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Introducing MINA - The molecularly Imprinted Nanoparticle Assay

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A totally abiotic ELISA-like assay, where no elements of biological origin are used, either for molecular recognition or for reporting purposes, is presented here. Using a novel synthetic approach, with immobilized template, surface-imprinted nanoparticles were prepared with a catalytic core capable of promoting a colorimetric reaction; these were used for combined target recognition and reporting. In microplate-competitive format the assay was capable of selectively determining vancomycin concentration from 10 nM to 1 mM, and with minimal modifications to standard assay protocols. In diluted serum, the assay could quantify vancomycin from 0.05 to 3 μ M. Results point towards the future development of a new generation of assays based entirely on stable and potentially more affordable synthetic reagents.

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Supporting Information

Supporting Information is available online from the Wiley Online Library or from the author.

Immunoassays have long been used in clinical, food, environmental and forensic industries to monitor a wide range of targets, ranging from proteins to DNA sequences and drugs.^[1, 2] Of these, the enzyme-linked immuno-sorbent assay (ELISA) is probably the most common method, based on the competition between free and enzyme-labeled ligand to bind to immobilized targets or antibodies.^[3] Although rapid, sensitive and cost effective for large sample loads, immunoassays have several disadvantages, mostly connected with limited stability/batch variability and the expense of production and use of antibodies and reporter enzymes.

Molecularly Imprinted Nanoparticles (MIN) capable of recognition of a target molecule have been identified as promising alternatives to antibodies in a range of applications^[4-6]. However, the added advantages (mainly storage/thermal stability and low cost) conferred by the use of imprinted materials as antibody replacements might not be effectively exploited if the assay system still requires the presence of a biological reporter enzyme. Accordingly, here we report the integration of catalytically active Fe_3O_4 with MIN as combined recognition and signaling functionalities in a core-shell nanoparticle format suitable for use in ELISA-like assays. The intrinsic peroxidase mimicking activity of $\gamma\text{Fe}_3\text{O}_4$ nanoparticles has been recently identified,^[7] making them attractive substitutes for the enzyme in a variety of assays, with stable catalytic activity over a broad range of temperatures, low cost/long shelf life and ease of manufacture. In order to produce the composite core-shell Fe_3O_4 -MIN, a variation of the solid-phase imprinting protocol^[8] was used, Scheme 1. This method was devised taking into consideration its potential for scale-up and automation, both important factors to consider regarding potential practical applications of the technology. That synthesis and purification time < 1 h per batch of particles is also advantageous. Briefly, the protocol consisted of the addition of monomer mixture and UV initiator, containing a dispersion of 3-(trimethoxysilyl) propyl methacrylate modified $\gamma\text{Fe}_3\text{O}_4$ nanoparticles ($d = 50$ nm), to the solid phase (glass beads) bearing the immobilized target template (vancomycin). After a short period of UV irradiation (2.5 min), nanoparticles were formed,^{[9][8]} and the solid phase containing the template and the bound MINs then functioned as an affinity support for the fractionation/purification of high-affinity MINs from the remaining polymer/unreacted monomers (see S. I. for full details), by washing with solvent at room temperature. High affinity materials were collected by elution at 60 °C. Subsequent magnetic separation of the high-affinity particles ensured that only those containing the magnetic Fe_3O_4 core were recovered. Consequently the collected MINs possessed both high-affinity (affinity separation step on the solid phase) and a Fe_3O_4 core (magnetic separation). Control (non-imprinted) particles were prepared in absence of immobilized template. Fe_3O_4 -MINs had diameters of 113 ± 23 nm and Fe_3O_4 -C (control) particles 81 ± 2 nm (by DLS). Particles possessed a core-shell structure, with a polymer shell surrounding the Fe_3O_4 core, Scheme 1. To increase polymer contrast during imaging, particles were stained with OsO_4 . A TEM image of the core Fe_3O_4 is presented as S. I (Figure S1) confirming particles have a diameter below 50 nm. To demonstrate that specific interactions between polymer and template were responsible for retention of the particles by the solid phase (during the cold column washing), control experiments were performed by loading the polymerisation mixture containing Fe_3O_4 nanoparticles onto the solid phase. In the absence of a polymerization step, 99.59 % of the Fe_3O_4 was eluted during the cold wash. Following

a polymerization step, only 82.75 % is eluted cold, with 17.25 % recovered during the hot wash (high affinity fraction) and included in the core-shell MINs. When the surface of the Fe₃O₄ core was not modified with methacrylate groups, a decrease in yield was observed (15.95 % recovered during hot wash) so modified Fe₃O₄ nanoparticles were used on subsequent experiments. The composition of the polymerization mixture was based on a published protocol^[8, 10] and included *N*-isopropylacrylamide, *N*-*tert*-butyl acrylamide, acrylic acid and the cross-linker *N*, *N*'-methylenebis(acrylamide). The full composition is given in S. I. Target recognition with this type of polymer can be attributed^[4] to a combination of multiple weak electrostatic and hydrophobic interactions and is especially suited to the imprinting of large molecules. The kinetics of the catalytic oxidation of TMB by Fe₃O₄ nanoparticles were studied by measuring the absorbance of the solution at 650 nm in 300 sec intervals, and compared for both free Fe₃O₄ and Fe₃O₄-MIN nanoparticles. Michaelis-Menten kinetics parameters (v_{max} and K_m) were calculated using the Lineweaver–Burk double reciprocal plot (see S. I, Figure S2). The catalytic constant (k_{cat}) was calculated according to Equation (1):

$$k_{cat} = v_{max} / [E] \quad (1)$$

where [E] is the Fe₃O₄ concentration (2.15×10^{-10} M). Catalytic parameters are summarized on Table 1. Results indicate that presence of the polymer shell does not significantly interfere with the catalytic activity of the nanoparticles. This can be attributed to the fact that the hydrophilic porous polymer shell, with low percentage of cross-linker (2%) is swollen in aqueous media, and so causes little resistance to the diffusion of the small-molecule reactants. Also, this is in accordance with Woo^[11] who observed small increases in V_{max} values for Fe₃O₄ nanoparticles after surface derivatization/coating with proteins.

Although imprinted polymers have the potential to directly replace natural receptors in assays such as ELISA, the materials produced using classical protocols have generally proved unsuitable for such applications due to a number of factors. Firstly, traditional MIPs are highly “polyclonal” in relation to their binding behavior (i.e. binding sites distributed throughout the polymer bulk and surface which have a wide range of affinities towards the target molecule) and suffer from high levels of non-specific binding. Secondly, the protocols for integration of imprinted polymers with assays are complicated and non-generic, requiring significant modifications to the procedure used for their manufacture or performance of the assay. Aiming to solve some of these problems we have developed the solid-phase imprinting method for synthesis of MINs with pseudo-monoclonal properties.^[8] As detailed in Scheme 1, by combining the affinity separation (for removal of low-affinity MINs) and the fact that particles have a limited number of binding sites (only at the point of contact with the solid-phase), the final product will have more uniform binding characteristics resembling those of monoclonal antibodies when compared with imprinted polymers prepared using traditional methods with free template in solution. MIN as produced here can be employed as direct replacements of antibodies/enzyme in standard ELISA-like assays with minimal modifications to the protocol. To demonstrate this, we selected the glycopeptide antibiotic vancomycin as analyte which also can point the way to the use of synthetic peptide epitopes instead of whole proteins in this type/format of

assay,^[12] again, to avoid using potentially unstable or expensive biomaterials. To develop the MIN-based assay, vancomycin was initially conjugated onto the well surface of a 96 well micro plate (via amine coupling, polystyrene plate surface derivatized with maleic anhydride). Then, in order to assess if imprinted nanoparticles could bind preferentially to this surface, as opposed to surfaces without derivatization, particles were loaded onto the wells which were subsequently washed. Random/irreproducible peroxidase activity values were detected on wells without immobilized vancomycin, ranging from no activity to values similar to those of Fe₃O₄-C on vancomycin-derivatized surface (see text below). High peroxidase activity was only detected on surfaces containing immobilized vancomycin and attached nanoparticles were then visualized by Atomic Force Microscopy (AFM), Figure 1. The incomplete coverage of the surface with particles might indicate that the performance of the assay can be improved by optimization of the surface characteristics. This can be related with incomplete surface coverage with reactive groups, or some of these are too close to the surface to allow for MIN binding. The use of spacer groups between target molecule and surface, or the use of different coupling techniques will be investigated in the future order to improve the amount of nanoparticles which can be bound to the surface, and so increase the amount of catalytic sites, with consequent increases in measured absorbances. The peroxidase activity was determined by addition of standard colorimetric substrate 3,3',5,5'-tetramethylbenzidine (TMB) for ELISA and hydrogen peroxide. TMB produces a blue color when oxidized by hydroxyl radicals produced in the presence of a catalyst (such as Fe₃O₄ or peroxidases) and H₂O₂.^[7] By measuring the rate of color development in the reaction system with TMB, H₂O₂ and Fe₃O₄-MINs at different concentrations it was found that the quantity of nanoparticles remaining in the wells after incubation and washing (in the absence of free vancomycin) is equivalent to ca. 20 µg mL⁻¹. The MIN-based assay was then performed in competitive mode. Upon addition of MINs and vancomycin solutions, the extent of nanoparticles bound to the well surface was found to be dependent on the amount of free vancomycin in solution (by measuring the peroxidase activity) after incubation and washing to remove unbound MINs. A good correlation was observed between the absorbance of the solution and the concentration of vancomycin over the range from 10 nM to 1 mM (Figure 2). When control Fe₃O₄-C particles were used, in the absence of free vancomycin, a response of 89 ± 11 mAU was recorded, as compared to 834 ± 47 mAU for the Fe₃O₄-MINs (both values obtained after 1 hour incubation with 100 µL nanoparticle solution, 50 µg/mL, loaded in each well and following washing). The absorbance values obtained with Fe₃O₄-C were not affected by the presence of vancomycin. While vancomycin was selected primarily as a model analyte, the obtained linear range is well within the recommended serum monitoring levels of 7 to 14 µM,^[13, 14] demonstrating potential for the development of vancomycin assays based on this methodology. The assay was found to be specific, with minimal response to other antibiotics, each present at 0.1 µM Figure 1 (inset). While there is no vancomycin analogue available, bleomycin and gentamycin share chemical similarities with vancomycin, hence their selection. In order to assess the performance of the assay in more complex sample matrix, vancomycin quantification was also performed in spiked porcine serum, diluted 1:100. Using this dilution, therapeutic monitoring levels of 7 to 14 µM (equivalent to 0.07-0.14 µM in the diluted serum) could in theory still be quantified, taking into account the initial calibration curve in Figure 2. The presence of serum proteins required the use of a spacer between

immobilized vancomycin and the well surface, possibly due to the formation of a layer of adsorbed protein on the surface restricting access of the Fe₃O₄-MINs to the vancomycin in the previous assay format. For spacer we used Bovine Serum Albumin (BSA) conjugated with vancomycin, this conjugate was then immobilized on the well surface. Using this approach, a new calibration curve was obtained for vancomycin from 3 to 0.05 μ M, Figure S3, which was then used to quantify vancomycin in spiked serum samples diluted 1:100 (final vancomycin concentration: 2.6, 1.3 and 0.66 μ M), with the following results: 2.6 μ M, found 2.1 ± 0.2 ; 1.3 μ M, found 1.2 ± 0.1 ; 0.66 μ M, found 0.20 ± 0.03 , measurements repeated five times. To increase the performance of the analysis in serum or similarly complex samples will probably require further optimization work related with type/length of spacer between target and plate surface, washing/blocking agents and assay protocols. Regarding stability, MINs are expected to be stable at room temperature, as recently demonstrated by Chianella *et al.*,^[15] who have studied the shelf-life of molecularly imprinted nanoparticles prepared with the same monomers as used here. It was found that after one month storage at room temperature (or one week at 40 °C) the molecular recognition properties of the nanoparticles were unaffected. In conclusion, we have demonstrated the possibility to perform an ELISA-like assay where no biological material was used, either for recognition or reporting purposes, with minimal modifications to the standard protocol. Peroxidase-mimicking imprinted nanoparticles were produced by the solid-phase imprinting method in the presence of Fe₃O₄ and used for combined target recognition and reporting by catalyzing the oxidation of TMB. The results are a starting point for the development of a new generation of assays using stable synthetic reagents, which could find uses especially in field/emergency conditions or in locations where a cold-chain for storage and transport is unavailable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Scheme 1.

Representation of the solid-phase synthesis protocol with addition of Fe₃O₄ for preparation of peroxidase-mimicking core-shell MIN (left) and TEM image of the obtained Fe₃O₄-MIN particles (right).

Figure 1. AFM images showing topography of the micro plate well surface before (left) and after 1 hour incubation with 100 μL Fe_3O_4 -MINs ($50 \mu\text{g mL}^{-1}$) and washing ($3 \times 100 \mu\text{L}$) with deionized water.

Figure2.

On the left, scheme of the assay in competitive mode, and on the right, calibration curve for vancomycin obtained with core-shell catalytic MIN (main) and (inset) normalized assay response (cross-reactivity) to different antibiotics, all experiments performed under physiological conditions (PBS 1 × , pH 7.2). Solutions of Fe₃O₄-MINs (100 μL, 50 μg mL⁻¹) were added to each of the vancomycin modified wells, followed by 50 μL vancomycin solution. After one hour incubation, wells were washed (3 × 100 μL PBS) and colorimetric TMB assay performed, full details in S. I. Error bars represent ± 1 standard deviation and are for experiments performed in triplicate.

Table 1

Apparent kinetic parameters of Fe₃O₄ nanoparticles and Fe₃O₄-MIN as peroxidase mimics.

Particle type	K_m [M]	V_{max} [M s ⁻¹]	k_{cat} [S ⁻¹]	(k_{cat}/K_m) [(M s) ⁻¹]
Fe ₃ O ₄	0.49	16.8×10^{-9}	77.8	156.3
Fe ₃ O ₄ -MIN	0.48	19.8×10^{-9}	92.1	190.6