Article type: Full Paper

A novel assay format as an alternative to ELISA – MINA test for biotin

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***Keywords*:** molecular imprinting, biotin, ELISA, MINA, abiotic

A novel abiotic assay based on biotin-specific fluorescent molecularly imprinted polymer nanoparticles (nanoMIPs) which acted as both reporter probes and binding agents, was developed. This is a first report of an assay which, unlike ELISA, required no washing steps or addition of enzyme substrates, making it more user-friendly. The components of the molecularly imprinted polymer nanoparticles assay (MINA) were assembled in microtiter plates fitted with magnetic inserts. The fluorescent nanoMIPs were bound to biotin-conjugated magnetic particles, which were attracted to the inserts. The addition of free biotin displaced fluorescent nanoMIPs into solution, generating a signal proportional to the concentration of biotin. The nanoMIPs had a dissociation constant (K*d*) of 14 nM, allowing the assay to detect biotin at nano-molar concentrations. The pre-assembled assay only required the addition of the sample and measurement of the fluorescence, and it functioned well after six weeks of storage without refrigeration. The assay did not show the susceptibility to several compounds which are known to interfere with avidin and streptavidin-based assays, such as mercaptoethanol and sugars. The protocols optimized in this work could be used to develop the abiotic assays for any other compound of interest.

1. Introduction

The detection and quantitation of molecules lies at the core of many industries, including clinical diagnosis, forensics, process control, food and environmental analysis.[1-4] Detection of target molecules is performed using assays based on molecular recognition (for example with antibodies, most commonly in an ELISA format), or using physical techniques such as chromatography, mass spectrometry, IR and NMR spectroscopy, and combinations thereof. These techniques suffer from various limitations: antibodies often denature under non-physiological conditions, whilst chromatographic and spectroscopic techniques require large, expensive equipment. Additionally, ELISA requires multiple steps including washing, blocking, and addition of enzyme substrates. As a result, these techniques require reasonably experienced personnel to operate. There is a need to develop inexpensive and robust assays which do not rely on cold-chain storage, and which can be employed by those with little training.

Biotin is ubiquitous within biochemistry primarily because its interactions with avidin and streptavidin are among the strongest and best known examples of molecular recognition.[5] Biotinylated probes, having been used to label the protein or DNA of interest, can be detected using enzyme conjugated avidin or streptavidin, which forms an extremely strong complex with biotin (Kd = 10^{-13} to 10^{-15} M).[6] There are multiple examples of ELISA being used for the detection of biotin and biotinylated products. These assays take advantage of the high binding affinity between biotin and avidin as a means for the immobilization of biotin. As biological molecules, avidin and streptavidin suffer from the same relatively poor stability in non-physiological conditions as antibodies. Another common tool for the determination of biotin concentration, which can also be performed in a microtiter plate format, is based on 4'-hydroxyazobenzene-2-carboxylic acid (HABA). HABA is a dye capable of forming a complex with avidin, occupying the same binding sites as biotin but with a significantly lower

binding affinity (K_d = 6 x 10^{-6} M) as compared to biotin.[6] This complex absorbs strongly at 500 nm, generating a yellow-orange color. Biotin displaces HABA, and hence the absorbance at 500 nm is inversely proportional to the concentration of biotin in a sample. The HABA-avidin assay suffers from relatively poor sensitivity, working optimally in the 2-16 μM range, and the analysis of biotinylated protein requires proteolysis in order to provide a quantitative measure of biotinylation.

The aim of this work was to develop an abiotic assay for detection of biotin using molecularly imprinted polymer nanoparticles (nanoMIPs) as both binders and reporters. Super paramagnetic iron oxide nanoparticles (SPIONs) conjugated to biotin (BIONs) were attracted to magnetic insert within a microtiter plate well. Fluorescent nanoMIPs bound to the immobilized target molecules, and so were removed from the aperture of the magnetic insert and did not contribute to the fluorescence signal. Addition of free target molecules displaced the bound target molecules, releasing the fluorescent nanoMIPs (Figure 1). The increase in fluorescence was then observed using direct spectroscopic analysis, with no washing steps required.

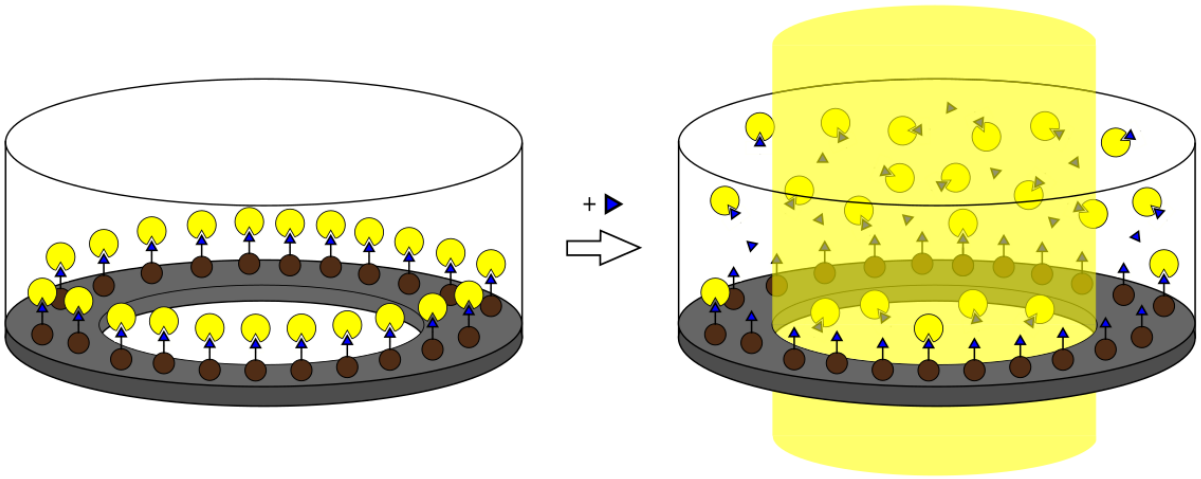

Figure 1. Schematic depicting proposed assay. Fluorescent nanoMIPs (yellow) bind to biotin (blue) immobilized on magnetic nanoparticles (brown). Addition of free target molecules displaces MIP nanoparticles, and the resultant increase in fluorescence is measured through an aperture in the magnetic insert.

2. Results and Discussion

In order to optimize the assay conditions all components were thoroughly characterized. The concentration of amine groups on silanized SPIONs and BIONs was determined using the Kaiser test, which is a common test for primary amines.[7] The limit of silanization described in literature for 5-nm diameter SPIONs was 300 mg APTES·g^{-1} iron oxide.[8] The particles used in this work were approximately 50 nm in diameter, corresponding to a surface area to volume ratio 10 times lower. In this case, a monolayer coverage would result in a predicted limit of 30 mg of APTES·g^{-1} iron oxide. The value observed during the Kaiser test was 19.6 mg APTES·g^{-1} iron oxide (146 μmol g^{-1}), indicating coverage approaching that of a monolayer.

The amine content of BIONs prepared using sulfo-NHS-LC-biotin was found to be 133 μmol g^{-1} SPIONs. This value was 13 μmol g^{-1} lower than that of the original silanized SPIONs, indicating the presence of 13 μmol g^{-1} of biotin. This corresponded to the immobilization of approximately 0.6 mg of sulfo-NHS-LC-biotin on 100 mg of silanized SPIONs; given that 2.3 mg of sulfo-NHS-LC-biotin was used for this reaction, this corresponded to a 26% coupling yield which was found to be sufficient for the purposes of this assay.

Successful biotinylation was also indicated by the appearance of peaks at 1650 and 1540 cm^{-1}, attributed to C=O (amide) stretches and N-H bends, respectively (Supporting Information, Figure 1S).

The size of SPIONs and BIONs was investigated using dynamic light scattering (DLS) and transmission electron microscopy (TEM). TEM images demonstrated that the non-functionalized SPIONs had a diameter of 25-60 nm (Supporting Information, Figure 2S). Suspensions of iron oxide nanoparticles in water were analyzed via DLS following brief sonication. Measurements were performed one minute after sonication. It was found that

immediately after sonication, the nanoparticles began to aggregate. As a result, the size observed via DLS gradually increased, and the following dimensions were indicative of the rate of aggregation rather than individual nanoparticle size (Table 1). Functionalization of the SPIONs surfaces appeared to lower the rate of aggregation, as silanized SPIONs showed less aggregation in the same time frame, and biotinylated SPIONs showed less still.

Table 1. Observed size of SPION and BION aggregates.

Particles	Diameter, nm
SPIONs	367 ± 26
Silanized SPIONs	211 ± 3
BIONs	158 ± 1

The fluorescent biotin-specific nanoMIPs were prepared using solid phase with immobilized biotin and polymerisable fluorescein (FlAm) as described in the Experimental section. The size of the nanoMIPs produced was investigated using DLS. Immediately following sonication, the nanoMIPs showed an average diameter of 230 nm (Supporting Information, Figure 3S).

The emission spectra of aqueous fluorescent nanoMIPs and FlAm were compared. As expected, the fluorescence spectrum of nanoMIPs closely matched that of the fluorescent monomer used in their synthesis, FlAm (Supporting Information, Figure 4S). The fluorescence of a given concentration of fluorescent nanoMIPs was approximately six times greater than that of the same concentration of FlAm. This provided justification for the use of fluorescent nanoMIPs as probes, as opposed to fluorescent dye conjugates, particularly when analyte concentrations were sub-nanomolar (Supporting Information, Figure 5S).

The binding of fluorescent MIP nanoparticles to biotin was analyzed using SPR. The dissociation constant (K_d) for biotin-specific fluorescent nanoMIPs towards free biotin was found to be 14 nM, correcting for drifting baseline (Figure 2).

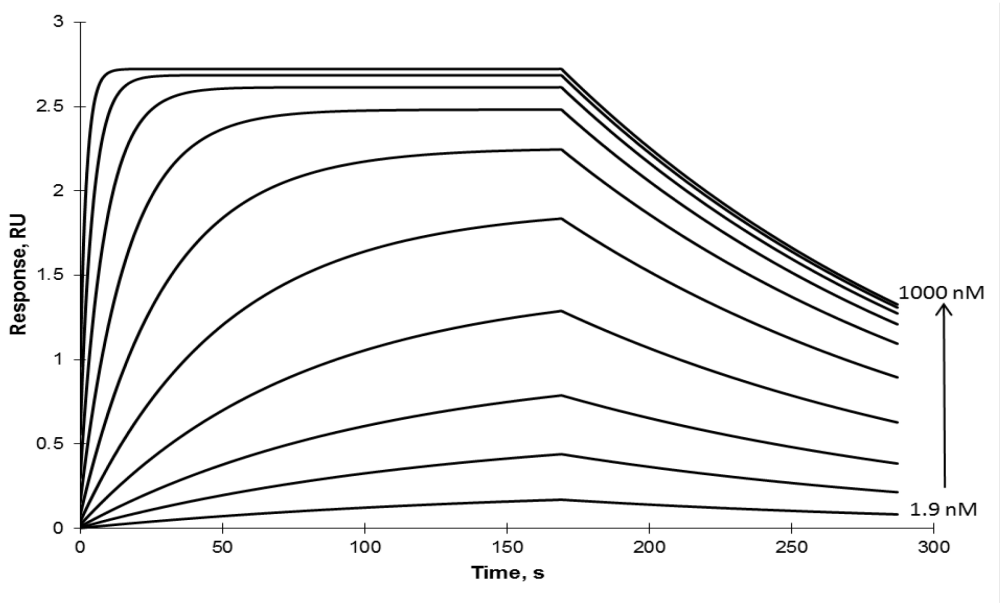

Figure 2. SPR analysis of interaction between nanoMIPs immobilized on the sensor chip and biotin with concentrations between 1.9 nM and 1000 nM (increasing by a factor of two).

By comparison, antigen binding fragments (Fab) typically possess K_d values in the pM - nM range.[9, 10] Monoclonal anti-biotin antibodies have a K_d value of 900 nM.[11] The interaction between biotin and avidin or streptavidin is considerably stronger still, with a K_d value of 10^{-13} to 10^{-15} M.[6] Previously observed K_d values for MIPs were in the nM-μM range, for example, nanoMIPs specific for atrial natriuretic peptide (K_d = 7.3 μM) and vancomycin (K_d = 3.4 nM), and bulk MIPs specific for theophylline (K_d = 0.3 μM) and diazepam (K_d = 65 μM).[12-14]

The level of immobilization of the target or receptor molecules on the surface of microtiter plate wells is one of the most important parameters determining the reproducibility and performance of ELISA and other assays. Typically, antibodies and other proteins are immobilized on the polystyrene surface using physical adsorption. Although this approach works in practice, it is not always possible to achieve a specific level or uniformity of coverage.

One immobilization approach, which has been previously explored, was the use of magnetic microtiter plate inserts.[15] These inserts are disk - shaped pieces of magnetic film

that can be placed into the bottom of a microtiter plate well. They feature an aperture in the center which allows optical measurements to be performed using a standard plate reader. With this format, target molecules (e.g. biotin) can be conjugated to paramagnetic particles, which are immobilized within the microtiter plate simply by adding them to the well and allowing them to be pulled towards the magnetic insert. Relatively large quantity of magnetic particles bearing target molecules can be immobilized, as there is no limitation based on available surface area.

The intended purpose of magnetic inserts is to simplify the immobilization process. Rather than purchasing a microtiter plate with a specific quantity of pre-immobilized target molecules (which may then require refrigeration), a generic magnetic-insert loaded plate can be ordered, and any amount of magnetic target-bearing particles can then be added. By coating the walls of microtiter plate wells with binding agents or target molecules, a surface area of approximately 7.4 x 10^{-5} m^2 is available per well. As described by Fisher Scientific pre-treated microtiter plates used for ELISA have a protein-binding capacity of at least 400 ng cm^{-1}.[16] For a monoclonal antibody (of mass ~150 kDa) this corresponds to a capacity of 2.5 pmol per well. If each antibody binds to a single fluorescent molecule (for example, labelled with a fluorescein derivative), and upon addition of analyte displaces into a volume of 100 µL, this would result in a fluorescent probe concentration of 25 nM.

However, monoclonal antibodies are relatively small compared to nanoMIPs, with a typical diameter of 7 nm.[17] If a nanoMIP has a diameter 100 nm, it will occupy a surface area approximately 200 times greater than that of a monoclonal antibody, resulting in a capacity of only 13 fmol per well. Displacement into a volume of 100 µL would therefore result in a probe concentration of 130 pM, below the limit of detection observed within this work. In this case, even total displacement would not result in a detectable signal. However, as magnetic inserts do not rely on covalent linkage or physical adsorption for target immobilization, they

are not limited by available surface area. 1 mg of 50 nm diameter BIONs possess a total surface area of 0.03 m^2, 400 times greater than that of the wall of one well. If this surface area were used optimally, 5.2 pmol of nanoMIPs can be immobilized. Displacement of these into a volume of 100 µL would result in a detectable probe concentration of 52 nM. This demonstrates the advantages of magnetic inserts and functionalized SPIONs as an immobilization platform, particularly when large particles such as nanoMIPs are used as binders.

Figure 3. Relative binding strength of BIONs to nanoMIPs at different pH values.

It is well established that fluorescein and fluorescein-derivatives such as FlAm are highly sensitive to pH. Fluorescein possesses a maximum fluorescence quantum yield at pH values of 8.0 and above that is 20% higher than at pH 6.5.[18] The assay system must be well buffered to prevent the analyte concentration affecting the pH (and therefore the fluorescence of the nanoMIPs). The binding interactions between BIONs and aqueous fluorescent nanoMIPs at different pH values were investigated. Of the pH values tested, the strongest binding between BIONs and nanoMIPs was found to occur at pH 6.5 (Figure 3), with relatively little dependence on pH (75% or greater binding performance at pH values within the range of 5.5-8.0).

The pH of the monomer mixture during polymerization was determined to be 6.3. As the binding sites form under these conditions, it was expected that deviation from this pH will result in different polymer/target charge distributions which will result in binding being less favorable. It is therefore as expected that the strongest binding would be observed at pH values of approximately 6.3. Furthermore, previous works have found that the optimal ratio of specific to non-specific binding of nanoMIPs occured at approximately pH 6.0.[19] All future assay optimization was performed using Tris-maleate buffer of pH 6.5.

The MINA assay developed here works as follows: BSA-treated BIONs bound to fluorescent nanoMIPs are attracted to magnetic inserts within a microtiter plate well, and so are not detected by the microtiter plate reader. Addition of free biotin results in the displacement of the fluorescent nanoMIPs, and a subsequent increase in fluorescence signal (Figure 1). As described in the Experimental section, this assay was tested with free biotin in a concentration range of 0.1 to 400 nM (Figure 4). Large background fluorescence was observed, which may imply the presence of some amount of non-bound nanoMIPs and could likely be reduced with more thorough washing.

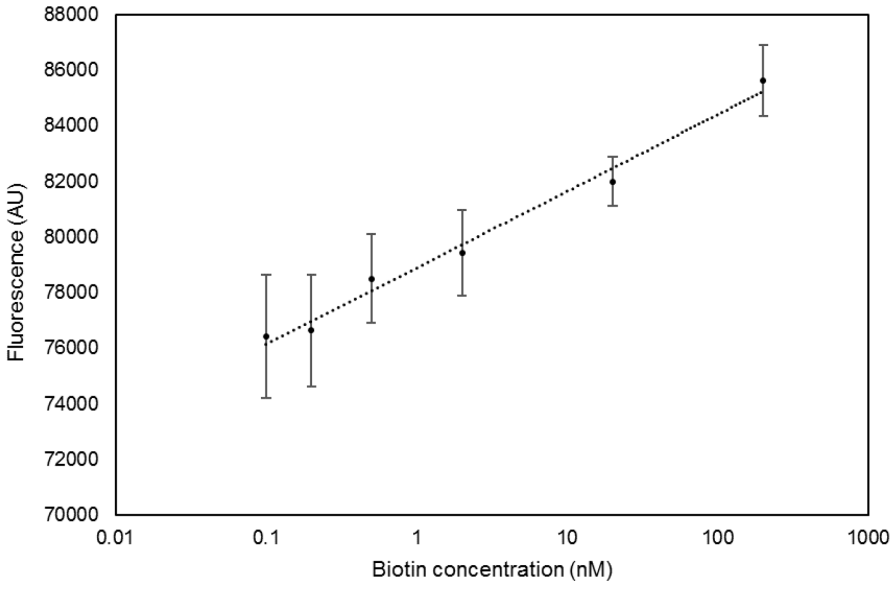

Figure 4. MINA response to free biotin solution in the concentration range between 100 pM and 200 nM.

There was a significant absolute difference in fluorescence between samples with low and high concentrations of biotin and the linear range of the assay was found to be between 200 pM and 200 nM. The limit of detection (LoD) of this assay was approximately 5 nM. The high standard deviation (approximately 1600 AU, 17% of the total increase in fluorescence upon addition of 200 nM biotin) was likely caused by poor distribution of BIONs bound to nanoMIPs prior to dispensing into wells.

Biotinylated and non-biotinylated IgG were also analyzed using the optimized protocol. According to the HABA test, 3 μM polyclonal IgG contained 3 μM biotin. Although there was a response for the presence of specific, biotinylated IgG and no response for the same concentrations of unmodified antibodies, the specific response was smaller than with free biotin. It correlates with the K_d of biotinylated IgG (713 nM) (Supporting Information, Figure 8S), which was 50 times higher than the K_d of free biotin. Such a difference in the binding affinity could be explained by considering the difference between the size of free biotin (244.31 Da) and biotinylated IgG (~150 kDa).[20]

The role of Kolliphor P188 during preparation of microtiter plates containing magnetic inserts was to reduce non-specific interactions between the analyte and the magnetic inserts and microtiter plate wells. As this took place prior to the addition of the BIONs, there were still some non-specific interactions between the analyte and BIONs. In order to reduce this, the BIONs were treated with BSA as described in the Experimental seciton. It was found that the binding between BIONs and nanoMIPs was reduced by a factor of two upon treatment of BIONs with BSA.

It is known that the binding of biotin to antibodies, avidin and streptavidin is inhibited by the presence of certain compounds. Reducing agents such as mercaptoethanol disrupt antibody-antigen binding by reducing disulfide bonds within the antibodies.[21] Saccharides can also interfere with binding between biotin and avidin/streptavidin. This is thought to be caused by interaction between the sugars and residues near the biotin binding site.[22] As the

monomers used to produce MIP nanoparticles in this work include carboxylic acid (acrylic acid), another concern was the formation of salts in the presence of metal ions such as magnesium. In order to investigate the susceptibility of nanoMIPs to these interference agents, the binding of BIONs to aqueous fluorescent nanoMIPs in the presence of three such agents was investigated. These agents included: 100 μM $MgCl_2$, 100 μM mercaptoethanol, and 100 mM sucrose.

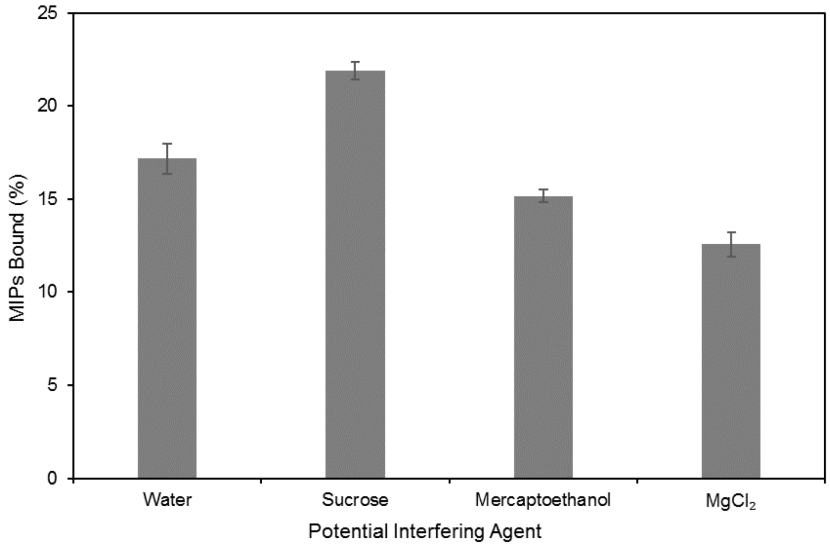

Figure 5. Binding of aqueous nanoMIPs to BIONs in the presence of various potential interference agents.

As expected, mercaptoethanol did not appear to significantly interfere with binding between BIONs and nanoMIPs, as this reducing agent primarily interferes with antibody binding via disruption of disulfide bridges, absent within the nanoMIPs. The presence of 100 μM $MgCl_2$ appeared to result in a lower level of binding, likely due to complexation of magnesium to negatively charged groups within the binding sites. Furthermore, the presence of sucrose noticeably increased the level of binding. Sucrose has previously been used to coat SPIONs in order to improve their stability and dispersibility in water.[23] It is likely that the presence of such high concentration of sucrose improved the dispersibility of BIONs during this experiment, disrupting layer packing of BIONs on the magnetic inserts, and allowing a greater level of binding to occur.

A displacement assay was prepared for measurement following long term storage as described in Experimental section. This plate was stored under ambient conditions (room temperature, exposed to air and protected from light) for six weeks.

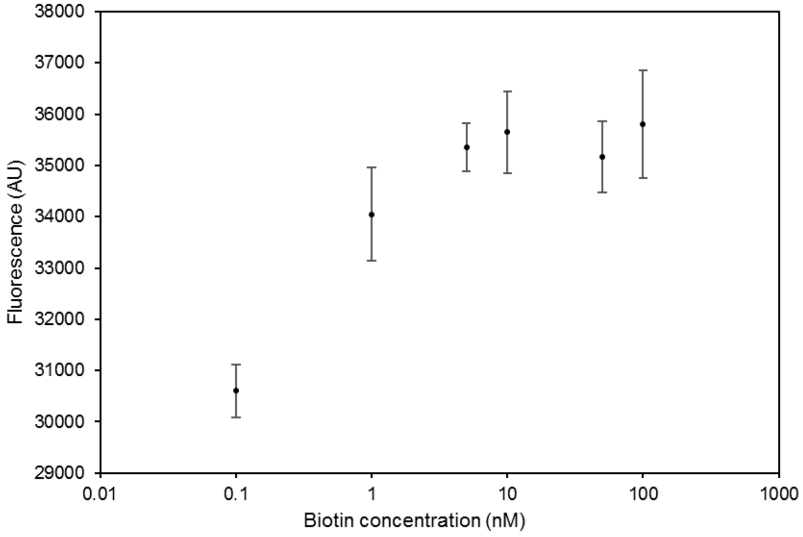

Figure 6. Displacement assay results following six weeks storage at room temperature.

It was noticed that the fluorescence signal observed in the absence of free biotin was lower than in the presence of concentrations exceeding 10 nM, at which point the fluorescence signal appears to plateau (Figure 6). The difference in the responses between the freshly prepared assay and the dehydrated assay can likely be attributed to the conditions under which the dehydrated microtiter plate were prepared. It is likely that drying has led to the pre-concentration of the nanoparticles and the formation of stronger complexes between nanoMIPs and BIONs during the incubation step. As a result, the emission intensity plateaus at lower biotin concentrations and the absolute fluorescence observed was lower for the dehydrated plate. Furthermore, the performance of the dehydrated assay was likely affected by the drying and rehydration process. It is known that SPIONs are difficult to suspend in solution once dried, and are typically coated with a hydrophilic agent such as PEG, poly(acrylic acid), polyethylenimine, or glutathione in order to improve colloidal stability.[24, 25] NanoMIPs still function following rehydration, and are often dried to facilitate long term

storage.[13, 26, 27] Finally, the aqueous fluorescent nanoMIPs produced for this work demonstrated excellent stability, as each batch produced consistent binding even four months after synthesis, despite spending prolonged periods without refrigeration.

3. Conclusions

A novel abiotic system for the detection of biotin was developed using fluorescent molecularly imprinted polymer nanoparticles in place of antibodies and enzymes as binders and reporters, respectively. The resultant assay used only low cost, high stability reagents, and required no washing steps, only the addition of analyte to pre-prepared microtiter plate wells. Furthermore, this assay can be used for the detection of any target molecule, as the imprinting process is not specific for biotin. The protocols developed here could potentially be used as a blueprint for the development of abiotic assays for any target of clinical, biotechnological or environmental interest.

4. Experimental section

Materials. (3-Aminopropyl)triethoxysilane (APTES), 3-(trimethoxysilyl)propyl methacrylate, *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA), mercaptododecanoic acid, ammonium persulfate (APS), biotin, *N,N'*-methylenebisacrylamide (BIS), dodecylamine, sodium hydroxide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), iron (II, III) oxide nanopowder (50-100 nm), magnesium chloride, mercaptoethanol, polyethylene glycol 2′000 monomethyl ether succinate, *N*-hydroxysuccinimide (NHS), ninhydrin, *N*-isopropylacrylamide (NIPAm), sucrose, D-(+)-trehalose dihydrate, *N*-tert-butylacrylamide (TBAm), *N,N,N',N'*-tetramethylethylene-1,2-diamine (TEMED), acrylic acid (AA), iron (III) chloride, magnesium chloride and glass beads (75 μm diameter) were purchased from Sigma-Aldrich. Polyclonal rat IgG were purchased from Bio-Rad AbD Serotek, UK. Sulfo-NHS-LC-biotin was obtained from ThermoFisher Scientific, UK. *N*-Fluoresceinylacrylamide (FlAm) was provided by Dr F. Canfarotta (MIP Diagnostics Ltd). Kolliphor P188 polymer was a gift from BASF, UK. Phosphate buffered

saline (PBS), consisted of phosphate buffer (10 mM), potassium chloride (2.68 mM) and sodium chloride (0.14 M), pH 7.4 (Gibco Life Technologies Ltd, UK). Adhesive-backed, 0.5 mm thick magnetic sheets with self-adhesive backing were purchased from Polarity Magnets, UK.

Preparation of microtiter plate with magnetic inserts. Disks with an internal diameter of 3 mm, an external diameter of 6.5 mm and of 0.5 mm thickness were cut from flexible magnetic sheets. These disks were inserted into the bottom of each well of a standard clear bottom microtiter plate. Each well was fully filled with a solution of 1% (w/v) Kolliphor P188 for 10 min, after which the plate was washed thoroughly with water and allowed to air dry.

Preparation of biotinylated iron oxide nanoparticles (BIONs). Super paramagnetic iron oxide (II, III) nanoparticles (SPIONs) (100.0 mg, 50 nm diameter) were dispersed in a solution of 2% (w/v) APTES in a 1:1 solution of water and ethanol (200 mL). The mixture was sonicated for 5 min and then maintained at 70 °C overnight. The silanized SPIONs were collected via magnetic decantation and washed with water (3 x 15 mL) and ethanol (15 mL) before being dried under N_2 and stored at room temperature.

Silanized SPIONs (100.0 mg) were then added to a solution of sulfo-NHS-LC-biotin (2.3 mg, 4.1 μmol) in PBS and dispersed through 1 min of sonication. This mixture was incubated at room temperature for 2 h. The resultant BIONs were collected on a magnet, washed with water (3 x 15 mL) and ethanol (15 mL) before being dried under N_2 and stored at room temperature.

Characterization of BIONs. The diameter of obtained BIONs was characterized via dynamic light scattering (DLS) using a Malvern Zetasizer Nano S Particle analyzer (Malvern Instruments, UK) equipped with a 633 nm laser. Nine measurements were performed on each sample, each consisting of 13 runs, at 25 °C. The biotinylation of SPIONs was confirmed using IR spectroscopy (RX FT-IR (ATR) spectrometer (Perkin-Elmer, UK)).

The extent of silanization of SPIONs was determined using the Kaiser test.[7] A calibration plot was generated by preparing dodecylamine in a range of concentrations from 5.00 mM, to 0.31 mM. 2 mL of each sample was then mixed with ninhydrin in ethanol (2 mM, 2 mL), and then incubated at 60 °C for 90 min. The samples were allowed to cool, and their absorption spectra were recorded between 400 and 700 nm. This process was then repeated using 4.0 mg of SPIONs (both silanized and biotinylated) in 1 mL of ethanol in place of 2 mL of dodecylamine solution, and the absorbance compared to that of the calibration spectra.

Synthesis of the biotin-specific fluorescent nanoMIPs. Fluorescent nanoMIPs were prepared using a previously described solid-phase synthesis method.[28, 29] Glass beads (60 g) were activated by boiling in NaOH (1 M, 24 mL) for 15 min. The beads were then washed with 10 bed volumes of distilled water, 1 bed volume of acetone, and dried under vacuum. A solution of 2% (w/v) APTES in dry toluene (24 mL) was prepared and added to the activated beads. Following overnight incubation, the beads were washed with acetone and dried once more under vacuum, generating amine-functionalized glass beads.

Silanized glass beads (60 g) were added to a solution of sulfo-NHS-LC-biotin (10.9 mg, 20.0 μmol) in PBS. This mixture was allowed to react at room temperature for 1 h with occasional swirling. The biotin-functionalized glass beads were then washed with 10 bed volumes of water, dried under vacuum, and stored at 4 °C.

The following monomers were dissolved in water (100 mL): NIPAm (39.0 mg, 340 μmol), BIS (6.0 mg, 39 μmol), TBAm (33.0 mg, 260 μmol) in ethanol (1 mL), AA (2.2 μL, 32 μmol), APMA (5.8 mg, 33 μmol), FlAm (3.0 mg, 7 μmol). Half of this monomer mixture (50 mL) was sonicated and purged with N_2 for 20 min to ensure removal of oxygen that would quench radical polymerization. Ammonium persulfate (APS) (30.0 mg, 130 μmol) and tetramethylethylenediamine (TEMED) (15 μL, 12.0 mg, 100 μmol) were dissolved in water (0.5 mL). This solution was then added to biotin-functionalized beads (60 g) and the

previously prepared monomer solution (50 mL). The resultant mixture was incubated at 20 °C for 90 min with swirling.

This mixture was then transferred to a solid-phase extraction (SPE) cartridge fitted with a 20 μm PE frit. The beads were washed with 20 °C water (10 x 50 mL) to remove low-affinity polymer and unreacted monomers. The cartridge was then transferred to a 65 °C water bath for 15 min. Upon reaching this temperature, the beads were washed with 65 °C acetonitrile (3 x 20 mL), and the eluent containing the high affinity polymer nanoparticles was collected.

In order to remove unreacted monomers, the polymer solution was reduced to 2 mL via rotary evaporation, added to water (18 mL), and dialyzed using a 50 kDa cellulose membrane in 10% ethanol (10:1 dialysis solvent : sample) for one week, with the solvent changed daily. The solution of fluorescent nanoMIPs was finally dialyzed against of two changes of distilled water, collected and stored in the dark.

Physical characterization of nanoMIPs. Fluorescence measurements were performed within clear bottom 96-well microtiter plates, both unaltered and fitted with magnetic microtiter plate inserts, using a Hidex microtiter plate reader (LabLogic, UK). Analysis of fluorescein-derivatives including FlAm and fluorescent nanoMIPs was performed using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Excitation was performed using high lamp power, and emission was measured from below the plate, to exclude emission of fluorescent material above the magnetic inserts. Fluorescent nanoMIPs (15.6 nM) and FlAm (38.0 nM) in water were repeatedly diluted by a factor of 3, to final concentrations of 21 pM and 470 pM. 100 μL samples of each concentration were added to a microtiter plate in triplicate, the fluorescence measured, and the background (solvent) fluorescence subtracted. All samples were prepared in triplicate.

DLS measurements of the fluorescent nanoMIPs were performed using a Malvern Zetasizer Nano S instrument (Malvern Instruments, UK) equipped with a 633 nm laser. Nine measurements were performed on each sample, each consisting of 13 runs, at 25 °C.

Absorbance measurements were performed using a UV-1800 spectrophotometer (Shimadzu, UK).

Measurement of nanoMIPs affinity using surface plasmon resonance (SPR). SPR measurements were performed using a Biacore 3000 (GE Healthcare Life Sciences, UK) at 25 °C using PBS as the running buffer at flow rate 35 μL min^{-1}. The self-assembled gold sensor chip was cleaned using an oxygen plasma and placed in a solution of mercaptododecanoic acid in ethanol (1.1 mg mL^{-1}) where they were stored until use. Before assembly the sensor chip was rinsed with ethanol and water and dried in a stream of air. The nanoMIPs were immobilized on the surface of the carboxylated chip using EDC/NHS coupling (0.4 mg and 0.6 mg per mL, respectively).

Biotin was diluted with PBS in the concentration range between 1.9 nM and 1000 nM. Sensorgrams were collected sequentially for all analyte concentrations running in KINJECT mode (injection volume 100 μL and dissociation time 120 s). Dissociation constant (K_d) was calculated from plots of the equilibrium biosensor response using the BiaEvaluation v4.1 software using a 1:1 binding model with drifting baseline fitting.

Biotinylation of antibodies using Sulfo-NHS-LC-Biotin. The solution of polyclonal IgG (1 mL, 1 mg mL^{-1}) was mixed with 2 mL of PBS containing 1 mg of sulfo-NHS-LC-biotin and incubated for 1 h. In order to remove unreacted biotin, the antibody solution was dialyzed using a 50 kDa cellulose membrane in 1 L of water for 1 week, with the solvent changed daily. The dialysis was conducted at 4 °C. The concentration of biotin on the surface of IgG was measured using the HABA method.[30]

Optimization of binding conditions. Dry BIONs (15.0 mg) were incubated in 1% (w/v) bovine serum albumin (BSA) in water (2 mL) for 10 min. The BIONs were then washed with water (3 x 2 mL) with brief sonication (30 s) and were re-dispersed in water (2 mL).

Fluorescent nanoMIPs in water (50 μL, 0.17 mg mL^{-1}, 5 nM) and Tris-maleate buffer (50 μL, pH 6.5, 20 mM Tris, 20 mM sodium maleate) were added to microtiter plate wells fitted with magnetic inserts. BIONs were prepared as described above using biotin and NHS/EDC coupling. The BSA-treated BIONs (10.0 mg, 63 nm) were dispersed in Tris-maleate buffer (1 mL, pH 6.5, 10 mM Tris, 10 mM sodium maleate) with brief sonication (30 s). The BIONs solutions were added to the wells containing fluorescent nanoMIPs, 5 μL at a time. In another series of wells, 10 mM Tris-maleate buffer (100 μL, pH 6.5) containing no BIONs was added as a control. The fluorescence was measured, and the decrease upon addition of BIONs was subtracted from the change in fluorescence upon adding buffer solution. The resultant decrease was used as an indicator of nanoMIPs binding. All samples were prepared in triplicate.

NanoMIPs in water (50 μL, 0.17 mg mL^{-1}, 5 nM) were added to microtiter plate wells containing magnetic inserts. BSA-treated BIONs (50 μL, 7.5 mg mL^{-1}, 48 nM) in 20 mM Tris-maleate buffer (50 μL) of pH 5.5, 6.0, 6.5, 7.2 and 8.0 was added to 5 sets of wells. The fluorescence was measured, and the decrease upon addition of BIONs was subtracted from the change in fluorescence upon adding buffer solution. The resultant decrease was used as an indicator of nanoMIPs binding. All samples were prepared in triplicate.

Development of the displacement-based assay. The experiments were conducted in microtiter plate wells modified with magnetic inserts as previously described.[15, 31] BSA-treated BIONs (15.0 mg) were prepared as described above and added to a solution of aqueous, fluorescent, biotin-specific nanoMIPs (2 mL, 0.83 mg mL^{-1}, 22 nM). The mixture was incubated for 1 h to allow binding between nanoMIPs and BIONs, then the nanoparticles

were collected and washed once with water (1 mL). These nanoparticles were then diluted further to a final volume of 2 mL (7.5 mg mL^{-1}, 48 nM). 50 µL of the BION/nanoMIPs solution was placed into each well of a microtiter plate containing magnetic inserts. Biotin solutions varying between 0.1 and 200 nM in Tris-maleate buffer were added to these wells (100 µL, pH 6.5, 15 mM Tris-maleate), the sample was shaken briefly, and the fluorescence measured 60 min later. All samples were prepared in quadruplicate.

Effect of potential interfering agents on binding. Fluorescent nanoMIPs in water (50 µL, 0.17 mg mL^{-1}) were added to microtiter plate wells containing magnetic inserts. Tris-maleate buffer (50 µL, pH 6.5, 30mM) was added to each well. The following solutions were prepared and 50 µL added to these wells in triplicate: water, 100 mM sucrose, 100 µM $FeCl_3$, 100 µM $MgCl_2$ and 100 µM mercaptoethanol. BSA-treated BIONs in 10 mM Tris-maleate buffer (100 µL, 7.5 mg mL^{-1}, 48 nM, pH 6.5) was added to half of these wells, and water (100 µL) to the remainder as a control. The fluorescence was measured, and the decrease upon addition of BIONs was subtracted from the change in fluorescence upon adding buffer solution. The resultant decrease was used as an indicator of nanoMIPs binding. All samples were prepared in triplicate.

Analysis of long-term stability of biotin assay. BSA-treated BIONs (30.0 mg) were incubated in a solution of nanoMIPs (2 mL, 0.83 mg mL^{-1}, 22 nM) for 1 h. The BION-nanoMIPs complexes were collected via magnetic decantation and placed in 4 mL of water containing 3% (w/v) trehalose. 50 µL of this mixture was then dispersed to each well of a microtiter plate fitted with magnetic inserts and dried *in-vacuo* overnight. The plate was left at room temperature under ambient conditions for 6 weeks, exposed to air but protected from light. After 6 weeks, biotin of concentrations between 0.1 and 400 nM in 10 mM Tris-maleate buffer pH 6.5 (100 µL) were added to each well, the sample was shaken briefly, and the fluorescence measured 60 min later. All samples were prepared in quintuplicate.

Supporting Information

It includes following information: infrared spectra of various SPIONs and BIONs; TEM image of unfunctionalized SPIONs; size distribution of fluorescent nanoMIP; normalized fluorescence spectrum of fluorescent monomer and nanoMIPs; comparison of fluorescence of fluorescent monomer (FlAm) and nanoMIPs; performance of displacement assay using BSA-treated and non-treated BIONs; displacement assay using biotinylated IgG and BIONS and SPR analysis of interaction between nanoMIPs immobilised on the sensor chip and biotinylated IgG with concentrations between 8 nM and 600 nM.

Acknowledgements

SSP would like to thank Zeyu Yang (Imperial College London) for performing IR analysis of magnetic particles. The authors are grateful to Francesco Canfarotta from MIP Diagnostics Ltd for providing the FlAm.

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A novel completely abiotic assay for biotin based on molecular imprinted polymer nanoparticles is developed. This is a first report of superior version of ELISA since it can be performed in one simple step without any washing or other liquid-handling steps, hence making it suitable for high throughput screening. It benefits from long shelf-life and does not require cold-chain supply.

Molecular imprinting, biotin, ELISA, MINA, abiotic

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A novel assay format as an alternative to ELISA – MINA test for biotin