# Affinity Mass Spectrometry Measurement of Targeted Cytokines under Physiological Conditions

Gregory Hurst,[1] Stephen Kennel,[2] Linda Foote,[2] Yongseong Kim,[1] Michelle Buchanan[1,2]

[1]Chemical and Analytical Sciences Division and [2]Life Sciences Division
Oak Ridge National Laboratory, Oak Ridge TN 37831-6365

Certain cytokine levels in serum can serve as sentinels of inflammatory function in animals. We are developing a method for large-scale screening of cytokine levels in serum samples from mutant mouse populations as a possible means to detect genetic conditions that lead to chronic inflammatory diseases such as Crohn's disease or arthritis. The goal of this work is to detect mutant animals which have relatively subtle abnormalities leading to chronic inflammatory diseases. To this end, we have developed a method for concentration and purification of tumor necrosis factor alpha (TNF-α), a 17 kDa proinflammatory cytokine that has a relatively high serum concentration in acute reactions and seems to be an early effector in inflammatory cytokine cascades. The method is based on affinity capture of cytokines from serum using an antibody-derivatized micron-sized bead support, followed by detection and semi-quantitation by MALDI-MS. The capture was optimized and characterized by using sensitive $^{125}$I labeling experiments.[1] The selectivity of affinity separations is complemented by the m/z measurement capability of mass spectrometry.[2]

Capture molecules were covalently linked to ca. 1 μm diameter aminopolystyrene or fragmented Sephacryl beads using glutaraldehyde linkages[3] or CNBr activation,[4] at 7-15 μg IgG per mg beads. TNF-α-specific capture molecules included purified rat monoclonal antibody to mouse TNF-α (RatxTNF-α), purified goat polyclonal antibody to mouse TNF-α (GxTNF-α), and a recombinant soluble TNF-α receptor protein (rhu TNFR:Fc). Non-specific capture molecules that served as controls for non-specific binding included a normal IgG and bovine serum albumin (BSA). To capture the analyte from solution, a suspension of antibody-derivatized microparticles was incubated with varying amounts of TNF-α in 100μL volumes of 5mg/mL BSA in phosphate-buffered saline (PBS, 0.15M NaCl, 0.010M sodium phosphate, pH 7.6). Beads were collected by centrifugation and washed with deionized water to remove BSA and salts prior to MALDI sample preparation. To release TNF-α from the beads, 2 μL of sinapinic acid (SA) matrix solution (in 1:1 $CH_3CN$:aqueous 0.1% TFA) were added to the beads with gentle mixing. One μL of the supernatant was then transferred to the MALDI sample plate, which was prepared in advance with either a "crushed-crystal" SA layer[5] or a thin layer of SA/nitrocellulose.[6] MALDI-MS was performed using a nitrogen-laser equipped PerSeptive Biosystems Voyager DE linear time-of-flight mass spectrometer in positive-ion mode. Parallel samples prepared with $^{125}$I-labeled TNF-α were analyzed with a Beckman 5500A gamma radiation counter to quantitate and optimize analyte binding by the derivatized capture beads.

MALDI signal can be detected reliably for as little as 1 ng (ca. 60 fmol) TNF-α in the initial 100 μL sample volume, carried through the capture/elution/MALDI procedure. The amount of TNF-α actually present on the MALDI plate is, at most, half of this amount. A comparison of capture molecules--RatxTNF-α, rhu TNFR:Fc and normal (non TNF-α-specific) rat IgG -- attached to polystyrene beads was performed using both radiotracer and MALDI detection. Radiotracer (Figure 1a) and MALDI (Figure 1b) response curves were obtained as a function of amount of TNF-α added to the capture molecule-derivatized beads. The radiolabel detection data in Figure 1a show that both RatxTNF-α and rhu TNFR:Fc capture up to 50% of the TNF-α from solution, while both radiotracer and MALDI detection show only low amounts of nonspecific capture of TNF-α by the IgG control. Figure 1c shows MALDI spectra obtained using the different capture molecules. Intense peaks resulted from the capture of 5 ng TNF-α by immobilized RatxTNF-α or rhu TNFR:Fc, but no response was noted when the immobilized normal IgG control was used to "capture" this amount of TNF-α. A very low-intensity TNF-α peak, representing non-specific interaction, was seen from the normal IgG beads when a five-fold larger amount of TNF-α (25 ng) was added.

We are currently working to improve sensitivity by eluting the TNF-α from the capture beads into smaller volumes, and to improve the precision of MALDI quantitation using more homogeneous sample preparation techniques and internal standards, with the goal of rapid throughput sample analysis at physiologically relevant concentrations.


1. Hurst, G.B.; Kennel, S.J.; Foote L.J.; Buchanan, M.V. *Anal. Chem.* 1999 (submitted).
2. Nelson, R.W.; Krone, J.R.; Bieber, A.L.; Williams, P. *Anal. Chem.* **1995**, *67*, 1153-1158.
3. Kennel, S.J. *J. Immunol. Methods* **1982**, *55*, 1-12.
4. Kennel, S.J.; Lankford, T.; Galloway, P.; Witschi, H.P. *Toxicol.* **1989**, *55*, 103-116.
5. Xiang, F.; Beavis, R.C. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 199-204.
6. Vorm, O.; Roepstorff, P.; Mann M. *Anal. Chem.* **1994**, *66*, 3281-3287.



We thank the Immunex Corp. for the generous gift of rhu TNFR:Fc. Research sponsored by the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory, managed by Lockheed Martin Energy Research Corp. for the U. S. Department of Energy under Contract No. DE-AC05-96OR22464.


**Radiolabel Detection**

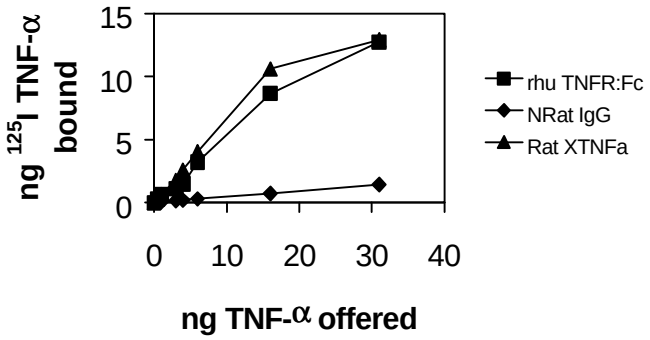


Fig. 1a

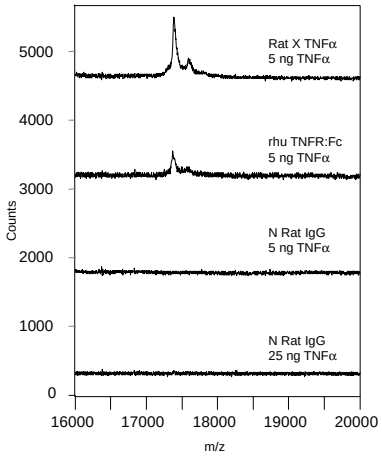


Fig. 1c

**MALDI Detection**

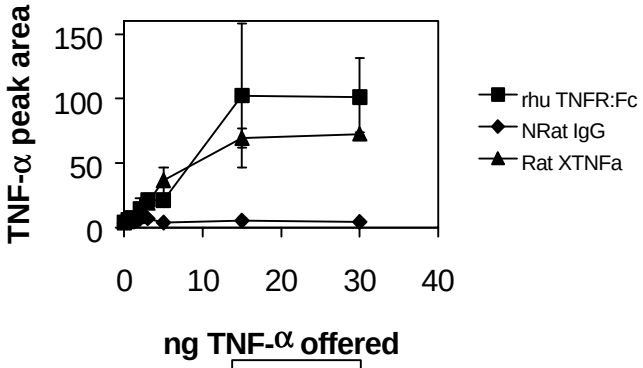


Fig. 1b