Sources of Immunogenic Nano- and Microparticles in Therapeutic Protein Products: Bioprocessing, Shipping, and Mishandling by Clinics and Patients

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Immunogenicity: Particles as Adjuvants

- Studies in mice with murine proteins show that protein nano- or microparticles and/or protein adsorbed to foreign microparticles stimulate immune response.
- Clinical studies with early human therapeutic protein products (e.g., growth hormone, interferon-alpha) showed immunogenicity rates correlated directly with levels of aggregates/particles.
- Even older human studies (60's and 70's) showed particles and aggregates stimulate immunogenicity
- Insights also gained by applying modern analytical methods to old protein products that are immunogenic.

Causes of Protein Aggregation

- Even without stress, aggregates can form during storage in solution; sufficient partially unfolded molecules even in "stable" formulation

- In addition, proteins are exposed to numerous stresses (e.g., light, surfaces) during production, purification, storage, shipping and delivery to the patient.

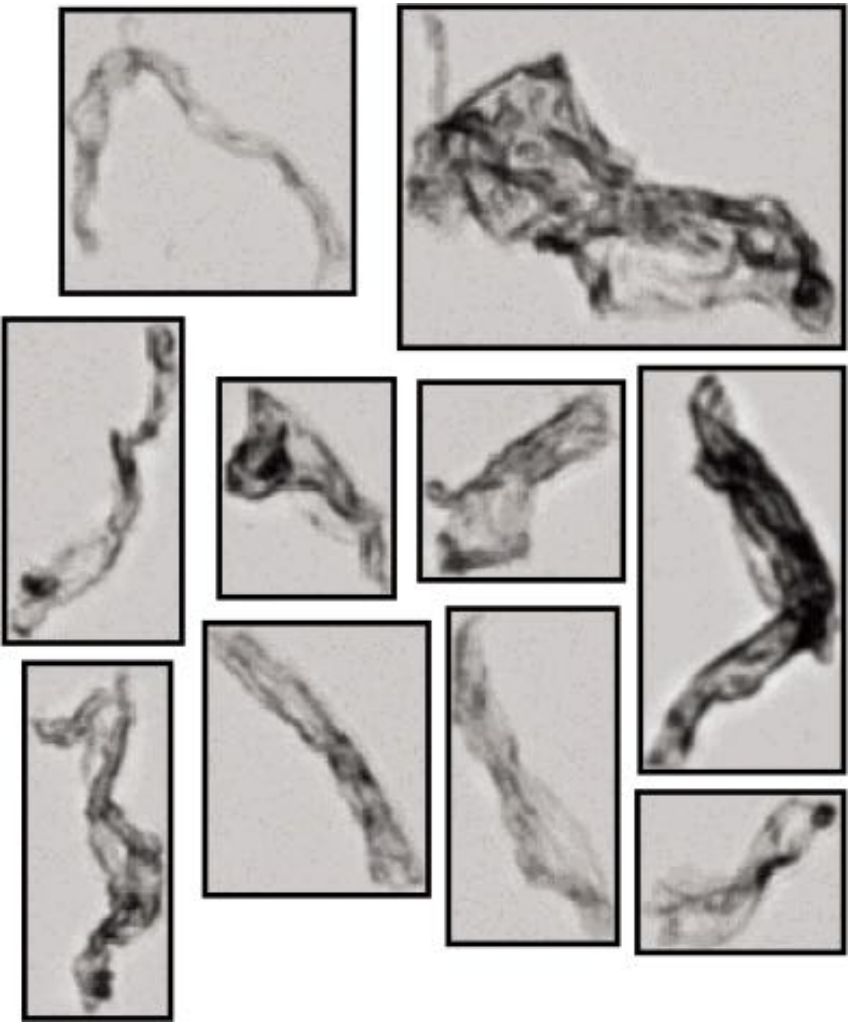
- Often, aggregation occurs because of exposure to air-liquid or solid-liquid interfaces.
  - bubble entrainment during mixing, filling & shipping
  - solution contact with pumps, pipes, vessels, filters, columns, ice, IV bags and tubing, etc.

- Handling and mishandling by pharmacy, clinic or patient

Mechanical Shock: Another Cause of Protein Aggregation and Degradation

- Recent work has documented that mechanical shock due to dropping a vial onto a surface causes violent fluid flows and cavitation.
- As a result a model IgG and rHGH formed visible and subvisible particles and were oxidized
- Much more work to be done on fluid dynamics modeling, mechanistic linkages between cavitation and protein degradation and effects of a wide range of parameters on protein aggregation and oxidation.
- Damage noted could be related to that seen some times during shipping; don't toss the box or drop the vial/syringe.

Why do subvisible protein particles so often look like the ones of the left?

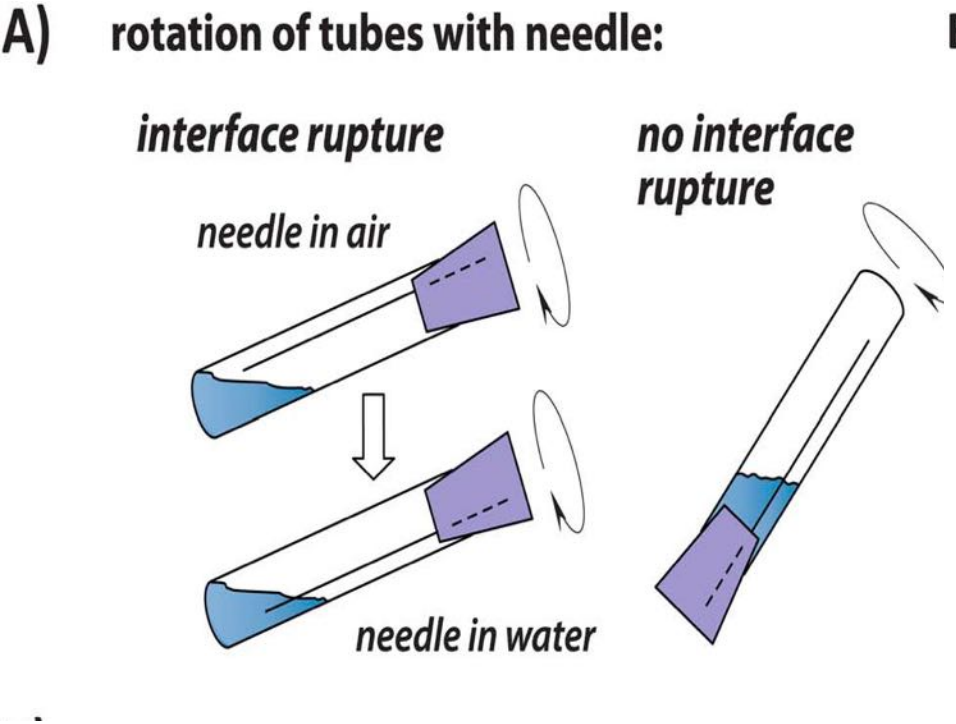


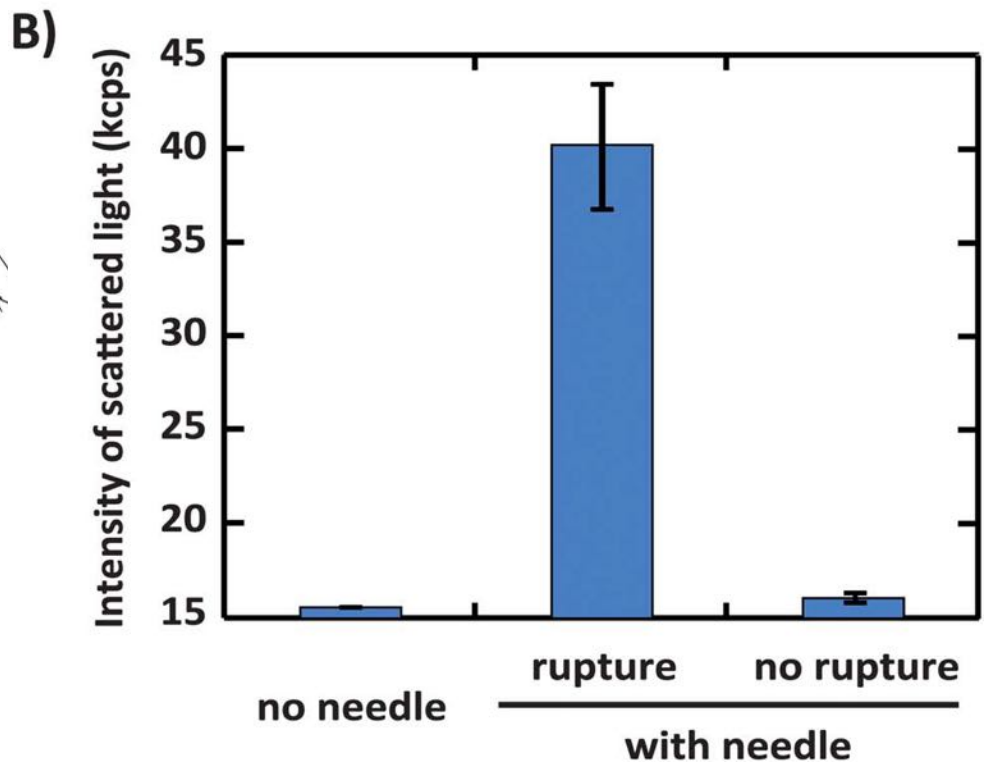
BioProcess International, 2009, 7:62–67

Causes of Protein Particles: Interfaces

- Protein molecules adsorb to most interfaces.
- Layers of adsorbed protein form gels or films.
- Rupture or sloughing off of film can lead to particles in the bulk solution phase.
  - During agitation and with bubble popping
  - Compression/dilation can disrupt film
  - Layers adsorbed (e.g., to syringe walls or to tubing used in processing or IV dosing) can slough off; facilitated by agitation, fluid movement or mechanical stress.
  - Also, multiple interfaces (e.g., silicone oil and air bubble in syringe)

Rupture of Air-Water Interface Causes Particle Formation from Adsorbed IgG



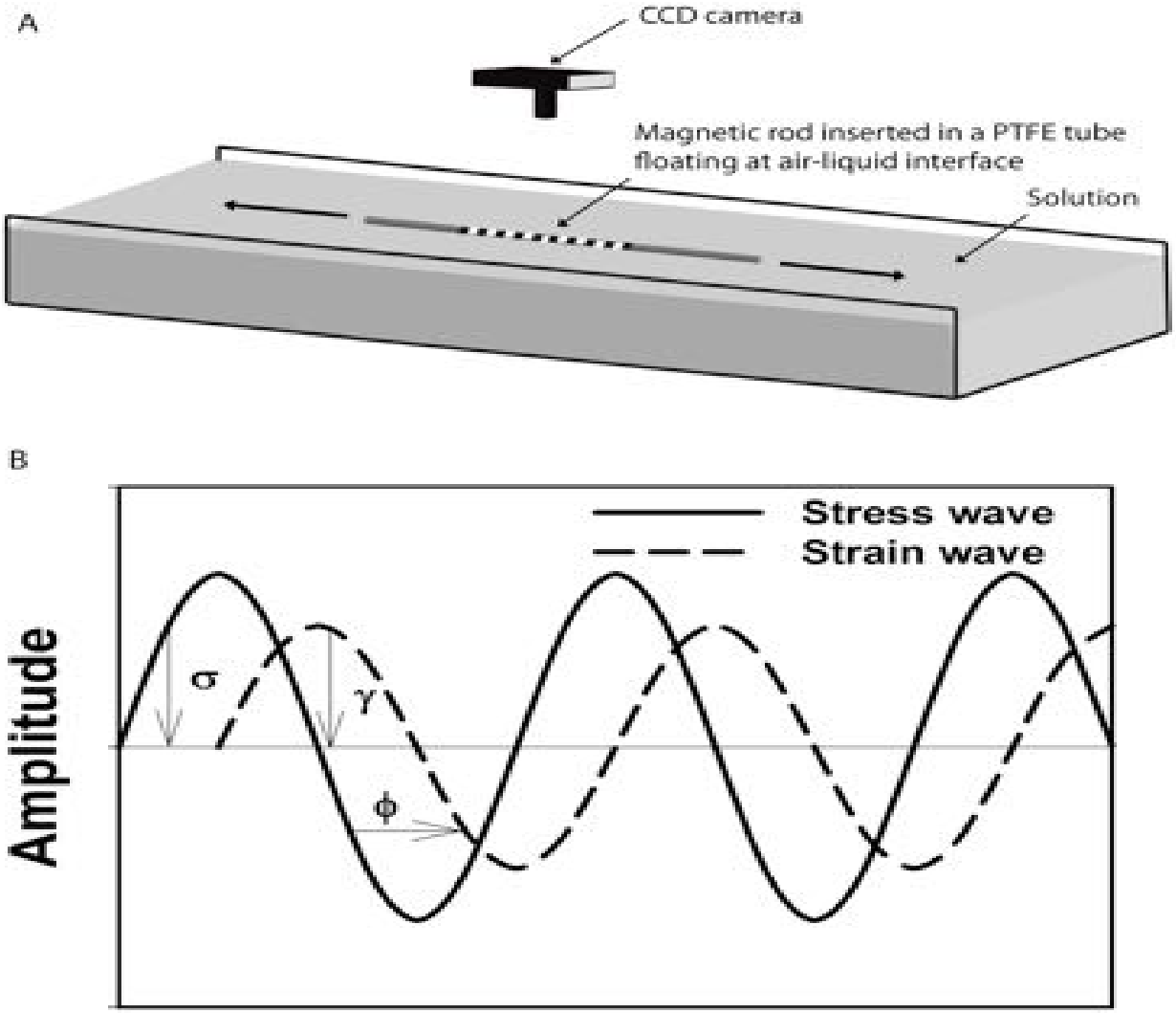


We have obtained similar results with rupture of IgG film at the silicone oil-water interface.

Particle formation was inhibited in presence of surfactants such as polysorbate 80.

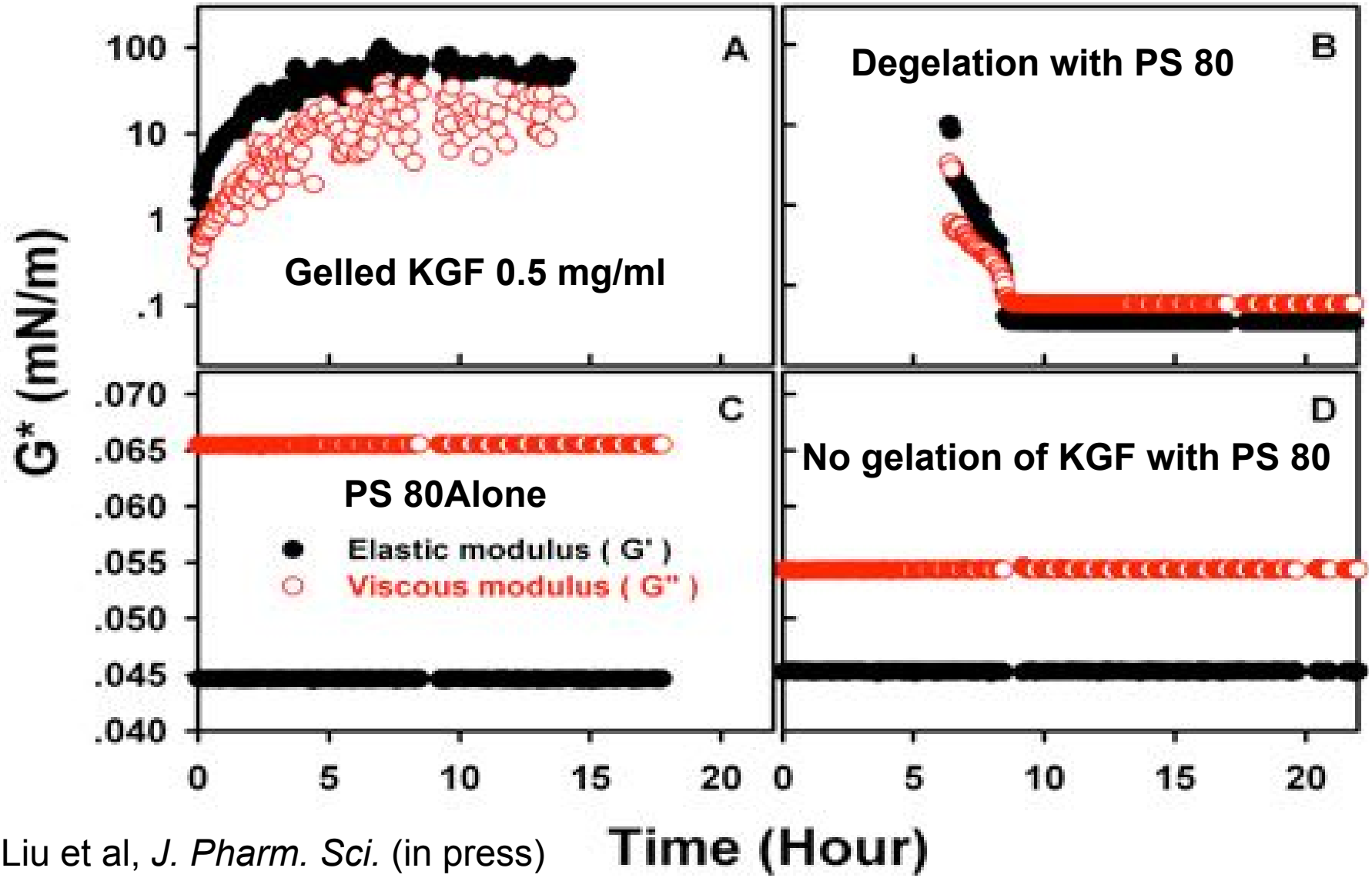
Rudiuk et al., *Soft Matter*, 2012, 8, 2651–2661

Interfacial Shear Rheology: Protein Gelation on Air-Water Interface



Liu et al, *J. Pharm. Sci.* (in press)

Inhibition of Protein Gelation on Air-Water Interface with PS80



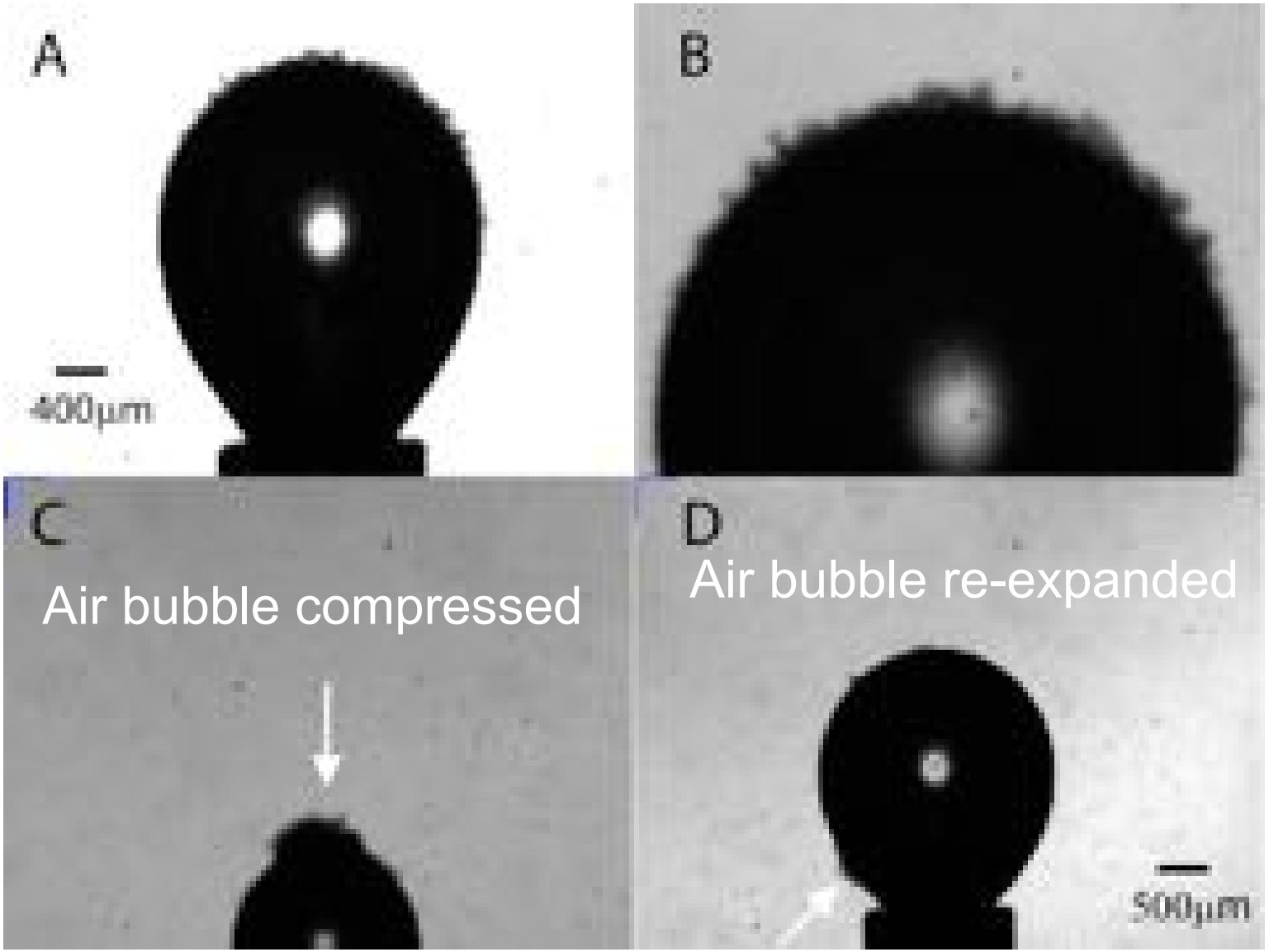
Liu et al, *J. Pharm. Sci.* (in press)

Agitation-induced Particle Formation

Sample		Particles/ml ≥ 1 micron	Soluble protein recovered (%)
KGF Alone	Time 0	1500 ± 800	100
	24 hr agitated	1,200,000 ± 300,000	98 ± 3
	24 hr quiescent	100,000 ± 20,000	102 ± 3
KGF + PS80	Time 0	8,000 ± 5,000	100
(only partial inhibition!!!!)	24 hr agitated	200,000 ± 100,000	106 ± 1
	24 hr quiescent	500,000 ± 100,000	105 ± 1

Particle counting is much more sensitive measure of aggregation than loss of soluble protein. Liu et al, *J. Pharm. Sci.* (in press)

Particles form on air-water interface



Foreign Particles from Container/ Closure/Filters/Pumps/Tubing/Bags

- Glass particles from containers.
- Glass cartridges and syringes are siliconized, and free silicone droplets can be generated.
- In syringes, there may also be tungsten particles and tungsten salts from needle insertion process.

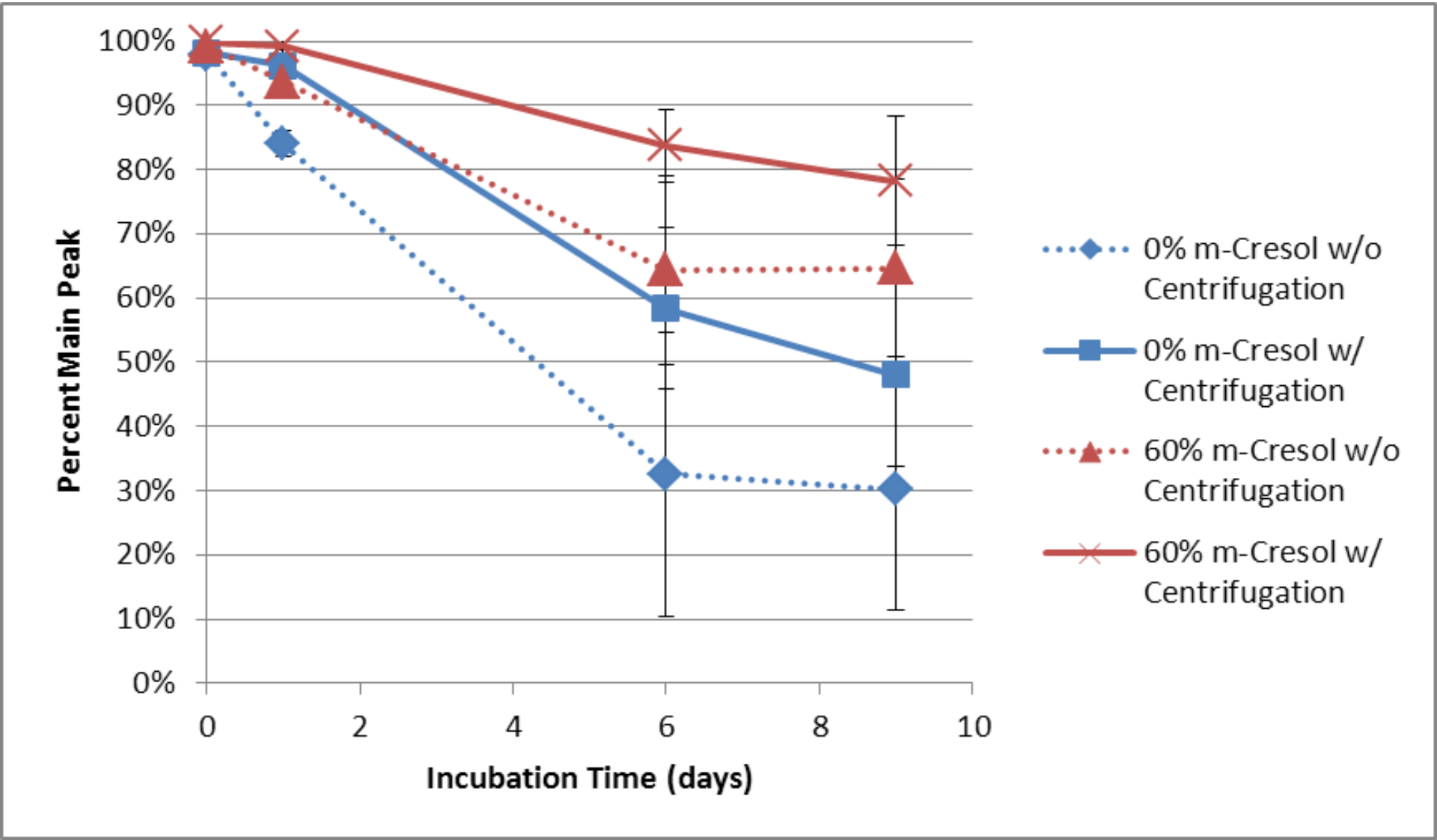
Foreign Particles

- **Rubber and/or silicone particles can come from stoppers.**
- **Plastic particles from bags used in processing, IV bags and IV tubing**
- **Stainless steel, silicone and other particles from filling pumps.**
- **Particles shed from filters during steps such as pre-filling sterile filtration**
- **Protein molecules adsorb to nano- and microparticles creating protein-coated particles, which may lead to further particle formation.**

Particles going along for the ride

- Particles shed during every step of processing.
- 0.2 micron cutoff filters will remove many particles.
- But smaller ones still “go along for the ride.”
- Viral filtration step for bulk drug (if used) would get rid of smaller particles, but sometimes at a cost of early filter fouling.
- But even after sterile filtration in fill-finish, huge numbers of particles may shed from equipment and container/closures or be formed due to stress to protein solution; injected into patients

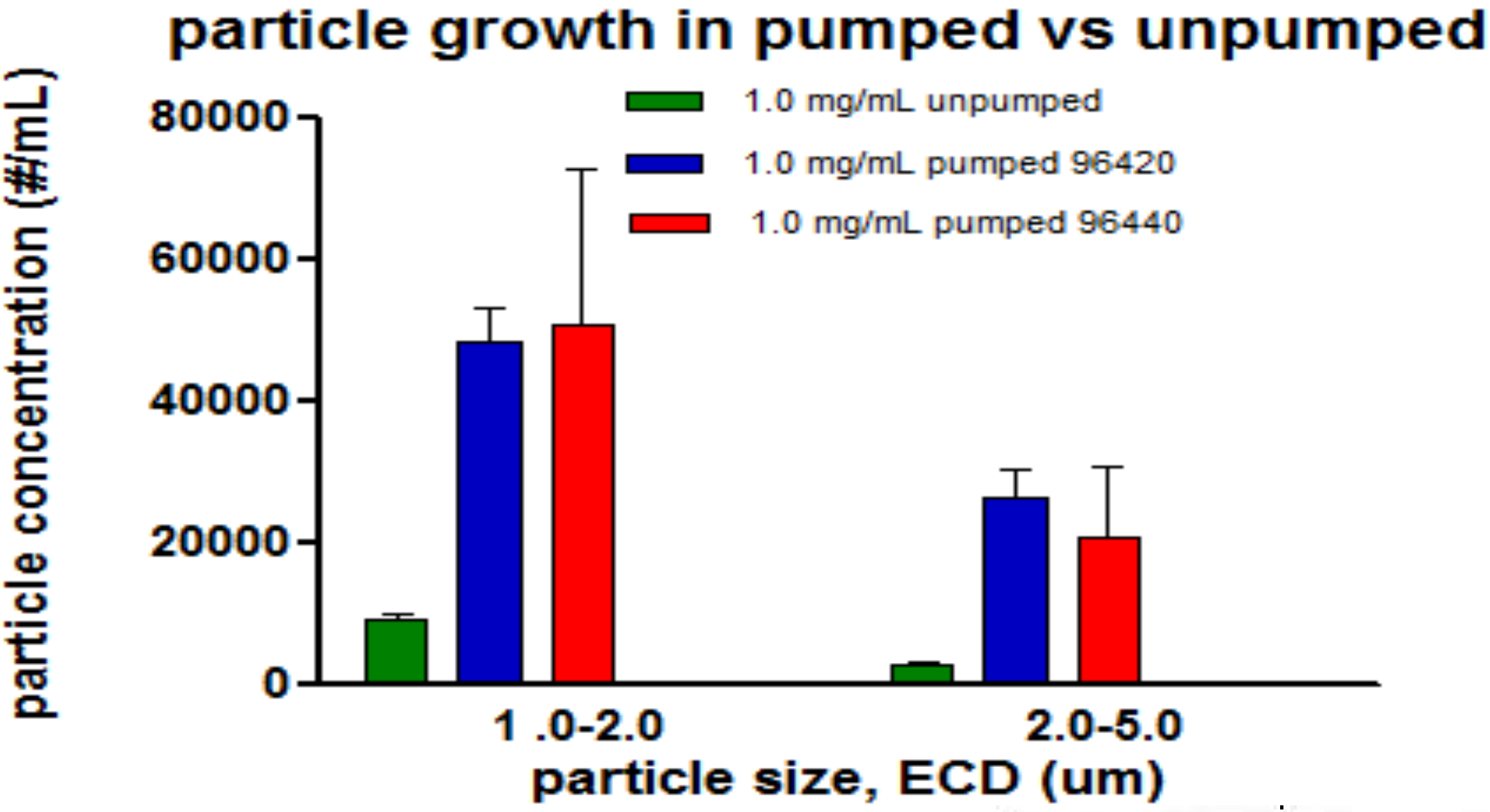
Particles from Desalting Column Promote Aggregation of Lispro Insulin during Quiescent Incubation

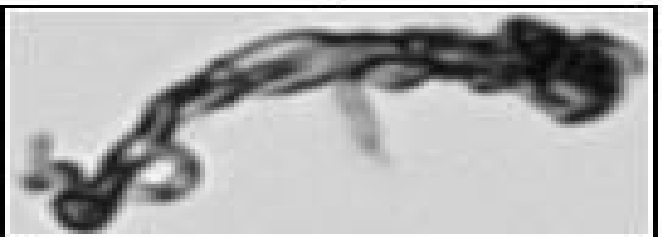


Particles Generated by Peristaltic Pumping: Preliminary Study

- Peristaltic pump and **platinum-cured silicone tubing** were purchased from Cole Palmer.
- Human IVIG was dialyzed into a 10 mm phosphate buffer and used at a concentration of 1.0 mg/mL.
- Samples were pumped through 2 feet of tubing at a rate of 1.0 mL/min, controls were not pumped.
- They were analyzed using Brightwell Microflow Imaging DPI 4200, Malvern ZetaSizer Nano and HPLC size exclusion chromatography.

Pumping causes particles





Images of typical particles

Role of Nano- and Microparticles on IVIG Particle Formation during Agitation

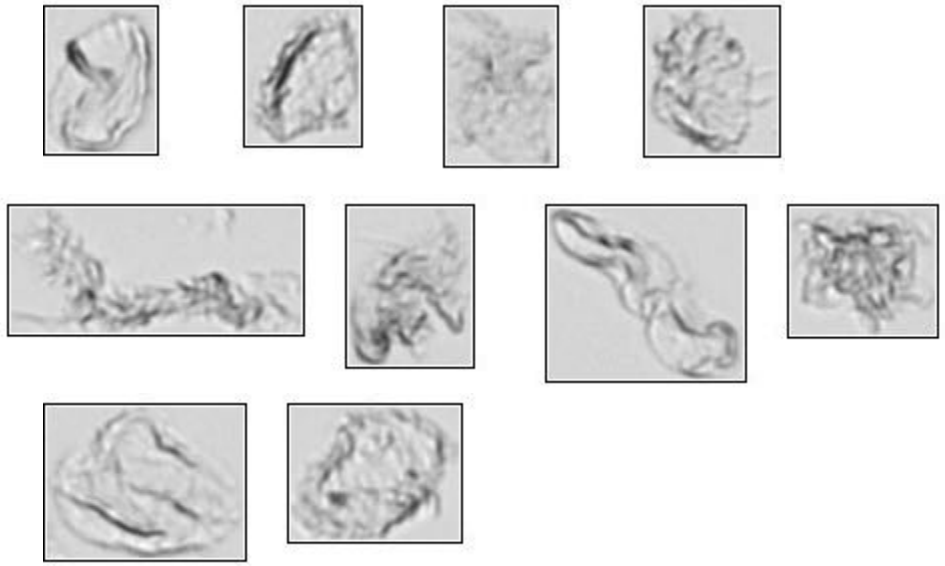
- Sample agitated for 48 hrs to create particles.
- Samples then subjected to three different centrifugation forces
  - 2350g removes microparticles
  - 30000g removes > ca. 0.2 micron particles
  - 112000g removes > ca. 25 nm particles
- Analysis using MFI and Nanosight

Pardeshi et al., unpublished

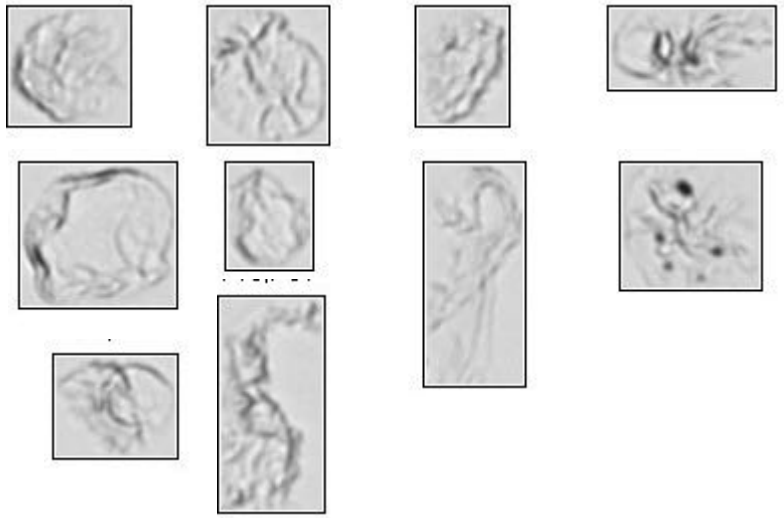
Images of IVIG Particles after Initial Agitation

MFI Screen Shot

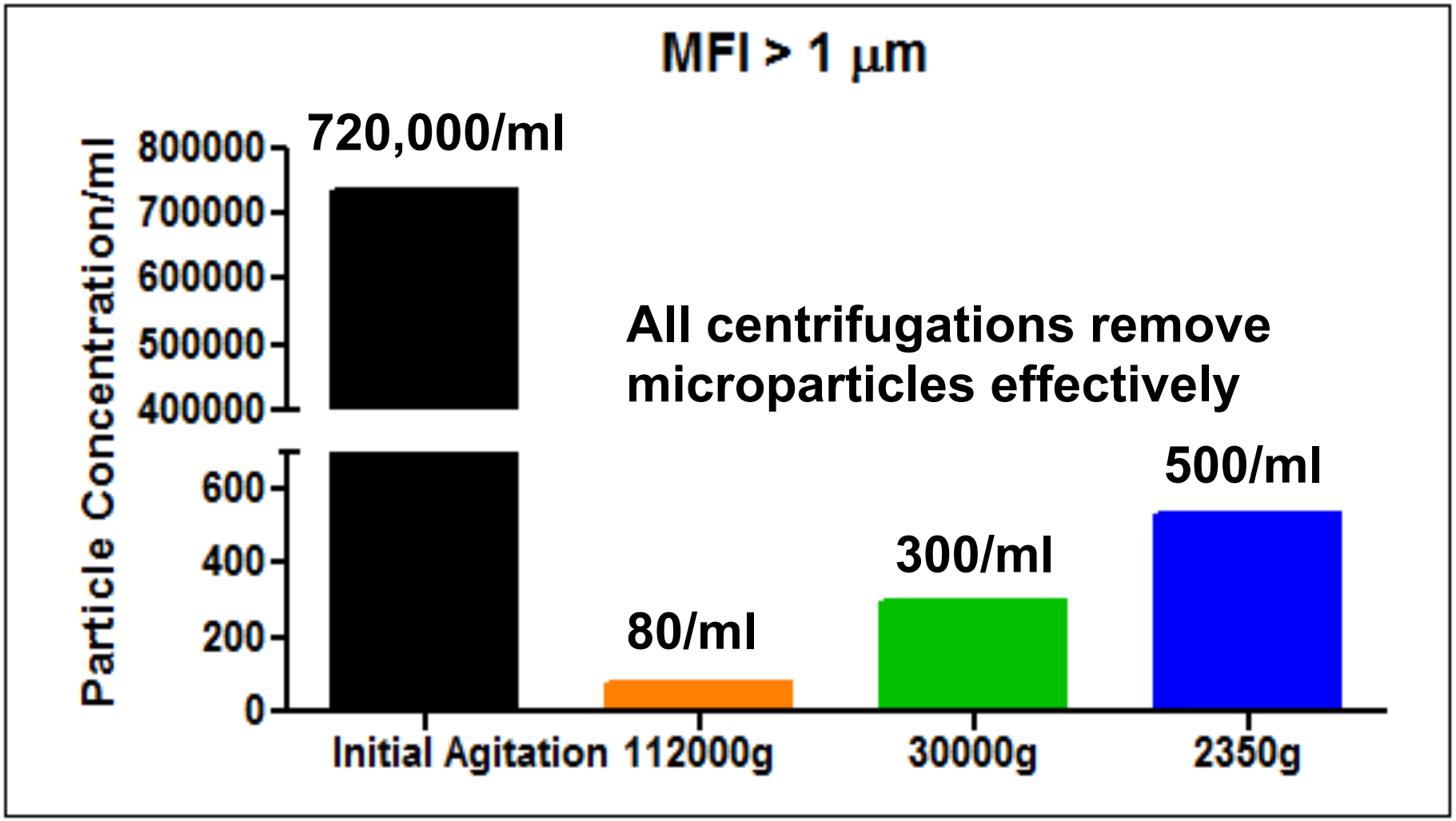
70-100 µm



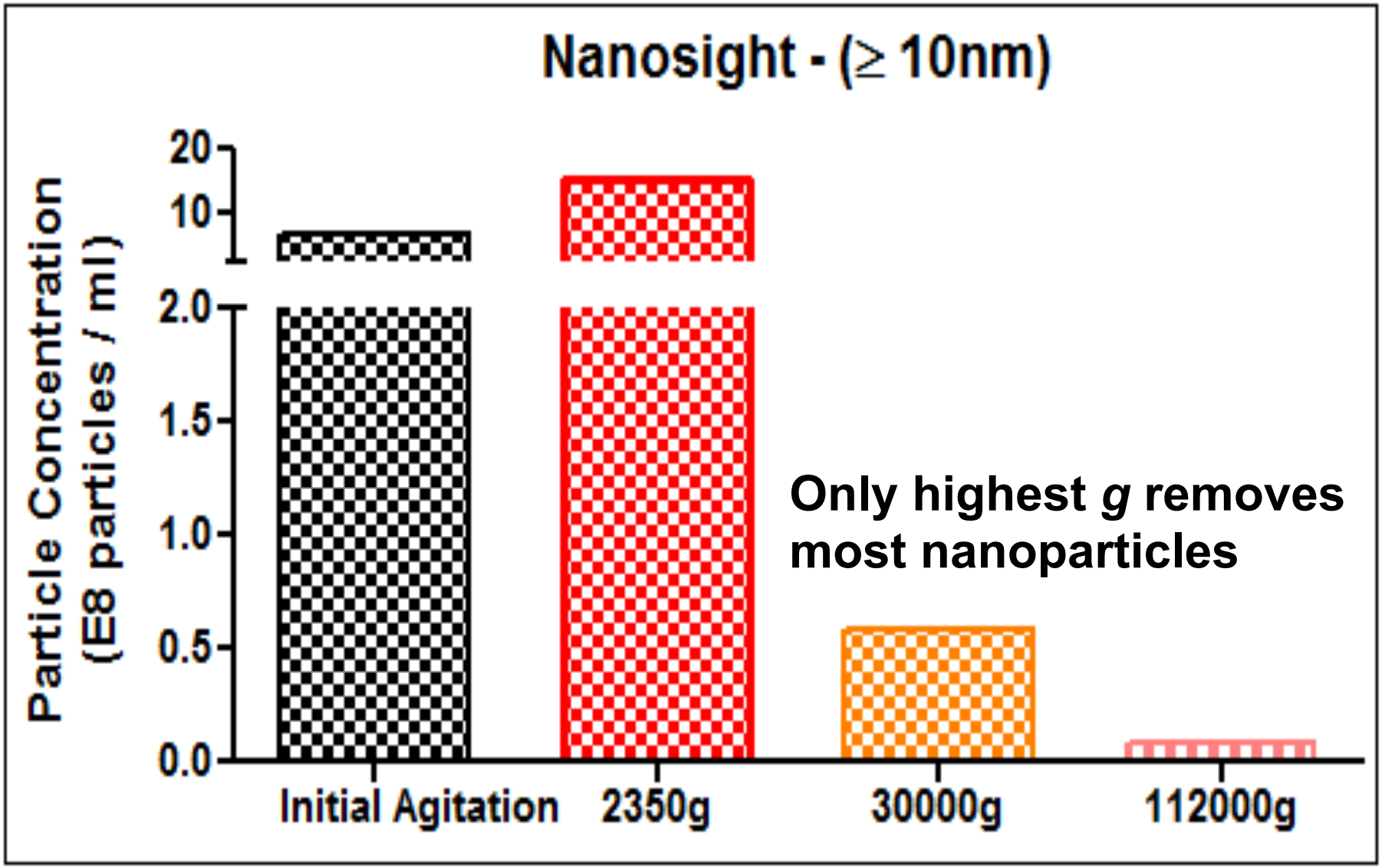
50-70 µm



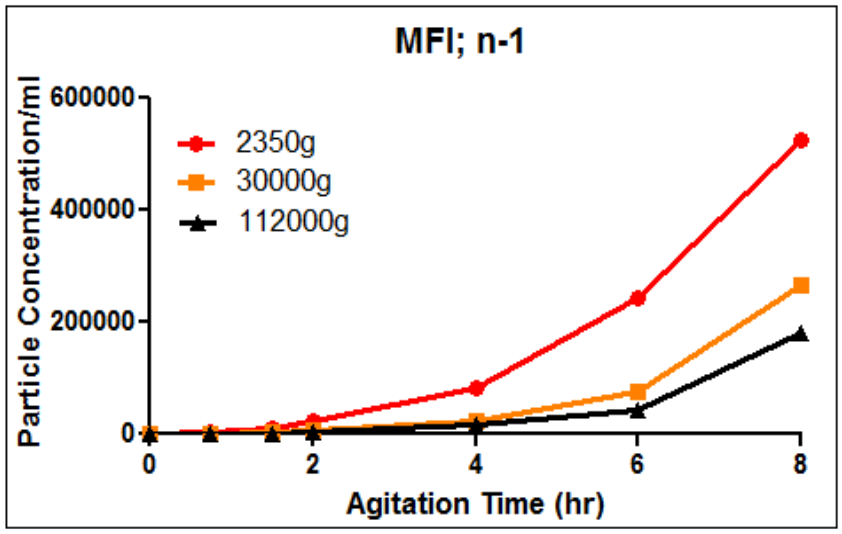
Microparticle Concentrations after Initial Agitation and Subsequent Centrifugations

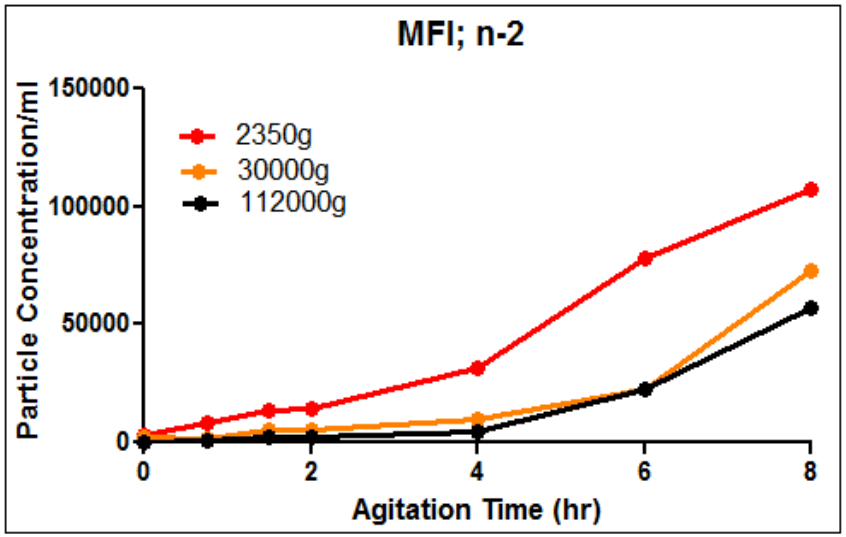


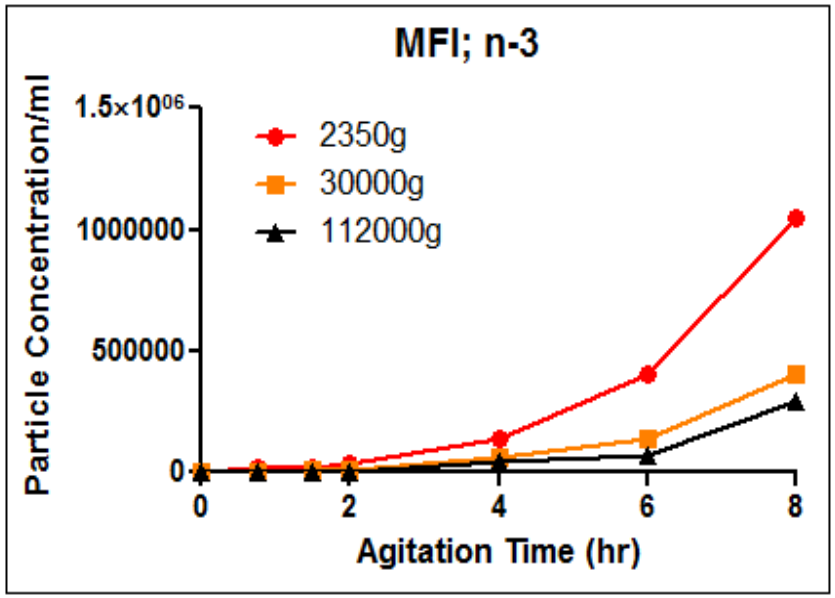
Nanoparticle Concentrations after Initial Agitation and Subsequent Centrifugation

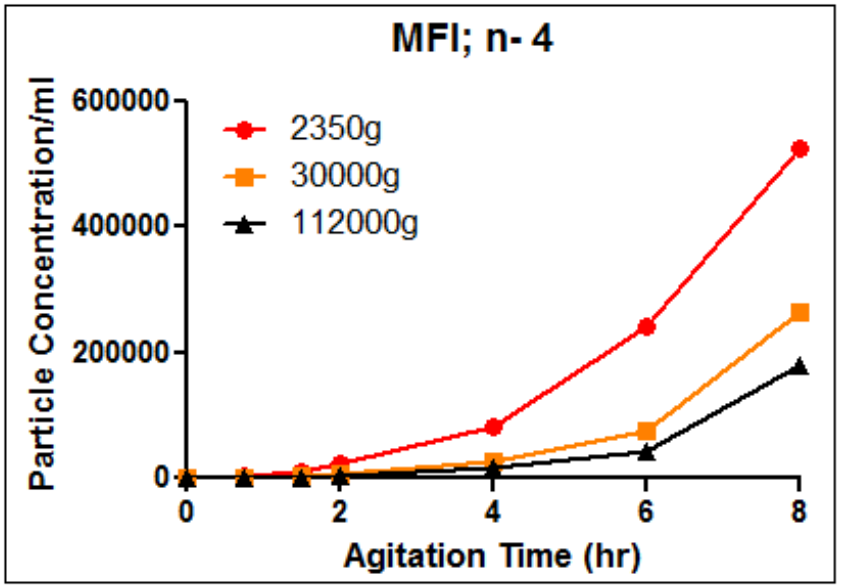


Microparticles during agitation



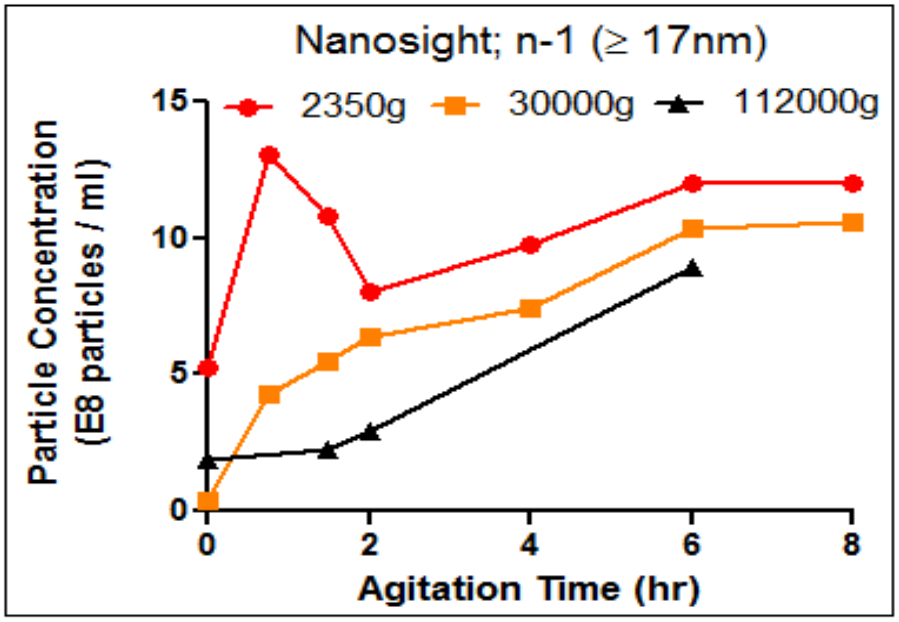


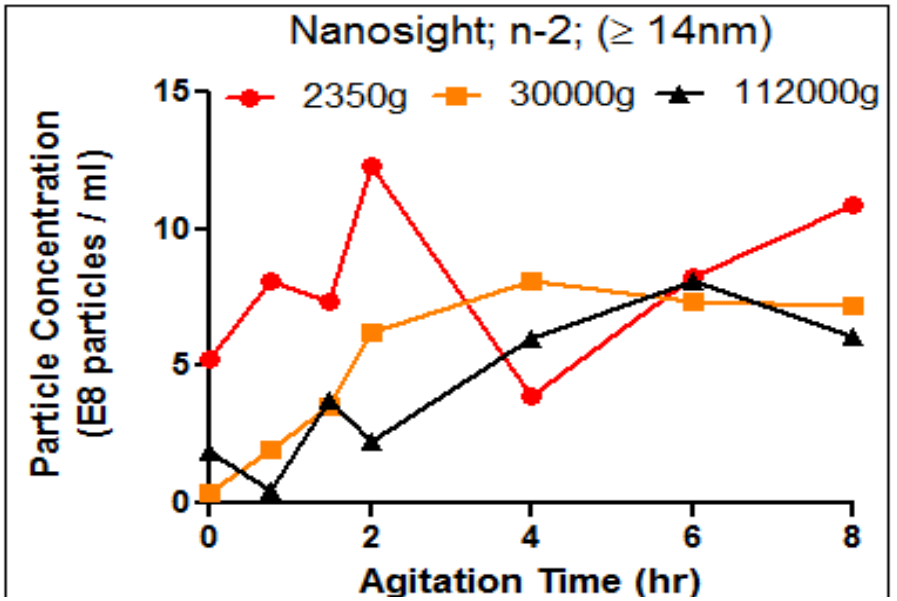


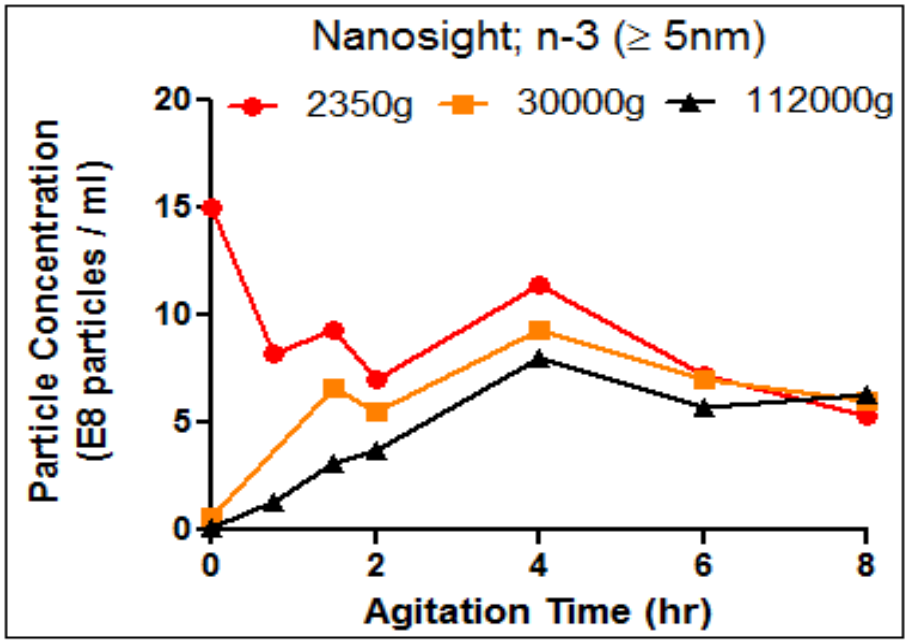


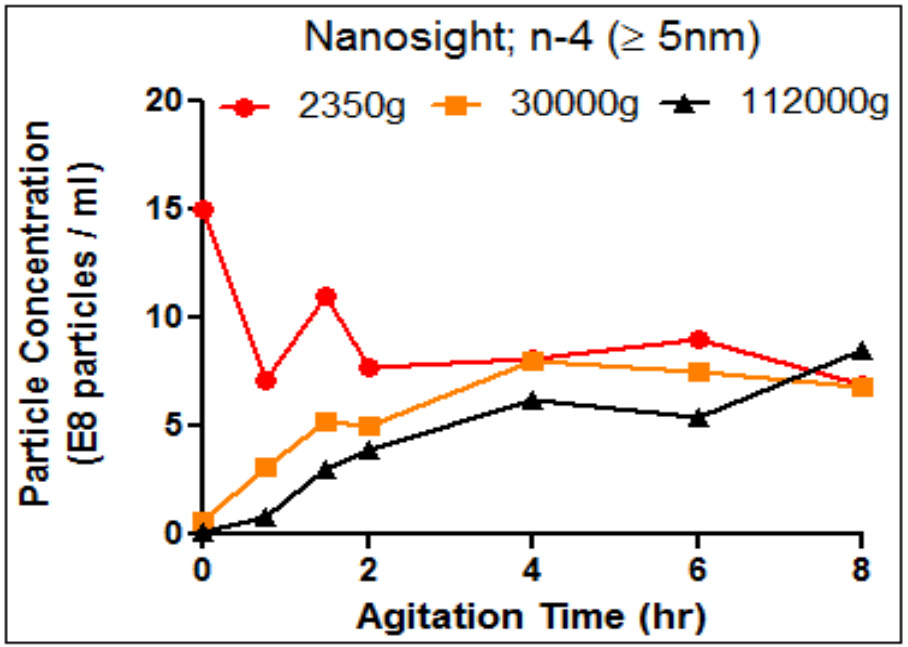
Results shown for individual sample vials.

Nanoparticle Concentrations during Agitation







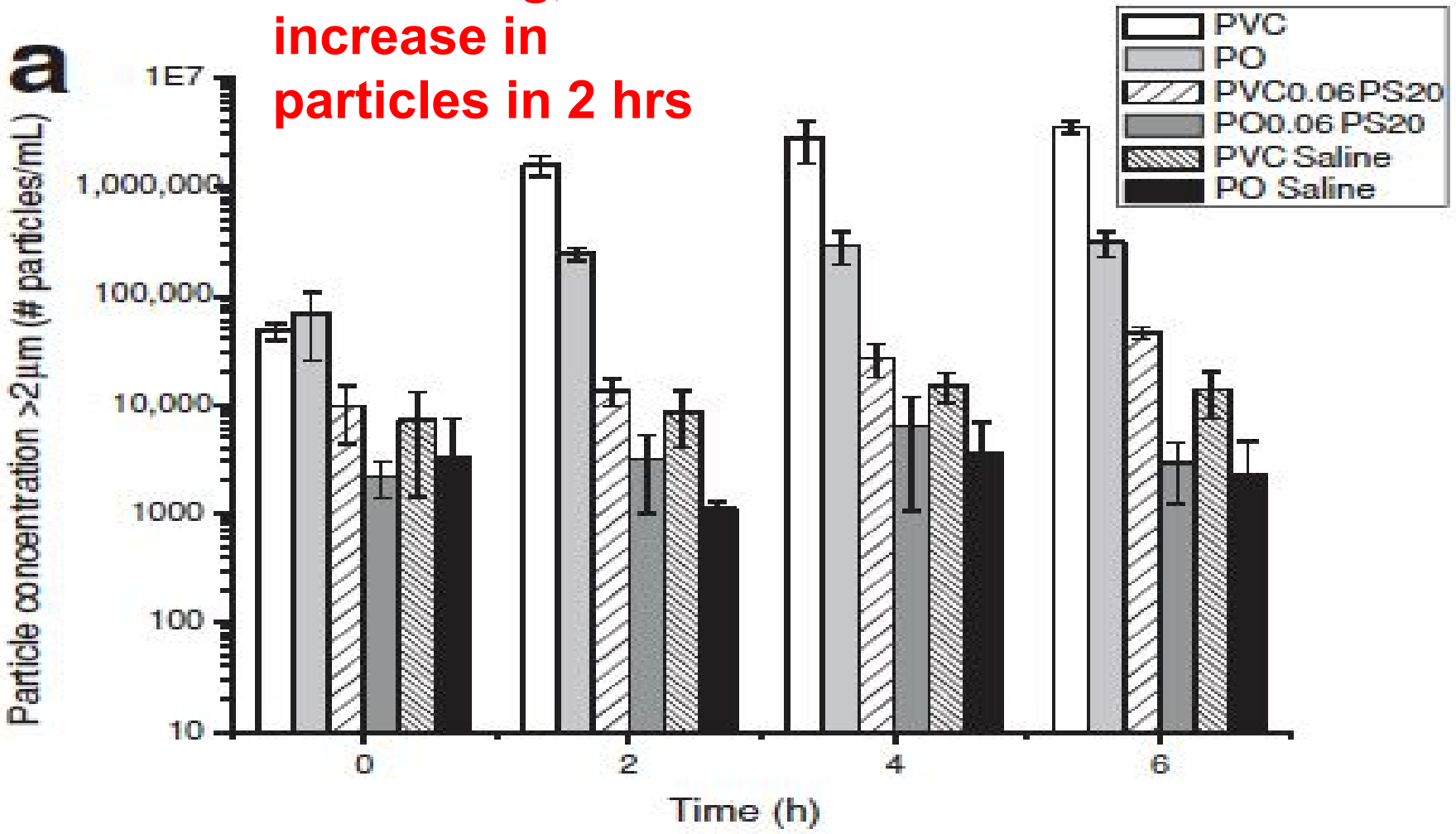


Stresses to Protein Therapeutics even during "Proper" Handling for IV Infusion

- Stabilizing excipients greatly diluted
- Air-water and solid-water interfaces for protein adsorption, aggregation and particle formation
- Particles from WFI, and IV bag, tubing, etc.
- Silicone oil from syringes used in rehydration and for final saline flush of line
- Peristaltic pump with silicone tubing
- With 0.2 and 1.2 micron in-line filters, plenty of particles can still get through system; **also filters can shed particles**
- Plasticizers leach into solutions

Microparticles in IgG4 formulation incubated in IV Bags

In PVC bag, > 20x increase in particles in 2 hrs



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Mishandling of Products by Pharmacies, Clinics and Patients

- Poor understanding and appreciation for proper handling of therapeutic protein products
- Could damage to a given dose play a role in adverse immunogenicity?
- Mishandling can cause particles and aggregates but extent of problem is unknown; studies needed mimicking situations in clinics, pharmacies, homes, cars, etc.
- We must better educate end users to minimize mishandling and associated product damage

Mishandling of Products by Pharmacies, Clinics and Patients

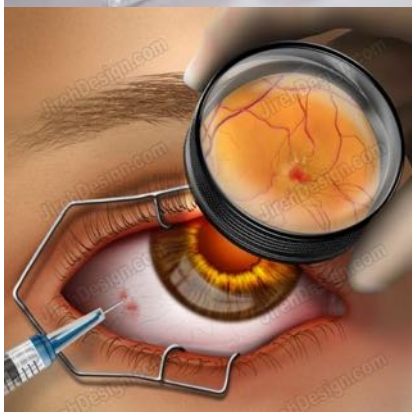
- IV administration
  - Prolonged exposure to light/elevated temperatures
  - Improper agitation or mechanical shock during transport (e.g., pneumatic tubes) from pharmacy to clinic
  - Minimal guidance from manufacturers
- Accidental freeze-thawing of prefilled syringes
  - “Blue ice packs” are at -20°C and can freeze products during shipping
  - Beware of cold spots in refrigerator

Mishandling of Products by Pharmacies, Clinics and Patients

- Exposure to high temperatures (e.g., transportation of Humira by pharmacies and patients).
- Mechanical shock; dropping box or syringe
- Exposure to light
  - Primary container left in sun light
  - With long enough exposure, even room lights can cause damage
- Incorrect rehydration of lyophilized products; e.g., vigorous shaking

Mishandling Case Study: Repackaged Avastin

- Avastin (Genentech)
  - rhmAb IgG1;
  - anti-Vascular Endothelial Growth Factor (VEGF);
  - Intravenous use in combination with chemotherapy for colorectal, lung, and kidney cancer;
  - Off-label intraocular use for wet age-related macular degeneration (AMD).
- Lucentis (Genentech)
  - rhmAb IgG1 fragment;
  - designed for intraocular use.
- Cost per dose: Avastin ≤$50 *vs* Lucentis >$2000
- Annual cost: Avastin ≤$650 vs Lucentis > $26,000



Gower, et al. *Retina*,2009,30(2),212-21
McGimpsey, et al, *Br J Ophthalmol* 2008 92: 436-437

http://images.usatoday.com/news/_photos/2006/07/10/avastin.jpg
http://bioethicsbytes.files.wordpress.com/2008/09/lucentis.jpg
http://i1.allaboutvision.com/i/me/amd-intravitreal-inject-231x260.jpg

Mishandling of Avastin

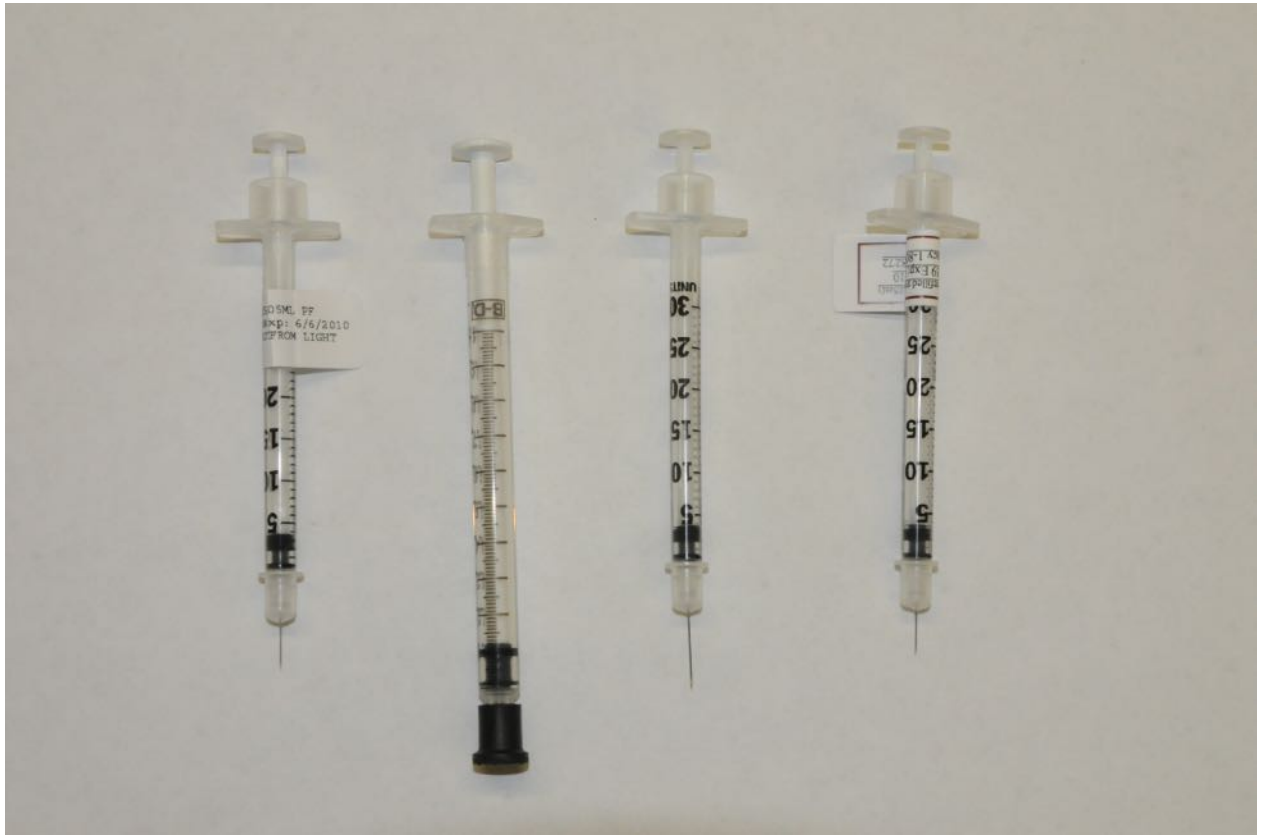
- The safety and efficacy of off-label use of intravitreal Avastin now documented in large randomized, double-blind, multi-center clinical trial

- But there have been reports of sustained elevation of intraocular pressure (IOP) and inflammation after the intravitreal use of Avastin; much lower apparent incidence with Lucentis.

- Mishandling of repackaged Avastin suspected
  - Off-label use of BD plastic insulin syringes
  - Multi-dose products created from single dose vial
  - No data from compounding pharmacies for stability, shipping or handling validation

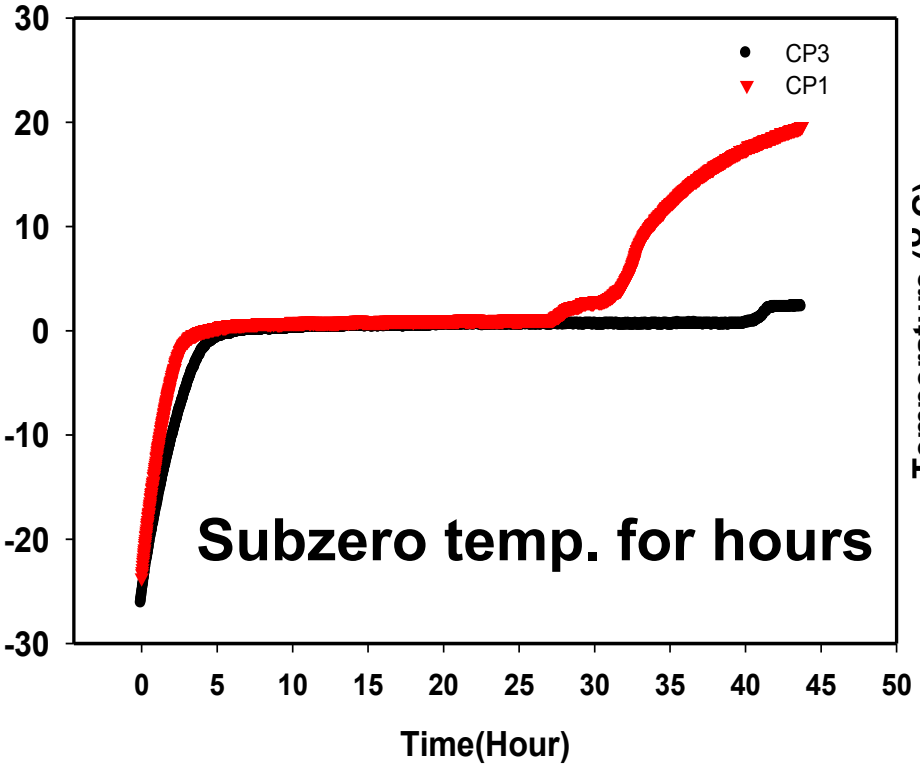
Jager, et al., *Retina*,2004,24(5),676-698; Manzano, et al., Retina,2006,26(3),257-261
Rich, et al., *Retina*,2006,26(5),495-511; Ness, et al., *Retina*,2010,3(2),332-338;
Artunay, et al, *Eye*, 2009, 23, 2187–2193; Yamashiro, et al, *Retina*,2010,30(3),485-490
Kahook,et al, *Ophthalmic Surg Lasers Imaging.* 2009, 40(3):293-295.

Avastin repackaged by compounding pharmacies in BD plastic syringes and shipped with polymer packs

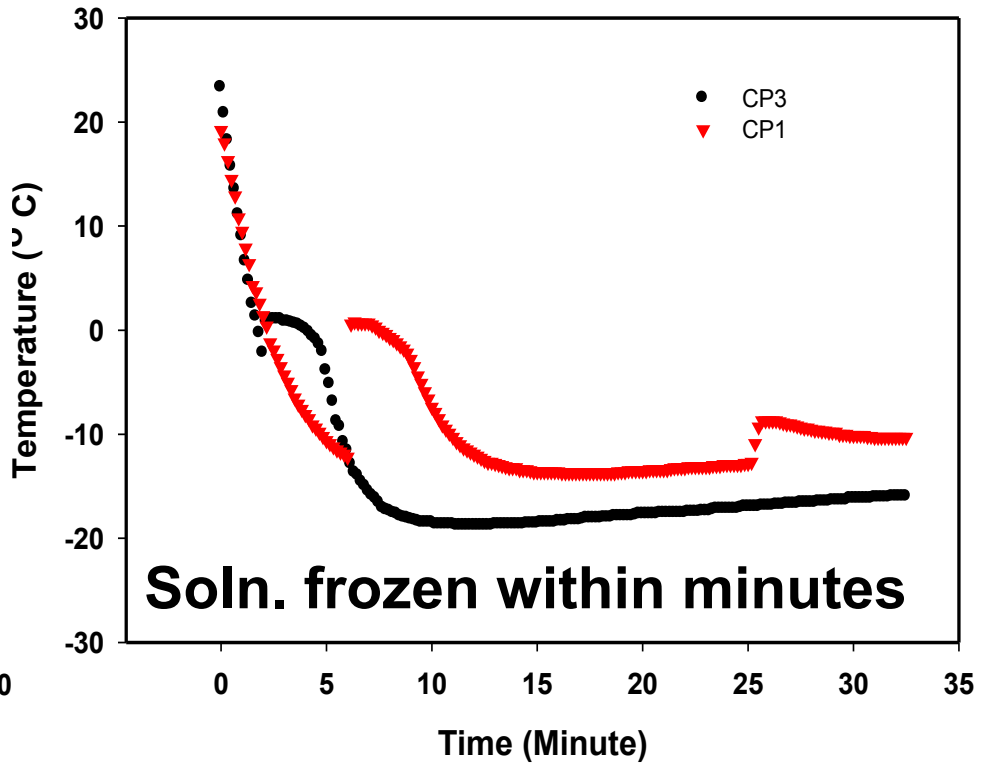


Possible freeze-thawing of repackaged Avastin during transportation

The surface temperatures of **gel packs** from CP 1 & 3 as a function of time.

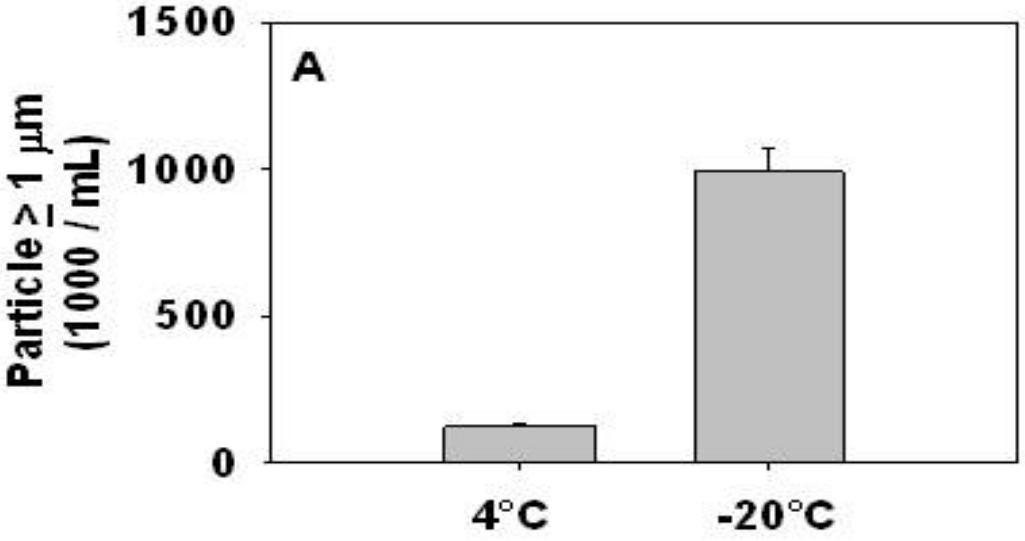


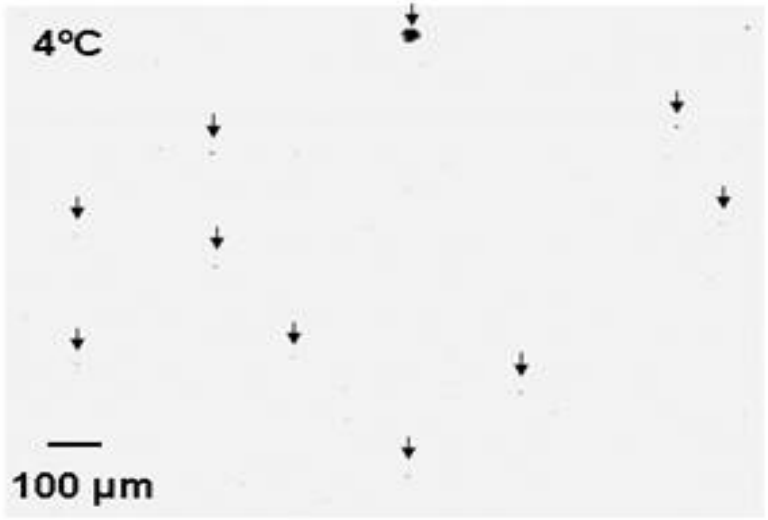
The temperatures of **placebo in the syringe** as a function of time.

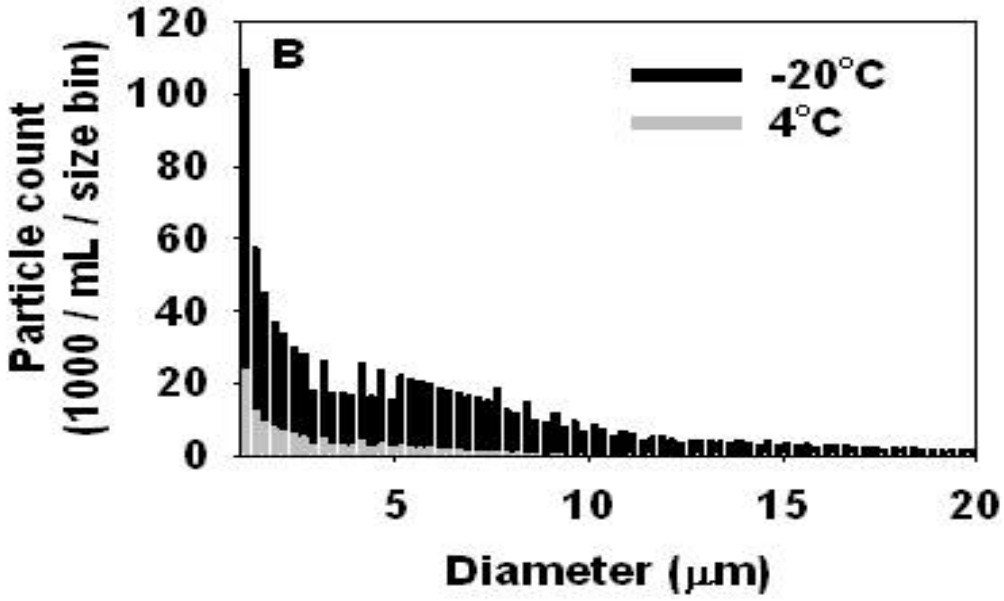


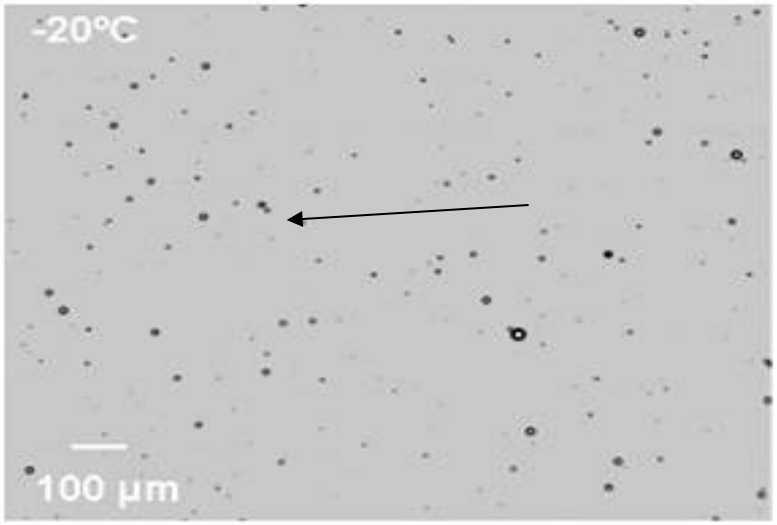
Liu et al., 2011, IOVS 52:1023

Freeze-thawing greatly increases particle levels in repackaged Avastin









Future Studies

- Effects of IV infusion procedure on aggregates and particles in **Remicade**
- Follow prescribing information as wells as protocol from CU Medical Center; run in lab
- Quantify and characterize particles, aggregates and leachates.
- Study as a function of time during run, saline bag type, filter size/brand, location in IV system, etc.

- Goal is to understand effects & **optimize proper handling** to minimize aggregation/particle formation during IV administration.

Conclusions

- Protein particle formation is ubiquitous in the production, shipping, storage and delivery of therapeutic proteins.
- Subvisible particles are critical species on protein aggregation pathway
- Particles serve as adjuvants and promote immunogenicity
- How do we optimize “proper” handling?
- How do we minimize mishandling of products in pharmacies, clinics and by patients?

2014 Workshop on Protein Aggregation and Immunogenicity

July 15-17

Breckenridge, Colorado

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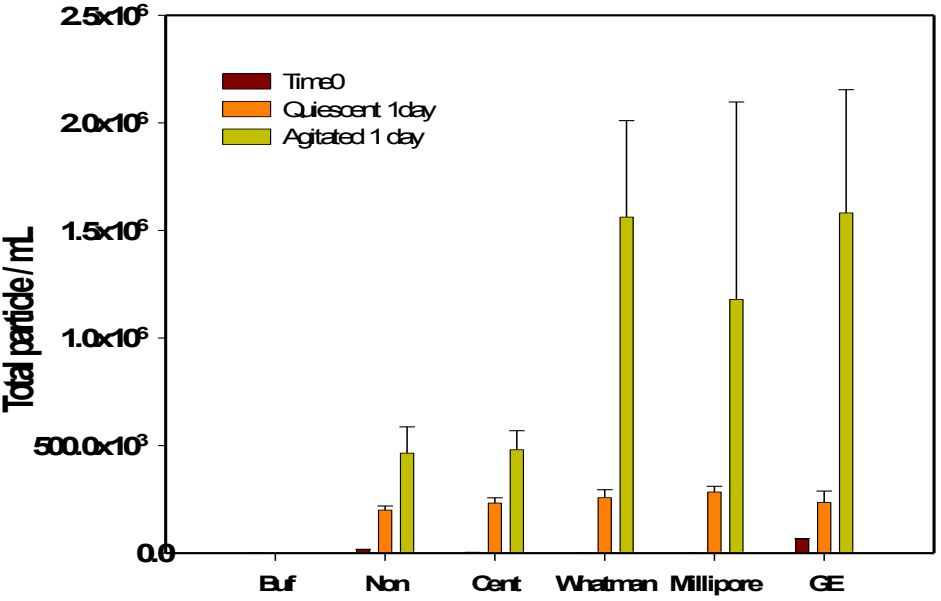
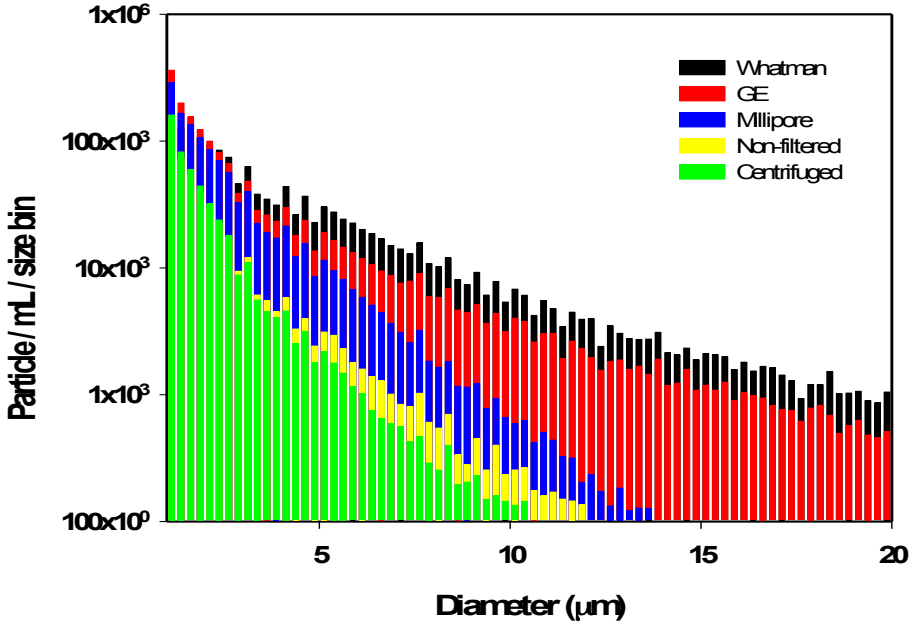
Effects of Particles Shed from Filters on Post-Filtration Aggregation

- For therapeutic protein manufacturing, filtration is used used for formulation exchange, virus removal and just before final filling.
- Filtration is also used to remove particles during IV administration of some protein drugs.
- But filters themselves can shed particles.
- And protein molecules can adsorb/desorb to/from filter materials.
- Both effects may cause protein aggregation.

Particles After 1-day of Agitation of Growth Factor: Induced by "unseen" nanoparticles?





Buf: 10mM Na Citrate buffer
Non: not filtered or centrifuged
Cent: centrifuged at 3000rpm(1400g) for 10 min
Whatman, Millipore and GE are PES membrane syringe filters without glass microfiber

Liu et al., unpublish