南京大学

Developments and challenges in the detection of doping with peptide hormones and related substances

New techniques for extraction, separation, and detection of EPO

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Outline

- Nanoparticles-based extraction-capillary zone electrophoresis-native fluorescence detection of EPO
- Boronate affinity monolithic capillaries for specific extraction of EPO

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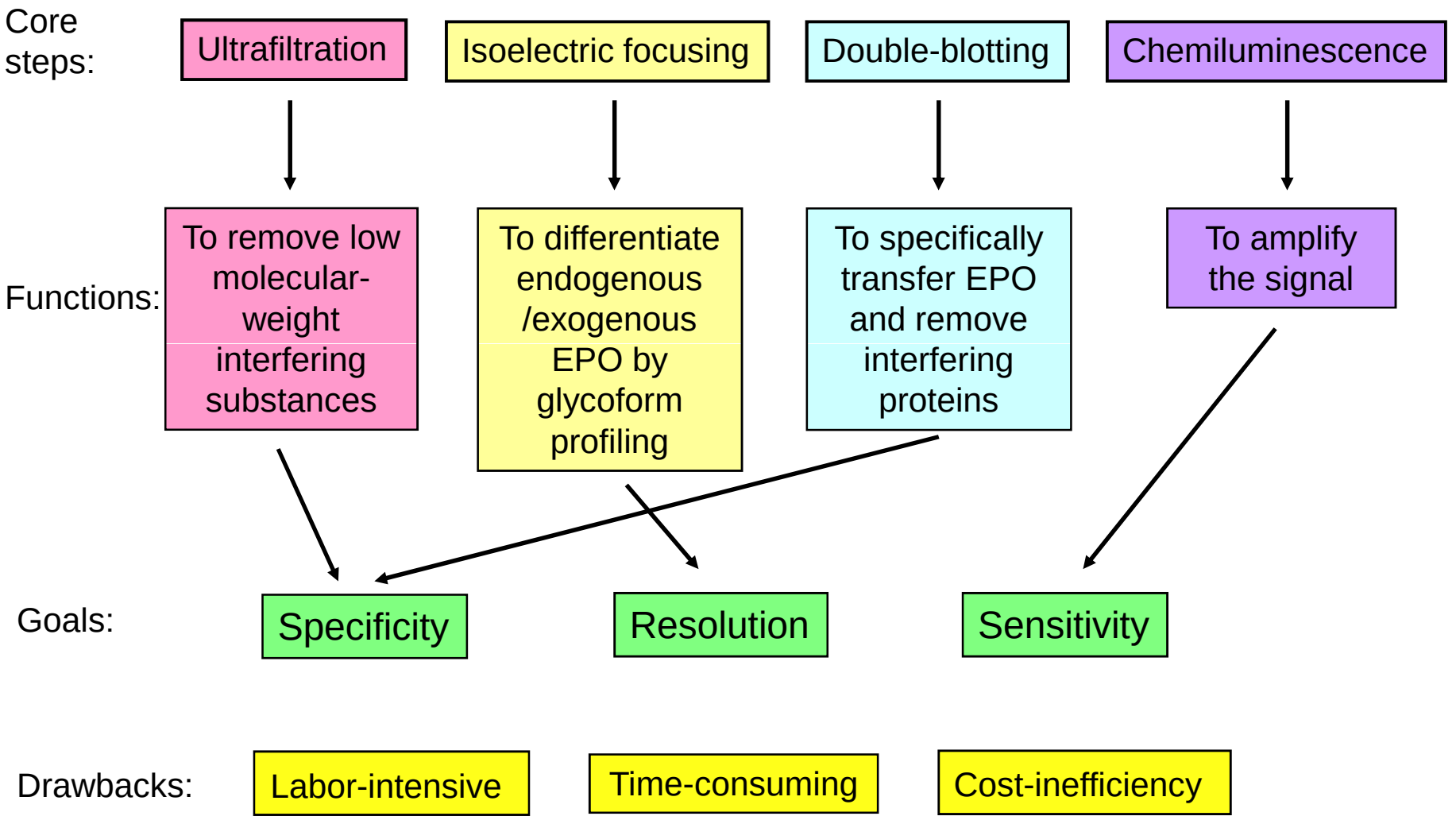
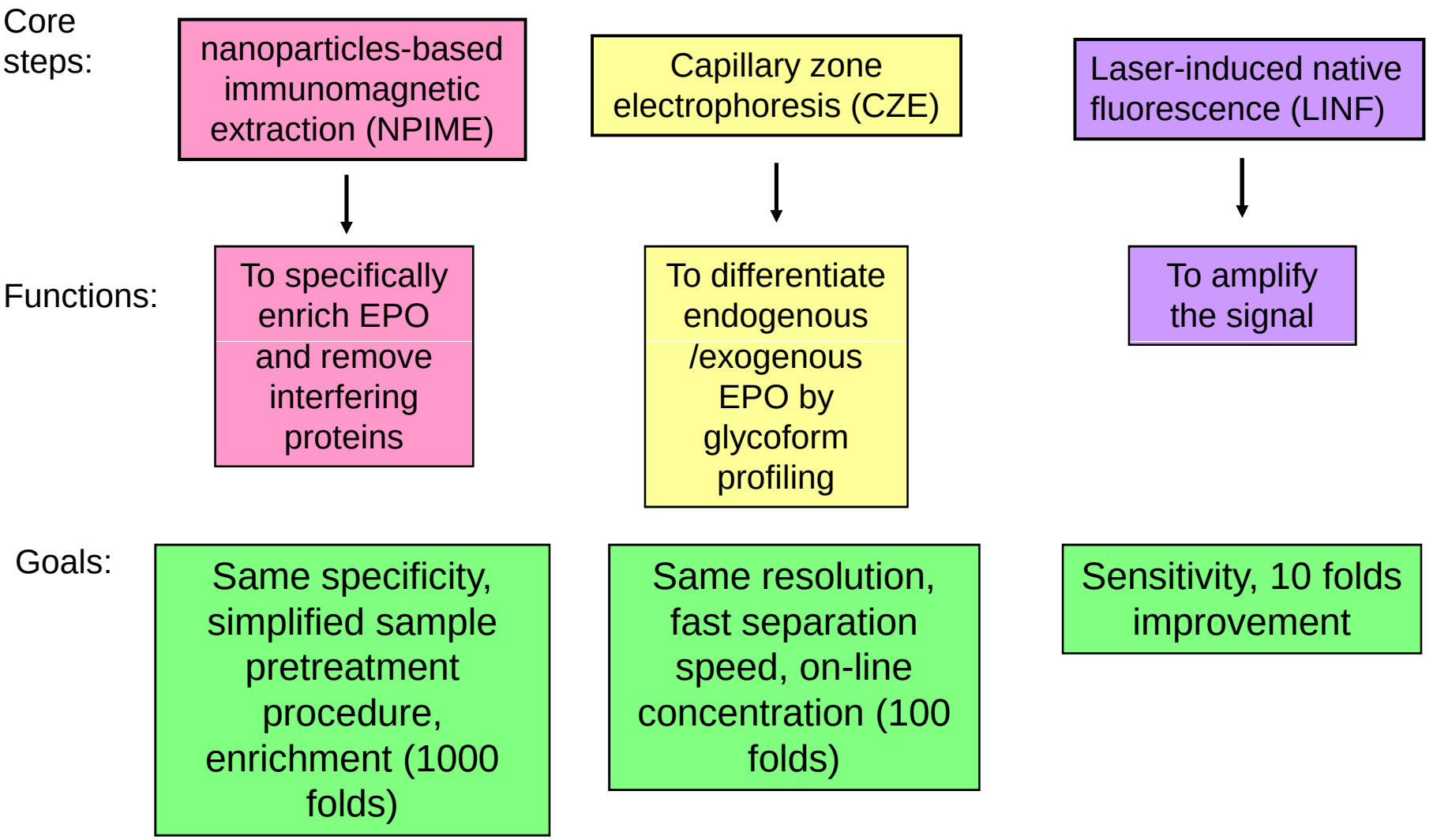
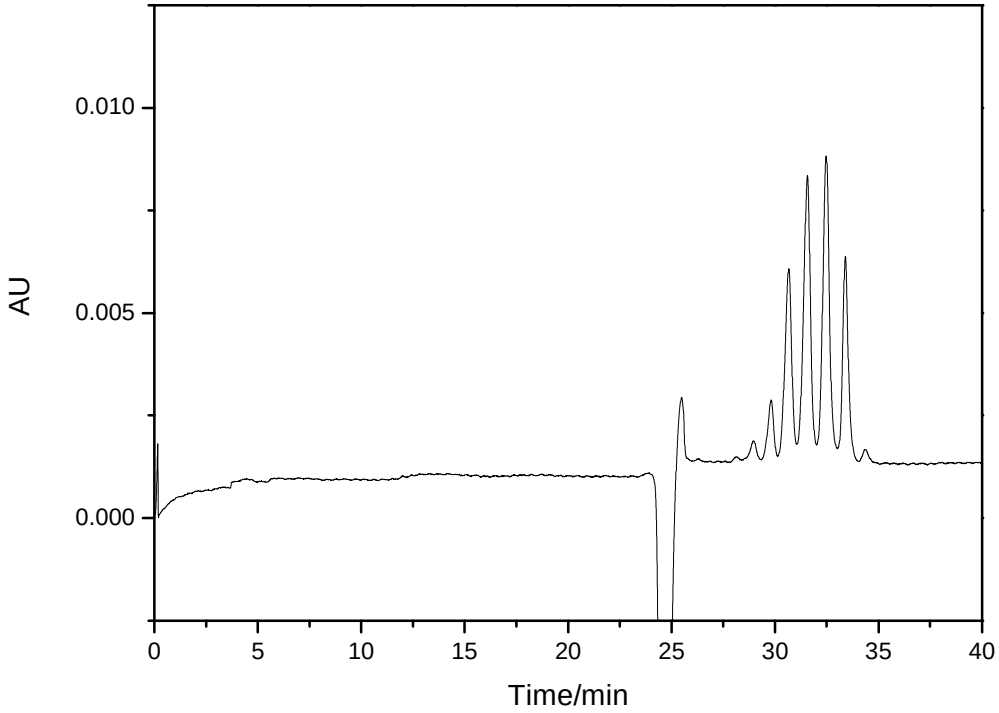
The IOC-validated EPO assay



Our proposed strategy

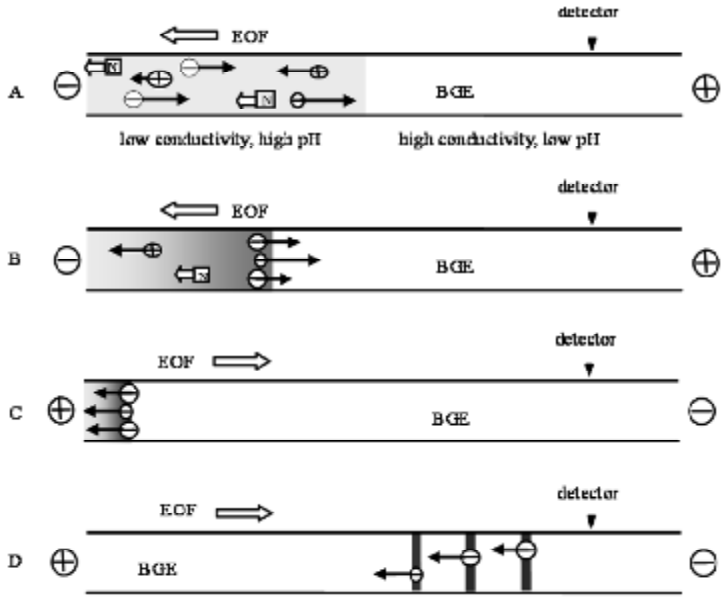


Capillary zone electrophoresis (CZE)-UV absorbance detection of EPO standard

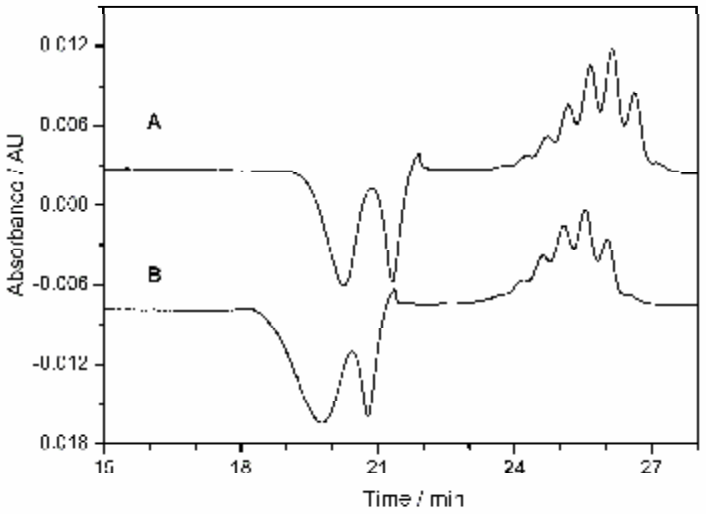


Capillary, bare fused-silica capillary with 60 cm total length (50 cm effective length) and 50 μm id; capillary temperature, 35 °C; separation voltage, 15 kV; BGE, 10 mM sodium acetate buffer containing 7 M urea, 10 mM Tricine, 3.9 mM putrescine, and 100 mM NaCl at pH 5.50; UV detection wavelength, 214 nm; sample, 1 mg/mL EPO, injected at 0.5 psi for 5 s.

Capillary zone electrophoresis (CZE) with on-line sample concentration of EPO standard



Principle of large volume sample stacking-reversed pH junction (LVSS-RPHJ)



CZE with LVSS-RPHJ of EPO

Sample and injected zone length (A) 0.02 mg/mL, 24.5 cm; (B) 0.01 mg/mL, 49 cm.

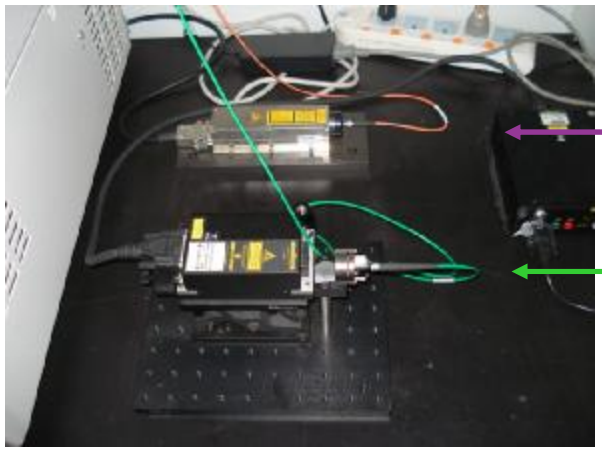
The detection sensitivity can be improved by 50–100 times, but at the price of resolution loss.

In-lab built deep UV LIF detection

(dual channel LIF)

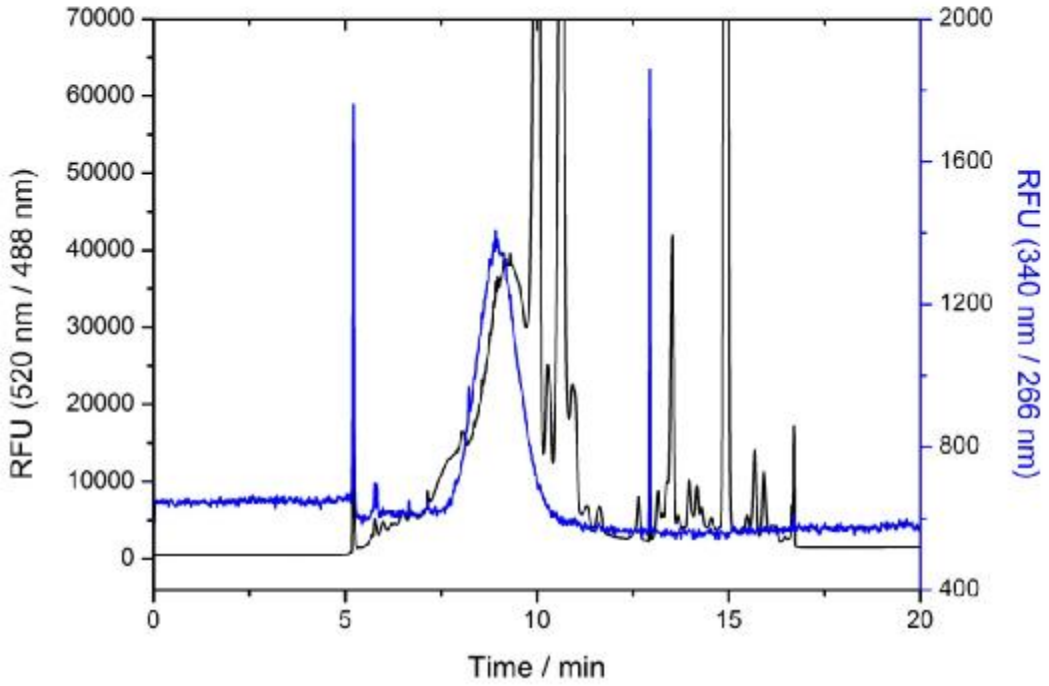
Beckman Coulter PACE MDQ system

Fiber and fiber adapter



Lasers

Comparison of the LIF detection of intact protein and fluorescent dye labeled protein



Native fluorescence

More sensitive as compared with UV absorbance (1 order of magnitude higher)

Original properties are preserved

Label-free

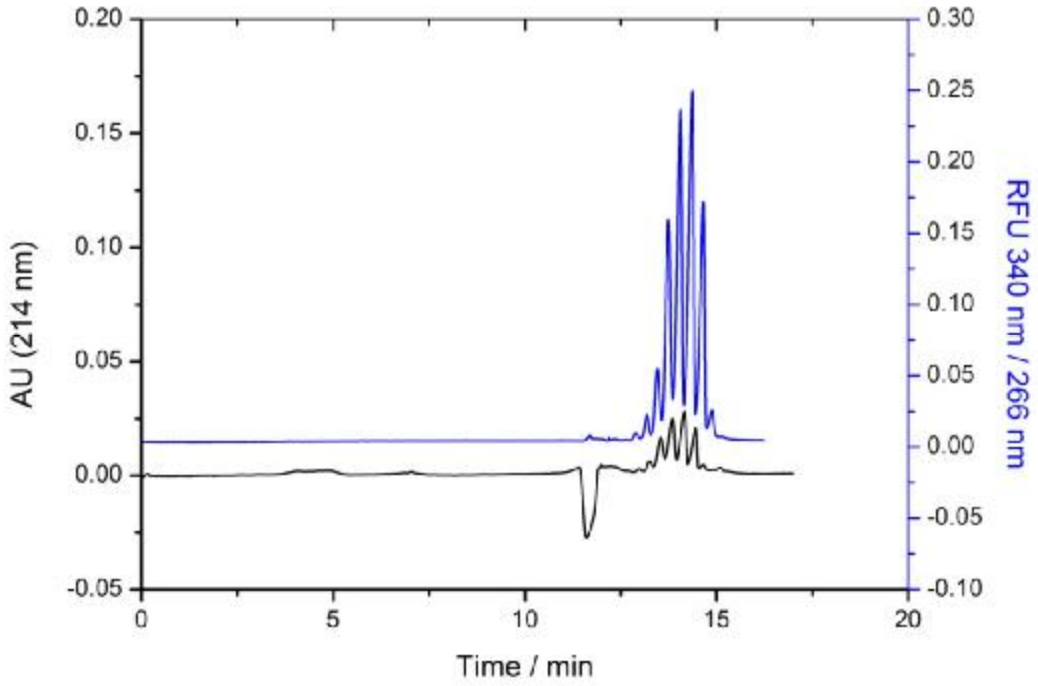
Fluorescent labeling

Much more sensitive as compared with native fluorescence (50 folds higher)

Variation of the original properties

Multiple labeling

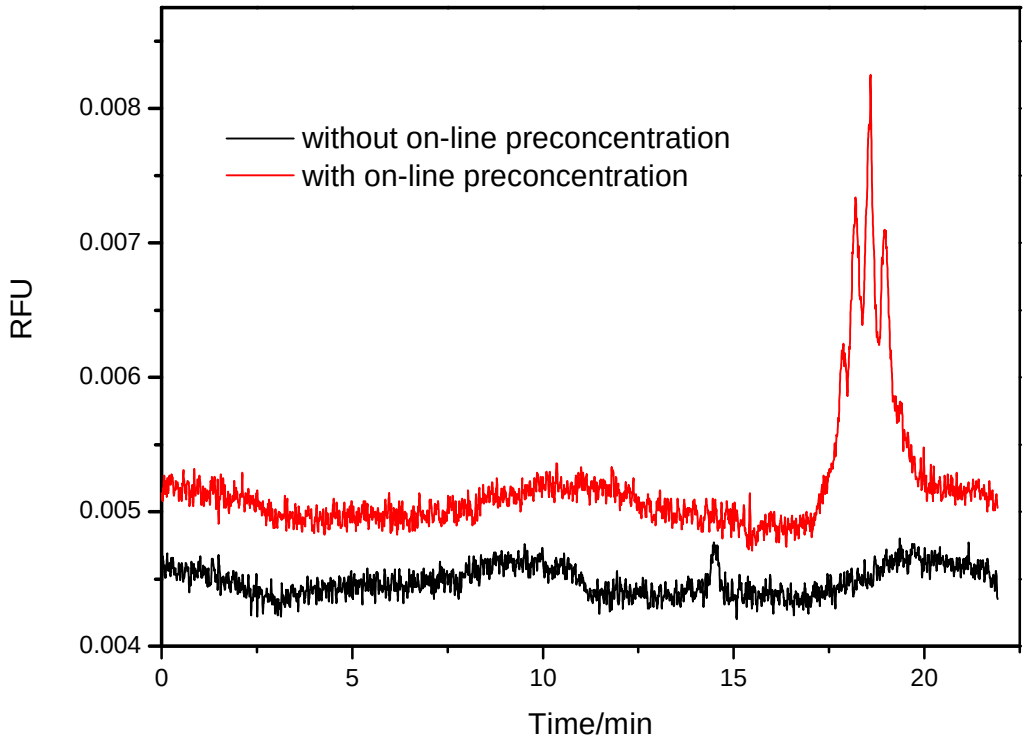
Deep UV LIF detection of EPO and comparison with UV absorbance



Label-free, identical finger-print profile

Improved detection sensitivity, 1 order of magnitude higher as compared with UV absorbance

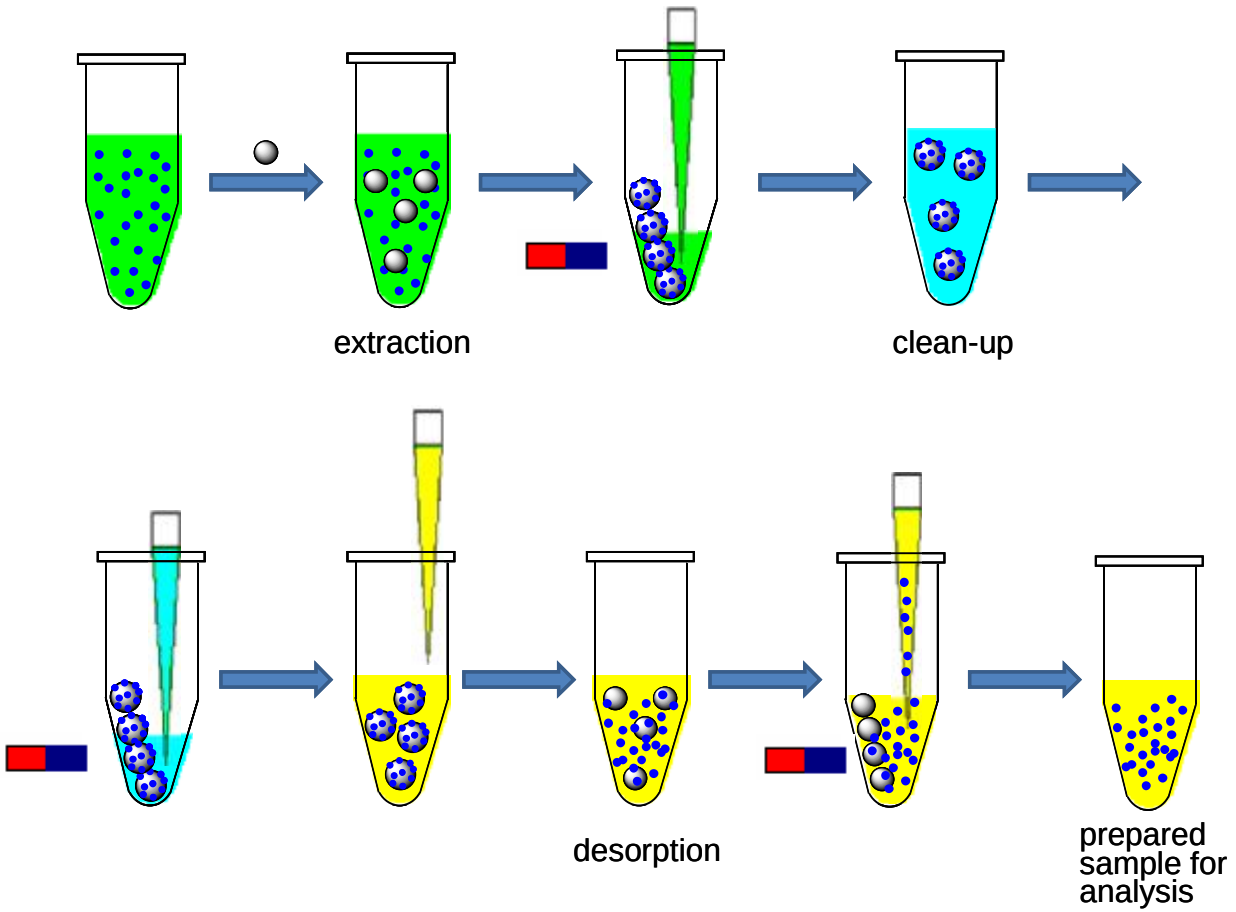
CZE-LINF with on-line sample concentration of EPO standard



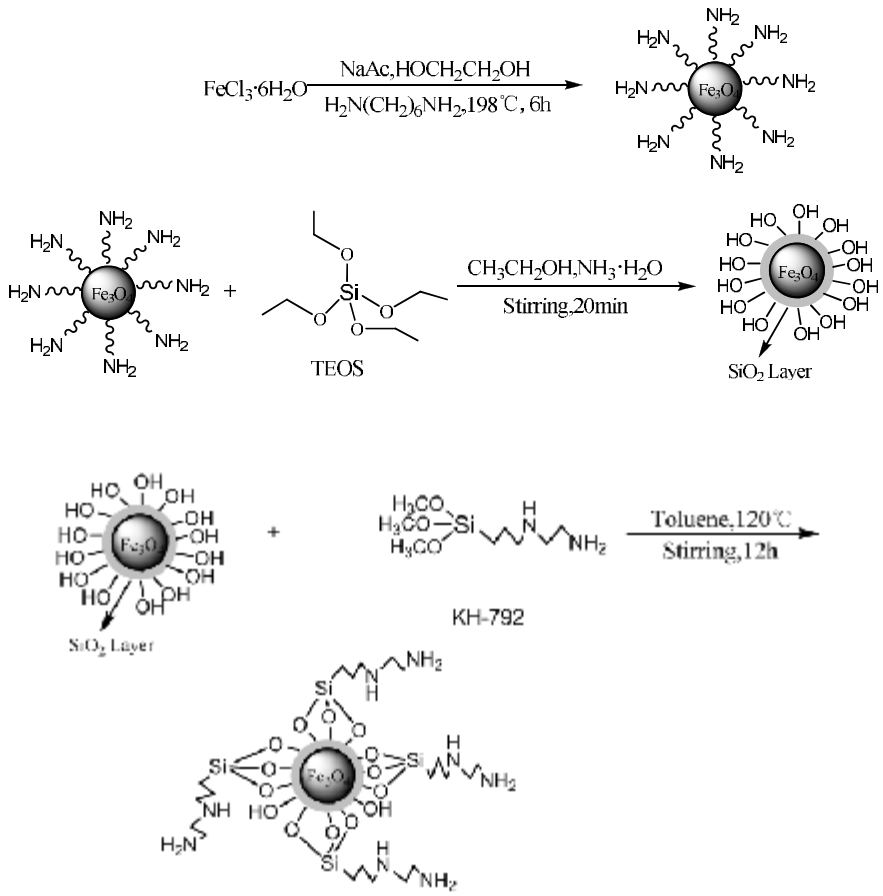
CZE with LVSS-RPHJ of EPO

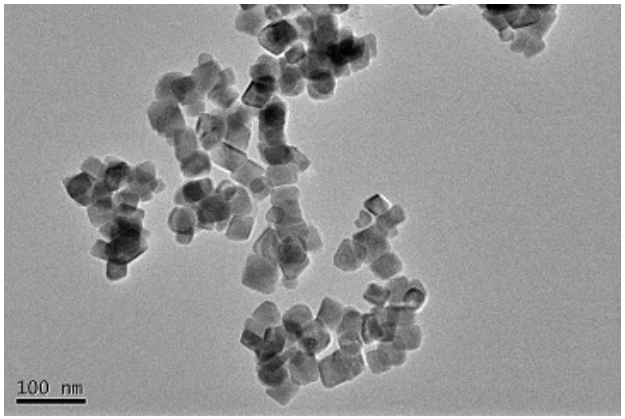
Sample concentration: 0.001 mg/mL (3×10^{-8} mol/L).

Procedure of magnetic particles-based extraction

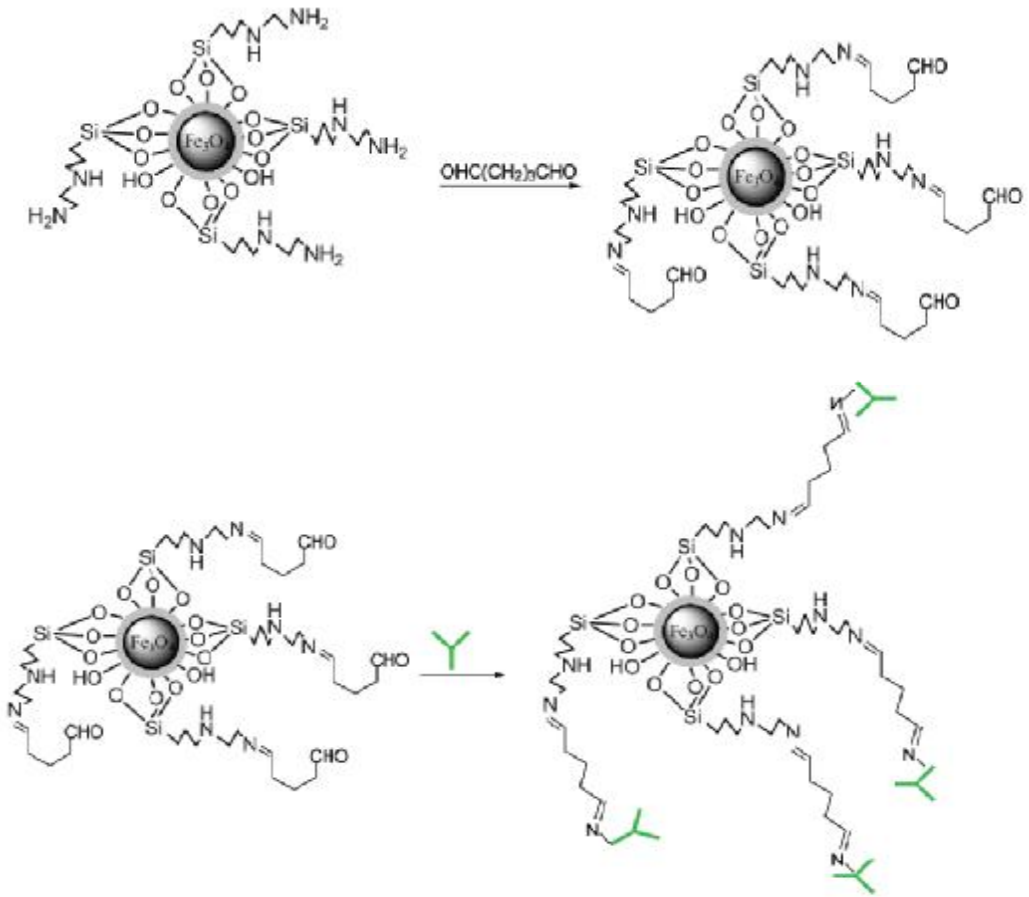


Amino-functionalized magnetic nanoparticles (Amino-MNPs)

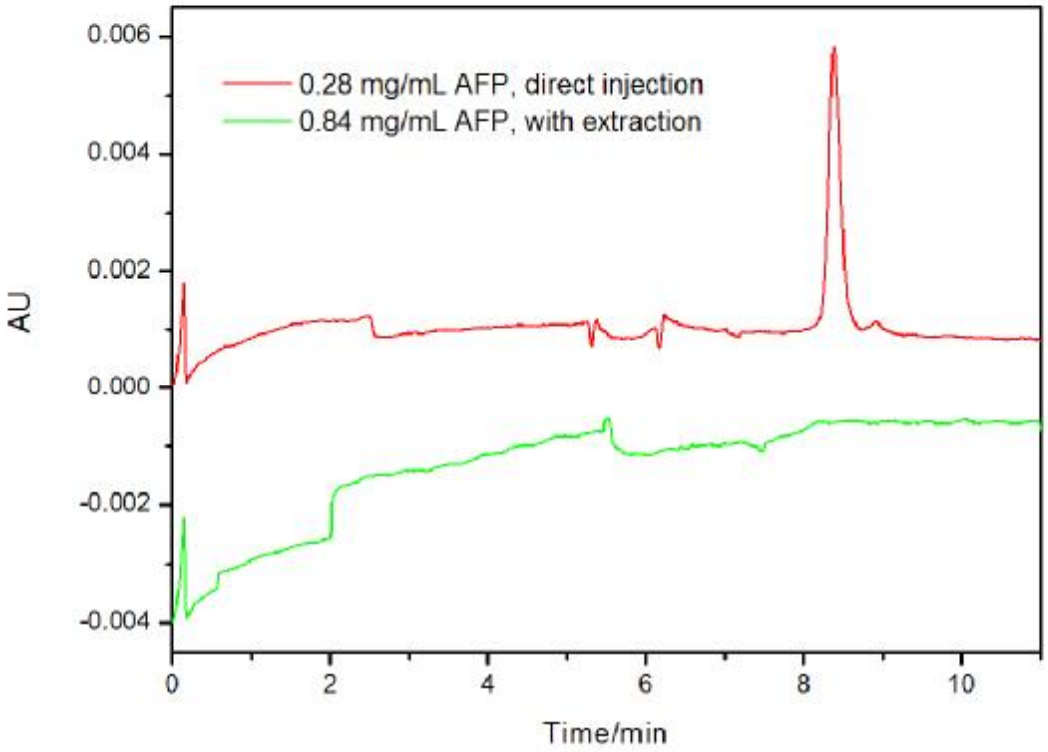




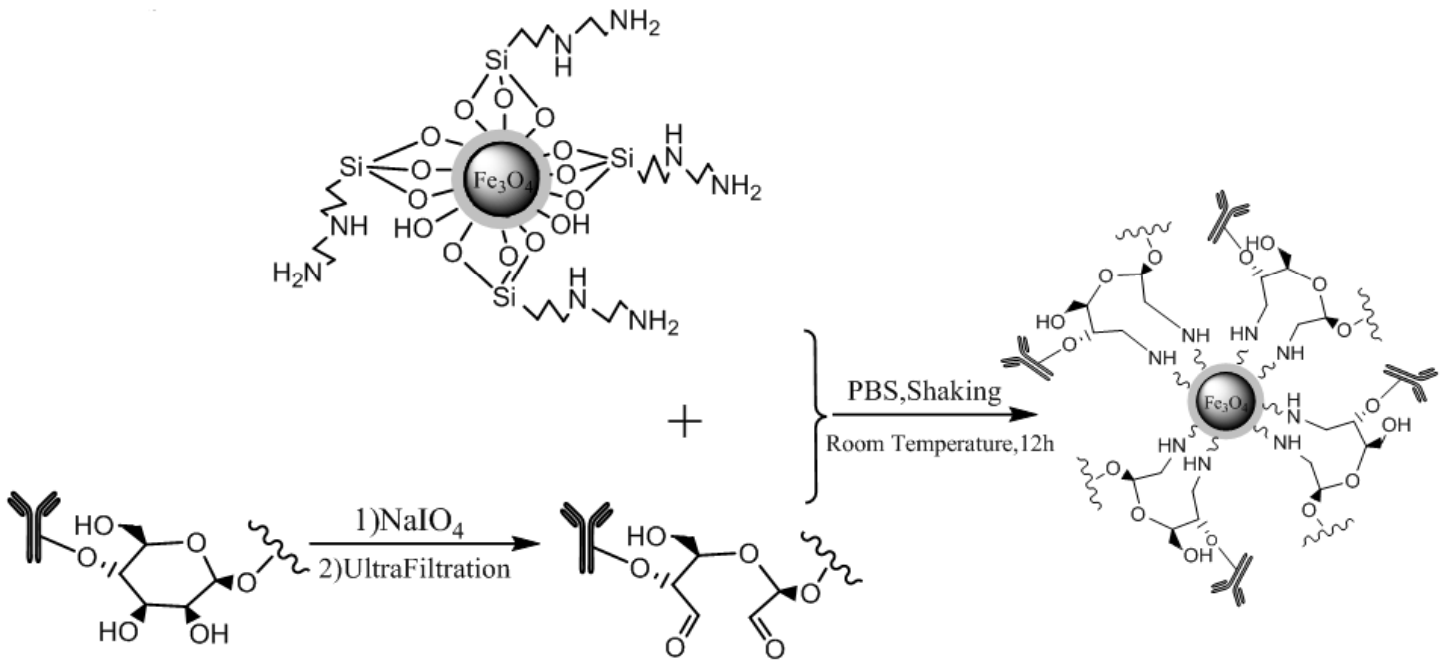
Random immobilization of antibodies through glutaraldehyde coupling



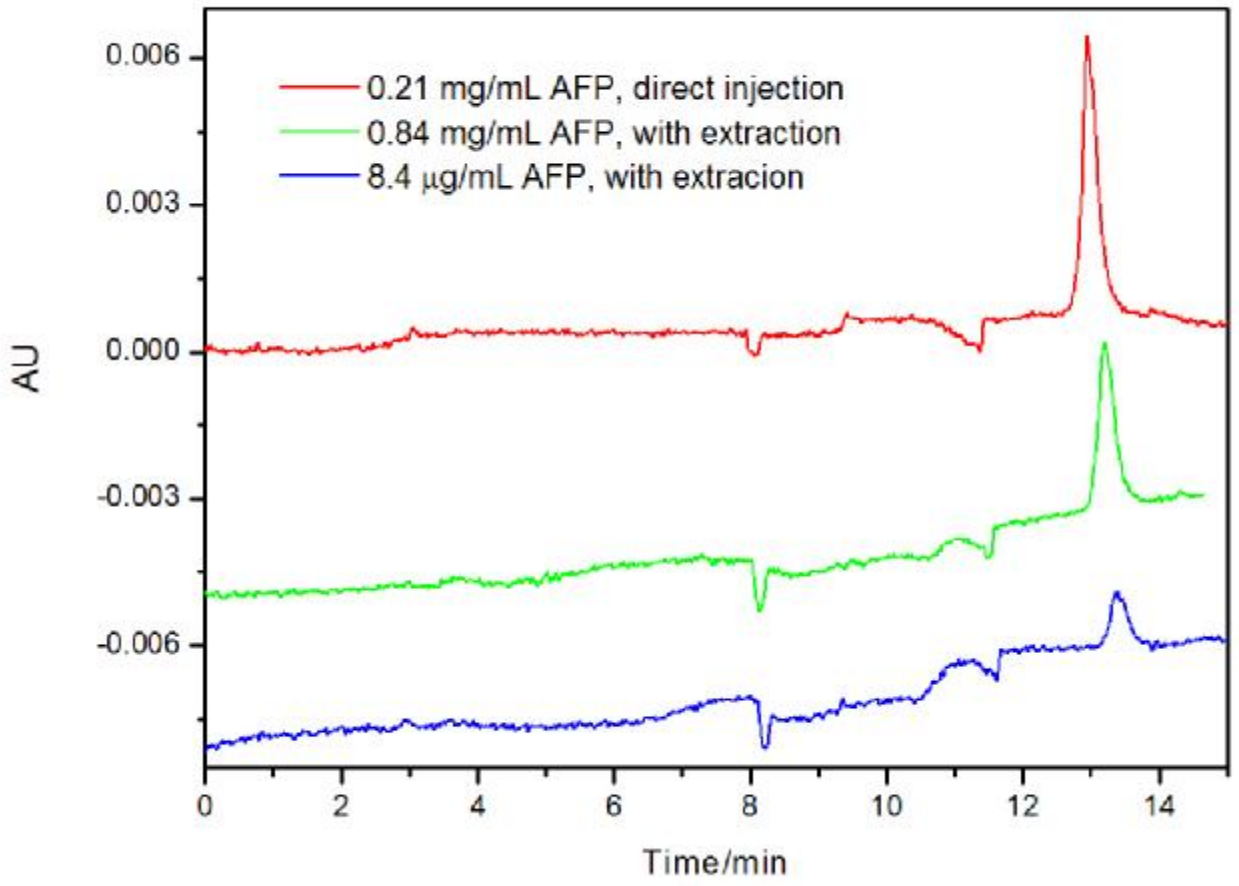
Extraction of AFP by anti-AFP immobilized MNPs



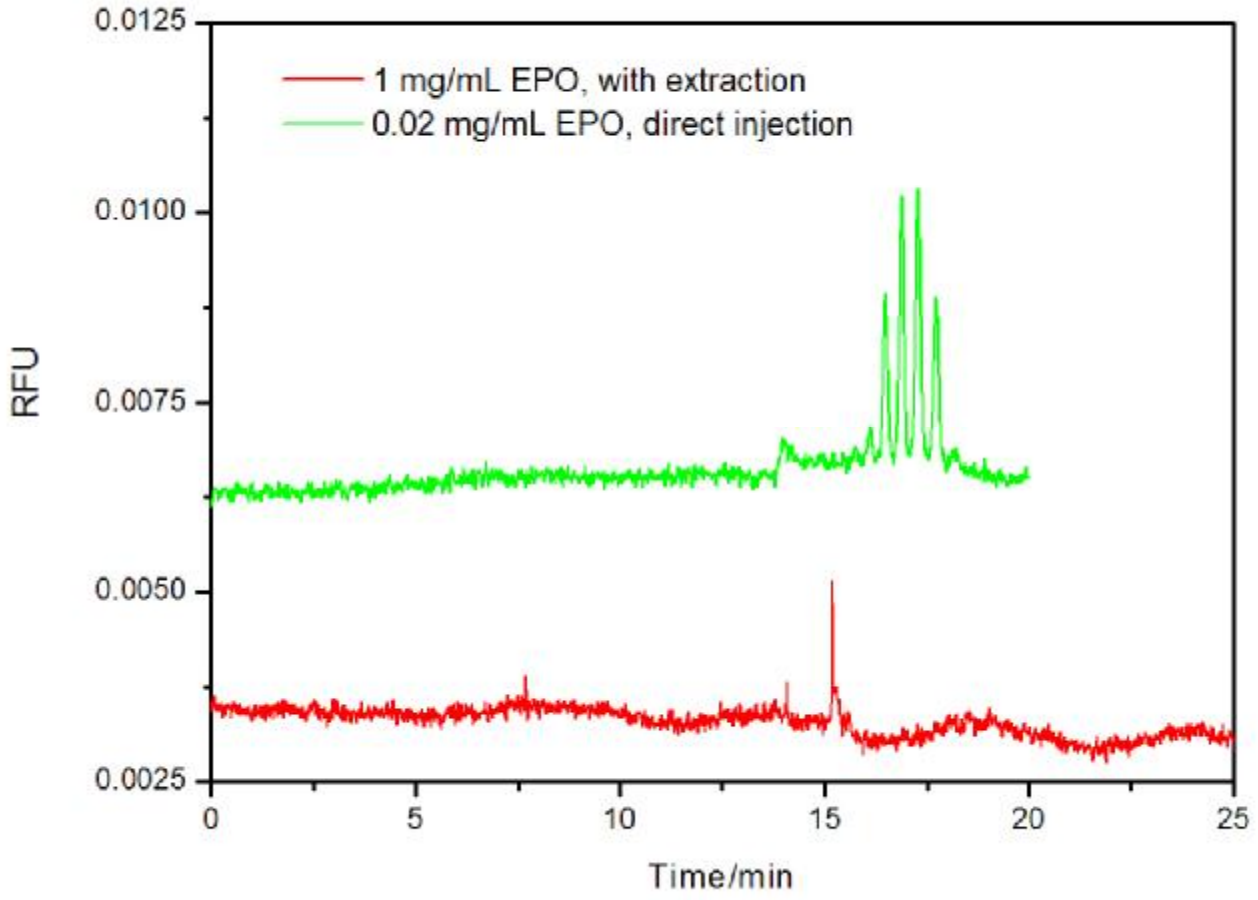
Oriented immobilization of anti-AFP IgG after oxidation of the carbohydrate moiety



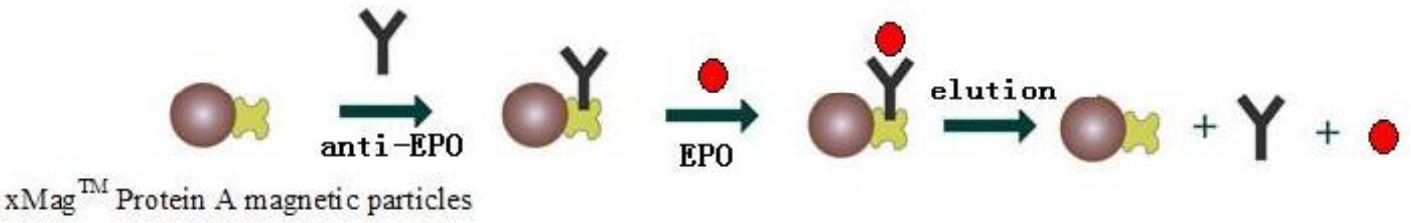
Extraction of AFP by anti-AFP immobilized MNPs



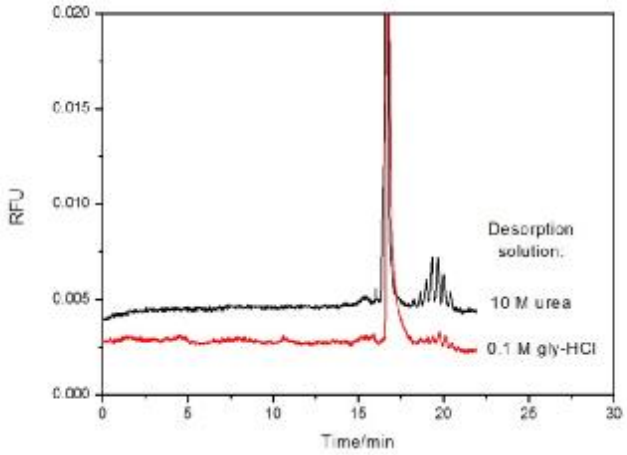
Extraction of EPO by anti-EPO immobilized MNPs

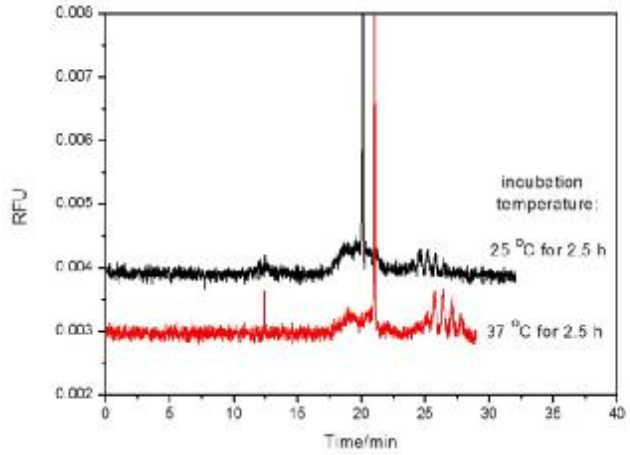


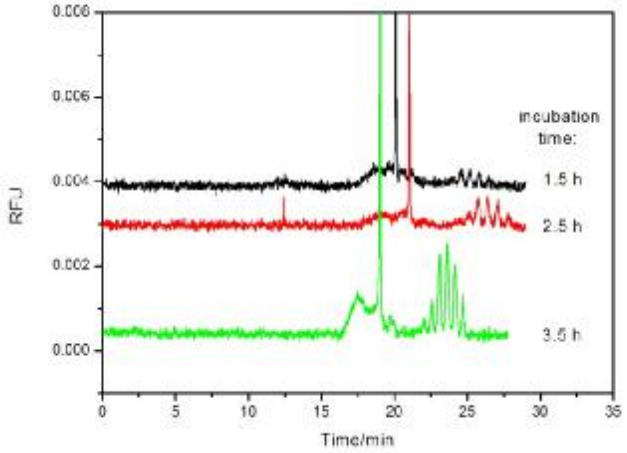
Oriented immobilization of Anti-EPO IgG to protein A-immobilized magnetic beads

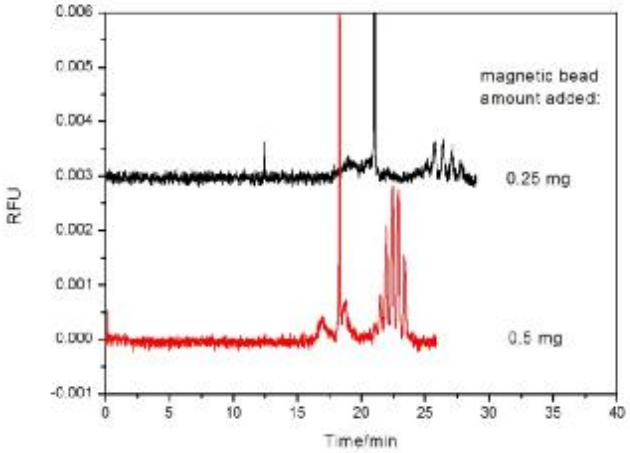


Optimization of experimental conditions (1)



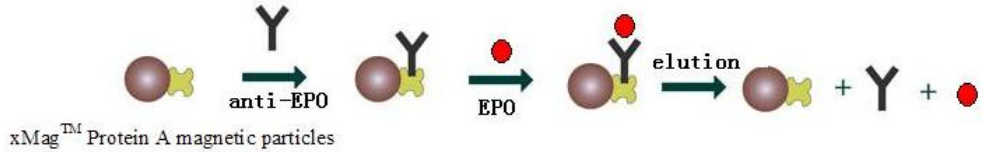




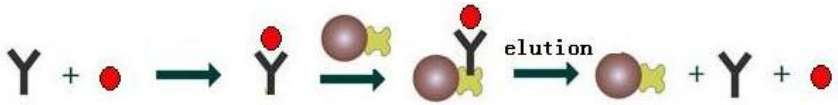


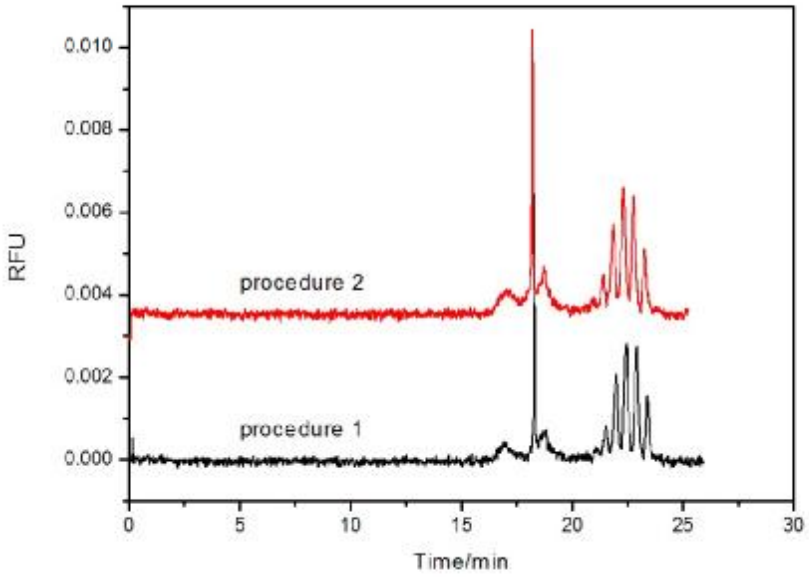
Optimization of experimental conditions (2)

Procedure 1:

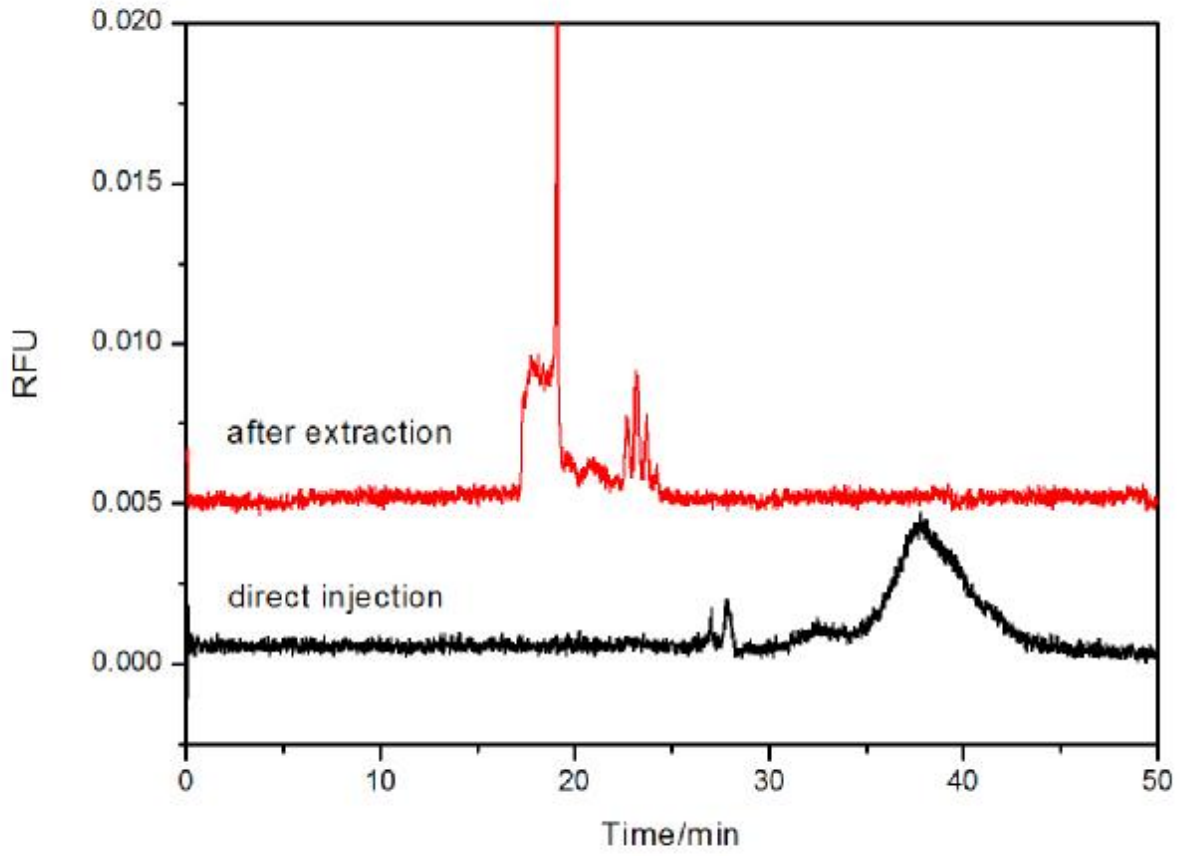


Procedure 2:

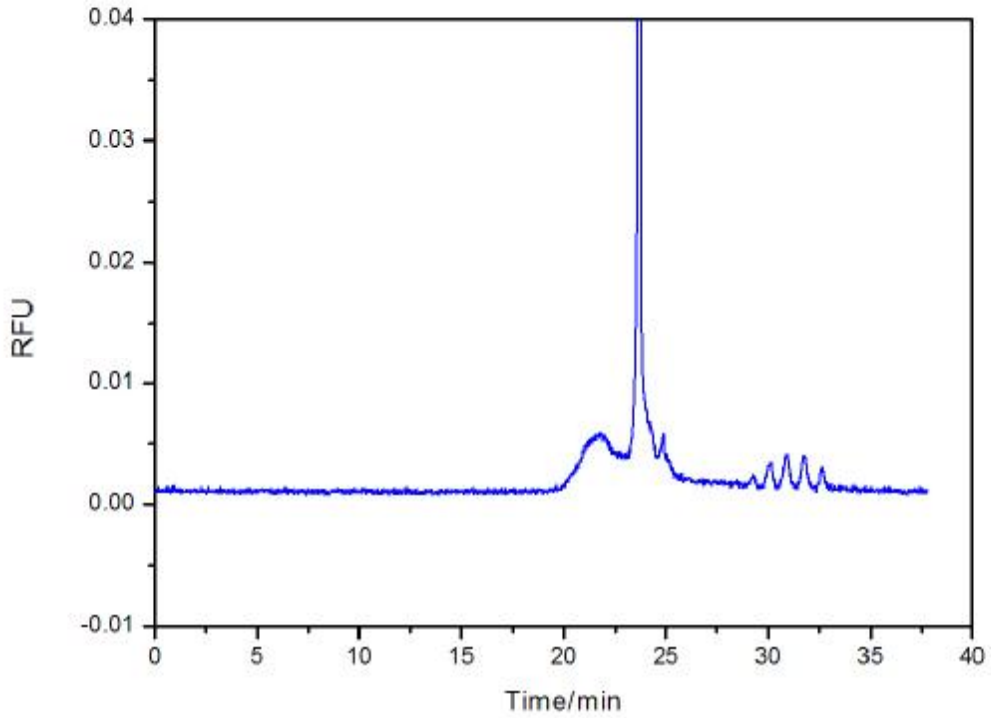




Extraction of EPO from clinical injection solution

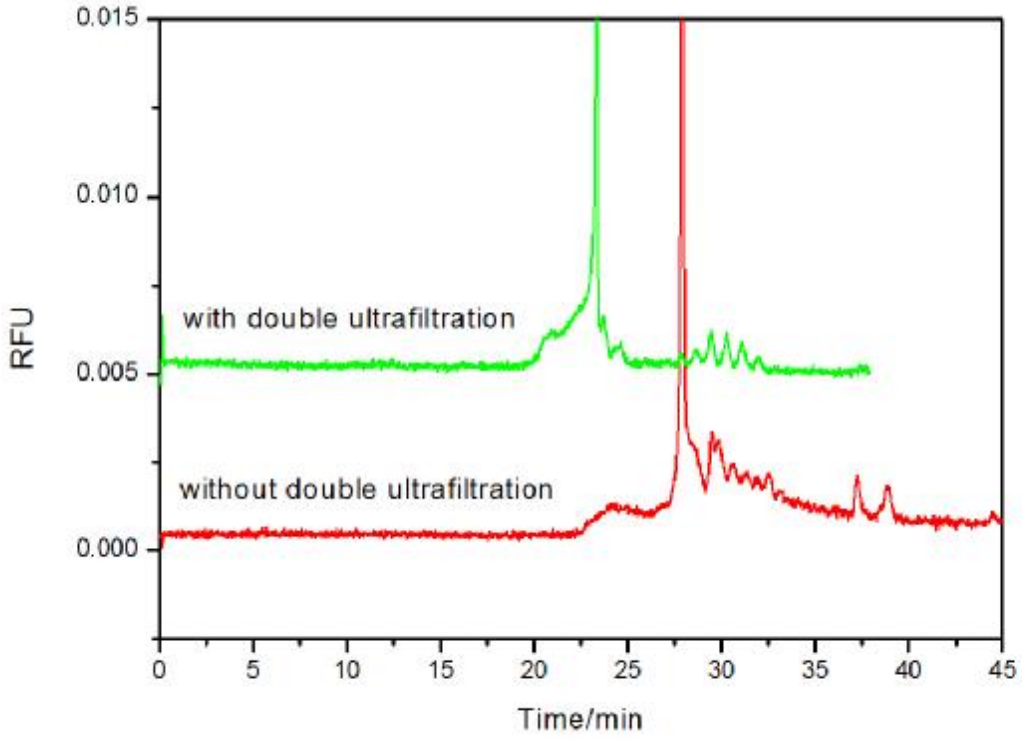


Limit of detection of EPO from aqueous solution



LOD = 14.7 nM (S/N = 3 for the smallest peak)

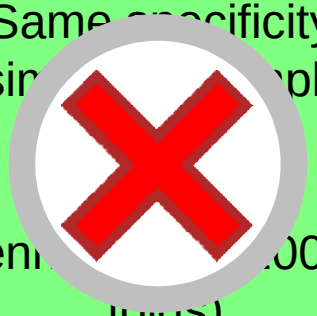
Detection of EPO from spiked urine sample



Ultrafitration 1: Amicon Ultra-15 (MWCO 10 kD), to remove low molecular weight interfacing species;
Ultrafitration 2: Amicon Ultra-0.5 (MWCO 50 kD) , to remove high molecular weight interfacing species, such as Tamm Horsfall glycoprotein (MW, 69 kD) and alpha-2-thiol proteinase inhibitor (MW, 72 kD).

Our proposed strategy

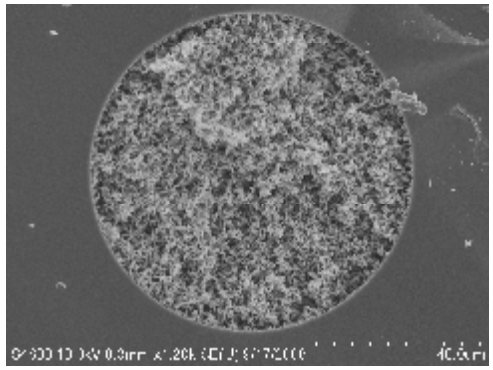
Core steps:	nanoparticles-based immunomagnetic extraction (NPIME)	Capillary zone electrophoresis (CZE)	Laser-induced native fluorescence (LINF)
Functions:	To specifically enrich EPO and remove interfering proteins	To differentiate endogenous /exogenous EPO by glycoform profiling	To amplify the signal
Goals:	Same specificity, sin ple en 000 folds)	Sam tion, f n co (100	Se olds t

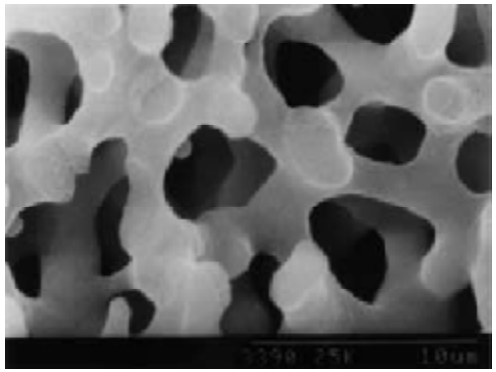


Outline

- Nanoparticles-based extraction-capillary zone electrophoresis-native fluorescence detection of EPO
- Boronate affinity monolithic capillaries for specific extraction of EPO

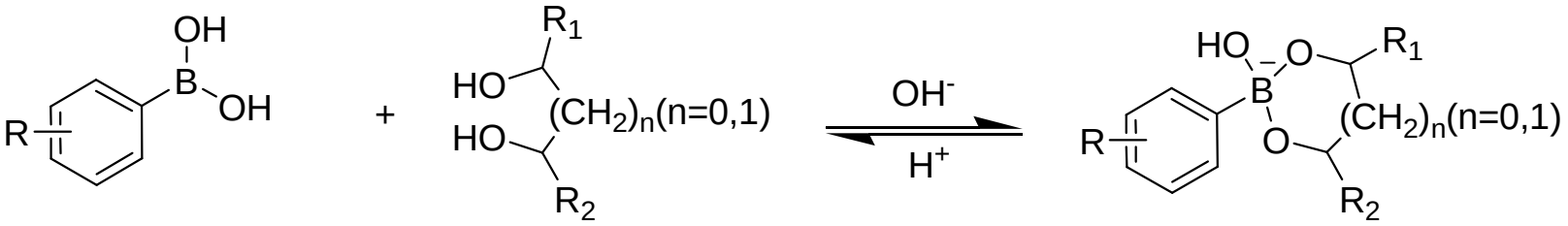
Why monolithic columns?





- Easy to prepare → low cost
- Fast convective mass transfer → high efficiency
- Open channel network → low back pressure, fast separation

Boronate affinity



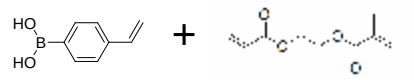
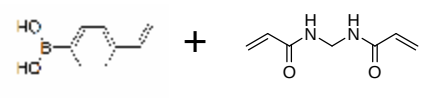
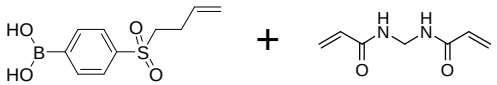
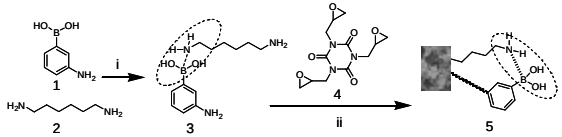
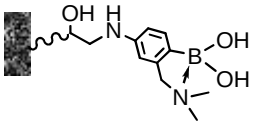
The cis-diol family:

Glycoproteins, glycopeptides, RNA, nucleosides, nucleotides, saccharides

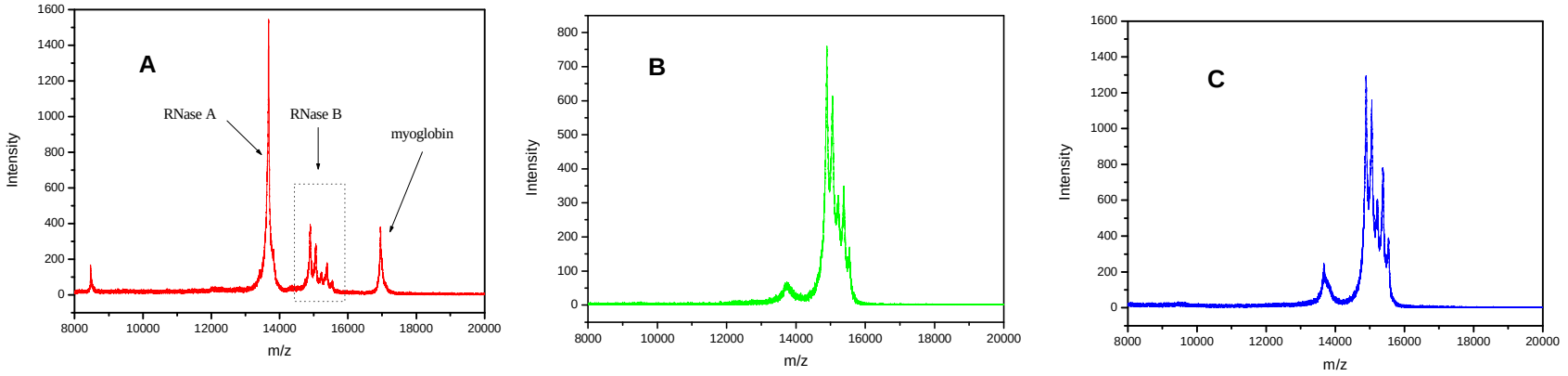
Attractive features:

- Broad-spectrum affinity → One ligand for all cis-diol biomolecules
- Covalent reaction → High specificity
- Reversible → Easy-to-control capture/release
- Fast desorption speed → Nice peak shape, low carryover
- Eluted under acidic solution → Compatible with MS

Boronate affinity monolithic capillaries with different features and functions

Name	monomer/crosslinker	characteristics	Specificity	Publication
Poly (VPBA-co-EDMA)		Hydrophobic, loading pH ≥ 8.0	Poor	J Chromatogr A, 2009, 1216, 4768–4774
Poly (VPBA-co-MBAA)		Hydrophilic, loading pH ≥ 8.0	Good	J Chromatogr A, 2009, 1216, 8421–8425.
Poly (SPBA-co-MBAA)		Hydrophilic, loading pH ≥ 7.0 Secondary separation capability	Good	Chem Commun, 2011, 47, 5067 - 5069.
Teamed boronate affinity		Weakly hydrophobic, loading pH ≥ 7.0	Good	Angew Chem Int Ed, 2009, 48, 6704-6707.
Wulff-type boronate		loading pH ≥ 5.5	Good	Chem Commun, in press (DOI:10.1039/c1cc11096a).
Protein A/G like		loading pH ≥ 7.0, Highly selective to antibodies	Specific to antibodies	under preparation

Selective extraction of glycoproteins by G2 monolith at basic pH



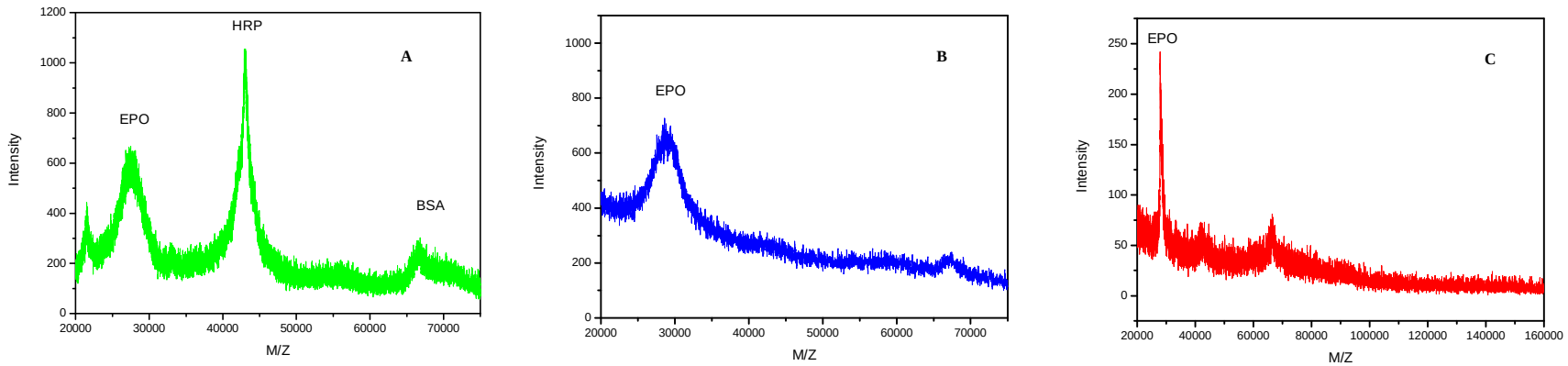
(A) Direct MALDI-TOF MS analysis of a 1:1:1 mixture of the glycoprotein RNase B with the nonglycoproteins RNase A and myoglobin; (B) Direct MALDI-TOF MS analysis of the glycoprotein RNase B; (C) Extraction of RNase B from the mixture in (A) using the poly (VPBA-co-MBAA) and followed with MALDI-TOF MS analysis.

LOD: 10^{-11}mol RNase B (S/N = 200)

Improved sensitivity, selective!

Unpublished data

Specific extraction of EPO by G2 monolith at physiological pH



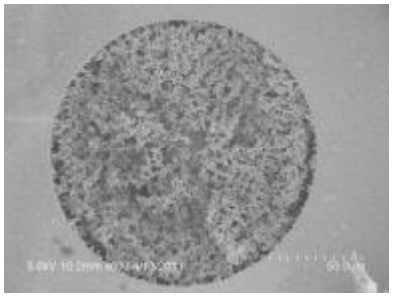
(A) Direct MALDI-TOF MS analysis of a 1:1:1 mixture of EPO, HRP and BSA; (B) MALDI-TOF MS analysis of EPO extracted from the mixture in A; (C) MALDI-TOF MS analysis of EPO extracted from EPO spiked human serum.

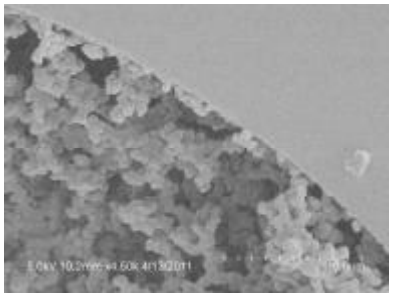
EPO: erythropoietin; HRP: Horseradish peroxidase; BSA: Bovine serum albumin

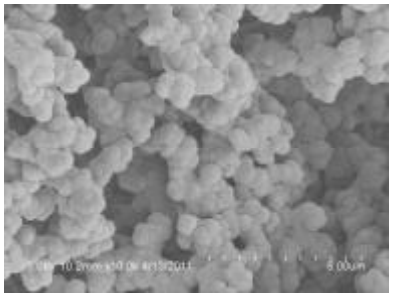
LOD: 10^{-10}mol EPO (S/N = 28)

Unpublished data

G6 monolith for antibody recognition, purification and immobilization



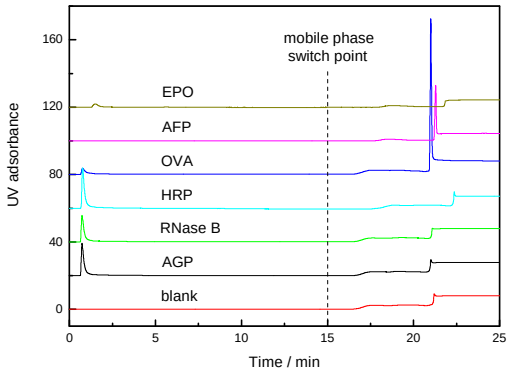




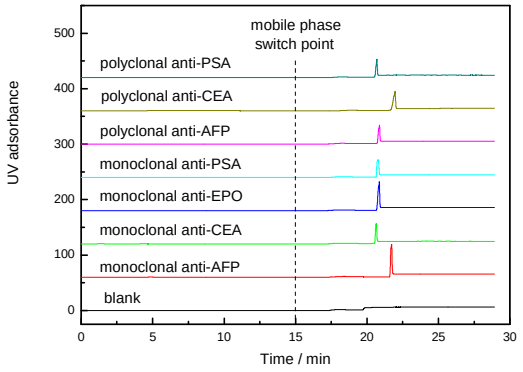
Working at neutral pH

A polymeric monolith exhibiting protein A/G-like affinity to antibody

Sharp peaks

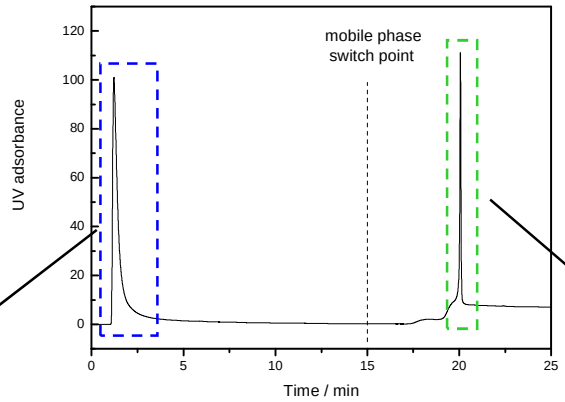


Most non-antibody glycoproteins tested were not retentive.

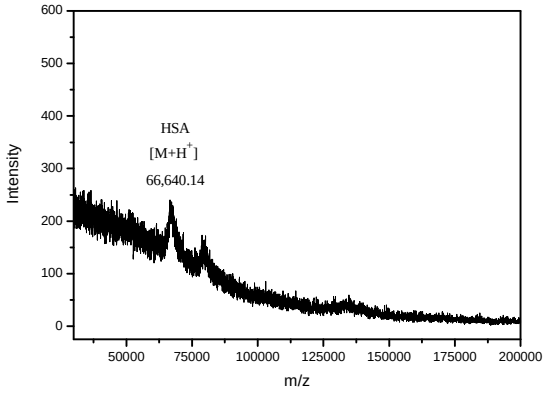


All antibodies tested were captured

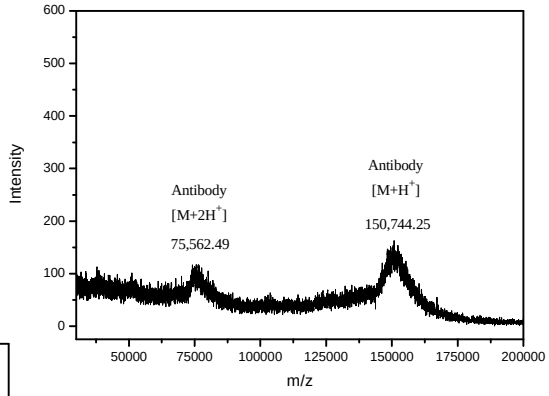
Identification of species extracted by G6 monolith from human serum



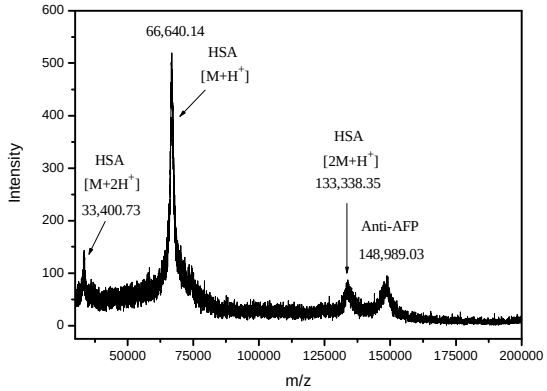
BAC of human serum



MALDI TOF MS of Frac 1

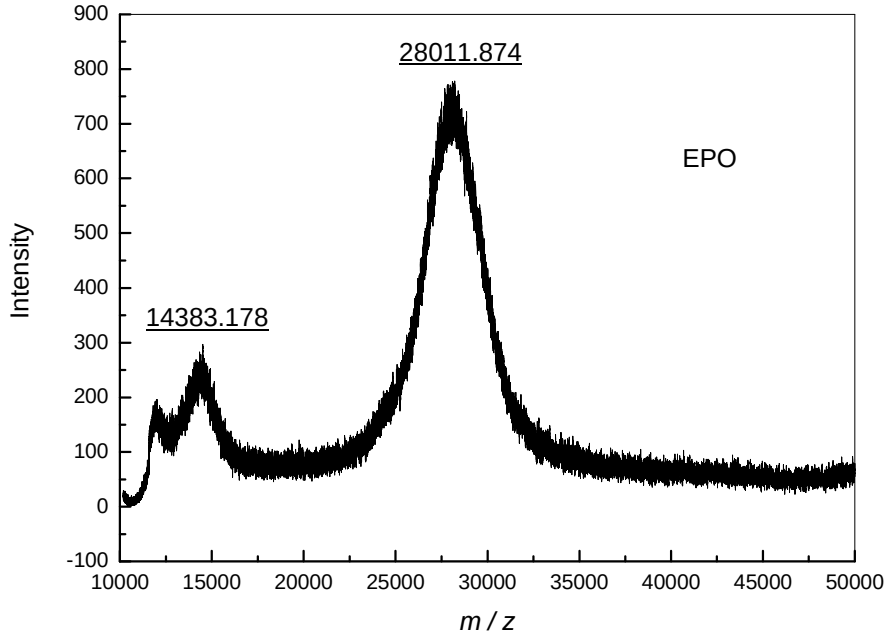


MALDI TOF MS of Frac 2



MALDI TOF MS of HSA + Anti-AFP

Specific extraction of EPO using Anti-EPO antibody immobilized monolith



MALDI TOF MS of EPO extracted by the anti-EPO antibody immobilized BA column from an aqueous EPO solution

Concluding remarks

1. A couple of new techniques have been developed for the extraction, separation and detection of EPO, including magnetic particles-based immuno-affinity extraction, monolith-based immuno-affinity extraction, capillary zone electrophoresis, and laser-induced native fluorescence. They provide useful tools for the analysis of samples with high EPO concentration and less complicated composition.

2. Due to the very limited concentration of EPO in real samples and the highly interfering matrix, anti-doping analysis of EPO is still a very challenging task. To address these issues, ultrasensitive detection and effective sample pretreatment approaches are highly desired.

3. Mass spectrometry-based proteomic analysis might be a useful alternative for anti-doping analysis of EPO, for which the boronate affinity monolithic capillaries might be a useful sample enrichment platform.

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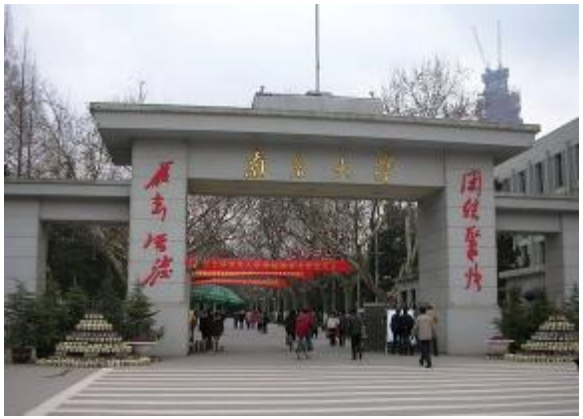



Thank you for your attention!