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Real-time observation of protein aggregates in pharmaceutical formulations using liquid cell electron microscopy†

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Abstract

Understanding the properties of protein-based therapeutics is a common goal of biologists and physicians. Technical barriers in the direct observation of small proteins or therapeutic agents can limit our knowledge of how they function in solution and in the body. Electron microscopy (EM) imaging performed in a liquid environment permits us to peer into the active world of cells and molecules at the nanoscale. Here, we employ liquid cell EM to directly visualize a protein-based therapeutic in its native conformation and aggregate state in a time-resolved manner. In combination with quantitative analyses, information from this work contributes new molecular insights toward understanding the behaviours of immunotherapies in a solution state that mimics the human body.

Introduction

The use of immunotherapies to specifically treat and manage human disease holds great potential to advance the biomedical field. With an increasing number of immunomodulators in the pharmaceutical pipeline, new technologies are needed to assess and characterize these protein-based reagents.¹ A major challenge that persists in pharmaceutical research is the problem of protein aggregation. Protein aggregates can cause a reduction in drug stability and efficacy, as well as immunogenic responses in patients.^{2,3} These considerations impact the safe administration of drug products highlighting the importance of understanding aggregation processes in greater detail.

Historically, protein aggregation is evaluated by the use of biophysical techniques including size exclusion chromatography (SEC), analytical ultracentrifugation (AUC), dynamic light

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scattering (DLS), high accuracy (HIAC) particle counting, and micro-flow imaging (MFI). These techniques can detect soluble proteins, particles, or aggregates in the sub-visible (1–100 μm) size range.^{2,4} There are limited technologies that can reliably detect protein species in the sub-micron (0.1–1 μm) particle size range.⁵ Two useful modalities to study proteins at this level include atomic force microscopy (AFM) and infrared nanospectroscopy.^{6,7} However, FDA guidelines are leaning toward assessing drug products at levels even smaller than the sub-micron range, creating a major impetus for new detection techniques.⁸

Electron microscopy (EM) is an ideal method to evaluate biological polymers through the direct observation of entities in the nanometer range. A current working hypothesis in the field is that protein aggregation occurs through a concerted series of protein unfolding steps. These steps are compounded by protein–protein interactions as new residues become exposed that are otherwise natively inaccessible.⁹ EM imaging can shed light on the nanoscale properties of protein aggregates to reveal their morphology and behaviours. While recent materials science applications have greatly improved our understanding of metallic nanoparticles and beam-induced effects,^{10–14} our work on biological materials provides a glimpse into the world of drug formulations while contributing new technical insights to the growing area of *in situ* EM research.

Cryo-EM specimen preparation preserves biological structures in a thin film of amorphous ice by a process known as vitrification.¹⁵ The vitreous ice also protects specimens while inserted in the high vacuum system of the microscope. Liquid cell EM that utilizes microfluidic technology offers an alternative approach to conventional preservation procedures.^{16,17} Using this new imaging modality biological specimens can be directly visualized in a liquid state within an EM. Under low-dose conditions (<1 electron per $\text{\AA}^2$) specimens containing fragile RNA strands remained intact while in mild buffer solution.^{11,18} Thus, local variances due to radiolysis can be somewhat attenuated by the use of solution that resists changes in pH, as recently demonstrated in theory and in practice.¹⁹

Here, we used liquid cell EM to study a protein-based therapeutic agent. We evaluated the effects of physical conditions that are considered harsh to proteins, commonly known as “stress conditions”. Stress effects can cause proteins to unfold and aggregate, and are a major concern to drug formulators. We further examined antibody interactions with the therapeutic agent at the nanoscale. For these assessments we used a drug-conjugate, PEGylated Interferon α_{2a} (Pegasys®; Roche²⁰) due to the following rationale: 1) the interferon monomer is 40 kDa and contains branched PEGylation sites of up to 40 kDa, giving it a sufficiently large overall molecular weight (~80 kDa) that can be viewed in the TEM as a discrete monomer, multimer, or protein aggregate; 2) it is a well-characterized formulation; 3) Pegasys® is FDA-approved and is being used to treat cancer and viral diseases including Hepatitis B/C in human patients; and 4) its association with a range of antibodies has not been evaluated at the molecular level using EM. Overall, the presented work demonstrates a new opportunity for real-time imaging that can help in understanding the temporal evolution of protein based therapeutic structures.

Materials and methods

A. Preparation of PEGylated interferon α_{2a} (Pegasys®)

PEGylated Interferon α_{2a} (Pegasys®) was prepared for stress treatment experiments using the following procedures. For acid-treated samples, aliquots (50 μ l) of the Pegasys® drug were mixed with 100 mM formic acid yielding a solution at pH 4.5. The solutions were incubated at 25 °C for 1 hour prior to dilution with 50 mM HEPES buffer (pH 7.5) containing, 150 mM NaCl, 10 mM CaCl_2 , and 10 mM MgCl_2 to reach a final concentration of 0.01 mg ml^{-1} . For freeze/thaw conditions, aliquots (50 μ l) of the Pegasys® drug were removed from storage at -20 °C, thawed to room temperature (25 °C) and returned to storage at -20 °C for a total of six cycles. Aliquots were mixed with HEPES buffer at a final concentration of 0.01 mg ml^{-1} prior to imaging experiments. For heat-stress conditions, aliquots (50 μ l) of the Pegasys® drug were mixed with HEPES buffer (0.01 mg ml^{-1} final concentration) and heated at 50 °C for one hour prior to imaging.

B. Preparation of protein aggregates for liquid cell TEM

The Pegasys® reagent (0.01 mg ml^{-1} in HEPES buffer) was incubated at 50 °C for 60 minutes. The resulting product was added to glow-discharged SiN base chips having a 10×10 array of microwells etched down to accommodate a depth of 150 nm. Each sample was incubated on the microchips for an additional minute then stained with 0.1–1% uranyl formate for 1 minute. Conventional staining methods utilize heavy metal salts (>2%) to encapsulate or fix EM specimens that can be subsequently dried at room temperature or flash-frozen in liquid nitrogen. By comparison, contrast enhanced specimens prepared for liquid cell TEM imaging require lower concentrations of stain (1%) to better visualize biological samples and soft materials.

A notable difference in conventional “positive” staining and “negative” staining methodologies relate to the manner in which the heavy metal ions interact with or preserve the specimen. During positive staining, metal ions associate with the specimen to produce dark contrast regions in comparison to areas that do not bind to the heavy metals. During negative staining, metal ions surround the specimen so that the sample appears white on a dark background. For liquid specimen, excess stain is removed then phosphate-free buffer solution is added to maintain hydration. The contrast observed in our liquid specimens presented here is consistent with previously described methodologies.¹⁶

To completely seal the microfluidic assembly containing the protein samples and contrast reagent, the top microchip was also glow-discharged and added to the assembly that was loaded into the tip of the Poseidon specimen holder (Protochips, Inc.) at room temperature. The microchip assembly was sealed with a metal face-plate held in place by three brass screws, illustrated in Fig. 1a–d.

C. Preparation of PEGylated interferon α_{2a} with antibodies

The Pegasys® formulation (0.18 mg ml^{-1}) was incubated with three different types of antibodies: 1) human anti-PEG (backbone specific) polyclonal IgG; 2) rabbit anti-PEG (methoxy endcap specific) monoclonal IgG; and 3) human anti-PEG (backbone specific)

monoclonal IgM. All antibody-Pegasys® mixtures were diluted to a final concentration of 0.01 mg ml⁻¹ in HEPES buffer. Aliquots (3 µl each) of the mixtures were applied to continuous carbon coated EM grids or silicon nitride microchips and incubated for one minute. Control samples contained the same concentration of antibodies, but lacked the Pegasys® reagent. For time course experiments, all samples were incubated on ice for 1 hour and prepared for TEM imaging at various time points.

D. Electron microscopy and cinematography

All specimens were examined using an FEI Spirit BioTwin TEM (FEI Company, Hillsboro, OR, USA) equipped with a LaB₆ filament operating at 120 kV under low-dose conditions (0.1–1 electron per Å²). Images were recorded using an FEI Eagle 2k HS CCD camera having a pixel size of 30 µm. For real-time acquisition, sequential images were collected at 1 s intervals for a cumulative dose of ~6–8 electron per Å² for the 30 and 40 s recordings. The image sequences were colored for visualization purposes and contour maps were computed and segmented according to statistical significance in electron density. Image sequences were imported into the iMovie 10.0.7 software package (Apple, Inc.) and the movies were compiled and exported (.mov format) for real-time observation. During data collection, we obtained multiple images for each stressed and unstressed condition and performed each experiment in duplicate. For the liquid cell imaging results, multiple images were collected and movies were produced from these imaging session. Velocity measurements were calculated for protein aggregates occurring in the movie frames using MATLAB.

E. Image quantification and particle analysis

Images for each stress condition were imported into the Clemex image analysis system (Clemex PSA, Version 6.0, Clemex Technologies Inc.). A calibration factor (µm/pixel) was assigned to each image by the software package on the basis of the width of a caliper (µm) being equivalent to the scale bar in each image. Particles in each image were individually measured across the long axis of each particle using the line measurement tool in the software package using previously described routines.²¹ To validate these measurements, we performed manual measurements using the SPIDER software package²² and automated measurements using the PARTICLE package (<http://www.image-analysis.net/EM/>). Data from these methods were nearly identical. Particles for each stressed condition along with the unstressed control were sorted by size and binned according to diameter measurements. The number of particles within each bin was plotted against the particle size. To summarize the data analysis, the number of particles within each bin was calculated as a percentage of the total number of particles measured for each stressed condition and control, then plotted against size dimensions. Statistical analysis performed on these measurements used the Schierer–Ray–Hare extension of the Kruskal–Wallace test²³ to delineate the size distributions. We implemented the criteria that if the p value is less than 0.05 within a bin, then the treatments have an effect on the measured particles (Fig. 1, ESI†).

Results and discussion

PEGylated interferon α_{2a} examined under stress conditions

PEGylation, or the attachment of polyethylene glycol (PEG) to a therapeutic protein, offers several advantages in drug delivery. Among these advantages are enhanced bioavailability, resistance to enzymatic degradation, slower renal filtration, and generally reduced immunogenicity.^{20,24,25} However, the manner in which PEGylated drugs behave in solution has not been widely studied using direct imaging techniques. To investigate this issue, we examined the Pegasys® formulation under a variety of conditions using liquid cell EM and the Poseidon system (Fig. 1).

The Pegasys® formulation was subjected to different stress conditions to provide insight for related manufacture, storage, and administration effects that protein-based therapeutics may encounter. These conditions include but are not limited to changes in pH, temperature, and immune response. To test for response to lower pH which would occur during endosomal processing in cells, aliquots (50 μ l) of the Pegasys® drug were mixed with 100 mM formic acid yielding a solution at pH 4.5. The solutions were incubated at 25 °C for 1 hour prior to dilution with HEPES buffer (pH 7.5) to reach a final concentration of 0.01 mg ml⁻¹. For freeze/thaw storage test conditions, aliquots (50 μ l) of the Pegasys® drug were stored at -20 °C, warmed to room temperature (25 °C) then placed back at -20 °C for six total cycles. An aliquot was mixed with HEPES buffer to a final concentration of 0.01 mg ml⁻¹. For heat-stressed conditions, aliquots (50 μ l) of the Pegasys® drug were mixed with HEPES buffer (0.01 mg ml⁻¹ final concentration) and heated at 50 °C for one hour. An aliquot of the control sample was diluted to the same concentration for comparison. EM specimens were then prepared using standard negative staining procedures.^{22,26} We also prepared contrast-enhanced specimens by placing the stained microchips in the Poseidon liquid specimen holder (Protochips, Inc.) and subsequently adding buffer solution to the specimen to maintain them in a hydrated state during imaging procedures.¹⁶

Images of specimens examined in the Poseidon specimen holder revealed that the acid-treated and freeze-thaw stress conditions had only a moderate effect on the integrity of the Pegasys® drug in comparison to untreated control specimens (Fig. 2a–c). Images of the drug reagent heated to 50 °C for 60 minutes showed larger protein aggregates of a variety of sizes that were smooth, round, and devoid of major structural features (Fig. 2d). Similar trends in size distribution analysis were observed by size exclusion chromatography (SEC) for each of the stressed and control samples (Fig. 2e; 2–5, ESI[†]). In this separation technique, larger items migrate around the beads that makeup the column while smaller items are retained in the beads. Hence, larger items found in the heated sample, showed a shorter retention time (~3.8 min) than smaller items found in the other conditions (~4.4 min).

Quantitative analysis performed on the images showed the peak in particle size was 3–8 nm for each sample (Fig. 2f; 1, ESI[†]). However, the size range distribution for the heated sample (2–72 nm) was quite different from the other samples. The size distribution for the control sample was 0.5–23 nm. For the acid-treated samples particles ranged from 2–12 nm; and from 0.5–17 nm for the freeze-thaw samples. Upon examining the crystal structure of a non-PEGylated form of interferon α_{2a} (Fig. 2g, pdbcode 1ITF²⁷), we found that the size of the

molecular model (3 nm) was consistent with the features of the particles observed in the TEM under the acid-treated, freeze–thaw, and untreated conditions. Combined analysis of these results suggested that oligomeric states of the Pegasys® were also likely present in the samples.

Real-time imaging of protein aggregates in solution

To observe the behaviour of aggregates in the drug formula, we performed liquid cell EM imaging. The Pegasys® formulation was heated to 50 °C for 60 min in an Eppendorf microfuge tube then aliquots (0.2 µl) were applied to glow-discharged SiN base chips. The microchips were assembled in the Poseidon specimen holder, stained with contrast reagent or left unstained, while hydrated using HEPES buffer solution, and examined under low-dose conditions using a FEI Spirit Bio-Twin TEM, operating at 120 kV. Images of the Pegasys® formulation revealed protein aggregates in the fluidic chamber migrated slowly in the surrounding liquid during a 40 second time course (Fig. 3a–c).

Images were colored and contour maps were calculated in a region of interest (ROI) based on statistical significance in electron density (Fig. 3d). Darker colors represent significant differences in density over the image background ($3-4\sigma$). Lighter colors represent less significant differences in density compared to the image background ($< 2\sigma$). Diffusion rates based on pixel displacements were quantified for aggregates numbered 1–7 having variable surface areas within the ROI (Fig. 3e). Overall, there was a strong relationship determined between aggregate size and fluid velocity ($R = 0.80$) during the 40 second time interval based on tracking 151 190 total pixel displacements. The larger aggregates travelled in solution at a slower rate than the smaller aggregates with overall movements being 0.5 nm per second (Fig. 3e). Time-resolved videos show movements of the protein aggregates with respect to the edge of an integrated microwell etched into the microchip that served as a reference point. (Movie 1–2, ESI†).

To ensure these observations were not due to imaging artefacts, we performed the same experiments on the heated samples prepared without contrast reagents. We identified regions of different liquid thicknesses and quantified migrating entities within a 40 s interval. Within one region of interest, we noted protein clusters, diffusible particles, and protein aggregates (Fig. 4a and b). Migration measurements were then performed on the different moving items (Fig. 4c).

Quantifying the overall displacement of each item showed the diffusible particles each moved ~7 nm, while the clustered protein material and aggregate moved ~3–4 nm (Fig. 4d). The aggregate migrated farther than the cluster, likely due to the thicker region of liquid surrounding the material that facilitated its travel. This migration trend was consistent with the aggregate movements observed in the samples prepared with contrast reagent.

To better understand how individual components in the Pegasys® formulation associated in solution following heat-induced aggregation, we evaluated additional regions of the specimens for quantification purposes and movie recordings. Individual frames from the movies are shown in Fig. 5a at representative time points (0, 20 and 40 seconds). Regions of interest were colored and contour maps were generated to evaluate individual aggregate

movements (Fig. 5b and c). Statistical differences in signal-to-noise in the images revealed the presence of denser protein aggregates. Darker colors represent significant differences in density over the image background ($3-4\sigma$). Lighter colors represent less significant differences in density ($< 2\sigma$) (Fig. 5d).

As the aggregates (numbered 1–6) in this ROI did not rapidly diffuse during the imaging process, we evaluated degrees of association within the movie recordings. Protein aggregates of varying surface areas (Fig. 5d, 1–6) were measured and each aggregate increased in size during the 40 s time interval (Fig. 5e). A heat map indicates cumulative changes in particle density for each of the six aggregates evaluated. This assessment showed a gain, loss, no change in pixel values with respect to the liquid background while tracking 93 440 total pixel displacements during the 40 second recording. Associated movies showed the increasing dimensions of the aggregates in solution (Movies 3–5, ESI†). Surprisingly, the propensity to associate was not based on size, but rather the proximity of other aggregates and the lack of rapid diffusion noted in the ROI.

To complement these experiments, we examined heat-stressed protein aggregates, prepared in the absence of contrast reagents (Fig. 6a). Images acquired over a 30 s time interval allowed us to evaluate the growth of protein aggregates (1–6) in solution, represented as contour plots in Fig. 6b. Colored renderings based on statistical differences in electron density, revealed changes in the surface area of each aggregate (Fig. 6c).

A heat map plot of the changes was superimposed over the contour plot. This data showed that aggregates 4 and 5 had the most growth, each between $100-150\text{ nm}^2$, while the other aggregates showed moderate changes in dimensions (Fig. 6d and e). These complementary results provide new insights for measuring unstained aggregate in a real-life manner involving drug storage or administration.

PEGylated adducts influence protein aggregation

Results from the present microfluidic imaging studies suggested that the Pegasys® drug may potentially elicit an immunogenic response in the body should aggregates form in regions of low velocity. To improve our understanding of binding interactions between the pegylated drug and immunogenic macromolecules, we prepared mixtures of the Pegasys® formulation with different antibodies having specificity for the PEG backbone or the functionalized terminal endcaps of the PEG chains. Antibody varieties used for these studies included polyclonal IgG antibodies (pAb), monoclonal IgG antibodies (mAb), and multivalent IgM antibodies. Experiments contained the Pegasys® formulation and each separate type of purified antibodies in solution prepared at a final concentration of 0.01 mg ml^{-1} . All mixtures were incubated at $4\text{ }^{\circ}\text{C}$ for up to 24 hours. At each time point, an aliquot of the mixtures was removed and examined using TEM imaging.

We acquired multiple images over several viewing areas. The images revealed no significant differences in the mixtures containing the drug and antibody controls under unstressed conditions. Also, no large aggregates formed in the antibody-drug mixtures during a 24 hour period (Fig. 7a–c and 6, ESI†). The IgM antibodies were somewhat unstable in the control samples as indicated by the appearance of smaller protein fragments. These observations

suggested that a branched distribution of PEGylated adducts on the interferon protein was an optimal design to minimize aggregation with immune proteins, including those with multiple binding epitopes such as IgM subtypes.

As an additional control, we examined PEG-antibody interactions by mixing the same antibody varieties with a biotin substrate having linear PEGylated chains that ranged in molecular weight from 2–40 kDa (Fig. 7, ESI†). This linear arrangement of PEG adducts is much different from the branched arrangement engineered onto the interferon protein in the Pegasys® formulation. Testing an alternative arrangement of adducts may provide potential insight toward understanding interactions during immune surveillance. The same experimental conditions were used for the pegylated substrate (0.01 mg ml^{-1}) and antibody (0.01 mg ml^{-1}) mixtures that we employed for the drug-antibody studies. EM images were acquired for these mixtures and compared with control images containing the antibodies lacking the pegylated substrate. No major aggregates were observed in the unstressed mixtures or the monoclonal or polyclonal IgG antibody control samples (Fig. 7, ESI†).

Unexpectedly, the IgM antibodies showed different morphologies following a 60 minute incubation with the various PEGylated substrates. Control IgM samples did not show aggregation-like effects (Fig. 7a–d, ESI;† right panels).

These results suggested that antibodies such as IgMs that contain a greater number of antigenic sites may be able to associate with multiple epitopes arranged linearly on the same substrate. Also, as the complexity in the underlying drug-antibody associations increased, aggregation potential was enhanced. Taken together, with this information suggested that linear pegylated chains may elicit undesirable immunogenic interactions in solution, but that branched pegylated chains of similar molecular mass as seen in the Pegasys® formulation, may go unscathed by a variety of antibodies in solution.

Conclusions

Compared to the current state of knowledge, these results demonstrate a new set of complementary imaging tools to identify and monitor protein-based drug formulas in a direct and rapid manner. The significance of the results is in the ability to observe real-time movements of therapeutic reagents in solution in a manner that mimics the body. Such insights can be further used to inform drug formulations by better defining how stress conditions promote protein aggregative effects, which need to be prevented according to FDA guidelines.

Harmful stressed conditions that we considered in our studies were related to issues of product storage and dose administration. These issues are important to consider as understanding the sources that lead to temporal changes in molecular structure can help minimize immunogenic responses. As we continue to develop more quantitative assessments of these effects, the next challenge will be to increase the time-frame of our real-time recordings to watch antibodies interact directly with aggregated reagents. As such, future experiments will involve determining chemical parameters to modulate aggregative effects to prevent such interactions. Implementing these procedures through the routine practice of

liquid cell EM technology can provide a direct screening method and cost saving measures for the pharmaceutical industry.

Overall, the presented work demonstrates a new means to visualize and assess the behaviour of protein-based therapeutics at the molecular level. Using liquid cell EM imaging tools, we present the first real-time movies of protein aggregates migrating in solution at the nanoscale. Further quantification and image analysis of proteins and modified substrates in solution revealed the manner in which PEGylated chains can influence aggregation-like properties. Importantly, we also found that branched chains of PEGylated adducts, as in the Pegasys® formulation, provided an optimal arrangement to minimize immune protein clustering and aggregation. Collectively, we demonstrate an alternative strategy to precisely examine protein-based therapeutic agents at the molecular level, which is of particular interest in designing drugs for extended circulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Das TK. Protein particulate detection issues in biotherapeutics development—current status. *AAPS PharmSciTech.* 2012; 13:732–746. [PubMed: 22566174]
2. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. *J. Pharm. Sci.* 2009; 98:2909–2934. [PubMed: 18823031]
3. Ratanji KD, Derrick JP, Dearman RJ, Kimber I. Immunogenicity of therapeutic proteins: influence of aggregation. *J. Immunotoxicol.* 2014; 11:99–109. [PubMed: 23919460]
4. U.S. Pharmacopeia 38 and National Formulary 33. Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections. 2015
5. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, et al. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. *J. Pharm. Sci.* 2009; 98:1201–1205. [PubMed: 18704929]
6. McAllister C, Karymov MA, Kawano Y, Lushnikov AY, Mikheikin A, et al. Protein interactions and misfolding analyzed by AFM force spectroscopy. *J. Mol. Biol.* 2005; 354:1028–1042. [PubMed: 16290901]
7. Ruggeri FS, Longo G, Faggiano S, Lipiec E, Pastore A, Dietler G. Infrared nanospectroscopy characterization of oligomeric and fibrillar aggregates during amyloid formation. *Nat. Commun.* 2015; 6:7831. [PubMed: 26215704]
8. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research. Guidance for industry, immunogenicity assessment for therapeutic protein products. 2014
9. Philo JS, Arakawa T. Mechanisms of protein aggregation. *Curr. Pharm. Biotechnol.* 2009; 10:348–351. [PubMed: 19519409]
10. Dukes MJ, Jacobs BW, Morgan DG, Hegde H, Kelly DF. Visualizing nanoparticle mobility in liquid at atomic resolution. *Chem. Commun.* 2013; 49:3007–3009.

11. Dukes MJ, Thomas R, Damiano J, Klein KL, Balasubramaniam S, Kayandan S, Riffle JS, Davis RM, McDonald SM, Kelly DF. Improved microchip design and application for in situ transmission electron microscopy of macromolecules. *Microsc. Microanal.* 2014; 20:338–345. [PubMed: 24331164]
12. Liao HG, Zheng H. Liquid cell transmission electron microscopy study of platinum iron nanocrystal growth and shape evolution. *J. Am. Chem. Soc.* 2013; 135:5038–5043. [PubMed: 23477794]
13. Grogan JM, Rotkina L, Bau HH. *In situ* liquid cell electron microscopy of colloid aggregation and growth dynamics. *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.* 2011; 83:061405.
14. Schneider NM, Norton MM, Mendel BJ, Grogan JM, Ross FM, Bau HH. Electron-Water interactions and Implications for Liquid Cell Microscopy. *J. Phys. Chem.* 2014; 118(38):22373–22382.
15. Dubochet J, Adrian M, Chang JJ, Homo JC, Lepault J, et al. Cryo-electron microscopy of vitrified specimens. *Q. Rev. Biophys.* 1988; 21:129–228. [PubMed: 3043536]
16. Gilmore BL, Showalter SP, Dukes MJ, Tanner JR, Demmert AC, McDonald SM, Kelly DF. Visualizing viral assemblies in a nanoscale biosphere. *Lab Chip.* 2013; 13:216–219. [PubMed: 23208001]
17. Peckys DB, Dukes MJ, de Jonge N. Correlative fluorescence and electron microscopy of quantum dot labeled proteins on whole cells in liquid. *Methods Mol. Biol.* 2014; 1117:527–540. [PubMed: 24357378]
18. Varano AC, Rahimi A, Dukes MJ, Poelzing S, McDonald SM, Kelly DF. Visualizing virus particle mobility in liquid at the nanoscale. *Chem. Commun.* 2015; 51:16176–16179.
19. Kim J, Jones MR, Ou Z, Chen Q. In Situ Electron Microscopy Imaging and Quantitative Structural Modulation of Nanoparticle Superlattices. *ACS Nano.* 2016; 10:9801–9808. [PubMed: 27723304]
20. Dhalluin C, Ross A, Leuthold LA, Foser S, Gsell B, Muller F, Senn H. Structural and biophysical characterization of the 40 kDa PEG-interferon- α 2a and its individual positional isomers. *Bioconjugate Chem.* 2005; 16:504–517.
21. Balasubramaniam S, Kayandan S, Lin YN, Kelly DF, House MJ, et al. Toward design of magnetic nanoparticle clusters stabilized by biocompatible diblock copolymers for T(2)-weighted MRI contrast. *Langmuir.* 2014; 30:1580–1587. [PubMed: 24479874]
22. Frank J, Radermacher M, Penczek P, Zhu J, Li Y, Ladjadj M, Leith A. SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J. Struct. Biol.* 1996; 116:190–199. [PubMed: 8742743]
23. Dytham, C. Choosing and using statistics: a biologist's guide. 3. Malden, MA; Wiley-Blackwell: 2011.
24. Bee JS, Chiu D, Sawicki S, Stevenson JL, Chatterjee K, et al. Monoclonal antibody interactions with micro- and nanoparticles: adsorption, aggregation, and accelerated stress studies. *J. Pharm. Sci.* 2009; 98:3218–3238. [PubMed: 19492408]
25. Inada Y, Furukawa M, Sasaki H, Kodera Y, Hiroto M, et al. Biomedical and biotechnological applications of PEG-and PM-modified proteins. *Trends Biotechnol.* 1995; 13:86–91. [PubMed: 7766222]
26. Ohi M, Li Y, Cheng Y, Walz T. Negative Staining and Image Classification - Powerful Tools in Modern Electron Microscopy. *Biol. Proced. Online.* 2004; 6:23–34. [PubMed: 15103397]
27. Klaus W, Gsell B, Labhardt AM, Wipf B, Senn H. The three dimensional high resolution structure of human interferon α -2a determined by heteronuclear NMR spectroscopy in solution. *J. Mol. Biol.* 1997; 274:661–675. [PubMed: 9417943]

Fig. 1.

The liquid cell imaging system for TEM imaging. (a) The microfluidic chamber of the Poseidon specimen holder is comprised of an O-ring fitting, base and top chips, and a metal face-plate held in place by 3 brass screws. (b) The O-ring is set into the holder (1), followed by the base chip that is wet by the liquid specimen (2–3). The top chip is set in place over the assembly (4), and that is sealed by the metal face-plate prior to entering the TEM vacuum system (5). (c) The base chip contained an array of microwells ($400 \times 400 \mu\text{m}$) that are transparent in the electron beam. Images of protein aggregates in the Pegasys® formulation were recorded in the EM. (d) Schematic of a cross-section through the fluidic assembly positioned in the electron beam. Integrated microwells ($10 \times 10 \mu\text{m}$) etched into the chips accommodate a solution thickness of 150 nm.

Fig. 2.

EM and quantitative analysis of the Pegasys® formulation under a variety of stressed conditions. (a) Images of the Pegasys® formulation under control conditions; (b) acid-treated conditions; and (c) six freeze–thaw (FT) cycles showed particles having similar features and dimensions. (d) Images of the Pegasys® formulation heated to 50 °C for 1 hour showed a larger distribution of particle sizes and features consistent with protein aggregates. Insets show magnified areas. Scale bars are 50 nm. The contrast in each image was enhanced by the use of uranyl formate during liquid cell preparation. (e) SEC chromatographs of the Pegasys® formulation evaluated under each stress condition. Heated samples showed a major peak at a retention time of ~3.8 min (black arrow), indicating higher molecular weight species (far right). All other chromatographs showed a major peak at ~4.4 min (black

arrows), indicating lower molecular weight species (also see Fig. 2–5, ESI[†]). (f) Plot to show the relationship between particle size and the number of particles present under each condition. (g) Crystal structure of interferon α_{2a} (40 kDa; pdbcode 1ITF²⁷). Scale bar is 1 nm.

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Fig. 3.

Protein aggregates in the Pegasys® drug formulation show bulk migration in solution. (a) Protein aggregates were formed upon heating the Pegasys® drug in solution at 50 °C. Scale bar is 100 nm. (b) Serial images of the protein aggregates in solution were collected over a 40 second time course and selected regions are highlighted (red boxes). Images of the proteins aggregates revealed their bulk migration in solution toward the edge of the microwell point of reference. (c) Colored images were produced by inverting the contrast and adjusting the temperature range of each image. Scale bar in each panel is 100 nm. Please see Movie 1, ESI.† (d) Contour maps of aggregates (1–7) show differences in density

ranging from $<1\sigma$ to 4σ at 0 and 40 s. Darker colors represent a greater difference in electron density with respect to the image background. Lighter colors represent a lesser difference in electron density. Scale bar is 50 nm. Arrows point to the same regions as in panel (c) and (d). Please see Movie 2, ESI.† (e) Quantitative measurements showed differences in local displacements and velocity values among aggregates of varying size relative to the liquid.

Fig. 4.

Unstained Pegasys® formulation shows bulk migration properties. (a) Protein aggregates were formed in solution upon heating the drug at 50 °C for 60 minutes. Thick patches of liquid were noted in surrounding thin solution. Protein constituents formed clusters (white box), diffusible particles (white arrows; labeled 1, 2), and aggregates (white dashed circle). (b) During a 40 s recording, items diffused toward the lower edge of the image in the *y*-direction. (c) measurements were performed on migrating items to reveal changes in the displacement for the noted cluster, particles 1 and 2, and aggregate material (d). Smaller entities (particles 1, 2) migrated a greater distance than larger items (cluster and aggregate) within same time course. Scale bar is 25 nm in (a) and (b).

Fig. 5.

Protein aggregates in the Pegasys® formulation show changes in associations. (a) Protein aggregates formed upon heating the Pegasys® drug in solution at 50 °C showed changes in associations within 40 s. Scale bar is 100 nm. (b) Serial images of protein aggregates were collected and regions are highlighted (red boxes). (c) A region of interest (ROI) showing the greatest change is circled in white. Scale bar is 20 nm. Images were inverted and colored using a temperature factor to adjust each image equally. Scale bar is 20 nm; (please see Movie 3, ESI[†]). (d) Contour maps of aggregates (1–6) show differences in density ranging

from <1 to 4σ at 0, 20 and 40 s. Darker colors represent greater differences in electron density while lighter colors represent a lesser difference in density. Scale bar is 25 nm; (please see Movies 4 and 5, ESI†). (e) Measurements show changes in aggregate associations during 40 s. Heat map of the ROI shows relative loss (blue), gain (red), or no change (black) in density with respect to the image background (green). Changes in surface area dimensions were tracked and quantified among aggregates of varying size.

Fig. 6.

Protein aggregates in the unstained Pegasys® formulation show growth in solution. (a) Serial images of aggregates formed in solution upon heating the interferon α_{2a} drug at 50 °C for 60 minutes. Images were equally adjusted and colored using the same temperature factor. Aggregates of varying size are numbered (1–6). White arrows point to smaller particles that cannot be quantified in the contour maps. (b) Contour maps of aggregates (1–6) at 0 and 30 s show differences in aggregate growth within the density range from <1 to 4σ . Darker colors represent a statistically greater difference in electron density with respect to the background, while lighter colors represent a statistically smaller difference in electron density. (c) The surface area of each aggregate shows growth over a 30 second interval. (d) Heat map indicates regions where relative density was gained (red), or unchanged (black) with respect

to the background image (green). (e) Changes in surface area were quantified among the aggregates. Scale bar is 100 nm in (a) and (b).

Fig. 7. EM analysis of antibody mixtures with the PEGASYS® formulation. (a) Antibodies against the PEGylated backbone or endcap epitopes (b) were mixed with the Pegasys® formulation and incubated over a 24 hour time course. The contrast in each image shown here at a 1 hour incubation was enhanced by the use of uranyl formate on TEM grids that were air-dried. EM images revealed that particles in the antibody-Pegasys® mixtures were similar to control samples of antibodies lacking Pegasys®. (c) No protein aggregates were formed within the

time course tested for each antibody mixture, including IgM antibodies that are multivalent. Scale bar is 100 nm. mAb (monoclonal antibodies); pAb (polyclonal antibodies).

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