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Molecularly Imprinted Nanoparticles for Inhibiting Ribonuclease in Reverse Transcriptase Polymerase Chain Reaction

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Contents

1. Materials.

Ribonuclease A (RNase A) from bovine, lysozyme from chicken egg white, β-Lactoglobulin from bovine milk, hemoglobin human, N-isopropyl acrylamide (NIPAm), methacrylamide (AAm), acrylic acid(AAc), N-tert-butylactylamide (TBAm), ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), QuantiPro™ BCA Assay Kit, phosphate buffer solution (PBS) (pH 7.4), ethanolamine hydrochloride, acetone, 60 mL SPE tubes and 20 μm pore frits and glass beads (75 μm) were purchased from Sigma-Aldrich DK. Q-Max® Cellulose acetate (CA) syringe filters 0.45 μm were from FRISENETTE (DK). Nunclon 96-well flat-bottom transparent microwell plates, Nunclon 96-well flat-bottom black microwell plates and RNaseAlert™ Lab Test Kit consisting of 25 tubes of Fluorescent Substrate, 250 μL of 10x RNaseAlert Lab Test Buffer, 500 μL of RNase A, 500 mL of RNase*Zap*® and 1.75 mL Nuclease-free Water were purchased from Thermo Scientific (DK). miRCURY LNA™ Universal RT microRNA PCR kit consisting of 128 μL of 5x Reaction buffer, 24 μL of 5x concentrated Enzyme mix, 12 fmol of 10x concentrated UniSp6, 200 r x n of UniSp6 RNA Spike-in control primer set, 200 r x n of hsa-miR-103a-3p primer set, 200 r x n of 2 primer setss, 500 μL of ExiLENT SYBR® Green master mix and 1.25mL of Nuclease-free water was purchased from Qiagen (Germany)**.** Deionized water was obtained from a Millipore (MilliQ) purification system. All chemicals were analytical or HPLC grade and were used without further purification.

2. Methods

2.1 Preparation of template-derivatized glass beads.

The protocol for immobilizing template was adapted from the method developed by Canfarotta *et al.*[1] RNase A was firstly immobilized covalently onto glass beads (Figure S1a). Twenty-five grams of glass beads were activated by boiling in 40 mL of 1 M NaOH solution for 15 min and thoroughly

rinsed with 300 mL of PBS, 100 mL of MilliQ water and 30 mL of acetone, and then they were dried in a vacuum oven at 80℃ overnight. To introduce epoxy groups to the surface of the glass beads, the dry beads were incubated overnight in a 40 mL of 4% v/v GPTMS/anhydrous toluene solution and refluxed at 110℃ overnight. The epoxy functionalized glass beads were washed eight times with anhydrous toluene and one time with acetone, and then dried in a vacuum oven at 80℃ for 12 hr. Ten mL of RNase A in PBS (0.5 mg mL^{-1}) was added to glass beads and incubated overnight at room temperature to immobilize RNase A on the glass beads. Afterwards, unbound RNase A has washed away by using MilliQ water. The amount of immobilized RNase A was confirmed by performing a BCA protein assay on an aliquot of GPTMS-derivatised glass bead, using bovine serum albumin (BSA) as the standard. Briefly, 550 mg GPTMS-functionalized glass beads were incubated with 0.44 mL of 1 mg mL^{-1} RNase A overnight. The supernatant of the mixture was subjected to a BCA protein assay. Through UV measurement, the amount of unbound RNase A was determined. Bound RNase A was calculated by subtracting the amount of unbound RNase A from the total amount of RNase A initially added to the glass beads. Finally, the glass beads were washed with MilliQ water, dried under vacuum and stored at 4 ℃.

2.2 Synthesis of RNase A NanoMIPs.

The protocol for solid phase synthesis was also adapted from the method developed by Canfarotta *et al.*[1] To prepare polymerization mixture for synthesizing RNase A nanoMIPs, TBAm (X mol %) was dissolved into 1 mL of ethanol and then was added into 99 mL of MilliQ water that contained AAc (Y mol %), AAm (Z mol %), NIPAm (98-X-Y-Z mol%) and BIS (2 mol%). The obtained solution has a total monomer concentration of 6.5 mM. Afterwards, the resulting solution was sonicated under the vacuum for 10 min followed by bubbling with a stream of nitrogen for 30 min. Meanwhile, 25 g of RNase A-derivatised glass beads were placed into a 50 mL falcon tube and

purged a stream of nitrogen for 40 min. After pretreatment, the glass beads were poured onto the polymerization mixture and purged by nitrogen for 5 min before adding a mixture of APS aqueous solution (30 mg/500 µL) and TEMED (15 µL) to initiate the polymerization reaction. The reaction was left to polymerize for 1 h at room temperature. After polymerization, all the content was transferred into a solid phase extraction (SPE) cartridge (60 mL syringe tube) fitted with polyethylene frit (20 µm porosity), and was washed using 15-bed volumes of MilliQ water at 20 ℃ to remove non-polymerized monomers and low-affinity nanoMIPs. Next, 60 ℃ MilliQ water was poured into the cartridge and incubated with RNase A-derivatised glass beads for 15 min in a 60 ℃ water bath. This procedure was performed several times to allow the elution of high-affinity nanoMIPs from the solid phase. The total eluted volume was about 50 mL.

2.3 The inhibitory potency of nanoMIPs towards RNase A.

In order to evaluate inhibition behavior of nanoMIPs towards RNase A, nanoMIPs (5 mg mL^{-1}) were incubated with RNase A (0.05 µg mL^{-1}) overnight in 2 µL of PBS solution. Five mL of the 10X RNase A Alert Lab Test Buffer was added into lyophilized fluorescent substrate tubes, and after vortexing, 40 µL RNase A free water was added. Eight µL of the fluorescent solution was pipetted into the 2 µL mixture of RNase A and nanoMIPs and incubated for 30 min at 37 ℃. After incubation, the 10 µL solution was added up to 50 µL with RNase A free water. The fluorescent measurements were done on a Spark® multimode microplate reader (Tecan, Sweden), with emission/excitation wavelengths set at 490nm/520nm. A negative control and positive control referred to fluorophore substrate mixed with RNase A nanoMIPs and RNase A, respectively.

2.4 Yield, dynamic light scattering (DLS) and scanning electron microscopy (SEM) of RNase A nanoMIP 4.

The yield of nanoMIPs was measured by calculating the weight yield value from a 10 ml aliquot of the nanoMIPs solution. In general, 10 mL of stock solution (in water) was evaporated under a stream of nitrogen in a pre-weighed empty vial placed in a 60 ℃ water bath. After evaporating, the glass vial was put into a vacuum oven at 80 ℃ for overnight to remove all the water. The yield can be calculated by subtracting the weight of pre-weighed empty vial from the overall weight of nanoparticle contained vial. Nanoparticle size and quality were determined by DLS using Malvern Instruments Nordic AB (Greve, Denmark). Two milliliters of a stock solution of RNase A nanoMIPs were sonicated for 20 mins. The dispersion was filtered through a 0.45 μm CA syringe filter to remove dust and possible aggregates. The filtered RNase A nanoMIPs were analyzed by DLS at 25 ^{o}C in a 3 cm^3 disposable polystyrene cuvette. The values were reported as an average of 3 measurements with each measurement consisting of 14 runs.

SEM analysis was carried out on a Quanta FEG 200 ESEM scanning electron microscopy (FEI, Oregon USA). Five milliliters of stock solution (in MilliQ water) was evaporated down to 0.5 mL. The solution was sonicated for 20 mins and filtered through a 0.45 μm CA syringe filter. A drop of RNase A nanoMIPs was placed on a silica wafer and dried in a desiccator overnight. Prior to SEM, the sample was sputtered with a thin layer of gold using a Carbon Coater (Quorum Technology, Denmark).

2.5 Binding capacity, kinetics, and selectivity of the RNase A nanoMIP 4.

Binding capacity, binding kinetics and binding selectivity of the synthesized nanoMIPs 4 were tested in Spark® multimode microplate reader in PBS buffer. For binding capacity, the nanoMIPs (5 mg mL^{-1}) was mixed with various concentrations of RNase A (5 μg mL^{-1}, 10 μg mL^{-1}, 20 μg mL^{-1}, 25 μg mL^{-1}, 40 μg mL^{-1} and 50 μg mL^{-1}, respectively) in 0.2 mL of PBS solution followed by

shaking for 20 h at room temperature. After incubation, the solutions were centrifuged at 15,000 g for 10 min and the supernatants were withdrawn (150 μL) and mixed with BCA kit reagents. The absorbance of each sample was measured at 562 nm using the Spark® multimode microplate reader. Based on RNase A standard curve obtained from the BCA protein kit, the amount of absorbed RNase A was calculated by subtracting the amount of unbound RNase A from the total amount of RNase A.

In order to determine the binding kinetics, the nanoMIPs (5 mg mL^{-1}) were mixed with RNase A (5 μg mL^{-1}) in 0.2 mL of PBS solution for different time periods, ranging from 5 min to 60 min. The samples were centrifuged at 15,000 g for 10 min. The binding kinetics was determined again by incubating the supernatant with BCA kit reagents, and the absorbance of samples was read at 562nm using the Spark® multimode microplate reader.

The specificity of the nanoMIPs was determined by incubating nanoMIPs (5 mg mL^{-1}) with 25 μg mL^{-1} of RNase A, Lysozyme, β-Lactoglobulin and Hemoglobin in 0.2 mL of PBS solution, respectively. The mixtures were shaken at room temperature overnight and the response of each protein was determined based on the change in absorbance before and after incubation by BCA protein kit.

2.6 The inhibitory potency of nanoMIP 4 or recombinant protein inhibitor towards RNase A.

The nanoMIPs (5 mg mL^{-1}) or recombinant protein RI (10 U μL^{-1}) were incubated with RNase A at various concentrations (0.025 μg mL^{-1}, 0.05 μg mL^{-1}, 0.5 μg mL^{-1}, 2.5 μg mL^{-1}, 5 μg mL^{-1} and 10 μg mL^{-1}, respectively) in 2 μL of PBS solution overnight. Afterward incubation, RNase A Alert™

Lab Test Kit was used to measure the amount of active RNase A. Detailed experimental step was performed as described in the section "**The Inhibitory potency of nanoMIPs towards RNase A**".

2.7 RT-PCR Assay.

The RT-PCR assays were carried out using the miRCURY LNA™ Universal RT microRNA PCR kit. The synthetic microRNA UniSp6 was used as the template to avoid any prior RNase A contamination, and the two-step assay was based on universal reverse transcription (RT) followed by real-time PCR amplification. Three types of RT assays were performed: 1) different concentrations of RNase A without adding any inhibitors; 2) different concentrations of RNase A mixed with protein inhibitor; 3) different concentrations of RNase A mixed with nanoMIPs.

The RT reaction mixture consisting of 2 μL of 5× Reaction buffer, 1 μL of Enzyme mix, 1 μL of microRNA UniSp6 (1 pg) was prepared. For the type 1 assay, 1 μL of RNase A (with concentrations of 0 pg μL^{-1}, 50 pg μL^{-1}, 100 pg μL^{-1}, 1 ng μL^{-1}, 10 ng μL^{-1}, 20 ng μL^{-1}, 50 ng $\mu L^{-1}$and 100 ng μL^{-1}) was added to the RT reaction mixture. For the type 2 assay, RNase A was first mixed with the protein inhibitor (10 U μL^{-1}) in a 2 μL-solution for 15 min, then the RT reaction mixture was added. The type 3 assay was similar to type 2, except the RNase A was mixed with the nanoMIP 4 (5 mg mL^{-1}). For all the reactions, an appropriate amount of nuclease-free water was added to adjust the final volume to10 μL. The RT step was performed on a Chromo4 real-time PCR cycler (Bio-rad, California, USA) at following conditions: 60 min at 42˚C, 5 min at 95˚C, and cooling to 4°C.

After the RT reaction, the cDNA products were diluted 10 folds in nuclease-free water. Four μL of the diluted cDNA template was transferred to the PCR reaction mixture which consisted of 5 μL of

PCR Master mix and 1 μL of UniSp6 RNA Spike-in control primer set. The PCR was carried out in the following condition: 95˚C for 10 min, 45 cycles at 95˚C for 10 s, 60˚C for 1 min followed by an optical read, and cooling to 4°C. The Ct values were calculated by the Opticon Monitor Software.

3. Reference:

[1] F. Canfarotta, A. Poma, A. Guerreiro, S. Piletsky, *Nature protocols* **2016**, *11*, 443

4. Experimental figures and tables

4.1 The illustation figure of nanoMIPs synthesis

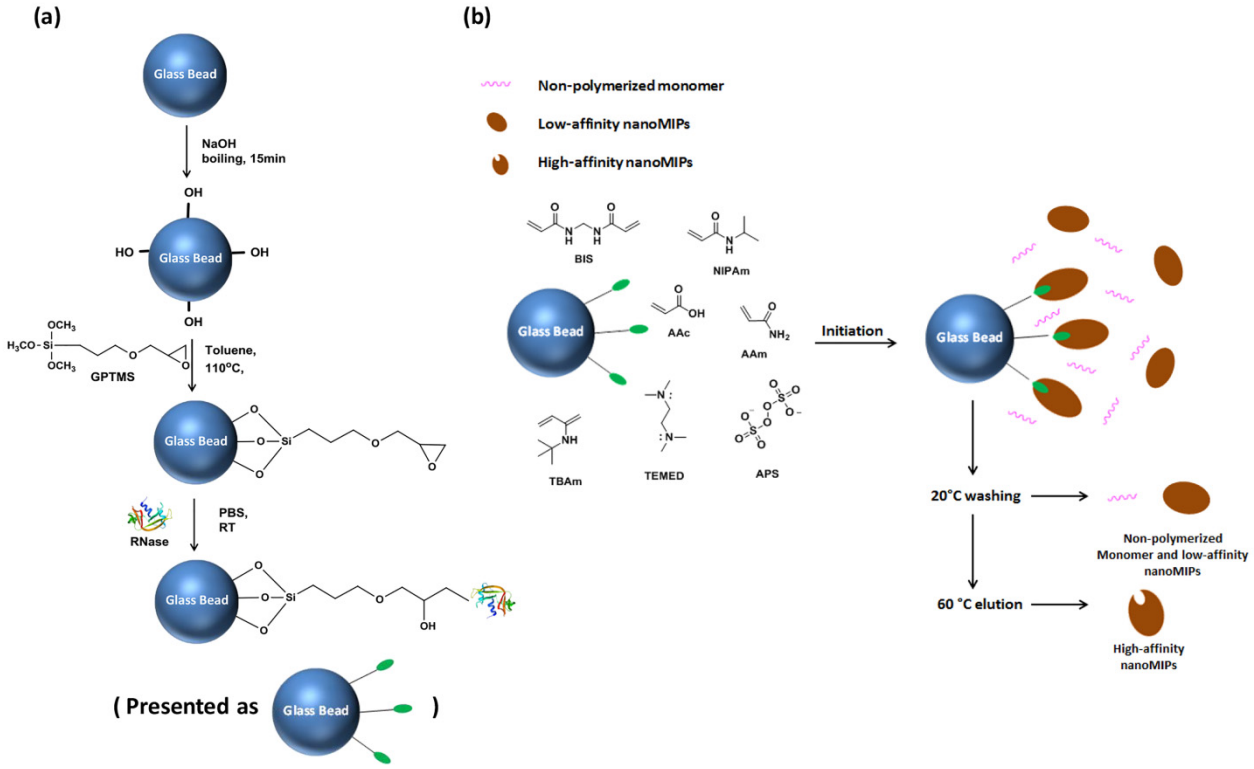


Figure S1. Illustration of (a) immobilization of RNase A on the surface of glass beads; (b) the solid-phase synthesis of the nanoMIPs.

4.2 Calibration of BSA

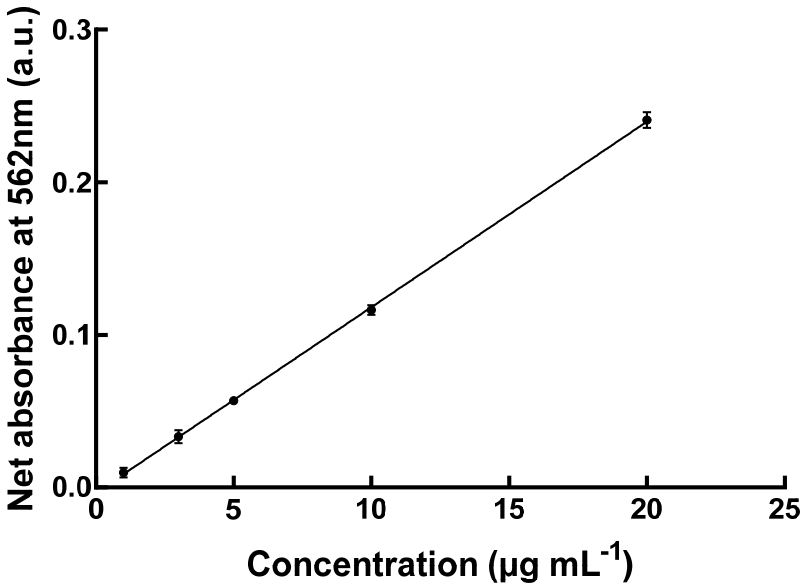
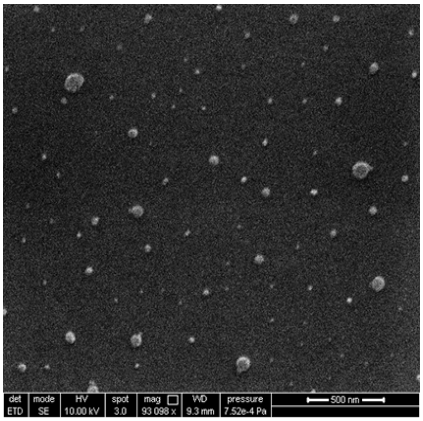


Figure S2. Calibration curve of BSA by BCA protein kit.

4.3 SEM image and DLS result and nanoMIP 4

(a)



(b)

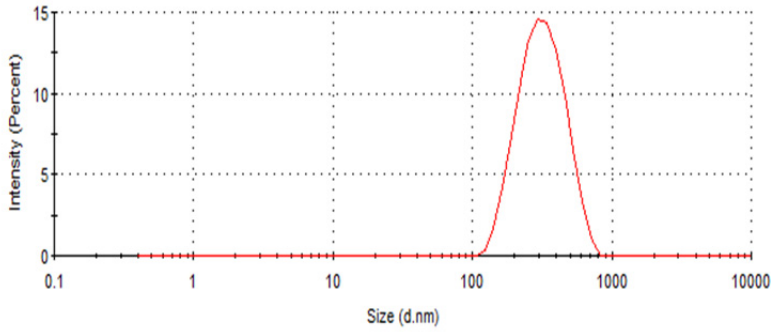


Figure S3. (a) SEM image of nanoMIP 4. (b) DLS measurement in 10mM of sodium phosphate buffer at pH 7.

4.4 Binding capacity, kinetics, and selectivity of nanoMIP4

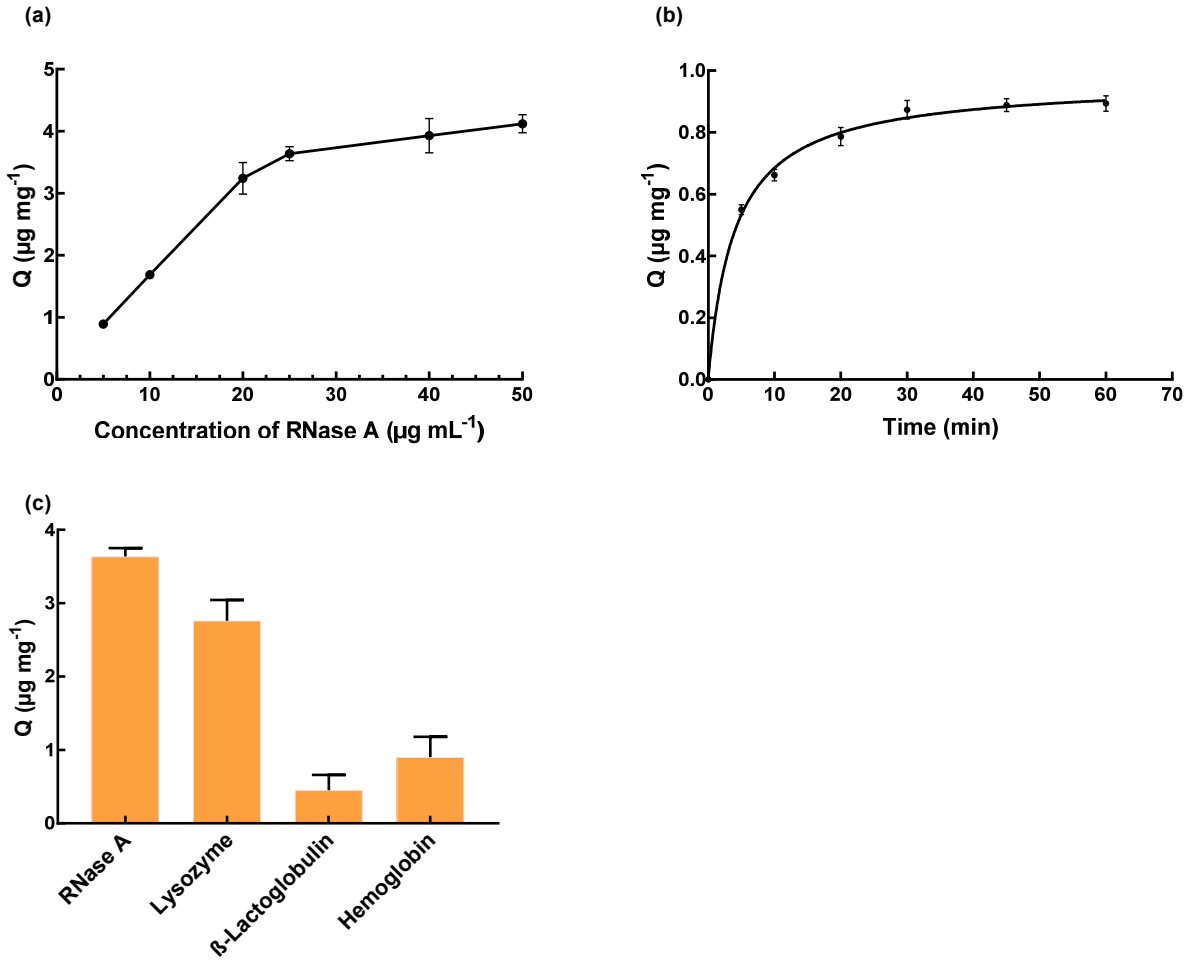


Figure S4. (a) The binding capacity of nanoMIP 4 towards RNase A. The nanoMIPs (5 mg mL^{-1}) was mixed with various concentrations of RNase A (5 μg mL^{-1}, 10 μg mL^{-1}, 20 μg mL^{-1}, 25 μg mL^{-1}, 40 μg mL^{-1} and 50 μg mL^{-1}, respectively) in a 0.2 mL-solution. (b) The binding kinetics of nanoMIP 4 towards RNase A. The RNase A nanoMIPs (5 mg mL^{-1}) was incubated with RNase A (5 μg mL^{-1}) in a 0.2 mL-solution for different time periods, ranging from 5 min to 60 min. (c) Binding selectivity of nanoMIP 4 towards RNase A. The selectivity of nanoMIPs were determined by incubating the nanoMIPs (5 mg mL^{-1}) with 25 μg mL^{-1} of RNase A, Lysozyme, β-Lactoglobulin, and Hemoglobin, respectively.

4.5 Binding affinity of nanoMIP4

In order to determine affinity constants and binding site concentrations, isothermal data (Figure 4Sa) was re-plot in the form of Scatchard plot by the Equation (1).

$$\frac{Q}{C_E} = \frac{Q_{max} - Q}{K_d} \qquad \text{Equation (1)}$$

Where, Q_{max} is apparent maximum number of binding sites, and K_d is equilibrium dissociation constant, and C_E is the concentration of free RNase A in solution, and Q is the concentration of bound protein to RNase A nanoMIPs. It is worth pointing out that this was a linearized form of the Langmuir equation and only assumed single affinity constant binding site populations. On the basis of this method, Scratchard profiles were plotted as shown in Figure S5. Apparent constant K_d and Q_{max} values were determined by the slopes and intercept from Scratchard equation $\frac{Q}{C_E}$ =-0.3082Q+1.536(r^2 =0.974), which were 3.2 μg mL^{-1} (237 nM) and 4.98 μg mg^{-1} (364 nmol g^{-1}), respectively.

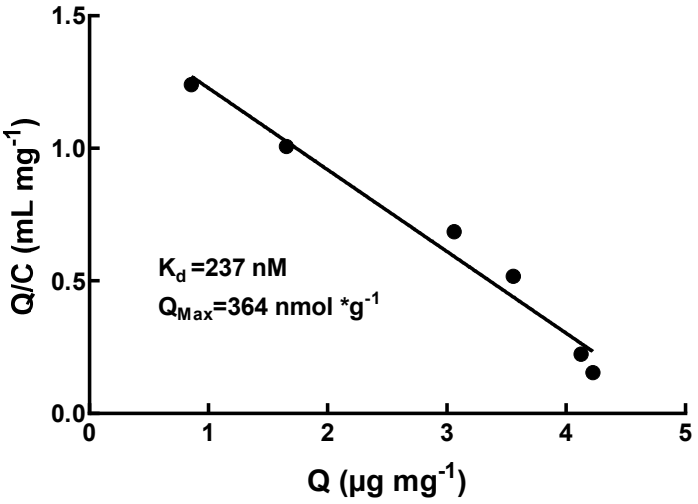


Figure S5. Scratchard Plot of binding of RNase A in PBS to RNase A nanoMIPs Q/C_e= -0.3082*Q + 1.563, (R^2=0.974)

4.6 Saturation curve and calibration curve of RNase to quantify RNase A

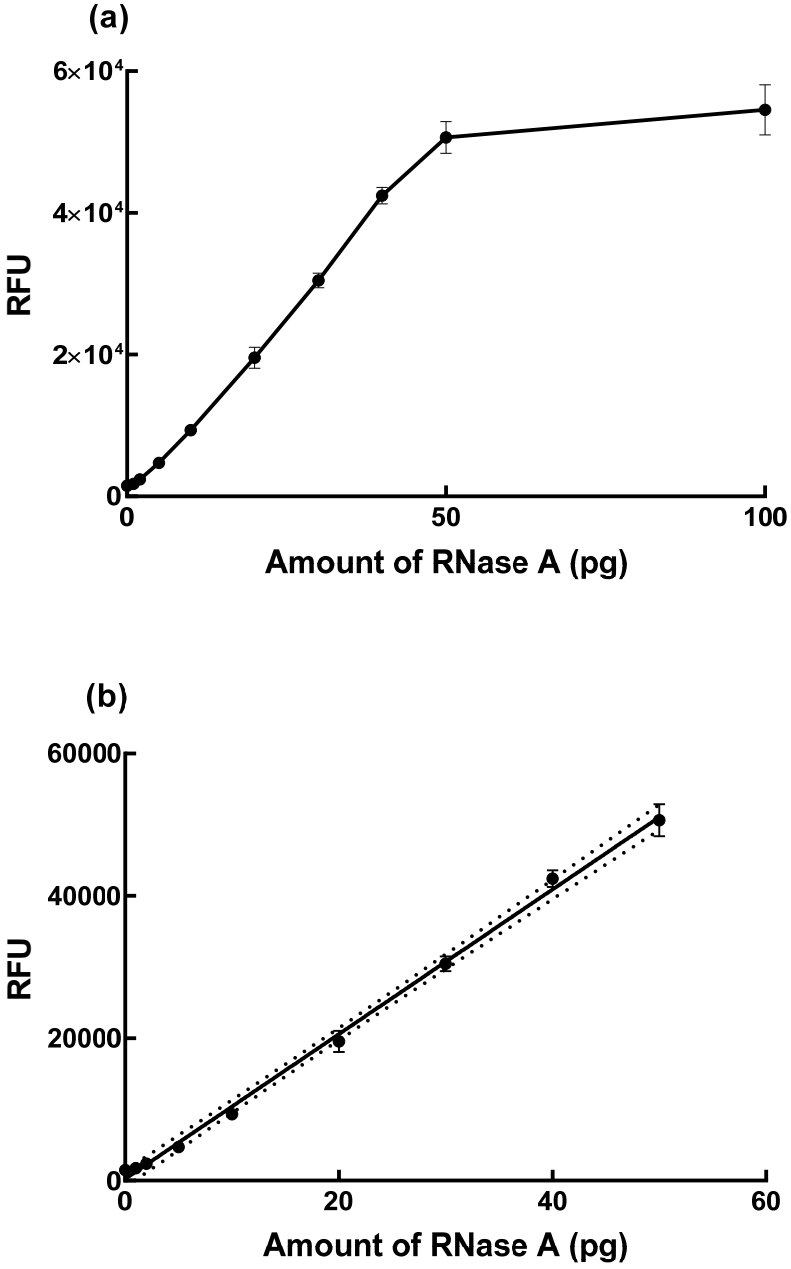


Figure S6. (a) Saturation curve and (b) linear calibration curve of RNase to quantify RNase A by RNase A Alert™ Lab Test Kit. The linear range is from 1 pg to 50 pg with the regression equation of $\boldsymbol{y = 1016x + 206.6}$ (R^2=0.996).

4.7 Monomer composition, hydrodynamic diameter and PDI of the nanoMIPs.

Table S1. Monomer composition, hydrodynamic diameter and PDI of the nanoMIPs.

No.	Functional monomer composition ratio (mol %)				Diameter (nm)	PDI
	NIPAm	AAm	AAc	TBAm		
1	40	0	58	0	333.50±8.21	0.368±0.039
2	50	0	43	5	220.13±0.78	0.152±0.009
3	50	0	5	43	271.30±3.82	0.364±0.033
4	50	5	5	38	302.03±7.01	0.284±0.024