High-Throughput Detection of Degraded Polysorbate in Biological Formulations with FMM

Introduction

The FDA requires that all biologic formulations be free of visible particles and has defined allowable levels of subvisible particles (SVPs) larger than 10 µm to ensure the potency, efficiency, and safety of these drug.[1] While most protein drug particle analysis focuses on particles formed due to formulation instability, SVPs formed through the degradation of formulation excipients must also be considered.

Polysorbates 20 (PS20) and 80 (PS80), best known as Tween-20 and -80, are excipients used in >70% of marketed parenteral biological drugs to improve product stability and shelf life.[2] However, when these formulations are stored for long periods of time (>6 months) at low temperatures (4 °C), visible and subvisible particles are formed due to the enzymatic hydrolysis of the polysorbates by host cell proteins (HCPs) such as esterases and lipases.[3,4] PS20 in particular has been found to be extremely prone to degrading into fatty acid particles.[5] Lauric, myristic and palmitic acids are the most common fatty acid degradation products, and there is a direct correlation between low fatty acid solubility and particle formation in common formulation buffers.[3–7] However, high-throughput, sensitive, and specific analysis of polysorbate particles has been difficult due to their complex chemistry and their low concentrations (<<0.5%) in high protein concentration (>100–200 mg/mL) formulation environments, making it a perennial needle in a haystack problem.

In this application note, we introduce the Aura™ polysorbate degradation assay, a specific and quantitative assay that in a few hours detects free fatty acid particles (FFAs) formed during polysorbate degradation in 96 samples using anywhere from 5 µL–10 mL of sample. The Aura uses backgrounded membrane imaging (BMI) and fluorescence membrane microscopy (FMM) to count, size and ID particles from 1 µm to 5 mm. Fluorescent labels utilized by FMM are selected based on thier ability to interact with particles of a certain nature, allowing for the generation of key information related to identity of the particles.

Method

BODIPY FL C_{16} Preparation

1. Dissolve 1 mg (1 vial) of BODIPY® FL C_{16} (ThermoFisher, catalog no. D3821) in 1 mL of DMSO to make "Solution A"
2. Mix 200 µL of "Solution A" with 643 µL of DMSO to make "Solution B"
3. Mix 20 µL of "Solution B" with 980 µL PBS (pH 7.4) to make 100 µM BODIPY FL C_{16}

Solutions

The following solutions were prepared in 50 mM acetate, 150 mM NaCl buffer to simulate different test samples.

1. Buffer with 0.04% (w/v) PS20
2. Buffer with 0.04% (w/v) PS20 and 2.5 µM human IgG (hIgG)
3. Buffer with 0.04% (w/v) PS20 and 3FFA

4 Buffer with 0.04% (w/v) PS20 and 2.5 µM human IgG (hIgG) and 3FFA

5 Buffer with 150 µM lauric acid

6 Buffer with 87.6 µM myristic acid

7 Buffer with 31.2 µM palmitic acid

We defined "three free fatty acids" (3FFA) as a supersaturated mixture of lauric, myristic and palmitic acids prepared using a combination of published protocols[3,4] to mimic their concentration in compendial grade PS20. The mixture contains 56% lauric acid, 32% myristic acid, and 12% palmitic acid. The % values were calculated based on the total fatty acid concentration (269 µM). All fatty acid solutions were stored at 4 ˚C for 3 weeks to generate particles and were used as a positive control for polysorbate particle formation. Buffers and water for injection (WFI) were all filtered (0.2 µm syringe filter) prior to use.

Aura Configuration

An Aura system with two fluorescence channels was used to perform all experiments described. Fluorescence Channel 2 (FL2: excitation 482/35 and emission 524/24 nm) was used to specifically ID fatty acid particles stained by the phospholipid targeting dye BODIPY FL C_{16}. The BODIPY FL C_{16} was prepared to final concentration of 10 µM in acetate buffer (2% DMSO). We recommend preparing the final staining solution in same buffer as the protein. All sample handling, preparation, and process operations were performed inside a laminar hood.

It is possible to specifically ID protein aggregation and excipient degradation in the same sample. In these situations, label the sample with 5 mM Thioflavin T (ThT) and measure the membrane plate using Fluorescence Channel 1 (FL1: excitation 440/40 and emission 500/40 nm) after labeling the sample with BODIPY FL C_{16} (See Application Note 7).

Polysorbate Degradation Assay Protocol

To perform the Aura polysorbate degradation assay (Figure 1):

1 Background Image a black membrane plate

2 Load and filter 40 µL of sample onto the backgrounded plate

3 Image the plate in brightfield (BF) mode to count and size all particles in solution

4 Label fatty acid particles with 40 µL of 10 µM BODIPY FL C_{16}. Incubate for 1 minute, then filter

5 Image the plate first in BF mode, then in FL2 channel

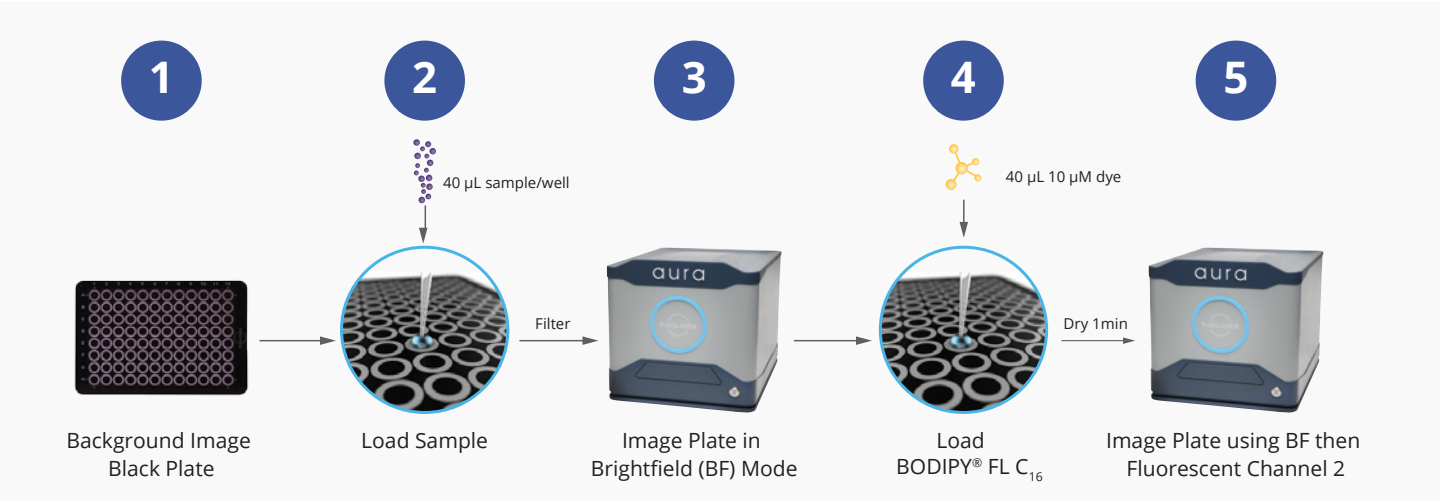

Figure 1: The polysorbate degradation assay workflow on the Aura detects degraded excipients, even in high concentration protein formulations. Samples are labeled with Thioflavin-T and BODIPY FL C_{16} to specifically ID protein aggregates and free fatty acids, respectively, with FMM.

Results and Discussion

Morphological Appearance of Different Fatty Acid Particles

The Aura images every particle, enables subvisible particle size distributions analysis, and makes morphological differentiation possible using built-in image analysis filters. When we analyze different free fatty acid solutions in the Aura, we find that each forms particles with different morphological characteristics. Lauric acid particles are large and irregular clusters, myristic acid particles are small and oval shaped, and palmitic acid particles form fibril-like particles and small circular clusters (Figure 2a-c). These unique morphological characteristics suggest the presence of free fatty acid particles that can then be confirmed with FMM using labeled fluorescence.

Fluorescent Identification of Fatty Acid Particles Using BODIPY FL C_{16}

BODIPY FL C_{16} is a high affinity stain specific to fatty acid particles. To evaluate its staining efficienty, we measured the percentage of particles of a given population that fluoresce over the dark membrane background. In the combined BF and FL images shown in Figures 3c and 3f, particles that are not stained by BODIPY FL C_{16} appear grey while the ones that are stained appear green. When the negative control hIgG sample was analyzed, only 28,776 counts/mL of particles of the total 442,600 counts/mL counted using BF fluoresced indicating a BODIPY FL C_{16} staining efficiency of 6% (Figure 3b, c). However, when the same BODIPY FL C_{16} staining solution

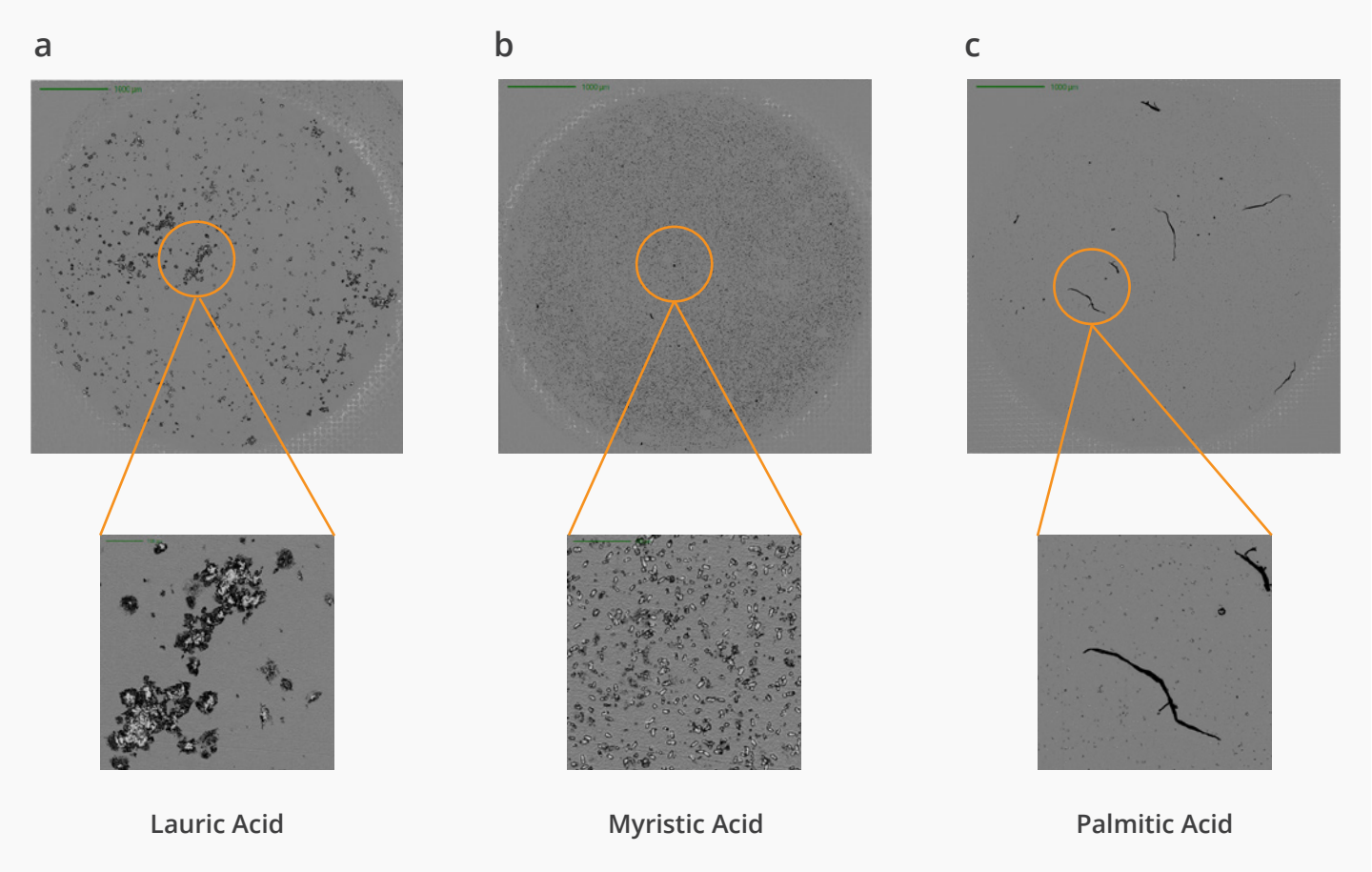

Figure 2: Morphological appearance of different fatty acids particles using brightfield image on the Aura. (a) Large, irregular particles of 150 µM of lauric acid. (b) Smaller particles of 87.6 µM of myristic acid. (c) Fibril-like particles of 31.2 µM of palmitic acid.

was added to the 3FFAs sample, where we expect to see particles attributed to excipient degradation (Figure 3d), 73% of the total particles fluoresced strongly and appeared as green (Figure 3e, f). It's also possible to individually identify the 3FFAs particles based on their size, shape, fluorescent intensity and even relative abundance, as demonstrated in Figure 3f. Here, we see that the larger lauric acid particles fluoresce more than myristic particles which in turn fluoresce more than palmitic acid particles when stained by BODIPY FL C_{16}.

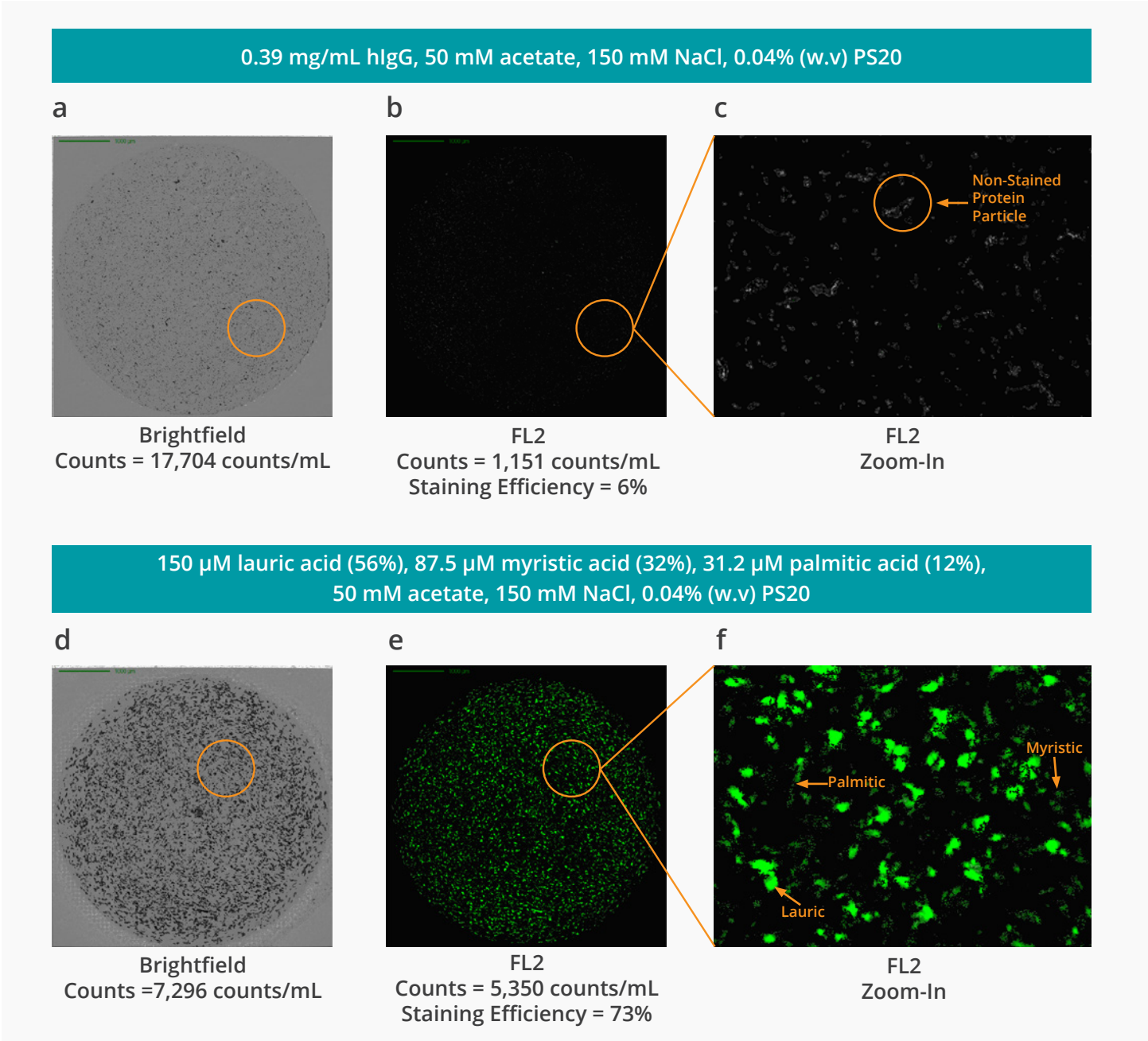

Figure 3: Images showing BODIPY FL C_{16} staining of IgG (negative control) and 3FFAs (positive control) (a) BF full well image of hIgG particles. (b, c) Combined FL2 images showing hIgG particles that were stained (no fluorescence). (d) BF full well image of 3FFAs. (e, f) Combined FL2 images showing 3FFAs particles that were stained (strong fluorescence).

Quantitative proof of BODIPY FL C_{16}'s higher selectivity to FFAs over proteins is shown in the Fluorescence vs. Size scatter plots shown in Figure 4. Figure 4a displays how FFA particles of every size fluoresce strongly when stained with BODIPY FL C_{16}, even reaching the saturation point (255-pixel intensities). In contrast, none of the labeled hIgG particles fluoresce above the background, with all their intensity values measuring along the background baseline (Figure 4b). We also see in Figure 4a that the larger the particle the larger the fluorescence intensity, likely due to the larger number of dye binding sites being filled.

Free Fatty Acid Limit of Detection (LOD) and Quantitation (LOQ) in the Presence of hIgG

We then determined the LOD and LOQ values of FFAs in the presence of hIgG. Figure 5 shows the FL2 particle counts (>5 µm) of a mixture of 0.049 mg/mL hIgG (~0.31 µM) and 9 different concentrations of 3FFAs. Both, hIgG and the supersaturated mixture of the three fatty acids were prepared with acetate buffer containing 0.01% (w/v) PS20. For simplicity, and because lauric acid is the major component of PS20, we graphed the concentration of lauric acid in the x-axis. The concentration of hIgG was kept constant at 0.049 mg/mL in all samples while the a titration series of the 3FFAs from 150 µM to 0.6 µM was created using 2-two serial dilutions.

Figure 5a, clearly demonstrats that the LOD of the 3FFA sample is 9.38 µM (>2183 counts/mL) and the LOQ is 18.75 µM (>2976 counts/mL). The linear fit of the logarithm of FL2 counts and the lauric acid's concentration yielded a straight line of R^2 = 0.9864 with an intercept of 1.828 ± 0.022 (negative control of 1682 ± 26 counts/mL) (Figure 5b).

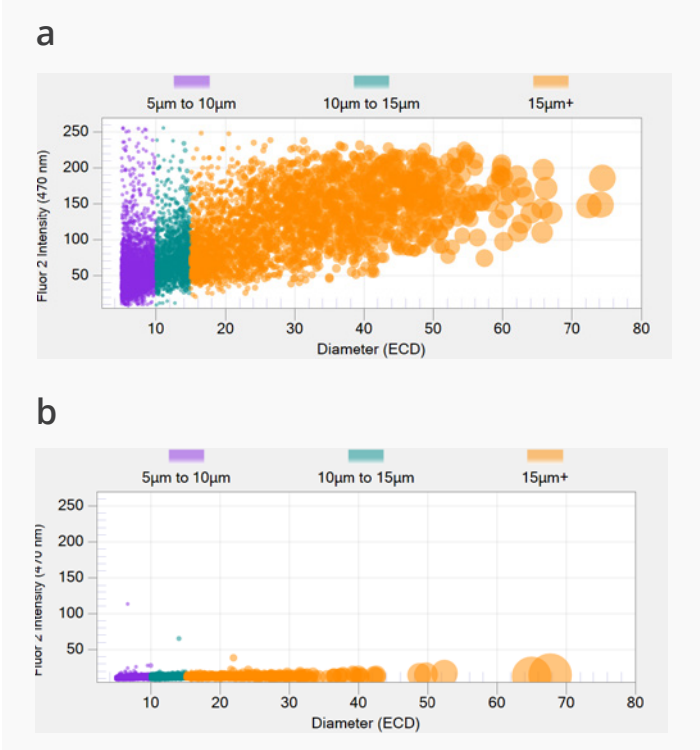

Figure 4: Scatter plots showing the FL2 signal (average particle FL2 intensity) vs diameter (ECD). Color represents particle size bins. (a) PS20+3FFAs and (b) PS20+hIgG.

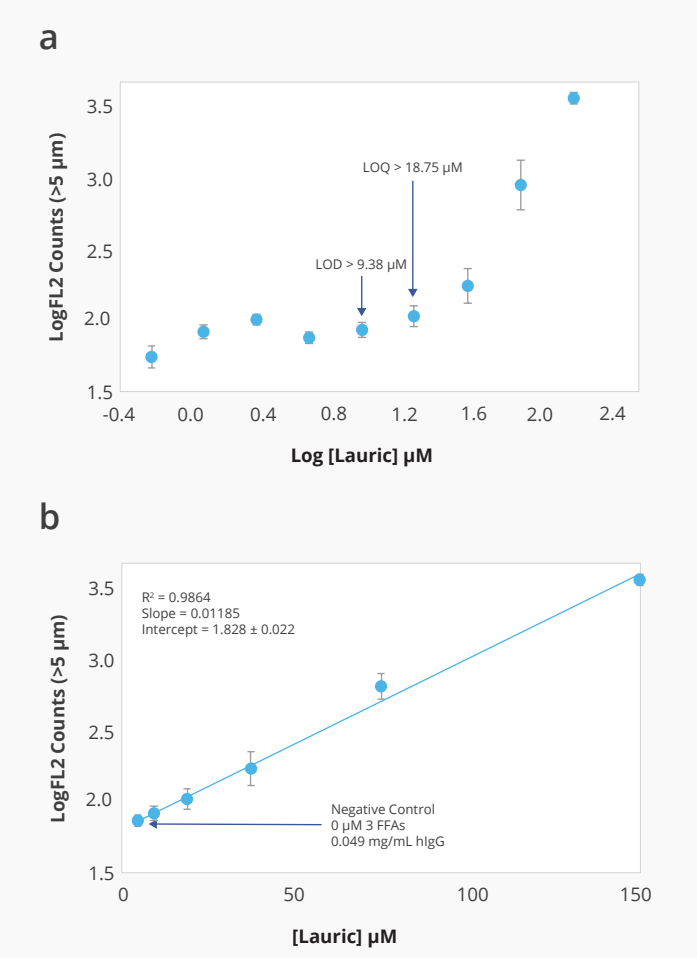

Figure 5: FL2 particle counts of the mixture hIgG+3FFAs determined after adding BODIPY FL C_{16}. The blue line represents the linear fit of the Log FL2 counts and concentration of lauric acid. (a) Log-Log plot of the FL2 particle counts of 9 different concentrations of 3FFAs. (b) Linear fit of the logarithm of the FL2 counts vs concentration of 3FFAs (expressed as concentration of lauric acid).

Figure 6 reveals that at a relative concentration of 9.38 µM lauric acid, the particles of the 3FFA mixture (Figure 6a) stains significantly more by BODIPY FL $C_{16}$with respect to the negative control (0.049 mg/mL hIgG) (Figure 6b). The power of the Aura polysorbate degradation assay is that the particle ID is visually verifiable by observing the resulting combined BF /FL images. Notice that in Figure 6b when hIgG is mixed with 3FFAs, more particles fluoresced more strongly than the controls shown in Figure 6a. This observation supports the data shown in Figure 5a, where the detection of 3FFAs is possible above 9.38 µM lauric acid. These limits of detection are more sensitive and are well in line with the solution phase polysorbate assays reported in the literature.[8]

Conclusions

The Aura can easily detect the major degradation components of PS20 in protein-containing samples at any stage of the drug manufacturing process. The method only requires 5 µL of sample, is specific and sensitive, and can analyze 96 samples in just a few hours, far outperforming other techniques. The Aura can also identify and differentiate the key degraded particulates from polysorbate formulation by their distinguishable shape, appearance, and specific labeling with BODIPY FL C_{16}. The Aura polysorbate assay can detect FFAs at concentrations relative to lauric acid above 9.38 µM (>2183 counts/mL) and quantitate above 18.75 µM (>2976 counts/mL).

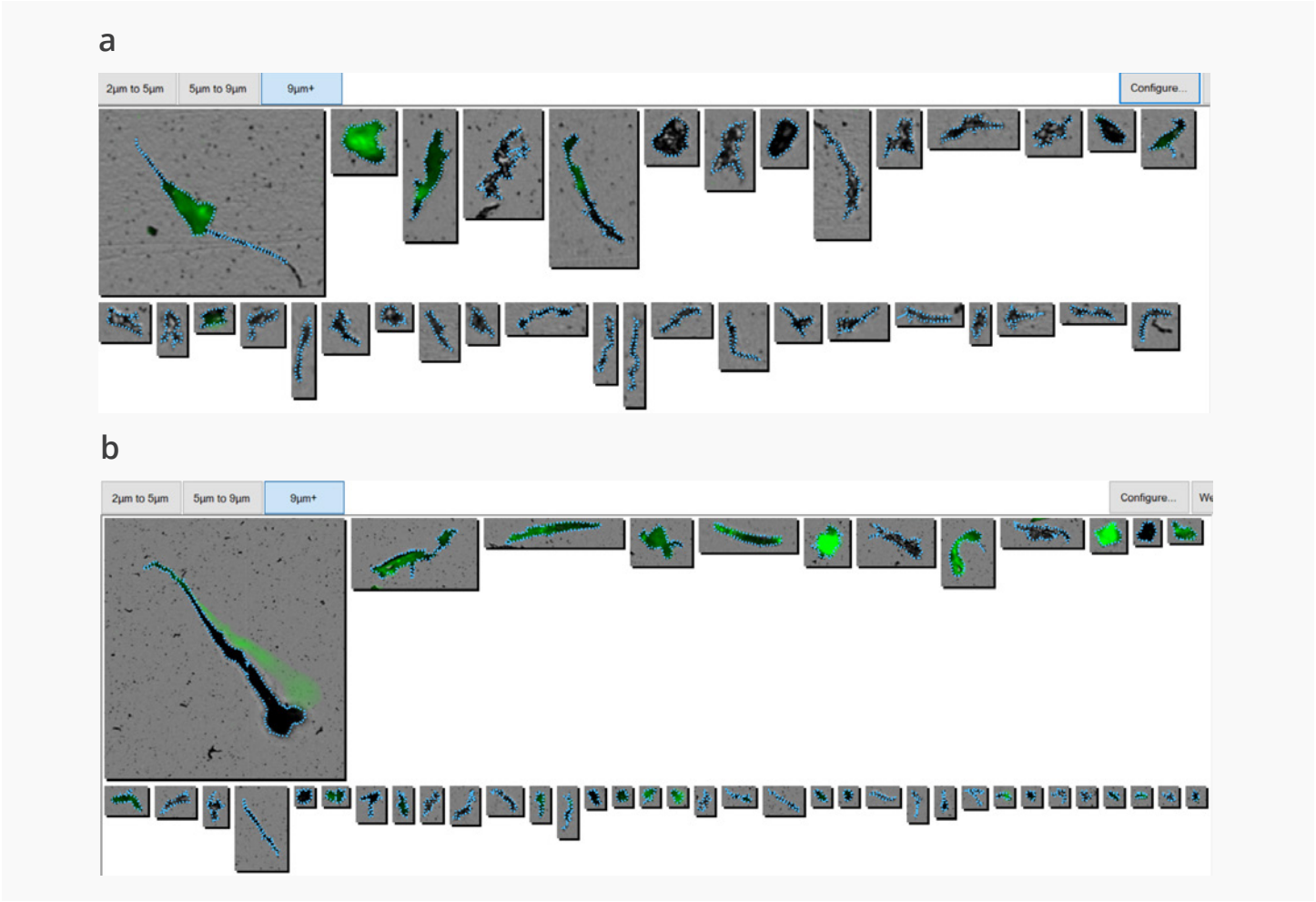

Figure 6: Image gallery showing particle staining by BODIPY FL C_{16}. (a) Particles of the negative control (0.049 mg/mL hIgG). (b) Particles of the mixture hIgG+3FFAs at a relative concentration of 9.38 µM lauric acid.

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