ARTICLE

Molecularly Imprinted Nanoparticle-Based Assay (MINA) – Detection of Leukotriene and Insulin

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Alvaro Garcia-Cruz*[a], Todd Cowen[a], Annelies Voorhaar[a], Elena Piletska[a] and Sergey A. Piletsky[a]

A novel molecularly imprinted polymer nanoparticle-based assay (MINA) performed in magnetic microplates was developed as an improved high-quality alternative to existing antibody-based immunoassays. MINA is a generic technology that can be adapted for biomarker detection in biological samples. Herein, we demonstrate the applicability of the MINA assay for the detection of leukotrienes and insulin in biological samples. MINA, used in a competition format, has allowed the detection of LTE4 in urine in a concentration range from 0.45 to 364 pM, with a LOD of 0.73 pM. MINA, used in a competition format, has allowed the detection of insulin in plasma in a concentration range from 25 to 2500 pM, with a LOD of 27 pM. This assay has shown comparable performance for the LTE4 and insulin detection to existing chromatographic techniques (LC-MS/MS) and immunoassays in clinically relevant concentrations. The main advantages of this assay are the efficient and low cost fabrication, preparation of synthetic binders without the use of animals, and fewer steps used in assay protocol as compared to traditional immunoassays.

Introduction

Design of the assay, principle of work.

Molecularly imprinted polymers (MIP) are synthetic (supramolecular) receptors used as recognition elements in assays[1, 2] and sensors[3-5] for a large variety of analytes including small molecules,[6-8] peptides,[9, 10] and proteins.[11-13] They have advantage over natural molecules including greater robustness, easy preparation, lower cost, efficient production and their capacity to bind "non-immunogenic" molecules.[14] The key difference to previous MIP assays is that the present MINA is completely abiotic, involving no antibodies or enzymes. In this format, fluorescent molecularly imprinted nanoparticles (nanoMIPs) are used as recognition elements and as reporters in combination with magnetic nanoparticles (MNP).

A solid phase approach was used to efficiently synthesise nanoMIPs.[22-23] During solid phase synthesis of MIP nanoparticles, controlled polymerisation is performed in the presence of a template immobilized onto a solid phase (Figure 1). The solid phase synthesis opens the possibility of using nanoMIPs in sensors and assays by directly replacing antibodies with MIPs using practically identical manufacturing protocols.[15] Particularly rewarding is the use of nanoMIPs in microplates containing disk-shaped inserts made of magnetic material. These inserts can capture MNP with immobilized analytes or magnetic@nanoMIPs added to microplates without chemical activation of their surface (Figure S1).

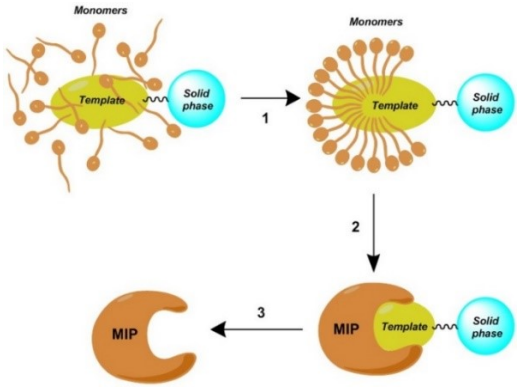


Figure 1. Schematics of solid phase synthesis of nanoMIPs: (1) Formation of a pre-polymerization complex between monomers and template; (2) Controlled polymerization; (3) Separation of synthesized nanoMIPs.

The apertures in the centre of the disks allow unhindered spectrometric measurements.[16, 17] The use of fluorescent nanoMIPs in microplates modified with magnetic inserts also allows measurement without requiring signal amplification, normally necessary for assays involving enzyme-antibody conjugates.[1, 17] Instead of enzyme amplification, fluorescent-labelled nanoMIPs acting as reporters can be detected in magnetic microplates with very high sensitivity.[18, 19] This form of abiotic assay offers high stability, longer shelf life and a simple operation procedure. MINA requires fewer steps than ELISA or LC-MS/MS, requiring no washing steps or addition of enzyme substrates, and so is more user-friendly and provides faster results.

[1]*Department of Chemistry, University of Leicester, University Road, LE1 7RH, Leicester (UK)*
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

The components of the MINA comprise of nanoMIPs, magnetic nanoparticles modified with the analyte and microtiter plates fitted with magnetic inserts. In this assay nanoMIPs can act as both reporter probes (fluorescent derivative) and binding agents (conjugated to magnetic beads). The present assay technology is a generic method for biomarker detection.

As an exemplification of this technology, we have developed two assays for the analysis of leukotrienes[20, 21] and insulin[22, 23]. The practical significance of this work is related to unmet need in assays capable of effectively measuring these targets in biological samples. The present work focuses on the application of MINA for the detection of insulin and LTE4 by employing specific fluorescent nanoMIPs in a microplates fitted with magnetic inserts. The detection of LTE4 was performed using a competitive assay comprising of LTE4-specific fluorescent nanoMIPs and LTE4-conjugated supermagnetic iron nanoparticles (MNP@LTE4). Insulin was determined using a sandwich version of MINA, comprising of insulin-specific fluorescent nanoMIPs and supermagnetic iron nanoparticles conjugated to imprinted insulin nanoMIPs (MNP@NanoMIPs).

Materials

Glass beads Spheriglass® A-Glass 2429 (150 – 200 µm diameter), were from Potters Industries LLC. Allylamine, ammonium persulfate (APS), [3-(2-aminoethyl amino)propyl]-trimethoxysilane (APTES), 1,2-Bis(triethoxysilyl)ethane (BTSE), dimethylformamide (DMF), 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNSCl). Ethylene glycol dimethacrylate (EGDMA); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), glutaraldehyde, N,N'- methylene-bis-acrylamide (MBA), N-isopropylacrylamide (NIPAM) and ethylene glycol methacrylate phosphate (EGMP) were acquired from Alfa-chemistry USA. Sodium hydroxide (NaOH), N-hydroxysuccinimide (NHS), phosphate buffered saline (PBS), tetramethyl-ethylene-diamine (TEMED), 3-(Trimethoxysilyl)propyl methacrylate (TMSMA), polyoxyethylene sorbitane monolaureate (Tween 20), itaconic acid (ITA); pentaerythritol tetrakis(3-mercaptopropionate) (PETMP); N-hydroxysuccinimide (NHS); N,N-diethyldithiocarbamic acid benzyl ester trimethylolpropane trimethacrylate (TRIM), Fluorescein o-acrylate (FAM), ethanol and acetone were purchased from Sigma-Aldrich (UK). Acetonitrile (ACN) was purchased from Fisher Scientific (UK). N,N'-diethyldithiocarbamic acid benzyl ester (iniferter) was obtained from TCI Europe (Belgium). Phosphate buffered saline (PBS), Polyoxyethylene sorbitane monolaureate (Tween 20) and sodium hydroxide were purchased from Thermo Fisher Scientific, UK. All chemicals and solvents were analytical or HPLC grade and used without any purification. Leukotrienes were purchased from Cayman Chemicals (USA). Urine (DIN EN 1616:1999) was purchased from Pickering Laboratories Inc. (U.S.A.). Leukotrienes (LTE4, LTD4 and LTB4), Human insulin, Human Proinsulin C-peptide (HPC) and Insulin-like growth factor 1 (IGF-1) were provided by Astra Zeneca.

Experimental Section

LTE4 computational modelling: the neutral LTE4 model was constructed with the Sybyl 7.3 software package, and underwent five cycles of 50 pico seconds (ps) molecular dynamics simulations and energy minimization (0.01 kcal/ mol Å) with applied Tripos force field and MMFF94 charges in vacuo. The lowest energy structure was then solvated in water and a further 10 ps dynamic at 300 K and analysed for conformity with the in vacuum results. The process was repeated with the zwitterion structure. Both molecules were then screened against a database of commonly used functional monomers using the previously described automated Leapfrog screening method.[24]

Insulin computational modelling: the crystal structure of insulin[25, 26] was obtained from the RCSB PDB (id. 3I40).[27, 28] Solvent water molecules were removed and the protein was screened against a database of common functional monomers used in MIP synthesis using the previously described automated Leapfrog method.[24] The database used consisted of 44 monomers, representing charged and neutral forms, and the screening was performed for 200,000 iterations.

Solid phase preparation: 20 g of glass beads (150-200 µm) were first activated by boiling in a 4M NaOH aqueous solution (1 mL per gram of glass beads) for 30 min, washed 3 times with deionized water (50 mL) and then incubated in 20% (v/v) sulphuric acid for 15 min. The beads were then washed 3 times with 5 mM PBS, double-distilled water and acetone (50 mL), then dried at 150 °C. Subsequently, glass beads were refluxed overnight in a 6% (v/v) APTES and 0.24% (v/v) BTSE solution in dry toluene. The system was cooled down and glass beads were washed 6 times with acetone and methanol (50 mL), then dried under vacuum for 15 min. Successively, glass beads were dried at 120 °C for 45 min and stored under nitrogen at 4°C until use (stable for 6 months).

LTE4 immobilization on solid phase: 20 g of silanized glass beads were incubated for 2 h in 7% (v/v) glutaraldehyde solution (in 0.01 M PBS, pH 8.5). The glass beads were then filtered, washed with deionised water and incubated overnight in 20 mL of a solution 0.05 mg/mL of LTE4, 50 µL of TEMED and 100 µL of water in DMF at pH 9.0. After incubation, the glass beads were filtered and incubated in a solution of ethanolamine (0.1 g/mL) in 10 mM PBS for 1 h. Afterwards, the glass beads are washed with distilled water and the reductive alkylation is completed by incubating LTE4-glass beads in a solution comprising (1 mg/mL) sodium cyanoborohydride and 10 mM PBS (0.8 mL of solution per gr beads) for 30 min at room temperature. Finally, LTE4-glass beads were filtered, rinsed with ultrapure water, acetone, and then dried using a SPE cartridge and stored at 4 °C.

Insulin immobilization on solid phase: silanized glass beads (40 gr) were incubated in 50 mL of 7% (v/v) of glutaraldehyde in 10 mM PBS (pH 7.2) for 2 h. Then glass beads were rinsed with double-distilled water. The glass beads were then incubated in an insulin solution (0.5 mg/mL in 5 mM PBS, pH 7.2) overnight at room temperature. Afterwards, glass beads were rinsed with double-distilled water and incubated in 50 mL of 0.1 mM

ethanolamine in 5 mM PBS during 15 min. Subsequently, glass beads were washed with distilled water. Then, glass beads were incubated in a 1 mg/mL sodium cyanoborohydride solution (in 0.01 M PBS, 1.5 mL of solution per g of beads) for 30 min at room temperature. Finally, the glassbeads@insulin were filtered and rinsed with ultrapure water, then dried and stored at 4 °C until use.

Solid phase synthesis of fluorescent LTE4 imprinted polymer nanoparticles (nanoMIPs): the monomer mixture was prepared by mixing ITA (3.0 g, 22.30 mmol) and allylamine (1.3 g,22.30 mmol) as functional monomers, TRIM (2.2 g, 6.38 mmol) and EGDMA (2.2 g, 11.0 mmol) as cross-linkers, FAM (8.0 mg, 0.02 mmol) as fluorescent monomer, PETMP (121 mg, 0.25mmol) as a chain transfer agent and diethyldithiocarbamic acid benzyl ester (501 mg, 2.10 mmol) as initiator, transfer agent, and terminator (iniferter) in 30 mL DMF. The monomer solution was purged in nitrogen for 5 min, then mixed with 20 g of degassed LTE4-glass beads. Subsequently, the polymerization was initiated by exposing the mixture to UV-light sources for 2 min (Philips model HB/171/A, 4 × 15 W/amps).

After polymerization, the crude mixture was transferred into a solid phase extraction (SPE) cartridge fitted with a polyethylene frit with 20 µm porosity. In order to collect the high affinity nanoMIPs their thermo-labile properties were employed. To perform this, the solid phase was washed firstly with DMF (3 × 30 mL) and then with acetonitrile (3 × 30 mL) at 0°C, in order to remove monomers, residues and low affinity nanoparticles. The high affinity nanoMIPs were recovered by elution using a solution of 20% ethanol in water at 45°C (3 × 25 mL). The crude mixture was lyophilized, then reconstituted in water, sonicated and filtered (PTFE Syringe Filters, 0.45 µm, 25 mm, Sterlitech).

Solid phase synthesis of fluorescent Insulin (nanoMIPs): the free radical polymerisation of nanoMIPs involves functional monomers, cross-linkers and initiator. The selected monomer mixture comprised of EGMP (16 mg, 0.08 mmol), NIPAM (39 mg, 0.34 mmol), MBA (52 mg, 0.34 mmol), allylamine (9 mg, 0.15 mmol), itaconic acid (15 mg, 0.08 mmol), and FAM (8 mg, 0.02 mmol).

All components were dissolved individually in 1 mL of DMSO, then added to 50 mL double-distilled ultrapure water. Afterwards, 50 mL of this polymerization solution was degassed with nitrogen and sonicated for 5 min and added to 60 g of glassbeads@insulin. Polymerization was initiated by addition of 0.5 mL of (60 mg/mL) ammonium persulfate and (30 µ L/ mL) TEMED. The polymerization was carried out at room temperature for 2 h. The polymerization was stopped by the addition of 50 mg of sodium nitrite and gassing with oxygen for 5 min. Then, the glass beads were transferred into a solid phase extraction (SPE) cartridge fitted with polyethylene frit (20 µm porosity). Unreacted monomers and other low-affinity materials were removed by eluting with cold water at 4°C (5 x 50 mL).

Subsequently, the cartridge was filled with 20 mL of ethanol and warmed up in a water bath to 60 °C for 10 min. Successively, the remaining fractions of high-affinity nanoMIP were collected by eluting with water at 60°C (4 x 10 mL). The collected nanoMIP solution was evaporated and reconstituted in water. The concentration was then calculated, for that, nanoMIP were lyophilized and the resulting solid weighted, then reconstitute in distilled water.

To remove the remaining insulin, nanoMIPs were subjected to a trypsin treatment (220 µg in 10 ml of PBS) for 72 h at 30 °C. After trypsinolysis, residues were eliminated by dialysis. For that, samples were transferred to a dialysis membrane tube with a cut-off of 50 kDa, dialysed for 72 h in 1.5 L of water and water was changed every 24 h. The nanoMIPs were then collected by magnetic decantation.

Purification of nanoMIPs: the purification was performed using a dialysis membrane (Spectra/Por 7 (Regenerated Cellulose, Ref. 132119, 10 kD MWCO, 11 cm Tubing Length, 32 mm Flat-width, 20.4 mm diameter, 3.3 mL/cm). NanoMIPs were dissolved in water (maximum concentration of ethanol tolerate is 10% for dialysis membranes). After, sonicated for 5 min. Subsequently, a dialysis tubing membrane was conditioned by washing with distilled water, then filled with the water and incubated in 0.5 L of water for 5 min. Subsequently, 10 mL nanoMIP solutions were transferred into the dialysis membrane and incubated in a constant water flow (50 mL / 3 min) in a 0.5 L volume for 2 h, dialysis was monitored using UV-vis spectroscopy. Then, nanoMIPs were lyophilized and stored at 4°C.

Synthesis of supermagnetic iron oxide nanoparticles (MNP): superparamagnetic iron oxide nanoparticles (MNP, Fe_3O_4) were prepared by a precipitation method. For that, $FeSO_4{\cdot}7H_2O$ (5.0 g, 18 mmol) of and $FeCl_3{\cdot}6H_2O$ (9.7 g, 36 mmol) were dissolved in 100 mL of water. The solution was added dropwise (5 mL/min) into a stirred (150 rpm) aqueous solution of ammonia (28% (w/w), 250 mL) at 90 °C. After the addition was completed, the mixture was agitated for 30 min at 90°C, the reaction lead to a change of colour from orange to black, then the crude was let to cool down at room temperature. Afterwards, magnetite nanoparticles were collected via magnetic decantation and washed (3 times × 50 mL) with water, 10 mM PBS solution and acetone and then dried under vacuum.

Silanization of MNP, incorporation of amino group (MNP@DAMO): the protocol was adapted from previous reports.[35] Firstly, 1 g of MNP was degassed under nitrogen, then dispersed by sonication (5 min) in a 6 % v/v (180 µL per mL) of DAMO and (10 µL per mL) 0.34 % v/v of BTSE stirring solution in toluene (45 mL per g of MNP), silanized by reflux during 30 min. Subsequently, the system was cooled down and MNP were washed (3 times × 50 mL) with pure toluene and acetone, after that collected via magnetic decantation, and then dried using a vacuum chamber for 1 h. The Kaiser test was used to confirm the silanization process was completed.

LTE4 immobilization on MNP (MNP@LTE4): 500 mg of MNP@DAMO were incubated for 2 h in 20 mL of 7% (v/v) glutaraldehyde solution (in 0.01 M PBS, pH 8.5). Then, functionalized MNP were decanted magnetically and filtered using a vacuum pump and washed with 5 mM PBS. Afterwards, functionalized MNP were incubated overnight in 20 mL of a solution 0.05 mg/mL of LTE4 in DMF. The resulting LTE4 coupled to MNP (MNP@LTE4) were washed and collected via magnetic

decantation. These were subsequently incubated in a solution of 1 mg/mL sodium cyanoborohydride in 5 mM PBS for 30 min at room temperature. Finally, MNP@LTE4 were collected magnetically, rinsed with ultrapure water, acetone, and then dried under vacuum and stored at 4 °C. Afterwards, MNP@LTE4 (1 mg/mL) were incubated in 1% BSA in water for 30 min, then sonicated (5 min). The excess was removed by washing with distilled water.

Silanization of MNP, incorporation of acryloyl group (MNP@TMSMA): Firstly, 1 g of MNP were degassed under nitrogen, then dispersed via sonication (5 min) in a 6 % (v/v) (180 µL per mL) of TMSMA and 0.34 % v/v (10 µL per mL) of BTSE stirring solution in toluene (45 mL per g of MNP), after that, silanized by reflux over 8 h. Subsequently, the system was cooled down and the resulting MNP@TMSMA were washed (3 × 50 mL) with toluene and acetone, collected via magnetic decantation, and dried using under vacuum for 1 h.

Solid phase synthesis of Insulin imprinted magnetic nanoparticles (MNP@NanoMIP): The polymerisation mixture comprised MNP@TMSMA (60 mg), EGMP (16 mg, 0.08 mmol), NIPAM (39 mg, 0.34 mmol), of MBA (52 mg, 0.34 mmol), allylamine (9 mg, 0.15 mmol), itaconic acid (15 mg, 0.08 mmol),. MNP@TMSMA were solubilized in 5 mL of DMSO and 10 µL of Tween 20. All monomers were dissolved individually in 2 mL of water. All components were then mixed and diluted to a total of 50 ml of water. Subsequently, the mixture was degassed with nitrogen and sonicated for 5 min and added to 60 g of glassbeads@insulin. Polymerization was initiated by the addition of ammonium persulfate (0.5 mL, 60 mg/mL) and (30 µL/ mL) TEMED and carried out for 2 h at room temperature. Subsequently, the polymerization was stopped by adding 50 mg of sodium nitrite and gassing with oxygen for 5 min. Then, the glass beads were transferred into a solid phase extraction (SPE) cartridge. Unreacted monomers and other low-affinity materials were removed by eluting with cold water at 4°C (5 x 50 mL).

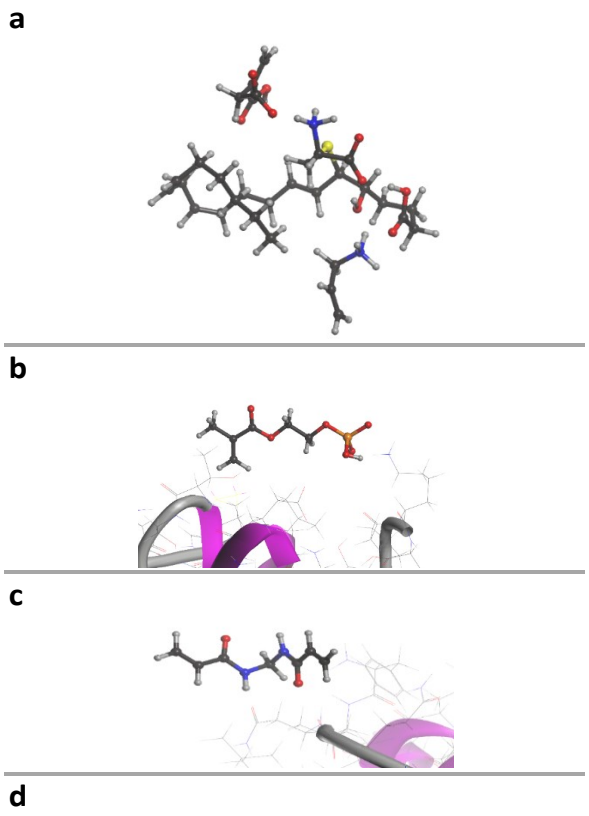
The cartridge was then filled with 20 mL of ethanol and warmed up in a water bath at 60 °C for 10 min. The remaining fractions of high-affinity MNP@nanoMIPs were collected by eluting with water at 60°C (4 x 10 mL). The solution was magnetically decanted and the resulting solid was dried under nitrogen and weighed, then reconstituted in distilled water.

Results and discussion

Molecular modelling and selection of monomers

Molecular modelling of imprinted polymers, which was pioneered by our group, is based on the screening and selection of functional monomers using molecular mechanics.[20] This approach is especially important for the design of the multicomponent polymeric mixture. Computational simulations and screening methods[29] were used here for the selection of monomers suitable for the specific recognition of LTE4 and insulin. Targets were screened against a database of 30 commonly used functional monomers for MIP synthesis. This screening was based on an automated process employing the Leapfrog algorithm (Tripos, USA) available with the Sybyl 7.3 molecular modelling package.[30] According to the molecular modelling results, a polymer formulation comprising allylamine and itaconic acid is favourable for the interaction with LTE4 (Figure 2a). The simulated recognition cavity showed a binding interaction with -79.76 kcal/mol energy between LTE4 and allylamine and -71.29 kcal/mol for the interaction of LTE4 and itaconic acid.

The insulin epitope mapping was performed by modelling computationally the crystal structure.[25, 26] Firstly, water molecules were removed from the insulin structure (3I40) and external residues were identified. For that purpose, insulin residues were initially examined to discard those that were internal or structural to the protein. After that, the protein was screened against a database of common functional monomers used in MIP synthesis using the previously described automated Leapfrog method.[24] The database used consisted of 44 monomers and the screening was performed for 200,000 iterations. Only exposed external residues were considered for this interaction, with the intention of identifying specific Insulin epitopes. The strongest interactions were obtained for Ethylene glycol methacrylate phosphate (EGMP), Allylamine and Itaconic acid (ITA) monomers as shown in Figure 2b-d.



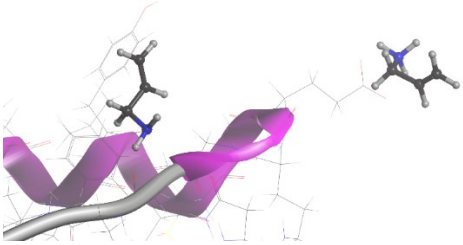


Figure 2. Molecular modeling for (a) LTE4 binding interaction with allylamine and itaconic acid. Insulin interaction with (b) EGMP, (c) MBA and (d) allylamine monomer.

The highest binding energy was exhibited by ethylene glycol methacrylate phosphate (EGMP) interacting with H and K external amino acid residues with a binding score of -526.6 kJ mol^{-1} and N,N'-methylenebisacrylamide (MBA) interacting with N and Q external residues with a binding score of -307 kJ/mol^{-1}. Two allylamine residues also scored -488.8 kJ mol^{-1} due to interactions with D and E external residues as shown in Figure 2d.

Solid phase synthesis of nanoMIPs

Glass beads were silanized and modified with glutaraldehyde for covalent immobilization of the analyte (Figure S2). The analyte immobilized on the solid phase serves as a template for the polymer imprinting process. The composition of the monomeric mixture was designed using molecular modelling.[31] Polymerisable fluorescein was added to the monomeric mixture to render the nanoMIPs fluorescent.

The nanoparticles were synthetized using controlled free radical polymerisation. The polymerization was performed in presence of the functional monomers, cross linkers, initiator and template-modified glass beads. All non-reacted monomers and low affinity nanoparticles were removed with solvent wash at a low temperature (20 °C). Afterwards, a high affinity nanoparticles were eluted at 60 °C. The purification of nanoMIPs is based on affinity separation - one of the key advantages of the solid phase synthesis approach. Additional purification of the nanoMIPs from the free fluorescein was conducted using dialysis and ultrafiltration.

NanoMIPs binding affinity and specificity

Surface Plasmon Resonance (SPR) was employed to evaluate the affinity and specificity of the nanoMIPs for analytes and interferences. The SPR response of specific LTE4-nanoMIPs was evaluated for LTE4, LTD4 and LTB4 (Figure S3, Figure S4a and Table 1). NanoMIPs had high specificity and satisfactory affinity to LTE4 with Kd = 26.3 nM (Chi^2= 3.1×10^{-5}). SPR results reveal that the relative cross-reactivity was estimated at 1.6 % and 39% for LTB4 and LTD4, respectively. These results are attributed to the structure homology of these compounds (Table 1).

Table 1. SPR response of nanoMIPs imprinted with LTE4

Molecule	Molecular weight (gr/mol)	Structure homology (%)	Kd in nM (Chi^2)	Cross reactivity
LTE4	439.6	-	26.3 (3.1×10^{-5}).	100%
LTB4	496.7	89%	1600 (86).	1.6%%
LTD4	336.5	77%	66 (0.52).	39.8%

Similarly, SPR analysis for nanoMIPs specific for insulin demonstrated high affinity and selectivity with a Kd = 0.74 nM (Chi^2=3.3) as shown in Figure S4b, Figure S5 and Table 2. The cross reactivity was found at 25% and 0.7%, for the insulin-like growth factor 1 (IGF-1) and human proinsulin C-peptide (HPC), respectively. Interaction between the nanoMIPs and IGF1 was caused by some degree of sequence homology (at 49%) between IGF1 and the insulin.

Table 2. SPR response for NanonoMIPs specific to Insulin

Molecule	Molecular weight (kDa)	Sequence homology (%)	Kd in nM (Chi^2)	Cross reactivity
Insulin	5.8	-	0.74 (3.3).	100%
IGF1	7.6	49	2.96 (3.2).	25%
HPC	3.6	0	110 (1.7).	0.7%

Competitive assay for LTE4

In competitive assay all components (nanoMIPs, analyte and MNP@LTE4) were mixed and added to the well. Consequently, the assay is based on the competition between the free analyte (LTE4) and the MNP@LTE4 for the nanoMIPs binding sites **(Figure 3)**. The assay is based on the change in the fluorescent directly related to the concentration of the analyte.

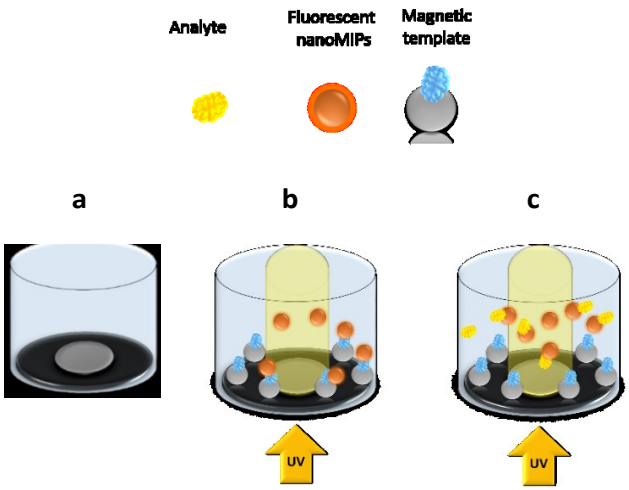


Figure 3. Competitive assay based on molecularly imprinted nanoparticles particles (MINA) and magnetic nanoparticles on microtiter plates fitted with magnetic inserts. (a) Magnetic insert in a well, (b) fluorescent nanoMIPs and magnetic template are added to the buffer solution. (c) The analyte is added to the well and fluorescent signal is measured.

Size estimation by DLS and microscopy characterization

Size of nanoMIPs was assessed using Dynamic Light Scattering (DLS), nanoparticles morphology and surface topography of the microplates was analysed using transmission electron microscopy (TEM) and atomic force microscopy (AFM), respectively. DLS measurements reveal an average diameter of LTE4 nanoMIPs as 517 ± 28 nm and a polydispersity index (PDI) at 0.307, indicating size homogeneity. The diameter of insulin nanoMIPs was calculated as 496 ± 2 nm with a PDI at 0.336. TEM analysis suggest that nanoMIPs and MNP are spherical particles (Figure S6a-b). The MNP size was measured as 40.6 ± 8 nm as shown in Figure S6c. The surface of the magnetic insert settled on the microplate well was characterize using AFM and optical microscopy on completely dried samples Figure S7. The naked insert presented a rough porous surface (5.2 ± 3 nm). After deposition of MNP the surface presented aggregates, the roughness was found as 48.5 ± 8 nm. Then, the complex of MNP-NanoMIP on the magnetic insert was analysed, as result agglomerates are present and the roughness was measured as 220.5 ± 68.5 nm.

Cross-reactivity and selectivity of the competitive MINA

The concentration of LTE4 and assay cross-reactivity was evaluated in competitive assay. The LTE4 assay response displayed a linear (R^2=0.98) response for LTE4 and negligible response for LTD4 and LTB4 as shown in Figure 4a. The assay response confirmed previously observed SPR results (Table 1).

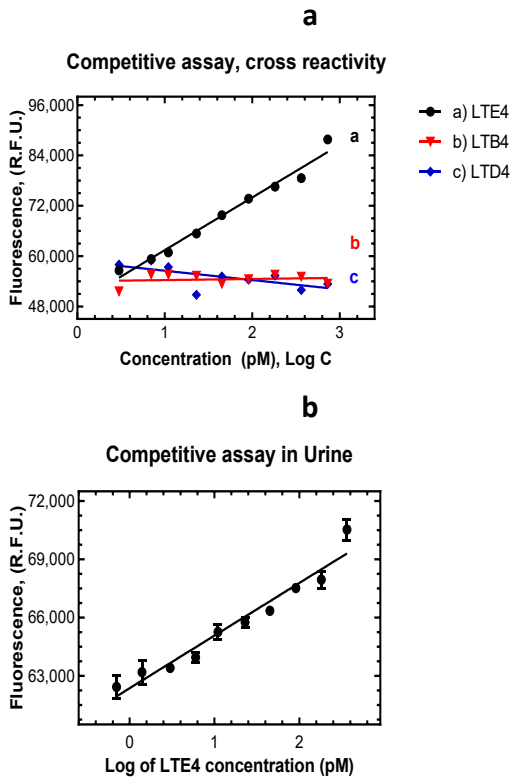


Figure 4. Competition assay response in (A) for (a) LTE4, (b) LTB4 and (c) LTD4 in buffer. The assay was performed using 100 µL of leukotrienes (1.1 to 730 pM per well). In (B) logarithmic assay response in spiked artificial urine evaluated in the concentration range from 0.45 to 730 pM of LTE4. The assay involved 100 µL of spiked urine. Assays were performed using 50 µL of 5 mM PBS buffer (pH=8.0), 50 µL of nanoMIPs (3.8 mg/mL per well) and 5 µL of MNP@LTE4 (0.4 mg/mL per well) in microplates with magnetic inserts, 45 min incubation and 3 replicates.

Assay performance in urine.

The performance of assay and interference of matrix has been tested using artificial urine (Pickering Laboratories Inc., U.S.A.). A linear regression analysis of fluorescence response for LTE4 (Log C) provided linearity, limit of detection (LOD) and limit of quantification (LOQ).[32] The LOD was calculated at 0.24 pM and LOQ measured at 1.02 pM in a LTE4 concentration range of (0.45 to 364 pM) as shown in Figure 4b. The competitive assay demonstrated applicability in clinically relevant concentrations as shown in Table 3.

Table 3. Optimal assay conditions for LTE4 quantification

Sample	Urine
*Linear range (pM)	0.45 to 364
Sensitivity (R.F.U./ pM)	2,707±220
*Linearity (R^2)	0.95
LOD, pM (S/N=3)	0.24
LOQ* (pM)	1.02
*Cross reactivity %	LTE4 (100 %) LTB4 (2.1 %) LTD4 (15 %)

*Experimental conditions: Competitive assay, 100 µL of sample, 50 µL of 5 mM PBS buffer (pH=8), 50 µL of nanoMIPs (3.8 mg/mL per well) and 5 µL of MNP@LTE4 (0.4 mg/mL per well), 45 min incubation.

MINA sandwich assay for insulin

Due to the relatively large size of insulin, the MINA could be performed in a sandwich format using MNP@nanoMIPs and fluorescent insulin imprinted nanoMIPs. Herein, both MNP@nanoMIPs and nanoMIPs contain binding sites for insulin, and could both bind at once forming a stable complex (sandwich) as shown in Figure 8. In this assay, MNP@nanoMIPs are added to microplates with magnetic inserts. Subsequently, fluorescent nanoMIPs and insulin are added. The mixture is incubated allowing formation of a complex nanoMIPs-insulin-MNP@nanoMIPs attracted to magnetic inserts. The assay response is measured as a decrement of the fluorescent signal proportional to the concentration of Insulin (Figure 5).

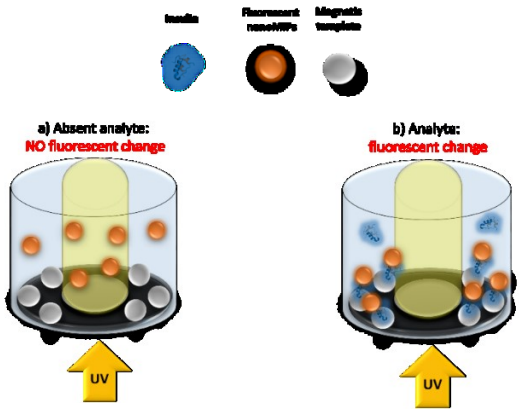


Figure 5. Sandwich MINA assay based on magnetic insulin imprinted nanoparticles (MNP@NanoMIPs) and fluorescent insulin imprinted nanoparticles (NanoMIPs) performed in microplates with magnetic inserts. (a) MNP@NanoMIPs and nanoMIPs in the well in the absence of insulin; (b) MNP@NanoMIPs and nanoMIPs in the well in the presence of insulin.

Performance of sandwich assay in plasma

The cross reactivity of the assay was evaluated by measuring assay response to insulin, potential interferent (BSA) and structurally related molecules with a different amino acid sequence (e.g. IGF1 and HPC) in plasma (Figure 6a and Table 4).

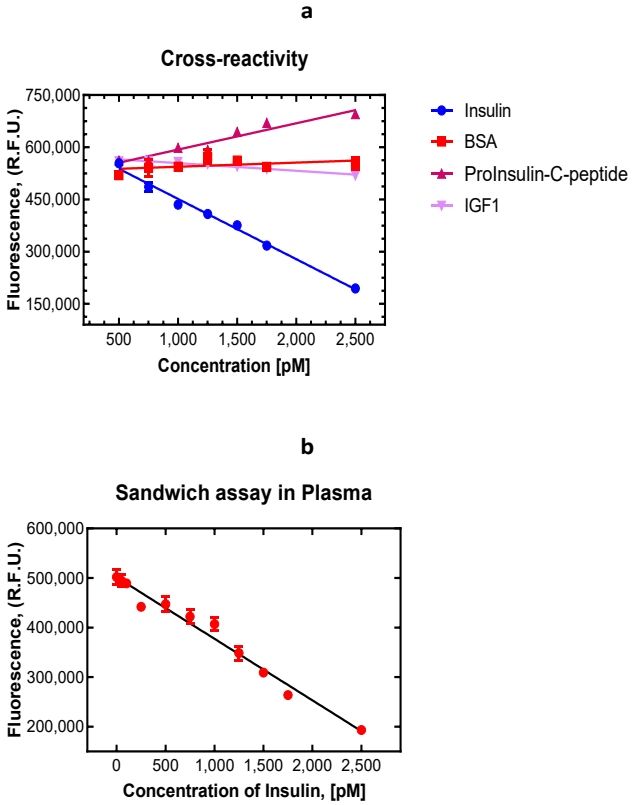


Figure 6. (a) Sandwich assay response to insulin, BSA, IG1, pro-Insulin and C-peptide in a concentration range of 500-2000 pM. The assay comprised 100 µL of the analyte solution, 50 µL of fluorescent nanoMIPs (5.5 mg/mL), 50 µL of MNP@NanoMIPs (2.7 mg/mL) and 100 µL of PBS, pH 8.0; b) Calibration curve for insulin (0-2000 pM) in plasma. All measurements are made in 3 replicates after 20 min incubation.

These results demonstrated negligible cross-reactivity for BSA (<6.4 %) and pro-Insulin-C-peptide (<11 %). Some degree of cross-reactivity was found for IGF1 (<30%) due to its sequence similarity (49% homology). These results are in agreement with the SPR results previously obtained (Table 2). The assay presented high selectivity for insulin in plasma in the linear range 500-2500 pM. The assay presented a satisfactory LOD at (27 pM) and LOQ at (41 pM) in a clinically relevant linear range from (25 to 2500 pM). The results in Figure 6b, demonstrate the applicability of the assay in plasma.

Table 4. Sandwich assay response to Insulin and related molecules

Analyte	Insulin	BSA	IGF1	HPC
MW(kDa)	5.8	66.5	7.6	3.6
Number of AA	51	583	70	35
AA Sequence Homology (%)	-	0	49	<11
Isoelectric pH	5.4	4.9	7.5	3.1
R^2	0.99	0.22	0.95	0.92
% Cross reactivity	100	6.4	30.3	11

Comparison with commercial assays

A significant challenge for measuring biomarkers is the availability of an appropriately sensitive and specific analytical method. To overcome these problems the use of MINA is proposed. The main advantage of MINA is the use of nanoMIPs, which are fabricated with a synthetic process over a short time frame and without the involvement of animals. These polymers are an alternative to replace traditional antibodies in conventional ELISA, overcoming poor reproducibility, costly and long manufacturing, ensuring a high sensitivity and specificity (Table 5).

Table 5. Comparison of the CysLTs assay in urine

	ELISA kit Detect all CysLTs (LTC4, LTD4, and LTE4)	MINA Selective to LTE4 (Customizable)
Origin	Animal: Bovine, Eel, Mouse	Polymers
Production time	6 months	2 weeks
Biohazards	Biosafety lab level 2 required	N/A
Sterilisation	sometimes not possible	UV, autoclave
Storage temperature	-80°C	4°C
Stability	≥ 6 months	≥ 12 months
Detection limit	40.5 pg/mL	0.32 pg/mL
Detection range	8.6-2,500 pg/ml	0.2 to 160 pg/mL
Cross rectivity to related components	Yes	No
Ease to use	12 steps protocol	3 steps protocol
Detection time	20 h	45 min
Price	£ 350-550	£ 50 (estimated)

Also, traditional ELISA assays present various disadvantages such as the need for refrigeration, short shelf life, variability, elaborate protocols and high cost of the assay. The MINA offers comparative advantages such as a rapid response, user friendly and short protocols, reduced use of reagents, long shelf life, easy storage, thermal stability, portability, low cost, and production without the involvement of animals. Additionally, the sensitivity and high selectivity are clinically relevant for an accurate medical diagnosis. Herein, the MINA assay has shown comparable performance to existing chromatography methods and immunoassays for LTE4 and insulin detection in urine and plasma samples, respectively. A comparative example between MINA and ELISA is displayed in Table 5.

Conclusions

A generic, abiotic high performance assay was developed. As a proof of concept, this was applied for the quantification of leukotrienes and insulin concentrations in urine and plasma. Fluorescent nanoMIPs for LTE4 and insulin were prepared using molecular modelling and solid phase synthesis. Their high affinity and low cross-reactivity were demonstrated using SPR. These nanoMIPs together with functionalized magnetic nanoparticles were used successfully in molecularly imprinted polymer nanoparticle based assays (MINA) in magnetic microplates. MINA demonstrated comparable performance for the LTE4 and insulin detection in artificial urine and blood plasma correspondingly with existing LC-MS/MS methods in clinically relevant concentrations. The present technology allows the production of a low cost assay, free of animal products, with a simpler and faster optimization protocol compared to standard immunoassays. The novel assay format requires fewer manipulations steps than standard immunoassays.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was funded in part by a grant from AstraZeneca (CW544005).

Notes and references

‡ Please check supplementary material

1. S. S. Piletsky, A. E. Cass, E. V. Piletska, J. Czulak and S. A. Piletsky, *ChemNanoMat*.
2. K. Smolinska-Kempisty, A. Guerreiro, F. Canfarotta, C. Cáceres, M. J. Whitcombe and S. Piletsky, *Scientific reports*, 2016, **6**, 37638.
3. K. Smolinska-Kempisty, O. S. Ahmad, A. Guerreiro, K. Karim, E. Piletska and S. Piletsky, *Biosensors and Bioelectronics*, 2017, **96**, 49-54.
4. E. Mazzotta, A. Turco, I. Chianella, A. Guerreiro, S. A. Piletsky and C. Malitesta, *Sensors and Actuators B: Chemical*, 2016, **229**, 174-180.
5. F. Canfarotta, J. Czulak, A. Guerreiro, A. G. Cruz, S. Piletsky, G. E. Bergdahl, M. Hedström and B. Mattiasson, *Biosensors and Bioelectronics*, 2018, **120**, 108-114.
6. S. S. Piletsky, S. Rabinowicz, Z. Yang, C. Zagar, E. V. Piletska, A. Guerreiro and S. A. Piletsky, *Chemical Communications*, 2017, **53**, 1793-1796.
7. D. López-Puertollano, T. Cowen, Á. García-Cruz, E. Piletska, A. Abad-Somovilla, A. Abad-Fuentes and S. Piletsky, *ChemNanoMat*, 2019, **5**, 651-657.
8. H. Munawar, K. Smolinska-Kempisty, A. G. Cruz, F. Canfarotta, E. Piletska, K. Karim and S. A. Piletsky, *Analyst*, 2018, **143**, 3481-3488.
9. L. Cenci, M. Bertolla, A. Anesi, E. Ambrosi, G. Guella and A. M. Bossi, *Analytical and bioanalytical chemistry*, 2017, **409**, 6253-6261.
10. L. Cenci, R. Tatti, R. Tognato, E. Ambrosi, C. Piotto and A. M. Bossi, *European Polymer Journal*, 2018, **109**, 453-459.
11. R. Schirhagl, U. Latif, D. Podlipna, H. Blumenstock and F. L. Dickert, *Analytical chemistry*, 2012, **84**, 3908-3913.
12. W.-H. Zhou, C.-H. Lu, X.-C. Guo, F.-R. Chen, H.-H. Yang and X.-R. Wang, *Journal of Materials Chemistry*, 2010, **20**, 880-883.
13. A. Rachkov and N. Minoura, *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 2001, **1544**, 255-266.
14. M. Wadhwa, I. Knezevic, H.-N. Kang and R. Thorpe, *Biologicals*, 2015, **43**, 298-306.
15. S. Piletsky, S. Rabinowicz, Z. Yang, C. Zagar, E. V. Piletska, A. Guerreiro and S. A. Piletsky, *Chemical Communications*, 2017, **53**, 1793-1796.
16. E. V. Piletska, S. S. Piletsky, A. Guerreiro, K. Karim, M. J. Whitcombe and S. A. Piletsky, *Journal of the Chinese Advanced Materials Society*, 2014, **2**, 118-129.
17. S. A. Piletsky, O. Piletska, A. Guerreiro, A. Poma, K. Karim and S. Piletsky, *Journal*, 2015.
18. L. Basabe-Desmonts, D. N. Reinhoudt and M. Crego-Calama, *Chemical Society Reviews*, 2007, **36**, 993-1017.
19. J. Xu, K. Haupt and B. Tse Sum Bui, *ACS Applied Materials & Interfaces*, 2017, **9**, 24476-24483.
20. R. Murphy, J. Maclouf and P. Henson, in *Eicosanoids and Other Bioactive Lipids in Cancer and Radiation Injury*, Springer, 1991, pp. 9-14.
21. J. L. Goulet, R. S. Byrum, M. L. Key, M. Nguyen, V. A. Wagoner and B. H. Koller, *The Journal of Immunology*, 2000, **164**, 4899-4907.
22. R. J. Santen, P. W. Willis and S. S. Fajans, *Archives of internal medicine*, 1972, **130**, 833-843.
23. G. Wilcox, *Clinical biochemist reviews*, 2005, **26**, 19.
24. S. A. Piletsky, K. Karim, E. V. Piletska, C. J. Day, K. W. Freebairn, C. Legge and A. P. F. Turner, *Analyst*, 2001, **126**, 1826-1830.
25. F. Sanger, *Science*, 1959, **129**, 1340-1344.
26. D. Nicol and L. Smith, *Nature*, 1960, **187**, 483.
27. V. I. Timofeev, R. N. Chuprov-Netochin, V. R. Samigina, V. V. Bezuglov, K. A. Miroshnikov and I. P. Kuranova, *Acta Crystallogr Sect F Struct Biol Cryst Commun*, 2010, **66**, 259-263.

28. H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov and P. E. Bourne, *Nucleic acids research*, 2000, **28**, 235-242.
29. A. R. Leach, *Molecular modelling: principles and applications*, Pearson education, 2001.
30. T. Cowen, K. Karim and S. Piletsky, *Analytica chimica acta*, 2016, **936**, 62-74.
31. F. Canfarotta, A. Poma, A. Guerreiro and S. Piletsky, *Nature protocols*, 2016, **11**, 443.
32. D. A. Armbruster and T. Pry, *The Clinical Biochemist Reviews*, 2008, **29**, S49.

Supporting Information

Molecularly Imprinted Nanoparticle-Based Assay (MINA) – Detection of Leukotriene and Insulin

Alvaro Garcia-Cruz, Todd Cowen, Annelies Voorhaar, Elena Piletska, Sergey A. Piletsky*

Dept. of Chemistry, University of Leicester, University Road, LE1 7RH, Leicester (UK)

Corresponding author emaill: agc14@leicester.ac.uk

1. Microplate inserts

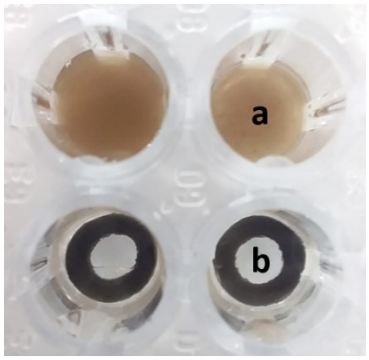


Figure S1. Microplate titter with 100 µL of MNP@LTE4 solution (0.4 mg/mL per well) (a) without and (b) with a magnetic insert. The MNP@LTE4 are suspended in solution (cloudy) without magnetic insert. When a magnetic insert is used, MNP@LTE4 are attracted to the bottom of the well and the solution is transparent.

2. Solid phase preparation.

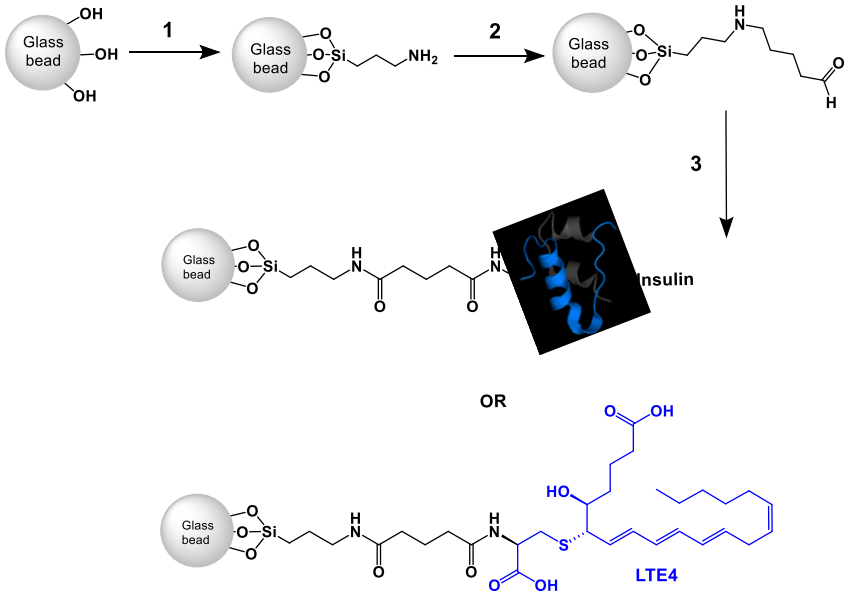


Figure S2. Preparation of the solid phase comprise activation, (1) silanization, (2) modification of glass beads using glutaraldehyde, and (3) analyte immobilization.

3. SPR experiments

The SPR response of specific LTE4-nanoMIP was evaluated against LTE4, LTD4 and LTB4 and the Kd calculated (Figure S3). The concentrations range of the injected leukotrienes ranged from 0.023 nM to 297 nM. The Kd for LTE4 was found 26 nM, which signifies a high affinity. Besides, LTB4 present a Kd of 1.6 µM (low affinity) and for LTD4 was 67nM (weak affinity). Moreover, the LTB4 relative cross-reactivity was estimated at 1.6 %. Therefore, the interaction between the nanoMIP towards LTB4 can be neglected (as shown in Figure S3). The LTD4 relative cross reactivity was found 39%. In summary, the LTE4 nanoMIPs have much higher affinity compare to LTB4 and LTD4.

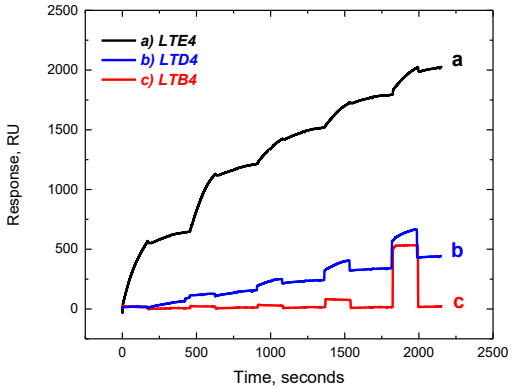


Figure S3. SPR response from NanoMIP towards leukotrienes (LTE4, LTD4 and LTB4). (a) High affinity is measured for the LTE4, (b) low response is observed for LTD4 and almost no response for LTB4.

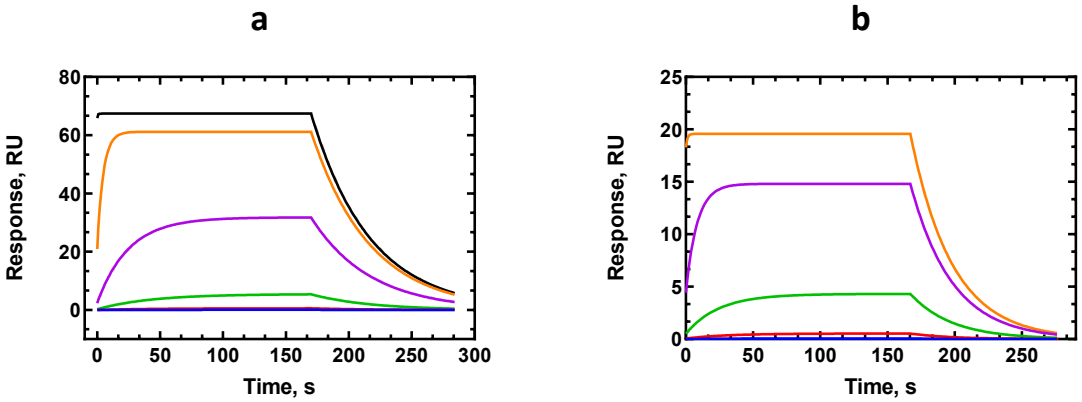


Figure S4. SPR response of corresponding nanoMIPs for (a) LTE4 and (b) insulin. LTE4 solutions were injected in the concentration rage 0.023-2300 nM) and insulin in 0.044-440 nM. All SPR experiments were performed in PBS buffer pH 7.4 at 25 °C.

The interaction of NanoMIP to insulin was found at 100 % with a Kd= 0.74 nM (Chi^2=3.3). The cross reactivity towards the Insulin-like growth factor 1 (IGF-1) was found at 25% interference with a Kd =2.96 nM (Chi^2= 3.2). Similarly, the Human Proinsulin C-peptide (HPC) cross reactivity was found at 0.7 % with a Kd =110 nM (Chi^2= 1.7) as shown in Figure S5. Interaction between the NanoMIP and IGF1 was caused by some degree of sequence homology (at 49%) between IGF1 and the insulin. The sequence homology with IGF1 are located in the A (at 62%) and B (at 40%) chain from insulin.

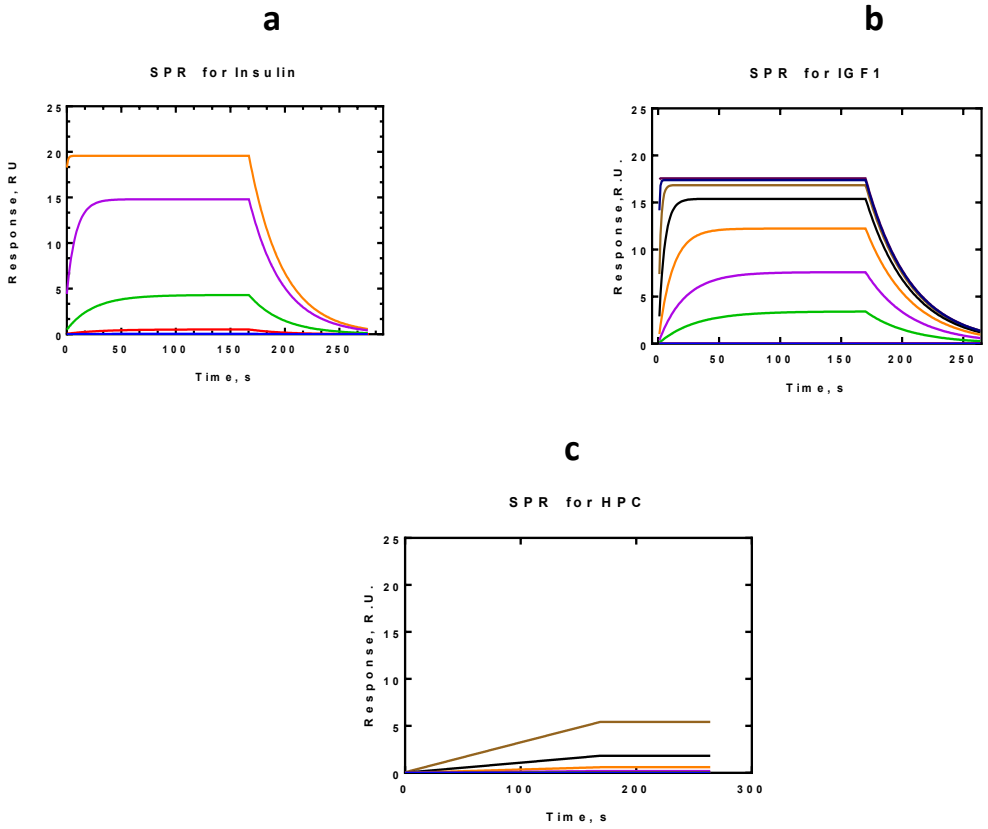


Figure S5. SPR response for NanoMIP to a) insulin, b) IGF and c) HPC in a concentration range of (0.044-440 nm) in PBS.

4. Microscopy characterization.

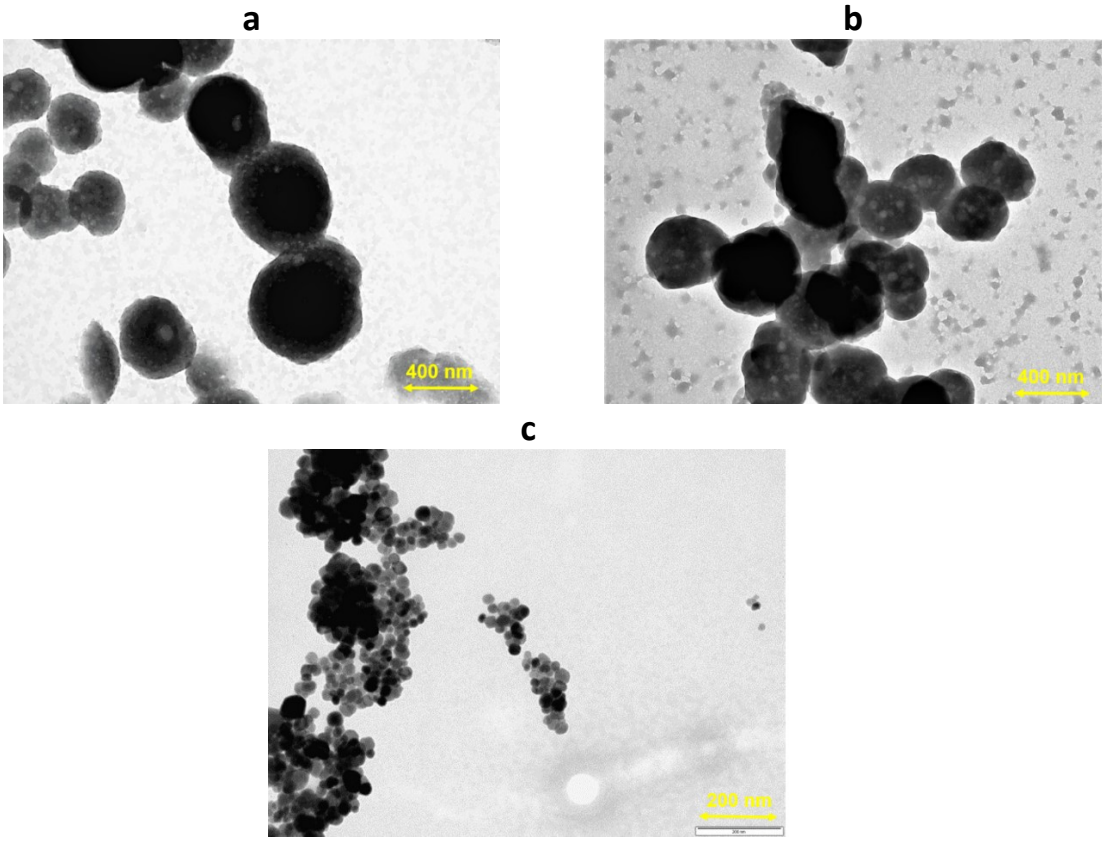


Figure S 6. TEM images obtained for NanoMIP specific for (a) LTE4 and (b) Insulin at 400 nm scale. In (c) image from MNP scale at 200 nm. Samples were viewed on a JEOL JEM-1400 TEM with an accelerating voltage of 100kV. Digital images were collected with a Megaview III digital camera with iTEM software.

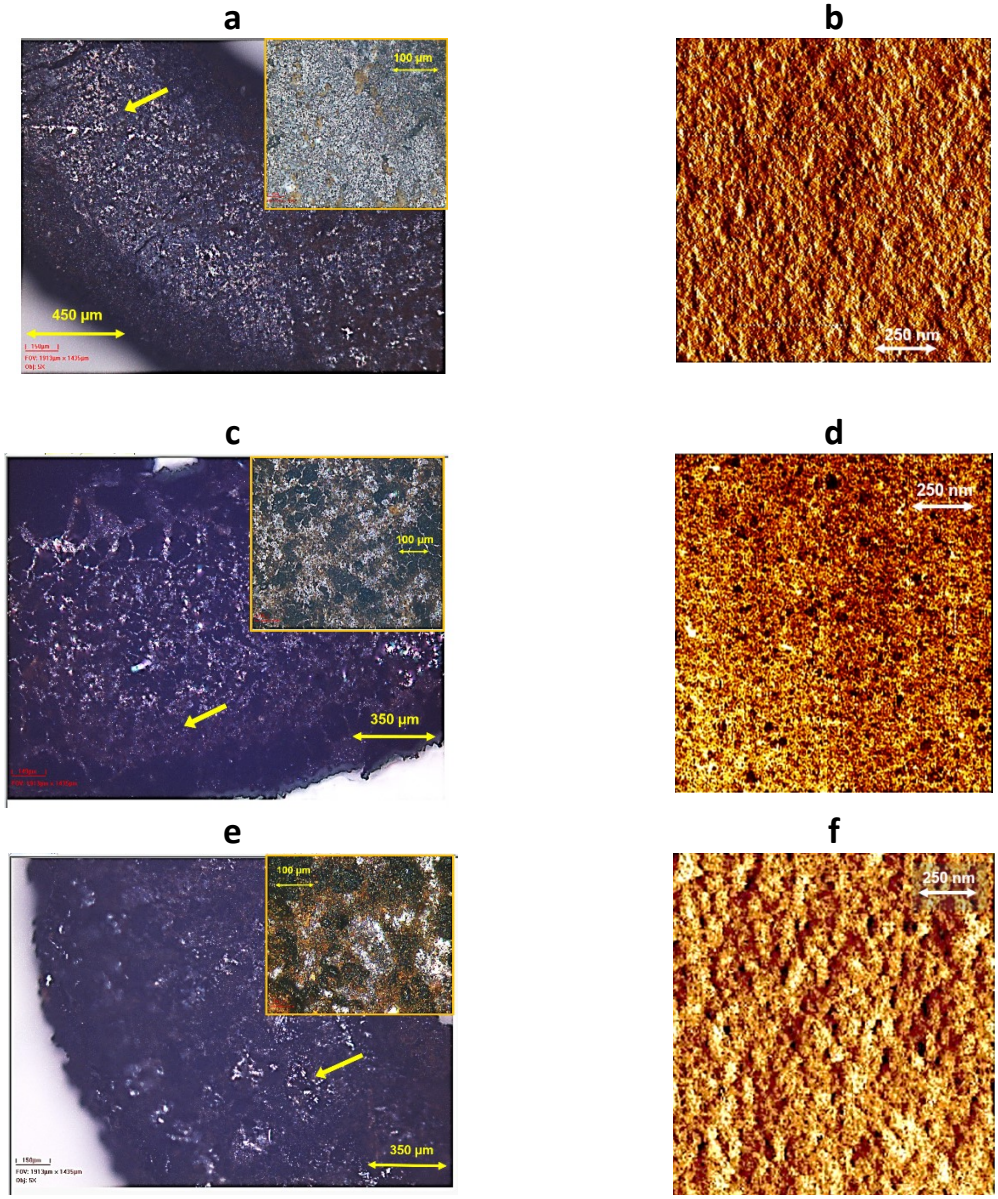


Figure S 7. Optical microscopy and AFM images for a magnetic insert (a & b) naked, (c & d) with immobilized MNP@LTE4 and (e & f) obtained for the complex between NanoMIP and MNP@LTE4. These samples were previously dried under nitrogen at 30^{o}C.

5. Instrumentation

5.1 Characterisation, size and FTIR

The particle size was determined using Dynamic Light Scattering (DLS) using a Malvern instruments, Zetasizer Nano (Nano-S) particle-size analyser from Malvern Instruments Ltd (UK). DLS measurements were perform in a 10 times diluted stock solution (0.01 mg/mL) previously sonicated for 5 minutes. The FT-IR spectra was acquired in a PLATINUM Diamond ATR accessory and INVENIO FTIR spectrometer equipped with the BRUKER FM optical components.

5.2 Microtiter plate preparation using magnetic inserts

96 well clear flat bottom polystyrene microtiter plates were purchased from Costar® and modified with magnetic disk inserts (3/6 mm internal and external diameter). Magnetic disk inserts were fabricated by laser cutting of magnetic sheets (0.5 mm thick, high magnetism=) (brown self-adhesive A4, 210 x 297 x 0.5 mm, with an adhesive force of 80 g/cm^2, purchased from Magnosphere) and placed manually on the bottom of each well.

5.3 Spectroscopy

The UV-Vis spectra of the nanoMIPs was analysed using a UV-1800 spectrophotometer SHIMADZU spectrometry in a sample diluted 10 times (0.01 mg/mL) before the analysis. Microtiter plate measurements were performed using Hidex Sense microtiter plate reader in Fluorescent mode with an excitation and emission at (485 ± 10) and (535 ± 20) nm, respectively, with number of flashes set to 30 and lamp power 200 (high), Flatbed laser model 4060 60W, TS-0034, QA8A-1105. NanoMIP fluorescence was measured using a 96-microtiter plate, 200 µL of nanoMIPs were dispensed in each well and dilutions were made from the stock solution (2.3 mg/mL).

5.4 Surface Plasmon Resonance (SPR)

NanoMIPs affinity and specificity was analysed using Biacore 3000 instrument (GE Healthcare Life Sciences, UK) at 25 °C using PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) as the running buffer at flow 35 µL/min. The self-assembled gold sensor chip (SA) was cleaned using plasma and placed in a solution of

mercaptododecanoic acid in ethanol (2.2 mg/mL) where they were stored until use. Before assembly the sensor chip was rinsed with ethanol and dried in a stream of air.

The equilibrium association and dissociation constants K_A and K_D, respectively, were calculated by the Langmuir binding theory. The K_D is related to the rate of complex formation (described by association rate constant k_{on}) and the rate of breakdown (described by dissociation rate constant k_{off}) such that $K_D=k_{off}/k_{on}$. The association constant (K_A) can be then calculated as K_D^{-1}.

Dissociation constants (K_D) were calculated from plots of the equilibrium biosensor response using the BiaEvaluation v4.1.1 software using a 1:1 binding model with drifting baseline (DB) fitting. The calculation of the dissociation constant was also done using Langmuir Blodgett (LB) algorithm using the AB (absorption) component of the SPR response, which was obtained after the subtraction of the drift and bulk effect.

For leukotriene nanoMIPs analysis: Both leukotriene-specific nanoMIPs were diluted in PBS and then immobilized *in situ* on the chip surface containing carboxyl groups using the EDC/NHS coupling (0.4 mg and 0.6 mg/mL, correspondingly). The leukotriene solutions were diluted in PBS in the concentration range between 0.023 and 2300 nM. Sensorgrams were collected sequentially for all nanoparticles concentrations running in KINJECT mode (injection volume- 100 µL and dissociation time- 120 s). Dissociation constants (Kd) were calculated from plots of the equilibrium biosensor response using the BiaEvaluation v4.1 software using a 1:1 binding model with drifting baseline (DB) fitting. The calculation of the dissociation constant was also done using Langmuir Blodgett (LB) algorithm using the AB (absorption) component of the SPR response, which was obtained after the subtraction of the drift and bulk effect.

For insulin nanoMIPs analysis: The solution of insulin nanoMIPs in PBS (1 mg/mL) was injected and immobilised *in situ* on the chip surface containing carboxyl groups using the EDC/NHS coupling (0.4 mg/mL and 0.6 mg/mL, correspondingly). The insulin was diluted in PBS in the concentration range between 0.044 and 440 nM. Sensorgrams were collected sequentially for insulin concentrations running in KINJECT mode (injection volume- 100 µL and dissociation time- 120 s).