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Development of Molecularly Imprinted Polymers specific for blood antigens for application in antibody-free blood typing

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A novel approach in antibody-free blood typing based on Molecularly Imprinted Polymeric nanoparticles is described.

Determining a patient's blood type is a vital and universal step in both blood donation and transfusion.[1,2] Existing blood type tests typically use antibodies immobilised on a solid material which cause blood cells to agglutinate upon making contact, and a clotted blood sample would indicate that the blood cells are of the blood type corresponding to the antibodies used.[3-5] Though antibodies display high affinity and specificity and are widely used as biological sensors, they are susceptible to denaturing caused by variation in temperature, pH, salinity and a number of other factors. If any of the mentioned requirements are not met the antibody-based blood typing test could potentially generate fatal errors. Therefore, development of synthetic receptors, which combine the recognition properties of natural antibodies with high stability, low cost and short production time would be very beneficial and desirable. In order to demonstrate the possibility of replacing natural antibodies in a blood typing application, MIP nanoparticles specific to blood B antigen have been created and tested.

For over forty years, molecular imprinting technology has been involved in the production of polymeric materials with receptors specific towards almost any target or 'template' - from small molecules to whole cells and viruses.[6] The first successful attempt of blood typing using MIPs was reported by Dickert and his group in 2005.[7,8] It was shown that it is possible to recognise different types of blood cells through the surface imprinting of whole erythrocytes on the sensor surface of microbalances covered with a 100-nm polyurethane layer. Nevertheless, the necessity to use whole blood cells in combination with microbalances was a rather complicated approach from manufacturing point of view and was not compatible with individual analytical tests, which should be portable, affordable and easy to use. Recent advances in the solid-phase synthesis of MIP nanoparticles have allowed us to come a step closer to this goal. In this work, blood type determinants, rather than whole red blood cells, have been selected as templates for imprinting. The blood group A and B determinants are two of the most important antigens present on erythrocyte membranes of humans.[1] Both of the determinants are oligosaccharide molecules. It is worth noticing that while peptide and protein imprinting is nowadays relatively well established process, oligosaccharide imprinting has not being described in the literature with exception of heparin.[9] Hence this paper is the first example of preparation of MIPs for trisaccharides and their use in diagnostic application. Blood type B trisaccharide consisting of galactose, fucose and galactose (Gal-a-1,3(Fuc-a-1,2)Gal) and has been selected as a template for the preparation of molecularly imprinted nanoparticles (Figure 1a).[10] The non-specific oligosaccharide maltotriose (Figure 1b) has been used for the preparation of control MIP nanoparticles, which are non-specific for blood. Additionally, the nanoparticles have been made magnetic by adding iron oxide to the monomeric mixture. The paramagnetic properties of the nanoparticles proved beneficial, offering easy handling and efficient immobilisation of the MIP nanoparticles on the magnetic surfaces of the assay system. It was expected that paramagnetic MIP nanoparticles would be drawn towards magnets, carrying any blood cells bound to them and causing the sample to decolourise. This change will only occur with blood samples of the same type as the MIPs used, and so it allows the determination of blood type.

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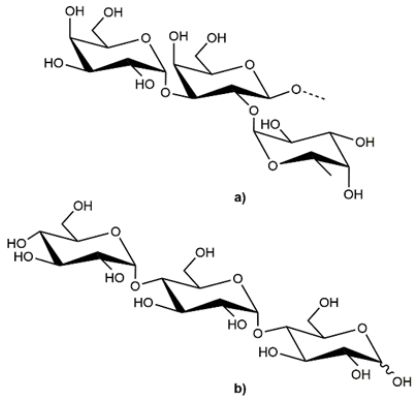
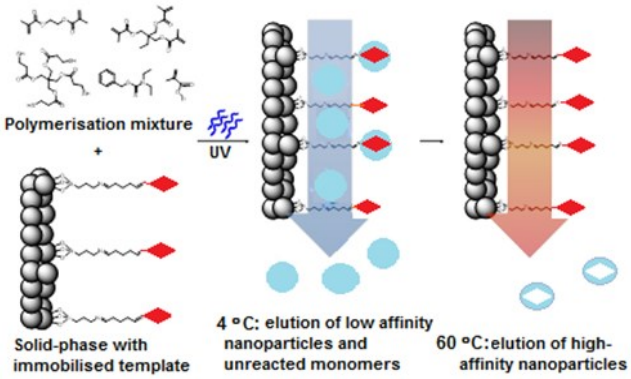


Fig. 1. Molecular structures of blood B antigen trisaccharide (a) and maltotriose (b).

The synthesis of blood-B-antigen-specific MIPs has been conducted using the solid-phase synthesis approach pioneered by the University of Leicester (Scheme 1).[11] It is important to highlight that this was the first reported case of a successful molecular imprinting of individual oligosaccharides.



Scheme 1. The schematics of the solid-phase synthesis of MIP nanoparticles.

In order to prepare the solid phase required for imprinting, the blood group B trisaccharide was immobilised onto the surface of amine-functionalised glass beads. The primary alcohol groups of the trisaccharide were coupled to the surface amine groups using *N,N'*-disuccinimidyl carbonate. The success of the immobilisation of the trisaccharide template was confirmed by reacting the trisaccharide-containing beads with Tollens Reagent, a test for the presence of reducing agents such as the aldehydes of the trisaccharides. For the solid phase synthesis of molecularly imprinted nanoparticles the following monomeric mixture was prepared: 90 mg of pentaerythritol tetrakis (3-mercaptopropionate (PETMP), 0.37 g of benzyl-dithiocarbomate iniferter, 1.62 g of trimethylopropane trimethacrylate (TRIM), 1.62 g of ethylene glycol dimethacrylate (EGDMA), 1.44 g of methacrylic acid, 11 g of acetonitrile, 50 mg iron (II) oxide.[12] The monomeric mixture was sonicated for 10 minutes and nitrogen gas was bubbled through the mixture for 5 minutes. Antigen-functionalised beads were degassed in a desiccator and saturated with nitrogen. The monomer mixture was then poured over the antigen-functionalised beads until it covered them completely. The beads were placed between two 60 W - UV light sources for 150 sec. In order to remove the unreacted monomers and low-affinity MIP nanoparticles the beads were washed with four bed volumes of acetonitrile at room temperature. The high-affinity nanoparticles were collected by washing the glass beads four times with 10 mL of acetonitrile at 65 °C, collecting and combining elution fractions each time. The high temperature wash enabled the separation of the high-affinity MIP nanoparticles from the template, therefore allowing their collection. The non-specific nanoparticles were prepared identically by imprinting of maltotriose, which is selected as a non-specific trisaccharide of similar size and functional groups to the B-antigen. The blood type B antigen and maltotriose-specific nanoparticles have been collected from the suspension using a magnet.

The physical properties of the produced magnetic nanoparticles have been characterised using a dynamic light scattering (DLS) technique. It was found that the particles with diameter between 70 and 300 nm were observed depending on the solvent used during the measurement. A scanning electron microscope was also used to inspect the nanoparticles (Fig. 2). These images demonstrate that approximate size of the nanoparticles in dry conditions is approximately 70 nm and particles are uniform in size and shape.

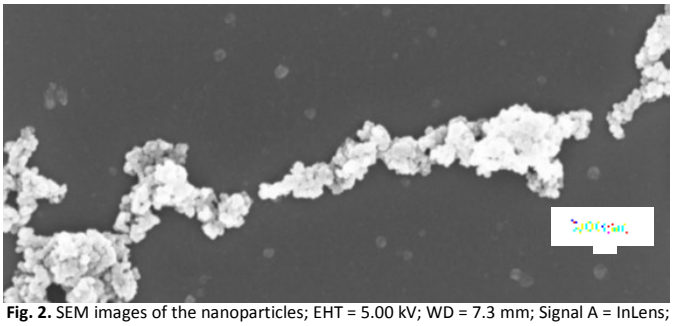
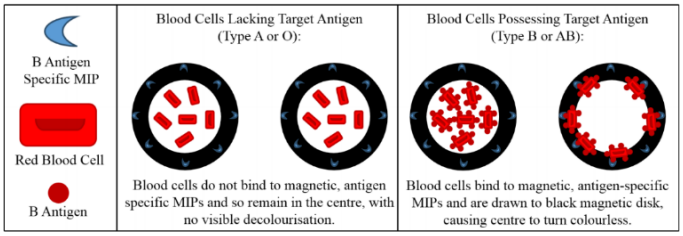


Fig. 2. SEM images of the nanoparticles; EHT = 5.00 kV; WD = 7.3 mm; Signal A = InLens; Mag = 63.15 K X.

In order to demonstrate specificity of MIPs to a single blood type, the following three sets of tests were performed: a) B antigen-specific MIP + blood type B; b) B antigen-specific MIP + blood type O; c) maltotriose-specific MIP + blood type B. The experiments were conducted in microtiter plate wells modified with magnetic inserts as previously described.[13] The optical density of solution was measured through the internal cavity to monitor the kinetics of the reaction between MIPs and blood. Due to the structure of the magnetic inserts, nanoparticles were spontaneously attracted to the inserts and the insert material did not interfere with the optical reading of the immobilised material performed using a standard microtiter plate reader. It was expected that specific blood cells will be bound to the corresponding specific MIP nanoparticles and be drawn to the magnetic inserts, and non-specific blood cells will remain distributed throughout the well (Scheme 2).



Scheme 2. Potential responses of different blood types to the presence of blood type B-specific MIP nanoparticles. The black circles are magnetic disks on the inside walls of the microtiter plate wells.

It was found that the results obtained for the experiments involving B antigen-specific MIPs with blood type B showed up to a 19% drop in optical density as expected for a successful result. The results for the following two experiments (B antigen-specific MIP nanoparticles with blood type O and maltotriose-specific MIP nanoparticles with blood type B) which were predicted to have only a minor decrease in optical density due to non-specific binding, instead showed a considerable increase (up to 18%). This allowed the test to provide results with greater clarity than anticipated; an indication of binding was the solution becoming clearer (Fig. 3, right), and an indication of lack of binding was the solution becoming darker (Fig. 3, left).

Fig. 3. Image of the microtiter plate, which contain blood type O samples (two left wells), and blood type B samples (two right wells) in the presence of B antigen-specific magnetic MIP nanoparticles. The black circles are magnetic disks on the inside walls of the microtiter plate wells.

This behaviour may be explained by a natural coagulation of the erythrocytes in the read-through area of the well. There are a number of causes of the observed gradual increase in optical density of blood outside the body. Before shaking occurs, the blood undergoes clotting due to the activation of the extrinsic pathway of the coagulation cascade. The activation of the cascade ultimately leads to fibrin polymerisation, which has been shown to cause the optical density of whole blood to increase.[14] Shaking can break apart the fibrin cross-links and therefore also lower optical density. When shaking takes place, the energy provided also causes haemolysis of some blood cells,[15] which contributes to the increase in optical density. Finally, oxygenated haemoglobin has a higher absorption coefficient of light of wavelength 405 nm and the blood may become increasingly oxygenated upon exposure to air.[16] All of these factors contribute to the gradual increase in optical density.

In order to study the kinetics of the interaction between the whole blood and nanoparticles, high-affinity MIPs were collected from 10 mL of the suspension using a strong magnet. They were then re-suspended in 1 mL of phosphate buffer saline solution, pH 7.4 (PBS). Two types of MIP nanoparticles have been tested- blood type B specific (B antigen trisaccharide-specific MIPs) and blood type B non-specific (maltotriose-specific MIPs). 10 µL of blood sample were mixed with 500 µL of PBS. In one series of experiments this was blood type B, and in the other, blood type O. 50 µL of the MIP solution in PBS was added to microtiter plate wells containing magnetic inserts followed by 50 µL of the blood solution in PBS. The optical density was measured for 40 min at a wavelength of 405 nm with 5-min intervals (Fig. 4).

It was demonstrated that the difference in optical density (between samples with specific MIPs, without specific MIPs and with non-specific MIPs) was greater when shaking took place after 20 minute of incubation. This is explained through the dynamics of the interaction between magnetically immobilised MIPs and red blood cells. During 20 min of incubation, blood cells had sufficient time to bind to one another and coagulate. Shaking after coagulation resulted in a single MIP nanoparticle binding to an entire cluster of coagulated blood cells. Removing clusters rather than single cells allowed each binding to cause a greater decrease in optical density. In the case of testing non-specific MIPs or non-specific blood types, coagulated erythrocytes were not attracted towards the magnetic inserts and optical absorbance increased. The responses of the test-system for specific and non-specific blood type confirmed that it is possible to determine the blood type without any need for natural antibodies using solely magnetic MIP nanoparticles.

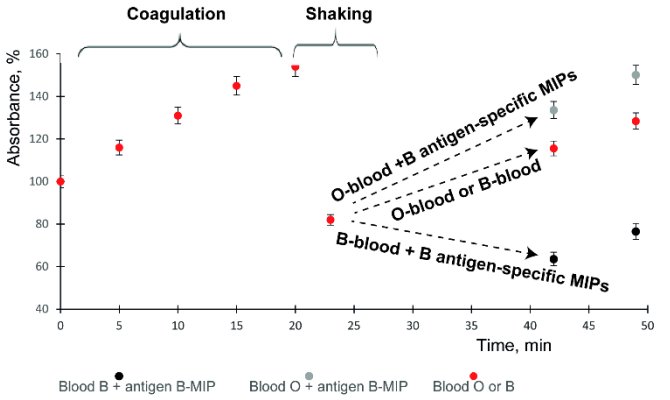


Fig. 4. Specific interactions between B antigen-specific MIPs and B-blood (black dots) and non-specific interactions between B antigen-specific MIPs and O blood (grey dots). All measurements in the microtiter plates were done in 6 replicates.

It is important to stress that this report does not constitute a complete development of the blood-testing system, but rather a feasibility study which demonstrates an alternative to use of antibodies in the blood typing system. It is possible to highlight that the protocols developed here could be used as blueprints for the preparation of other blood antigen-specific synthetic receptors. For example, using type A trisaccharide as a template will allow the creation of MIP nanoparticles that bind

selectively to blood-type A antigens. Having presented here an optimised synthesis of blood type B antigen-specific MIP nanoparticles, it will be simple to create a test kit that can differentiate between type A, type B, type AB and type O blood using visually observed differences in optical densities. Further steps would include the imprinting of the protein-based D antigen. Combined with A and B antigen-specific MIPs, this will allow the creation of a test for the determination of ABO/Rh blood type using no antibodies. This system can then be generalised to other blood types which use erythrocyte surface antigens as biomarkers. Additionally, since the proposed assay relies on blood coagulation it would be possible to reduce the test time by using appropriate clotting activators.

It was shown that blood antigen-specific molecularly imprinted nanoparticles can be created that can replace natural antibodies in blood type analysis. The new format of the test system is based on magnetically-induced decolourisation rather than existing agglutination-based principles. The creation of these B-antigen specific magnetic MIPs is the first step towards a general system for the easy determination of blood type using synthetically produced polymers, which may then be expanded for use in other biological sensors.

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