SUPPLEMENTARY INFORMATION

In Situ Real Time Monitoring of Emulsification and Homogenization Processes for Vaccine Adjuvants

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S1. Full Spectral Range of Selected Raman Spectral for Each Monitored Process Step

To obtain *in situ* Raman spectra, the Raman Rxn2 (Kaiser Optical Systems, Inc.) analyzer directly attached to the RamanRxn probe (Kaiser Optical Systems, Inc.) was used. A spectral range of 150-3425 cm^{-1} was probed using a 785 nm laser. The spectral range of 400-1800 cm^{-1} was considered for all analyses, due to the high number of unique Raman bands used for identification as well as the increased level of biochemical information available in this range.[1-4] Throughout each monitored step, spectra were collected every thirty seconds using iC IR 7.0 (Mettler Toledo). Raman spectra collected at selected time points evenly spaced throughout each step were analyzed using PLS_Toolbox (Eigenvector Research, Inc.) which operates within MATLAB (MathWorks). The selected Raman spectra were baseline corrected via an automatic weight least squares algorithm (order = 2) and normalized by total area, which adjusts obtained spectra to the same scale, in an unweighted fashion, to correct for scaling effects.[5] The full range of selected Raman spectral data is provided in the Supplementary Information (**Fig. S1**).

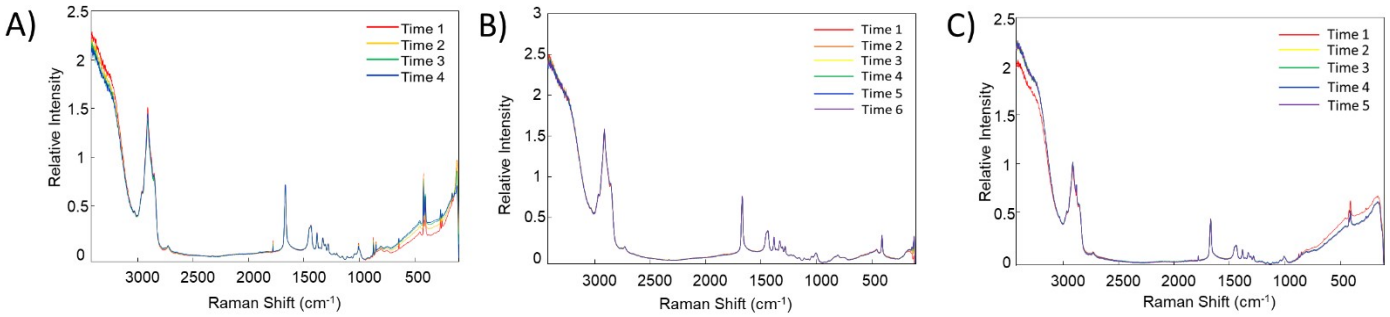

Figure S1. Full spectral range of Raman spectral data obtained at selected time points for each of the three monitored processes, including the emulsification step (A), the stability assessment of the coarse emulsification product (B), and the homogenization step (C).

S2. Reaction Monitoring of the Emulsification Step

The results of *in situ* reaction monitoring of the emulsification step show several important conclusions (**Fig. 2**). PAT information at four evenly spaced time points throughout the process were compared. Based on the optical images obtained using the optical particle imaging probe (**Fig. 2A**), there is a visual decrease in particle size; the turbidity was also tracked throughout the reaction, indicating the process was progressing and turbidity increased with time (**Fig. S2A**). The decrease in particle size is confirmed upon viewing the chord length distributions (CLD) which were calculated at the same four time points using *in situ* particle analysis (**Fig. 2B**). Here, the CLDs indicate an increase in the number of medium particles (between 10-15 μm) with time and a decrease in the size population of small particles, which shift from ~4 μm to ~2 μm (**Fig. 2B**). Reaction monitoring using *in situ* particle analysis throughout the entire process confirms these results (**Fig. S2B** and **Fig. S2C**), with the mean size of particles in the reaction decreasing with time.

In situ Raman spectroscopy was additionally used to monitor potential molecular changes occurring during the emulsification step. Reference Raman spectra of all materials involved in the first step were obtained (**Fig. S3**). The Raman spectra of neat reference materials indicates all materials involved in this process are represented by unique Raman spectra, suggesting univariate analysis via reaction monitoring of individual Raman bands could be possible. Raman spectra were collected every minute throughout the emulsification. Selected Raman spectra from the same previous four time points were then compared to determine if molecular changes were occurring (**Fig. 2C**). Based on the collected spectra, it can be deduced that molecular changes do not occur – no new Raman bands appeared within the spectra, no Raman bands disappeared, and the relative intensity of the bands within the spectra remain consistent. Reaction monitoring of selected individual Raman bands also shows that no changes occur over time, other than an initial change in intensity before and after the process starts due to the intense mixing (**Fig. S2D**); thus, the Raman spectra indicate molecular changes do not occur.

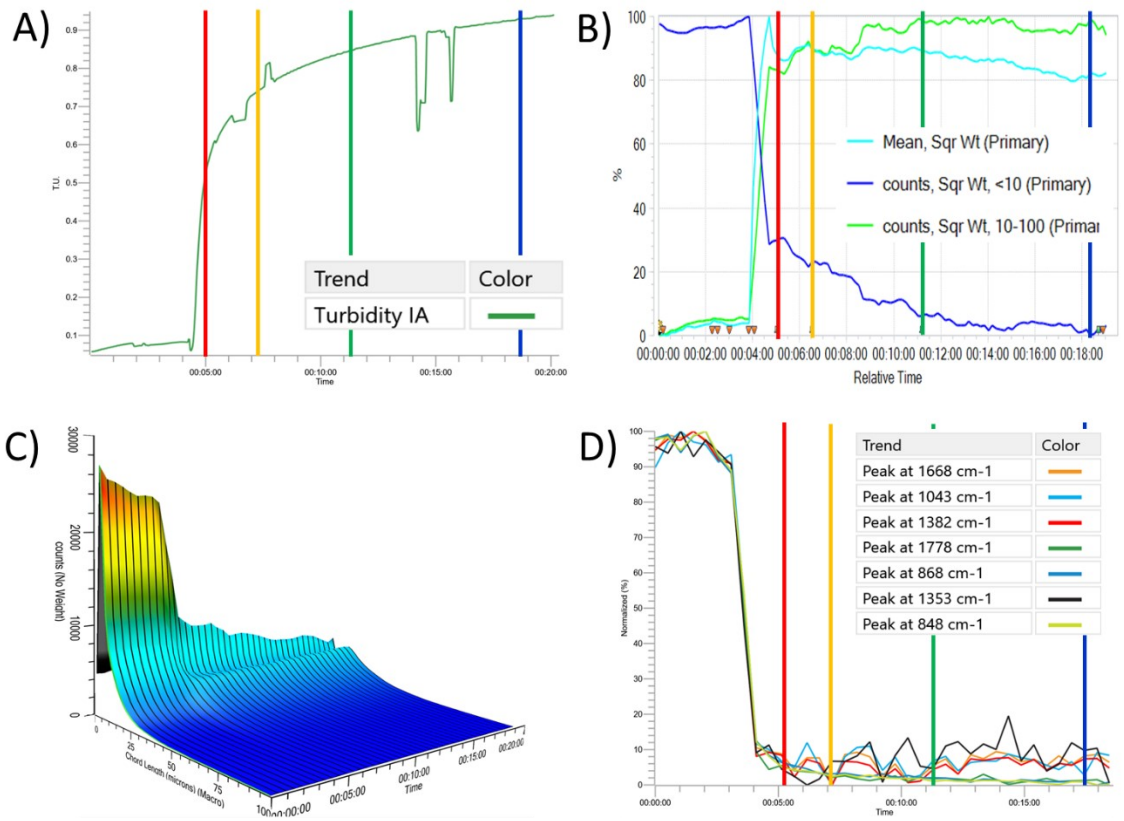

Fig. S2 Reaction monitoring trends obtained using three different PAT tools during the emulsification step, including the turbidity trends obtained via optical particle imaging (A), the counts of different sized particles obtained using *in situ* particle analysis (B), the full set of *in situ* particle analysis data illustrating how the counts of chord length changes throughout the entire monitored step (C), and selected Raman band trends obtained using Raman spectroscopy (D), with the colored vertical bands corresponding to the selected analyzed time points.

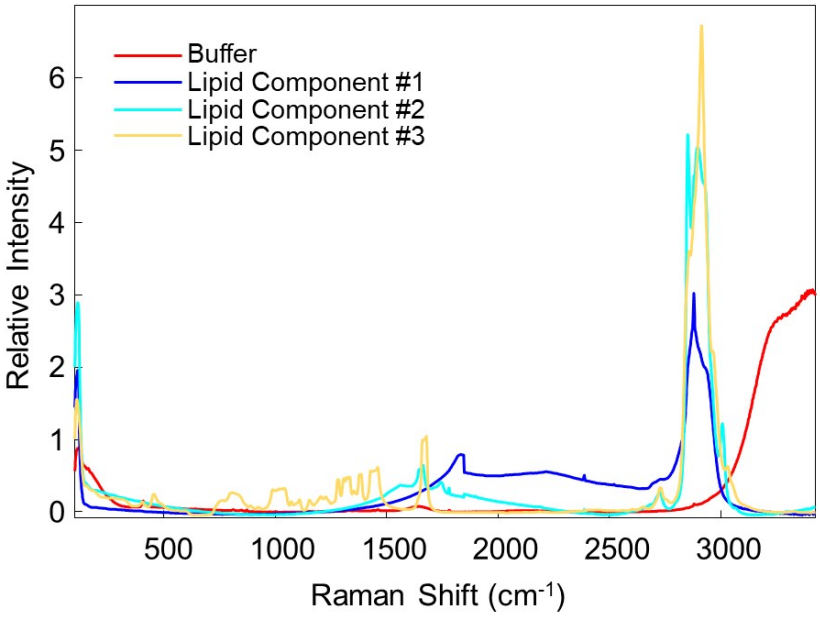

Fig. S3 Reference Raman spectra obtained from neat reference materials involved in the initial step, including the buffer (red), and three different lipid components (dark blue, light blue, and yellow).

S3. Stability Assessment of the Coarse Emulsification Product using PAT

A small amount of the coarse emulsion product from the emulsification step (~ 30 mL) was set aside to investigate its stability over time. Here, the product was monitored for 24 hours while stirred at constant rate of 200 rpm. Optical particle imaging, *in situ* particle analysis, and Raman spectroscopy were used to monitor potential changes in the sample's molecular structure or particle size and count. The OptiMax 1001 controllable automated reactor was used; all three probes were inserted into the reaction vessel, in addition to temperature and pH probes; the vessel was capped with a Teflon lid to prevent outside contamination.

Overall, the results indicate the product is stable with mild mixing. PAT data from six evenly spaced time points throughout the 24-hour reaction time were compared (**Fig. 3**). The optical images indicate the particle size appears to remain consistent throughout the monitored 24 hours (**Fig. 3A**); all trends, including particle count and turbidity, obtained from the image analysis further indicate that the overall particle size and count is unchanged, considering the scales shown (**Fig. S4A**). Results from *in situ* particle analysis indicate the chord length distributions over time remain unchanged (**Fig. 3B**) and the number of particles that are <10 µm or between 10-100 µm does not change throughout the entire 24-hour process (**Fig. 3C** and **Fig. S4B**). Based on these observations, it can be deduced that the number and the size of the particles within the emulsion product do not change when allowed to mix at 200 rpm.

Further, six Raman spectra collected at the same previously investigated time points were compared. These Raman spectra overlap significantly with each other, with no changes observed between individual spectra (**Fig. 3D**). Reaction monitoring of various Raman bands throughout the 24-hour reaction was accomplished, with the trends indicating no significant changes occurring (**Fig. S4C**). The intensely noisy trends suggest that there are no differences, even in intensity, between the Raman spectra obtained throughout the entire 24-hour reaction time.

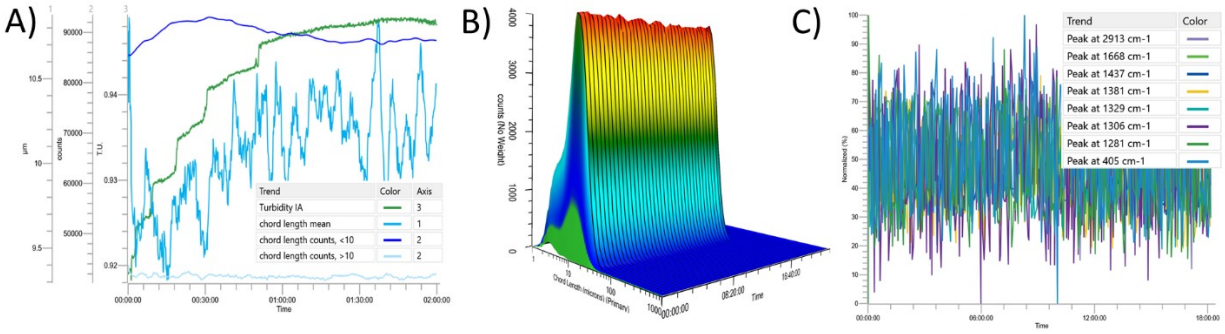

Fig. S4 Reaction monitoring trends obtained using three different PAT tools during the emulsification product stability assessment, including the turbidity and chord length trends obtained using optical particle imaging (A), the full set of *in situ* particle analysis data illustrating how the counts of chord length changes throughout the entire monitored step (B), and selected Raman band trends obtained using Raman spectroscopy (C).

S4. Reaction Monitoring of the High-Pressure Homogenization Step

The last monitored step is the high-pressure homogenization. Here, the coarse emulsion product from the emulsification step is further mixed under continuous high pressure to homogenize the sample and form a stable emulsion product. All three PAT tools – including optical particle imaging, *in situ* particle analysis, and Raman spectroscopy – were again used to monitor this step. Data was continuously measured; for analysis, five evenly spaced time points throughout the process were reported herein.

The optical images obtained from the optical particle imaging probe indicate a continuous decrease in particle size with time, as evidenced by the visual increase in density of the particles (**Fig. 4A**). The reaction trends confirm an increase in turbidity and in the number of small particles (< 10 µm) as well as a decrease in the number of particles between 10-100 µm, with the turbidity leveling out after about 5 minutes into the process (**Fig. S5A**). The results of *in situ* particle analysis suggest overall particle size is decreasing, and the number of medium particles is increasing with time (**Fig. 4B**). Reaction monitoring using *in situ* particle analysis indicates there is an increase in the number of particles that are smaller than 10 µm and a decrease in the number of particles which are between 10-100 µm (**Fig. 4C** and **Fig. S5B**). Lastly, five Raman spectra were selected at the same time points throughout the high-pressure homogenization and compared. The resultant Raman spectra overlap significantly, suggesting molecular changes are also not occurring during this step. Reaction monitoring of several different Raman bands further indicate molecular changes do not occur (**Fig. S5C**); although the change in intensity of the Raman spectra obtained before the process starts, when the process starts, and the end of the process affects the Raman reaction monitoring trends, the trends do not suggest that molecular changes are occurring (**Fig. S5C**). The intensity difference is not surprising, due to the dramatic high pressure mixing which occurs during the step.

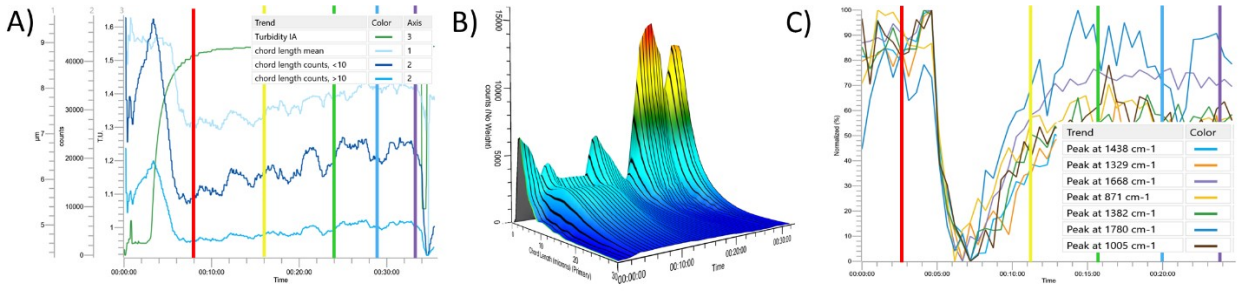

Fig. S5 Reaction monitoring trends obtained using three different PAT tools during the homogenization process, including the turbidity trends and chord length trends obtained using optical particle imaging (A), the full set of *in situ* particle analysis data illustrating how the counts of chord length changes throughout the entire monitored step (B), and selected Raman band trends obtained using Raman spectroscopy (C), with the colored vertical bands corresponding to the selected analyzed time points.

References

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