Final report

Title: Molecularly imprinted magnetic nanoparticles for selective protein recognition
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Summary

Molecularly imprinted polymers(MIPs) targeting large macromolecules like proteins are envisioned as cheap and robust substitutes for biological antibodies in affinity separations, biosensors, ligand binding assays, biomedical imaging and targeted drug delivery. One will, however, encounter problems during their preparation [1].

(i) The main obstacle is the restricted movement of the large biomacromolecule in a highly crosslinked polymer network hindering its removal after polymerization and its rebinding to the MIP.

(ii) As opposed to small molecule imprinting which is usually carried out in aprotic, relatively apolar solvents, protein imprinting should be performed in aqueous media due to the delicate nature of protein molecules. Water, however interferes with most of the secondary forces prevalent in non-covalent imprinting. This is partially counteracted by the large number of interactions that a protein molecule can establish through its surface amino acid residues with the functional monomers, but, in turn, these weak multi-point interactions will lead to an increased non-specific binding.

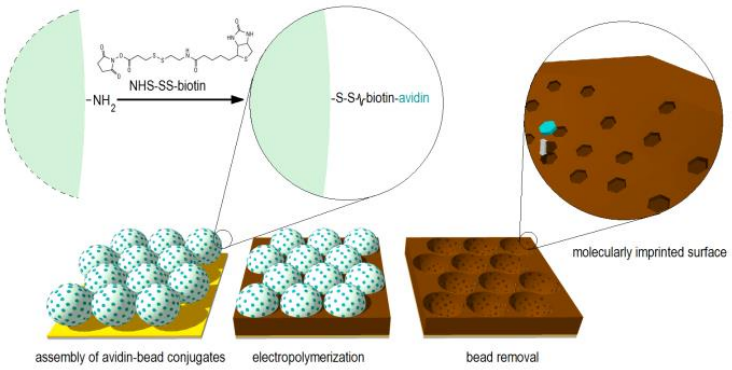
In this project we have offered solutions to the above problems by (i) designing novel polymer formats for protein imprinting whereby the imprints are formed exclusively on the surface of the *nanostructured polymer* or in *very thin polymer layers, core-shell particles* or *nanoparticles* where the dimensions of the imprinted polymer is comparable to the size of the protein molecule. (ii) Secondly, to mimic nature's molecular recognition in DNA-protein interactions we have synthesized *novel functional monomers based on nucleotides and nucleobases* to be incorporated into MIPs. Moreover, we have proposed a novel strategy to

prepare *photoswitchable* MIPs whereby template binding and release can be controlled by light. In order to characterize the selectivity of the above functional polymers a deep theoretical *study of selectivity* in general, and in terms of MIPs have been carried out and new strategies have been proposed.

Surface imprinted nanostructured polymer films by nanosphere lithography

Surface imprinting localizes the protein-selective binding sites exclusively on the surface of a polymer network. As this can lead to a decreased binding capacity it is plausible to design nanostructured MIP materials in which the surface to volume ratio and consequently the number of binding sites are significantly increased.

We have introduced a novel approach to create surface-imprinted nanostructured polymer films for selective protein recognition based on nanosphere lithography. Nanosphere lithography is a widely known technique to generate periodic patterns by using a mask of monodisperse nanospheres and depositing the desired material through the mask. The mask is then removed and the layer keeps the ordered patterning of the mask interstices[1]. Avidin, a protein abundant in egg white has been conjugated to 750 nm diameter polystyrene beads through its biotin binding sites with a cleavable crosslinker, succinimidyl-2-(biotin-amido)ethyl-1,3-dithiopropionate.



This ensured the oriented immobilization of avidin on the surface of the latex beads making site-directed imprinting and possibly more homogeneous binding site distribution feasible. The beads were drop-casted onto the surface of a gold coated quartz crystal. A conducting polymer film (poly(3,4-ethylenedioxythiophene) poly(styrenesulfonate) PEDOT/PSS) was electropolymerized in between the particles with a thickness of the bead radius, under mild, aqueous conditions.

[1] Colson P; Henrist C; Cloots R: Nanosphere Lithography: A Powerful Method for the Controlled Manufacturing of Nanomaterials, Journal of Nanomaterials, 2013: 19 pages, Article ID 948510, 2013

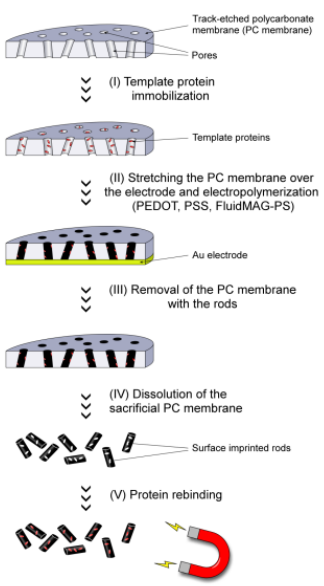
Removal of the protein conjugated beads was a two-step process: firstly the protein-nanosphere linkage was cleaved then the beads were dissolved in an organic solvent. This resulted in 2D arrays of periodic complementary cavities in the polymer layer. QCM-based nanogravimetry was used to measure the template binding to the imprinted and non-imprinted films. It was shown that imprinting takes place at the molecular level and the avidin coating of the beads generated selective recognition sites on the surface of the PEDOT/PSS film. The binding capacity of the surface-imprinted polymer was ca. 6.5 times higher than that of films created with unmodified beads. It also exhibited excellent selectivity against analogues of avidin, and other proteins with similar size. The work appeared as a cover page article in *Advanced Functional Materials* [2].

In the non-imprinted polymer film the latex beads showed periodic hexagonal closely packed arrangement but the avidin-coated particles deposited quite randomly and in a loose packing. This gave rise to a relatively large non-imprinted surface in the imprinted MIP film increasing its nonspecific protein binding. To lower this non-imprinted area a deposition method was sought to achieve highly ordered closely packed arrangement of the avidin coated beads. Different nanoparticle deposition techniques were tried such as dip coating and horizontal dragging of the nanoparticle suspension between two plates, but none of them brought about the desired result. We assumed that the close packing was hindered by the fact that the settled avidin coated particles stuck to the gold surface and could not move freely to self-assemble upon evaporation of the solvent. Therefore, we have attempted to make the gold substrate very hydrophilic by self-assembling a monolayer of thiolated ethylene glycol onto it. This layer did improve the packing of the beads, but unfortunately it deteriorated the adhesion of the successively electropolymerized PEDOT/PSS film. Finally, an optimized drop-coating procedure has led to the desired result. Extreme care was taken that the nanoparticle suspension was placed onto the gold substrate immediately after UV/ozone cleaning of the gold and the solvent was evaporated in a controlled humidity chamber. Electropolymerization of PEDOT/PSS in the voids and dissolution of the avidin-PS particles revealed that a highly ordered hexagonally packed negative imprint of the beads was formed in the polymer film. Multi-layers of the beads have been deposited with the optimized method and the film thickness was controlled by the

electrical charge passed during electropolymerization. In this way 3-D arrays of the bead cavities could be formed resulting in a much higher protein binding capacity of the sensing film. (Results to be published soon.)

Surface-imprinted protein selective magnetic microrods [3]

The substitution of antibodies with protein imprinted polymer micro/nanoparticles in binding assays or sample clean-up requires the separation of these particles from the sample after reaction with the analyte. Often centrifugation is not a viable alternative due to the small size of the particles or the length of the process. Filtration can result in the loss of the free protein or the polymer particle by adsorption on the filter material. The introduction of magnetic properties into the MIP enables its straightforward and fast separation by an external magnetic field. For this purpose we have synthesized protein selective magnetic microparticles by electrochemical polymerization using a sacrificial microreactor based on our earlier work[2].



Avidin as a model protein was immobilized onto the pore walls of a track-etched polycarbonate membrane by physisorption. A conducting polymer (PEDOT/PSS) was electrodeposited into the pores together with PSS-coated superparamagnetic nanoparticles having a hydrodynamic diameter of 100 nm. Dissolution of the membrane at the end of the polymerization resulted in cylindrical PEDOT/PSS microrods with protein recognition sites confined to the surface of the polymer. The incorporation of the magnetic nanoparticles into the polymer matrix was quantified by atomic absorption spectrometry measuring the iron content. The dimensions of the microrods were matching the pore size of the track-etched membrane used as microreactor. The surface imprinted microrods could selectively bind the imprinted protein and showed superparamagnetic properties. The selectivity was demonstrated by comparative binding experiments of a fluorescent labelled

[2] Menaker A, Syritski V, Reut J, Öpik A, Horváth V, Gyurcsányi RE: Electrosynthesized Surface-Imprinted Conducting Polymer Microrods for Selective Protein Recognition, Adv. Mater., 21 (22): 2271-2275, 2009

avidin to the surface-imprinted and the reference magnetic polymer microrods. The magnetic properties of the microrods allowed their efficient collection and manipulation in magnetic field even in highly diluted solution. This property can be advantageously utilized in ligand binding assays or in sample clean-up for the separation of bound and unbound protein.

Thin protein-imprinted polymer film arrays by microelectrospotting [4]

Electropolymerization is a powerful tool in the imprinting of proteins [5] having distinct advantages over the traditional free-radical polymerization, especially in chemical sensing applications. Most of the electropolymerizable monomers can be deposited under mild conditions from aqueous solutions without the need of external initiaton (high temperature, UV light or strong oxidizing agents) which may cause structural changes in the protein template. The precise control over electrosynthesis enables the fine tuning of the polymer layer thickness. Electrosynthesis can be used straightforward to create several nanometer thick polymer films from which proteins can freely move in and out. There is a wide variety of electropolymerizable monomers (moreover new monomers can be custom synthesized) from which one should chose the most suitable one for the target protein. The polymerization conditions (monomer/template ratio, monomer and template concentration, film thickness etc.) should also be varied to achieve optimal template recognition. Finally, the selectivity of the obtained MIP films has to be tested with the template and other proteins. This requires a huge number of experiments which calls for a high-throughput methodology. In order to accelerate the development (optimization and characterization) of protein imprinted polymers we have elaborated a novel approach to prepare thin MIP films on surface plasmon resonance imaging (SPRi) chips by electropolymerization. The technique is based on microelectrospotting arrays of 500 μm diameter and several nanometer thick polymer films. As a proof of concept, the imprinting of ferritin in poly(scopoletin) film has been elaborated. A plastic pipette tip was loaded with the mixture of the monomer and protein and a quasi-reference electrode was immersed into it. When it contacted the gold surface of the SPRi chip which served as a working electrode a potential pulse program was applied causing the polymerization of scopoletin. The procedure was repeated at different positions of the chip creating an array of electropolymerized spots.

The optimal template removal and rebinding conditions were established simultaneously on the polymer spots in the flow system of the SPRi device. The optimal MIP composition under the optimized measuring conditions showed 13 times higher protein rebinding in comparison with the non-imprinted polymer which is one of the highest ratios achieved so far in protein imprinting. The ferritin MIP also exhibited good selectivity against other proteins. This work confirms that microelectrospotting is a viable technique to generate protein imprinted polymer arrays and combined with the high multiplexing capability of SPRi it allows for the fast, simultaneous optimization of monomer compositions and polymerization conditions for different protein templates.

Molecularly imprinted micro/nanospheres formed at high monomer concentrations by precipitation polymerization [6, 7]

Conventional precipitation polymerization suits well to the preparation of spherical MIP particles, because it requires high crosslinking degree, a common feature in molecular imprinting and it does not need any surfactant or stabilizer which would interfere with the formation of the template-functional monomer complex during imprinting. However, the solvent need is very high (monomer concentrations are below 5%) and the template-monomer complexation is not favored in the dilute medium which suppresses the formation of the selective binding sites.

We have shown that this problem can be tackled by using carefully chosen mixtures of a nonsolvent and a co-solvent as the polymerization diluent and introduced a *modified precipitation polymerization* method. Monomer concentrations up to 25-30 w/v% still resulted in uniformly sized microspheres and full conversion of monomers could be obtained. We could explain this behaviour by the fact that the co-solvent was enriched in the growing particles and swelled them providing a steric stabilization against coagulation. The type of functional monomer, crosslinker and co-solvent, and the non-solvent/co-solvent ratio were influential parameters on the microparticle morphology. The proposed method can be applied to a wide range of solvent-monomer systems to prepare molecularly imprinted micro- or nanoparticles.

Novel functional monomers based on nucleotides or nucleobases

As single nucleotide containing monomers are not available commercially and their synthesis has not been published yet, we designed a synthesis route for their production based on the current phosphoramidite-based synthesis methods of oligonucleotides. To incorporate a polymerizable group a methacrylic phosporamidite has been synthesized first which was further modified with the nucleoside phosphate group.

The commercially available 6-aminohexanol was reacted with methacryloyl chloride in the presence of triethyl amine in dichloromethane at 0°C to yield 6-methacrylamidohexyl methacrylate. The obtained ester was hydrolysed with sodium hydroxide (15% aqueous solution) in ethanol to *N*-(6-hydroxyhexyl)methacrylamide. Latter amide was converted to 2-cyanoethyl 6-methacrylamidohexyl diisopropylphosphoramidite in a reaction with 2-cyanoethyl diisopropyl chlorophosphoramidite in the presence of *N,N'*-diisopropyl ethyl amine in acetonitrile at 0 °C. In the next step a model reaction was attempted. To the above mentioned methacrylamidohexyl diisopropylphosphoramidite derivative tetrahydrofurfuryl alcohol was added in the precence of 1*H*-tetrazole in acetonitrile. We afforded an unstable phosphite compound which was oxidized to a phosphate derivative with the help of *tert*-butyl hydroperoxide. After the latter successful test reaction, we used a trityl protected DNA nucleoside, the trityl thymidine, as a product of a reaction between thymidine and trityl chloride in pyridine. The methacrylamidohexyl diisopropylphosphoramidite derivative was reacted with trityl thymidine in the same conditions as in the earlier mentioned analogue reaction. The gained trityl protected nucleoside phosphite was oxidized to a stable phosphate derivative. In the last step the trityl group was removed with trifluoroacetic acid. All the above prepared compounds were purified by column chromatographic methods and were characterized by infrared and NMR spectroscopies. The thymidine containing methacrylic phosporamidite was copolymerized with N-isopropylacrylamide to yield thermoresponsive nanogels. However, the amount of the thymidine monomer was too small to confirm its incorporation into the polymer particles by NMR spectroscopy. Its synthesis has been repeated to produce larger quantities. Unfortunately, this complicated 10 step synthesis produced the polymerizable thymidine

phosphoramidite in very low overall yield, because a rather expensive reagent, 2-cyanoethyl diisopropyl chlorophosphoramidite and the final product were both very unstable and easily autopolymerized. We only gained 11 mg of this product, which was not enough for further experiments.

We have decided that instead of preparing the polymerizable nucleotide we follow a simpler route and modify only the nucleobase with a polymerizable side chain since the nucleobase itself is expected to interact with the protein molecules during the imprinting process. We tried to prepare methacryloylamido adenine according to a reported procedure[3]. Adenine was deprotonated with aqueous NaOH, and then reacted with the earlier prepared methacryloyl benzotriazole in dioxane. This reaction, however, resulted in very low yield and again we did not obtain enough functional monomer for polymerization. This kind of reaction (regioselective methacryloyl group transfer from benzotriazole to adenine) could not be found anywhere else in the literature, which rises some suspect whether it really can take place.

According to another method[4] adenine was functionalized successfully with a polymerizable side chain by a regioselective *N*-alkylation reaction, but with an additional propylene-linker. The commercially available 3-bromopropanol was reacted with methacryloyl chloride in the presence of triethyl amine in dichloromethane. Adenine was treated with sodium hydride in DMF then alkylated with the above prepared 3-bromopropyl methacrylate serving enough adenine-based functional monomer for the polymerization experiments in only three steps.

Copolymerization experiments were carried out with the new functional monomer in dilute (0.5% w/w) solutions to see whether it can be incorporated into a lightly crosslinked polymer nanogel together with other monomers. N,N-methylene bisacrylamide (BIS) was used as a crosslinker (5 mol%) and N-isopropylacrylamide (NiPAm) as the backbone monomer in varying ratios compared to the adenine-based monomer. After polymerization the residual monomer concentration in the supernatant was measured by HPLC and the conversion was calculated. When the polymerization was not taken to full conversion we found that the adenine based

[3] Diltemiz SE; Hür D; Ersöz A; Denizli A; Say R: Designing of MIP based QCM sensor having thymine recognition sites based on biomimicking DNA approach, Biosensors and Bioelectronics, 25: 599–603, 2009

[4] Spijker HJ; van Delft FL; van Hest JCM: Atom Transfer Radical Polymerization of Adenine, Thymine, Cytosine, and Guanine Nucleobase Monomers, Macromolecules, 40 (1): 12–18, 2007

functional monomer incorporated into the polymer in a much higher ratio than it was in the starting monomer mixture. These experiments verified, first of all, that the new monomer can be copolymerized with monomers frequently used in protein imprinting. Secondly, they showed the higher reactivity of the new monomer compared to NiPAm and BIS, meaning that in the first phase of the polymerization it is preferentially incorporated into the polymer.

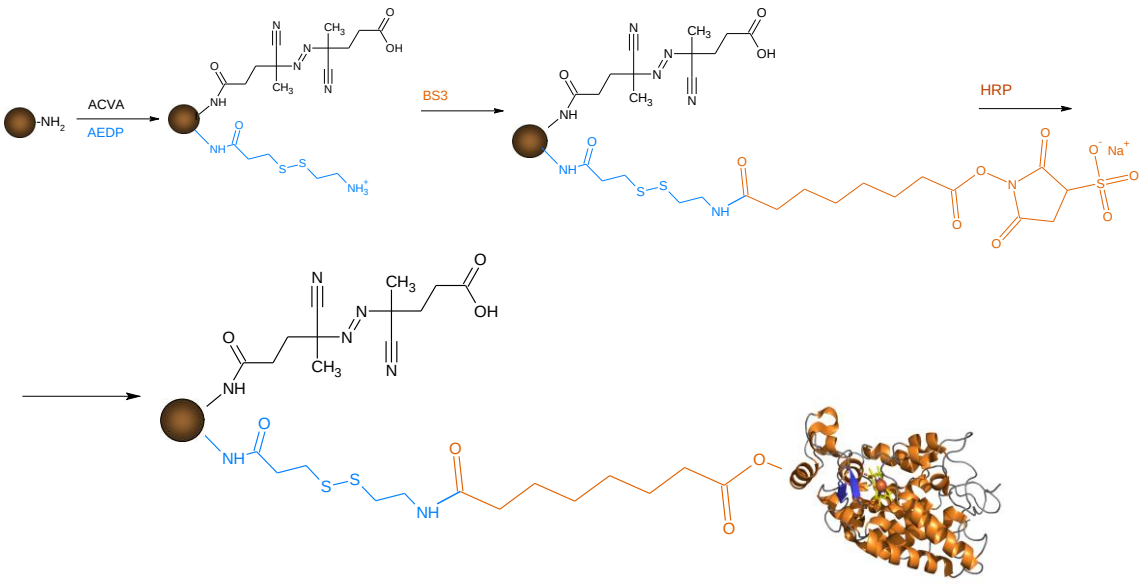
To exploit the recognition capability of the designed new functional adenine based monomer two different polymer formats have been explored in the project; namely nanogels synthesized from dilute monomer solution and core-shell particles. Nanogel particles having dimensions in the 50 to 200 nm range are lightly crosslinked and swollen in aqueous solution facilitating the access of protein molecules to the binding sites. Core-shell particles exhibit a thin imprinted polymer shell the thickness of which is tuned to be comparable to the hydrodynamic diameter of a protein.

Core-shell molecularly imprinted magnetic nanoparticles [8-11]

Grafting of a MIP shell onto core-particles is usually performed by attaching either a polymerizable unit or the initiator molecule on the core particle, while in other approaches template immobilization is chosen to promote print formation. We have designed a scheme (Scheme 1) that combines the benefits of (i) immobilizing the initiator on the core particle in order to confine polymer growth to the surface[5] and (ii) tethering the template molecules to the surface in order to enhance print formation[6]. We have chosen a cleavable spacer (3-[(2-aminoethyl)dithio]propionic acid, AEDP) for protein immobilization to facilitate template removal. As a model template we have chosen horseradish peroxidase (HRP).

[5] Q.Q. Gai, F. Qu, Z.J. Liu, R.J. Dai, Y.K. Zhang: Superparamagnetic lysozyme surface-imprinted polymer prepared by atom transfer radical polymerization and its application for protein separation. *Journal of Chromatography A* 2010 (1217) 5035-5042.

[6] R.X. Gao, X. Kong, X. Wang, X.W. He, L.X. Chen, Y.K. Zhang: Preparation and characterization of uniformly sized molecularly imprinted polymers functionalized with core-shell magnetic nanoparticles for the recognition and enrichment of protein. *Journal of Materials Chemistry* 2011 (21) 17863-17871.



Scheme 1: Procedure to co-immobilize initiator and template to the core particles using a cleavable spacer (BS3 (bis[sulfosuccinimidyl] suberate) links the cleavable spacer to a surface amine-group of the enzyme)

During the experimental realisation of the above scheme we have faced several obstacles which led us to gradually modify the immobilization procedure.

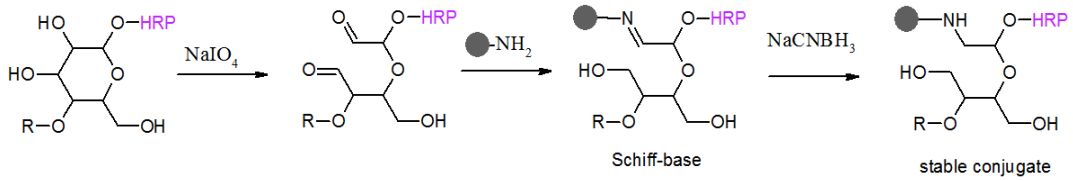
Initial experiments were made by immobilizing only the initiator (4,4'-azobis(4-cyanovaleric acid), ACVA) onto commercial cross-linked dextran coated magnetic particles with 300 nm nominal diameter. The amount of ACVA on the particles was too low to be detected by FTIR. Quantification using a radical scavenger (2,2-diphenyl-1-picrylhydrazyl) was also not successful despite thorough optimization of the assay procedure. The fact that polymerization occured in the presence of the particles was, however, sufficient proof of the successful coupling of ACVA to the particles.

Next the full scheme was applied but coupling of HRP had very low efficiency as demonstrated by the negligible peroxidatic activity of the modified particles. A non-specific protein assay based on copper reduction and reaction with bicinchoninic acid (BCA) showed sufficiently high coverage values but these were not reliable due to the very high background signal of the unmodified core particles.

In parallel, the composition of the polymerization mixture, in terms of monomer and particle concentration, crosslinking ratio and monomer-to-particle ratio, had to be optimized to avoid visible aggregation of the particles. Scanning electron microscopy revealed that even seemingly colloidal polymer suspensions contained aggregates. SEM further showed that the core particles themselves were not spherical but consisted of clusters. This inapt morphology and the incompatibility of the dextran shell with the BCA protein assay forced us to switch to a different core particle.

We continued optimizing the HRP-coupling procedure on commercially available aminosilica-coated magnetic particles with 500 nm nominal diameter and on amine-functionalized, polystyrene-coated magnetic beads of 2 µm nominal diameter. We found that the ratio of ACVA:AEDP during the first, co-immobilization step strongly influences the amount of HRP finally obtained on the particles. This was explained by the much higher reactivity of ACVA which led to its over-representation on the particle surface compared to its ratio in the modifying cocktail. The co-immobilization of ACVA and AEDP was therefore split into separate steps but still only about 3% protein coverage was reached (compared to the calculated monolayer coverage of the particle surface with HRP molecules).

We obtained much better results (22% coverage) using a different protein coupling chemistry: the glucose moieties of HRP were oxidized by sodium periodate to give aldehyde groups which readily form a Schiff-base with the amine groups on the surface. This can in turn, be mildly reduced into a stable covalent bond (Scheme 2). In this case, the periodate-activated HRP was coupled directly to AEDP, omitting the BS3 linker.



Scheme 2: Covalent coupling of periodate-activated HRP to the particles through reductive amination

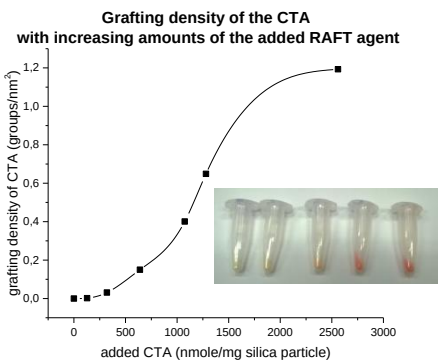
Experiments aimed at removing the protein from the surface (and eventually, from the MIP) by reducing the spacer's disulphide bond with tris-(2-carboxyethyl)phosphine (TCEP) revealed that at least a portion of the protein molecules are not attached to the spacer but possibly to remaining amine groups of the original surface and cannot, therefore, be removed by TCEP. Liberation of the binding sites is crucial in MIP preparation and failure to remove all of the template molecules leads to a decrease in binding capacity. To overcome this problem, we resorted to a different template removal strategy: that of digesting the protein into small fragments which are expected to be easily washed out of the MIP network. At this point the use of AEDP was abandoned as no longer necessary for template removal and the periodate-activated HRP was coupled directly to the particles' amino groups.

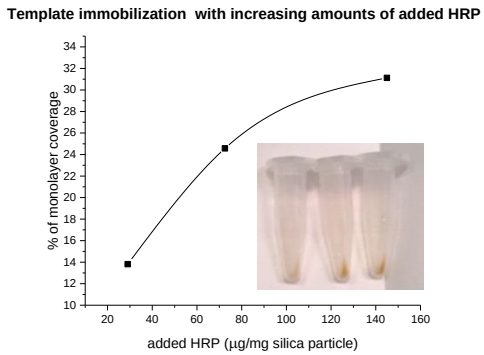
Proteinase K, a protease enzyme with broad cleavage specificity was chosen to digest the template protein. We first optimized the digestion of free, dissolved HRP in order to obtain the largest number of amino acid fragments in the digest (indication of more complete digestion), as monitored by HPLC-MS-MS. We found that the native form of HRP resists decomposition by proteinase K. Denaturing with a surfactant as well as disruption of the disulphide bridges in the protein was necessary to ensure access for proteinase K to the cleavage sites [11]. The optimized protocol was then applied to extract the HRP template from the molecularly imprinted polymer shell. Template recovery was found to increase with repeated digestions up to three cycles. The protein fragments remaining attached to the particle after digestion are expected to be maximum 7 amino acids long (simulation by Expasy Peptide Cutter)[7] and are assumed not to interfere with rebinding; in fact some even suggest that these fragments contribute to rebinding[8].

On the other hand, better control of polymer formation was expected from immobilizing a mediator of reversible addition-fragmentation chain transfer (RAFT) polymerization[9] instead of ACVA on the particles. Unlike ACVA which decomposes into two identical radicals (only one of which remains tethered to the particle, the other will initiate polymerization in the solution

[7] http://web.expasy.org/peptide_cutter/
[8] Zdyrko B; Hoy O; Luzinov I: Toward protein imprinting with polymer brushes. Biointerphases, 4: FA17-FA21, 2009
[9] Chiefari J et al.: Living free-radical polymerization by reversible addition-fragmentation chain transfer: the RAFT process, Macromolecules, 31: 5559-5562, 1998

phase), our chain transfer agent of choice (CTA; 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid) decomposes into an active and an inactive radical. By immobilizing this CTA to the particles through its carboxyl group, the active radical will remain surface-bound while the inactive radical is released into the solution. We adapted a colorimetric assay[10] to measure the amount of CTA immobilized on the surface: a strongly acidic dye is adsorbed to the particles at pH 3 through ion pairing with the protonated surface amine groups followed by elution at basic pH. Assuming a 1:1 association between the dye and amino groups, the amount of amino groups on the surface can be calculated from the absorbance of the eluate. The best assay results were obtained using Eriochrome Black T dye, and the amount of immobilized CTA was calculated as the difference between the number of amino groups before and after modification. The coverages obtained were not sufficient for the subsequent polymerization to be governed by RAFT mechanism, due to the low specific surface area of the particles. Small enough particle size would be below 100 nm where particles with magnetic core are generally nonspherical. We therefore adapted Stöber's method[11] to synthesize non-magnetic silica particles with 50-60 nm diameter, as determined by dynamic light scattering. CTA-coverages obtained on the silica nanoparticles were sufficiently high and were even visible to the naked eye as the CTA's red colour appeared on the white silica particles.



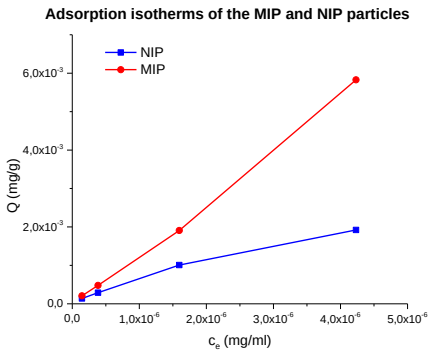


The HRP-coupling to the Stöber silica has been optimized also. Here, again the reaction could also be verified visually due to the brown colour of the protein. Polymerization with UV or thermal initiation using azo-type radical sources proved unsuccessful, possibly due to the retarding effect of the applied chain transfer

[10] Uchida E; Uyama Y; Ikada Y: Sorption of low-molecular-weight anions into thin polycation layers grafted onto a film, Langmuir 9: 1121-1124, 1993

[11] Stöber W; Fink A; Bohn E: Controlled growth of monodisperse silica spheres in the micron size range, Journal of Colloid and Interface Science, 26: 62-69, 1968

agent. Chemical polymerization with APS and TEMED resulted in polymer formation in the solution phase as well, and the poly(methacrylamide-*co*-bisacrylamide) gel was not separable from the silica particles. Exchanging MAAm to NiPAm seemed to solve this problem: the NiPAm-based nanogel (that formed in the solution to some extent) is swollen at room temperature and should remain in the supernatant upon centrifugation. FTIR confirmed the presence of a polymer layer on the particles which was estimated to be 3 nm thick based on thermogravimetry.



Batch rebinding assays revealed very low adsorption capacity towards HRP of the obtained core-shell particles with little difference between imprinted and non-imprinted polymers.

We suspect that this is due to inadequate or uneven polymerization on the particle surface. 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid retards the polymerization to a great extent, therefore the polymerization process was very slow, and, unfortunately quite random. In some batches we obtained sufficient monomer conversion after 1 day, but in some instances we had to add more initiator consecutively to achieve polymerization. We have also tested another, less retarding CTA, namely 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid, but in this case a lot of polymer was formed in the solution that we could not separate, so we could not verify the formation of the polymer shell.

In summary, all the steps of the core-shell particle synthesis are in hand now, except for the polymerization due to its uncertainty. To resolve this problem we plan to incorporate magnetic nanoparticles into the Stöber silica which helps to separate the particles from the polymer formed in solution and permits unquestionable measurement of the polymer shell.

Protein imprinted nanogels

At the start of the project there has been no literature example on the use of nanogels in protein imprinting. Shea et al have earlier successfully imprinted a peptide using a thermoresponsive NiPAm based nanogel[12]. As a starting point we have adapted the polymer composition from this work and prepared nanogels at room temperature from NiPAm, acrylic acid and tert-butylacrylamide (TBAm) as functional monomers and BIS as crosslinker using chemical initiation and sodium dodecyl sulfate (SDS) surfactant as stabilizer. The obtained nanoparticles were highly monodisperse with a hydrodynamic diameter of 40 nm.

By the addition of a model template, horseradish peroxidase (HRP) to the monomer solution we attempted protein imprinting. After synthesis the nanogel solution was tested for HRP enzymatic activity to see how the polymerization conditions influenced the protein. The test has revealed that HRP was denatured during the polymerization process. Further investigations proved that this was due to the presence of acrylic acid and the surfactant (SDS) used for particle stabilization. As protein denaturation can have a detrimental effect on the imprinting process the polymerization method was changed to surfactant-free emulsion polymerization (SFEP) and acrylic acid was excluded from the polymer recipe. SFEP of poly(NiPAm) is generally carried out at 60-70^{o}C to accelerate the decomposition of the peroxide initiator. Because this high polymerization temperature would denature the protein, we, therefore, added a catalyst (tetramethyl ethylenediamine) and carried out the polymerization at 42^{o}C, slightly above the lower critical solution temperature (LCST) of NiPAm. It was found that HRP did not lose its activity under these polymerization conditions.

With this system non-imprinted nanogels were successfully prepared. To minimize the nonspecific adsorption on the non-imprinted nanogels we have systematically varied the monomer composition. Different amounts of the hydrophobic TBAm (0; 20 and 40 mol%) were copolymerized with NiPAAm and BIS was substituted with a more hydrophilic crosslinker, polyethyleneglycol diacrylate (PEG-700). The particle size and the LCST of the non-imprinted polymers were measured by dynamic light scattering. Increase in the TBAm content from 0% to

[12] Hoshino Y; Kodama T; Okahata Y; Shea KJ: Peptide Imprinted Polymer Nanoparticles: A Plastic Antibody, J. Am. Chem. Soc., 130: 15242–15243, 2008

20% and to 40% led to a decrease of the LCST from 34°C to 19°C and to 5°C, respectively, indicating the formation of a more hydrophobic polymer network. The type of crosslinker did not influence the LCST. The size of the nanoparticles above the LCST was few hundred nanometers. We have assessed the binding of HRP on the purified non-imprinted particles. Particles with PEG-700 crosslinker showed much smaller HRP adsorption than BIS containing ones, supporting our expectations. Increasing the TBAm content of the nanogel gave rise to a substantially increased nonspecific protein adsorption. Therefore, NiPAm crosslinked with PEG-700 was proven a promising backbone polymer for the incorporation of the adenine-based functional monomer.

The purification of the imprinted nanogels after polymerization posed irresolvable difficulties to us due to the similar size range of the nanogel and the protein template. The unreacted monomers could be removed from the non-imprinted nanogels by dialysis through a 100kDa membrane. However, this pore size was insufficient to remove the 44 kDa HRP. To remove HRP from the imprinted nanogels they were dialyzed through a 1000 KDa membrane (pore size corresponding to an approx. 14 nm globular protein) but this procedure was not successful either as most of the nanogel particles were also removed. Ultracentrifugation through a 100kDa ultrafiltration membrane resulted in complete blockage of the filter pores and organic solvents could not precipitate the nanogels.

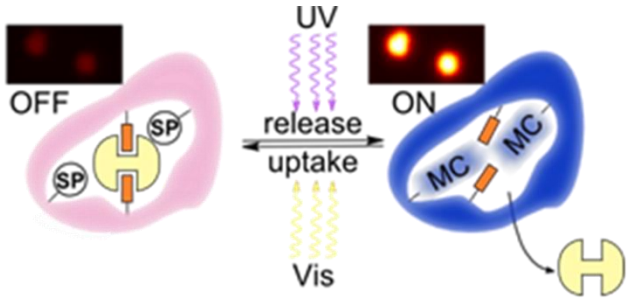
At this time a promising method appeared in the literature for the "solid-phase synthesis" of protein-imprinted nanogels[13]. In this approach the protein template is immobilized onto a solid phase and the nanogel synthesis is carried out in the presence of the protein modified beads. Imprinted nanogels form around the immobilized template, and therefore can be separated from the nanogels formed in solution by simple washing. We have adapted this system for the imprinting of a model template lysozyme. First, 100 µm glass beads were derivatized with 3-aminopropyltriethoxysilane to introduce reactive amino groups onto their surface. The coupling reaction resulted in a 0.2 µmol NH_2/g bead concentration. These were in turn activated with glutaraldehyde to which lysozyme could be coupled through its surface

[13] Poma A; Guerreiro A; Caygill S; Moczko E; Piletsky S: Automatic reactor for solid-phase synthesis of molecularly imprinted polymeric nanoparticles (MIP NPs) in water, RSC Advances, 4: 4203-4206, 2014

amino groups. The amount of lysozyme immobilized onto the beads was measured to be 3.2 nmol/g by BCA colorimetric protein assay. Poly(NiPAm) nanogels were prepared in the presence of the protein modified glass beads and during this process nanoparticles were formed both in the solution and also around the immobilized template. Washing the beads with room temperature water removed the unreacted monomers and non-imprinted or low-affintity nanogel particles. Elution with 4^{o}C water disrupted the interaction between the protein and high-affinity imprinted particles due to the swelling of poly(NiPAm) below its LCST. The particle size of the collapsed imprinted particles was around 80 nm measured by dynamic light scattering. The nanogel particles showed high affinity towards lysozyme in batch rebinding experiments. After the successful adaptation of the "solid-phase synthesis" method for protein imprinting we will use the novel adenine-based functional monomer in the imprinting of different proteins.

Molecularly imprinted microparticles with light controlled template binding and release [12]

Endowing MIPs with photoresponsive properties enables their use in remote controlled chemical sensors moreover it offers a mild way to remove the template after polymerization. Photoswitchable MIPs are scarcely reported in the literature and generally suffer from low selectivity. This is because the molecular recognition process is based on the photoswitchable monomer (mainly azobenzene type) that does not have adequate functional groups for interaction with the template.



We have introduced a new strategy that overcomes this limitation. Beside a spiropyran (SP) monomer that was planned to be responsible for the light induced template binding and release we have incorporated another well-established functional monomer, methacrylic acid to form a complex with the template. It was anticipated that photoinduced structural changes in the spiropyran unit (ring opening) would result in

drastic changes in the conformation of the polymer network, thereby also changing the spatial arrangement of the binding sites and expelling the template. Terbutylazine, a triazine type pesticide was chosen as a model template. Spiropyran methacrylate has been synthesized according to a previously reported procedure[14]. To prepare micron sized spherical particles precipitation polymerization was used and the type of crosslinking monomer and the ratio of the monomers have been thoroughly optimized to achieve simultaneous selective template binding and photoresponsive properties. The resulted 1.7 μm particles showed a highly increased binding of the template compared to a reference, non-imprinted polymer prepared in the same manner but omitting the template. Upon UV illumination of the particle suspension the absorbance peak of the open, merocyanine form appeared. Visible light illumination gradually decreased the absorbance peak of merocyanine and the solution returned to its original color indicating that the incorporated SP units underwent photoisomerization. The reversibility of the photoswitching was confirmed with several alternating UV-Vis cycles. The photoactivatable properties of the MIP microspheres were characterized and visualized also by fluorescence microscopy. The effect of photoswitching on the template binding property of the particles was investigated with repeated UV-Vis illumination cycles. This experiment has proven that the template uptake and release in the MIP could be photoregulated due to the incorporated photoswitchable monomers. By separating the template recognition from the photoswitching function our approach offers a generic method to synthesize photocontrollable MIPs with a wide selection of targets. This approach eliminates the need to decorate photochromic monomers with functional groups that are capable of binding with the template. Instead, well-established MIP recipes with common functional monomers can be used with the addition of a polymerizable photoswitchable molecule in an optimal amount.

Assessment of the selectivity of MIPs

The most important step in the characterization of protein imprinted polymers (and in general MIPs for any kind of template) is the measurement of their selectivity. There are

[14] Friedle S, Thomas SW: Controlling Contact Electrification with Photochromic Polymers, Angew. Chem., Int. Ed., 49: 7968–7971, 2010

different experimental approaches in the literature to assess the selectivity of MIPs. If the MIP is used as an HPLC column packing selectivity is characterized by differences in retention factors of different compounds. If the MIP is used in solid phase extraction then selectivity is demonstrated by the different recoveries of different compounds. The selectivity of MIP sensors is characterized by differences of the sensor signal in solutions of different compounds at the same concentration. In batch adsorption measurements the adsorbed concentrations of different compounds in otherwise identical extraction experiments are compared. As one can see, in different analytical applications the selectivity is differently interpreted. Moreover, one cannot easily (or at all) transfer the results between different applications.

These observations about the selectivity of MIPs prompted us to study the general concept of selectivity in analytical chemistry. We have shown [13] that the most important definitions of selectivity [14,15] are compatible only if the analytical response function is linear or quasi-linear. When the response function is non-linear the traditional definition of selectivity may become inapplicable. We have also shown [16] that in the case of multivariate (multichannel) linear methods, like absorption spectrum measurements, the chemometric selectivity definition used in the ordinary least squares (OLS) method (based on the net analyte signal) contradicts the selectivity concept used in single channel methods. We recognized that this contradiction is due to overlooking the possibility of bias-variance tradeoff with the OLS methods. The selectivity of biased methods (including ridge regression and partial least squares regression) is dependent on the composition of the future samples, a perhaps unexpected result for analytical chemists.

Following this generalized study of analytical selectivity we could return to the problem of MIP selectivity [17]. We have shown that measurement of MIP adsorption isotherms is the most generally useful way of presenting MIP selectivity. The individual isotherms of two compounds are useful for predicting chromatographic selectivity. The combined (competitive) isotherm of two compounds is needed to predict the selectivity of batch extractions and MIP sensors. We have found simple graphical methods to describe MIP selectivity in these cases.

For future technological applications of MIPs it is of great concern to know the maximum loadability of MIPs with the target compound(s) and the MIP selectivity at high loadings. We

could show by direct measurements [18] that the binding capacity of MIPs is nearly equivalent to the functional group concentration of MIPs based on methacrylic acid (the most common functional monomer of MIPs). This value is much higher than indicated by earlier results, which were based on extrapolations. The selectivity between some basic compounds could also be retained at high concentration levels.

PUBLICATIONS

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[3] Ceolin G; Orbán Á; Kocsis V; Gyurcsányi RE; Kézsmárki I; Horváth V: Electrochemical template synthesis of protein-imprinted magnetic polymer microrods, J. Mater. Sci., 48(15): 5209-5218, 2013

[4] Bosserdt M; Erdőssy J[15]; Lautner G; Witt J; Köhler K; Gajovic-Eichelmann N; Yarman A; Wittstock G; Scheller FW; Gyurcsányi RE: Microelectrospotting as a new method for electrosynthesis of surface-imprinted polymer microarrays for protein recognition, Biosens. Bioelectron., 73: 123–129 2015

[5] Erdőssy J; Horváth V; Yarman A; Scheller FW; Gyurcsányi RE: Electrosynthesized molecularly imprinted polymers for protein recognition, Trends in Analytical Chemistry, 79: 179–190, 2016

[6] T. Renkecz, K. László, V. Horváth: Molecularly imprinted microspheres prepared by precipitation polymerization at high monomer concentrations, Mol. Impr., 2: 1-17, 2014, Open Access journal

[15] Júlia Bognár participating researcher has changed her name to Júlia Erdőssy during the project.

[7] Renkecz T; Horváth V: Preparation of molecularly imprinted microspheres by precipitation polymerization, Methods in Molecular Biology - Synthetic Antibodies (accepted for publication), 2017

[8] Bognár J; Horváth V: Molecularly imprinted magnetic nanoparticles for the recognition of horseradish peroxidase. “Mátrafüred 2014” International Conference on Electrochemical Sensors, Visegrád, June 15-20, 2014 (poster)

[9] Erdőssy J, Kassa E; Horváth V: Molecularly imprinted magnetic microparticles prepared by immobilization of both template and initiator, Graduate Student Symposium on Molecular Imprinting August 27-28 2015, Kent, UK (oral presentation)

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The results of the project have also been presented at 21 international and 8 national conferences including the ones in the above list of publications.

PH.D. DISSERTATIONS AND DIPLOMA WORKS PREPARED IN THE FRAMEWORK OF THE PROJECT

1. Renkecz T: Novel molecularly imprinted polymers – membranes, microspheres, photoswitchable particles, 2013, Ph.D. dissertation
2. Tamás B: Selectivity of molecularly imprinted polymers, 2014, diploma work
3. Szakolczai A: Interaction of weak acids and bases in non-aqueous media, 2015, diploma work
4. Róka Z: Preparation of nanogels for the molecular imprinting of proteins, 2014, diploma work
5. Farkas A: Molecular imprinting of proteins using nanosphere lithography, 2016, diploma work
6. Kassa E: Development of a protein digestion method for the removal of the template from molecularly imprinted polymers, diploma work