FIGURE LEGEND

Figure 1. PUC-ERLIC proteomics workflow showing plasma sample preparation for the simultaneous isolation of secretory and extracellular vesicle-enriched glycoproteins prior to mass spectrometry. Yellow suspension collected after PUC was found to be enriched in glycoproteins.

Figure 2. Plasma extracellular vesicles isolated using prolonged ultracentrifugation (PUC). (A) Electron micrographs of harvested extracellular vesicles (sizes 50–100nm, recorded at 23,500 X magnification with a defocus of -6µM). (B) Electron micrographs of extracellular vesicles recorded on carbon (23,500 X magnification with a defocus of -6µM). (**C**) Western blot analyses of harvested proteins using extracellular vesicle markers Alix, CD9 and CD81. PUC-01 and PUC-02 are biological replicates.

Figure 3. Prolonged ultracentrifugation (PUC) enables simultaneous recovery of secretory and extracellular vesicle-enriched glycoproteins from human plasma. (A) Comparison of fractions recovered from 5mL total plasma after 18h or 2h of UC. Yellow pellet recovered after PUC (18h) was significantly larger and more visible than the pellet recovered after UC (2h). (B) Proportion of unique N-glycoproteins, N-glycopeptides and N-glycosylated sites obtained when using PUC-ERLIC or standard UC-ERLIC approaches (both with PNGase F treatment). Significant glycoprotein enrichment was observed after PUC-ERLIC. The glycosylation percentage was calculated using the number of unique glycosylated products divided by the sum of all unique glycosylated and unmodified peptides.

Figure 4. PUC-ERLIC facilitates enrichment of the N-glycoproteome of human plasma. (A) Proportion of unique N-glycoproteins, N-glycopeptides and N-glycosylated sites obtained using PUC-ERLIC, PUC alone, or ERLIC alone (all with prior PNGase F treatment). The glycosylation percentage was calculated using the number of unique glycosylated products divided by the sum of all unique glycosylated and unmodified peptides. (B) Venn diagram of overlapping plasma N-glycoproteins obtained using PUC-ERLIC, PUC alone, or ERLIC alone.

Figure 5. Venn diagram showing the N-glycosylated plasma proteins identified using PUC-ERLIC (current study), SPEG approach (Yang *et al (56)*) or affinity capture method (Drake *et al* (57)).

Figure 6. Gene Ontology (GO) functional annotation showing the proportionate distribution of identified glycoproteins according to their (A) molecular functions, (B) biological processes and (C) cellular localization. Predictions were obtained using STRAP v1.5(45), AmiGO v2(46, 47) and DAVID v6.7(48, 49).