Application of High Pressure and Highly Stable Trypsin Aggregate Coating on Superparamagnetic Magnetite/Silica Nanoparticles for High Performance Proteomics

Daniel López-Ferrer[1], Byoungsoo Lee[2], Jungbae Kim[2] and Richard D. Smith[1]
[1]Pacific Northwest National Laboratory, Richland, WA 99352; [2]Korea University, Seoul, Korea

Pacific Northwest NATIONAL LABORATORY

Overview

- High stable and active trypsin coated magnetic nanoparticles (EC-TR/NPs) have been developed via a simple crosslinking procedure
- The performance of the EC-TR/NPs showed a negligible loss of trypsin activity after repeated use, while a control sample (trypsin attached covalently to nanoparticles) subjected to the same experienced a rapid inactivation due to trypsin denaturation and autolysis.
- Digestion efficiency using EC-TR/NPs subjected to pressure cycling technology was evaluated using both standard proteins and a complex global brain proteome.
- Pressure Cycling Technology (PCT) enhanced the speed of the enzymatic reaction as well as the reproducibility among different replicates, improving the overall quality of the experiments in a reduced amount of time compared to standard protocols.

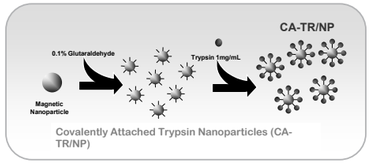
Introduction

- Protein digestion for bottom-up proteomics has traditionally been performed using a relatively lengthy incubation period (6-12 h).
- Substantial energy and efforts have been devoted to improved protein digestions using immobilized enzymes.
- By using immobilized enzymes, the enzyme-substrate ratio increases dramatically and in some cases the immobilization method increases the stability of the protein
- The combination of PCT and ultra-stable immobilized enzymes allows for fast and reproducible digestions as well as enhanced stability of the enzyme making it suitable for repeated use.

CONTACT: Daniel Lopez-Ferrer, Ph.D.
Biological Sciences Division, K8-98
Pacific Northwest National Laboratory
P.O. Box 999, Richland, WA 99352
E-mail: daniel.lopez-ferrer@pnl.gov

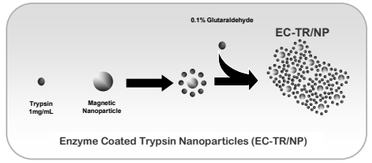
Methods

Development of immobilized trypsin on magnetic nanoparticles

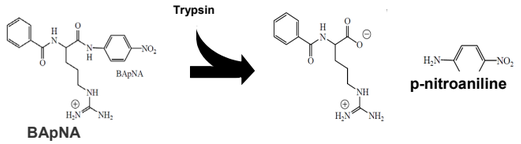


Schematic diagram of the synthesis of covalently attached trypsin nanoparticles (NPs). NPs are first functionalized with 0.1% glutaraldehyde followed by the covalent attachment of trypsin and free amine capping by Tris-HCl treatment.

Schematic diagram of the synthesis of trypsin aggregate coating on NPs. A layer of trypsin is first covalently attached to the NPs' surface. Then more glutaraldehyde and trypsin are added to form the enzyme aggregates. Finally the free amines are capped by Tris-HCl treatment.



Activity measurements

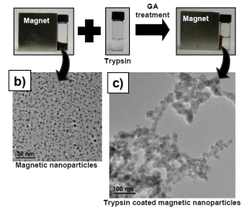


Trypsin activity was measured using benzoyl L-arginine p-nitroanilide hydrochloride as a substrate (L-BAPNA). L-BAPNA is a colorless chromogenic substrate that releases p-nitroaniline (yellow) when digested with trypsin.

Pressure cycling protein digestion system

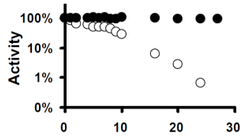
Results

Enzyme loading onto magnetic nanoparticles



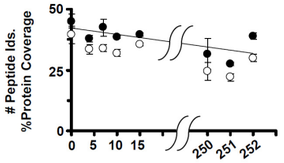
a) Magnetic capture efficiency of magnetic nanoparticles and EC-TR/NPs. Transmission electron microscopy (TEM) images of **b)** magnetic nanoparticles (scale bar, 50 nm) and **c)** EC-TR/NPs (scale bar, 100 nm)

Enzyme activity and stability of enzyme coated magnetic nanoparticles



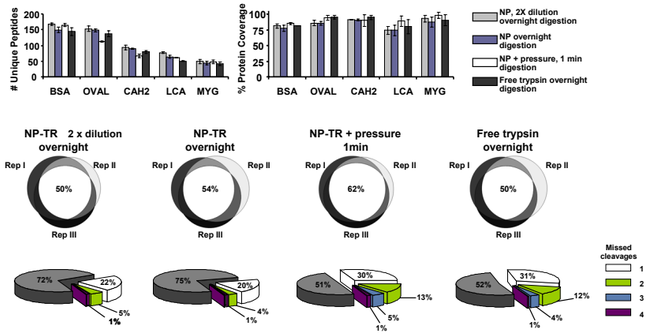
Enzyme activity of CA-TR/NP (empty circles) and EC-TR/NP (filled circles) after repeated uses and storage at room temperature. The activities of CA and EC-TR/NP were measured by the hydrolysis of BAPNA. The generation p-nitroaniline was monitored by measuring the increase in absorbance at 410 nm (A410) and the enzyme activity was calculated from the time-dependent increase of A410.

Enzyme stability study using microgram quantities of protein



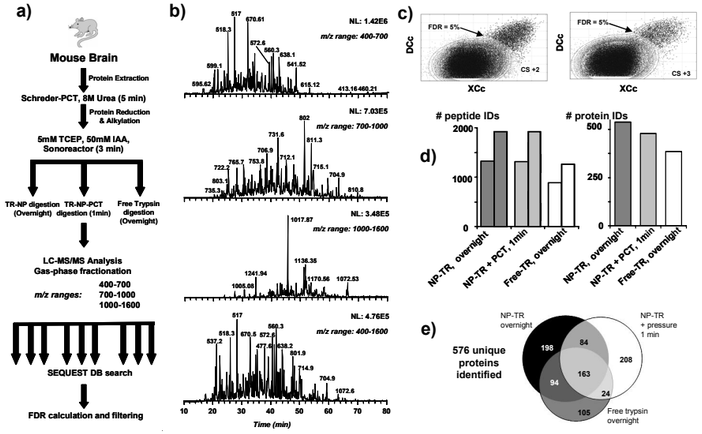
Time course analyses of iterative 100 ug BSA digestions using EC-TR/NPs as a function of unique identified peptides (empty circles) or protein coverage (filled circles).

Pressure-assisted digestions with EC-TR/NPs (5 protein standards)



Comparison of EC-TR/NP pressure assisted digestions with overnight digestion performances for EC-TR/NPs and free trypsin. A five standard protein mixture was used for the experiment. **a)** Histograms comparing the number of unique peptides and the percent protein coverage for three digestion replicates. **b)** Venn diagrams showing the peptide overlap between the three digestion replicates. **c)** Pie charts showing the percentage of missed cleavages for the different digestion methods.

Application of pressure-assisted digestion with EC-TR/NPs to a global mouse brain proteome analysis



a) Schematic of the analysis workflow. **b)** Representative chromatograms obtained from EC-TR/NPs-PCT digestions of mouse brain samples after gas-phase fractionations. **c)** Scatter plot of the corrected scores obtained after SEQUEST database searches from the generated MS/MS spectra. **d)** Histograms comparing the number of unique identified peptides (empty bars), total identified peptides (diagonal filled bars) **e)** Venn diagrams showing the overlap between three technical digestion methods.

Conclusions

- The enzyme coating strategy to create immobilized magnetic NPs represents a promising new material for robust high-throughput digestion platforms.
- The EC-TR/NPs displayed a high degree of stability that gives them long lasting attributes, even when stored at room temperature. After repeated uses, activity is not lost, and the magnetic core makes the particles amenable to use in a robotic station that uses a 96-well plate format.
- The combination of PCT and EC-TR/NPs has been demonstrated to be an effective approach for fast protein digestion.
- By integrating EC-TR/NPs with PCT, proteolysis is significantly accelerated and excellent mixing is achieved, which translates into higher reproducibility.
- The new EC-TR/NPs coupled with PCT offer significant enhancements that can aid in the development of ultra fast, reproducible, automated digestion platforms for high throughput proteome sample processing.

Acknowledgements

The authors thank Ron Moore and Mikhail E. Belov for helpful suggestions and technical support, and Vlad Petyuk for providing the mouse brain sample and the script for counting the number of missed cleavages.

Portions of this research were supported by the NIH National Center for Research Resources (RR018522), NIH National Cancer Institute (R21 CA12619-01), the Pacific Northwest National Laboratory's (PNNL) Laboratory Directed Research and Development Program, and grants from the National Research Foundation funded by the Korean Ministry of Education, Science & Technology (No. 2009-0082314, No. 2009-0059861, and No. 2009-0075638).

A significant part of the work was performed in the Environmental Molecular Science Laboratory, a U. S. Department of Energy (DOE)/Office of Biological and Environmental Research national scientific user facility located on the campus of PNNL in Richland, Washington. PNNL is a multi-program national laboratory operated by Battelle for the DOE under Contract DE-AC05-76RLO1830.

The authors also wish to acknowledge Pressure BioSciences Inc. for providing the PCTMicroTubes used in these experiments and for their helpful suggestions.

References

1. D López-Ferrer, *et al.* Application of Pressurized Solvents for Ultra Fast Trypsin Hydrolysis in Proteomics: Proteomics on the Fly. *J Proteome Res* 2008, 7:3276-3281.
2. D López-Ferrer, *et al.* Highly stable trypsin-aggregate coatings on polymer nanofibers for repeated protein digestion. Proteomics. 2009 7:1893-1900.