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Probing the Surface Structure of Monoclonal Antibody Aggregates with Multiscale Microscopy

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Therapeutic monoclonal antibodies suffer from poor stability and shelf life due to their propensity to form protein aggregates under thermal, light, and mechanical stresses [1]. There are persistent concerns about increased immunogenicity of these drugs due to the existence of sub-visible (< 50) protein aggregates [2, 3]. While the mechanisms and kinetics of protein aggregation are well-known in literature, the structure of protein aggregates is poorly understood [4]. A broad range of protein aggregate sizes and morphologies are observed in therapeutic mAb formulations, making it difficult to classify and characterize the higher order structure of aggregates. Ensemble characterization of protein aggregate structure with techniques like x-ray scattering or IR spectroscopy only provides approximate measurements of structure due to the diversity of protein aggregate morphology. Recent *in vitro* and mouse studies have found that both the size and structure of mAb protein aggregates impact their immunogenicity. Specifically, researchers have recently found that sub-micron protein aggregates are capable of directly activating Fcγ-receptors on antigen presenting immune cells [5]. As the structure of protein aggregates mediates how they interact with the immune system [6], a more detailed fundamental understanding of mAb protein aggregates beyond their size and gross morphology is warranted to enhance the safety of this important class of therapeutics.

Here we describe multiscale imaging experiments to probe the size and surface structure of sub-micron sized mAb protein aggregates. We utilized NISTmAb, the NIST standard reference mAb (RM 8671), to demonstrate this measurement. NISTmAb is a humanized IgG1k mAb developed specifically for testing new analytical techniques. The protein was provided at a concentration of 100 mg/mL and was diluted to 1 mg/mL in histidine buffer. Protein aggregates were formed by magnetically stirring the solution at 1000 rpm for ~5 hours, which formed a broad distribution of protein aggregate sizes ranging from 100 – 1000 nm as measured by light scattering and scanning electron microscopy (SEM) [7].

We have recently adopted a super-resolution optical imaging technique, interferometry scattering microscopy (IFS), to quantify the size distribution of sub-micron protein aggregates [7]. IFS utilizes a widefield optical microscope and a layered silicon sensor to enable visualizing and quantifying the size of low contrast nanoparticles down to sizes of ~100 nm [8]. The intensity of particles in an IFS image (I_{IFS}) is directly proportional to the volume (or radius cubed) of the nanoparticle, enabling intensity based measurement of particle size (Figure 1a,b). IFS combined with fluorescence imaging enables investigating the effect of protein aggregate size on the surface structure (Figure 1b,c). In particular, recent reports correlating Fc binding affinity of mAbs to the immunogenicity of protein aggregates (*e.g.* [6]) have motivated us to investigate how protein aggregate size affects the surface concentration of solution facing Fc domains. We fluorescently labeled Fc domains on the protein aggregate surface using Alexa fluor 488 conjugated anti-human IgG1 and standard immunolabeling protocols (Figure 2a). Quantitative analysis of the fluorescence intensity (I_{fl}) of each protein aggregate enables calculating the relative concentration of bound labels per protein aggregate (Figure 2b,c). For these protein aggregates, we find that the smallest aggregates (~100 nm) have a decreased concentration of surface bound labels, indicating they have fewer

solution facing Fc domains per surface area of the protein aggregate. The advantage of this low spatial resolution optical approach is that it provides ample statistics to establish new structural features of protein aggregates, aside from particle size, that might lead to immunogenicity. Finally, we will present recent electron microscopy studies that utilize gold nanoparticles to label Fc domains on the surface of protein aggregates, enabling nanometer spatial resolution imaging of the surface structure of protein aggregates [9].

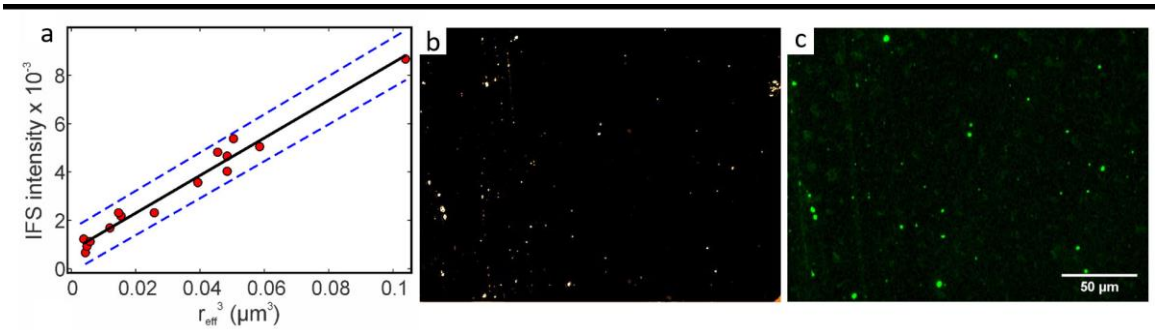

Figure 1. (a) Correlation between particle IFS intensity and particle volume for NISTmAb protein aggregates. The particle size was measured by SEM imaging (not pictured). (b) False colored IFS image of NISTmAb protein aggregates. (c) Correlative fluorescence microscopy image of Alexafluor 488 conjugated IgG labeled NISTmAb protein aggregates (Same area as (b)).

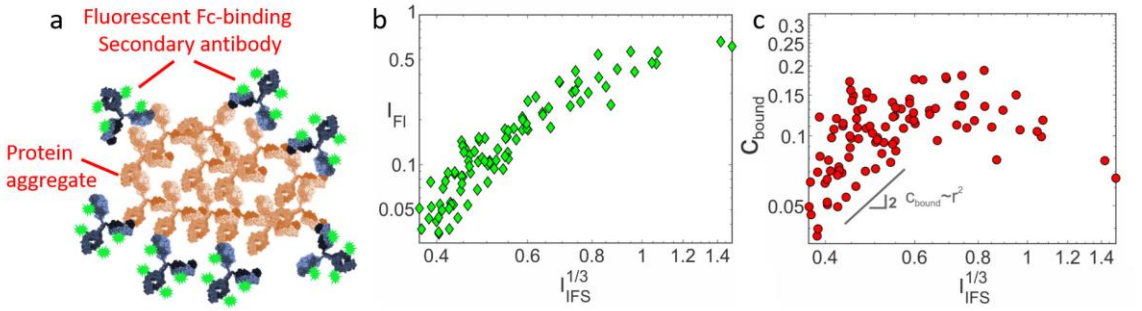

Figure 2. (a) Schematic of binding experiment. (b) Normalized fluorescence intensity of labeled NISTmAb protein aggregates as a function of the cube root of IFS scattering intensity (proportional to the radius of the protein aggregate). (c) Relative surface concentration of bound fluorescent IgG labels as a function of the cube root of IFS scattering intensity.

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