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Published in:
Chemical Communications

Link to article, DOI:
10.1039/c8cc00343b

Publication date:
2018

Document Version
Peer reviewed version

Journal Name

COMMUNICATION

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

DOI: 10.1039/x0xx00000x

www.rsc.org/

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We describe a novel dispersive solid-phase imprinting technique for the production of nano-sized molecularly imprinted polymers (NanoMIPs) as plastic antibodies. The template was immobilized on in-house synthesized magnetic microspheres instead of conventional glass beads. As a result, high-affinity and template-free MIPs were produced in high yield.

Natural antibodies are the most utilized affinity agent due to their high affinity and selectivity towards biomolecules of interest. However, they are expensive to produce, and are unstable due to their biological origin.[1-3] As a result, developing alternative antibody mimics have gained increasing prominence.[4,5] Molecularly imprinted polymers (MIPs), also known as plastic antibodies, are synthetic affinity agents which can be tailor-made for a wide range of analytes. They are synthesized by mixing the template with functional monomers to form non-covalent interactions. Upon crosslinking, a molecular-sized plaster cast of the template is formed which can be utilized as the recognition site. While the imprinting of small molecules has become almost routine, the imprinting of large biomolecules especially proteins remains a significant challenge.[6]

Hoshino *et al* demonstrated the use of mild acrylamide-based free-solution precipitation polymerization to imprint proteins.[7] Nano-sized MIPs (NanoMIPs) were successfully formed. However, a significant problem of this method was the removal of the template from the polymer matrix. Dialysis was time-consuming and required a few days. Centrifuge membrane filters offered a quick solution, but the nanoMIPs could stick to the membrane, resulting in the loss of the product.

Recently, Poma *et al*. and Ambrosini *et al*. both demonstrated the use of solid-phase imprinting as an effective method for the synthesis of protein nanoMIPs.[8-11] Instead of having the template in solution, the proteins were immobilized onto glass beads. The beads were then packed into a reactor where the reaction mixture containing monomers, initiator and crosslinker was added. After synthesis, the unreacted monomers and low affinity nanoMIPs were washed away, and high-affinity nanoMIPs were separated from the templates by hot washing or thermo-responsive swelling. The solid-phase synthesis technique greatly simplified the template removal process and ensured only high-affinity nanoMIPs were collected. However, despite the promise, the use of glass beads as solid support has caused a number of drawbacks. In all previous examples, commercial glass beads with average diameter of 70-100 µm were employed. The relatively large diameter led to low surface area, such that only a small amount of protein could be immobilized. In order to provide adequate template, a typical reaction required as much as 20 - 80 g of glass beads.[12] Another problem with the glass beads was that they were prone to abrasion and could not be stirred during reaction, since stirring could cause the formation of nanoglass particles (nanodust) as well as the leaching of the template. Consequently, the interactions between the protein templates and monomers were adversely affected by the close proximity of glass beads, resulting in low reaction efficiency and low yields (0.15-0.3 mg of MIPs per gram of glass beads).[13] The need for a large quantity of solid support as well as the low yield has prevented the technique from being widely adopted by the MIP community. One potential strategy to address these challenges would be to use alternative solid-phase materials.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Journal Name

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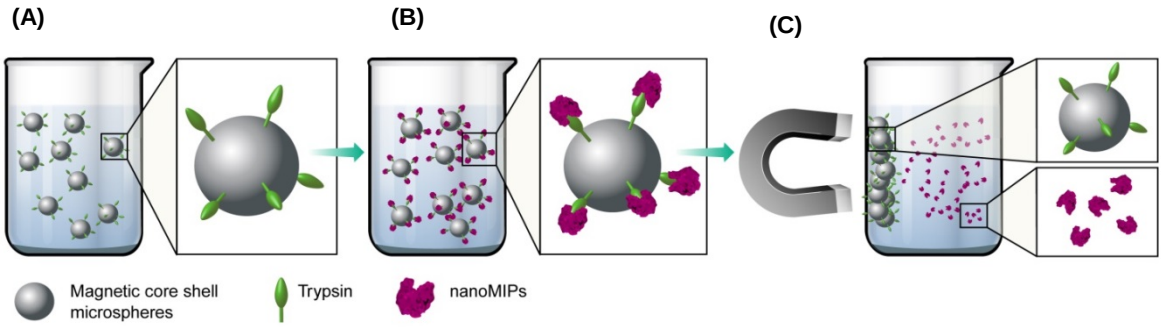

Scheme 1: Overview of dispersive molecular imprinting. (A) The protein template was immobilized on the magnetic microspheres. (B) During the polymerization, the magnetic microspheres were dispersed throughout the mixture. (C) After the reaction, the microsphere-MIP conjugates were collected by a magnet, and high-affinity MIPs were eluted by hot washing.

In this paper, we developed a new imprinting method termed "dispersive solid-phase imprinting" (**Scheme 1**). In contrast to previous solid-phase synthesis, we immobilized the proteins on magnetic microspheres with a diameter of 600-700 nm (**Scheme 1A**). The high surface-to-volume ratio of the magnetic microspheres due to their lower diameter and higher surface area resulted in higher degree of template immobilization per gram of solid phase material, hence a much smaller volume of solid support was needed for each reaction. Moreover, the magnetic microspheres were not sensitive to abrasion due to their small molecular sizes. Thus, the magnetic microspheres could be dispersed throughout the mixture during the polymerization, allowing for more thorough monomer-template interactions (**Scheme 1B**). After the reaction, the microsphere-MIP conjugates were simply collected by a magnet attributed to the magnetic property of the microspheres, and high-affinity nanoMIPs were eluted by hot washing (**Scheme 1C**). The novel dispersive solid-phase imprinting technique combined the advantages of both free-solution polymerization and solid-phase synthesis, and allowed for the production of high-affinity and template-free nanoMIPs in high yield. We demonstrated that by using the magnetic microspheres, the yield improved 83-167 folds (**Table 1**). In addition, as the method could be easily scaled up, it is promising for commercial purposes.

Core-shell magnetic microspheres were synthesized. Firstly, iron oxide cores with a size of 260 nm were produced using the thermal-solvent method as previously described.[14] The thermal solvent approach was chosen since it allowed larger microspheres to be formed by assembling a number of smaller individual nanoparticles. Next, a SiO_2 layer with a radius of 200 nm was added by a sol-gel process. The microspheres were then modified by grafting an epoxy functional group onto the surface.

Table 1. Comparison of dispersive solid-phase imprinting and conventional solid-phase synthesis.[9,13]

	Solid support	Amount of solid phase per reaction	Amount of immobilized protein template	Yield of nanoMIPs
Solid-phase synthesis	Glass beads (70-100 µm)	40 g	1.7 nmol g^{-1} of glass beads	0.15-0.3 mg of MIPs g^{-1} of glass beads
Dispersive solid-phase imprinting	Magnetic microspheres (671 nm)	300 mg	237 nmol g^{-1} of microspheres	25 mg of MIPs g^{-1} of microspheres

We used epoxy group instead of conventional glutaraldehyde functional groups because the latter had longer linker which tended to cause aggregation of the microspheres. The size and zeta potential measurement are listed in **Table S1**. The total size upon the addition of the epoxy silane was approximately 671 nm with a PDI of 0.051. An increase in the zeta potential was observed when the SiO_2 layer and the epoxide monolayer were added sequentially. The TEM images of FeO_x, FeO_x@SiO_2 and FeO_x@SiO_2-epoxide are shown in **Figures S1.** XPS and IR analysis confirmed the formation of both FeO_x and FeO_x@SiO_2 (**Figure S2 and S3**). The size of the resultant microspheres was about 3 times as big as that of the nanoMIPs. We did not synthesize microspheres smaller than that, as it could increase the chance of encapsulation of the microspheres into the MIPs

during polymerization. Trypsin was used as the model template in this study. The protein was immobilized onto the magnetic microspheres *via* an amine coupling reaction to the epoxide grafted magnetic microspheres in carbonate buffer pH 9.0. The degree of immobilization was characterized by a BCA protein assay (**Figure S4**). The amount of protein immobilized was 237 nmol per gram of microspheres, which increased 140 folds when compared to the immobilization on the glass beads (1.7 nmol per gram of glass beads).[9] The high degree of immobilization matched well with the increased surface of the microspheres, as the surface-to-volume ratio of the microspheres (8.5 μm^{-1}) was 85-140 times as much as that of the glass beads (0.06-0.1 μm^{-1}). The amount of protein immobilized on 300 mg of microspheres was thus equivalent to that immobilized on 40 g glass beads. This means that by using the microspheres, much less solid-phase material was required per reaction.

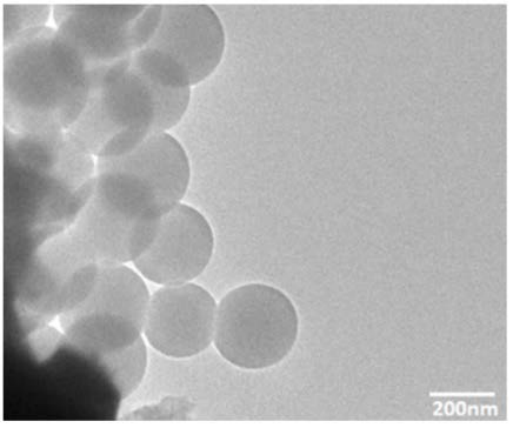

Figure 1. TEM image of trypsin Imprinted nanoMIPs.

NanoMIPs were formed using the hydrogel-based precipitation polymerization.[7] A typical reaction involved the addition of acrylic acid (Acc) and N-tetra-butylacrylamide (TBAm) as the negatively charged and hydrophobic functional monomers respectively, N-isopropylacrylamide (NIPAM) as the back bone monomer, N,N'-methylenebisacrylamide (BIS) as the cross-linker. N-(3-Aminopropyl)methacrylamide (APM) hydrochloride was also added to introduce primary amine groups. In addition, sodium dodecyl sulfate (SDS) was used as a surfactant in the reaction in order to stabilize the nanoMIPs upon formation as well as prevent encapsulation of the microspheres. 300 mg of magnetic microspheres with immobilized protein templates were well mixed with the monomers by dispersing the microspheres into the reaction mixture. The polymerization was initiated using N,N,N'N – tetramethylethylenediamine (TEMED) as the catalyst and ammounium persulfate as the Initiator (APS). Constant orbital stirring was applied during the synthesis. The resultant microsphere-MIP conjugates were collected using a magnet, then washed several times with water at room temperature to remove all non-reacted monomers and low affinity nanoparticles. The TEM image of the synthesized nanoMIPs eluted off the solid phase by a 60°C hot elution is shown in **Figure 1**.

The nanoMIPs had an average size of 200-220 nm, while the size reported by DLS was 206 ± 4.2 nm with PDI of 0.3 ± 0.04 (**Figure S5**). Both TEM and DLS analysis demonstrated that the nanoMIPs had well-controlled spherical structures and were very homogenous in size. The yield of the nanoMIPs in presence of 300 mg microspheres was 7.5 mg, or 25 mg g^{-1} of microspheres. In contrast, when the same amount of protein template (immobilized on 40 g of glass beads) was used in the conventional solid-phase synthesis, only 5 mg nanoMIPs was obtained.[15-17] This was due to the fact that the templates interacted much more efficiently with the monomers when they were dispersed in the solution. As a result, more monomers were transferred into high-affinity nanoMIPs. To further increase the amount of the nanoMIP product, the dispersive solid-phase imprinting could be easily up scaled by increasing the quantity of the microspheres. Comparison of dispersive solid-phase imprinting and conventional solid-phase synthesis was summarized in **Table 1**. The binding between the nanoMIPs and trypsin was characterized using the surface plasmon resonance (SPR). The nanoMIPs were immobilized on the SPR surface using an amine coupling. The corresponding NIP (MIP for another analyte) was immobilized as a control. Trypsin solutions were injected over the surface from the lowest to highest concentrations, and the accumulative sensorgram is shown in **Figure 2A**.

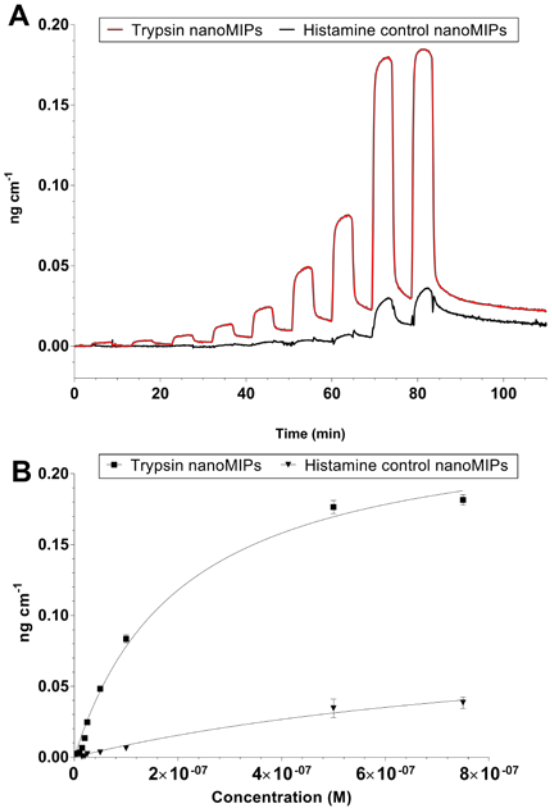

Figure 2. (A) SPR sensorgram showing the response of nanoMIPs and control NIPs. (B) Equilibrium analysis of trypsin binding to the NanoMIPs and the histamine control MIPs.

The K_D was determined to 2 x 10^{-7} M while R_{max} was 0.186 ng cm^{-1} (**Figure 2B).** The selectivity of the nanoMIPs was confirmed on SPR by incremental injections of trypsin, RNase, β-lactoglobulin, and hemoglobin, respectively (**Figure S6**). The responses of each protein at the highest concentration tested are shown in **Figure 3.** Trypsin showed much higher response compared to other proteins, suggesting good selectivity towards trypsin.

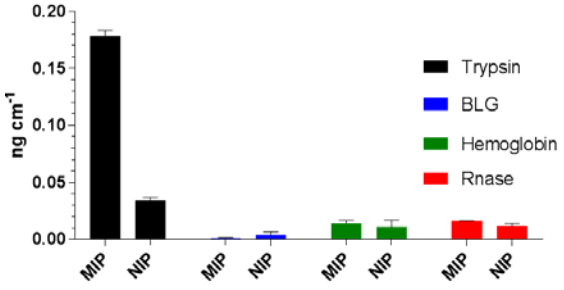

Figure 3. SPR response of proteins towards trypsin nanoMIPs

In summary, a new dispersive solid-phase imprinting technology was successfully developed to synthesize protein nanoMIPs. By using the magnetic microspheres as a new type of solid support material, nanoMIPs with high affinity and high selectivity towards the protein template were obtained, while the yield increased 83-167 folds when compared to conventional solid-phase synthesis. The dispersive solid-phase imprinting technique combined the advantages of both free-solution polymerization and solid-phase synthesis, and would make a big step forward for the production of protein MIPs. In addition, this technique is also compatible with small molecule imprinting and epitope imprinting.[14-16] We are currently expanding the imprinting methodology for imprinting biological relevant macromolecules and investigating the use of chemical approaches and low temperature methods for the elution of high affinity nanoMIPs to allow for the Solid-Phase to be reused even for temperature sensitive templates.

Conflicts of interest

The authors declare no conflict of interest

Acknowledgements

This work was financially supported by the Villum Fonden, Denmark, Project No. 13153.

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