Molecular Imprinting

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# High-Capacity Hierarchically Imprinted Polymer Beads for Protein Recognition and Capture**

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Key life science disciplines (e.g. diagnostics, proteomics, protein purification) rely on selective protein binders that serve the purpose of specifically capturing a protein in a complex matrix for either analytical or preparative use.[1,2] These binders are commonly of biological origin, for example, antibodies for protein fractionation or detection in proteomics or antibody-binding proteins (e.g. Protein A) for antibody capture in downstream processing. Common to most biologically derived protein binders are their lability, high cost, denaturation tendency, and intrinsically low binding capacity.

Robust artificial protein binders in the form of molecularly imprinted polymers (MIPs) could potentially overcome these limitations, thus offering a step change in the above disciplines.[3] In spite of numerous reports that describe protein-imprinted hydrogels, advances towards generic and robust imprinting techniques have been slow. One reason is the need for employing a low cross-linking level in order to provide a mesh size of the network large enough for the protein to penetrate. The memory effects of these gels are thus easily erased, thereby preventing repeated use of the gels. Various forms of surface-imprinting techniques have been used with promising results to address this problem.[4] However, robust imprinting techniques that afford materials, which can compete with established bioaffinity media in terms of both affinity and capacity, are still lacking.

By combining our previously developed hierarchical imprinting technique[5] for small molecules with protein imprinting we herein demonstrate a general technique (Figure 1) to obtain protein-imprinted separation media that could potentially serve this purpose. As model proteins in this proof of concept study, the human blood plasma proteins,

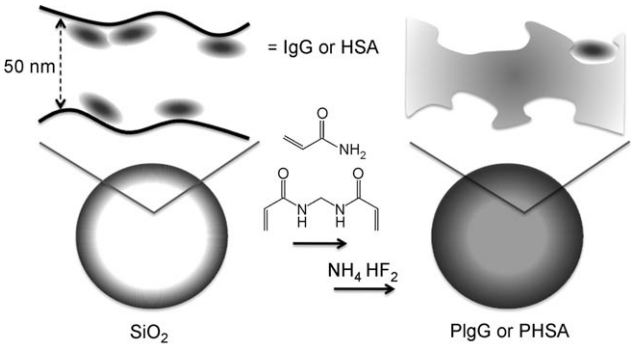


***Figure 1.*** Hierarchical protein imprinting starting from wide-pore silica modified with a submonolayer of adsorbed protein (IgG or HSA), filling of the pores of the protein–silica template with a monomer solution, polymerization, and subsequent removal of the protein and silica porogen by fluoride etching and washing. The last step leaves behind an inverse replica of the silica template that features highly accessible protein-complementary binding sites at the pore walls of the polymeric beads.

human serum albumin (HSA; 68 kDa, pI = 4.7), and immunoglobulin G (IgG; 150 kDa, pI ≈ 9 for the monoclonal antibody in this study)[6,7] were chosen. As a first step, the proteins were physically immobilized on wide-pore silica ($d_p = 50$ nm) at or close to their isoelectric points. This procedure is known to result in the formation of a strongly adsorbed monolayer with only minor loss of the native protein structure.[6] HSA was nearly quantitatively adsorbed under these conditions, whereas IgG showed a somewhat lower affinity (Figure S1 in the Supporting Information). Saturation of HSA adsorption was reached at approximately 100 mg g$^{-1}$, which corresponds to a protein surface density of approximately 2 mg m$^{-2}$, consistent with a monolayer coverage. HSA- and IgG-modified silica templates were then prepared by adjusting the protein surface coverage of approximately 20%—the submonolayer coverage being chosen in order to reduce surface protein–protein interactions.

In order to benchmark the hierarchically imprinted polymers in comparison to reported bulk materials, we chose the common redox-initiated polymerization of methylenebisacrylamide (MBA) as cross-linking monomer (10% w/w) and acrylamide (AAm) as functional monomer (90% w/w), but replaced water with dimethyl formamide (DMF) as

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solvent.[8] Chiral resolution tests (Figure S2) and attenuated total reflectance (ATR)-FTIR (Figure S3) performed before and after the protein-modified silicas were brought in contact with DMF showed that the protein higher-order structure had not been severely perturbed. The filling of the silica pores was then performed by injecting the polymerization solution into a packed bed of protein-modified silica beads (2 mL $g^{-1}$ beads) in a glass column and by allowing it to enter into the pores at elevated pressure (29 psi; Figure S4). This procedure was followed by removal of the interstitial polymerization solution by washing with trimethylpentane and thereafter by polymerization at 37 °C for 16 h. The silica could be quantitatively removed by overnight etching in an $NH_4HF_2$ solution, whereas protein removal proved to require a more extensive washing protocol. Thus, after a series of consecutive washing steps using water, a concentrated solution of sodium chloride, and an acidic modifier (trifluoroacetic acid, TFA), approximately 90 % of HSA and 80 % of IgG could be removed from the beads (Figure S5). Nonimprinted polymers (NIPs: $P_N$HSA and $P_N$IgG) were prepared analogously to the MIPs, but by using bare silica as template.

The SEM images in Figure S6 and the nitrogen sorption analysis results in Table S1 (see the Supporting Information) show that the poly(acrylamide) beads were macroporous with slightly inferior pore parameters ($S$, $d_p$, $V_p$) compared to the silica template. Not surprisingly, the polymer beads expanded strongly in contact with solvent, thus resulting in swelling factors in the range 3–5. When imprinted and nonimprinted beads were compared, the latter showed a markedly higher swelling factor than the imprinted beads, despite their similar physicochemical properties (Table S1, Figure S7).[9,10]

We subsequently tested the polymers for their ability to bind the template proteins. The polymers were incubated with buffer solutions (pH 7.4) of the template proteins followed by HPLC quantification of the supernatant concentrations of the free protein at equilibrium. Figure 2 shows the dependence of binding of the two structurally similar proteins HSA and BSA (0.5 mg $mL^{-1}$) to the polymers on the HSA coverage of the silica template. The coverage of the surface has a pronounced effect on the selectivity for the template protein; the highest selectivity is observed for PHSA prepared by using the lowest HSA coverage (20 %). The selectivity then decreases with increasing coverage ultimately resulting in a complete lack of selectivity for PHSA prepared by using the saturated silica template.[11] This effect probably reflects the presence of more isolated proteins at low surface coverage, thus featuring a larger solvated surface for imprinting. Further experiments were therefore performed with polymers (PHSA and PIgG) prepared by using silicas with a low protein surface coverage.

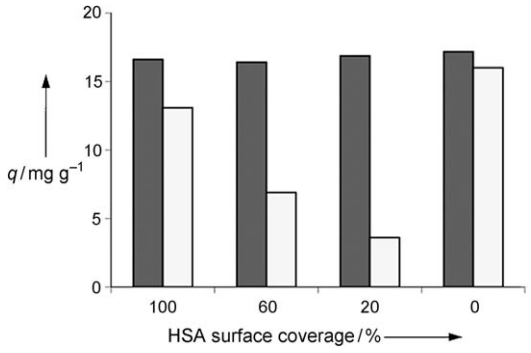


***Figure 2.*** Amount ($q$) of HSA (dark gray bars) or BSA (light gray bars) adsorbed to hierarchically imprinted polymers suspended in a solution of protein (0.5 mg $mL^{-1}$) in phosphate buffer (pH 7.4, 50 mM). The polymers were prepared from silicas with different surface coverages of HSA as indicated.

The protein-binding isotherms exhibit a multiple sigmoidal shape with an onset that occurs at a lower concentration for the MIPs than the NIPs; the MIPs retain considerably more protein at the saturation level (Figure 3). This result

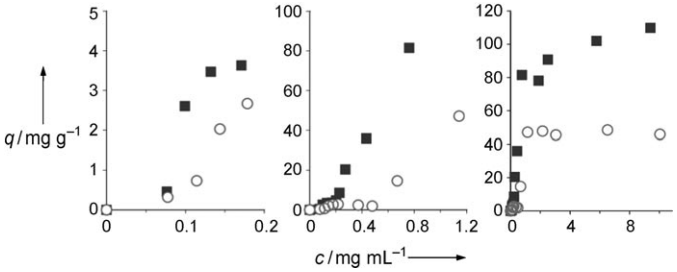


***Figure 3.*** Adsorbed amount ($q$) of IgG on PIgG (solid squares) or $P_N$IgG (open circles) versus the concentration of free protein in phosphate buffer (pH 7.4). The left and center graphs represent enlarged sections of the right graph.

precludes curve fitting to any of the common adsorption models. PIgG shows at least two sigmoidal steps, with the first step occurring between 0.05 and 0.15 mg $mL^{-1}$ of free IgG, thus resulting in a first plateau that corresponds to an adsorbed amount of approximately $q = 3.5$ mg $g^{-1}$ sorbent, which indicates the presence of binding sites with $K_d$ in the range 500 nM–1 μM. The next clear step occurs in the region 0.2–1 mg $mL^{-1}$, hence resulting in a steep increase in $q$ up to 80 mg $g^{-1}$. The final maximum $q$ value on PIgG was nearly 100 mg $g^{-1}$, which corresponds closely to the maximum binding-site occupancy when assuming a quantitative imprinting yield. The HSA imprinted polymer (PHSA) showed a qualitatively similar adsorption behavior but binding appeared to be weaker, whereas the maximum $q$ value of nearly 150 mg $g^{-1}$ was higher than for PIgG (Figure S8). Exposing PHSA to the structurally similar protein BSA ($M_w = 66$ kDa, pI = 4.7) resulted in a much shallower curve with $q$ values lower than that observed for BSA added to the nonimprinted polymer.[11]

A plausible origin of the multiple sigmoidal binding curve is the tendency of plasma proteins to aggregate in the adsorbed state.[7] For instance, HSA and IgG do not form homogeneous monolayers on hydrophilic surfaces, but tend to adsorb in the form of clusters, also at submonolayer coverage. Templating of such clusters will inevitably lead to binding sites that accommodate more than a single protein molecule, where affinity depends on site occupancy. However, the nonimprinted polymer also displays this behavior and it is therefore reasonable to assume that clustering occurs on both surfaces, but is facilitated on the MIP.[12]

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To gain further insight into the protein-binding mechanism we followed the time course of binding after bringing a dilute protein solution in contact with the polymers. Figure 4

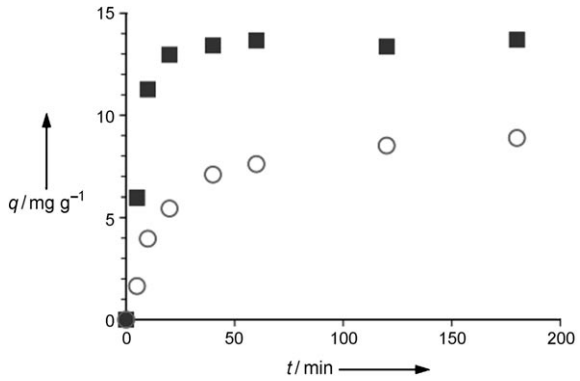


***Figure 4.*** Adsorbed amount ($q$) of HSA (0.4 mg mL$^{-1}$) versus time on PHSA (solid squares) and $P_N$HSA (open circles) (ca. 75 mg hydrated polymer) in tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 7.4).

(and Figure S9) shows that protein binding in all cases reached an equilibrium within 200 minutes, but the kinetics depended on both the protein and the adsorbent. First considering protein binding to the NIPs, HSA here exhibited a slower adsorption rate than IgG, which is consistent with the known adsorption kinetics of these two proteins to both hydrophilic and hydrophobic surfaces.[7,13] For the MIPs however, HSA displayed a faster adsorption, whereas IgG adsorbed more slowly than on the NIPs.

Measurement of protein fluorescence in the gels during adsorption provided further insights into this behavior. Figure S10 shows the fluorescence emission spectra of the polymers in the previous experiment prior to and after protein addition. The enhanced emission intensity of the imprinted polymers compared to the nonimprinted polymers before protein addition can be attributed to the residual nonextractable template. Exposure of the polymers to the template proteins resulted in a further enhancement of the emission intensity that was not directly proportional to the amount of adsorbed protein. Furthermore, the emission maximum of HSA exhibited a blue-shift upon adsorption, which was most pronounced (8 nm) when the protein was adsorbed to its complement PHSA. These changes can be interpreted in terms of changes in protein conformation and in the local environment.[14] Hence, soft proteins like HSA and BSA exhibit blue-shifts and normally lower intensities after adsorption, thereby indicating conformational changes, whereas adsorption of hard proteins is usually accompanied by intensity changes only. In the case of HAS, it is noteworthy that the complementary surface of the MIP seems to influence the protein conformation the most. The time course of these changes (Figure S10C) indicated a slower process than that of the protein adsorption shown in Figure 4. Possibly, the MIP-induced structural change reflects the state of the protein during imprinting, where some conformational change is likely to have occurred.[6]

To explore the use of the MIPs as capture phases, we first focused on blood serum, which is a matrix that contains both template proteins. A method was developed based on a benchmark method by using AntiHSA antibodies or Protein A affinity for the capture and depletion of these plasma proteins from blood plasma.[15] The stained gels obtained by SDS-PAGE of the native serum and the eluted fractions are shown in Figure 5. Although the MIPs fail to deplete the

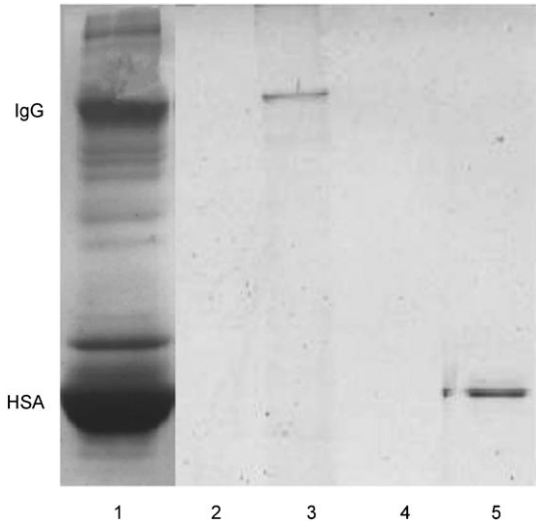


***Figure 5.*** SDS-PAGE gels after separation under nonreducing conditions of a native serum sample (lane 1), the elution fraction after treatment of serum with $P_N$IgG (lane 2), PIgG (lane 3), $P_N$HSA (lane 4), and PHSA (lane 5). The proteins were visualized with Coomassie brilliant blue.

proteins from the sample, in contrast to the bioaffinity depletion method, the elution fractions clearly prove that the polymers can operate as capture phases. The only noticeable bands appear at identical positions as the template proteins, that is, HSA is only found in the eluate from PHSA and IgG from PIgG, whereas no detectable protein was eluted from the nonimprinted polymers.

In conclusion, hierarchical imprinting affords protein-discriminative phases for both soft and hard proteins of large sizes that present a high density of accessible binding sites. The loading capacity is high and, in the context of antibody purification, rivals the capture phases used in downstream processing.[2] Protein binding to the phases is reversible and the proteins can be specifically extracted from blood serum and then recovered in functional form upon a reasonably mild elution step. These features are encouraging for applications of protein-capturing MIPs in biotechnology and life sciences.

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[1] M. J. Taussig et al., *Nat. Methods* **2007**, *4*, 13.

[2] A. A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, *J. Chromatogr. B* **2007**, *848*, 28.

[3] a) N. W. Turner, C. W. Jeans, K. R. Brain, C. J. Allender, V. Hlady, D. W. Britt, *Biotechnol. Prog.* **2006**, *22*, 1474; b) M. E. Byrne, V. Salian, *Int. J. Pharm.* **2008**, *364*, 188; c) D. E. Hansen, *Biomaterials* **2007**, *28*, 4178; d) T. Takeuchi, T. Hishiya, *Org. Biomol. Chem.* **2008**, *6*, 2459; e) Y. Ge, A. P. F. Turner, *Trends Biotechnol.* **2008**, *26*, 218.

[4] a) H. Nishino, C.-S. Huang, K. J. Shea, *Angew. Chem.* **2006**, *118*, 2452; *Angew. Chem. Int. Ed.* **2006**, *45*, 2392; b) Y. Li, H.-H. Yang, Q.-H. You, Z.-X. Zhuang, X.-R. Wang, *Anal. Chem.* **2006**, *78*, 317; c) H.-Y. Lin, C.-Y. Hsu, J. L. Thomas, S.-E. Wang, H.-C. Chen, T.-C. Chou, *Biosens. Bioelectron.* **2006**, *22*, 534; d) X. Hu, G. Li, J. Huang, D. Zhang, Y. Qiu, *Adv. Mater.* **2007**, *19*, 4327; e) R. Ouyang, J. Lei, H. Ju, *Chem. Commun.* **2008**, 5761; f) Y.-J. Zhao, X.-W. Zhao, J. Hu, J. Li, W.-Y. Xu, Z.-Z. Gu, *Angew. Chem.* **2009**, *121*, 7486; *Angew. Chem. Int. Ed.* **2009**, *48*, 7350; g) A. Cutivet, C. Schembri, J. Kovensky, K. Haupt, *J. Am. Chem. Soc.* **2009**, *131*, 14699.

[5] M. M. Titirici, A. J. Hall, B. Sellergren, *Chem. Mater.* **2003**, *15*, 822.

[6] E. P. Vieira, S. Rocha, M. Carmo Pereira, H. Möhwald, M. A. N. Coelho, *Langmuir* **2009**, *25*, 9879.

[7] J. L. Ortega-Vinuesa, P. Tengvall, I. Lundström, *Thin Solid Films* **1998**, *324*, 257.

[8] For a typical benchmark procedure for protein imprinting in poly(acrylamide) hydrogels, see: S. Hjertén, J. L. Liao, K. Nakazato, Y. Wang, G. Zamaratskaia, H. X. Zhang, *Chromatographia* **1997**, *44*, 227. The lower surface tension of the prepolymerization mixture using DMF facilitates pore intrusion.

[9] This observation may be due to residual nonextractable protein which as a cross-linker may enhance network stiffness. This hypothesis was further supported by the template-induced shrinkage observed for PHSA (see Figure S11).

[10] Z. Chen, Z. Hua, L. Xu, Y. Huang, M. Zhao, Y. Li, *J. Mol. Recognit.* **2008**, *21*, 71.

[11] The high uptake of proteins displayed by the NIP shows that a simple comparison of template binding to the MIP and NIP is misleading in this case.

[12] We note that the behavior is contrary to comparable bulk-imprinted hydrogels, which commonly show smooth continuous curves. For example, see Ref. [10].

[13] M. Malmsten, B. Lassen in *Proteins at Interfaces II*, American Chemical Society, Washington, DC, **1995**, p. 228.

[14] S. R. Clark, P. Billsten, H. Elwing, *Colloids Surf. B* **1994**, *2*, 457.

[15] SIGMA ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit—Technical Bulletin.


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