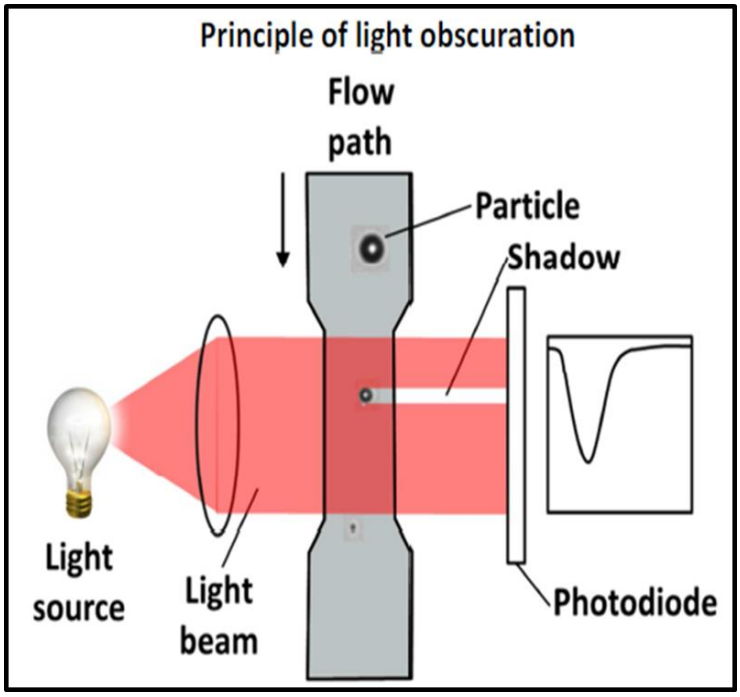
Light microscopy is a very effective technique for the analysis of particulate matter in to protein sample. It allows visualization, counting and sizing of particles in the range of 1 μm to several mm. The method is mentioned in the US and EU Pharmacopeia for the analysis of subvisible particles along with light obscuration. (Corvari et al., 2015; Demeule et al., 2010)

4.6.2. Visual inspection:

The USP requires parenteral preparations to be "essentially free from visible particulates". This method is limited to use with particles that are greater than 80 mm in size. By definition describes the examination of particles detectable by the human eye without any auxiliary equipment. As a limitation, it only distinguishes between absence and presence of visible particles and does not provide information about particle properties such as number, structure or origin. (Corvari et al., 2015; Demeule et al., 2010)

4.6.3. Light Obscuration:

At initial stages of the formulation development, light obscuration was the only method to measure sub-visible particles in biopharmaceutical products. This technique mainly depends on the ability of a particle to decrease the measured light intensity when passing through a light beam. Meanwhile this standard light obscuration instrument has detection range between 2-400 μm. Basically, it reports results in the form of particle concentration i.e. (counts/mL). These instruments are calibrated with polystyrene standards. Moreover, the technique is sensitive to air bubbles, which could be introduced during sample preparation or analysis. Analysis of highly-concentrated protein solutions or formulations can be difficult. It is incapable for the analysis of protein formulation with high viscosity. The main limitations of this method are to discriminate between the proteinaceous and non-proteinaceous particles. Another major disadvantage is that it is unable to differentiate between transparent and translucent particles. So, to overcome these hindrances, there is need for an orthogonal method which can endure over such difficulties. (Sharma et al., 2010; Zölls et al., 2013, Narhi et al., 2009)



(Zölls et al., 2013)

Figure 4: principle of Light Obscuration

4.7 Orthogonal method:

Orthogonal methods are methods that use fundamentally different principles. In this context, MFI is the orthogonal method that provides very different selectivity to the primary method i.e. LO.

4.7.1. Micro Flow Imaging:

Flow microscopy techniques have gained attention in the area of formulation development from past two decades due to its sensitivity, ability to capture digital images of the particles from the parenteral products and robustness. It does not only give accurate counting but also discriminates particles based on their morphology. It works on the principle of flow imaging in which continuous sample is pumped by a peristaltic pump through a flow cell and bright-field images are captured in successive frames by a high-speed sensitive digital camera and the images are analyzed based on variations in the transmitted light intensity. This system permits counting and examination of all particles present in the protein formulation based on morphological filters. It is able to detect particles in the range of 1 to 70 µm. it has minimum sample volume requirement of 0.5 mL, which is more suitable for the parenteral products and full fill the need for small volume requirements. It has many advantages over light obscuration such as the ability to discriminate between the proteinaceous and non-proteinaceous particles. Another benefit is to differentiate between transparent and translucent particles. A good example is silicon oil droplet which is well characterized in the images captured by the MFI. It can also endure sample with high viscosity and has no effect on refractive index of the medium. (Sharma et al., 2010; Zölls et al., 2013, Narhi et al.,)

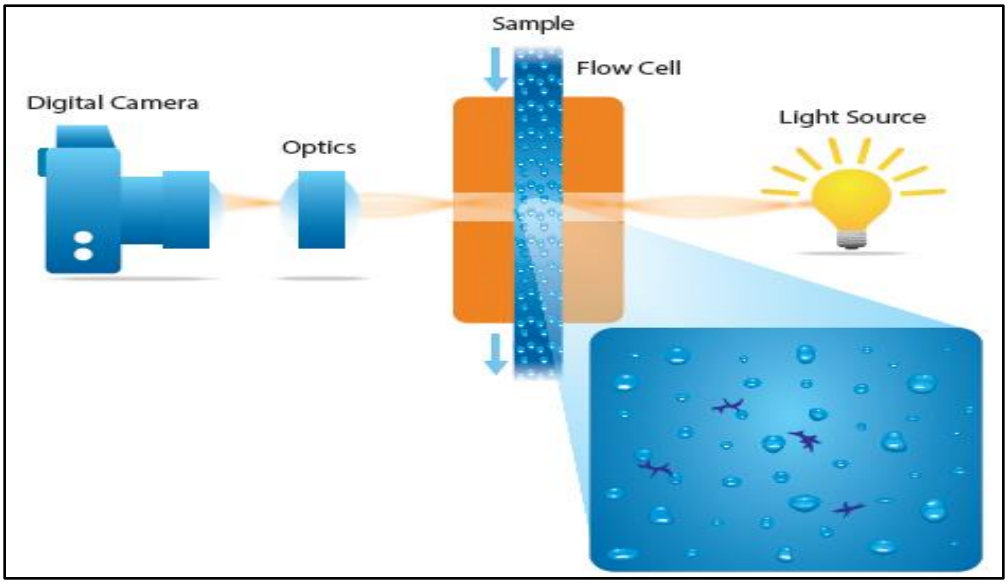
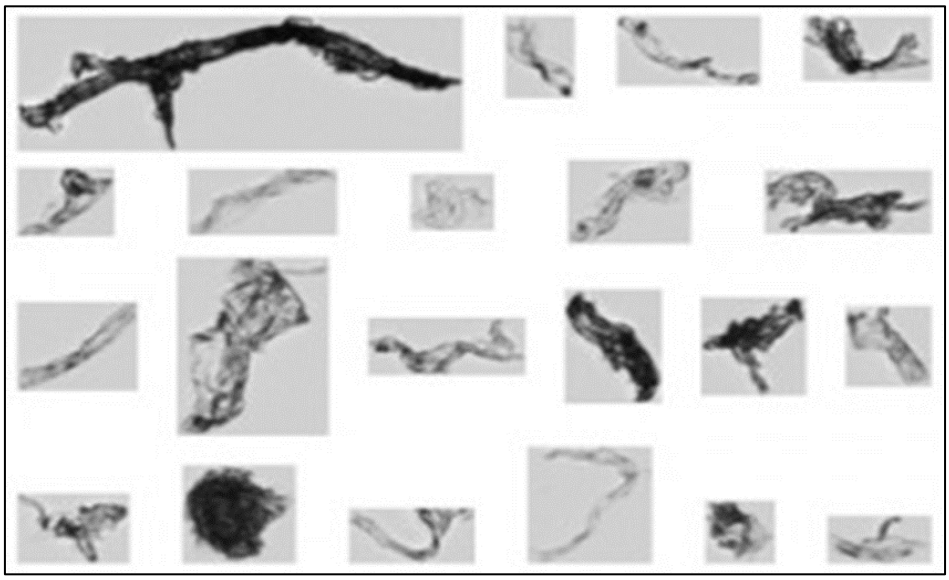


Figure 5: Principle of Micro Flow Imaging

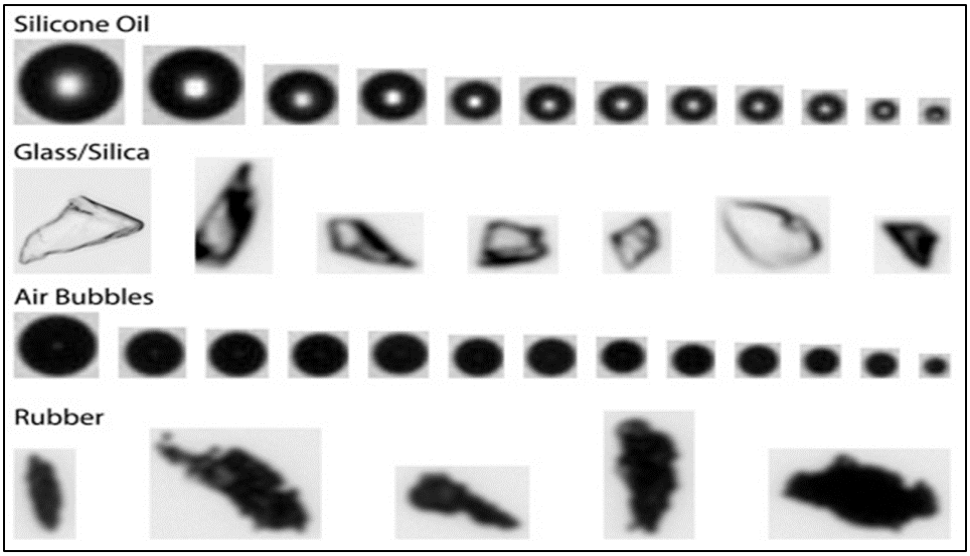
The below diagram shows the images of typical protein aggregates, which are captured by the MFI software. It is able to detect and identifies various types of protein aggregates from the protein formulation irrespective of its shape.



(Protein simple)

Figure 6: Depicts various types of typical aggregates found in the protein formulation

The images captured by MFI software and how it can differentiate silicon oil droplets, glass or silica, air bubble and rubber.



(Protein simple)

Figure 7: Shows the difference between subpopulation of particles captured by MFI

MFI reports results mainly on basis of following parameters (Zölls et al., 2013),

ECD (Equivalent circular diameter): It is expressed in microns and represents the diameter of a sphere that occupies the same two-dimensional surface area as the particle.

Aspect Ratio: A value between zero and one (unit-less) that represents the ratio of the minor axis length over the major axis length of an ellipse that has the same second-moments as the particle.

MFD (Maximum Feret Diameter): The **Maximum Feret Diameter** is expressed in microns and represents the longest dimension of the particle independent of its angular rotation at the time the image was captured.

Circularity: A value between zero and one (unit-less) that represents the ratio of the circumference of an equivalent area circle over the measured perimeter**.**

4.9. Comparison of Light Obscuration and Micro Flow Imaging

Table 5: Comparison of LO and MFI

Aspects	Light obscuration (LO)	Micro flow imaging (MFI)
Principle	Light obscuration	Microscopic imaging
Result reporting	▪ Particle concentration (counts/mL)	▪ Particle concentration (counts/mL) ▪ Shape/ morphology
Detection range	2 to 400 µm	1 to 70 µm
Minimum sample volume	25 mL (for 5 mL syringe) 2.5 mL (for 1 mL syringe)	0.5 mL
Sampling efficiency	≤ 60%	80-85%
Particle concentration range	≤ 18,000 counts/mL	≤ 850,000 counts/mL
Effect of RI	Yes	No

(Zölls et al., 2013)

Above table gives comparative information on different aspects of MFI and LO. As from the literature MFI was proved to have high throughput due to various aspects such as, high detection range, minimum sample volume requirement, high sampling efficiency and high particle concentration range than LO.

Objectives

4. Objective:

- To analyze particles in protein formulations using Compendial and orthogonal techniques
- Comparative evaluation of light obscuration and micro flow imaging methods
- To determine the size, shape and morphology of particles
- Separation of proteinaceous and non- proteinaceous particles using micro flow imaging

Materials and Methods:

6.1. Materials:

i. Polystyrene Standards:

COUNT-CAL Polystyrene NIST particle size standards were obtained from Thermo Scientific with particle sizes of 2, 5, 10 and 25 μm and concentrations of 3000 particles per ml. Standards were vortexed for 1minute and then allowed to settle down. Before the initiation of analysis, standards were tumbled 15-20 times.

ii. Protein formulations:

Placebo and Protein formulations were provided by the Drug Product Development (DPD) department of Intas Biopharma division from previously prepared batch, termed as protein formulation - I, Protein formulation - II and Protein formulation - III respectively. Protein formulations were allowed to keep at RT prior 10 minutes before analysis. Then it was vortexed for 1minute and then it was allowed to settle down. Before the initiation of analysis, formulations were tumbled 15-20 times.

6.2. Methods:

i. Liquid Particle Counter:

LPC is the standardized instrument works on the principle of Light Obscuration, which determines the particle count, particle concentration and size of the particles (visible and sub visible) within the standard range of 10µm and 25µm and also up to size range of 5µm.

The analysis was performed using a HIAC/Royco 3000A Liquid Syringe Sampler (termed HIAC) with a HRLD-150 sensor with a 780 nm laser light with maximum particle concentration limit of 18,000/ml. The software PharmSpec version 2.0 under laminar air flow (LAF) conditions.(Demeule et al., 2010; Frahmet al., 2016; Sharma et al., 2010; Zölls et al., 2013)

Normally test is done for the Small Volume Injection(SVI) and Large Volume Injection (LVI). As per the USP Guidelines, there are general limits for particles per container and particles per mL respectively, For SVI 6000 → >10µm and 600 → >25µm And for LVI 25→ >10µm and 3 → >25µm. As it is the preferred method for the determination of Subvisible particles in therapeutic protein injection. It comprises of (1) Blank test which is done in order to check the Environment suitability, Glassware cleanliness and water or suitable solvent which involves (a) Environment test and (b) Procedural Blank test. The syringe is stored under 70% IPA and Between each sample analysis, the system was rinsed with water for injection. for system suitability system is flushed with 100% IPA prior to analysis. Sample measurement consisted of four injections at a volume of 5 ml each. For the small sample volume, each sample measurement was performed three times at a volume of 0.4 ml as stated in the USP general chapter <787>. The first injection was discarded and the result is reported in cumulative counts/ ml. (Sharma et al., 2010; Zölls et al., 2013)

ii. Micro Flow Imaging:

An MFI5200 system (Protein Simple) equipped with a 100 µm flow cell and controlled by the MFI View System Software (MVSS) version. The system was flushed with 0.6 mL purified water at maximum flow rate. Samples of 0.6 mL were analyzed at a flow rate of 150 µL/min and a fixed camera rate leading to a sampling efficiency of about 80–85%. MFI works on the broad spectrum of Digital microscopy, Micro- Fluidics and Image processing, which is an automatic instrument for the analysis of particles in the suspended liquid (protein formulation), It captures the Real Time Bright field images as sample passes through the flow cell sensing zone and it analyzes the particle size, count, transparency and morphology, ECD(Equivalent circular diameter), MFD (Maximum Feret Diameter), aspect ratio particularly for subvisible particle ranging from 1 µm to 70 µm (Zölls et al., 2013, Demeule et al., 2010; Frahmet al., 2016; Sharma et al., 2010; Zölls et al., 2013)

For Particle data analysis MFI uses MFI View Analysis Suite (MVAS) version 1.2. Particles stuck to the flow cell wall were only counted once and edge particles were excluded from analysis. As it is highly sensitive technique, it offers high sampling efficiency, low sample volumes, minimizes shear stress on sample and robust sample introduction method for Drug Product and Formulation studies. System suitability is performed on each day of analysis using polystyrene beads to ensure that the sizing and counting accuracy is within predefined limits. (Demeule et al., 2010; Frahm et al., 2016; Sharma et al., 2010)

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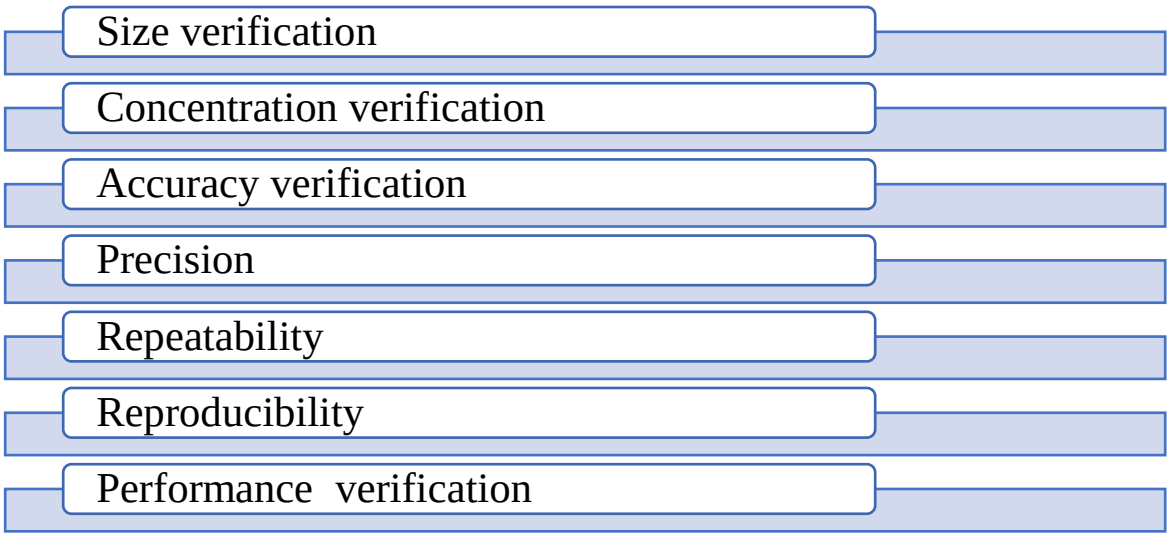
- **Method Development**

Considering all the information with respect to MFI and LO, the requirement of method development for MFI would be useful to overcome limitations faced by compendial methods.

Therefore, method development for polystyrene standards and protein formulations were done using following parameters,



Results and Discussion

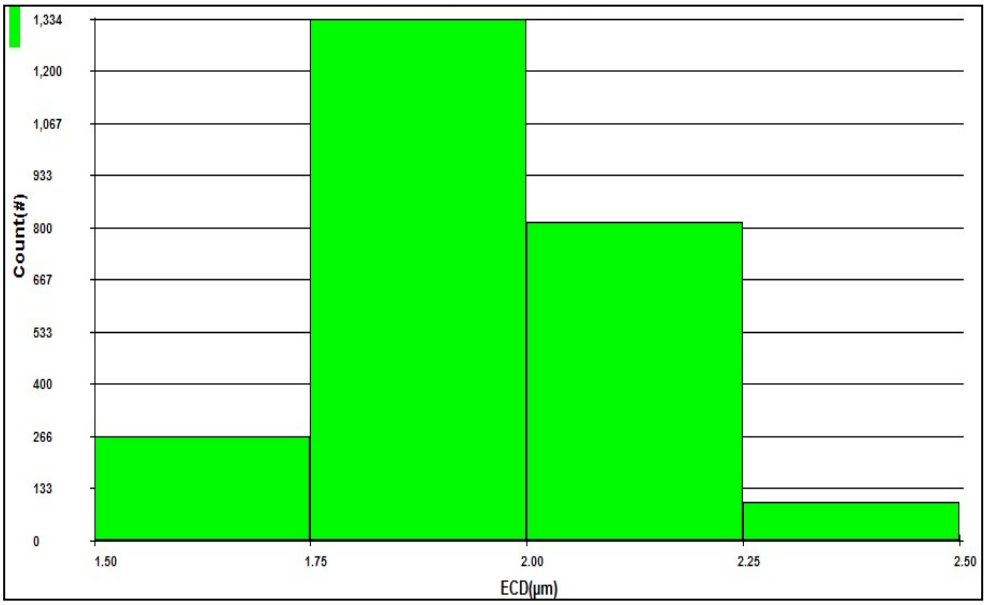
7.1. Results

Analysis of polystyrene standards:

- Polystyrene standards of 2 µm, 5 µm, 10 µm and 25 µm were evaluated and passed with the predefined limits of 3000 ± 600 particles/mL
- Polystyrene standards of 5 µm and 10 µm were comparatively analyzed with LO and MFI and results were within the average range of predefined limits
- Polystyrene standards of 2 µm and 25 µm were passed at the higher side and the lower side of predefined limits respectively
- Polystyrene standards of 2 µm, 5 µm, 10 µm and 25 µm were evaluated for size, concentration, accuracy, precision, reproducibility, repeatability and performance verification and results were best achieved for MFI

- **Size and concentration verification:** Standards of 2 µm (Acceptance range 2400-3600 particles/ mL)

  - Mean size: 1.95
  - Count: 2507
  - Volume analysed: 0.7850
  - Concentration: 3193.53 particles/ mL



(a)

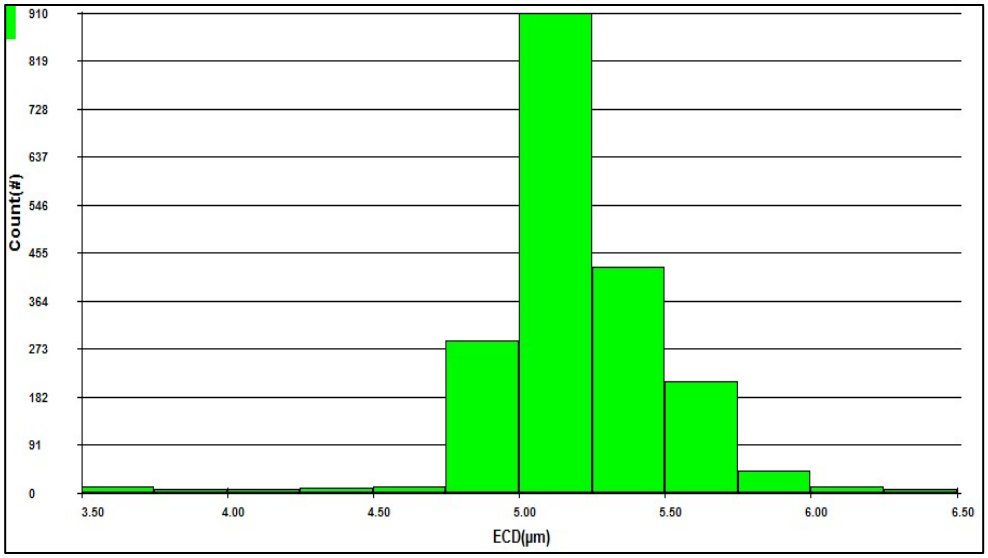
ECD(µm)	Count(#)	Cumulative (%)
1.63	265	-
1.88	1334	-
2.13	812	-
2.38	96	-
2.63	0	-
-	-	-
-	-	-

(b)

Figure 8: (a) Depicts analysis of polystyrene standards of 2 µm, of ECD v/s Particle concentration (counts/mL) (b) Shows individual count for each particle in ECD

- **Size and concentration verification:** Standards of 5 µm (Acceptance range 2400-3600 particles/ mL)

  - Mean size: 5.20
  - Count: 1938
  - Volume analysed: 0.6103
  - Concentration: 3175.34 particles/ mL



(a)

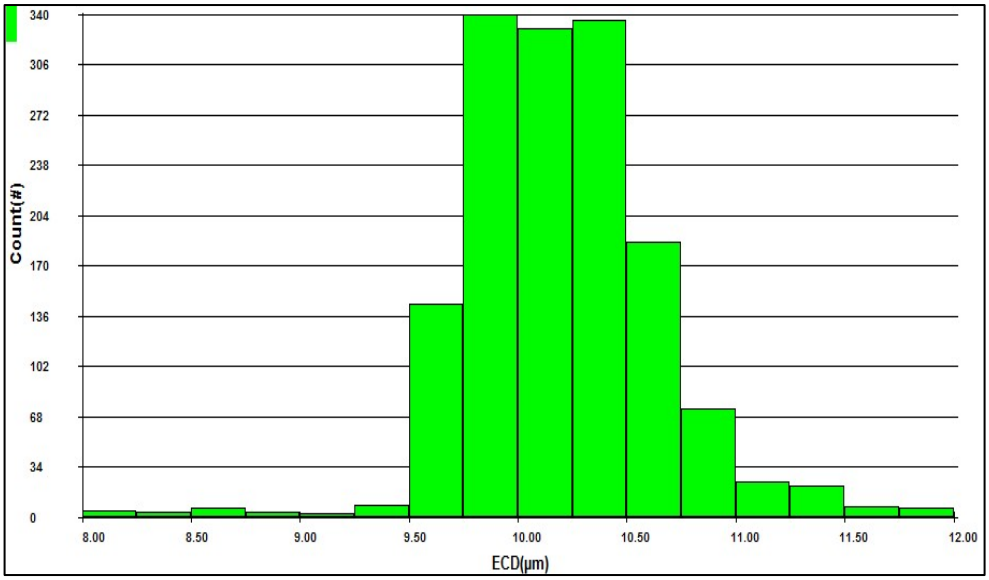
ECD(µm)	Count(#)
3.63	11
3.88	7
4.13	7
4.38	8
4.63	11
4.88	287
5.13	910

(b)

Figure 9: (a)Depicts analysis of polystyrene standards of 5 µm, of ECD v/s Particle concentration (counts/mL) (b) Shows individual count for each particle in ECD

- **Size and concentration verification:** Standards of 10 µm (Acceptance range 2400-3600 particles/ mL)

  - Mean size: 10.20
  - Count: 1493
  - Volume analysed: 0.6101
  - Concentration: 2447.21 particles/ mL



(a)

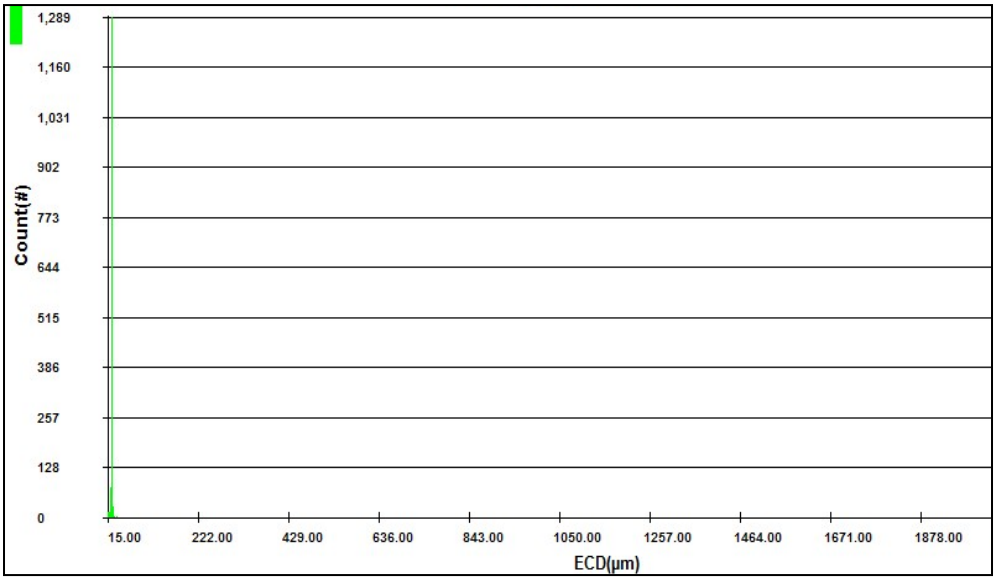
ECD(µm)	Count(#)
8.13	4
8.38	3
8.63	6
8.88	3
9.13	2
9.38	8
9.63	144

(b)

Figure 10: (a) Depicts analysis of polystyrene standards of 10 µm, of ECD v/s Particle concentration (counts/mL), (b) Shows individual count for each particle in ECD

- **Size and concentration verification:** Standards of 25 µm (Acceptance range 2400-3600 particles/ mL)

  - Mean size: 24.67
  - Count: 2234
  - Volume analysed: 0.7850
  - Concentration: 2845.77 particles/ mL



(a)

ECD(µm)	Count(#)	Cumulative (%)
15.50	13	-
16.50	11	-
17.50	9	-
18.50	7	-
19.50	12	-
20.50	11	-
21.50	11	-

(b)

Figure 11: (a) Depicts analysis of polystyrene standards of 25 µm of ECD v/s Particle concentration (counts/mL) (b) Shows individual count for each particle in ECD

- **Image description of polystyrene standard beads:**

Figure shows polystyrene beads captured by MFI,

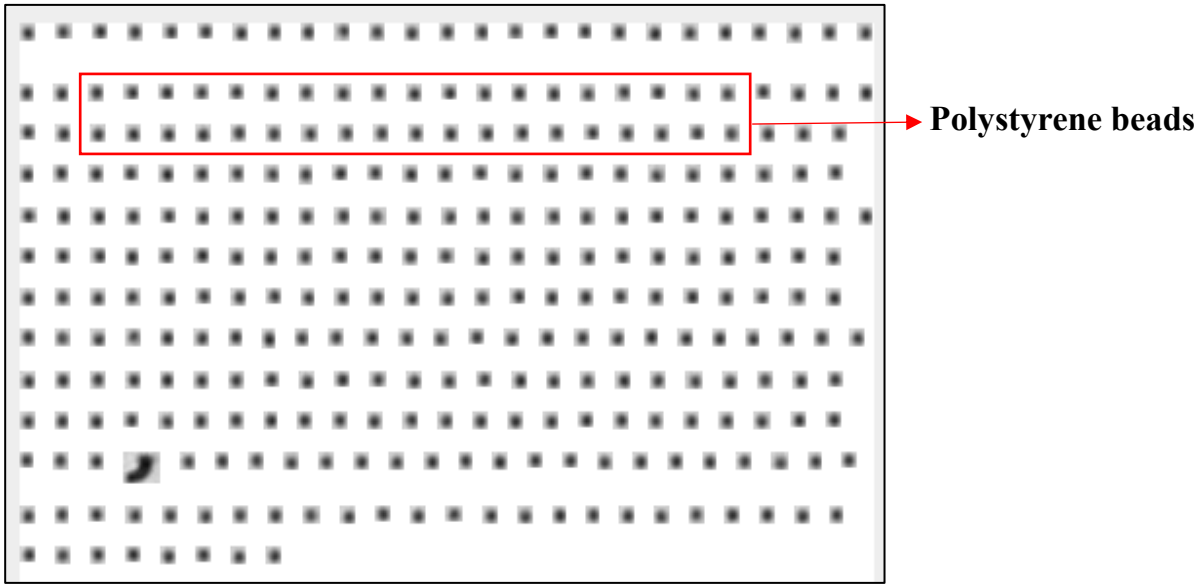


Figure 12: Shows the images captured by MFI of Polystyrene beads standards

Analysis of placebo:

Comparative analysis of placebo was done using MFI and LPC and there was big difference found between concentration of particles.

Sr.No.	Sample	Run	2 µm	5 µm	10 µm	25 µm	Total
			MFI	MFI	MFI	MFI	MFI
1	Placebo	1	633.30	974.49	4.91	0.00	1020.35
		2	6361.21	7953.15	26.20	1.64	8148.05
		3	5358.88	9938.16	127.75	3.28	10380.37
		4	4377.97	8977.13	324.42	13.11	10284.62
		Avg	4182.84	6960.73	120.82	4.51	7458.35
Sr.No.	Sample	Run	2 µm	5 µm	10 µm	25 µm	Total
			LPC	LPC	LPC	LPC	LPC
1	Placebo	1	17.33	0	0	0	17.33

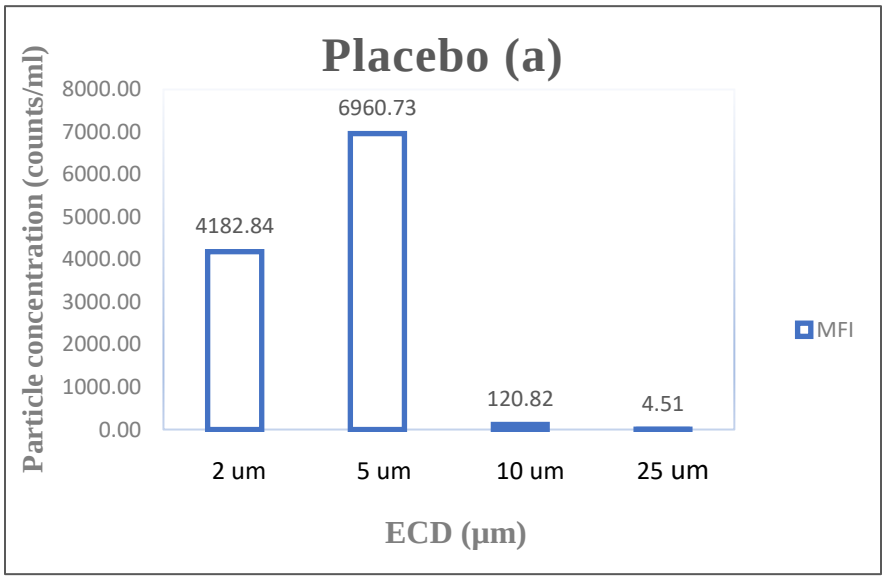


Figure 13 (a): Depicts analysis of placebo of ECD v/s particle concentration for MFI

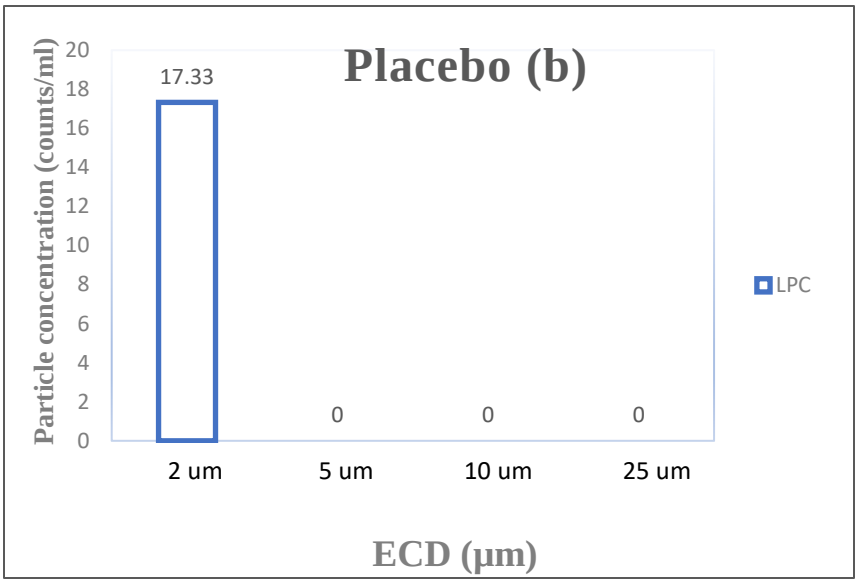


Figure 13 (b): Depicts analysis of placebo of ECD v/s particle concentration for MFI

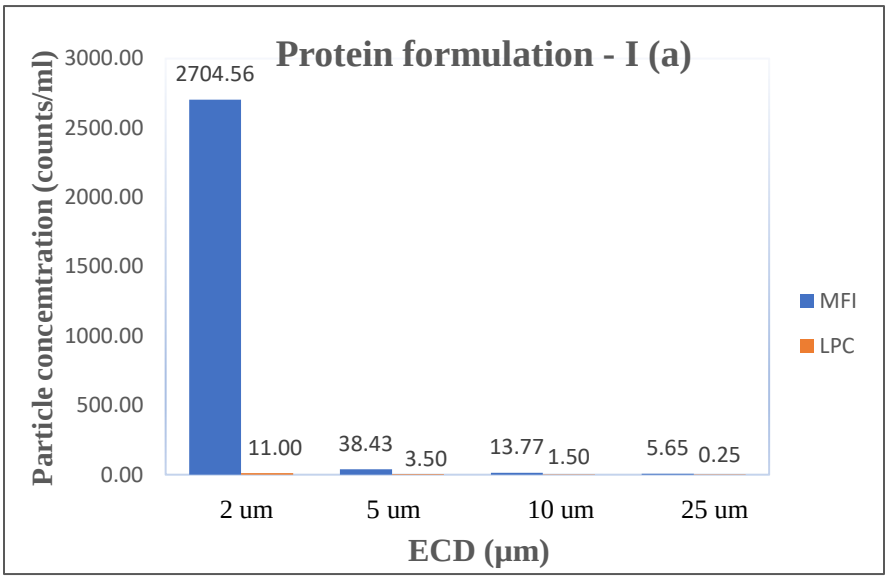
Analysis of Protein formulations:

- Comparative evaluation of LO and MFI were done using three protein formulations
- Protein formulations with particle size of 5 µm and 10 µm were passed and results were within the average range. In case of MFI, Sensitivity was found for 2 µm and 25 µm sized particles
- Below data represents analysis of protein formulation – I, II and III in MFI and LPC;

Sr.No.	Sample	Run	2 µm		5 µm		10 µm		25 µm		Total	
			MFI	LPC	MFI	LPC	MFI	LPC	MFI	LPC	MFI	LPC
1	Protein Formulation - I	1	3202.84	11.00	52.77	5.00	25.24	3.00	20.29	1.00	3301.14	20.00
		2	3319.73	7.00	36.71	4.00	13.77	0.00	0.00	0.00	3370.21	11.00
		3	2474.06	15.00	29.81	3.00	11.46	0.00	2.29	0.00	2517.62	18.00
		4	1821.61	11.00	34.41	2.00	4.59	3.00	0.00	0.00	1860.61	16.00
		5	1586.16	46.00	3616.03	6.00	12.75	2.00	1.28	0.00	5216.22	54.00
		6	2544.58	45.00	826.72	11.00	3.82	0.00	0.00	0.00	3375.12	56.00
		7	3053.50	48.00	133.75	13.00	5.10	3.00	0.00	0.00	3192.35	64.00
		8	3115.99	72.00	81.58	9.00	11.47	0.00	1.27	0.00	3210.31	81.00
		Avg	2639.81	31.88	601.47	6.63	11.03	1.38	3.14	0.13	3255.45	40.00
2	Protein Formulation - II	1	295.71	8.00	4079.12	4.00	0.00	0.00	0.00	0.00	4374.83	12.00
		2	82.55	4.00	1744.91	0.00	2.29	0.00	0.00	0.00	1829.75	4.00
		3	94.06	7.00	199.60	2.00	4.59	0.00	0.00	0.00	298.25	9.00
		4	105.53	6.00	82.59	3.00	11.47	0.00	6.88	0.00	206.47	9.00
		5	184.88	131.00	117.30	13.00	12.75	1.00	2.55	0.00	317.48	145.00
		6	270.31	136.00	44.63	13.00	19.13	0.00	1.28	0.00	335.35	149.00
		7	1577.73	125.00	100.76	16.00	19.13	2.00	1.28	0.00	1698.90	143.00
		8	1077.08	136.00	107.07	17.00	26.77	3.00	0.00	0.00	1210.92	156.00
		Avg	460.98	69.13	809.50	8.50	12.02	0.75	1.50	0.00	1283.99	78.38
3	Protein Formulation - III	1	53.54	10.00	70.11	3.00	8.92	1.00	0.00	0.00	132.57	14.00
		2	53.54	5.00	81.58	2.00	12.75	0.00	0.00	0.00	147.87	7.00
		3	78.56	6.00	67.60	0.00	21.68	1.00	0.00	0.00	167.84	7.00
		4	88.98	13.00	103.31	3.00	30.64	2.00	1.28	0.00	224.21	18.00
		5	700.61	42.00	174.52	13.00	20.38	0.00	1.27	1.00	896.78	56.00
		6	820.36	44.00	272.60	5.00	3.82	0.00	0.00	0.00	1096.78	49.00
		7	954.11	50.00	467.50	6.00	70.06	1.00	6.37	0.00	1498.04	57.00
		8	1084.04	49.00	551.57	7.00	34.39	2.00	5.10	0.00	1675.10	58.00
		Avg	479.22	27.38	223.60	4.88	25.33	0.88	1.75	0.13	729.90	33.25

- **Repeatability and Reproducibility of protein formulations -I and II:**

  Reproducibly and repeatability were best achieved in protein formulation – I (a) and (b), whereas, compromised for protein formulation – II (c) and (d) by the polystyrene standards for both instruments.



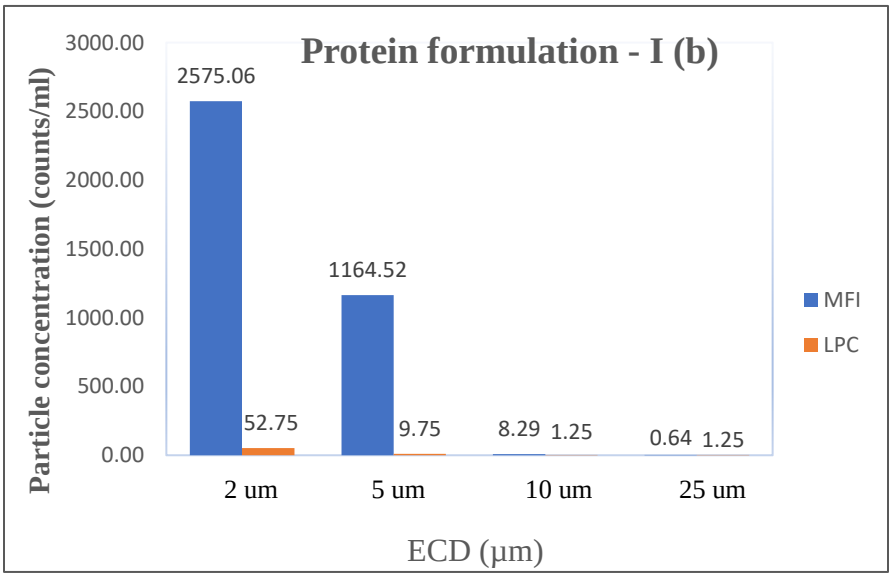
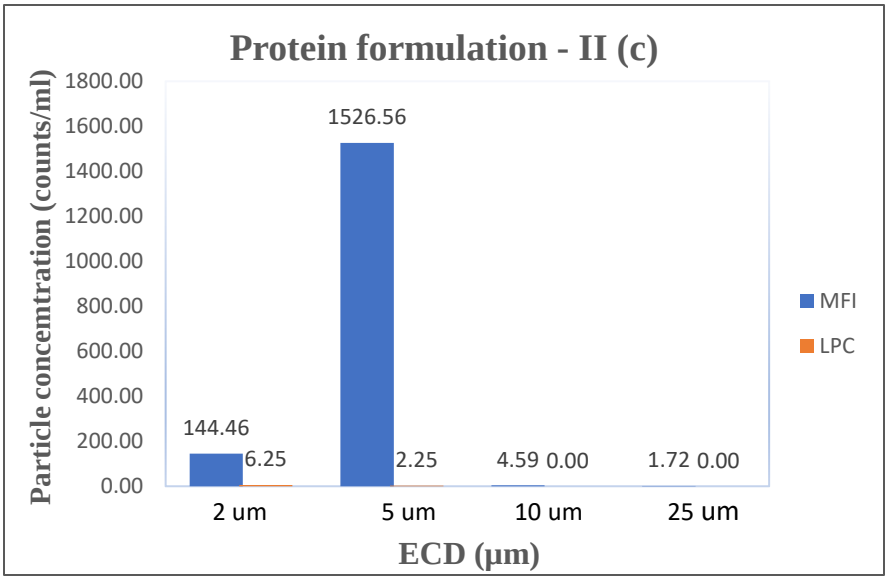


Figure 14: Graph **(a)** and **(b)** represents analysis of protein formulation for repeatability and reproducibility



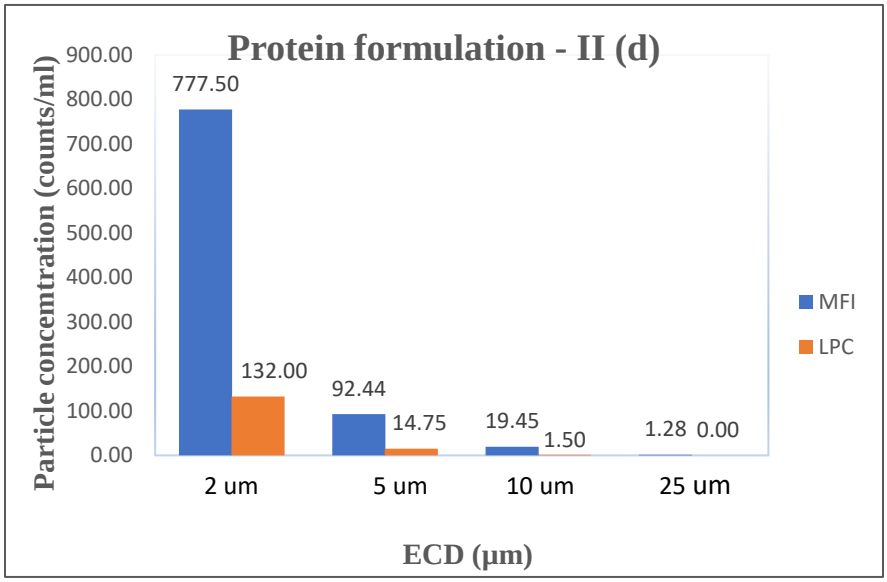
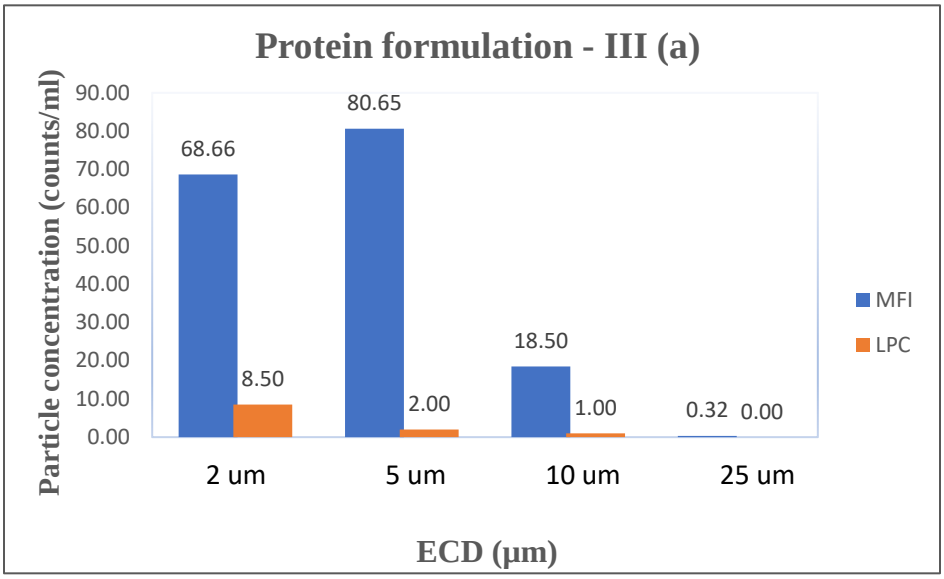


Figure 15: Graph **(c)** and **(d)** represents analysis of protein formulation for repeatability and reproducibility.

- **Precision and Accuracy verification using protein formulation – III:**

  Precision and accuracy verification were achieved with protein formulation – III (a) and (b) for both instruments.



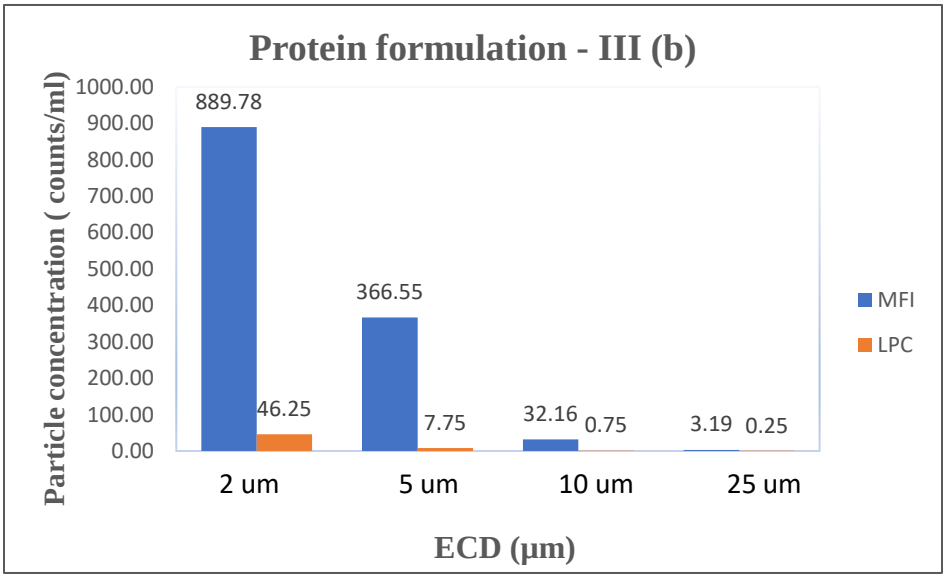


Figure 16: Graph **(a)** and **(b)** represents analysis of protein formulation for precision and accuracy verification

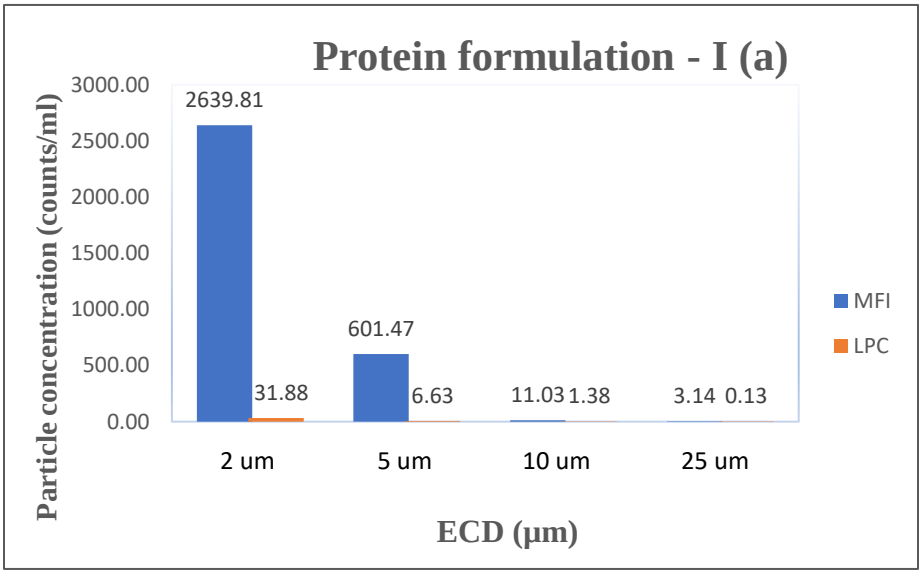
- **Performance verifications for protein formulations – I, II and III:**

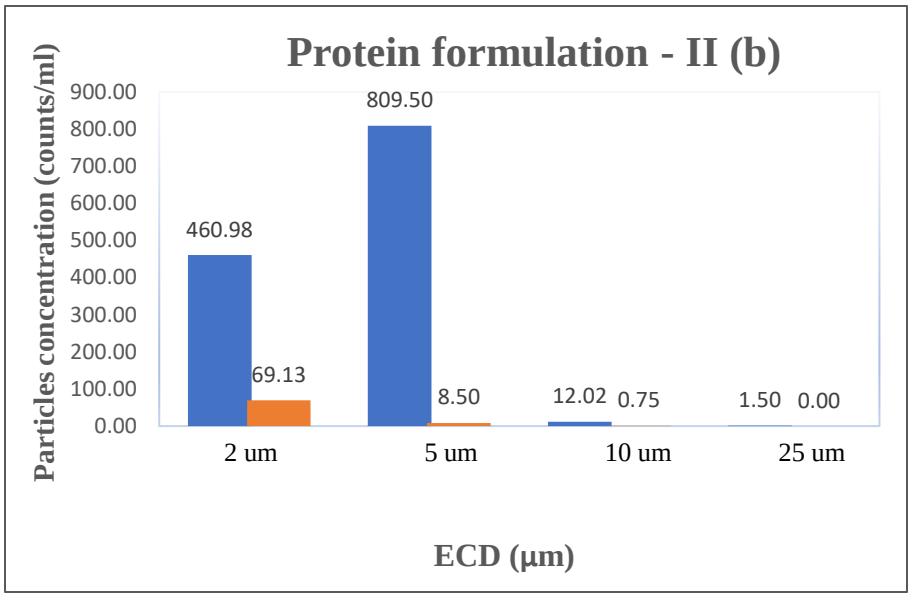
Performance verification was found to better for LPC in case of 10 µm and 25 µm particles but in case of MFI was found sensitive for extremes.

The best counting efficiency was obtained for MFI in the range of 1 to 10 µm, and LO had showed good results for particles above 10 µm.

The below data represents average value of protein formulations for performance verification;

Sr.No.	Samples	Run	2 µm		5 µm		10 µm		25 µm	
			MFI	LPC	MFI	LPC	MFI	LPC	MFI	LPC
1	Protein formulation – I	Avg	2639.81	31.88	601.47	6.63	11.03	1.38	3.14	0.13
2	Protein formulation – II	Avg	460.98	69.13	809.50	8.50	12.02	0.75	1.50	0.00
3	Protein formulation – III	Avg	479.22	27.38	223.60	4.88	25.33	0.88	1.75	0.13





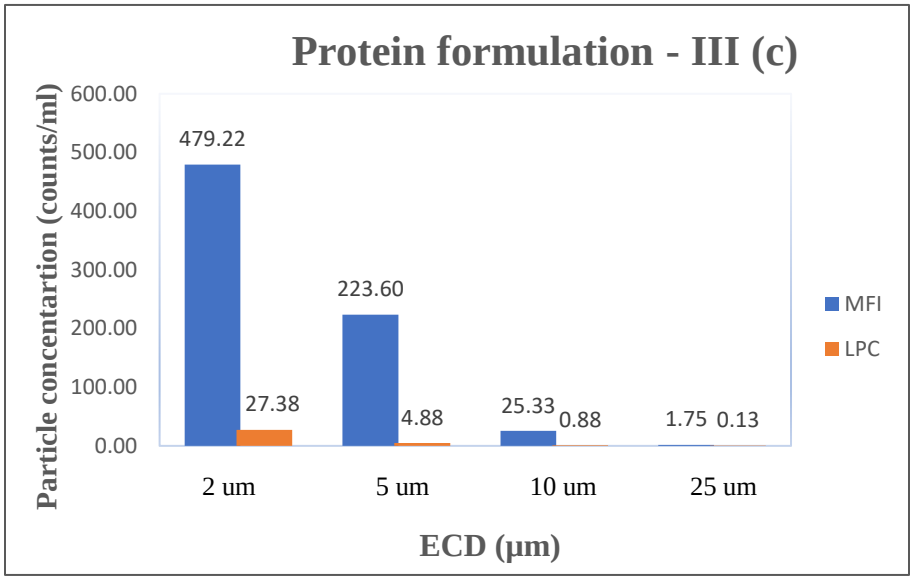


Figure 17: Graph **(a), (b)** and **(c)** represents analysis of protein formulation for performance verification

- **Image description of protein formulation – I, II and III:**

Figure 18 and 19 shows different images of silicon oil droplets, air bubbles, contaminant and protein aggregates in protein formulations

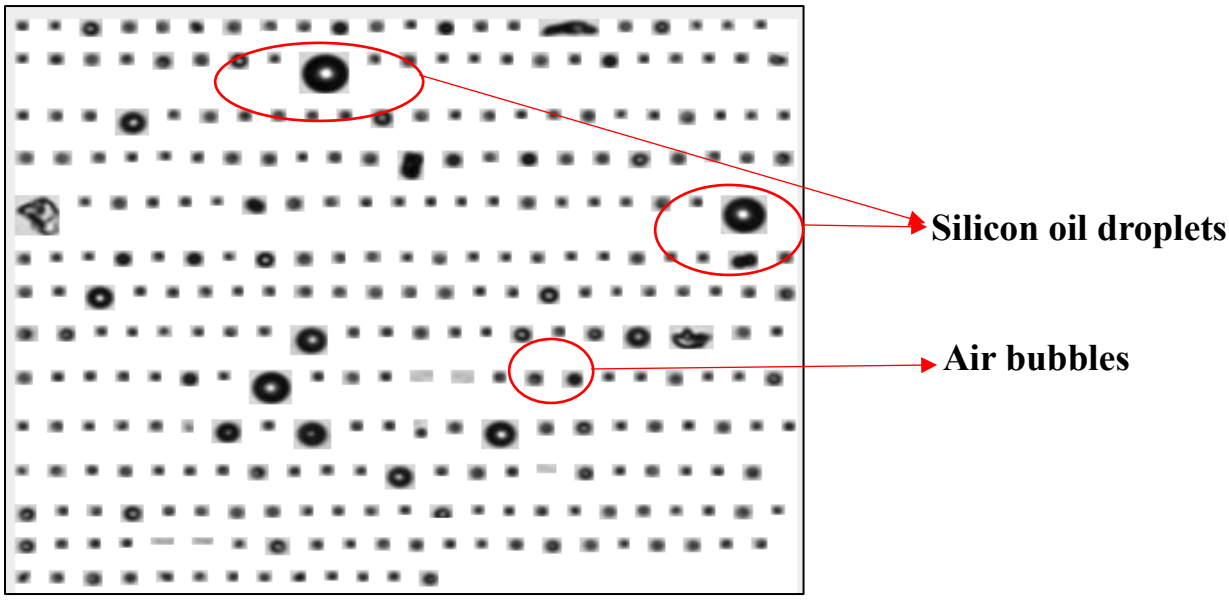


Figure 18: Represents Images captured by MFI showing silicon oil droplets and air bubbles

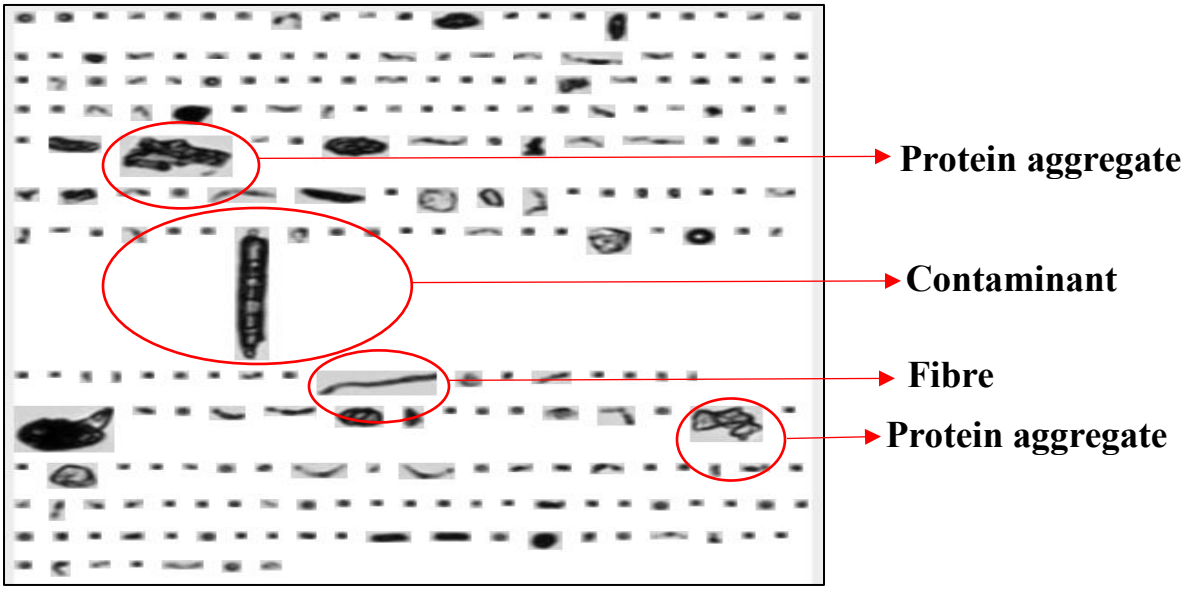


Figure 19: Represents Images captured by MFI showing contaminant, Fibre and protein aggregates

7.2 Discussion:

Flow imaging microscopy techniques are considered to better method for both counting and visualization of particles in therapeutic protein formulations as compared with other analytical techniques for subvisible particles analysis. due to the capability to characterize particles based on images. Parameters such as shape and transparency can be used to differentiate between different particle subpopulation. Here LO as compendial method and MFI as orthogonal method were comparatively evaluated for their suitability for protein particle analysis and characterization (Corvari et al., 2015; Frahm et al., 2016).

Polystyrene standards of 2 µm, 5 µm, 10 µm and 25 µm were evaluated for size, concentration, accuracy, precision, reproducibility and repeatability were best achieved for both the methods. results were comparable due to the nature of the polystyrene beads used for the evaluation. upon performance verification, result was found to better for LPC in case of 10 µm and 25 µm particles but in case of MFI was found to be sensitive for extremes. Reason could be determined as due to the high magnification power of MFI towards lower range particles.

In case of analysis of placebo, there was huge difference found between results of both methods, reason might be concluded as the difference in the composition of the placebo with respect to protein formulations.

Upon comparative evaluation of LO and MFI using three protein formulations, Sensitivity was found for 2 µm sized particles and reason behind such sensitivity might be due to scattering of small sized particles and another reason could be Brownian effects on particles. And also, accuracy and precision were compromised for 25 µm particles, might be due to particles get sticked into the flow cell. And to overcome this hurdle, washing of flow cell was done with any surfactant. E.g. micro 90.

Repeatability and reproducibility were performed using all protein formulations but were not best achieved due to certain complications found during method development in the instruments.

Precision and accuracy verification were done for all protein formulation, it was obtained at lesser extent. This was might be due to sample handling or instrument handling error.

On performance verification of both instruments, the best counting efficiency was obtained for MFI in the range of 1 to 10 µm, and LO had showed good results for particles above 10 µm.

MFI was able to well characterized particle subpopulations i.e. proteinaceous and non-proteinaceous and transparent and translucent particles based on morphological filters available.

8. Conclusion:

The number of analytical methods for the quantification and characterization of protein particles has continuously increased during the last few decades. Numerous characteristics of particles in therapeutic protein formulations such as size, shape, chemical composition or structure, can be determined based on different measurement principles. However, no single method is capable of providing information on all desired parameters for the complete size range, which makes a combination of several methods based on different measurement principles necessary for a comprehensive characterization. So, it can be concluded from the study that, selection of appropriate method depends strongly on the main parameters of interest and the intended application. Each technique shows its pros and cons in different aspects (Sharma et al., 2010; Zölls et al., 2013).

High-efficiency in terms of particle counting accuracy and precision was best achieved by the MFI system due to which it is a preferred system among various techniques.

LO will remain the standard method for the particles range above 10 µm but to quantify and characterize particles below 10 µm MFI will be the sensitive method for analysis.

For data analysis, in most of the cases two methods will not show exactly the same result for one parameter due to a different underlying measurement principle.

In regards to the comparison of different analytical methods, more proteinaceous particle standards to be used rather than polystyrene standards.

Characterizing protein formulation with Micro Flow Imaging has provided with more information regarding protein particles, non -protein particles, silicon oil droplets, glass etc.

Comparative evaluation of Light Obscuration and Micro Flow Imaging has provided more insight into the particle characteristics, morphology and nature development of new methods for more effective solution.

Characterizing protein formulation with Light Obscuration and Micro Flow Imaging, will provide more information regarding the particle morphology and other parameters, which leads towards less aggregation propensity with less immunogenicity

9. References:

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