CHARACTERIZATION OF THE GLOBULE SIZE DISTRIBUTION OF CYCLOSPORINE OPHTHALMIC EMULSION BY CRYOGENIC ELECTRON MICROSCOPY

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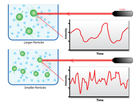
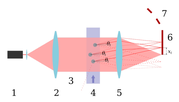
BACKGROUND

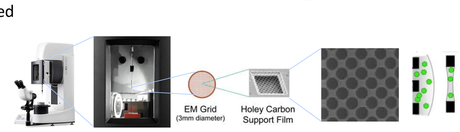
- **Cyclosporine 0.05%** (0.5 mg/mL) emulsion is indicated for treating inflammation caused by keratoconjunctivitis sicca (dry eye syndrome).
  - ➢ The emulsion contains cyclosporine, castor oil, glycerin, polysorbate 80, carbomer copolymer type A, purified water, and sodium hydroxide to adjust pH.
  - ➢ The emulsion is white and opaque in color.
- According to the Bioequivalence (BE) draft guidance on cyclosporine ophthalmic emulsion, the U.S. Food and Drug Administration (FDA) recommends for the *in vitro* option that a generic test product should:
  - (i) be Q_1/Q_2 the same as the reference listed drug (RLD);
  - **(ii) have comparable physicochemical characteristics**;
  - (iii) have comparable *in vitro* drug release rate to that of RLD.
- The recommendations state that the globule size distribution (GSD) will be the basis for establishing BE. Since GSD of the commercial cyclosporine emulsion has not been comprehensively investigated, this study sought to characterize globule size and report a suitable method for comparing generic products to an RLD.
- To characterize emulsion globule size, some commonly used methods include Dynamic Light Scattering (DLS), Laser Diffraction, and Transmission Electron Microscopy (TEM).
- Dynamic Light Scattering
  - ➢ Correlates size from Brownian motion of particles.
  - ➢ Accurate viscosity input required.
  - ➢ Sphere approximation; intensity based.
- Laser Diffraction
  - ➢ Size from angular variations in intensity.
  - ➢ Refractive index required.
  - ➢ Volume equivalent sphere size model.
- Transmission Electron Microscopy
  - ➢ Negative Stain: increases contrast of a specimen by staining the background supporting film with heavy metal staining agents, such as uranyl acetate (UA) and methylamine tungstate (Nano-W).
  - ➢ Cryo-TEM: plunge freezing allows direct investigation of emulsion in the vitrified, frozen hydrate state, i.e., very close to the native state of emulsion droplets in solution.





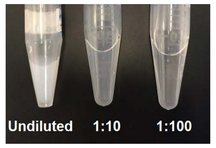
MATERIALS AND METHODS

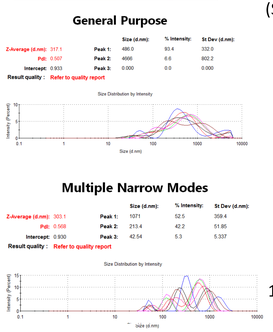
- Dynamic Light Scattering (DLS)
  - Instruments
    - Zetasizer Nano ZS (Malvern Instruments), backscattering detector (173°), quartz cuvette.
  - Follow recommendations from NIST-NCL PCC-1 protocol
    - 0.1 µm filtered 10 mM NaCl solution was used for all dilutions, unless specified.
- Laser Diffraction
  - Instrument: Mastersizer 3000 with Hydro EV wet dispersion (Malvern)
    - DI water used for dilution (approx. 300 mL); refractive index of castor oil used n=1.48.
    - All instruments validated with 20 – 20000 nm diameter NIST™ traceable polystyrene size standards (3000 and 4000 Series, Thermo Fisher).
- pH was monitored before and after dilution of cyclosporine emulsion with 10 mM NaCl using a benchtop meter and a micro pH electrode (Orion, Thermo Scientific).
- Viscosity was measured using an AR G-2 Rheometer (TA Instruments) with a 2° cone and plate setup.
  - Oil-free stock was prepared by mixing inactive ingredients, by % w/v of 0.05% carbomer copolymer type A (Pemulen™ TR-2 NF, Lubrizol), 1 % Polysorbate 80 (Tween 80, Fisher Scientific), and 2.2 % Glycerol (Fisher Scientific).
- Transmission Electron Microscopy (TEM) was performed on a Jeol 1400 TEM/STEM at 120 kV.
  - Negative Stain: A glow-discharged Formvar carbon coated grid (EMS) or a NanoPlus SMART silicon grid (Dune Sciences) with the emulsion was stained with 10 µL of either 1% Uranyl Acetate (Sigma Aldrich) or Nano-W stain (Nanoprobes Inc.) for 1 min, blotted, and observed in the TEM at 120 kV.
  - Cryo-TEM: The emulsion samples were plunge-frozen using a Leica EM GP plunge freezer. Briefly, 2 µL of emulsion sample was placed on a glow-discharged (EMS TS-150) copper grid (Quantifoil R 2/1, 200 mesh), back-blotted for 9 sec at 25 °C and 82% humidity, then plunged into a bath of liquid ethane at −175 °C, transferred to a Cryo-TEM holder (Gatan 914) and observed in the cryo-TEM at 120 kV under Minimum Dose System.



RESULTS

Size Measurement with Dynamic Light Scattering (DLS)

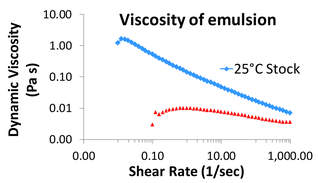




Dilution Factor	Intensity-weighted distribution (nm)	Z-Average (nm) (Viscosity 0.89 cP used)	PdI	Intensity Peaks (nm)
1X (Stock)		301.2 ± 11.4 (Error due to incorrect viscosity)	0.56 ± 0.03	877.6 (52%) 260.5 (39%) 67.1 (9%)
10X		103.7 ± 0.9	0.28 ± 0.01	180.0 (67%) 53.6 (33%)
100X		101.2 ± 1.3	0.27 ± 0.01	149.7 (78%) 46.3 (22%)
1000X		111.0 ± 4.2	0.30 ± 0.04	145.4 (83%) 41.5 (10%) 663.5 (6%)

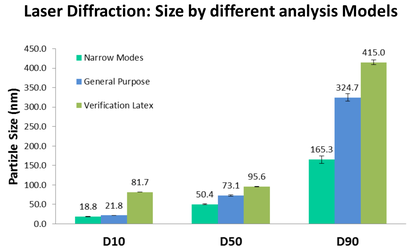
- Undiluted 1X stock solution shows significantly inconsistent peak position and histogram shape among measurements.
- 10X, 100X and 1000X diluted emulsions show slightly inconsistent intensity distribution among measurements but Z-average size and polydispersity index (PDI) remain similar.
- Inconsistency in intensity-weighted distribution among measurements for emulsions (undiluted, 10X, 100X and 1000X diluted) suggests carbomer copolymer type A , a high molecular weight viscosity modifier in the formulation, likely interferes with the DLS measurement.

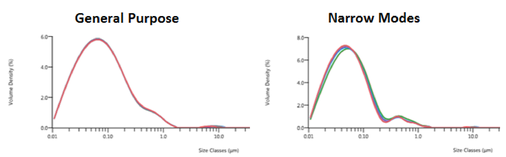
Dilution Effect on Viscosity of Emulsion



- The viscosity of 1X (stock) emulsion is much higher than water due to the presence of carbomer copolymer type A and Polysorbate 80.
- Shear thinning behavior - viscosity decreases with increased shear rate.
- Dilution (1:10) viscosity is closer to water (0.00089 Pa s); Castor oil = 0.650 (Pa s).

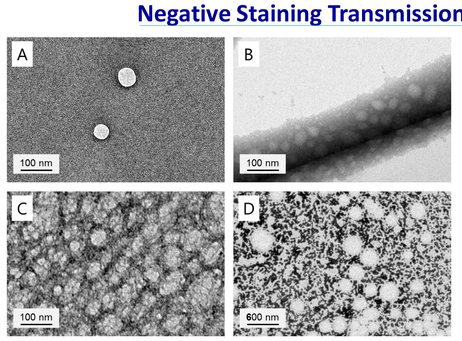
Size Measurement with Laser Diffraction





- Narrow modes analysis was arbitrarily selected for the emulsion, D_{10} = 18.8, D_{50} = 50.4, and D_{90} = 157 nm.
- Peaks: 49 nm (93% volume) and 433 nm (7% volume).

Negative Staining Transmission Electron Microscopy

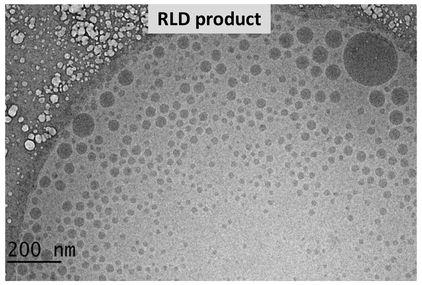


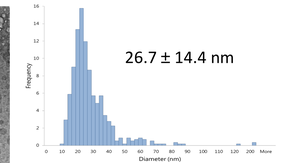
	Number-averaged Size
A. UA stain on formvar	50.1 ± 24.4 nm
B. Nano-W stain on formvar	36.9 ± 26.5 nm
C. UA stained on NanoPlus	N/A
D. Nano-W stain on NanoPlus	217.7 ± 61.4 nm

- Selection of staining agents and TEM grids affects size, morphology and distribution of oil globules.
- TEM Grid handling and rinsing during the staining also affect the result.

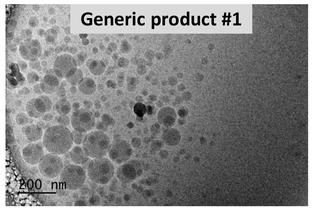
RESULTS

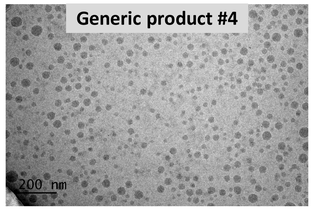
Cryogenic Transmission Electron Microscopy

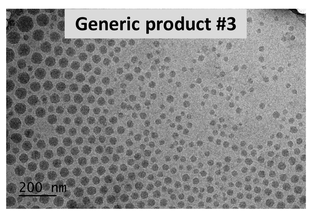




- Spherical oil globules of cyclosporine ophthalmic emulsions can be visualized with cryo-TEM.
- Most of globules in RLD product are in the range of 10-50 nm. Occasionally, globules above 100 nm can also be seen.







	Number-averaged size
RLD	26.7 ± 14.4 nm
Generic Product #1	45.9 ± 27.2 nm
Generic Product #2	41.8 ± 43.5 nm
Generic Product #3	42.2 ± 56.8 nm
Generic Product #4	26.5 ± 10.5 nm
Generic Product #5	33.2 ± 18.5 nm

CONCLUSIONS

- DLS, Laser diffraction and cryo TEM results show cyclosporine emulsion is in the nanometer range.
- DLS is a robust technique for measuring the Z-Average size and PdI of the cyclosporine emulsion, which is unaffected by analysis mode.
- DLS is not robust for producing size distribution of cyclosporine emulsion due to peak fluctuation, histogram variation among measurements, and a lack of size peak resolution.
- Issues with DLS measurement arise from the non-Newtonian viscosity from the carbomer copolymer Type A in the formulation (DLS requires a point value input for viscosity).
- Laser diffraction suggests emulsion contains nanometer globules. However, accurate sizing is questionable as the size of oil globules is near its limit of precision.
- Negative staining TEM method often generates inconsistent result.
- Cryo-TEM clearly visualizes the cyclosporine ophthalmic emulsions with spherical shape and a narrow globule size distribution of 26.7 ± 14.4 nm (ranging from 10 to 220 nm).
- Cryo-TEM can be reliably used to compare globule size distribution among the RLD product and generic products.

ACKNOWLEDGEMENTS

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