

OVERVIEW

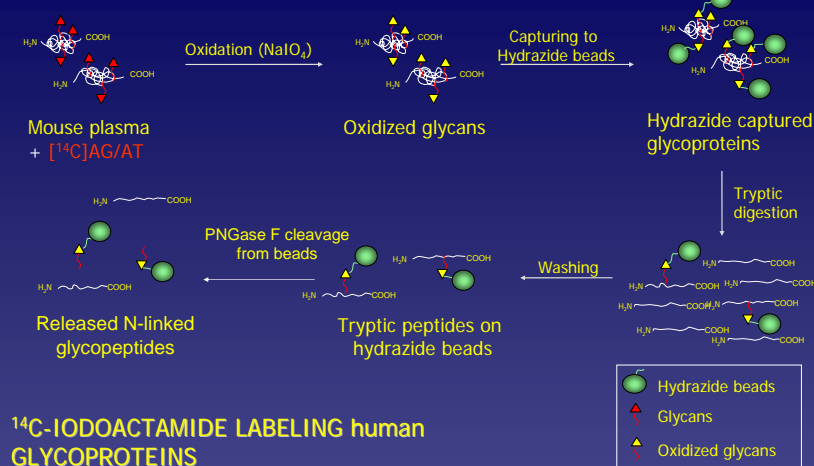
Optimizing the recently developed Solid-Phase Extraction of (N-linked) Glycopeptides (SPEG) procedure for blood plasma analysis by spiking in [¹⁴C]-labeled human glycoproteins significantly enhanced the specificity and yield of the isolated N-linked glycopeptides. This will improve the sensitivity of discovery of clinical biomarkers from blood.

INTRODUCTION

There is growing interest in discovery of disease biomarkers from blood plasma. For this reason, quantitative analysis of plasma proteins has been the focus of different proteomic technologies. The challenges faced by all quantitative plasma proteomic methods include complexity and the high dynamic range of the plasma sample. Therefore, a desirable proteomic technique for plasma profiling must be sensitive, reproducible, and robust. Recently, we have developed a method for Solid-Phase Extraction of N-linked Glycopeptides (SPEG), and we have shown that analysis of plasma using SPEG improves dynamic range and sensitivity. Here, we optimized each step of the method and developed a standard procedure for plasma analysis using SPEG and mass spectrometry by spiking in two [¹⁴C]-labeled human glycoproteins.

METHODS

SPEG METHOD [1]



¹⁴C-iodoacetamide labeling human GLYCOPROTEINS

α-1-acid glycoprotein 1 (AG) (Positive Control: 2 Cys in; 2 Cys out)
MALSSWLTVL**SLLPLLEA**QIP**L**CANL**V**VPV**IT**MA**TL**DQ**IT**GW**F**YIASAF**R**NEBY**NS**VQ
EIQATFF**F**TP**IK**ED**T**IFLREY**Q**TR**Q**DC**Y**IT**Y**TLNV**Q**RE**IG**TI**S**RYVGG**Q**EHFA**HL**L
 IL**R**DT**K**Y**M**LAF**D**V**N**DE**K**N**W**LS**V**Y**A**DK**P**ET**T**K**Q**L**G**F**Y**E**A**L**D**CL**R**IP**K**SD**V**Y**V**Y**T**D**W**KK
 DK**C**E**P**L**E**K**Q**HE**K**ER**K**Q**E**EG**S**

α-1-antitrypsin (AT) (Negative Control: 0 Cys in; 1 Cys out)
MPSS**V**SW**G**ILL**LAG**L**C**QL**V**VP**V**SL**A**ED**P**Q**G**DA**A**Q**T**DT**S**HD**Q**D**H**PT**F**N**K**IT**P**N**L**AE**F**AF**S**
 LY**R**QL**A**H**Q**S**NE**T**NI**FF**S**P**V**SI**A**T**A**F**A**ML**SL**GT**K**AD**T**H**D**E**I**LE**GL**N**F**IL**T**IP**E**A**Q**I**H**E**G**F
 Q**E**LL**L**ET**L**N**Q**PD**S**QL**Q**L**T**TC**N**GL**FL**SE**GL**K**L**V**D**K**F**LE**D**V**K**LY**H**SE**A**FT**V**N**F**GD**T**EE**A**KK**Q**
 IN**D**Y**V**E**K**GT**Q**G**K**IV**D**L**V**K**E**LD**R**DT**V**F**A**LV**NY**IF**F**KG**K**W**E**R**P**F**E**V**K**D**T**EE**D**F**H**V**D**Q**V**TT**V**
 K**V**PM**M**K**R**L**G**MP**NI**Q**H**CK**KL**SS**W**LL**M**K**Y**L**C**NA**T**A**IF**FL**P**DE**G**K**L**Q**H**LE**N**EL**T**H**D**I**T**K**F**L
 EN**E**DR**R**S**A**SL**H**LP**K**LS**I**T**G**TY**D**L**K**SV**L**G**Q**L**G**IT**K**V**F**NS**G**AD**LS**GV**T**EE**A**PL**K**LS**K**AV**H**KA
 V**L**T**I**DE**K**TE**A**AG**A**MF**LE**AI**P**MS**I**PE**V**K**F**N**K**PF**V**FL**M**I**EQ**NT**K**S**PL**FM**G**K**V**V**NP**Q**K**

RESULTS

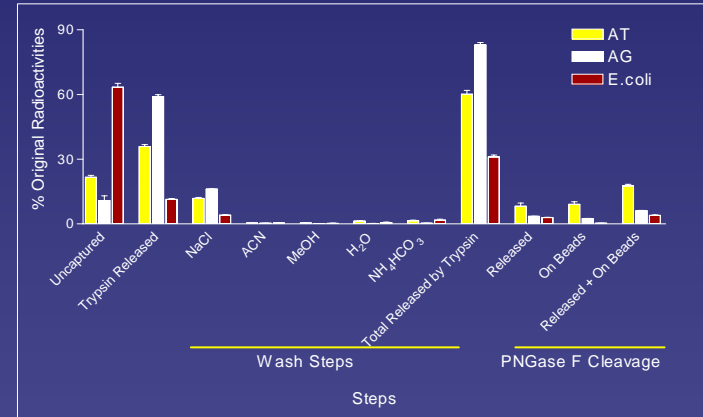
1, Evaluating the performance of each step of SPEG procedure for plasma protein analysis

The specificity and efficiency of N-linked glycopeptide isolation was evaluated by using original SPEG procedure for mouse plasma spiked in [¹⁴C]-labeled E.coli lysate and two human glycoproteins — AG and AT.

For standard procedure, aliquots of 20 μl mouse plasma were spiked with ~10⁴ cpm [¹⁴C]-labeled AG/AT/E.coli lysate, and oxidized in 10 mM NaIO₄ for 1h at RT, then coupled on 200 μl Affi-gel slurry (50% v/v). The bound proteins were digested by trypsin, washed, and N-linked glycopeptides were released from the slurry by 0.6 μl of PNGase F overnight at 37°C.

For peptide-level SPEG, protein mixtures were digested to peptides by trypsin prior to SPEG.

Data are shown as percentage of [¹⁴C] radioactive activities from mobile fractions in each step versus total radioactive activities used initially.



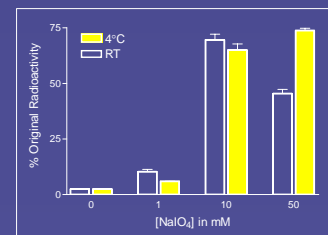
Concerns:

- 1) Specificity needs improving: the recovered [¹⁴C]-labeled AT tryptic peptide is located outside of the N-linked glycopeptides and can indicate the specificity of N-linked glycopeptide;
- 2) Efficiency: the final yield of AG is less than 10%. For AG, 2 of 4 [¹⁴C]-labeled tryptic peptides are within the N-linked glycopeptides and can indicate the yield of the glycopeptides.

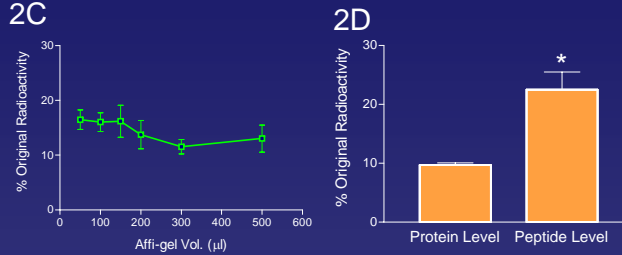
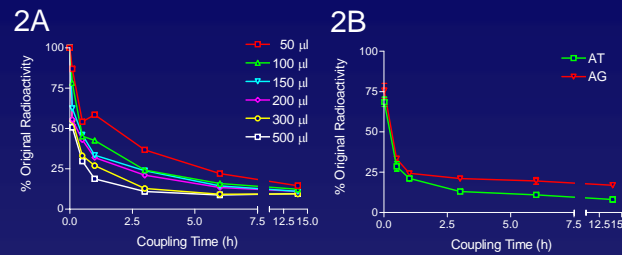
2, Optimizing each step

1) Oxidation:

10mM NaIO₄ at room temperature or 50mM at 4°C got the best coupling efficiency of AG.



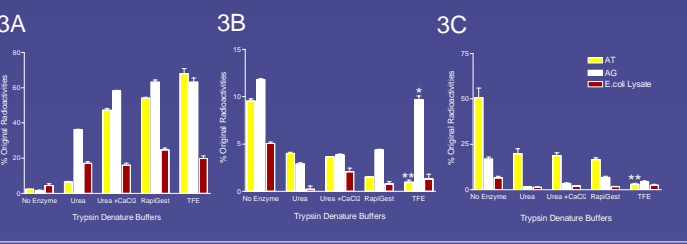
2) Coupling:



- 2A. Higher slurry volume, faster glycoprotein captured; while 50μl slurry was sufficient for 20 μl mouse plasma if coupled overnight;
- 2B. The coupling is near completion in three hours comparing to the overnight coupling in the original procedure;
- 2C. Peptide-level SPEG: 50μl Affi-gel slurry was sufficient for 20 μl tryptic digested mouse plasma after overnight coupling;
- 2D. Higher yield from peptide-level SPEG (>120% increase).

3) Trypsin Digestion:

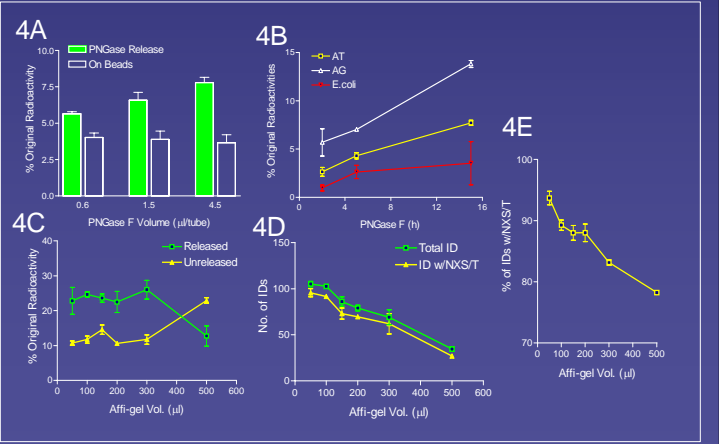
Here, five conditions were tested, including:
 100 mM NH₄HCO₃;
 8M Urea in 100 mM NH₄HCO₃ (original);
 8M Urea in 100 mM NH₄HCO₃, plus 2 mM CaCl₂;
 0.1% RapiGest in 100 mM NH₄HCO₃;
 and 50% TFE (Trifluoroethonal) in 100 mM NH₄HCO₃.



- 3A. Released [¹⁴C] radioactive activities from beads by tryptic digestion;
- 3B. PNGase F released [¹⁴C] radioactive activities;
- 3C. PNGase F unreleased [¹⁴C] radioactive activities.

4) PNGase F Release:

- 4A. Higher PNGase F results in higher yield;
- 4B. Longer incubation time increases the yield of recovered N-linked glycopeptides;
- 4C. Peptide level SPEG-Final yield of N-linked glycopeptides by different volume of slurry: 50 μl slurry is sufficient for 20 μl mouse plasma, while 500 μl slurry induced sharply decrease of yield;
- 4D. Number of identified N-linked glycopeptides by MS/MS— numbers of total IDs and IDs with NXS/T motif;
- 4E. Specificity of N-linked glycopeptides—percentage of IDs with NXS/T motif over total IDs: excess amount of bead increases the non-specific peptides identified.



CONCLUSION

The comparison experiments have shown that 200 μl of hydrazide resin, 10mM NaIO₄, and 4.5 μl PNGase F for 14 hours at 37 °C give the most satisfying results in terms of capturing N-linked glycopeptides from 20 μl mouse blood plasma. The buffer system for trypsin digestion is a critical factor for SPEG specificity and efficiency—50% TFE performed the best results. Finally, peptide-level glycopeptide capture have shown itself as one effective way to significantly increase the yield of SPEG.

REFERENCES

[1] Hui Zhang, Xiao-jun Li, Daniel Martin and Ruedi Aebersold, Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. Nature Biotechnology. 2003, 21:660-666.