Electronic Supplementary Information

Solid-phase synthesis of molecularly imprinted nanoparticles for protein recognition

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1. Materials

Glass beads (GBs) were obtained from Roth Sochiel E.U.R.L (Lauterbourg, France). Nanosep membrane centrifuge filter devices (MWCO: 100 kDa), PD-10 gel filtration columns, Bradford and Bicinchoninic acid protein assay reagents were purchased from VWR International (Fontenay sous Bois, France). *p*-Aminobenzamidine dihydrochloride (PAB), Orange II sodium salt, (3-aminopropyl)triethoxysilane (APTES), glutaraldehyde, *N*-isopropylacrylamide (NIPAM), *N,N'*-ethylenebisacrylamide (EbAm), potassium persulfate (KPS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), N_{α}-*p*-tosyl-*L*-arginine methyl ester hydrochloride (TAME), trypsin (porcine pancreas, type IX-S), kallikrein (porcine pancreas), thrombin (bovine plasma), fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). Magnetic silica beads functionalized with benzamidine (SiMAG-Benzamidine) were supplied by Chemicell GmbH (Berlin, Germany) and a magnetic separator (DYNAL MPC®-6) was purchased from Dynal A.S. (Oslo, Norway). Buffers were prepared with Milli-Q water, purified using a Milli-Q system (Millipore, Molsheim, France).

2. Preparation of the *p*-aminobenzamidine functionalized glass beads (PAB-GBs)

Glass beads (100 µm diameter, BioSpec Product Inc.) were activated by incubation in a 4 M NaOH solution at 100 °C. After boiling for 10 min, they were washed with water, acetone and then dried in an oven at 100 °C. The activated GBs were then incubated in a solution of APTES in toluene 2 % (v/v), with shaking, overnight at room temperature. After silanization, the GBs were rinsed with toluene, acetone and the coupling of PAB was performed by applying the procedure used by Yang *et al.*[1] Briefly, the silanized GBs were incubated in a 5 % (v/v) glutaraldehyde solution in 0.1 M Na phosphate buffer + 0.15 M NaCl pH 7.4 (PBS buffer). After stirring at 30 °C for 12 h, the GBs were washed with water. The glutaradehyde-activated glass beads were dispersed in a 0.1 M PAB solution at pH 7.4. The mixture was incubated at room temperature for 5 h. Then the PAB-GBs were washed with PBS buffer and immersed in 1 mg/mL solution of $NaBH_4$ in PBS buffer for 30 min at room temperature. The unreacted carbonyl groups were blocked by incubation in 40 mM ethanolamine for 30 min. Finally, the obtained PAB-GBs were washed with buffer, water and stored at 8 °C in 1 M NaCl containing 20 % ethanol until use.

3. Characterization of the PAB-GBs

After silanization, the amino groups available on the silanized glass bead surface were determined using a colorimetric assay with the anionic dye Orange II.[2,3] The silanized glass beads (100 mg) were incubated in 1 mL of 40 mM Orange II solution (acidic solution: Milli-Q water adjusted to pH 3.0 with 1 M HCl) and stirred for 30 min at 40 °C. The sample was then intensively rinsed with the acidic solution to remove unbound dye. Once air-dried, the colored glass beads were immersed in 1 mL of alkaline solution (Milli-Q water adjusted to pH 12 with 1 M NaOH) to release the adsorbed dye. The desorbed dye solution was adjusted to pH 3.0 by adding 15 µL of 1 M HCl. The concentration of the desorbed dye was determined by measuring the absorbance of the solution at 485 nm.

The affinity of PAB-GBs for trypsin was evaluated by incubating the PAB-GBs (150 mg) in 1 mL trypsin solution (10 µM) in 25 mM Na phosphate buffer pH 7.0, referred as buffer A, at room temperature for 30 min. This amount of trypsin is in excess with respect to the amount of amino groups determined on the surface of the glass beads. After removing the supernatant, the functionalized glass beads were washed with buffer A to remove the unbound trypsin, and incubated in 1 mL of 10 mM HCl + 0.5 M NaCl solution for 10 min to desorb bound trypsin. The supernatant was collected and subjected to enzyme activity assay with TAME to quantify the bound trypsin. The calibration curve in Fig. S2 was used for quantification.

4. Solid-phase synthesis of MIP-NPs

All the steps of the synthesis were carried out in a glass column (2 cm internal diameter x 6.5 cm height) provided with a glass coarse frit and packed with PAB-GBs (37.5 g). Initially the packed column was washed with 75 mL of milli-Q water, followed by 100 mL of buffer A. Then 10 mL of a solution of trypsin (1 mg/mL) was percolated through the column. The flow-through was collected and passed through the column again four more times. The excess trypsin was removed by rinsing the column with 75 mL of buffer A.

The polymerization mixture was prepared by mixing NIPAM (95 mol%) as functional monomer, EbAm (5 mol%) as crosslinker, KPS/TEMED (7.5/1 molar ratio (for optimization see Tab. S1); where the amount of KPS was 3% mol/mol with respect to polymerizable double bonds) as initiation system. All these components were dissolved in buffer A to achieve the desired monomer concentration of 0.5% (w/w). After purging with nitrogen for 15 min, an excess of the pre-polymerization solution (41.7 mL) was percolated in the reactor which was then transferred to an oven at 37°C for 24 h. At the end of the polymerization, the column was washed with 200 mL of buffer A at 37°C and the MIP-NPs were eluted with 45 mL of buffer A at room temperature. Finally the trypsin bound to the PAB-GBs was eluted by washing the column with 150 mL of 10 mM HCl + 0.5 M NaCl. The different fractions were collected: the washing step at 37°C (4 x 50 mL), the elution step at room temperature (3 x 15 mL) and the final acidic washing (3 x 50 mL). They were dialysed against water for 2 days (3 times change per day) with a MWCO dialysis membrane of 6000-8000 Da, and then lyophilized. They were then subjected to enzymatic activity assay and protein determination assay with Bradford (Amresco) and Bicinchoninic acid (G-Biosciences) reagents in order to monitor eventual leakage/release of the protein template. A non-imprinted polymer (NIP) was prepared using the same protocol but in the absence of the template trypsin. It should be noted that only the first 15 mL fraction of the eluate contains the MIP-NPs.

The yield of polymerization was calculated by taking into account that the void volume of the column (and therefore the volume of polymerization solution in the glass column) was 5 mL.

Polymer	KPS/TEMED (molar ratio)	Hydrodynamic diameter nm ± sd (n=3)	PdI ± sd (n=3)
1	KPS (without TEMED)	880 ± 48	0.50 ± 0.04
2	KPS/TEMED 10/1	582 ± 127	0.45 ± 0.31
3	KPS/TEMED 7.5/1	59 ± 2	0.24 ± 0.01
4	KPS/TEMED 5/1	34 ± 3	0.26 ± 0.04
5	KPS/TEMED 1/1	82 ± 22	0.46 ± 0.01

Tab. S1 *Optimization of the molar ratio of KPS/TEMED initiation system to obtain nanoparticles. The polymerization conditions are the same as those described above, except that the polymerization was conducted in a glass vial.*

5. Particle size determination

The hydrodynamic size of the MIP-NPs was measured directly on the eluate solution from the column, by dynamic light scattering using a Zeta-sizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) at 25 °C. For scanning electron microscopy (SEM) imaging, the eluted fraction was diluted 100 times with water (Fig. S1). SEM imaging was carried out on a Philips XL30 Field Emission Gun Scanning Electron Microscope (Amsterdam, Netherlands). Polymer particles were sputter coated with gold prior to the SEM measurement.

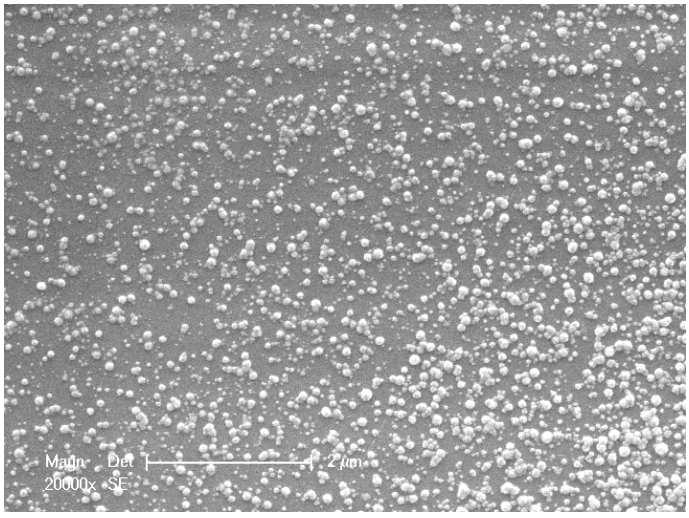

Fig. S1 *SEM of the MIP-NPs.*

6. Enzymatic activity measurements

Trypsin enzymatic activity measurements were done spectrophotometrically on a Uvikon XS spectrophotometer. Trypsin activity assays were carried out with TAME as substrate in 50 mM TRIS HCl + 10 mM $CaCl_2$ pH 8.0 (referred as buffer B) at 25 °C.[4] A 1 µM stock solution of trypsin was prepared in a mixture solution containing 1 mM HCl + 10 mM $CaCl_2$. From this stock solution, trypsin concentrations between 15 nM and 100 nM were pipetted to a standard 1-cm path length quartz cuvette containing TAME (500 µM), in a final volume of 1 mL. After mixing, the hydrolysis of TAME was monitored by the change in absorbance at 247 nm

against a reference cuvette (without trypsin) for 1.5 min. A calibration curve of trypsin activity, represented by (Δ Absorbance/min) was hence constructed (Fig. S2).

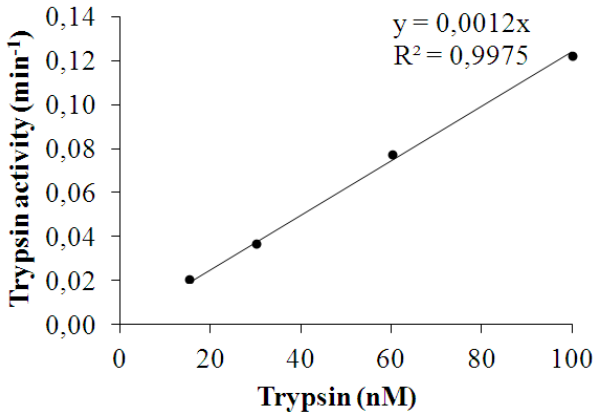

Fig. S2 *Calibration curve of trypsin between 15 and 100 nM in 50 mM TRIS HCl + 10 mM $CaCl_2$ pH 8.0. Data are the mean from three independent measurements for each concentration.*

7. Coupling of trypsin to SiMAG-Benzamidine

100 µL of SiMAG-Benzamidine particles (binding capacity (84-210 µM) according to the manufacturer), were washed three times with 0.5 mL of buffer B by vortexing and recovering the particles by magnetic separation. To attach trypsin, the particles were resuspended in 1 mL of trypsin solution (1 mg/mL) in buffer B and left to incubate at room temperature for 30 min on a tube rotator. Excess trypsin was removed by washing the particles successively four times with buffer B, until no trypsin was detected in the supernatants. At the end, the SiMAG-Benzamidine-trypsin beads were resuspended in 100 µL of buffer B. Bound trypsin was calculated by subtracting the unbound trypsin in the collected supernatants from the initial trypsin amount added to the beads. Enzyme activity was measured as described above. Bound trypsin was found to be 62 µM, which is in accordance with the specifications given by the manufacturer.

8. Binding experiments of MIP-NPs with SiMAG-Benzamidine-trypsin

MIP-NPs and NIP-NPs were solubilized in buffer B to obtain a final concentration of 6 mg/mL. In 1.5 mL polypropylene microcentrifuge tubes, polymer concentrations varying from 0.2 to 1 mg/mL were added to a SiMAG-Benzamidine-trypsin suspension (final concentration of trypsin: 600 nM) and the final volume was adjusted to 500 µL with buffer B. The samples were incubated for 30 min at 35 °C on a tube rotator. The tubes were then placed in the magnetic separator and the supernatant was collected. The supernatants were subjected to static light scattering measurements (SLS) to determine the concentration of unbound polymers. The % of bound polymer was calculated using the equation $(C_{initial}\text{-}C_{Unbound}/C_{initial})*100$, where C_i: initial polymer concentration; $C_{unbound}$: unbound polymer concentration.

9. Static light scattering measurements (SLS)

Static light scattering measurements (SLS) were done on the same Zetasizer NanoZS instrument used for particle size determination. All measurements were performed at 25 °C using toluene as standard and a refractive index increment (dn/dc, the change of refractive index (n) as a function of the change in polymer concentration (c)), value of 1.627 mL/g for PNIPAM in aqueous solution.[5] A calibration curve for both MIP and NIP-NPs, represented by the intensity of the scattered light at different polymer concentrations was hence constructed (Fig. S3).

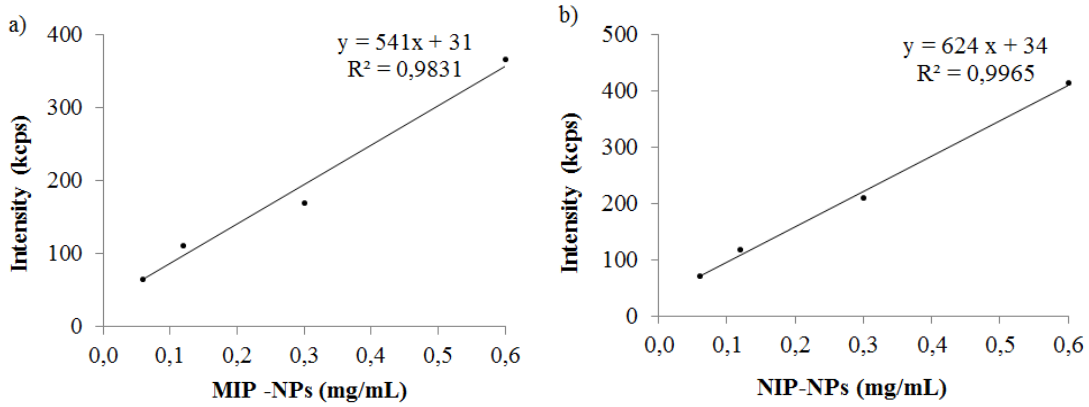

Fig. S3 *Calibration curves of (a) MIP-NPs and (b) NIP-NPs between 0.06 and 0.6 mg/mL in* 5 mM TRIS HCl + 10 mM $CaCl_2$ pH 8.0. *Data are the mean from three independent measurements for each concentration.*

10. Preparation of fluorescein isothiocyanate-labeled trypsin (FITC-trypsin)

FITC-trypsin was prepared according to J. R. Lakowicz *et al.*[6] Since the reaction was performed in 100 mM Na bicarbonate buffer pH 9.2 at room temperature for 2 hours, the stability of trypsin in those conditions was checked beforehand. The residual activity of trypsin was 99 ± 3% (n=4), thus confirming the possibility to apply this protocol. Trypsin (2 mg/mL) was dissolved in 100 mM Na bicarbonate buffer pH 9.2 and mixed with 78.5 uL of FITC solution (10 mg/mL in DMSO). The reaction mixture was incubated for 2 h at room temperature and the labeled protein was separated by gel filtration on a PD-10 column (GE Healthcare) equilibrated with 0.1 M Tris-HCl buffer+0.15 M NaCl pH 7.2. To avoid trypsin autoproteolysis, the pH of the fraction containing FITC-trypsin was brought to 3.0 by adding a solution of 2 M HCl.[7] The fraction was divided into aliquots which were then stored at -20 °C. To determine the fluorescein-trypsin ratio, trypsin concentration was measured with the Bradford method, whereas the bound fluorescein was determined via its absorption at 495 nm using a molar extinction coefficient of FITC $\varepsilon_{495\ nm}$= 68000 M^{-1} cm^{-1}.

11. Competitive binding experiments of MIP-NPs with FITC-trypsin in presence of unlabeled trypsin, kallikrein or thrombin

In 0.5 mL polypropylene microcentrifuge tubes, 20 µL of MIP-NPs stock solution (10 mg/mL) were added to 3 µL of FITC-trypsin and the final volume was adjusted to 500 µL with buffer B (final concentrations: FITC-trypsin 200 nM; polymer 0.4 mg/mL). The samples were incubated for 30 min on a tube rotator.

To investigate the selectivity of the MIP-NPs in presence of the competitor at a final concentration of 1 µM, 8.5 µL of a 60 µM protein stock solution (or buffer for the control) was added to the polymer samples previously incubated with FITC-trypsin. The samples were left to incubate for 2.5 h on a tube rotator. The solution was then transferred into a Nanosep (100K omega) and centrifuged at 13800*g* for 30 min. The filtrate was diluted twice with buffer B and the emission fluorescence intensity of the unbound FITC-trypsin was measured on a FluoroLog-3 spectrofluorimeter (Horiba Jobin Yvon, Chilly Mazarin, France). The λ excitation/emission were set at 492/517 nm, respectively. The binding efficiency was calculated using the equation $(I_b-I_s)/I_b*100$, where I_b: fluorescent intensity of 3 µL of FITC-trypsin in 1 mL of buffer B; I_s: fluorescent intensity of FITC-trypsin in the sample.

12. Equilibrium binding assays - Scatchard plot

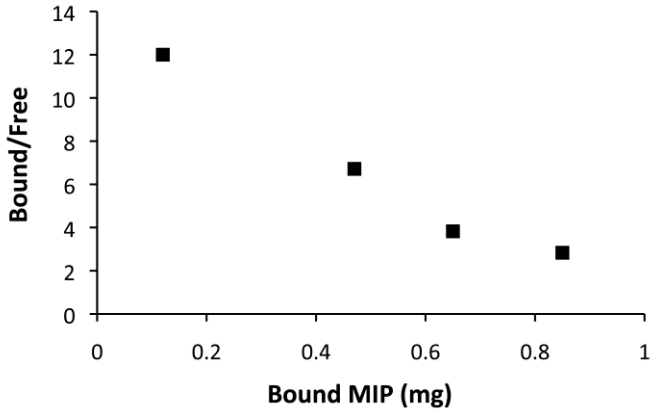

Fig. S4 *Scatchard plot of an equilibrium binding assay of trypsin binding to MIP (for details see experimental section, point 8).*

12. References

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