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Contract work for the September - December time period was directed at continuing the effort to reduce Liposome Encapsulated Hemoglobin (LEH) mean size. This included evaluating the effects of lipids, lipid ratios, hemoglobin (Hb) concentration, buffers, and process parameters.

A summary of relevant experiments is presented below:

1. Dilution and ionic strength effects Sept. 91
   LEH was diluted with phosphate saline buffer or phosphate sucrose buffer to evaluate the effect of dilution and ionic strength on aggregation of the liposomes. Saline buffer had no noticeable effect as measured by mean size. However sucrose buffers seemed to cause some LEH disaggregation.

2. LEH processed at different ionic strengths Sept. 91
   LEH was produced using bovine Hb in 30 mM phosphate with 250 mM saline buffer at pH 7.4 and 5 mM phosphate with 263 mM sucrose buffer at both pH 7.4 and pH 8.3. Effects of pH and ionic strength were evaluated along with Hb recovery and in-process losses. Little effect was seen on mean size. However blood characteristics such as P50 were improved at pH 7.4, and the Hb recovery was 22% with sucrose vs. 12.6% with saline.

3. External buffer Oct. 91
   Dialysis of LEH against 9% sucrose, 5mM lactobionate with 9% sucrose, 30 mM phosphate with 9% sucrose showed that phosphate/sucrose buffer gave the best size results.
4. Ionic strength

Oct. 91

Adding LEH to 5mM phosphate/9% sucrose at pH 7.4 buffer with NaCl varied from 0 to 200 mM demonstrated a decrease in mean size from 654 nm to 263 nm. LEH is apparently affected by electrostatic interactions.

5. Preclinical trial lot

Oct. 91

A 3 L lot was produced on large scale equipment with Somatogen recombinant Hb (rHb). Physical characteristics were typical of previous lots with recovery of Hb at 13.4%. However met Hb formation was more rapid with storage than has usually been experienced with bovine Hb.

6. Lipid and Hb assay interactions

Oct. 91

Fully characterized empty liposomes were spiked with a range of Hb concentrations to assess the effect of Hb on lipid assays. Hb interferes with lipid assays causing low results.

7. Hb concentration effect on encapsulation and recovery

Nov. 91

LEH processed with bovine Hb at 87, 124, and 141 g/L Hb showed optimal recovery and encapsulation at 124 g/L initial Hb. Other product characteristics were unaffected.

8. Free Hb separation

Dec. 91

A Superose 6 column was tested for its ability to separate free Hb from LEH and found to be very effective. A separation height of 5 cm is required.

9. Temperature effect on empty liposome size

Dec. 91

LEH lipids sonicated at 0°C and 50°C showed that colder processing produces broader size distributions with larger mean size.

10. Detergent effects on lipid assays

Dec. 91

The detergent octyl-beta-glucopyranoside (OBG) was used to dissolve LEH prior to HPLC lipid assay to assess whether OBG can reduce lipid-Hb interaction. The detergent was judged ineffective.

11. rHb concentration effect on encapsulation and recovery

Dec. 91

A repeat of experiment #7 but with rHb at 67, 121, 171, and 219 g/L confirmed that 120-125 g/L is optimal for recovery and encapsulation of rHb.
12. Formulation work via sonication Sept. 91 - Dec. 91

A sonication protocol was developed for controlled evaluation of a large matrix of formulations utilizing the standard LEH components, DSPC, DMPG, and Cholesterol with rHb. Effect of lipid chain length was evaluated by substituting DMPC and DPPC for DSPC. Buffers utilized were sucrose/phosphate and sucrose/phosphate buffered rHb. This work is still ongoing and starting to show some interesting trends as of late December.

Because the Nov. 91 meeting at NRL led to an agreement to explore formulations in order to solve the size/filtration problem, that has been the major thrust of effort most recently. Research will continue on a small volume basis to identify and optimize the most promising formulations.

Sincerely,

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