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Surface-imprinted polymers in microfluidic devices

SCHIRHAGL Romana†, REN KangNing & ZARE Richard N.*

Department of Chemistry, Stanford University, Stanford, CA, 94305-5080, USA

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Molecularly imprinted polymers are generated by curing a cross-linked polymer in the presence of a template. During the curing process, noncovalent bonds form between the polymer and the template. The interaction sites for the noncovalent bonds become "frozen" in the cross-linking polymer and maintain their shape even after the template is removed. The resulting cavities reproduce the size and shape of the template and can selectively reincorporate the template when a mixture containing it flows over the imprinted surface. In the last few decades the field of molecular imprinting has evolved from being able to selectively capture only small molecules to dealing with all kinds of samples. Molecularly imprinted polymers (MIPs) have been generated for analytes as diverse as metal ions, drug molecules, environmental pollutants, proteins and viruses to entire cells. We review here the relatively new field of surface imprinting, which creates imprints of large, biologically relevant templates. The traditional bulk imprinting, where a template is simply added to a prepolymer before curing, cannot be applied if the analyte is too large to diffuse from the cured polymer. Special methods must be used to generate binding sites only on a surface. Those techniques have solved crucial problems in separation science as well as chemical and biochemical sensing. The implementation of imprinted polymers into microfluidic chips has greatly improved the applicability of microfluidics. We present the latest advances and different approaches of surface imprinting and their applications for microfluidic devices.

molecular imprinting, microfluidic devices, surface imprinting, bioanalysis, separation, sensors

1 Introduction

Natural receptor molecules, such as enzymes or antibodies, can have extremely high selectivity to their respective substrate or antigen [1, 2]. The high selectivity of these compounds creates an enormous number of applications in analytical chemistry [3, 4], diagnostics [5, 6], environmental science [7–9] and many other fields. However, in many cases, they are relatively difficult to produce or are highly expensive [10, 11]. Their lifetime and reusability are limited because they are degraded by oxygen or microorganisms [12]. Additionally, they cannot be used in harsh environments, such as in acids, bases, or organic solvents [13].

Molecularly imprinted polymers (MIPs) are attractive alternatives for natural receptors because they are more robust [14, 15] and relatively easy to synthesize [16]. More than 4000 polymerizable compounds are commercially available [17], which allows for tuning of certain material properties to a given analyte. Their successful use in catalysis [18, 19], separations [20–23], drug delivery [24, 25] and selective sensing [26, 27] have already been reviewed in previous articles.

MIPs are generally created by curing a prepolymer in the presence of a template. The main challenge during the synthesis of MIPs which are selective to biomolecules or macromolecules is that they need to be imprinted at conditions close to the natural environment of the biomolecules to ensure conformational integrity [28]. During the curing process, the groups interacting between the polymer and the template are aligned by self-assembly. It is believed that complexes are formed between the template and one or more functional polymer monomers. The polymer becomes highly cross-linked, which keeps the complexes in position

*Corresponding author (email: zare@stanford.edu)
†Current address: Department of Physics, ETH-Zürich, 8093 Zürich, Switzerland

after the template is removed. Thus, it is crucial to choose monomers that complement the chemical moieties on the template molecules [29, 30]. The cavities left behind by the template selectively reincorporate the respective analytes.

The traditional approach of bulk-imprinting, reviewed in [31–33], generates these types of imprinted polymers. The template, typically a small molecule, is simply added to the prepolymer mixture [34–36]. The cavities are generated throughout the bulk material. In order to remove the analyte molecules after successfully creating binding sites, the polymer needs to meet certain requirements. The material must be porous enough to allow molecules to diffuse through it [37, 38]. To facilitate template removal the imprinted polymer is usually either washed in solvents [39–41], acids or bases [42, 43], or detergents [44, 45]. The polymer can also be heated for template removal [46, 47]. Another elegant method for template removal is the addition of digesting enzymes like proteases [48]. Additionally, imprinted hydrogels [48–50], which allow the diffusion of slightly larger molecules, can be used. Alternatively, substances can be added that increase the porosity of the polymer [51, 52]. Furthermore, the surface-to-volume ratio of the polymer can be increased; polymer nanoparticles provide a large surface which eases template removal [53, 54] or a bulk imprinted material can be ground to expose the imprinted site to the surface [55, 56].

At the same time the polymer must be sufficiently cross-linked so that the binding sites retain their shapes. It is obvious, that this requirement is the reason why these methods are limited to molecules that are relatively small [57]. For large molecules, high cross-linking densities seriously hinder mass transfer of the template, leading to slow template removal and rebinding kinetics or, in the worst case, permanent entrapment of the template in the polymer network by physical immobilization [58]. An easy method to overcome this difficulty is to simply perform bulk imprinting and only use the imprints that formed on the surface. This method was successfully used to generate imprinted surfaces for protein crystal growth [59, 60], chemical sensing [61–63], and protein recognition [56]. However, it is often not favorable to lose a lot of template in the bulk because it is irreversibly entrapped. To circumvent this problem and to expand the method to large analytes, such as viruses, proteins or cells, a method called surface imprinting or two-dimensional imprinting was developed where imprints are only on the surface.

Although molecular imprinting was less effective for larger molecules, e.g., proteins (because of their variant conformations), with even bigger templates, the cellular imprinted polymers were able to selectively capture the template cells from a mixture. This interesting phenomenon promises a new platform for biological and clinical assays, but its physical basis was still unclear. Recently, we coated the inner surface of the imprint cavity with a thin and uniform layer of cross-linked alkylsilane groups, which leaves the surface morphology intact. We found that this coating greatly reduced the selectivity of the template surface, thereby demonstrating that chemical recognition as well as physical shape controls cell capture [64].

An appealing platform to perform the desired experiments with MIPs is a microfluidic device. These devices are widely used whenever small volumes of an analyte are used, e.g. for separations [65, 66], single-cell analyses [67–70] or single-molecule handling [71, 72], as well as synthesis [73]. Specialized reviews on microfluidic platforms in cell biology [74–76], particle synthesis [77], diagnostics [76–78], chromatography [79] or electrophoresis [80, 81] were recently published. The materials used in microfluidic platforms are more or less inert, transparent, and nontoxic, which allows their coupling to all kinds of analytical techniques, such as mass spectrometry [82], optical detection [83–85], mass sensitive detection [86, 87], electrical readout [88, 89], or surface plasmon resonance spectroscopy [90–92]. Additionally, valves can be implemented in the microfluidic device which allows directed flows into certain areas of the chip [93]. The main advantage of incorporating MIPs into microfluidic devices is that tiny channels reduce diffusion from a solution to the imprinted surface [94]. This leads to a significant decrease of response times for sensors or an increase of throughput for separations [95]. Here we review the methods of surface imprinting and their combination in microfluidic devices. We compare the different methods and describe the most promising ones for different applications.

2 Direct imprinting

Binding sites can be created either by direct imprinting with the desired analyte or through indirect imprinting. Direct imprinting is generally easier so it is more common than indirect methods.

2.1 Stamp-coating

The most common technique for surface imprinting is stamp-imprinting because it is one of the easier and more flexible approaches (Figure 1). The desired analyte is spread on small (3–8 mm) stamps. The solvent is removed either by simple drying (for solutions without buffer) or spin-coating. In particular, buffer-containing solutions must be spin-coated to remove surplus buffer, which can form crystals and cover the template molecules. The stamps are then pressed into a prepolymer. One of the most important parameters that must be optimized for stamp-coating is the viscosity of the prepolymer [96, 97]. When the analyte is pressed into the polymer, it needs to be viscous enough so that the template can make an impression. But the prepolymer should not be so soft that the template sinks too deeply into the material and makes a poor impression and causes

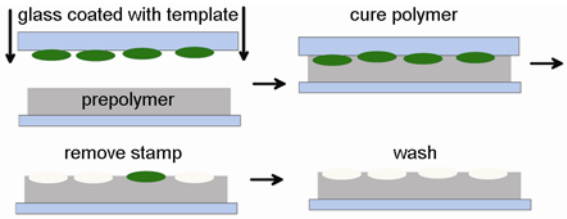

Figure 1 Schematic representation of stamp-coating. First the template is spread on a stamp which is then pressed into a prepolymer. The prepolymer is allowed to cure and the stamp is removed. Most people also add a washing step to remove template residue.

great difficulty in removing the template after the polymer has cured. The removal of the template from the cured polymer is normally easier for smaller analytes. Additionally, smaller stamps might further ease template removal. For larger analytes, longer template removal steps or harsher conditions might need to be used.

This surface-imprinting technique was used by Lieberzeit *et al*. [98] to generate insulin-imprinted polymers. The temperature-treated, and therefore denatured, insulin showed remarkable selectivity (factor of 6) compared to a nonimprinted reference. A similar approach was used by Darder *et al*. [99] for imprinting chlorella vulgaris and anabaena sp. PCC7120. Instead of glass plates, they used polydimethylsiloxane (PDMS) pieces for the stamp material. They showed that algae could make an impression into sol-gels consisting of methyltrimethoxysilane, phenyltrimethoxysilane and tetramethoxysilane. Stamp-coating was also used by Jenik *et al*. [100] to create surface-imprinted layers for the detection of picorniaviridiae (cause of common cold, foot and mouth disease and other illnesses). They achieved at least a factor of two higher sensor response for the respective template compared to different subgroups of rhinovirus as well as a factor of eight higher sensor response when compared with a different virus species. Furthermore, a decrease of the sensor response was observed after heat degeneration of the viral template. To achieve sensor response times (measured by a quartz crystal microbalance (QCM)) in the range of minutes and to reduce the sample volumes required per experiment, they implemented their sensor surfaces into a microfluidic channel enabling the consumption of only 40 μL for each experiment.

The group of Peter Ertl at the Austrian Institute of Technology implemented tobacco mosaic virus-imprinted polymers into a microfluidic sensor chip [101]. They generated imprints of tobacco mosaic virus by stamp-coating the virus into a polyvinylpyrrolidone polymethacrylic acid copolymer spin-coated onto interdigitated capacitor (IDC) structures (See Figure 2). After removing the template, they applied a PDMS top layer with the microfluidic channels. In this manner, the authors were able to detect and differentiate between different viruses by measuring impedance changes caused by the incorporation of virus into the imprinted polymer.

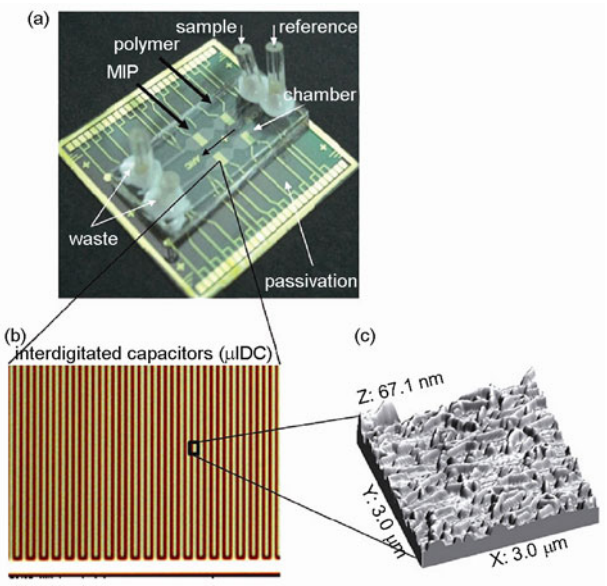

Figure 2 (a) Impedance chip with integrated MIP, reprinted with permission from [101]; (b) zoom on the coated interdigitated capacitor (IDC) structure; (c) tobacco mosaic virus on the MIP surface.

Wangchareansak *et al*. [102] used the stamp-coating method to create polymers imprinted with wheat germ agglutinin (WGA). WGA, a model compound for the interaction between viruses and cells during infection, was pressed into a copolymer consisting of acrylamide, methacrylic acid and methylmethacrylate. The investigators were able to differentiate between WGA and bovine serum albumin (BSA) with a QCM-based sensor using a simple microfluidic channel (80-μL volume). They found that the adsorption of proteins to the sensing surface could be described by a Brunnauer-Emmet-Teller model. Based on this model, the investigators described a multilayer formation of template on imprinted polymers.

Interesting work has been published by Bossi *et al*. [103] who used stamp-coating to imprint into molten gallium. Gallium has a melting point below 37 °C so it can be used without thermally damaging a biological template. The method is promising for future applications because it combines the conductive properties of metals with the molecular selectivity of biological entities. In their work, Bossi *et al*. adhered proteins, such as horseradish peroxidase, BSA, RNA polymerase, urease from jack bean, and DNA-binding proteins, to function as templates onto a smooth mica surface. Then the investigators poured pure molten gallium (T = 37 °C) over the stamps and left it to solidify at room temperature for 30 min in a humidity-controlled chamber. The metal surface was then peeled from the template. The imprints left behind clearly represented the sizes and shapes of the various proteins. The investigators rebound either the template protein or control proteins and found remarkable differences (a factor of 19) between the dissociation constants. Furthermore, they demonstrated that the template stamp

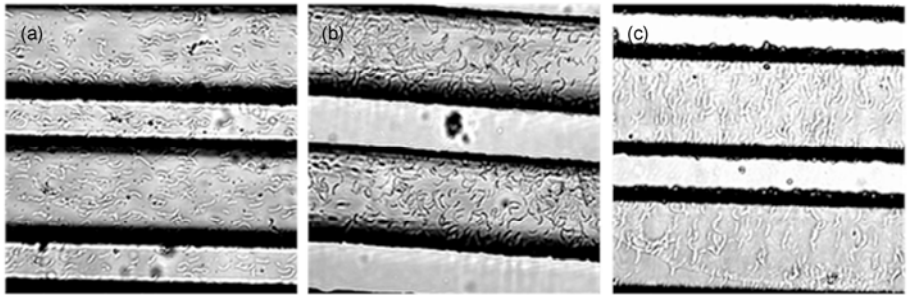

Figure 3 Imprints oriented in different directions [105]. Imprints were oriented (a) parallel, (b) randomly or (c) normal to the flow vector of a microfluidic channel.

could be reused twice without a loss in recognition ability.

A useful technique to characterize molecularly imprinted surfaces was demonstrated by El Kirat *et al*. [104]. They studied the binding specificity of imprinted polymers toward their template by atomic force microscopy (AFM). As a proof of concept, El Kirat *et al*. stamp-coated *cytochrome c*, an important protein in the mitochondrial electron transport chain, to a mica surface. They removed the template by washing with detergents followed by trypsin digestion. The investigators observed a distribution of binding forces between 85 and 95 pN by attaching a template molecule to an AFM tip, rebinding to the MIP and directly measuring the binding forces. Additionally, different types of cross-linkers were tested and compared to determine which cross-linkers had the better performance.

One downside of the method is that it is unsuitable for very fragile templates. Additionally, reincorporation of large and asymmetric templates declines because it is unlikely that the analyte is captured in the same orientation as the imprint. Thus, an epitope approach might be favorable. For methods with potentially higher selectivity or sensitivity see section 3.1 (sub-structure imprinting) and 3.3 (antibody replica). Another alternative is presented in section 2.2 (oriented imprinting).

2.2 Oriented imprinting

When our group implemented surface-imprinted polymers into microfluidic chips, we observed that the orientation of imprints matters. Rod-shaped templates are preferentially reincorporated into cavities pointing in the same direction as the flow vector in the microfluidic channel. As a result, randomly oriented imprints caused sub-optimal capture of asymmetric templates. So we developed a method to orient the template before imprinting [105]. We temporarily bound a PDMS layer with microfluidic channels to a polylysine-coated glass stamp by electrostatic interaction. We forced cells of a rod-shaped cyanobacterium, though the channels as the template and captured them on the positively charged polylysine in the direction of the flow. The PDMS was removed and the oriented cells (adhered to glass plates) were used in stamp-coating. We incorporated the imprinted surface into a microfluidic chip to perform cell capture. The capturing efficiency was significantly larger than observed for randomly oriented imprints. An even more pronounced difference was observed compared to a control experiment where all imprints were oriented normal (unfavorable) with respect to the flow. Imprints in all three orientations can be seen in Figure 3. Because the imprinted surface captured significantly more of the respective template, we were able to differentiate and separate different bacteria strains with 90% sorting efficiency. This is the first successful approach to applying a "chromatography-like" adhesion-based separation to bacteria cells. We also discovered a strong pH dependence of the efficiency for bacterial capture.

2.3 Drop-coating

Drop-coating is a method for creating imprints of very fragile bioanalytes. The prepolymer is spin-coated on a surface in a concentrated form where one of the monomer components can function as solvent without any further dilution. Immediately after spin-coating, a drop of the analyte solution is injected onto the curing polymer; and imprints are formed by the sedimenting analytes within minutes. This method is very convenient for fragile cells that would decompose during longer imprinting times. This concept was developed by Seifner *et al*. [106] by imprinting with blood cells on linked polyvinylpyrrolidone. Unlike other prepolymers in molecular imprinting that are diluted to slow the polymerization process, vinylpyrollidone was used both as the monomer and the solvent. A cross-linker and a radical starter were dissolved into the vinylpyrollidone and the mixture was polymerized within minutes. Seifner *et al*. in-

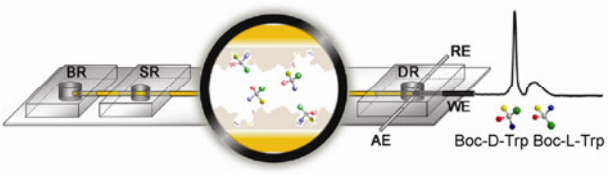

Figure 4 Schematic of MIP-based electrophoretic separation on a microfluidic chip, reprinted with permission from [107]. BR, buffer reservoir; SR, sample reservoir; DR, detection reservoir; WE, working electrode; RE, reference electrode; AE, auxiliary electrode.

corporated their polymer surface in a simple microfluidic channel to create a sensor that was able to categorize blood subgroups that only differed in the amounts of certain surface antigens.

Qu *et al*. [107] implemented this technique into a microfluidic chip (Figure 4) which could be used for the enantiomeric separation of the L and D enantiomers of tertbutoxycarbonyl-tryptophan (BOC-Trp). The investigators used a prepolymer mixture in which acrylamide was the functional monomer and ethylene glycol dimethacrylate was the cross-linker and incorporated the mixture into the microfluidic system. They optimized the polymeric composition to be able to sort standard analytes by enantiomeric separation (Boc-D-Trp, Boc-L-Trp) within 75 s. A drawback of the method is the short polymerization times. Long curing times are generally favored in order to give the template and prepolymer parts more time to form the monomer-template complex because it is thought that this leads to more and stronger binding sites [108].

2.4 Sacrificial layers

Another elegant way to create binding sites is to use a sacrificial layer that is applied between the analyte and the prepolymer and covalently bound to the forming polymer. Its dual purpose is to prevent the analyte from reacting with the monomers and to introduce new functional groups that can interact with the template. Shi *et al*. [109] used this approach to create a disaccharide layer in between proteins and the polymer (see Figure 5). After spreading protein on a mica surface, they coated the protein layer with a disaccharide layer that reacted with the polymer. The mica was peeled off and the proteins were removed by a solvent. The investigators demonstrated selective recognition for albumin, immunoglobulin-G, lysozyme, ribonuclease, and streptavidins as well as competition to the natural receptor counterparts. A similar approach was used by Dickert *et al*. [110] to prevent covalent linking of a proteinogenic virus

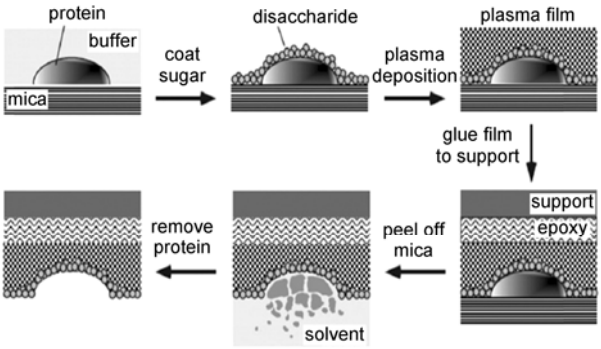

Figure 5 Sacrifical layer assisted imprinting, reproduced from [109]. First, the template (proteins in this case) is adhered to a mica (or glass, see other references) surface and coated with a disaccharide layer. Then a polymer-coating and a support are deposited (here via plasma deposition) on top of the template molecules. Finally, the mica surface is removed and the template can be removed by a solvent. Disaccharide-coated cavities remain that are able to selectively reincorporate their respective template.

surface to a prepolymerized polyurethane (reaction of –N=C=O of isocyanate with $–NH_2$, or –OH). They used glucose, 4-aminophenol, or 4-aminobenzoic acid as a sacrificial layer to react with the polymer and interact with the tobacco mosaic virus. They used a simple PDMS-based microfluidic channel to inject 250 μL samples into their sensors. The investigators were able to differentiate between saps drawn from infected and uninfected tobacco plants.

2.5 Polymer-brush imprinting

Zdyrko *et al*. [111] introduced a method to generate imprinted polymers based on polymer brushes. Protein molecules were first chemically bound to a reactive polyglycidylmethacrylate polymer layer. Next, the space surrounding the adsorbed biomolecules was modified with a grafted polyethylene glycol (PEG) layer (Figure 6).

The template was removed by enzyme treatment, which led to the formation of islands complementary to the protein shape. Unlike the PEG-coated surface, the imprinted surfaces were able to adsorb to the protein template. Additionally, they were able to differentiate between bovine serum fibrinogen and BSA.

A similar approach was used by Wang *et al*. [112] who created a thiol film, which was analogous to PEG, on a gold surface. In contrast to the previously described method, the template was bound directly to the surface and there was no polymer in between. Wang *et al*. used their system to detect a cancer biomarker, carcinoembryonic antigen, in solutions of the purified biomarker as well as in a culture medium of human colon cancer cell line (selectivity was tested with hemoglobin). In order to demonstrate the general applicability of the method, they also created a poliovirus-imprinted (selectivity was tested with adenovirus) and an amylase-imprinted surface.

Turner *et al*. [113] used a lipid monolayer consisting of a cationic lipid and a nonionic lipid to create a recognition surface for ferritin. The cationic molecules interacted with the negatively charged protein surface. To improve protein affinity for the monolayer and to reduce protein-protein aggregation, lipids modified with PEG were introduced into the monolayer. The investigators formed a monolayer of

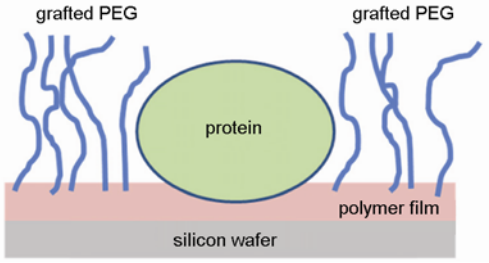

Figure 6 Scheme of creating a polymer-brush imprinted surface. First a polymer film is spread on a substrate followed by adsorption of the template. Polyethylene glycol (PEG) chains are next grafted around the template.

lipid containing the template at a water/air interface and transferred the lipid layer onto a hydrophobic substrate. This immobilization was shown by fluorescence correlation spectroscopy to significantly hinder further diffusion of lipid molecules. Rebinding studies demonstrated as much as a six-fold increase in ferritin adsorption to imprinted versus control monolayers. Cross selectivity was shown to be minimal to BSA.

A similar technique can also be used on the surface of nanoparticles instead of a planar substrate. Gültekin *et al*. [114] produced gold-silver nanoparticles with a dipicolinic-acid-imprinted shell. Dipicolinic acid plays an important role in bacterial spore formation. The investigators used methacryloyliminodiacetic acid-chrome which forms metal chelates. The chelates within the imprinted cavities interacted with dipicolinic acid and its structural analogues [115] and generated a better binding of the template. If a template was incorporated into the beads, the fluorescence of the beads was quenched. This technique was used to create a sensor for bacterial spores from Bacillus cereus. Selectivity was tested with phthalic acid, which is known to build complexes similar to the spores with the chelating monomer, and a selectivity factor of 28 for the template molecule was found.

A drawback of the method is its limitation to polymers that form brushes. Additionally, differentiating analyte species of comparable sizes may be difficult because the recognition is mainly based on size. This is particularly the case for gold because it nonspecifically adsorbs many different kinds of bioanalytes.

2.6 Thin-film imprinting

A similar approach to the polymer-brush imprinting method is thin-film imprinting. In this method, which was used by Cohen *et al*. [115], an imprinted polymer is created by spreading a thin prepolymer film (not branched polymer chains that grow from the surface) around the template. The thin polymer film, consisting of tetraethoxysilane, must be placed below the template height so that the template can be removed after curing. Cohen *et al*. used this method to generate polymer films imprinted with several different bacteria (*D. radiodurans*, *S. natans*, *E. coli* CN13, *B. subtilis*) and oocytes of the protozoanparasite cryptosporidium parvum. The template was removed by washing with concentrated salt solutions so that the cells shrink in the hyperosmotic media. The imprinted surfaces were able to reincorporate their respective templates but the quantitative extent of selectivity was not stated.

Fujikawa *et al*. [116] cast a polymer monolayer around tobacco mosaic virus imprints. They deposited a thin, completely cured polymer layer underneath the actual imprinted polymer to facilitate binding of the imprinted polymer to the supporting substrate. Then the template virus was spread and surrounded by a monolayer of titanium oxide, the functional monomer. Finally, the template was removed from the cavities by oxygen plasma treatment. The investigators showed that imprints clearly reproduced the size and shape of the respective analyte virus. It must be noted that the treatment degrades the template virus as well as all kinds of organic materials so it cannot be used for most polymer compositions.

The group of Evgeny Vulfson at Polytechnic Institute of New York University discovered a method to apply thin-film imprinting on the surface of polymer particles [117, 118] to form an emulsion of organic polymer particles surrounded by the aqueous phase. A hydrophilic shell was then polymerized around the organic cores. This allowed the template species, *Listeria monocytogenes* and *Staphylococcus aureus*, that were in the aqueous phase to adhere to the particles. Unprotected areas of the polymer surface were modified with perfluoropolyether to activate those areas for further reaction. The bacteria were then removed and another polymer shell was created only at the areas not previously covered with bacteria. The resulting cavities reproduced the size of the template species. However, it must be noted that the method only makes use of the size of the templates and not the surface chemistry, unlike other described approaches.

Harvey *et al*. [119] modified the same method to generate imprinted polymers for spores (Figure 7). First, they precipitated polymer particles. The template spores *Bacillusthurgiensiskurstaki* as a surrogate to *Bacillus anthracis*, were then allowed to attach to the beads. The molecularly imprinted polymer was formed by adding poly(allylamine), which self-assembled around the spores. The polymer was cured by UV light. The beads were deactivated to prevent the amine groups on the bead surface from nonspecifically reacting. The beads were next suspended in 1,1,2-trichlorotrifluoroethane, and Cytonix Fluor N2340 perfluoroetherdiisocyanate was added. Finally, the spores were removed and the cavities were allowed to react with concanavalin A. Concanavalin A is a protein that is known to bind to different saccharides so it should facilitate binding. The investigators showed that the imprinted beads bound to their respective template spores. They observed 25%

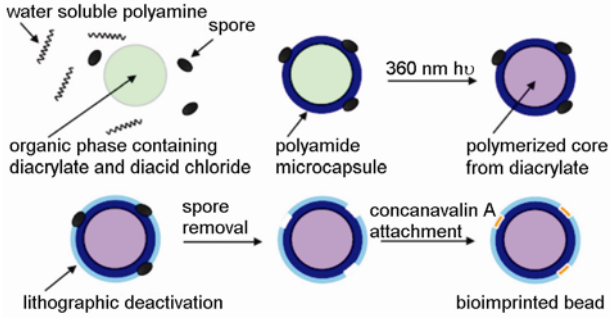

Figure 7 Scheme for thin-film imprinting of polymer particles (containing adipoyl chloride, hexanedioldiacrylate and dibutyl ether), reprinted with permission from [119].

stronger binding to the imprinted beads compared to non-imprinted beads.

A drawback of the method is that size is mostly used to differentiate species and not the chemical properties of the surface. It can only be applied to templates which are large enough to be thicker than the polymer film that is spread around them. Furthermore, some authors have reported that membrane fragments from the template bacteria remain in the polymer surface [117]. They claim that the membrane fragments help to gain selectivity; however, the fragments might not be favorable in other cases. It is possible to perform the method on a particle surface but the described protocols are rather complicated compared to other described methods.

2.7 Photoinduced polymer deformation

Another approach (Figure 8) exploits the deformation of azopolymers when they are irradiated by light. Narita *et al.* [120] synthesized an azopolymer that consisted of azobenzene-bearing acrylate copolymers which deformed when irradiated by light.

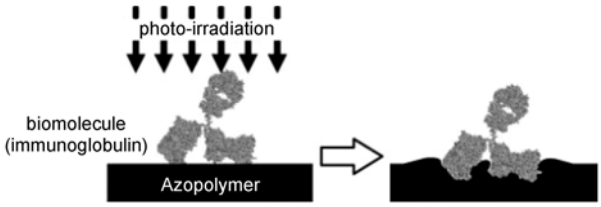

Figure 8 Photoinduced polymer formation, reprinted with permission from [120].

Irradiation of the azobenzene-bearing acrylate copolymer leads to conformational changes in the azogroups (*cis-trans*). This material deformation allows the template molecule (immune globulins) to make an impression in the azopolymer. They compared different azopolymers and showed that the antibodies they imprinted retained their biological function for the capture of antigens.

The main drawbacks of the method are the restrictions of the polymer systems that can be used for the technique as well as the need to irradiate the template molecule which might lead to denaturation. This issue was not observed for immunoglobulins but might be an issue for more fragile templates.

2.8 Polymer microbands and microrods

Molecularly imprinted polymer microbands were developed by Lautner *et al.* [121]. Polymer microbands are produced by a special technique which was invented to meet the requirements of surface plasmon resonance (SPR) measurements. In SPR, analytes can be only detected if they very close to the surface; many polymers cannot be used because they are not thin enough and the recognition surface is simply not within the penetration depth of the evanescent wave. Instead of printing the top of a polymer, Lautner *et al.* found a way to imprint the sidewalls of their microbands. They coated polycarbonate microbands on defined areas by photolithography, and adhered the protein template to the microbands. A conductive polymer was electrodeposited between the bands. The polycarbonate was dissolved and the protein template was removed, resulting in microbands imprinted on the sidewalls.

Menaker *et al.* [122] used a similar approach to produce a selective protein recognition material (Figure 9). Instead of microbands, they used spherical rods that were surface-imprinted. Those rods were polymerized in surface-modified polycarbonate membranes instead of microbands. The investigators used avidin, fluorescently labeled avidin, and BSA as target proteins for their MIPs. The MIPs were evaluated by fluorescence imaging after binding of fluorescently labeled proteins. A disadvantage of the

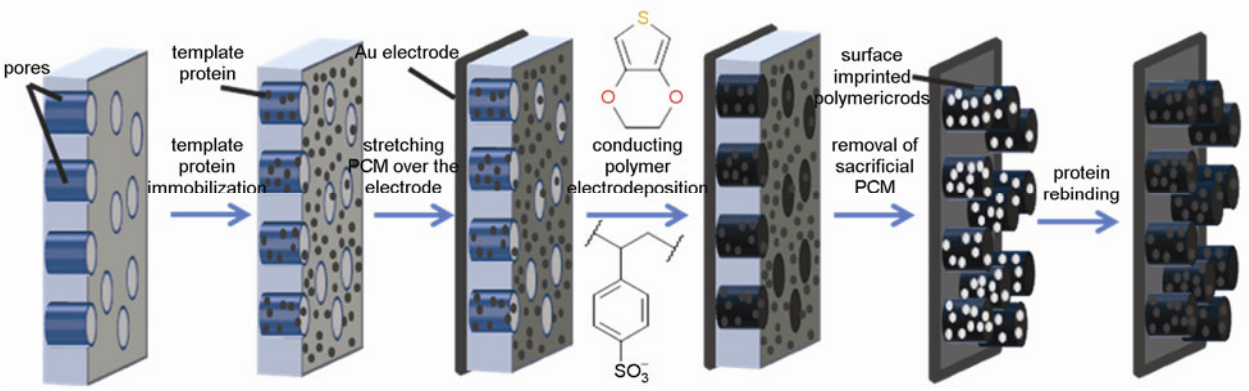

Figure 9 Schematic representation of polymer-microrod fabrication with permission from [122]. First, the template is immobilized within the pores of a polycarbonate membrane. Then, the membrane is mounted on a gold electrode and a conducting polymer is deposited which fills the surface modified pores. The polycarbonate is removed by washing with chloroform leaving surface imprinted rods behind. Finally, the remaining cavities can be used to reincorporate the protein template.

method might be that rods are more fragile than a surface.

2.9 Sol-gel template synthesis

Sol-gel template synthesis combines the advantages of surface imprinting, which makes it easier to remove the template, with the advantages of bulk imprinting of 3D structures, which have more room for binding sites. In sol-gel template synthesis, which was first shown by Ylmaz *et al*. [123], a template is first covalently bound to a silica substrate. The silica substrate is then mixed with a prepolymer which is then allowed to cure. Finally, the silica substrate is destroyed by etching with aqueous HF. A fiber network with recognition cavities on the surface remains. Ylmaz *et al*. used the method to create a polymer network that selectively bound to caffeine, theophylline, and theobromine. These molecules were bound to aminopropyl-derivatized silica gel. Remarkably, the researchers achieved less than 2% cross selectivity. This protocol was further developed by Sellergrens' group [124] for the imprinting of amino acids and peptides. They immobilized precursors, (9-(2-bromoethyl)adenine and 6-chloro-2,4-diaminopyrimidine), of their adenine and triaminopyrimidine templates on the surface of amino-functionalized silica particles. The investigators used these particles to make an impression into a prepolymer containing ethyleneglycol dimethacrylate and methacrylic acid. To dissolve the silica substrate, they washed their imprinted material with $(NH_4)HF_2$ solution. They used their imprinted material as stationary phases for chromatography and evaluated their imprinting effect by separation of the respective template materials as well as structural analogues.

Li *et al*. [125] used the method to generate a nanowire network for affinity chromatography of different proteins, such as cytochrome *c* from different species, hemoglobin from different species and insulin. A drawback of the method might be that the fragile polymer network could be less robust than a simple surface or bulk imprinted material. Additionally, the harsh treatment with HF might not be feasible for some polymer compositions although monolithic MIPs appear stable toward similar treatments [126].

2.10 Phase-boundary imprinting

An interesting surface-imprinting method for small molecules was shown by Araki *et al*. [127]. They modified the standard precipitation polymerization process [128–130] to generate binding sites solely on the surfaces of polymer particles. Normally, the generation of binding sites is done by curing and cross-linking the functional monomers (divinylbenzene) in organic droplets within an aqueous phase. Hydrophobic templates are simply added to that mixture and can be found in the whole polymer particles (bulk imprinting). In order to generate imprints only on the surface of organic droplets, Araki *et al*. used a bifunctional molecule, benzyldimethyl-n-tetradecylammonium chloride, a detergent that orients the template molecule toward the surface. The bifunctional molecule had a charged head and a nonpolar tail so it oriented toward the phase boundary (Figure 10).

The investigators were able to create surface-imprinted polymer beads that targeted the amino acid derivative, *N*-benzyloxycarbonyl-glutamic acid. The beads demonstrated chiral recognition and some selectivity toward structural analogues in aqueous media. A drawback of the method is that it is restricted to template molecules with a bipolar structure.

2.11 Voltammetric-deposition imprinting

Rick *et al*. [131] showed a method that was ideal for combining molecularly imprinted polymers with cyclovoltammetric detection of biomolecules. The method relied on voltammetric deposition which allowed precise control of the layer thickness and conductivity of the imprinted polymer. An initial layer of polypyrrole was first deposited on screen-printed platinum supports. This layer functioned as a supporting layer and provided electric conductivity. On top of that layer, two layers of polyaminophenylboronic acid were formed. The first of these layers was not imprinted and formed a barrier between the polypyrrole and the outer layer. The outer layer was deposited in the presence of a protein template so it functioned as the recognition surface. Finally, the template was removed. The authors used current transmission reductions caused by the bonding of the nonconductive protein to the polymer surface as a measure of re-binding. They were able to detect 1 part per million of lysozyme and cytochrome *c* in solution. This approach has the potential disadvantage that it is restricted to conductive polymers.

3 Indirect imprinting

Although direct imprinting is the easiest way in most cases

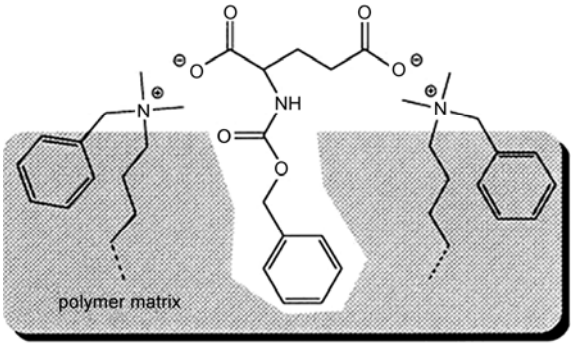

Figure 10 During phase-boundary imprinting [127] the template (at the center) is oriented at the phase boundary between organic (grey) and aqueous (white) phase. The template is surrounded by the bifunctional monomer benzyldimthyl-n-tetradecylammonium chloride.

to generate selectivity, it has its limitations. In situations where the desired analyte is simply not available or hard to work with it (e.g. pathogens or unstable molecules), indirect imprinting is an attractive alternative. The polymer is not imprinted with the desired analyte itself but with other molecules which give the desired selectivity. Furthermore, if very large particles are the target, removal of templates is often an issue.

3.1 Sub-structure imprinting

An elegant way of creating selectivity toward a desired biomolecule is to create imprints with a characteristic substructure of the molecule. This approach is very similar to how natural antibodies bind to a small substructure of their target, the epitope. Small structures tend to produce more selective imprints because large imprinted sites may be seen as general nanopores that can bind a range of smaller polypeptides and result in reduced selectivity. The technique is attractive if the captured molecules have a certain orientation. This orientation aspect was used by Tai *et al*. [132] who imprinted with a 15-amino acid surface antigen of dengue virus to create selectivity toward a virus protein. After capturing the protein at a specific site, they read out their signal by using an antibody targeted to the opposite side of the protein. The researchers were able to detect concentrations down to 5 ng dengue virus per mL.

Nishino *et al*. [133] published some generally useful advice for sub-structure imprinting with proteins. Terminal peptides make better imprinting targets because their structure is unambiguously defined. In contrast to other regions of the target protein, they have fewer interactions with the protein secondary structure, which may hinder or frustrate binding. According to Nishino *et al*, the minimum length of peptide necessary to create ''unique'' recognition for the target protein has been estimated to be around 9 amino acids. The authors also stated that an exposed C-terminus is preferable, because this site is less prone to post-translational modifications [134]. In their experiments, Nishino *et al*. were able to extract their target proteins (*cytochrome c*, alcohol dehydrogenase, and bovine serum albumin) from mixtures with C-terminal-imprinted acrylamide/ethylenebisacrylamide polymer thin films. They used stamp coating (see section 2.1) in which they immobilized the template peptide sequence with the N-terminus to a silanized silicon surface functioning as the stamp.

Titirici *et al*. [135] combined sol-gel template synthesis (for a detailed description see section 3.9) with substructure imprinting. They modified the method in order to create peptide-imprinted microspheres. First, peptide was immobilized on porous silica beads. Methylmethacrylate was polymerized and cross-linked in the presence of those silica particles, leading to surface imprints of the peptide sequence. The investigators removed the silica part of the particles by etching with HF so that the peptide-imprinted polymer portion remained. The imprinted polymers recognized several peptides with the template substructure. Another advantage of this approach applies if selectivity toward a group of targets sharing a certain surface structure is desired; the common structure can be used to create imprints that recognize a whole group of targets.

The disadvantage of the method is that one needs to know which substructure of an analyte is present on the surface, which is often nontrivial [136–139]. Even if a feasible surface structure is known, it might be difficult to synthesize [140]. Furthermore, the HF treatment might not be possible for some polymers or analytes.

3.2 Artificial template stamps

For highly complex templates, such as entire cells, the quality of the stamp (see section 2.1) can vary substantially.

Furthermore, if the template organism is pathogenic or difficult to culture, one might want to avoid contact with it as much as possible. Motivated by these drawbacks in microorganism imprinting, the group of Franz Dickert at the University of Vienna created a double-imprinting process (Figure 11) to create artificial template stamps [141]. The main advantages of this technique are its high reproducibility and reusability of the artificial templates (fresh cells are only needed once).

To obtain artificial template stamps two imprinting steps are required (Figure 11). First, the natural template is printed into a polymer. After removing the template, the cavities are filled with a second prepolymer. The second polymer forms the artificial template. After the two polymers are separated, a replica of the natural template is generated which can be used several times to create imprints in a third imprinting step.

Two different approaches for different cell types were shown in [141] to create "artificial cells" made out of polymer. In both methods, the cells are first pressed into a prepolymer. Polyurethane was used analogue to stamp coating (section 2.1) for relatively robust yeast cells. For imprinting with the fragile red blood cells that were fixed to improve their durability, PDMS, was used because of its softness. After removing the template, the cavities generated by both

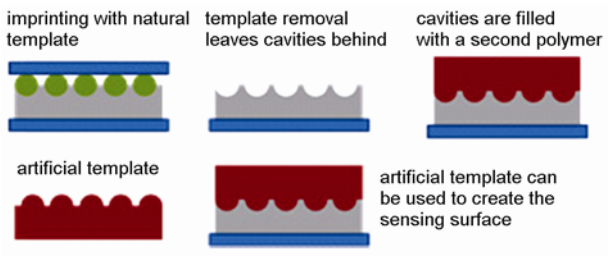

Figure 11 Artificial template production. First cells are pressed into a prepolymer. Then the cellular template is removed to leave cavities that are filled with a second polymer leading to the "artificial template" which can be used several times to generate the capturing surface.

cell types were casted in a second prepolymer. As artificial cell material for both cell types, a single component epoxy, SU8-2025 (Microchem), was chosen. The viscosity of the resin was adjusted with cyclopentanone. To favor the formation of hydrogen bonds, bisphenol A was added. After separating the two polymers, the "artificial cell" stamp (called master by the authors) was used to imprint a third polymer on the surface of a transducer that functioned as a sensor layer. With those recognition layers, Jenik *et al*. [141] were able to differentiate different yeast strains and blood groups (Figure 12).

Seidler *et al*. [142] used the same method to generate artificial yeast cells imprints by binding to cells in different states of their life cycle. They synchronized cells by preventing certain steps in the life cycle and used those cells as the template for the first imprinting step. As described in [141], the authors filled the cavities with a second polymer leading to the artificial template stamps. The stamps were used for a third imprinting step into a prepolymer on the gold surface of a QCM. The authors created an array and incorporated their sensor into a simple microfluidic channel that allowed them to detect various fractions of cells in different states of the life cycle.

One disadvantage of the method is that imprinting must be performed three times so making it very time-consuming.

3.3 Antibody replicas

Antibody replicas, which were first produced by Schirhagl *et al*. [143], are an alternative for direct imprinting if the analyte is unstable and will denature during the imprinting process. Like the previous method of artificial cell templating, the amount of analyte used during the fabrication can be minimized. The analyte's antibody serves as template in this double imprinting approach (Figure 13).

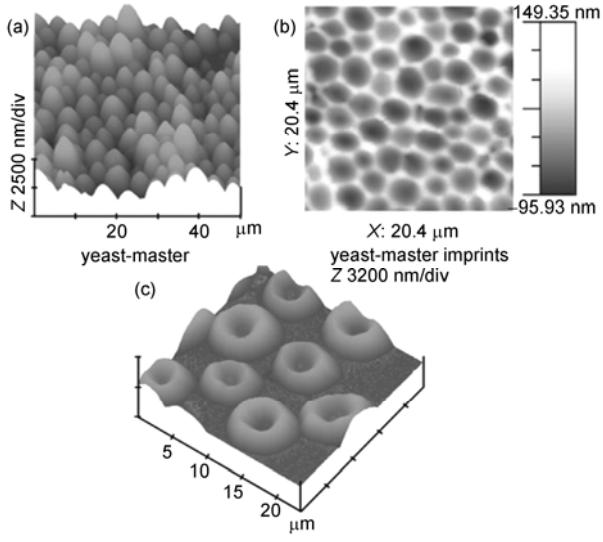

Figure 12 Double imprinting, with permission from [141]. An artificial yeast cell stamp consisting of a commercial epoxy (SU 8 2025) shown in (a) was produced by double imprinting, (b) shows the final recognition surface created by imprinting with the artificial yeast cells. A similar approach was used to create the artificial erythrocytes consisting of the same epoxy, as shown in (c).

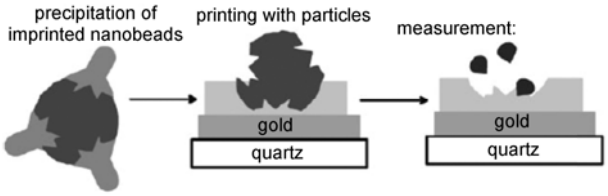

Figure 13 Antibody replicas, modified from [144]. First, particles are precipitated together with natural antibodies. After removing the antibodies these particles are used for a second imprinting step leading to positive replicas of the antibodies. These patterns on a surface can be used to selectively capture the respective antigens.

The method is based on the epitope in a similar way as in natural antibodies and sub-structure imprinting (see section 3.1). First, polymer particles are precipitated in presence of the antibody with the desired selectivity. When the antibody is removed, nanoparticles with cavities representing the negative structure of the template are left behind. These polymer particles adhered to a glass plate (analogous to stamp coating in section 2.1). It should be noted that only a small amount of the antibody is required because these nanoparticle stamps are reusable. This glass plate is used for a second imprinting process leading to positive structures of the antibody on a polymer surface. Schirhagl *et al*. were able to differentiate several types of picorniaviridiae, and generated a sensor for rhino virus (the cause of common cold). Simple microfluidic channel architecture was used as measuring chamber during virus sensing.

In the group of Dickert, the same method was used to produce a sensor based on a QCM for the glucose-regulating hormone, insulin [144]. The investigators compared their method with natural antibodies and obtained comparable selectivities to several proteins (glargine, pepsin, trypsin, lysozyme). They also showed that their replicas had higher sensitivities toward the respective analytes compared to a conventionally imprinted polymer.

Additionally, antibody replicas were used to detect allergenic proteins in food extracts [145]. The authors showed a comparison of their method to a surface-imprinted polymer as well as an immobilized antibody. In agreement with the work from Dickert's group, they obtained selectivities that were slightly worse than with a natural antibody but with improved sensitivities.

Recently, it was shown that the method can also be applied to the detection of small molecules [146]. A sensor for atrazine (a pesticide) was developed and evaluated by measuring cross selectivities with several other structurally related pesticides (propoxur, atrazinedesethyldesiisopropyl, atrazinedesisopropyl, methoxychlor, atrazinedesethyl, terbutylazine and even simazine and propazine only differing from atrazine by a methyl group). At least a selectivity fac-

tor of 4 was found for all tested compounds. In order to improve the sensor response times, the investigators incorporated the molecularly imprinted surface into a simple microfluidic environment of an inlet, an outlet, and a detection chamber.

Drawbacks of the method are the more complex synthesis of the material as well as the need for a specific antibody for the desired analyte.

4 Conclusions and outlook

In the last two decades, several surface imprinting methods have been developed. Besides chemical sensing, which is the most prominent surface-imprinting application, other avenues of investigation have become important. These analytical techniques demand new imprinting approaches that meet their requirements. Table 1 summarizes the described methods, their advantages and drawbacks as well as their most important applications. The combination with microfluidic devices has led to improvements in capturing efficiency and response times by reducing diffusion effects. Furthermore, microfluidic devices have successfully decreased the necessary sample volumes and eased sample handling. But to date, only relatively simple microfluidic architectures have been used together with surface imprinting so there is much room for improvement. Bulk imprinting of small molecules has achieved selectivity factors as high as 100 [147], so in comparison, the field of surface-imprinting of molecules is still in its infancy. Perhaps much more promising is surface imprinting of larger objects, such as viruses, bacteria, and cells.

Table 1 Summary of described methods for surface imprinting

Method	Specialities	Drawbacks	Applications	Microfluidic combination
Stamp-coating	easy, usable over a wide range of template sizes (small molecules to entire cells)	not optimal for some applications	sensing (mass sensitive, optical, electro chemical)	several microfluidic designs were realized
Oriented imprinting	for template with a preferred orientation in a flow (e.g. rod shaped bacteria)	more difficult than stamp coating	sensing (optical), separation	a serpentine channel was used
Drop-coating	very fragile analytes that would be decomposed during other methods can be used (e.g. red blood cells)	short curing times are generally not favorable	sensing, sample preparation for artificial templates	very simple architectures (inlet chamber and outlet)
Sacrifice layers	for templates with a special affinity to some non polymeric material or templates that would react with one of the monomers (e.g. most biomolecules with polyurethanes)	more difficult than stamp coating	sensing	very simple architectures (inlet chamber and outlet)
Polymer brush imprinting	easy, usable over a wide range of template sizes (small molecules to entire cells), can also be done on particles (better for small molecule capturing due to higher surface)	restriction to brush forming polymer	separation, sensing (optical)	no
Thin film imprinting	easy, can also be done on particles	not usable for small molecules	sensing (optical)	no
Polymer microbands	optimal for coupling with surface plasmon resonace (SPR)	structures more fragile than a film on a surface or bulk material	sensing (SPR)	no
Sol-gel template synthesis	3D network is formed combines advantages of bulk-imprinting with advantages of surface imprinting	harsh treatment with HF not feasible for all polymers and templates	separation	no
Sub-structure imprinting	large non symmetric targets, has an epitope (substructure of the analyte that is used as template) similar a natural antibody	it is often not easy to find a feasible epitope	separation	no
Artificial template stamps	large templates which are difficult to handle and not always available in reproducible quality (cells), stamps are reusable	more difficult, requires several imprinting steps.	sensing (optical, mass sensitive)	very simple architectures (inlet chamber and outlet)
Phase boundary imprinting	for small molecules with polar and apolar groups	limited to small bipolar molecules as templates	separation	no
Antibody replicae	large non symmetric targets, has an epitope (substructure of the analyte that is used as template) similar a natural antibody, stamps reusable	more difficult, requires several imprinting steps, natural antibody is required	sensing (mass sensitive)	very simple architectures (inlet chamber and outlet)

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