**Preservation of Quaternary Structure in Thermostable, Lyophilized Ebola Glycoprotein Vaccines**

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**Motivation**

Zaire ebolavirus (EBOV) is one of the most lethal infectious agents known, but despite disease severity, there are still no EBOV vaccines currently approved by the United States Food and Drug Administration. All currently licensed vaccines for other diseases, as well as all experimental EBOV vaccines, require storage under a continuous cold-chain to ensure efficacy is maintained. One such experimental EBOV vaccine is SSV-ZEBOV, which has been used during the ongoing Ebola outbreak in the Democratic Republic of the Congo and requires storage at -60 to -80°C. These cold-chain requirements are difficult to maintain in low- and middle-income countries with unreliable electricity and poor healthcare infrastructure, especially in remote African villages where Ebola is endemic.

To potentially eliminate cold-chain requirements and improve manufacturability, a subunit vaccine consisting of EBOV glycoprotein was formulated as an amorphous glass through lyophilization. The thermostable vaccine and liquid controls were incubated for up to 12 weeks at both 25°C and 40°C to accelerate degradation. We intended to find a stability-indicating assay that would be sensitive enough to show protein degradation over time and would help to predict immunogenicity and conformational long-term stability.

**Vaccine Formulation**

The EBOV glycoprotein (EBOV-GP) was formulated as a lyophilized vaccine with liquid controls in both ammonium acetate/trehalose buffer and phosphate buffered saline (PBS).

- **EBOV PBS** = EBOV-GP at 0.1mg/mL in 10mM PBS, pH 7.4 (standard formulation)
- **EBOV LIQ** = EBOV-GP at 0.1mg/mL with 9.5% (w/v) trehalose in 10mM ammonium acetate, pH 7
- **EBOV LYO** = lyophilized presentation of EBOV LIQ (thermostable formulation)

Prior to use for analysis or administration to mice, lyophilized vaccine formulations were reconstituted with filtered water for injection.

**Vaccine Immunogenicity**

- Swiss Webster mice were vaccinated with 3 doses in 3 week intervals. Vaccines contained a nanoemulsion adjuvant, CoVaccine HT, that was added to formulations immediately before injection in mice.
- Lyophilized formulations showed no decrease in immunogenicity compared to PBS formulations and 12-week incubation did not affect this immunogenicity.
- After incubation in PBS, but not in LYO formulations, we observed increased cross-reactivity with Marburg virus glycoprotein (MARV-GP), potentially indicating conformational changes in EBOV-GP leading to non-antibody formation.

**Size Exclusion Chromatography**

- EBOV-GP naturally forms trimer, which can oligomerize into dimer of trimer. Previous work showed these higher molecular weight species are more immunogenic and effective at eliciting specific antibodies than monomer.
- EBOV LYO and PBS formulations had overall protein retention after incubation.
- EBOV LYO maintained high MW species over incubation period, while EBOV LIQ and PBS had some dissolution into monomer, especially at 40°C

**Near-UV Circular Dichroism**

- Near-UV CD is sensitive to changes in secondary structure with relation to environment around aromatic amino acids
- Little difference in spectral shape was observed after incubation, indicating minimal changes in tertiary structure of EBOV-GP

**Intrinsic Fluorescence Quenching**

- Stern-Volmer constants (Ksv) indicate relative accessibility of solvent to tryptophan residues.
- After incubation for 12 weeks at 40°C, significantly different (p<0.05) Ksv values were observed for EBOV LYO and PBS compared to unincubated controls, indicating changes in tertiary structure based on tryptophan accessibility.
- Higher Ksv values showed a positive correlation with relative amounts of monomer from Fig. 2C

**Far-UV Circular Dichroism**

- In far-UV range, CD of a protein indicates secondary structure through amide chromophores along the backbone.
- Lyophilized formulations maintained spectral shape and overall minima.
- Minima of EBOV LIQ and EBOV PBS samples shifted to lower wavelengths over incubation period, potentially indicating changes in oligomeric state from Fig. 2C

**Conclusions**

By monitoring variations in secondary, tertiary, and quaternary structure as well as vaccine immunogenicity, it was discovered that differences in oligomerization state observed via size-exclusion chromatography was the best indicator of glycoprotein stability in the various formulations. Not only did the thermostable, lyophilized vaccine retain its secondary and tertiary protein structure, but it also maintained its quaternary structure over the 12-week incubation, whereas the liquid controls saw a marked shift to less-immunogenic low molecular weight species.

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