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 rVSV-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 thermostability, 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-specific antibody formation

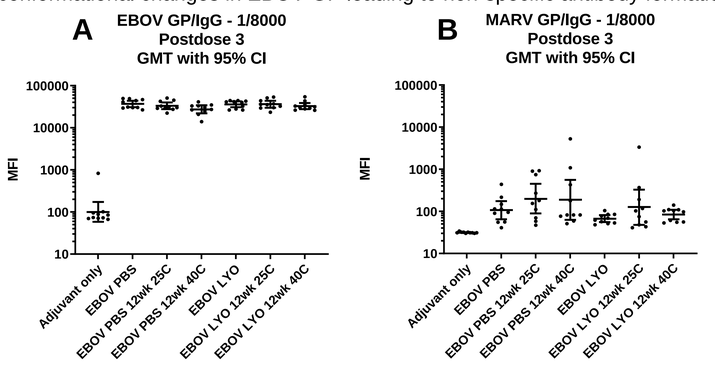

Figure 1. Mouse immunogenicity of EBOV PBS and EBOV LYO formulations before and after incubation showing (a) anti-EBOV-GP IgG and (b) anti-MARV-GP IgG antibody titers.

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 HMW species over incubation period, while EBOV LIQ and PBS had some dissociation into monomer, especially at 40°C

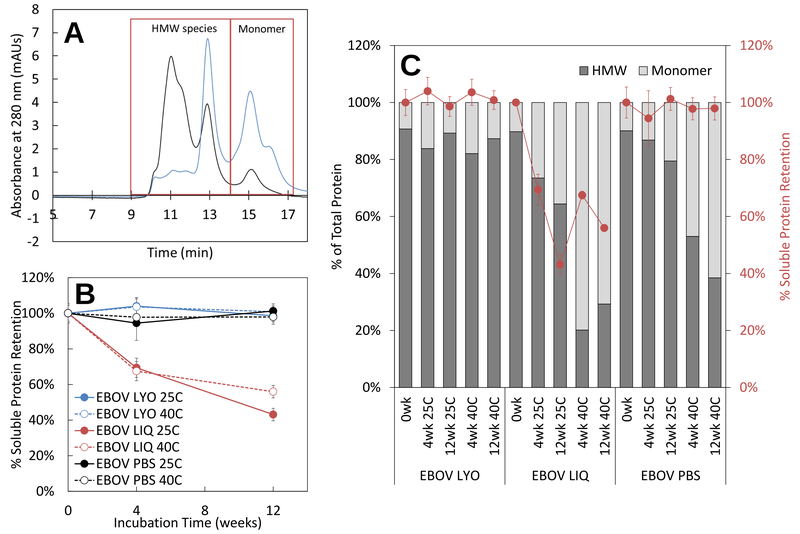

Figure 2. (a) Representative size-exclusion chromatogram of unincubated liquid in PBS formulation (black) and 4 week incubated liquid in PBS at 40C (blue). (b) Area under the entire chromatogram was calculated and normalized to areas of each unincubated formulation to represent protein retention over time. (c) Total soluble protein area was divided into HMW species and monomer species and is shown as the percent of the total area (gray bars, left axis) compared to soluble protein retention (red markers, right axis)

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

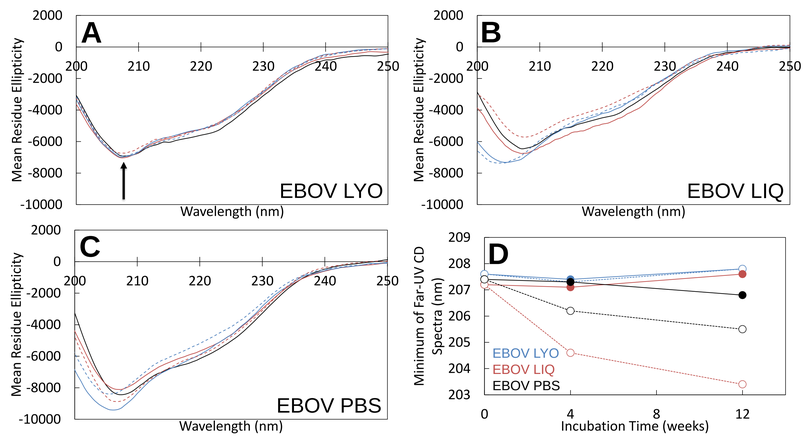

Figure 3. Far-UV circular dichroism spectra of (a) lyophilized formulations, (b) liquid in ammonium acetate formulations, and (c) liquid in PBS formulations that were freshly prepared (black solid line), incubated for 4 weeks (red solid line for 25C and blue solid line for 40C), or incubated for 12 weeks (red dashed line for 25C and blue dashed line for 40C). Changes in overall peak minimum were monitored over the incubation period, shown in (d) where solid markers represent incubation at 25C and open markers represent incubation at 40C.

Near-UV Circular Dichroism

- Near-UV CD is sensitive to changes in tertiary 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

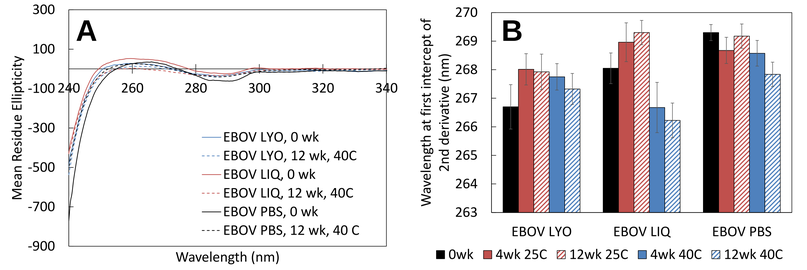

Figure 4. (a) Near-UV circular dichroism spectra for unincubated and incubated for 12 weeks at 40C samples. (b) Changes in Near-UV CD were assessed by calculating the second derivative spectra and monitoring the wavelength corresponding to the first intercept (an inflection point) over the incubation period.

Intrinsic Fluorescence Quenching

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

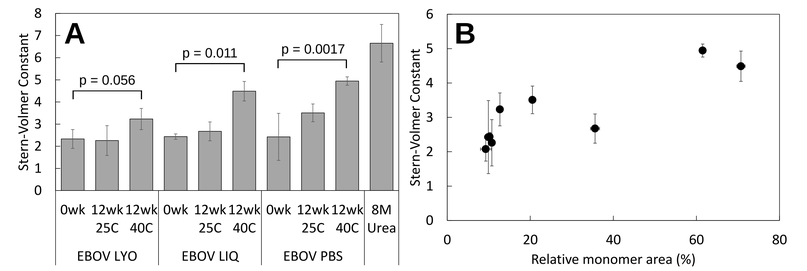

Figure 5. (a) Stern-Volmer constants for freshly prepared and incubated samples for lyophilized (EBOV LYO), liquid in ammonium acetate (EBOV LIQ), and liquid in PBS (EBOV PBS) compared to unfolded control in 8M urea. (b) Comparison of the Stern-Volmer constant with monomer area from size-exclusion chromatograms shows a positive correlation between degree of unfolding and relative amount of monomer.

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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