Electronic Supplementary Information (ESI)

Automatic reactor for solid-phase synthesis of molecularly imprinted polymeric nanoparticles (MIP NPs) in water

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Experimental section

Reactor. All system components were purchased from HEL Ltd. (UK). Standard components which were used "off the shelf" include the control software, PC interface, syringe pumps, cooler/heater, thermocouple and multi-way valve. The reactor consists of a jacketed glass column (120 mL) with a polypropylene frit (porosity: 20 μm) at the bottom to retain the solid phase, which is pre-loaded before assembly onto the machine. The column is mounted on a stand which includes a shaker for the homogenisation of the reactor contents. Connected to the stand and the column jacket, a water circulator controls the internal temperature of the reactor, monitored via an internal thermocouple. Monomers, initiator and washing solvents are delivered to the reactor via a set of syringe pumps with four lines. An additional inlet at the top of the reactor provides for nitrogen flushing, used before polymerisation to remove oxygen and to pump out the reactor contents. At the outlet, a multi-way valve with six outlets provides for separation of waste streams from product fractions, which are collected in glass bottles. All the mentioned components are computer-controlled, using proprietary software (WinISO®) developed by HEL Ltd. (UK).

Chemicals. *N*-isopropylacrylamide (NIPAm), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), acrylic acid (AAc), *N,N′*-methylenebisacrylamide (BIS), *N*-*tert*-butylacrylamide (TBAm), PBS, tris(hydroxymethyl)aminomethane (TRIS), 3-aminopropyltrimethyloxysilane (APTMS), glutaraldehyde (GA), calcium chloride, cysteamine, α-amylase, pepsin A, ethanol, toluene and acetone were purchased from Sigma-Aldrich (UK). Trypsin and bicinchoninic acid (BCA) Protein Assay Kit were purchased from Thermo Fisher Scientific (UK). Sodium hydroxide was obtained from Fisher Scientific (UK). Double-distilled ultrapure water (Millipore) was used for analysis. All chemicals and solvents were analytical or HPLC grade and were used without further purification.

Preparation of derivatised glass beads as template support and affinity media. Glass beads (300 mL, 53 μm < diameter < 106 μm, purchased from Blagden Chemicals, UK) were first vibrated with abrasive ceramic beads to remove any surface coating, and then activated by boiling in NaOH

(1 M) for 1 min. Then, they were washed thoroughly with double-distilled water at 60 °C, then acetone and finally dried at 80 °C for 2 h. The beads were then incubated in a solution of APTMS (2 %, v/v) in toluene overnight, then thoroughly washed with acetone and subsequently incubated in a GA solution (7 %, v/v) in PBS (0.01 M, pH 7.2) for 2 h, after which they were rinsed with double-distilled water. Pepsin A and α-amylase templates were then immobilised by incubating the glass beads with a solution (0.5 mg/mL) of each template in PBS (0.01 M, pH 7.2) overnight at 4 °C.[1] In the case of trypsin, the immobilisation has been performed by using TRIS/HCl buffer (0.05 M, pH 8.0) with $CaCl_2$ (0.02 M) at 4 °C.[2] The derivatised beads were washed with double-distilled water and stored at -18 °C until usage. Immobilisation has been confirmed by performing a BCA Protein Assay on an aliquot (100 mg) of derivatised glass beads, according to the specifications of the manufacturer. The amount of protein immobilised was determined spectrophotometrically (280 nm) by analysing the amount of protein unbound to the glass beads. For this, the beads were washed to remove any adsorbed protein and the amount of total free protein determined.

Synthesis of MIP NPs using derivatised glass beads. The procedure has been adapted from Hoshino *et al.*[38] The following monomers were dissolved in H_2O (100 mL): NIPAm (39 mg), BIS (2 mg), TBAm (33 mg) and AAc (2.2 µL). TBAm was previously dissolved in EtOH (2 mL) and then added to the aqueous solution. The total monomer concentration was 6.5 mM. The solution was degassed under vacuum and sonication for 10 min, and then purged with N_2 for 30 min. After this time, the container with the polymerisation mixture was connected to one of the pumps of the automatic synthesiser and 80 mL of it injected in the reactor vessel containing template-derivatised glass beads (60 g). The polymerisation was started by adding an APS aqueous solution (800 µL, 60 mg/mL) and TEMED (24 µL), after which the solution was drawn through the bulk of glass beads down to a total active (final) polymerisation volume of 25 mL. The polymerisation was then carried out at room temperature for 2 h. Following this the reactor was washed out with double-distilled water (100 mL, delivery flow rate: 2 mL/min). The high-affinity nanoparticles were eluted from the affinity media by passing three fractions (50 mL) of double-distilled water at 60 °C (delivery flow rate: 2 mL/min). The concentration of the nanoparticles fractions has been evaluated by freeze-drying an aliquot (13 mL) of each solution. Apparent molarities of the nanoparticles fractions were calculated using the Equation (1)[3] which approximates NPs as regular spheres:

$$[NPs] = \frac{6}{\pi N_A d^3 \rho} X \quad (1)$$

where N_A is Avogadro's constant, d is the hydrodynamic diameter of particles found by DLS (nm), ρ is polymer density of particles (g/cm^3) and X is the weight concentration (mg/mL) found after freeze-drying. According to Debord and Lyon,[4] the ρ value for non-swollen particles with a similar composition is about 0.08 g/cm^3. Absence of protein template in the nanoparticles fraction has been confirmed by BCA Protein Assay performed in accordance with the protocol of the manufacturer.

Effect of the amount of derivatised glass beads on yield of MIP NPs. To study effect of the amount of derivatised glass beads on the yield of MIP NPs four different amounts of trypsin-derivatised glass beads (20 g, 40 g, 60 g and 80 g) have been tested in the presence of a constant amount of polymerisation mixture (25 mL). At the end of each cycle, the yield of the nanoparticles

fractions has been evaluated by freeze-drying an aliquot (13 mL) of each solution, and reported in weight percentage of the total amount of monomers present in the active polymerisation mixture. All the results have been corrected by subtracting the amount of material obtained after a blank run performed just with double-distilled water on the same amount of derivatised glass beads.

Dynamic Light Scattering (DLS) size analysis of MIP NPs. To verify the size of the synthesised nanoparticles, the eluted fractions were sonicated for 20 min, then filtered through 1.2 μm glass fibre syringe filters and analysed in 3 cm^3 disposable polystyrene cuvettes at 25 °C using a Zetasizer Nano (Nano-S) from Malvern Instruments Ltd (Malvern, UK).

Transmission Electron Microscopy (TEM) imaging of MIP NPs. TEM images of MIP NPs were taken using a Philips CM20 Transmission Electron Microscope. Samples for the analysis have been prepared by depositing the MIP NPs solution (5 μL), previously filtered through a 1.2 μm glass fiber syringe filter, on a copper grid holder and leaving them to dry overnight.

Treatment of BIAcore gold chips and surface immobilisation of templates. Au-coated chips (SIA Kit Au, BIAcore) were cleaned by immersion in Piranha solution [H_2SO_4/H_2O_2, 3:1 (v/v)] for 5 min. **Caution! This mixture is highly corrosive, hence extreme care is required during this process.** Then they were thoroughly rinsed with double-distilled water and left in EtOH overnight. The immobilisation of the templates has been performed by incubating the chips in a solution of cysteamine (0.2 mg/mL) in EtOH at 4 °C for 24 h, after which they have been washed with EtOH and incubated in a solution of GA (7 %, v/v) in PBS (0.01 M, pH 7.2) for 2 h. After this step, the chips were washed with PBS (0.01 M, pH 7.2) and immersed in a 1.2 mg/mL solution of each template in PBS (0.01 M, pH 7.2) for 24 h at 4 °C.[1] In the case of trypsin the immobilisation was performed in TRIS/HCl buffer (0.05 M, pH 8.0) with $CaCl_2$ (0.02 M).[2] Once the immobilisation was completed, the chips were assembled in holders and stored under Ar at 4 °C to be used within the following 24 h.

Surface Plasmon Resonance (SPR) affinity analysis of MIP NPs. Affinity analysis has been performed using a BIAcore 3000 SPR system (BIAcore, Sweden). The Au-chips with templates immobilised on their surfaces were used for the experiments. All the affinity experiments were performed in PBS (0.01 M, pH 7.4) as mobile phase at a flow rate of 35 μL/min and a temperature of 25 °C, sequentially injecting each NPs solution (100 μL) and analysing the sensor response for 2 min after every injection. The specificity of the MIP NPs was assessed by cross-testing their affinity on all the chips prepared. Kinetic data have been fitted using BIAEvaluation Software v4.1 (BIAcore, Sweden) assuming a Langmuir binding isotherm and baseline correction if required.

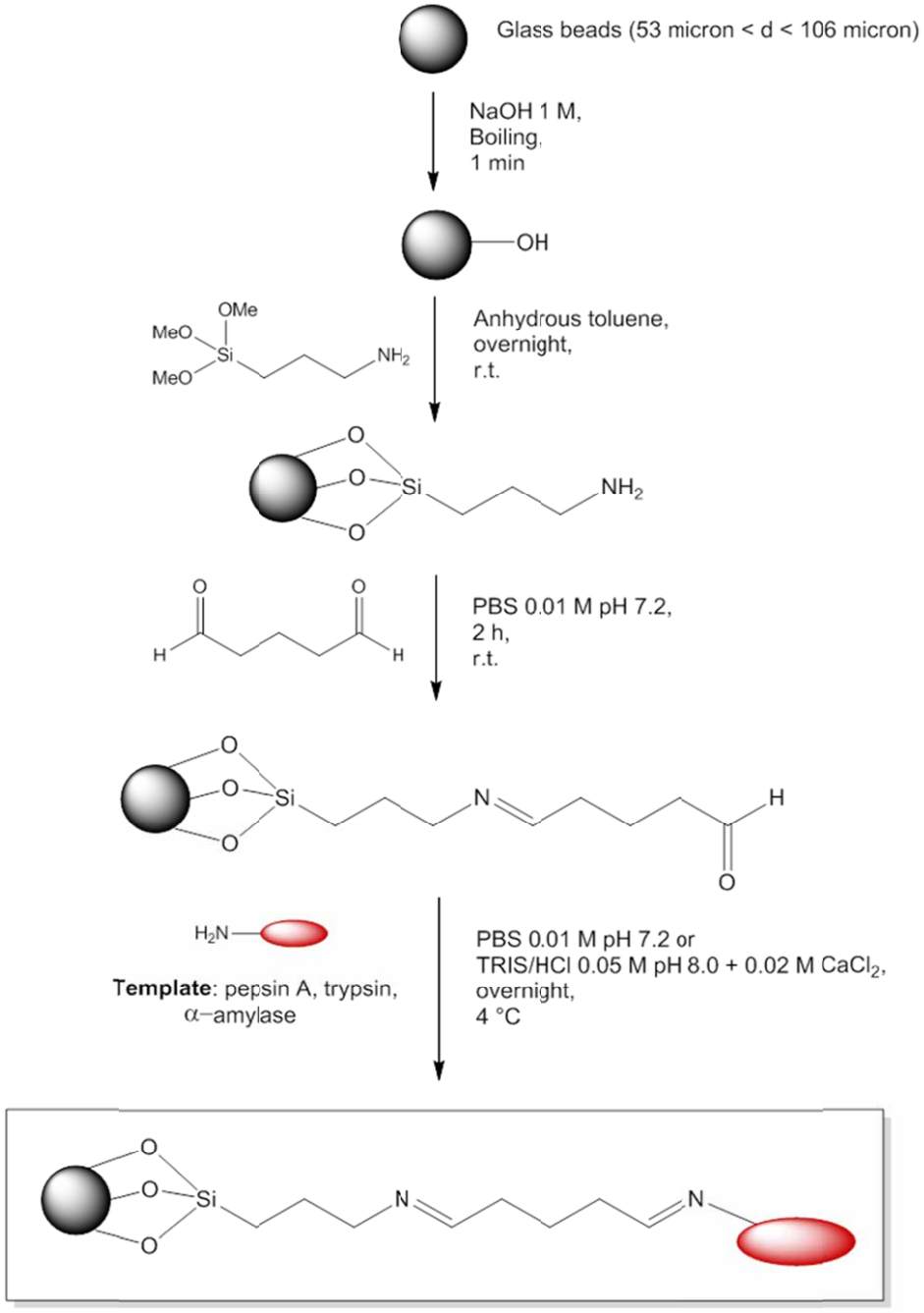

Fig. S1 Schematic of the synthetic protocol for the immobilisation of the template on the glass beads surface. The activation of the glass beads in boiling NaOH increases the amounts of reactive OH groups on their surface, so that the following silanisation reaction with APTMS is facilitated, resulting in a good coverage of the glass surface with silane containing primary amino groups. In the subsequent steps, GA acts as a linker between the two primary amino groups (one on the glass surface, the other on the template) through the formation of Schiff base bonds.

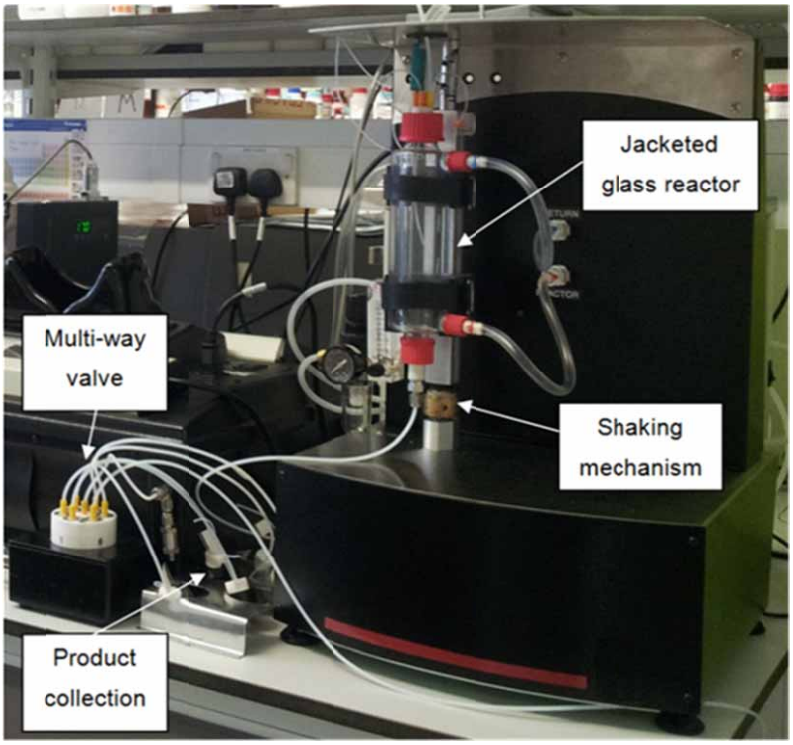

Fig. S2 Photo of the automatic reactor setup developed and used for the synthesis of protein-imprinted MIP NPs on solid phase.

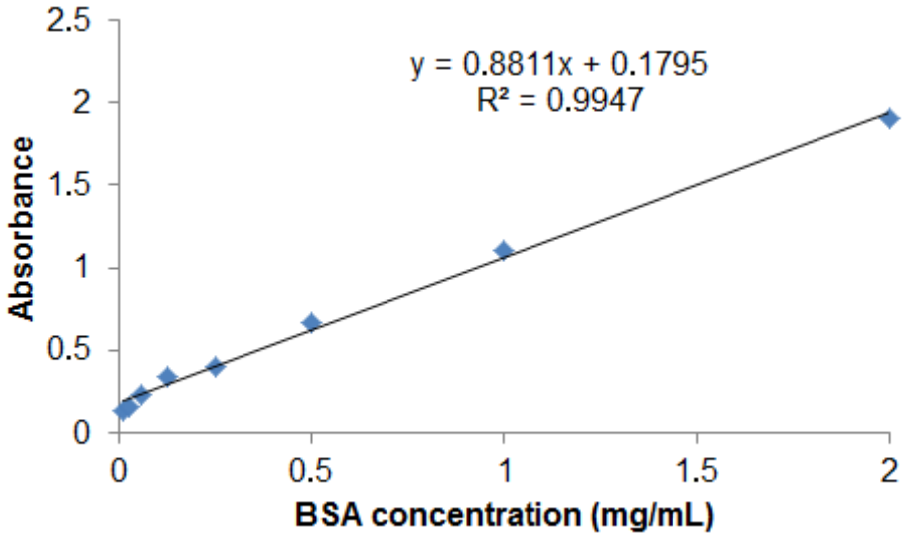

Fig. S3 Calibration curve for the BCA assay performed to assess the presence of residual protein into the nanoparticles fractions. The concentrations range from 0.015 to 2 mg/mL. At 0.015 mg/mL the absorbance value is 0.135, same as the blank (just water) and the NPs fractions analysed exhibited an absorbance of while the blank is 0.1 and the NPs 0.130±0.002.

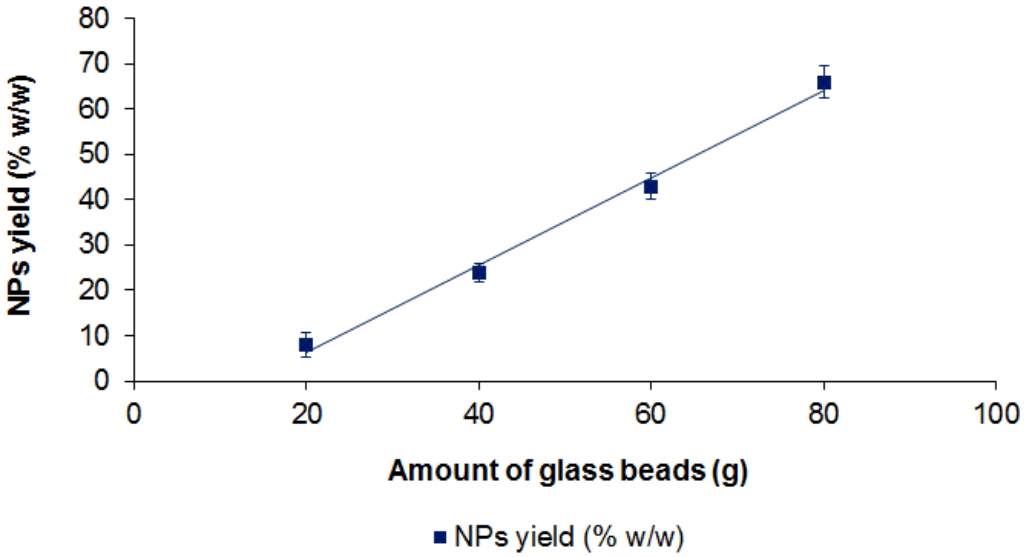

Fig. S4 Effect of the amount of template-derivatised glass beads on MIP NPs yield, expressed as mass of NPs produced per mass of monomers, and for the same volume of polymerisation mixture (25 mL). Error bars represent standard deviation, SD (n = 3).

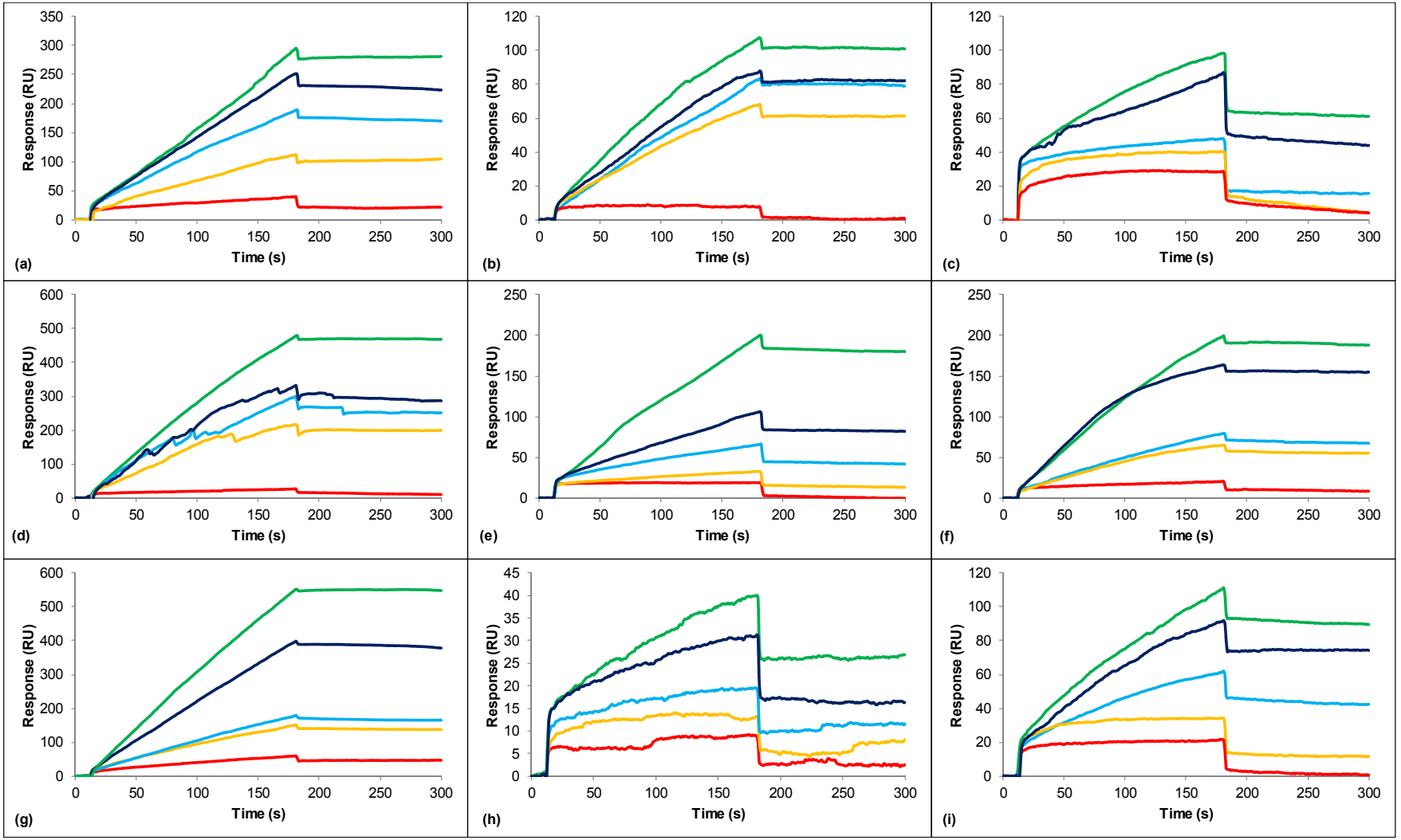

Fig. S5 SPR sensorgrams for: pepsin A MIP NPs injected on pepsin A (a), trypsin (b) and on α-amylase (c) coated sensor surfaces; α-amylase MIP NPs injected on α-amylase (d), pepsin A (e) and on trypsin (f) coated sensor surfaces; trypsin MIP NPs injected on trypsin (g), pepsin A (h) and on α-amylase (i) coated sensor surfaces. Concentration of MIP NPs ranged from 4×10^{-4} nM to 4×10^{-8} nM (10-fold dilutions). SPR tests were performed in PBS (0.01 M, pH 7.4) at 25 °C.

References:

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4 J. D. Debord and L. A. Lyon, *Langmuir*, 2003, **19**, 7662–7664.