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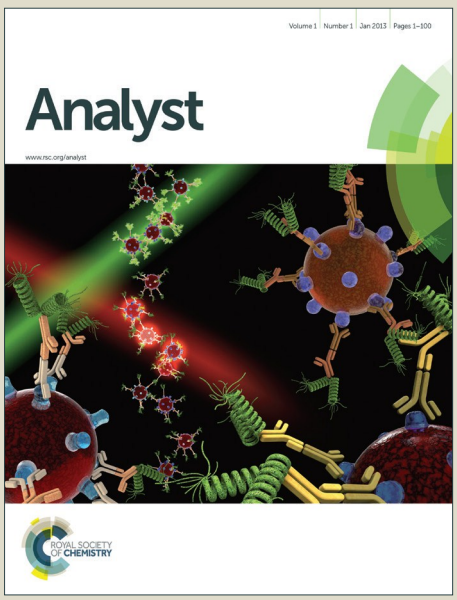

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ARTICLE

Does size matter? Study of performance of pseudo-ELISAs based on molecularly imprinted polymer nanoparticles prepared for analytes of different sizes

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Abstract. The aim of this work is to evaluate whether the size of the analyte used as template for the synthesis of molecularly imprinted polymer nanoparticles (nanoMIPs) can affect their performance in pseudo-enzyme linked immunosorbent assays (pseudo-ELISAs). Successful demonstration of a nanoMIPs-based pseudo-ELISA for vancomycin (1449.3 g mol^{-1}) was demonstrated earlier. In the present investigation, the following analytes were selected: horseradish peroxidase (HRP, 44 kDa), cytochrome C (Cyt C, 12 kDa) biotin (244.31 g mol^{-1}) and melamine (126.12 g mol^{-1}). NanoMIPs with a similar composition for all analytes were synthesised by persulfate-initiated polymerisation in water. In addition, core-shell nanoMIPs coated with polyethylene glycol (PEG) and imprinted for melamine were produced in organics and tested. The polymerisation of the nanoparticles was done using a solid-phase approach with the correspondent template immobilised on glass beads. The performance of the nanoMIPs used as replacement for antibodies in direct pseudo-ELISA (for the enzymes) and competitive pseudo-ELISA for the smaller analytes was investigated. For the competitive mode we rely on competition for the binding to the nanoparticles between free analyte and corresponding analyte-HRP conjugate. The results revealed that the best performances were obtained for nanoMIPs synthesised in aqueous media for the larger analytes. In addition, this approach was successful for biotin but completely failed for the smallest template melamine. This problem was solved using nanoMIP prepared by UV polymerisation in an organic media with a PEG shell. This study demonstrates that the preparation of nanoMIP by solid-phase approach can produce material with high affinity and potential to replace antibodies in ELISA tests for both large and small analytes. This makes this technology versatile and applicable to practically any target analyte and diagnostic field.

1. Introduction

The enzyme-linked immunosorbent assay (ELISA) is probably one of the most recurrent and important tests in diagnostics.[1-3] So far, a large number of ELISAs has been developed for different classes of analytes such as proteins, drugs, viruses, and DNA. ELISA tests can be performed in different formats with the most common being direct, indirect, competitive and sandwich assays. In the direct format, either the antibodies or the antigen are immobilised on microplate wells and the direct binding of antigen or antibody produces the analytical signal. In the indirect format, the antigen is immobilised on the microplate wells, followed by the binding of the antibodies, which are subsequently bound by an anti-species antibody labelled with an enzyme to produce the analytical signal. In the competitive format, the antibodies are immobilised on the microplate wells and competition for the binding to the antibodies is between the free analyte and the analyte conjugated with an enzyme, which gives the analytical signal. In the sandwich format, an antibody (capture) is immobilised on the microplate wells and after the binding of the antigen, a detection antibody, often labelled with an enzyme and capable to bind a different epitope of the antigen, is used to get the analytical signal. Regardless of the format, these assays are used for a quantitative determination of the target analytes usually exploiting a colorimetric reaction.[4] Despite having great advantages such as high sensitivity, high selectivity and easy operation, ELISAs have several drawbacks such as high costs (due to the price of the antibodies), and poor stability of the reagents involved, which need to be kept refrigerated.

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For these reasons, scientists have been trying to develop new materials to replace antibodies in ELISAs and overcome the aforementioned drawbacks. In this regard, molecularly imprinted polymers (MIPs), thanks to their stability, low cost and easy production, are considered a very promising alternative to antibodies in many different fields such as diagnostics, pharmacology, biotechnology and chemical manufacturing.[5-7] However, traditional methods of MIP synthesis yield bulk products or microparticles, which are difficult to integrate into assays or sensors. One of the major drawbacks to work with MIPs in ELISA is the absence of a reproducible and straightforward method for coating microplate wells with the material. In the literature, there are several papers describing the applications of MIPs in ELISA, [8-15] but only a few demonstrate direct application of MIPs in the assays for quantitative detection of the target analytes.[8-9, 16] In one example, the surface of the microplate wells was modified with a homopolymer of 3-aminophenylboronic acid, which was imprinted with ephedrine[15]. The MIP-coated microplate was used successfully in an enzyme-linked assay for detection of epinephrine at micromolar concentrations. The assay sensitivity was in the range of 1-100 μM. In a second example, Zhao and colleagues modified the surface of a microplate with a hydrophilic molecularly imprinted film, prepared *in situ*[16]. The MIP film was capable of recognising olaquindox with a limit of detection of 17 ± 1.6 mg L^{-1}. In another example, the surface of the microplate was modified with a hydrophilic molecularly imprinted film prepared *in situ*, this time specific for estrone[9]. In this case, the limit of detection for estrone was 8.0 ± 0.2 µg L^{-1}.

The fact that very few examples of MIP-based assays are reported is due to several reasons. Firstly, the MIPs used in these assays resemble polyclonal antibodies, which can give high levels of nonspecific binding. Secondly, their preparation relies on manual, labour-intensive methods of synthesis. Thirdly, the immobilisation protocols are often complex, affecting the reproducibility of the tests and hence producing a high degree of variability between measurements.[17] One of the possible solutions which allows the preparation of reliable MIPs for ELISA is the use of the solid-phase synthetic approach for producing MIP nanoparticles (nanoMIPs) with pseudo-monoclonal binding properties.[18] Recently, several research groups have reported examples of protocols for the synthesis of MIP nanoparticles.[19-20, 22] In our case the nanoparticles, which can be produced either in aqueous or organic media depending on the template molecule, exhibit uniform binding sites and high affinity for the target analyte. The main advantage of materials prepared by such solid-phase approach is the possibility to replace directly antibodies with MIPs in standard pseudo-ELISA, with minimal modification of the immobilisation and assay protocol. Recently we have described the use of nanoMIPs, produced by solid-phase approach in a microplate-based assay for accurate measurements of vancomycin (1449.3 g mol^{-1}) with a limit of detection of 2.5 pM.[17] In the present work, we have decided to investigate the effect of the size of the template on the performance of MIP nanoparticles in ELISA. For this, we have selected a variety of target analytes with different size: horseradish peroxidase (HRP, 44 kDa), cytochrome C (Cyt C, 12kDa), biotin (244.31 g mol^{-1}) and melamine (126.12 g mol^{-1}) and we have used them as templates to produce nanoMIPs with the same synthetic protocol (Figure 1). The resulting nanoparticles were then tested in pseudo-ELISA to elucidate and understand whether templates of any size can be used to prepare, by employing the same general protocol, nanoMIPs capable of replacing successfully antibodies in ELISA tests.

2. Experimental Section

2.1 Materials

Biotin, 4-aminophenol, melamine, acrylic acid (AA), N-isopropylacrylamide (NIPAm), poly ethylene glycol (PEG) 1100 MW, N,N'- methylene-bis-acrylamide (BIS), glutaraldehyde (GA), N-tert-butylacrylamide (TBAm), ammonium persulfate (APS), tetramethylethylene- diamine (TEMED), cytochrome C (Cyt C), 3-aminopropyltrimethyloxysilane (APTMS), sodium hydroxide (NaOH), bovine serum albumin (BSA), horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB liquid substrate for ELISA), Tween 20, sodium dodecyl sulfate (SDS), acetone, 2-[morpholino]ethanesulfonic acid (MES), N-hydroxy-succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich, UK. Phosphate buffered saline (PBS), pH 7.4, containing phosphate buffer (0.01 M), potassium chloride (0.0027 M) and sodium chloride (0.137 M) was also from Sigma-Aldrich, UK. In all experiments double-distilled ultrapure (DI) water (Millipore, UK) was used. All chemicals and solvents were of analytical or HPLC grade and used without further purification. Microplates, Nunclon 96 microwell, were purchased from Thermo Scientific, UK.

2.2 Preparation of solid-phases for different templates

The protocol for the preparation of the solid-phases with immobilised templates was adopted from Surugiu and colleagues.[12] Glass beads were activated by boiling in NaOH for 10 min and washed with DI water and acetone. After drying, the beads were incubated overnight in a 2 % solution of APTMS in dry toluene and then washed with acetone and dried. For the immobilisation of biotin template, the latter (25 mg) was first activated using EDC/NHS (196 mg/177 mg) at pH 6.0 for 15 min. The activated template was then mixed with the glass beads, the pH was adjusted to 7.4, and the reaction was left running overnight. Finally, the glass beads were washed with DI water, dried, and stored at 4°C until further use.

The protocol for immobilisation of HRP, Cyt C and melamine, which has been already reported elsewhere,[17] was based on glutaraldehyde (GA) coupling chemistry. Briefly, after incubation with a 2 % solution of APTMS in dry toluene, followed by washing with acetone and drying, the glass beads

were left for 2 hour at room temperature in a solution of GA in PBS (pH 7.4). Then HRP (0.5 mg mL^{-1}), Cyt C (0.5 mg mL^{-1}) and melamine (5 mg mL^{-1}) were added to the corresponding glass beads mixture and incubated overnight. A small amount (10 % v/v) of *N*-methylpyrrolidone was added to help dissolving melamine in PBS. At the end of the incubation period, the glass beads were washed with double-distilled water, dried, and stored at 4°C until use.

2.3 Synthesis of nanoMIPs for different templates by chemical polymerisation

The polymerisation of nanoparticles for HRP, Cyt C, biotin and melamine was performed by mixing NIPAM (39 mg), BIS (2 mg), TBAm (33 mg dissolved in 2 mL of ethanol), and AA (2.23 g) in 100 mL of DI water (100 mL). The solution was ultra-sonicated for 10 min and then degassed with nitrogen for 30 min. Then 60 g of template-functionalised glass beads were added to the reaction cylinder containing 60 mL of polymerisation mixture and were stirred briefly to homogenise the contents. The mixture was again degassed with nitrogen and the polymerisation reaction was initiated by adding 600 µL of APS (60 mg mL^{-1}) and 18 µL TEMED and left to polymerise for 1.5 h at room temperature. Before the collection of the high affinity nanoparticles, the solid-phase was washed to remove unreacted monomers and low affinity nanoparticles using DI water (60 mL x 8 times) at low temperature (20 °C). The high affinity nanoparticles were collected by using DI water (60 mL x 5 times) at 60 °C. The solutions of high affinity MIP nanoparticles were concentrated to a final volume of 100 mL by ultrafiltration on a Millipore Amicon Ultra centrifugal filter unit (30 kDa MWCO) and used in pseudo-ELISA tests.

2.4 Synthesis of nanoMIP for melamine with and without PEG-shell by UV polymerisation

The composition of the polymerisation mixture for the synthesis of nanoMIPs specific for melamine with a PEG-shell was adapted from Guerreiro and colleagues.[21] The polymerisation mixture consisted of 1.44 g MAA, 1.62 g TRIM and 1.62 g EGDMA, 0.377 g N,N-diethyldithiocarbamic acid benzyl ester (Iniferter) and 0.09 g of CTA in 5.26 g of ACN. The mixture was degassed with nitrogen for 20 minutes. Melamine-derivatised glass beads (30 g) were then placed in a flat glass vessel and purged with nitrogen for 5 min. This was followed by UV polymerisation for 1 min and 30 s. After polymerisation, the content was transferred into a SPE cartridge fitted with a polyethylene frit and placed in an ice bath (0 °C) for 10 min. Washing steps were carried out with ACN at 0 °C in order to remove non-polymerised monomers and low affinity nanoparticles. Afterwards, post-derivatisation of melamine-MIP nanoparticles (addition of PEG-shell) was performed on the same solid-support with the high-affinity nanoMIPs still attached. For this purpose, poly(ethylene glycol) methacrylate (75 mg, MW= 1100) was dissolved in ACN (8 mL) and degassed with N_2 for 10 min. This solution was then added to the glass beads, degassed with nitrogen for 5 min and irradiated with UV light for 1 min and 30 s. After polymerisation, the content was washed again with low temperature ACN to remove non- and low affinity polymerised material, followed by a series of hot wash using either ACN or water at 60 °C. This allows high affinity MIP to be eluted from the solid-phase. The total collected volume of high affinity fraction of nanoMIPs with PEG-shell was about 120 mL.

To obtain the melamine nanoMIPs without the PEG-shell the procedure was exactly the same, but without the post-derivatisation process and high affinity nanoparticles were collected with hot water straight after the first series of cold washings with ACN.

In the final step of the preparation of both types of nanoparticles (with and without PEG) eluted in hot ACN, the solvent was exchanged to water using the Amicon Centrifugal Filter Units (MWCO 30 kDa). This was done both because of the incompatibility of the plastic microplates with organic solvents and the possible interference of the hydrophobic solvent with the immobilisation of the nanoparticles on the microplate wells. To exchange the solvent, 20 mL of the nanoMIP solution in ACN was concentrated down to 3 ml by evaporation. Then 10 mL of water was added into a Amicon Centrifugal Filter Unit (MWCO 30 kDa) followed by 1 mL of melamine nanoMIPs in ACN and the filter was centrifuged at 3500 rpm for 3 minutes. After performing this step for the 3 ml of nanoMIPs, 14 ml of water were added 4 times, so that nanoMIP free of ACN and therefore safe to be used on plastic microplates were obtained.

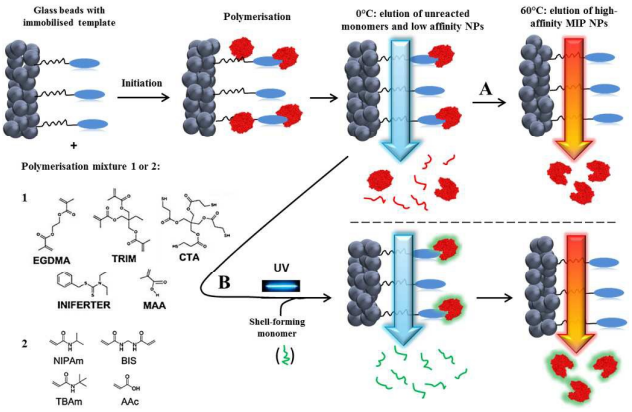

Figure 1. Scheme of the solid-phase synthesis of bare (a) and core-shell (b) nanoMIPs. Polymerisation mixture 1 and synthetic route A were employed for the synthesis of melamine nanoMIPs without PEG. PEGylated nanoMIPs were produced using the same mixture 1 and route B. Polymerisation in water was performed through synthetic route A and polymerisation 2.

2.5 Characterisation of nanoMIPs

The size of all the nanoMIPs was determined by dynamic light scattering (DLS) using a Zetasizer Nano (Nano-S) from Malvern Instruments Ltd. (Malvern, UK). For further confirmation, nanoMIPs were also characterised using a Philips CM20 transmission electron microscope (TEM). Prior to DLS and TEM measurements, the solution of nanoMIPs was ultra-sonicated

for 3 min to disrupt possible aggregates. To perform DLS, a small aliquot of nanoMIPs in water (1 mL) was tested. The dispersion was analysed by DLS at 25 °C in a 3 cm^3 disposable polystyrene cuvette. Attenuator position, measurement duration and number of runs were automatically chosen by the instrument. For TEM a drop of the nanoMIPs solution was placed on a carbon-coated copper grid and dried in a fume hood before measurement.

2.6 Preparation of the HRP–biotin (HRP–B) conjugate

A stock solution of biotin (1 mL, 0.2 mg mL^{-1}) was prepared in 0.1 M MES buffer pH 6, to which EDC (17.7 µL from a stock solution of 10 mg mL^{-1} in water) was added, followed by NHS (1.72 mg). The reaction was allowed to proceed at room temperature for 15 min. Then the solution was mixed with 20 mL of HRP (0.6 mg mL^{-1}) in PBS buffer at pH 7.4 and incubated for 2 h. The HRP–biotin conjugate (HRP–B) was then washed to remove free biotin using a Millipore Amicon Ultra centrifugal filter unit (30 kDa MWCO). For this procedure, 10 washes with PBS (5 mL) were performed. After washing, the conjugate was dissolved in DI water (2 mL), its concentration was estimated by comparison with the enzymatic activity of the free enzyme, and it was stored at −18 °C until further use. This conjugate solution was used as the stock solution for the pseudo-ELISA tests.

2.7 Preparation of the HRP–melamine (HRP–M) conjugate

HRP (10 mg) was dissolved in 0.1 M MES buffer, pH 6 (1 mL), to which EDC (0.4 mg) followed by NHS (0.6 mg) was added. The reaction was allowed to proceed at room temperature for 15 min. At this point, the buffer was removed by ultrafiltration on a Millipore Amicon Ultra centrifugal filter unit (30 kDa MWCO). Activated HRP was collected from the ultrafiltration unit and immediately incubated with melamine (10 mL, 1 mg mL^{-1}) in PBS buffer at pH 7.4 for 2 h. The HRP–melamine conjugate (HRP–M) was then washed to remove free melamine on a Millipore Amicon Ultra centrifugal filter unit (30 kDa MWCO). For this procedure, 10 washes with PBS (5 mL) were performed. After washing, the conjugate was dissolved in DI water (2 mL), its concentration was estimated by comparison with the enzymatic activity of the free enzyme, and it was stored at −18 °C until further use. This conjugate solution was used as the stock solution for the pseudo-ELISA tests.

2.7 Immobilisation of nanoMIPs onto the surface of microplate wells

The immobilisation of nanoMIPs onto the microplate well was performed by direct deposition of the nanoparticles' solutions (40 µL, 0.056 mg mL^{-1}) into the wells of a 96-well microplate. After dispensing, the solvent was allowed to evaporate overnight at room temperature.

2.8 Optimisation of the assays conditions

Several parameters, such as composition of blocking and washing buffers, quantity of nanoMIPs, time of incubation, and concentration of HRP–conjugate was adopted from Chianella and co-workers.[17]

2.9 Direct assay for enzymes

Microplate wells were coated with nanoMIPs by dispensing undiluted stock solution (40 µL, 0.056 mg mL^{-1}) into each well followed by overnight evaporation. Each well was then conditioned by washing with PBS (2 × 250 µL) followed by 1 h blocking with 300 µL of PBS containing 0.1% of BSA and 1% of Tween 20. After further washing with PBS (3 × 250 µL), a small volume (100 µL) of several concentrations of HRP (0.1-45 nM) or Cyt C (10-1000 nM) in PBS were added to the wells in triplicates and incubated in the dark at room temperature for 1 hour. After washing with PBS (3 × 300 µL), containing 0.1% of BSA and 1% of Tween 20, TMB reagent (100 µL) was added and incubated for 15 min. The enzymatic reaction was then stopped by the addition of H_2SO_4 (0.5 M, 100 µL) and the absorbance of each microplate well was measured at 450 nm using a UV/Vis microplate reader (Dynex, UK).

2.10 Competitive assay for biotin and melanime

Microplate wells were coated with nanoMIPs by dispensing undiluted stock solution (40 µL, 0.056 mg mL^{-1}) into each well followed by overnight evaporation. Each well was conditioned by washing with PBS (2 × 250 µL) followed by 1 h blocking with 300 µL of PBS containing 0.1% of BSA and 1% of Tween 20. After further washing with PBS (3 × 250 µL), a solution containing HRP–T (100 µL, 1:800 dilution from stock solution) and free analyte in the concentration range between 0.001 and 6000 nM was added to each well. Plates were incubated in the dark at room temperature for 1 h. After washing with PBS (3 × 300 µL), containing 0.1% of BSA and 1% of Tween 20, TMB reagent (100 µL) was added and incubated for 10 min. The enzymatic reaction was then stopped by the addition of H_2SO_4 (0.5 M, 100 µL) and the absorbance of each microplate well was measured at 450 nm using the UV/Vis microplate reader.

3. Results and discussion

3.1 Synthesis and characterisation of nanoMIPs

The synthesis of nanoMIPs for each template was performed manually using a solid-phase approach. For this, melamine, HRP and Cyt C were immobilised onto the surface of amine-derivatised glass beads through their amino groups by glutaraldehyde coupling. Biotin was immobilised onto the surface of amine-derivatised glass beads through its carboxylic group by EDC/NHS coupling chemistry. The polymer composition for the preparation of the nanoMIPs was adapted from Hoshino and colleagues.[22] In addition to this, nanoparticles for melamine were prepared in an organic solvent (ACN) by UV polymerisation with and without a PEG shell. In general the interactions between all the imprinted polymers nanoparticles and the different templates were due to a combination of multiple electrostatic and hydrophobic interactions. The final concentration of the stock solution for each type of nanoMIP was determined by weighing a freeze-dried aliquot of the nanoparticles solution and, if needed, it was adjusted to 0.056 mg mL^{-1}. This was done to allow a direct

comparison of the data obtained in this work, with the results of the pseudo-ELISA developed for vancomycin in a previous work.[17] The size of nanoparticles prepared in water was analysed by DLS and measured as 250 ± 30.1 nm, 170 ± 21.8 nm, 148 ± 7.3 nm, 133.4 ± 19.6 nm for HRP, Cyt C, biotin and melamine respectively. The value indicates that in general larger templates produce larger nanoMIPs. To confirm the size obtained with the DLS, melamine nanoparticles were also analysed by TEM (Fig. 2).

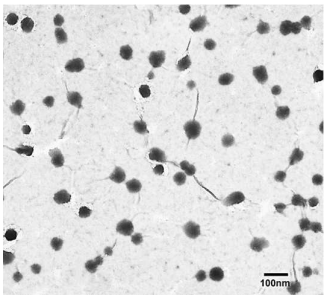

Figure 2. TEM image of nanoMIPs specific for melamine.

3.2 Direct and competitive pseudo-ELISA performed using nanoMIPs

After synthesis and characterisation, the nanoMIPs were utilised as replacement for antibodies in pseudo-ELISAs to develop quantitative assays for different target analytes. The immobilisation of the nanoparticles onto microplate wells was kept as simple as possible and similar to the antibodies immobilisation, which are frequently immobilised onto microplates by physical adsorption.

The immobilisation of the nanoMIPs, achieved by a simple overnight evaporation, seemed to be efficient and stable, as nanoparticles remained attached to the surface even after several washes with PBS. Once immobilised, the nanoMIPs were used in a direct pseudo-ELISA to quantify the two enzymes as well as in a competitive pseudo-ELISA to quantify biotin and melamine, with competition between HRP-template conjugates (HRP-B and HRP-M) and free analytes. Both assays were performed using the same conditions as in the assay developed for vancomycin.[17] These included the same blocking and washing steps, as well as adjusting the concentration of the stock solution of the two conjugates (for the competitive mode) to 2.5 mg mL^{-1} (before diluting them 800 times for the competition reaction) .

To assess specificity of corresponding nanoMIPs we have incubated enzymes (45 nM) and conjugates (3.1 µg mL^{-1}) for 1 hour in a microplate with the corresponding nanoparticles. Following a further washing step, the substrate TMB was added and incubated for 10 minutes. After stopping the enzymatic reaction with H_2SO_4, the colour of the microplate wells was read at 450 nm using the UV/visible microplate reader. The results are reported in Fig. 3. The figure shows that specific binding to the nanoparticles was observed for HRP, Cyt C and HRP-B, as there is a significant difference in signal between wells with and without nanoMIPs. NanoMIPs for melamine prepared in water by chemical polymerisation did not show specific binding to HRP-M conjugate. However, nanoparticles prepared in organic solvent by UV polymerisation with and without PEG did show affinity for the corresponding conjugate. Therefore only the melamine nanoMIPs prepared in organic solvent were used for further investigations.

The following experiment involved the assessment of the response of pseudo-ELISAs to increasing concentration of analyte. For the enzymes, HRP (0.1-45 nM) and Cyt C (10-1000 nM) were incubated with the nanoMIPs immobilised on the plate for 1 hour and excess of unbound protein washed out as described in Experimental section.

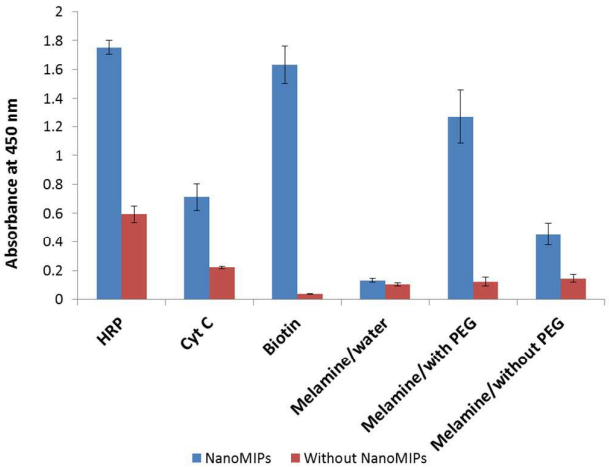

Figure 3. Binding of enzymes (HRP and Cyt C) and conjugates (HRP-B and HRP-M) to the corresponding nanoMIPs. Bare microplates wells (blocked and washed as described earlier) were used as controls. Error bars represent ±1 standard deviation for experiments performed in triplicate.

The amount of analyte bound by nanoMIPs was quantified by reading the plate at 450 nm after colour development resulting from the addition of the enzymes' substrate (TMB). The selectivity of the nanoMIPs was tested by performing assays with nanoMIPs imprinted for a different protein (IgG) immobilised on the microplate wells. The results are reported in Figure 3A for HRP and Figure 3B for CytC.

These figures show linear calibration plots for both enzymes with a linear range between 0.1-2.3 nM for HRP and 10-1000 nM for Cyt C. For both enzymes, saturation was observed at higher concentrations. The limits of detection, calculated as the concentration value obtained from 3 times the standard deviation of the control (in absence of enzymes) were 0.5 nM for HRP and 25 nM for Cyt C. The disparity in sensitivity between the two tests was mainly due to a difference in activity between the two enzymes. Cyt C is not as active as HRP in reacting with the substrate TMB and higher concentrations are needed to develop an appreciable colour. Figure 4A and 4B also show that the interaction between the nanoMIPs and the enzymes is selective, as the binding to control nanoMIPs prepared for a different protein (IgG) is much lower, especially in the case of Cyt C.

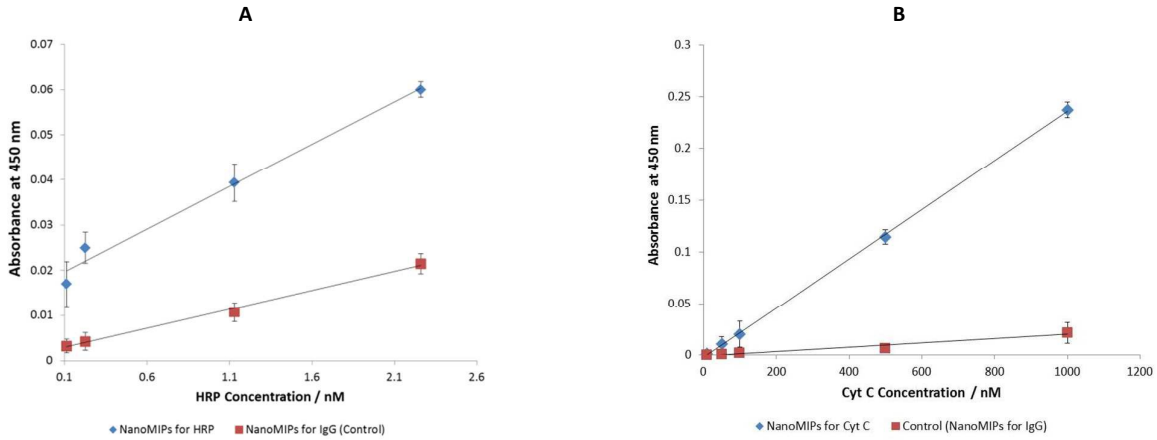

Figure 4. Direct peudo-ELISA carried out with nanoMIP specific for HRP (A) and Cyt C (B). Binding selectivity was tested by incubating the two enzymes with nanoMIPs specific for IgG. Absorbance values were obtained after subtraction of background (in absence of enzyme). Error bars represent ±1 standard deviation for experiments performed in triplicate.

As mentioned above, for the smaller analytes (biotin and melamine) the pseudo-ELISA was performed in a competitive mode with competition between the HRP-template conjugate (HRP-B and HRP-M) and the free analytes (biotin, melamine). For biotin ELISA, free biotin in concentration 0.0001 nM-0.0250 nM was added to the wells at the same time as the conjugate HRP-B (3.1 µg mL^{-1}, 1:800 of stock solution). The results, shown in Figure 4, clearly indicate that free biotin can be detected over 3 orders of magnitude concentration range. The assay response was linear to the analyte concentration (r^2 = 0.9846) when plotted in logarithmic scale in the range from 0.0001 nM to 0.025 nM. The limit of detection of the assay, calculated from the value of 3 times the standard deviation of the control (absence of biotin) was 7.4 pM. The competitive assay showed saturation at concentrations of biotin higher than 0.025 nM.

In order to test the selectivity of the nanoMIPs synthesised for biotin, the same competitive experiments were performed with nanoMIPs prepared for melamine immobilised on microplate wells (Figure 5). The curve indicates that HRP-B and free biotin do not compete for the binding to nanoMIPs specific for melamine, as the response of the assay is flat, indicating selectivity of the nanoMIPs made for biotin.

In our initial experiment we did not observe any specific binding of melamine-HRP conjugate to melamine-imprinted nanoMIPs made in water (data not shown). This is not surprising since melamine is a small molecule and it cannot form strong bonds with MIP in water due to competition with water molecules themselves. On the other hand, the particles imprinted for melamine by polymerisation in organic solvent also did not show good performance in ELISA (Figure 5) due to the fact that they do not stick to the surface of the microplate (relatively hydrophilic) because of their hydrophobicity. Following this concept, we have produced nanoMIPs with a PEG shell, which would increase their surface hydrophilicity and offer some protection of polymer binding sites.[23,24]

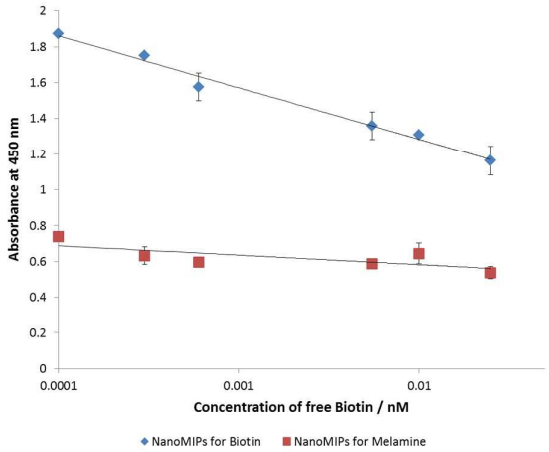

Figure 5. Competitive pseudo-ELISA for biotin performed with nanoMIPs specific for biotin and with nanoMIPs specific for melamine to assess selectivity. Error bars represent ±1 standard deviation for experiments performed in triplicate.

PEG coated nanoMIPs were prepared in acetonitrile by UV polymerisation as described in the Experimental section and eluted in ACN. The competitive pseudo-ELISA was carried out with concentration of free melamine varied between 10 and 6000 nM added to the microplates at the same time as the conjugate. The results, shown in Figure 6, revealed a significant improvement of the assay. For these nanoMIPs, successful competition was observed over 3 orders of magnitude, with linearity from 10 nM to 6000 nM and a limit of detection of 25 nM (calculated as explained above). The competitive assay started to show saturation at concentrations of analyte higher than 6000 nM.

As observed here in the presence of a PEG shell there is evidence of a successful competitive binding between the free analyte and the HRP-M conjugate to the nanoMIPs immobilised on the microplate, whereas in the case of nanoMIPs without PEG the results did not show successful competition.

The results shown here prove that coating of nanoparticles with PEG by UV allows the synthesis of monodisperse imprinted nanoparticles with good affinity for small molecules in aqueous environment and therefore capable of good performance in pseudo ELISA.[24]

Conclusions

In this work we have investigated whether the size of the template, used to produce molecularly imprinted polymers nanoparticles (nanoMIPs) by solid-phase synthesis can affect the performance of the resulting material in pseudo-ELISAs. The detection limits of the pseudo-ELISA tests performed with nanoMIPs prepared for templates of different sizes, including the pseudo-ELISA for vancomycin carried out in a previous work,[17] are summarised in Table 1.

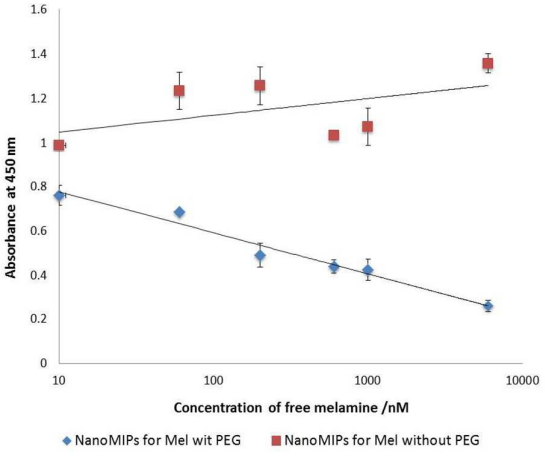

Figure 6. Calibration curves obtained using a competitive pseudo- ELISA for melamine performed with nanoMIPs specific for melamine with and without PEG.Error bars represent ±1 standard deviation and are for experiments performed in triplicate.

Table 1. Limit of detections (LOD) of pseudo-ELISAs performed with different nanoMIPs.

NanoMIPs template	Polymerisation Method	Pseudo-ELISA Format	LOD
HRP	Chemical /Water	Direct	0.5 nM
Cyt C	Chemical /Water	Direct	45 nM
Vancomycin*	Chemical /Water	Competitive	2.5 pM
Biotin	Chemical /Water	Competitive	7.4 pM
Melamine	Chemical/Water	Competitive	-
Melamine with PEG	UV	Competitive	25 nM

*Data from Chianella and co-workers.[17]

The table shows that the size of the templates, whether these are large proteins or small molecules such as biotin and melamine, has little effect on the sensitivity of pseudo ELISA based on nanoMIPs. In case of melamine, due to its small size, a modification of the protocol by grafting nanoparticles with PEG shell is necessary to improve assay performance.

This work proves that the solid-phase approach with controlled radical polymerisation in water or acetonitrile can be used to prepare nanoparticles for a wide range of target analytes. In the few cases where this protocol does not work, for example for small analytes (MW < 500 Da), UV grafting of a PEG shell can be used to improve MIP performance, without any specific optimisation. This gives the possibility to develop assays for a vast range of analytes and possibly different analytical applications in relatively short time.

References

1 R. M. Lequin, *Clinical Chemistry*, 2005, **51**, 2415-2418.
2 L. W. Solomon, P. C. Stark, L. Winter, V. Kumar and S. Sinha, *Oral Diseases*, 2010, **16**, 151.
3 S. X. Leng, J. E. McElhaney, J. D. Walston, D. Xie, N. S. Fedarko and G. A. Kuchel, *Journal of Gerontology*, 2008, **63**, 5.
4 A. Vieira, *Molecular Biotechnology*, 1998, **10**, 4.
5 D. A. Spivak, *Advanced Drug Delivery Reviews*, 2005, **57**, 1779.
6 A. G. Mayes and M. J. Whitcombe, *Advanced Drug Delivery Reviews*, 2005, **57**, 1742.
7 G. Wulff, *Microchimica Acta*, 2013, **180**, 1359.
8 S. A. Piletsky, E. V. Piletska, B. Chen, K. Karim, D. Weston, G. Barrett, P. Lowe and A. P. F. Turner, *Analytical Chemistry*, 2000, **72**, 4381.
9 S. Wang, Z. Xu, G. Fang, Y. Zhang, B. Liu and H. Zhu, *Journal of Agricultural and Food Chemistry*, 2009, **57**, 4528.
10 W. Ben, Z. Qiang, C. Adams, H. Zhang and L. Chen, *Journal of Chromatography. A*, 2008, **1202**, 173.
11 Y. Tang, G. Fang, S. Wang, J. Sun and K. Qian, *Journal of AOAC International*, 2013, **96**, 453.
12 I. Surugiu, B. Danielsson, L. Ye, K. Mosbach and K. Haupt, *Analytical Chemistry*, 2001, **73**, 5.
13 Z. X. Xu, H. J. Gao, L. M. Zhang, X. Q. Chen and X. G. Qiao, *Journal of Food Science*, 2011, **76**, R69-75.
14 Y. Yonamine, Y. Hoshino and K. J. Shea, *Biomacromolecules*, 2012, **13**, 2952..
15 S. A. Piletsky, E. V. Piletska, A. Bossi, K. Karim, P. Lowe and A. P. F. Turner, *Biosensors & Bioelectronics*, 2001, **16**, 701-707.
16 D. Zhao, X. Qiao, Z. Xu, R. Xu and Z. Yan, *Journal of Immunoassay & Immunochemistry*, 2013, **34**, 16.
17 I. Chianella, A. Guerreiro, E. Moczko, J. S. Caygill, E. V. Piletska, I. M. De Vargas Sansalvador, M. J. Whitcombe and S. A. Piletsky, *Analytical Chemistry*, 2013, **85**, 8462.
18 A. Poma, A. Guerreiro, M. J. Whitcombe, E. V. Piletska, A. P. F. Turner and S. A. Piletsky, *Advanced Functional Materials*, 2013, **23**, 7.
19 Y. Ma, H. Li and L. Wang, *Journal of Materials Chemistry*, 2012, **22**, 18761.
20 Y. Ma, H. Li, S. Peng and L. Wang, *Analytical Chemistry*, 2012, **84**, 8415.
21 A. R. Guerreiro, I. Chianella, E. Piletska, M. J. Whitcombe and S. A. Piletsky, *Biosensors and Bioelectronics*, 2009, **24**, 2740.
22 Y. Hoshino, T. Kodama, Y. Okahata and K. J. Shea, *Journal of the American Chemical Society*, 2008, **130**, 15242.

23 E. Moczko, A. Guerreiro, E. Piletska and S. Piletsky, *Langmuir*, **29**, 9891.
24 E. Moczko, A. Poma, A. Guerreiro, I. Perez de Vargas Sansalvador, S. Caygill, F. Canfarotta, M. J. Whitcombe and S. Piletsky, *Nanoscale*, 2013, **5**, 3733-3741.