Automatic reactor for solid-phase synthesis of molecularly imprinted polymeric nanoparticles (MIP NPs) in water

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Electronic Supplementary Information (ESI) available: experimental details, schematic of the immobilisation of the templates on solid phase, photo of the reactor, BCA assay calibration curve and results, SPR sensorgrams.

Abstract

We report the development of an automated chemical reactor for solid-phase synthesis of MIP NPs in water. Operational parameters are under computer control, requiring minimal operator intervention. In this study, "ready for use" MIP NPs with sub-nanomolar affinity are prepared against pepsin A, trypsin and α-amylase in only 4 hours.

The extent of research into biomedical and diagnostic nanotechnology is impressive,[1] despite currently very few examples of biomedical nanomaterials have been approved by the FDA.[2] Apart from the potential toxicity, the usage of nanomaterials in therapy and diagnostics finds a strong opponent in the well-established leadership of "safer" natural antibodies.[3] Industrial manufacturing of antibodies, however, is logistically complex and expensive, especially for applications in therapy.[4] Potential immunogenic adverse reactions, low stability and poor performance in non-physiological conditions restrict the application of antibodies to specific niche areas and shorten their shelf-life.[5,6] Finally, natural antibodies can be difficult to integrate with assays and sensors.[6,7]

A possible alternative are MIP NPs, which share key characteristics with antibodies such as aqueous solubility, size, affinity and selectivity for the target. Additionally, solutions of MIP NPs can be handled similarly to solutions of antibodies, with the advantage of stability and robustness.[8] Moreover, operational parameters for producing MIP NPs can be carefully controlled.[9] There is, however, lack of a generic protocol for the synthesis of MIP NPs with specific characteristics,[8] which restricts their practical applications, despite recent reports of biological activity[10] and *in vivo* detoxification[11] hint at a great potential of MIP NPs in the nanomedicine arena.

With the aim to provide a method that is scalable, reproducible and controlled, we have focused our efforts on the development of synthetic strategies for producing high-performance MIP NPs. The most suitable protocol should in our view include affinity purification with immobilised template to remove monomers/low-affinity NPs from the product.[12] Even better results would be obtained if the immobilised template is used also in polymerisation, as this will ensure high-binding site affinity and prevent the contamination of products with the template.[13] This approach would also add advantages of easy automation and the potential for scaling-up production.

Recently we have reported the first successful example of solid-phase synthesis of MIP NPs using living-polymerisation chemistry, performed in organic solvent under UV irradiation – conditions which are favourable for the imprinting of small molecules.[14] Imprinting of high-molecular weight

targets such as proteins, polysaccharides and DNA, however, would require an aqueous environment to preserve their structure during polymerisation.[15]

Here we report the development of a reactor for MIP NPs preparation using chemical polymerisation performed in specifically mild aqueous conditions (monomer concentration: 6.5 mM) for proteins such as trypsin, pepsin A, α-amylase.

The solid-phase synthesis of MIP NPs is performed in the presence of template previously immobilised on glass beads according to a well-established technique for coupling of proteins to silica surfaces (Fig. S1 in the Electronic Supplementary Information, ESI).[16] The amount of template immobilised, determined spectrophotometrically, was 1.7 nmol/g of glass beads for trypsin, 2.8 nmol/g for pepsin A and 2.9 nmol/g for α-amylase.

The principle behind MIP NPs solid-phase production is summarised in Fig. 1 with a schematic of the new reactor presented in Fig. 2 (a picture of the reactor can be found as Fig. S2 in the ESI). The developed prototype (Fig. 2) consists of a temperature-controlled reactor, which is loaded with the template-derivatised solid phase and fitted on a stand with a shaking mechanism to ensure adequate mixing of the polymerisation mixture. A set of pumps delivers the monomer mixture, initiator and washing and elution solvents, while on the outlet a fraction collector separates waste streams from high-affinity product fractions. The machine also includes a N_2 inlet to flush the reactor before polymerisation and to force out the liquid and empty the reactor under positive pressure. All the parameters and components of the reactor are computer-controlled and can be programmed in advance by the operator.

The following generic protocol has been applied for the automated synthesis and purification of MIP NPs for proteins: the first step involves loading the monomer mixture dissolved in water onto the temperature controlled reactor containing the template-derivatised affinity media. A mild polymerisation process is then initiated by ammonium persulfate (APS) and *N,N,N′,N′*-tetramethylenediamine (TEMED) and carried on for the desired reaction time. At the end of the polymerisation, the reactor likely contains a mixture of high-affinity MIP NPs, low-affinity MIP NPs and unreacted monomers/oligomers which needs purification. To this purpose, the reactor temperature was kept at 15 °C to allow removal of all the unreacted monomers/oligomers and other low-affinity MIP NPs, while keeping high-affinity MIP NPs attached to the immobilised template. These latter were subsequently eluted by washing at 60 °C (Fig. 1). The increase in temperature disrupts the interactions between the immobilised target and the high-affinity MIP NPs, thus assisting in their collection. The whole procedure lasts for about 4 h, after which an average yield of 43 ± 2.8 % (w/w) of high-affinity product with respect to initial monomer mass was obtained (when 60 g of solid phase were used). The yield of MIP NPs did not change significantly by changing the polymerisation time. Reactions for 2, 4, 10 or 20 h have been attempted in presence of 20 g of derivatised glass beads and the yield ranged from 8 to 10.5 % (w/w), hence we decided to carry out the polymerisation for the shortest time possible with the aim to potentially perform multiple cycles per day.

The technology developed here is extremely convenient for the production of high-affinity MIP NPs, offering several advantages in comparison with the classical preparation procedures:[8] i) it produces one fraction of high-affinity MIP NPs with narrow distribution of binding sites;[12] ii) MIP NPs are easily separated from the non-polymerised monomers, initiator and template using automatic

temperature-controlled elution steps,[14] as confirmed by the BCA assay (detection limit: 5 μg/mL) which did not display any protein contamination in the product (Fig. S3 in the ESI); iii) by using an immobilised template, the polymerisation process can be performed even if the template is poorly soluble in water; iv) the time for synthesis and purification of MIP NPs is significantly reduced (4 h) when compared with other available protocols, such as dialysis which can take days;[11] v) high binding site accessibility is achieved due to the "surface-imprinting" procedure.[12,17]

Since proteins can be easily denatured, it was not our intention to re-use the templates following MIP NPs preparation. It is, however, possible to use this approach in the imprinting of immobilised recyclable protein epitopes which would offer a convenient way to synthesise large quantities of protein specific MIP NPs.[14,15]

Optimisation experiments were performed to assess the effect which the modification of the quantity of glass beads has on the yield of MIP NPs, demonstrating that the amount of product increases proportionally with the amount of derivatised solid phase used in synthesis (Fig. S4 in the ESI). With this setup, the maximum dry weight of high-affinity material produced cycle is 12.5 mg (66 % yield with 80 g of solid phase). Increasing the volume of the reactor and quantity of solid phase will considerably increase the amount of product, an important aspect to be considered for scaling-up the procedure for commercial purposes. Other potential strategies might be the use of a solid support with a different morphology/surface area or running several reactors in parallel.

According to DLS analysis, MIP NPs had hydrodynamic diameters below 240 nm (208 ± 3 nm for pepsin A MIP NPs, 207 ± 12 nm for trypsin MIP NPs and 236 ± 4 nm for α-amylase MIP NPs, n = 3), which made them 4 times larger than those synthesised by Hoshino *et al.* using a similar monomer mixture.[11] The presence of solid phase and the absence of ionic surfactant might have an effect on the concentration and aggregation of monomers during the phase separation stage, which results in larger particles.

TEM analysis showed very similar images (see Fig. 4 for a typical sample of NPs made for trypsin). MIP NPs exhibited a dry size of ca. 80-100 nm and a spherical shape (Fig. 4). Higher values of measured diameters (by DLS) can be attributed to decreased motion in solution due to the swelling of the low cross-linked NPs gels in water, and/or to the presence of irregularities or exposed polymer chains on the particle surface.

The affinity and specificity of trypsin, α-amylase and pepsin A MIP NPs were investigated using a BIAcore 3000 SPR system by immobilising each template onto the gold sensors surface. Several concentrations of the high-affinity MIP NPs (from 4×10^{-4} nM to 4×10^{-8} nM) were sequentially injected on each chip bearing one of the templates, and their binding behaviour was recorded. The results of this study are shown in Fig. S5 in the ESI (a to i). All the MIP NPs synthesised specifically recognised and bound their target. Apparent dissociation constants (K_D) of 1.7×10^{-11} M, 4.1×10^{-11} M and 3.4×10^{-10} M were calculated for pepsin A, trypsin and α-amylase MIP NPs, respectively.

In our experiments we did not analyse low-affinity MIP NPs due to the difficulty with separation from the mixture of co-eluted monomers/oligomers. It was also not possible to synthesise and test non-imprinted (NIP) NPs since their preparation in the absence of template could not be performed using the protocol described in this paper. In order to prepare NIP NPs the accepted method is to prepare polymer under exactly the same conditions as the MIP but in the absence of template. Even

in the case of traditional "bulk" MIPs this is not ideal, since changes in surface area and morphology are often evident when the template is excluded, making the comparison with NIPs a compromise at best. In the case of immobilised templates it is not possible to prepare a NIP under the same conditions as the MIP, since separation of the monomer mixture and elution under the same conditions as the MIP cannot be achieved. For these reasons, the specificity of MIP NPs was assessed in a cross-reactivity study of the MIP towards other proteins which were not used in imprinting (e.g., trypsin MIP NPs were also injected onto the pepsin A and α-amylase-derivatised chips, see Fig. S5 in the ESI). Despite presence of some non-specific interactions, their analysis resulted in K_D values about 2 orders of magnitude higher than the specific ones. A higher selectivity could be achieved by attempting an oriented immobilisation of the template on the solid phase during the imprinting process, or by performing a rational selection of the monomers to be used for the NPs production. Both these aspects are currently under investigation.

In summary, we produced MIP NPs for pepsin A, α-amylase and trypsin using a newly designed automatic reactor containing template-derivatised glass beads as a solid support. Our automatic method offers short synthesis/purification times, thus making it potentially suitable for industrial applications and production. We believe that MIP NPs prepared with this reactor might challenge antibodies for *in vitro* as well as *in vivo* applications (e.g., in drug development/delivery or in imaging). We are actively pursuing demonstrations of the potential of these MIP NPs[18] as well as developing further improvements in reactor design.

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Figures

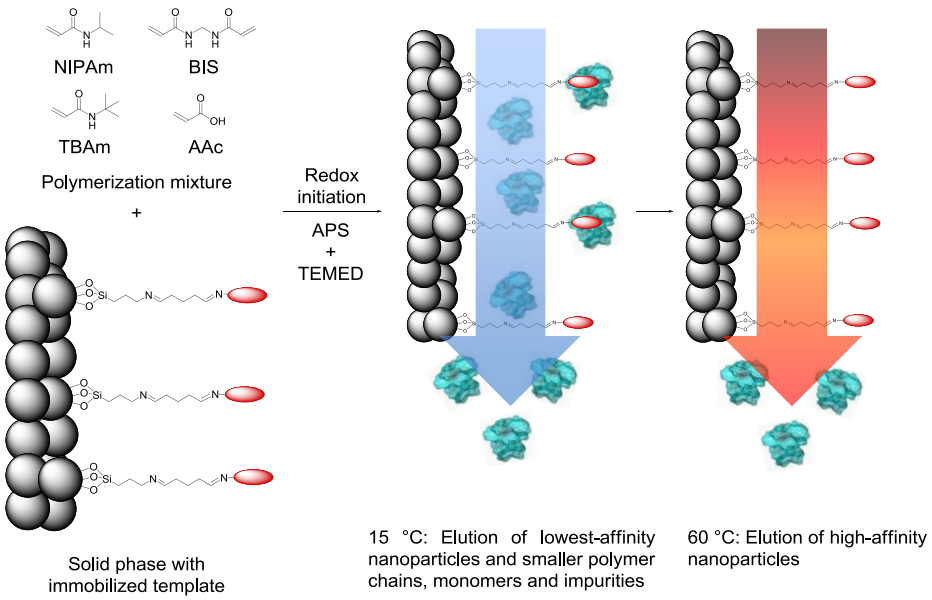

Fig. 1 Schematic representation of the solid-phase synthesis and purification of the high-affinity MIP NPs exploiting the different interaction strength at different temperatures.

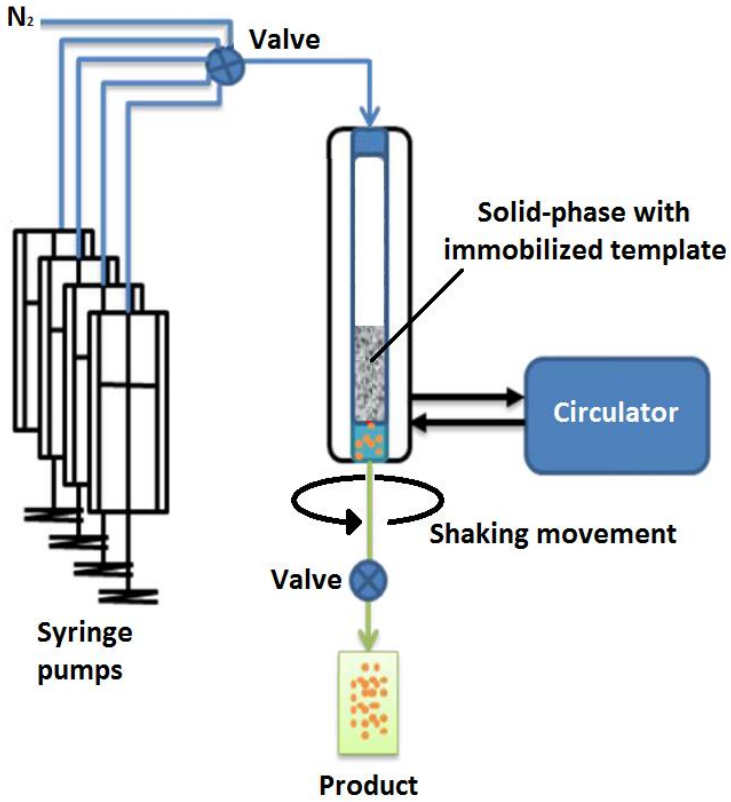

Fig. 2 Scheme of the automatic reactor setup developed and used for the synthesis of protein-imprinted MIP NPs on solid phase.

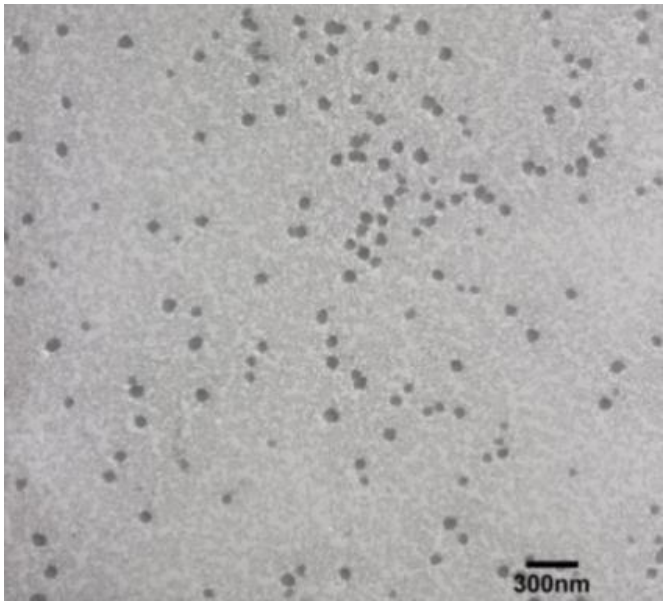

Fig. 4 TEM image of MIP NPs for trypsin at 20000× magnification.

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