Dispersive Solid-Phase Imprinting of Proteins for the Production of Plastic Antibodies

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Table of Contents

Experimental Section

General Information

Ferric chloride $FeCl_3 \bullet 6H_2O$, ethylene glycol, sodium acetate, polyethylene glycol (PEG) MW 2000, tetraethoxysilane (TEOS), 35% ammonium hydroxide, (3-Glycidyloxypropyl)trimethoxysilane (GPTMS), N-isopropylacrylamide (NIPAm), acrylic acid (Acc), N-tetra-butylacrylamide (TBAm), N-(3-Aminopropyl)methacrylamide hydrochloride, N,N'-methylenebisacrylamide (BIS) N,N,N'N – tetramethylethylenediamine (TEMED), ammounium persulfate (APS), trypsin from porcine pancreas and ethanolamine hydrochloride were purchased from Sigma Aldrich. All stabilisers were removed from monomers prior to use via an inhibitor removal columns supplied from Sigma Aldrich. N-hydroxyl-succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and were purchased from Thermo scientific. All protein samples were standardised using BCA assay kit supplied from Sigma Aldrich and all absorbance experiments were performed on a Spark plate reader (TECAN). All hydrodynamic size and zeta potential measurements were performed on a Zetasizer (Malven, UK). IR spectra were taken using a Spectrum 100 (Perkin Elmer, MA, USA). All transmission electron microscopy (TEM) images were taken on a Tecnai T20 G^2 (FEI, Oregon USA). XPS spectra were measured using a K-alpha XPS (Thermo Fisher Scientific, MA, USA**)** All surface plasmon resonance experiments were performed on a MP-SPR Navi^{TM} 200 OTSO instrument (Bionavis, Finland). Bare gold chips were purchased from Bionavis.

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Supporting information for this article is given via a link at the end of the document.

Synthesis of trypsin-immobilised magnetic microspheres

The magnetic microspheres were synthesized using the core-shell technique. Firstly, iron oxide microspheres were synthesized as the magnetic core using the thermal solvent method.[1] Briefly,

$FeCl_3 \bullet 6H_2O$ (1.35 g) and dissolved in ethylene glycol (40 ml). Anhydrous sodium acetate (3.6 g), and PEG, MW 2000 (0.1 g) were added sequentially and the mixture was stirred for 30 minutes to form a homogenous orange suspension. The solution was then degassed under vacuum for 30 minutes, transferred to a 100 ml autoclave reactor and heated in a furnace oven at 200 ^oC for 10 hours. The resultant black precipitate was washed several times with ethanol and dried under vacuum for 6 hours. Next, a SiO_2 layer was added to the magnetic core**.** 0.5 g of the resultant magnetic microspheres was re-suspended in ethanol (60 ml) and water (12 ml). The black solution was then sonicated using a probe sonicator for at least 15 minutes followed by the addition of 35% ammonia hydroxide (4 ml), TEOS (4 ml). The solution was allowed to react with pulse sonication for 4 hours under dry ice before transferring to an orbital shaker overnight. The resultant particles were washed several times with water until the pH had returned to neutral. This was followed by washing with ethanol several times and dried under vacuum for 12 hours. After depositing the SiO_2 layer, the microsphere surface was modified with MPA to introduce a polymerizable group to the surface**.** The $FeOx@SiO_2$ microspheres (0.4 g) were mixed with 4% (v:v) GPTMS in anhydrous toluene. The solution was sonicated for 15 minutes, followed by heating to 60 ^oC and overhead stirring overnight under nitrogen. The $FeOx@SiO_2$-epoxide microspheres were washed several times using anhydrous toluene followed by ethanol before drying for 10 hours under vacuum. The protein was immobilized onto the $FeOx@SiO_2$-epoxide by incubating a 10 mg ml^{-1} of trypsin (10 ml) in 10 mM carbonate buffer pH 9.0 with the $FeOx@SiO_2$-epoxide microspheres (300 mg) for 16 hours. The resultant $FeOx@SiO_2$-trypsin protein was washed several times with PBS (8 x 20 ml) using magnetic separation between each wash. Unreacted epoxide groups on the microspheres were then incubated with 1M ethanolamine pH 9.0 (10 ml) for 2 hours followed by washing with PBS buffer (8 x 20ml) using magnetic separation between each wash. The resultant trypsin microspheres were stored dried at 4 ^oC until further use. The theoretical amount of protein covalently attached to the magnetic microspheres can be estimated from the **Equation 1**.[2]

$$S = (6 / \rho Sd)(C)$$

Where S is the amount of representative protein required to achieve surface saturation (mg protein /g of microspheres, ρS is the density of solid spheres (g/cm^3), d is the mean diameter (µm) and C is the capacity of the microsphere surface for a given protein (mg protein/m^2). The capacity of a protein can be estimated by comparing its molecular weight to that of BSA or IgG.[3] The actual amount of trypsin was estimated from a BCA assay.

Characterization of trypsin immobilized microspheres

Magnetic microspheres were synthesized and characterized by TEM, dynamic light scattering (DLS) and FT-IR. TEM images were shown in **Figure S1**. **Table S1** shows the relative hydrodynamic radius and zeta potential. Upon the addition of GPTMS, the zeta potential of the microspheres increases suggesting that the epoxide has a stabilising effect on each microsphere. The XPS and FT-IR spectrum in **Figure S2** and **S3A** show differences between the FeO_x and $FeO_x@SiO_2$. However there is no observed difference between $FeO_x@SiO_2$ and $FeO_x@SiO_2$-epoxide. This may be due to the lack of penetration depth in ATR based setups. IR also confirmed the elution of the nanoMIPs, after several cold washes and several hot washes (**Figure S3B**).

The template was immobilised onto microspheres via bio-conjugation of primary amines on the protein with the epoxide on the microspheres using pH 9.0 buffer. The amount of immobilised

protein was determined by measuring the UV absorbance before and after incubation using BCA assay kit according to the manufacturer's instructions. The difference in absorbance was compared to a BSA standard calibration plot as shown in **Figure S4**.

Synthesis of trypsin nanoMIPs.

NanoMIPs were synthesized using a precipitation based polymerization in the presence of the solid-phase.[4,5] A 100 ml aqueous solution containing NIPAm (0.34 mmol), ACC (0.032 mmol), TBAm (0.26 mmol), APM (0.06 mmol) and BIS (0.013 mmol) was sonicated under vacuum followed by degassing by nitrogen. The solution was then incubated with FeOx@SiO_2-trypsin microspheres (300 mg) for 30 minutes on an orbital shaker to form a pre-polymerization mixture. The polymerization was initiated by the addition of TEMED (30 µl) followed by 1ml of APS (30 mg ml^{-1}). The solution was stirred on an orbital shaker for 1 hour. The solution was then removed and the microspheres were washed several times with water at room temperature. The nanoMIPs were eluted off the nanoMIPs by incubating the microspheres with water (10 ml) at 60 ^{O}C for 15 minutes with shaking followed by separation with a magnet. This was repeated a further 2 times. The complete elution of the nanoMIP product was confirmed by freeze drying the additional hot wash fractions until no solid product was observed. Non-Imprinted nanoNIPs were prepared in the same manner as described for nanoMIPs using histamine templated magnetic microspheres.

Characterization of nanoMIPs

A 10 ml aliquot of nanoMIPs was dried using a freeze dryer to determine the yield of nanoMIPs synthesis and concentration of the stock solution. The size of the nanoMIPs in solution was determined by DLS (**Figure S5**). A 10 ml solution of nanoMIPs was reduced in volume using a rotary evaporator to about 1ml. The resultant solution was then filtered using a 1.2 µm diameter filter and sonicated. The same solution was also characterized using TEM by evaporating 10 µl of each sample onto a copper grid and analysing immediately.

The binding characteristics of each batch of nanoMIPs were studied using an SPR binding assay. The sensor surface was prepared by incubating bare gold chips with 5 mM MUA in ethanol (10 ml) for 24 hours. Samples were degassed by sonication and the headspace of the container was filled with nitrogen. The self-assembly monolayer surface was then washed with water, then ethanol and finally dried using nitrogen. The chips were promptly docked into the machine and the instrument was primed three times prior to the immobilization of the MIP-NPs and analysis. Immobilization of the nanoMIPs were performed by firstly setting the flow rate to 20 µl min^{-1} with 10 mM PBS as the run buffer and injecting a mixture of 0.1 M NHS and 0.4 M EDC for 4 minutes to activate the carboxylic group on the SAM. The nanoMIPs from each batch were exchanged in MES buffer pH 6.0 and then injected 100 µl (100 µg ml^{-1}) on channel 1 of the instrument. The concentration of MIP was estimated from yield and hydrodynamic radius using the equation proposed by Debord and Lyon *et al.*[6] By immobilizing the MIP nanoparticles and injecting a known concentration of analyte, we could more accurately determine the K_D. The histamine nanoNIPs were injected in the same manner as for the nanoMIPs with the exception that they were immobilized on channel 2 of the sensor chip. Both channels were blocked by injection of 1M ethanolamine (100 µl) onto the surface. Binding assays were then performed at a flow rate of 20 µl min^{-1} with 10 mM PBS. 100 µl of trypsin (5 - 750 nM) was injected over the surface of the chip starting from the lowest concentration and the response measured over time. Each injection was followed by a dissociation phase of 4 minutes. The

response was recorded and the binding affinity K_D was determined using the equilibrium affinity binding model on the trace drawer analysis software.

Selectivity experiments were performed by measuring the response of β-lactoglobulin, RNase, and haemoglobin to the MIP immobilised surface respectively. 100 µl of each protein (0 - 750 nM) was injected sequentially followed by a 4 minute dissociation phase. The response of different proteins is shown in **Figure S6**. The response of the highest concentration injected (750 nM) was recorded for selectivity analysis.

Table S1. The sizes and zeta potential of FeOx, FeOx SiO_2 and FeOx SiO_2 epoxide microspheres.

	DLS Size (nm)	PDI	Zeta Potential (mV)
FeO_x	260	0.085	12.25
FeO_x-SiO_2	652	0.068	-47.20
FeO_x-Epoxide	671	0.051	-60.77

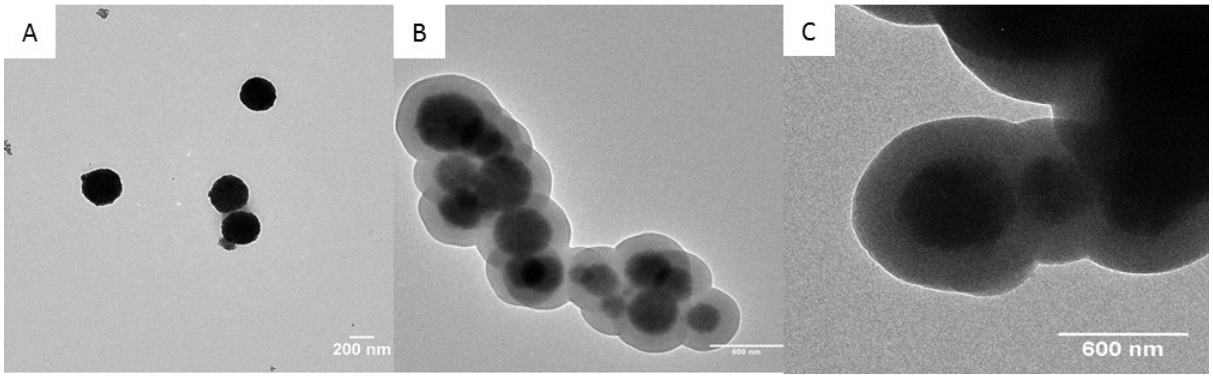

Figure S1. TEM images of (A) FeO_x; (B) $FeO_x@SiO_2$ (Scale bar = 600nm); and (C) $FeO_x@SiO_2$-epoxide microspheres.

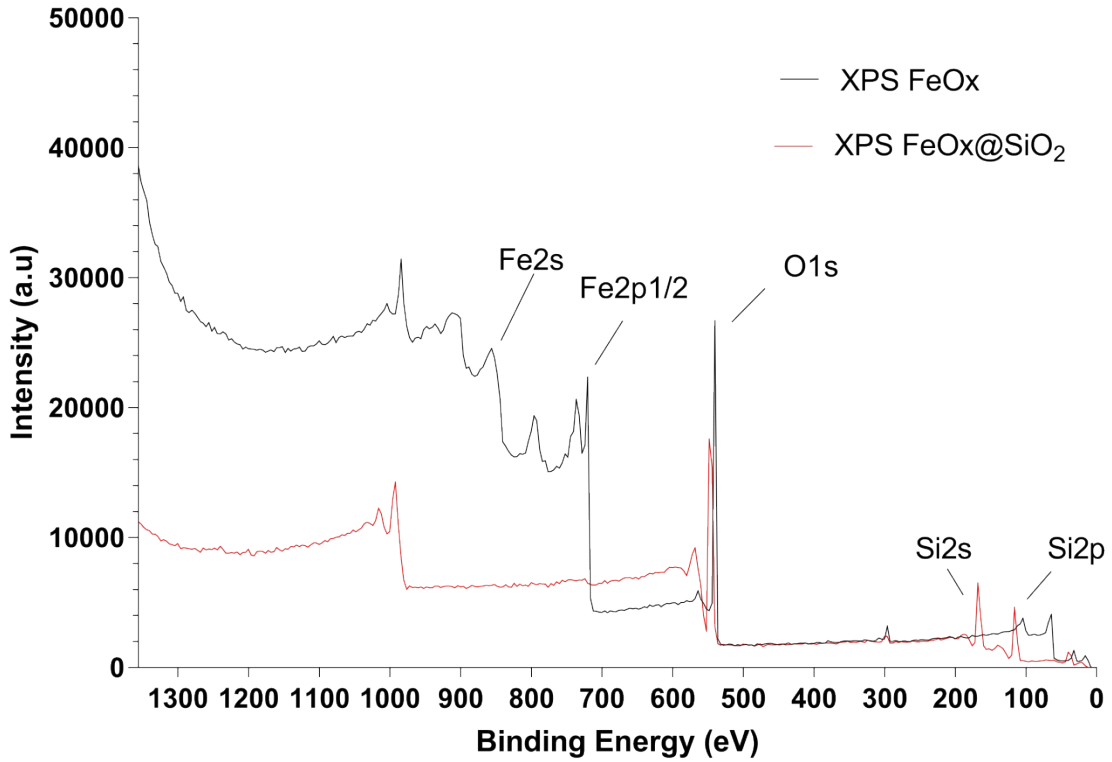

Figure S2. XPS of (A) FeO_x and (B) $FeO_x@SiO_2$ microspheres.

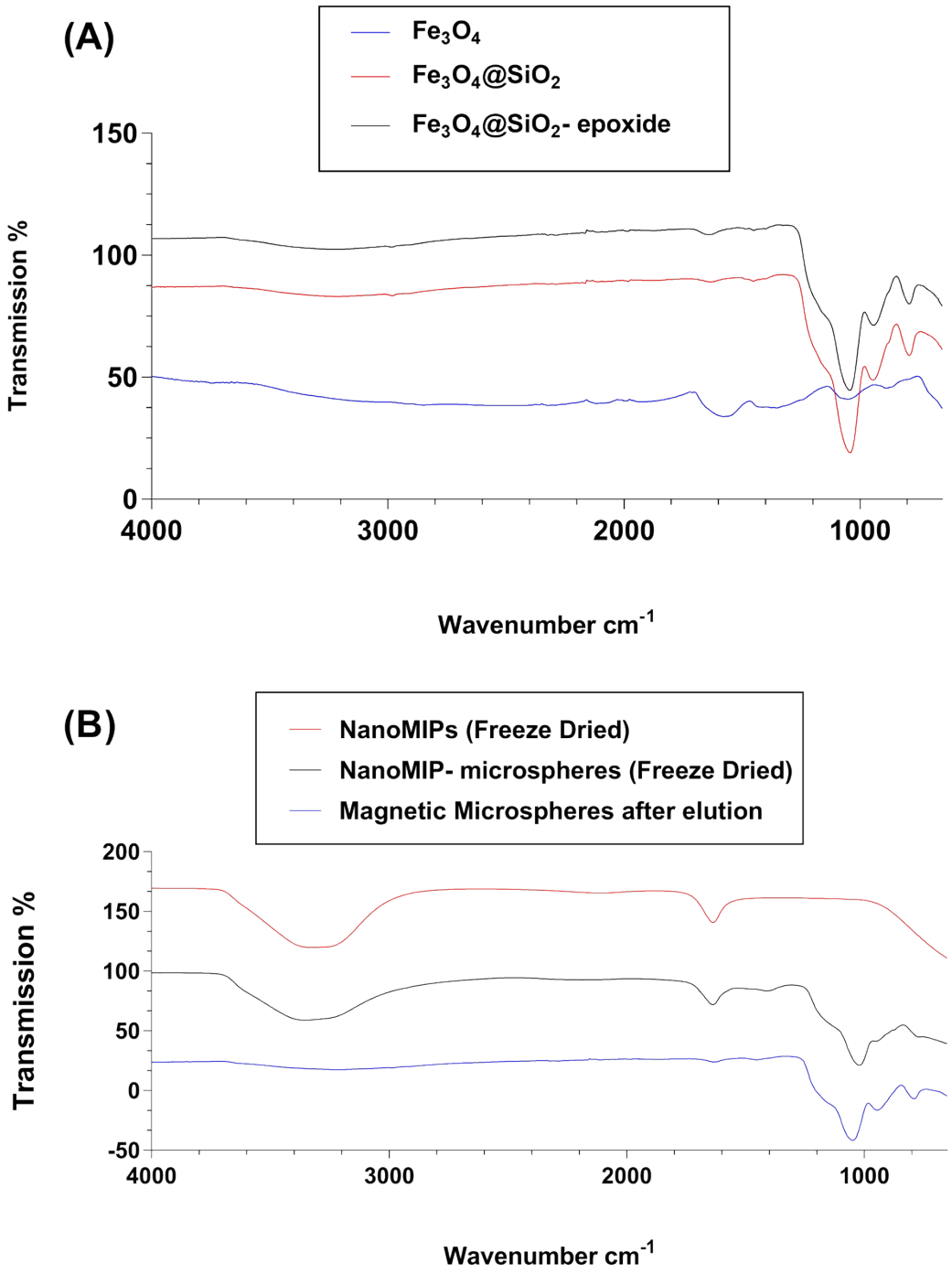

Figure S3. (A) IR spectra of FeO_x, $FeO_x@SiO_2$ and $FeO_x@SiO_2$-epoxide microspheres; (B) IR spectra of the freeze dried nanoMIP, freeze dried NanoMIP-Microsphere complex and magnetic microspheres after several cold washing steps and several hot washing steps.

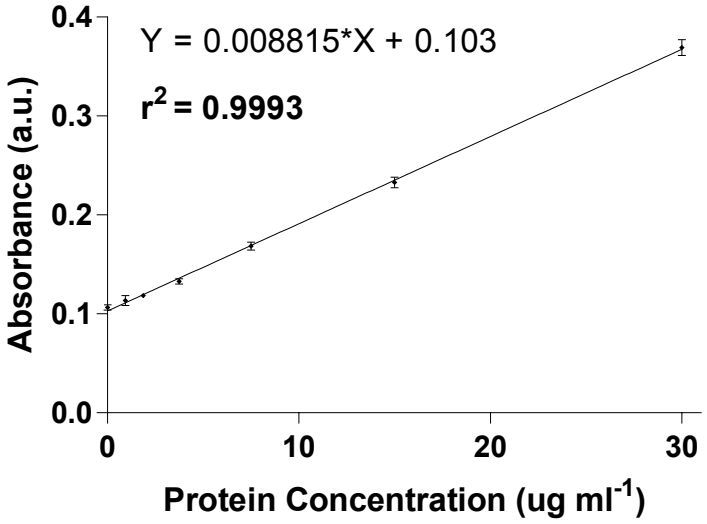

Figure S4. Bovine serum albumin (BSA) standard calibration plot obtained using a bicinchoninic acid (BCA) kit.

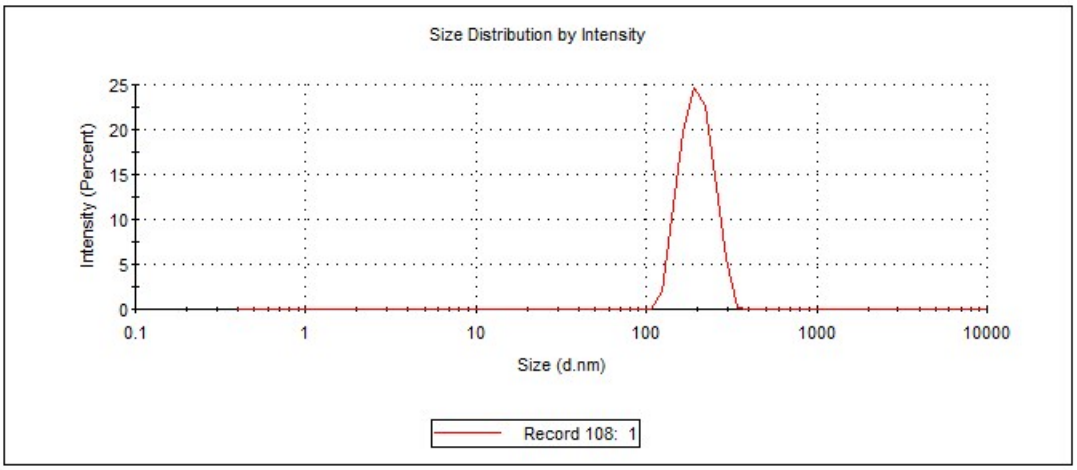

Figure S5. DLS size measurement of the nanoMIPs.

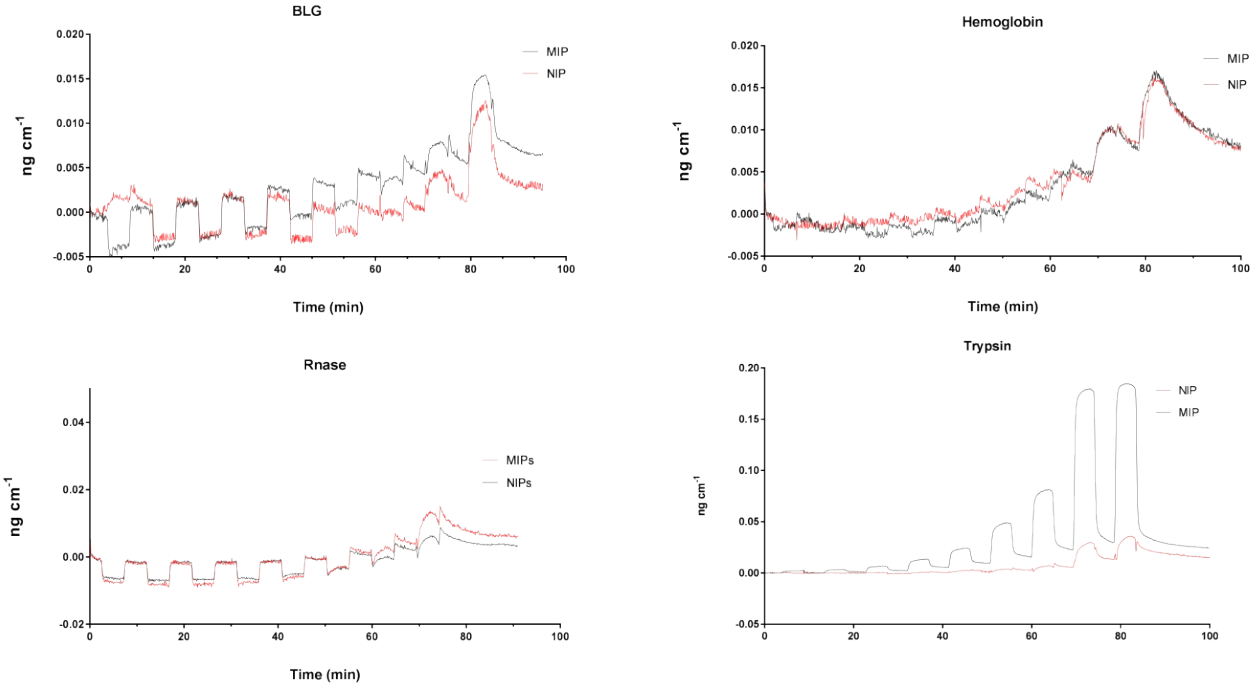

Figure S6. Sensorgrams for BLG, Hemoglobulin, RNase and trypsin.

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