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COMMUNICATION

Epitope imprinted polyethersulfone beads by self-assembly for target protein capture from the plasma proteome

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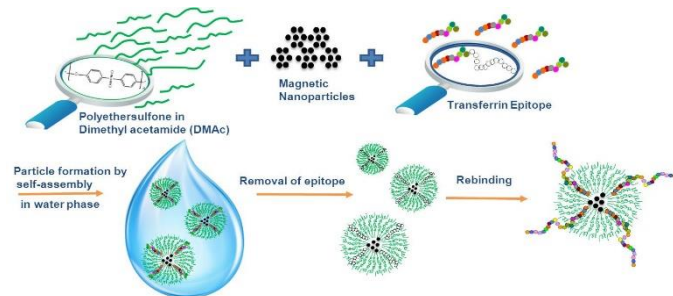
Polymer self-assembly was developed as an epitope imprinting strategy involving facial processes and high recognition site density. As a model, transferrin epitope-imprinted polyethersulfone (PES) beads were successfully fabricated using this technique. The imprinted beads demonstrated excellent selectivity toward the transferrin epitope and transferrin even in the real sample.

Protein capture *in vitro* is important for the detection and examination of clinically significant biomarkers. Antibody-based techniques that involve antibodies affixed to columns, membranes, glass, microplate wells, particles or other surfaces have been utilised for this task.¹ However, producing antibodies to certain biomarkers or target chemicals is impossible using current antibody production techniques. For example, most antibodies toward posttranslational modification proteins such as glycoproteins, phosphoproteins, acetylated proteins and hydroxylated proteins remain scarce.² Furthermore, antibody-based protein capture is limited by certain inherent characteristics.³ Therefore, the development of artificial antibodies is essential to overcome the aforementioned problems.

Protein imprinted materials are those that contain specific recognition sites formed through interactions with template proteins, which direct the position and orientation of the material's structural components.⁴ As artificial antibodies, protein-imprinted materials offer many advantages such as long-term storage stability, potential re-usability, resistance to harsh environments and low cost.^{3c} Several forms of protein-imprinted materials have been used for protein purification/isolation, protein crystallisation and as artificial antibodies in immunoassays.⁵ However, obtaining enough purified protein for protein imprinting remains difficult, especially with the low-abundance proteins in proteomic sample.⁶ Epitopes are the regions within the structures of globular proteins that can be classified as antigenic determinants. Epitope-imprinted materials were originally proposed by Rachkov and Minoura.⁷ Kenneth J. Shea *et al.* verified that synthesised peptides (i.e., the epitope) could replace

proteins as the template during protein imprinting.⁸ A few imprinting formats have recently been developed for the epitope.⁹ However, these imprinting strategies have been limited to unique epitopes, and their delicate fabrication protocols have hindered the extension of these methods to additional epitope categories. With the development and application of protein imprinting, the general consensus is that a characteristic fragment (i.e., an epitope) of the target protein should be the alternative to the whole protein as the template.^{3c, 6} Therefore, there is a pressing need for a general and straightforward imprinting method for the epitope.

Polyethersulfone (PES) possesses outstanding biocompatibility and controlled self-assembly properties and is among the most promising artificial polymeric materials for use in clinical research and therapy.¹⁰ Therefore, we attempted to employ PES polymer self-assembly to develop a universal strategy for epitope imprinting that is independent of the epitope category.



Scheme 1. The fabrication of transferrin epitope-imprinted polyethersulfone particles via polymer self-assembly and their application.

The epitope imprinted PES particles (MIP) were prepared using a self-assembly method based on a PES phase inversion in its non-solvent. As shown in Scheme 1, PES was dissolved to a concentration of 20 wt% in dimethyl acetamide (DMAc). An epitope from transferrin, the N-terminal sequence MRLAVGALL, was employed as the model template and dispersed with a concentration of 3 wt% in

the PES solution. Magnetic Fe_3O_4 nanoparticles were also dispersed in the PES solution for easy isolation of the particles from the incubation solution and further facile target protein capture. The resulting polymer solution was added dropwise to distilled water using a 0.6-mm diameter syringe needle at room temperature to fabricate the epitope-imprinted particles via phase inversion. The non-solvent water was selected as a coagulation medium, which is commonly used when preparing PES membranes.¹¹ The water exhibited high solubility for DMAc but not for the PES or the magnetic nanoparticles. Therefore, when the mixture was added to the water, a phase inversion occurred due to the rapid exchange of the DMAc and water. Imprinting occurred simultaneously with the PES solidification, and the transferrin epitope was retained in the solidified PES microspheres due to the non-covalent interactions between the PES and the transferrin epitope. Next, these particles were incubated in water for over 24 h to remove the DMAc from the microspheres. After the complete exchange between the solvent and the non-solvent, the preparation of the particles imprinted with the template was complete. The template molecules were separated from the solidified polymers by washing the polymers with methanol and a basic solution at 40°C until no epitope peaks could be detected in an HPLC elution. Simultaneously, non-imprinted PES particles (NIP) were prepared with a concentration of 24 wt.% PES solution in the same manner without the addition of the template.

Scanning electron micrographs (SEM) were acquired for the cross-sections of the prepared particles. As shown in Fig. 1(a–c), a hierarchical pore structure was observed, and macrovoids were distributed throughout the porous PES microspheres. A finger-like structure, which is typically seen during the phase inversion of PES, was observed under the outer skin layer as shown in Fig. 1(b). As shown in Fig. 1(c), many small pores were observed inside the microspheres, which could facilitate their application as an adsorbent material. The morphology, as determined by SEM, suggested that the PES transformation from the polymer solution to the solid state occurred quickly in the poor aqueous solvent, followed by the instantaneous PES precipitation. A similar hierarchical pore structure was also found in the non-imprinted microspheres as shown in Fig. S1. According to the drying loss measurements,¹¹ the average diameters of the MIP and NIP beads were 4.37 and 4.47 mm, respectively, while the porosities of the MIP and NIP beads were 84.75% and 83.46%, respectively.

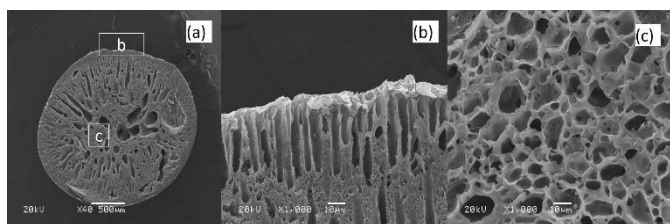


Figure 1. SEM photographs of the cross-sections of MIP: the entire particle (a), the skin layer (b) and the porous internal structure (c). Voltage: 20 kV

To study the recognition abilities of the transferrin epitope-imprinted particles, binding experiments were conducted in transferrin epitope (0.25 mg/mL) and transferrin (0.25 mg/mL) aqueous solutions at 20°C. Then, the concentration of the epitope or protein was measured via HPLC ($n = 3$) at different time intervals. Figure 2 (a) shows the quantities of transferrin epitope bound to the imprinted and non-imprinted PES particles. The quantities bound to

the imprinted particles increased over time becoming saturated at 11.43 $\mu\text{mol/g}$ after 20 h. Meanwhile, quantities bound to the non-imprinted particles remained low, only 1.21 $\mu\text{mol/g}$ after 20 h. The low epitope binding to the NIP could be attributed to the antifouling property of the PES matrix toward the biological compounds that are abundant in the amino and hydroxyl groups.^{10, 12} The imprinted particles required 20 h to reach saturated binding. This relatively slow mass transfer was likely caused by the condensed skin layer on the exterior of the particles. The mass transfer ratio could be increased by further optimising the proportions of PES and non-solvent solution to control the structures of the particles.

To confirm the ability of the epitope-imprinted materials to recognise the related protein, the protein adsorption kinetics was plotted as shown in Fig. 2(b). The quantities of protein bound to both the imprinted and non-imprinted particles increased as a function of time with saturation occurring between after 70 h. the binding quantity reached 0.47 $\mu\text{mol/g}$ at saturation for the MIP particles, but only 0.21 $\mu\text{mol/g}$ for the NIP particles. Compared with the epitope binding, the protein binding required more time to reach saturation due to its slow mass transfer caused by its larger volume in the hierarchical pore structure. The NIP particles still bound a certain quantity of transferrin,

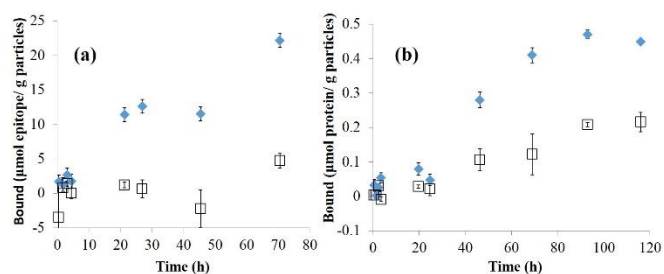


Figure 2. Time course of the binding of the imprinted (◆) and the non-imprinted (□) particles in a transferrin epitope aqueous solution (a) and a transferrin solution (b). The initial concentration of each test compound was 0.25 mg/mL, and the incubation temperature was 20°C.

likely due to the large porosity of the PES particles.

The recognition coefficient (α), which is the ratio of the quantity bound with the imprinted materials ($[S]_{(\text{imprinted})}$) to that with the non-imprinted materials ($[S]_{(\text{non-imprinted})}$), is typically applied to evaluate the recognition abilities of the molecularly imprinted materials.¹³ However, this coefficient is potential to be influenced by the denominator effect, especially when the non-imprinted materials bind very less amount of the template. Therefore, a measurement of the binding amount to the recognition sites ($[S]_{(\text{sites})}$), $[S]_{(\text{sites})} = [S]_{(\text{imprinted})} - [S]_{(\text{non-imprinted})}$, was developed to evaluate the recognition ability.¹¹ The epitope recognition and protein recognition were compared in terms of both α and $[S]_{(\text{sites})}$. For the epitope recognition (at 70 h), the α and $[S]_{(\text{sites})}$ were 4.70 and 17.47 $\mu\text{mol/g}$, respectively; for the protein recognition (at 70 h), the α and $[S]_{(\text{sites})}$ were 3.36 and 0.29 $\mu\text{mol/g}$, respectively.

The value of α in the epitope recognition was larger than that in the protein recognition. This result demonstrates that the recognition sites exhibited increased selectivity toward the epitope over that of the protein. We deduced that not all recognition sites formed by the epitope could be applied to selectively bind the protein due to steric hindrance. The efficiency of the epitope imprinted sites for protein recognition was calculated in the Supplementary Information.

To examine the selectivity, transferrin epitope-imprinted microspheres were applied toward the recognition of transferrin from a protein mixture consisting of transferrin (TRF, *pI* 5.5), ribonuclease B (RNB, *pI* 8.8), cytochrome C (CYC, *pI* 10.6) and β -lactoglobulin (β -LG, *pI* 5.1), which covered a wide range of *pI* values to mimic real samples. Each protein was present at 0.25 mg/mL in the solution. The recognition coefficients (α) of the TRF, RNB, CYC and β -LG were 2.66, 0.16, 0.06 and 1.42, respectively. Clearly, the recognition of the TRF was the highest, and the coefficients for the RNB and CYC were negligible. A slight recognition of the β -LG was also observed. We deduced that the recognition of the β -LG was caused by two factors: First, β -LG is a major whey protein in many mammalian species including rodents and humans. Its amino acid sequence and three-dimensional structure indicate that it is a lipocalin, which can bind hydrophobic ligands and act as specific transporters during biological processes.¹⁴ Thus, in this case, the β -LG could easily bind the hydrophobic phenyl ring on the PES chain, which could lead to high non-selective binding on the PES-based materials. Second, the N-terminal amino acid of β -LG, methionine, is the same as that of the template, which could lead to a slight recognition of the β -LG by the recognition sites.

During the protein recognition, special cavities are typically employed to explain the selectivity of the imprinted materials toward the protein.¹⁵ In a previous study, we demonstrated that imprinted cavities alone are insufficient to achieve the recognition of template proteins with strong affinity and high specificity, while other interactions between the target and recognition sites are indispensable.¹⁶ In this study, the S(=O)₂ group in the PES could accept electronic charge via the resonance effect thus stabilising the negative charges from the phenyl and pyridine rings. Furthermore, hydrogen bonding between the S(=O)₂ group in the PES and both the amino and carboxyl groups could play essential roles in stabilising the transferrin epitope in the DMAc system and sculpting the recognition sites during the phase inversion. However, during the protein recognition, the imprinted particles must be incubated in the aqueous media to maintain their protein conformation. Clearly, water, which competes with the protein in forming hydrogen bonds, could weaken the hydrogen interactions between the protein and the MIP. The hydrogen bonding could play a role in the MIP fabrication, but it was most likely not a factor during the recognition process in the aqueous solution. We also measured the recognition coefficient, α , and the binding amount to the recognition sites, [S]_(sites), under a lower epitope concentration of 0.10 mg/mL after 20 h. Compared with those under the higher epitope concentration of 0.25 mg/mL, the recognition coefficient increased from 9.47 to 13.84, and the value of [S]_(sites) did not change significantly (9.95 μ mol/g at 0.10 mg/mL and 10.22 μ mol/g at 0.25 mg/mL). This phenomenon in which the selectivity increased with decreasing incubation concentration is not unusual for small molecule imprinting,¹⁷ studies of which have demonstrated that the epitope imprinting of at least 9 amino acids followed small molecule imprinting and recognition models. Therefore, the epitope-based recognition sites bind the particular proteins not only through special cavities that are complementary in shape to the protein terminal but also through intermolecular forces such as charge transfer, which are complementary to the protein in their chemical functionality.

Considering the binding capacity, adsorption kinetics and selectivity, the MIPs prepared via PES phase inversion with an

epitope as the template have demonstrated the potential for target protein recognition. Therefore, we applied MIPs and NIPs for human plasma proteome analysis and quantification. The MIPs and NIPs were incubated with the human plasma and diluted by a factor of 50; then, the supernatant was collected. The supernatants were analysed with a typical shotgun proteome analysis protocol (see Supplementary Information). The false positive rate was set to less than 1% for the human plasma proteome analysis, while all results were obtained in triplicate. A normalised, label-free quantitative method—termed the normalised spectral index (SI_N), was used to quantify the human plasma proteome in this study.¹⁸ In our previous study, the SI_N allowed a quantitative comparison of the biologically distinct data sets with high confidence and relative ease because the SI_N largely eliminated variances between the replicate MS measurements, improving the quantitative reproducibility and highly significant quantification of thousands of proteins detected in the replicate MS measurements of the same and distinct samples.^[24a] Figure 3 shows the quantitative comparison of the human plasma treated with the MIPs and NIPs, while the SI_N was applied to quantify the human proteome. The quantitative changes in the top ten proteins were calculated and are listed in the Supplementary Information. The SI_N of the transferrin decreased by 25.97% after the real sample was incubated with the MIPs, but decreased by only 0.42% after the human plasma was incubated with the NIP. Compared with those using the NIP, as shown in Fig. 3, the largest SI_N changes were found for the transferrin. The ratio of the MIP changes to those of the NIP was 61.57. This high value demonstrates that the transferrin epitope-imprinted materials could selectively capture the target proteins, even from plasma, for proteomic analysis.

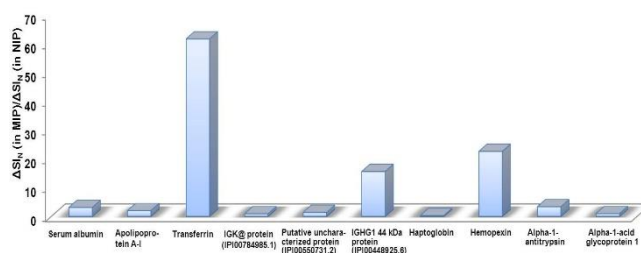


Figure 3. Quantitative changes in the top ten proteins in the human plasma after treatment with MIP and NIP particles.

Conclusions

In conclusion, a PES self-assembly was successfully applied to prepare transferrin epitope-oriented MIPs. The MIPs exhibited excellent selectivity, not only toward the transferrin epitope but also toward transferrin. Furthermore, to the best of our knowledge, the present study was the first to apply imprinted materials to analyse and quantify a human plasma proteome. Considering its selectivity, non-discrimination toward epitopes and environmentally friendly fabrication protocols, this self-assembly strategy could become a general and straightforward method for developing artificial antibodies to capture proteins, especially proteins whose antibodies cannot be produced by current biological processes.

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Notes and references

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