Title: Molecularly Imprinted Polymers for Cell Recognition

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Abstract:

Since their conception fifty years ago, molecularly imprinted polymers (MIPs) have seen extensive development both in terms of synthetic routes and applications. Perhaps the most challenging target for molecular imprinting are cells. Though early work was based almost entirely around microprinting methods, recent developments shifted towards epitope imprinting to generate MIP nanoparticles. Simultaneously, the development of techniques such as solid phase MIP synthesis have solved many historic issues of MIP production. This review briefly describes various approached used in cell imprinting with a focus on applications of the created materials in imaging, drug delivery, diagnostics and tissue engineering.

1 The drive to recognize and interact with cells

The living functions in organisms arise from specific cell cross-talks which ultimately rely on macromolecular interplays. Dysfunctional molecular interactions at cellular level are often responsible for cell malfunctioning and the consequent onset of a disease [1]. Biomimetic tools that explore molecular interactions have been used for cell imaging, improving drug delivery, tissue engineering and diagnostics [2]. Design of such tools however is not easy due to complex nature of molecular interactions and lack of affordable generic protocols suitable for the development of supramolecular receptors with ordered system of functional groups that mimic natural molecules. The present review focuses on molecularly imprinted polymers (MIPs), as an alternative to biomimetics and biosimilars. We shall discuss here historical foundations and the recent technology advancements for the preparation of MIPs suitable for cell recognition, the frontier applications to cells and in cell biology, highlighting the achievements, the current limitations and the future trends.

2 Molecularly imprinted polymer: the concept.

MIPs are recognition materials prepared by a template-assisted synthesis [3, 4]. The imprinting process, schematized in Figure 1, consists in the polymerization of the monomers and the cross-linker in the presence of a target molecule that acts as a template. Driven by thermodynamics the template interacts with the monomers forming a pre-polymerization complex, stabilized by molecular interactions, that is later “frozen” by polymerization. As a result, molecular impressions of the template are stamped into the formed polymeric material creating specific binding sites capable of recognition of template and its analogues.

Insert Figure 1.

MIPs are robust, and possess affinity and selectivity for the template comparable to that of natural receptors. Small molecules, peptides, nucleic acids, proteins, cells and viruses have been imprinted, confirming the versatility of the MIP approach [5-7]. With the recent progress in development of MIP nanoparticles (nanoMIPs) [8,9], this technology became suitable for frontier applications in the domain of life science and medicine.

3 The development of whole-cell imprinted MIPs.

Whilst the molecular imprinting of small molecules, peptides and even proteins is well-established counting many examples in literature, patents and even commercial products (e.g. SupelMIP® by Sigma-Aldrich, www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/supelmip.html), the holy grail of MIP technology is the imprinting of complex template structures such as whole cells. These MIPs would have a broad range of applications, including use in environmental and clinical assays, targeted therapeutics and imaging, cell separation and tissue culturing. Over the past two decades much effort has been put towards the successful achievement of this goal with successful examples such as cell-imprinting using stamping of the whole cells [7]. The proof of concept was performed by Vulfson and colleagues in 1996 [10,11]. It involved cell lithography for preparing polymers with affinity for bacteria. Since then, micro-contact stamping has seen extensively development, along with alternative strategies such as the preparation of MIPs from self-assembling silica nanoparticles, and the use of cell epitopes in place of whole cells.

2 Micro-contact stamping

Micro-contact stamping, otherwise referred to as microprinting, is the most frequently explored technique to generating MIPs using whole cells as templates [7]. It involves deposition of the

target cells on a flat solid support layer and then topping them with monomers or a soft polymer, such as pre-polymerised polyurethane (PU). The polymer is then cured, sandwiching cells between the support layer and the formed polymer (Figure 2). Whole-cell MIPs exhibited shape, size and functional selectivity for the cell templates [11, 12]. Key example of cell-recognizing MIPs is found in using imprinted poly-vinylpyrrolydone (PVP) for the selection of erythrocyte subtypes [13]. Developed MIPs have shown outstanding selectivity towards erythrocyte subgroups A1 and A2, despite both types exposing the same antigens on the surface, differing solely in the density of glycolipids on the respective cells. These results permitted to conclude that in contrast to antibodies, whose recognition ability relies on the presence of a defined antigen on the cell surface, MIPs instead are able to interact with the entire cell surface showing sensitivity to quantitative differences in surface chemistry [14]. A broad range of targets and materials have already been imprinted using this approach, including bacteria, mammalian cells and yeast with key examples reported in Table 1 [15, 16].

Insert Figure 2.

Microcontact stamping was successfully exploited for cell recognition, cell selection and sensing purposes (Table 1). Microcontact stamping provided also surfaces suitable for controlled cell growth. Interestingly the comparison of cells grown on flat and imprinted surfaces showed that MIP surfaces promoted higher expression levels in adhesion proteins, confirming the MIP substrate elicits biochemical response in the growing cell [17-19].

Microcontact printing can be performed using both, organic and inorganic polymers. Commercial ready-to-use organic polymers such as polystyrene (PS), polyacrylate, polyvinylpyrrolidone (PVP), polyacrylamide, PU and Epon1002F were used to generate imprinted surfaces for *Bacillus cereus* [20]. The best performance was achieved with PU and Epon1002F. This is an important result, as it allows replacing self-synthesised polymers with

well characterised commercial materials, with the outcome to enable use of this technique by non-specialists in polymer synthesis.

More recently, Dulay and colleagues assessed the ability of a polydimethylsiloxane (PDMS) layer created by micro-contact stamping of bacterial cells to distinguish between living and inactivated cells [14]. These polymers showed significantly higher affinity for inactivated cells prepared using the same technique as was used for polymer imprinting [14,17]. But, due to the synthetic limitations of PDMS, authors moved to organosiloxane polymers made by sol–gel chemistry. The broad selection of available silanes allowed to benefit from a plethora of functionalities whilst retaining optical transparency and mechanical resistance [21]. Although the mechanical stability of inorganic materials is usually higher than their organic counterparts, it is important to consider the mechanical stress which the cells undergo during the stamping procedure, might be damaging more delicate targets, such as human cells.

A superior strategy in cell imprinting lies in generating a polymer layer using cells as a template, and then using this as a mould to generate a second polymer layer. This layer can then act as a "master mould" that can be used to use as a template instead of bacteria. This may improve the ease, reproducibility and safety of making imprinted polymer layers, as no living bacteria are needed after the first imprint [18].

Imprinting of sections of cell membranes - Cell recognition can also be achieved by imprinting sections of cell membrane. It is known that charged proteins exposed on the cell membrane have a key role in adhesion, proliferation, interaction and localization of the cell. Bao and colleagues reported a novel method to produce bacteria-imprinted polymers by exploiting the bacterial surface-charge heterogeneity using charged methacrylate ethyl trimethyl ammonium chloride and 3-dimethyl (methacryloyloxyethyl) ammonium propane sulfonate fixed in

polymer network by surface-initiated atom transfer radical polymerisation (ATRP) [22]. The charge distribution on the imprinted cavities complemented the charge distribution of the bacteria surface, allowing for stronger electrostatic-mediated recognition. Borovicka and colleagues fabricated "colloid antibodies" by coating microbial cells with a silica shell that was subsequently fragmented to create complementary shell fragments [23,24]. Authors demonstrated that the recognition is mediated by the size and shape of the imprints but also electrostatic interactions and the surface charge of the microbial cells.

A sophisticated whole-cell imprinting approach was developed by Alexander and colleagues, who exploited bacterial redox systems to induce copper-mediated ATRP of cationic 2-(methacryloyloxy)-N,N,N-trimethylethanaminium chloride and zwitterionic 2-(N-3-sulphopropyl-N, N-dimethyl ammonium) ethyl methacrylate at the surface of *E. coli* and *P. aeruginosa* cells, thus generating polymers directly *in situ* at the surface of the microorganisms [25]. The cells also doubled as a solid-phase to isolate high-affinity from low-affinity polymer products, similar to the technique pioneered by Piletsky and colleagues [8, 26–27]. A "click" chemistry reaction was used to attach fluorescent reporters on the polymers, to simultaneously bind and visualise the pathogens (Figure 3).

Insert Figure 3.

3. Epitope imprinting

The whole-cell imprinting approach produce a shape-recognition material, that might not be optimal when the goal to achieve is the recognition of a specific type of human cell, such as the ability to differentiate or locate cancer cells in a tissue or in an organ. Given the high plasticity of mammalian cells, the sole shape recognition is not always offering the level of

discrimination required for success. Moreover, MIPs intended for cell recognition *in vivo* should have the size of natural macromolecules (nanometers), so to be suitable for circulation within vessels, within the lymphatic system and for the intracellular space diffusion, whereas the imprint of a whole cell inevitably results in micrometer size. For all these reasons, alternative imprinting approaches had to be proposed. In the case, attention should focus on particular molecular components present on the cell surface such as proteins, lipids, saccharides and their derivatives.

Saccharides - When targeting the glycomoieties typically present on the cell surface, the imprinting process was performed by stamping portions of glyco-architecture, in a process analogous to the epitope imprinting [28]. Monosaccharides such as sialic acid and mannose have been used most frequently as representative targets [29–33]. In another example Kinoshita and colleagues have created core-shell imprinted gold nanoparticles bearing thermo-responsive *N*-isopropylacrylamide (NIPAm) imprinted with *E. Coli* O157 lipopolysaccaride [34]. The target bacteria bound to the nanoparticles with excellent selectivity (>15) against other types of *E. coli*. NanoMIPs, prepared using a solid-phase approach with immobilised trisaccharide of the blood type B-antigen, were able to distinguish between erythrocytes of different blood types [35]. Similarly, MIPs made for glycans were able to differentiate between different types of cancer cells [36].

Proteins - Proteins of cell membranes are obvious targets for cell imprinting. Imprinting of entire proteins or corresponding peptide epitopes is a well-established technique [28,37]. For example, the whole protein was imprinted in the preparation of a fibronectin (FN)-imprinted polysiloxane membrane, made using silanes as functional monomers and calcium alginate hydrogel membrane as the substrate. The FN-imprinted polysiloxane membrane provided improved cell adhesion and favourable cell growth for mouse fibroblasts (L929) [38]. Unfortunately, most membrane proteins are prohibitively expensive and for this reason are

rarely used as templates in molecular imprinting. A much more exploitable concept is the imprinting of a small peptide sequence - epitope that is characteristic for a particular protein and exposed on its surface. Finding such epitopes due to extreme complexity of the proteome, is a difficult task. A short summary provides an outline of the strategy currently used in the rational selection of epitopes for molecular imprinting (Box I and II).

A recent example of epitope imprinting describes the use of the peptide arginylglycylaspartic acid (RGD) with well-known cell-adhesive function. An RGD-imprinted surface was successfully designed to anchor RGD and consequently cells [39]. In another example, the progastrin-releasing peptide was used as template to prepare molecular imprinting sites of zeolite-chitosan-TiO_2 microspheres for dot-blot immunoassays with multiple native antigens for rapid serodiagnosis of human lung cancer [40].

An epitope imprinting approach was exploited to generate amoxicillin delivery systems aimed at *Helicobacter pylori* [41,42]. In this system, the primary template was a modified epitope sequence of Lpp20, a membrane lipoprotein specific to *H. pylori*. The modification exploited the conjugation of a lipophilic chain to guarantee the presence of the template at the surface of the nanoparticles during the inverse microemulsion polymerisation method.

Similarly, cancer cells overexpressing epidermal growth factor receptor (EGFR) have been successfully targeted by imprinting NIPAm-based MIPs with an epitope of EGFR [43]. The resultant MIPs were able to differentiate between cells with differing levels of EGFR expression. These MIPs were prepared by first immobilising the template peptide on glass beads prior to polymerisation. Using this solid-phase approach, it was possible to remove low-affinity polymers and monomers with a low-temperature washing step, and easily separate high affinity MIPs from template molecules (Figure 4).

Insert Figure 4.

To conclude – two approaches continue to dominate cell imprinting – microprinting and epitope imprinting. A range of organic and inorganic polymers were employed to imprint bacterial and mammal cells successfully. While microprinting is perfectly suited for producing cell-specific surfaces, epitope imprinting can be used to produce nanoMIPs capable of addressing cell targets *in vivo*.

4 Applications

Cell concentration and separation– Most successful examples of the use of cell-imprinted MIPs in separation are related to capturing and separating bacteria. The possibility of separating different strains of bacteria by electrophoresis was demonstrated in 2006 [44]. Imprinted gel granules were synthesized from acrylamide and N,N'-methylenebisacrylamide in the presence of *E. coli* as a template. The electrophoretic migration of the gels was affected by the presence of the template, showing good discrimination between *E. coli* MRE-600, and *E. coli* BL21. Specific capturing of *Deinococcus radiodurans*, *E. coli*, *Sphaerotilus natans* and *Bacillus subtilis* by imprinted films was achieved by Cohen and colleagues [45]. Surface imprinted PU films were used for selective capturing of methanotrophs from paddy soil [46]. The use of virulent bacteria during the production of the cell-imprinted polymer thin films and the cell-capture process bears an obvious and persistent risk of infection, which could be a major hurdle for the implementation of this method. A successful attempt was made to remove the potential biohazard risk by using inactivated bacteria, when poly(dimethylsiloxane) films were imprinted with inactivated *M. smegmatis* [14].

Apart from bacteria, MIPs were used for spore capture and concentration within an integrated biological detection system for *Bacillus anthracis* [47]. The binding assay showed strong spore-binding capability and a robust imprinting effect that accounted for 25% additional binding over non-imprinted controls. This process was rapid, taking only 30 minutes.

In a different example, cell adhesion was improved by the imprinting of FN and cell-adhesive peptide Arg-Gly-Asp-Ser [48,49]. Template-enhanced adhesion of fibroblasts, MC3T3-E1, and L929 cells was observed after 24 hours (Figure 5).

Insert Figure 5.

Tissue engineering - Numerous studies have previously indicated that stem cell fate is regulated by a combination of intrinsic (e.g., specific transcription factors) and extrinsic mechanisms invoked by the local microenvironment [50,51]. Stem cells sense different mechanical cues that guide rearrangement of adhesion proteins and the cytoskeleton, which in due course affect intracellular processes [52]. The predictive design of tissue scaffolds is difficult due to limited understanding of microenvironment patterns that guide cell differentiation. Molecular imprinting may offer a solution to this problem.

In one study, tissue-specific substrates were prepared by imprinting mature and dedifferentiated chondrocytes. Rabbit adipose derived mesenchymal stem cells seeded on cell-imprinted substrates were driven to adopt the specific characteristics of the cell types used as templates for cell imprinting [53]. Besides residual cellular fragments presented on the template surface, the imprinted topography of the templates played a role in stem cells differentiation. In a similar study, mature human keratinocyte cells were used in the imprinting of PDMS. Human adipose-derived stem cells (ADSCs) seeded on cell imprinted substrates were driven

to adopt the specific shape and characteristics of keratinocytes [54]. The observed morphology of the ADSCs grown on the keratinocyte casts was noticeably different from that of stem cells cultivated on the stem cell imprinted substrates. Authors speculate that mechanical deformation caused by cell-imprint interaction may induce transduction by affecting the chromatin arrangement inside the stem cell nucleus. ADSCs, semifibroblasts and tenocytes were differentiated, redifferentiated and transdifferentiated, respectively, into chondrocytes after being cultured for 2 weeks onto chondrocyte-imprinted PDMS substrates [55]. A similar effect was also observed when ADSCs were cultured on keratinocytes-imprinted substrates [54] or on chondrocytes or fibroblasts-imprinted substrates [53]. Although the aim of these works was to develop an efficient and cheap approach for regenerative medicine and wound healing, it is likely that MIP-guided cell differentiation can be used on a large scale for growing more complex tissues, and potentially whole organs.

The advantage of using molecular imprinting in guiding cell differentiation lies in the relatively simple procedure for creating topographical cell fingerprints for directed tissue growth. In clinical usage, an opportunity exists for the use of MIPs in the enrichment of cell populations, for example, separation of leukocytes by aphaeresis or enrichment of haematopoietic stem cells, and aiding repopulation of the immune system, for example, in multiple sclerosis patients who have undergone immunoablation treatment [56–58]. In these applications, MIPs have to compete with antibody-binding methods such as fluorescence-activated and magnetic-activated cell sorting [59]. It should be noted that in most cases the selective recognition of nanoMIPs is at least in line with that of the antibody [60], moreover the possibility to produce fluorescent nanoMIPs and/or core-shell magnetic nanoMIPs is well recorded [61], therefore the MIP technology is sufficiently mature for the challenge.

Drug delivery - There is a current trend in pharmacology represented by the increasing number of FDA-approved nanoparticle formulations, amounting to ~50 in 2017 [62]. Currently, several

types of nanoparticle-based drug carriers are available on the market. They are based on solid dispersion (Gris-PEG, Sandimmune, Intelence etc.), self-emulsifying drug delivery systems (Neoral®, Agenerase, Aptivus etc.) or nanocrystals (NanoCrystal®, Rapamune, Megace® ES) [63]. The polymer architecture of nanoparticles dictates drug loading effectiveness, drug-release rate and biodistribution [64]. Nanoparticles (NPs) smaller than 8 nm are cleared rapidly from the blood stream by the renal system and NPs larger than 200 nm are sequestered by the mononuclear phagocytic system in the liver and spleen [65,66]. NanoMIPs represent an entirely new compound class which can now be deployed to address both extracellular protein targets (as an alternative to biological antibodies), and potentially to currently intractable intracellular proteins [67]. Potentially nanoMIPs can assist with increasing a drug's half-life within the body, increasing drug payload, facilitating targeted drug delivery, improving drug permeability through cell membranes and offering the possibility of oral delivery.

One particularly important subject in NP research is the oral delivery of macromolecules. The main mechanism for NPs transport is adsorptive endocytosis [68]. Summarizing numerous absorption studies, there seems to be an agreement that the optimum size of NPs suitable for drug delivery via oral route is 10-100 nm [69]. The extent of systemic appearance of this type of NP after gastrointestinal absorption has been reported as 10-15% [70]. NanoMIPs, in contrast to antibodies and aptamers, are capable of penetrating cell membranes by endocytosis, and even reaching nuclei [67,71]. The same mechanism is used for oral delivery of drugs assisted by nanoMIPs. In one such example nanoMIPs were made by precipitation polymerization and used for the oral delivery of insulin through a transmucosal oral route (Figure 6) [72].

Insert Figure 6.

Recently, Piletsky and colleagues have compared intravenous and oral delivery of nanoMIPs and their impact on the clearance of nanoparticles through kidney and bile. Interestingly, the nanoMIPs were successfully excreted in both urine and faeces (Figure 7). Oral administration showed an increased level of faecal excretion, in line with other clearance data for NPs through the hepatic route. No particles were detected one week after administration.

Insert Figure 7.

Due to their size and the large number of functional groups available for entrapment/conjugation of drug molecules, nanoMIPs have great potential as drug carriers. Most papers published on this topic describe entrapping drug molecules in the bulk of polymers. The delivered/released quantity of drugs varies from 0.5-180 μg/mg of nanoparticles, depending on the drug type and synthetic protocol used in the nanoMIPs preparation [71,73–75]. The imprinting process ensures a 2-3 time increase in the quantity of entrapped drugs as compared to non-imprinted particles [75]. The half-time drug release in these experiments varied from 2-20 hours based on the drugs polarity and its affinity to the polymer carrier. This is significantly shorter than the circulation time of synthetic particles demonstrated in clinical trials, which is under 12 days [76]. The average results obtained for nanoMIPs circulation in the body are 7 days which is an improvement as compared with circulation of small drug molecules [77].

Targeted drug delivery originates from MIPs ability to interact specifically with cell receptors. Most therapeutic agents (90% or more) will inevitably be concentrated in the reticuloendothelial organs such as the liver and spleen due to clearance by mononuclear phagocytes [78]. Active targeting is being explored as a method to achieve spatial localization

of drugs in diseased organs while eliminating off-target adverse effects in normal tissue. The ligands used to modify nanoparticles include antibodies, their fragments, proteins, peptides and aptamers [79]. NanoMIPs can also be decorated with specific ligands to achieve a targeting effect. Thus nanoMIPs containing folic acid showed a greater amount of intracellular uptake in folate receptor-positive cancer cells (MDA-MB-231 cells) in comparison with the non-folate nanoparticles and free paclitaxel, with half maximal inhibitory concentrations (IC50) of 4.9 ± 0.9, 7.4 ± 0.5 and 32.8 ± 3.8 nM, respectively [74]. Sialic acid-coated nanoMIPs with S-nitrosothiols were used for nitric oxide-release as chemotherapy agents [80]. Specific targeting of cancer cells was achieved by nanoMIPs imprinted with EGFR epitope [81]. In a similar way, senescent cells were targeted by dasatinib-bearing nanoMIPs imprinted with epitope of senescent markers B2M [82]. NanoMIPs loaded with drugs were able to specifically kill senescent cells, showing significantly greater level of binding within organs of older animals. Targeted delivery can be achieved using external factors such as magnetic field [77]. In this work, nanoMIPs with magnetic cores were prepared via co-precipitation polymerization in the presence of olanzapine as a template, and used for magnetic field-guided drug delivery of olanzapine to rat brains.

So far, most examples related to drug delivery describe drug loading through the binding to imprinted sites in the polymer matrix. This may not be the most desirable way, as the produced nanoparticles typically release their drug cargo too quickly, within 4-7 hours. The covalent attachment of drugs through cleavable linkers would be preferred. This approach follows similar trends with the conjugation of drugs with antibodies [83–85].

In a rare example, nanoMIPs themselves were used as a drug [86]. In this work, nanoMIPs, imprinted with the quorum signalling peptide SNGLDVGKAD, prevented the translocation of pneumococci from lungs to blood and improved the survival rate of infected mice.

In a very interesting example of a theranostic application, amphiphilic lipopolysaccharides, derived from *Pseudomonas aeruginosa,* were used as a template in the preparation of nanoMIPs by the inverse emulsion method [87]. Fluorescent nanoMIPs, labelled with IR-783, showed selective recognition of target bacteria in keratitis and meningitis models (Figure 8). *P. aeruginosa*-targeted nanoMIPs encapsulated with a photosensitizer (methylene blue) were used also for *in vitro* photodynamic therapy. Compared to non-imprinted NPs, an almost two order of magnitude difference in cell counting was noted, indicating the higher efficacy of nanoMIPs against bacteria after laser exposure. The nanoMIPs formulation was very stable, showing similar performance after six months storage.

Insert Figure 8

There are a number of issues to be resolved and questions to answer before practical application of nanoMIPs in drug delivery can be considered. Among these are:

- How safe are nanoMIPs?
- Should nanoMIPs be biodegradable?
- How do nanoMIPs properties influence their biodistribution and clearance?
- What is the best way to conjugate drugs to nanoMIPs?
- How can nanoMIPs be produced on a large scale and in accordance with quality-control guidelines such as Good Laboratory Practice?

So far nanoMIPs were tested mainly *in vitro*. Cell viability tests (NIH-3T3) using human embryonic kidney (HEK293) suggested that the developed material did not present any detectable cytotoxicity at <100 μg mL^{-1} nanoMIP concentrations [71,77,88]. Limited *in vivo* tests also showed that nanoMIPs had no visible impact on the hepatocytes and the structure of the kidney. No sign of toxicity was found, no body weight changes or clinical symptoms (i.e. diarrhea, fever) were found 14 days after the experiment [72].

The answer to the question of whether MIP formulations should be biodegradable is not straightforward. Potentially, biodegradable nanoMIPs might have simplified clearance process. However, the byproducts of polymer degradation might be more toxic than the nanoparticles. Monomers such as methacrylic acid, methyl methacrylate and ethylene glycol dimethacrylate are biocompatible and non-toxic [89,90]. The same is not true for some other monomers such as acrylamide [91]. The examples shown in this paper, as well as many other relevant examples from literature, imply that non-degradable polymers might be safer for use in medical devices and drug delivery [92,93]. Besides the residual monomers, other toxic impurities can be present in a plastic product, including oligomers, low molecular weight polymer fragments, catalyst remnants and surfactants [94]. It is therefore essential to ensure the complete removal of non-polymerised components from MIPs formulation.

Besides the complications in the experimental design of nanoparticles, there are multiple challenges in the manufacturing, regulation, and approval of nanoparticles for clinical use. The majority of protocols describing the synthesis of nanoMIPs cannot be easily adapted to large-scale manufacturing. A major breakthrough was therefore the combination of nanoMIP synthesis with an affinity separation step into a single procedure, using an immobilised template for MIP formation [95]. The resulting process allowed the construction of the first

prototype automatic nanoMIP synthesiser [8]. The process of MIP synthesis using the automated reactor is shown schematically in Figure 3. This approach represents the state-of-the-art in nanoMIP synthesis: not only can soluble particles with a well-defined size (30-100 nm) and a narrow size distribution be produced in a matter of one hour, but they possess nanomolar dissociation constants for their respective targets, there is no residual template present and the immobilised template can be re-used. This automated process overcomes all of the historic drawbacks of bulk MIPs, and raises the exciting possibility of deploying nanoMIPs in therapeutic applications. Despite this success, bringing manufacturing protocol to compliance with Good Laboratory Practice and Good Manufacturing Practice, as well as passing FDA Investigational New Drug trials, will be challenging.

Imaging - In many ways, drug delivery and imaging are connected. Both applications should address safety issues and the issue of targeted delivery to specific cells and organs. For imaging applications, nanoMIPs should have fluorescent, magnetic or positron-emitting tags. So far only fluorescent labels were used in combination with nanoMIPs, including pyrene, fluorescein and rhodamine derivatives [29,96], quantum dots [97] and carbon dots [33]. In one study, two differently colored nanoMIPs were imprinted with D-glucuronic acid and N-acetylneuraminic acid. Both MIPs were found to be highly selective towards their target monosaccharides, as no cross-reactivity was observed with other sugars present on the cell surface [32]. Fluorescently-labeled nanoMIPs were used for multiplex imaging of fixed and living human keratinocytes, to localize hyaluronan and sialylation sites (Figure 9). Monodispersed 400 nm sized particles bound their targets located in the extracellular region. In contrast, 125 nm particles were able to stain the intracellular and pericellular regions as well.

Insert Figure 9.

In a similar work, fluorescent nanoMIPs were imprinted with sialic acid and used for the imaging of cancer cells [31]. These nanoMIPs exhibited selective staining for DU 145 cancer cells and did not enter HeLa cells even after long incubation times. In a previously mentioned work fluorescent nanoMIPs were imprinted with a linear epitope of EGFR and used in confocal microscopy [81]. A strong fluorescent signal was detected from the MIPs in MDA-MB-468 cells over-expressing EGFR, whereas almost no signal was observed in MDA-231 or SKBR3 cells. These results show that nanoMIPs can potentially be used as a cell imaging tool against difficult targets such as membrane proteins.

Very few papers actually describe the use of nanoMIPs *in vivo*. In a rare example, nanoMIPs were imprinted with human VEGF and coupled with quantum dots (QDs) for cancer imaging [98]. The composite nanoparticles exhibited specific binding toward human melanoma cell xenografts, overexpressing hVEGF, in zebrafish embryos. In another work, fluorescein-labelled nanoMIPs, imprinted with senescence membrane marker B2M, were used for the selective targeting of senescent cells [82]. NanoMIPs were able to detect senescent cells in aged mice without eliciting any apparent toxicity (Figure 10).

Overall nanoMIPs are promising materials which can be considered for advancing imaging, in particular when antibodies are less desirable due to their immunogenicity or long production time. Moreover, one of the main limitations associated to the state of art in imaging techniques is the detection limits of fluorescent antibodies, currently set to antigens expressed on the target cell more than 1000 times/cell, whereas key inflammatory and cancer markers, such as interleukins, are often present in just few hundreds of copies on the cell membrane, therefore escaping the current detection limits [99]. Yet the nanoMIPs, given their larger dimensions (10-400 nm), contain significant number of fluorophores *per* nanoparticle, surpassing the

aforementioned limitations, without need for secondary bindings or amplifying catalytic events. Despite holding great promises, nanoMIP-based bioimaging is still in its infancy and more work is required before it can be considered for practical applications. The research focus in this area should shift from fluorescence to MRI and PET imaging. It is critically important that safety issues are addressed and manufacturing problems solved for this technology to advance.

Insert Figure 10.

Sensing - In diagnostics cell-imprinted MIPs are used almost entirely for the detection of microorganisms. Currently, laboratory-based biochemical methods for microorganism analysis are performed by means of standard antibody assays and polymerase chain reaction [100]. Cell culture still remains a standard technique for identifying bacterial species; however, it usually requires 24–48 hours, depending on the growth speed of the target bacterium [101]. These methods generally require a high level of technical skill, and complex sample preparation. There is therefore an industry-driven requirement to design novel, rapid and reliable analytical detection methods for microorganisms.

A QCM sensor platform was developed for the detection of *Escherichia coli*, *Bacillus Cereus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* [101-103]. Imprinted PPy and PU were generated directly on sensor surface. The QCM device allowed detection of microorganisms at concentrations of 1.4×10^8 cells/mL within 2-3 minutes. Overall, the QCM sensors have shown similar sensitivity to SPR, afforded 10 regeneration cycles and worked for at least 3 months [104]. In an example of practical application, PU-based QCM sensor was used to follow growth processes of *Escherichia coli* and *Saccharomyces cerevisiae* in a

bioreactor [105,106]. The sensor was able to identify different stages of the cell cycle, with a LoD of 1.6×10^8 cells/mL.

Electrochemical sensors based on conducting MIP materials, such as electropolymerised 3-aminophenol and 3-aminophenylboronic acid, were used for the detection of *Staphylococcus aureus* and *Staphylococcus epidermidis* [107,108]. Cyclic voltammetry and electrochemical impedance spectroscopy in the presence of redox probes were explored for specific detection of the target bacteria at 10^3–10^7 cfu/mL concentrations. Imprinted PPy/poly(3-methylthiophene) was used in impedance detection of *Bacillus subtilis* endospores at 10^4-10^7 cfu/mL concentration [109]. Surface-imprinted polydopamine was used for yeast sensing, allowing a LoD of 50 cfu/mL with excellent selectivity against smaller *Vibrio alginolyticus, Escherichia coli* and *Staphylococcus aureus* [110]. A microprinting method was used to develop a capacitive sensor for *E. Coli* with a LoD of 70 cfu/mL [111]. This sensor was able to detect the target in river water. Electropolymerised 3-aminophenylboronic acid was used to create a sensor for *Staphylococcus epidermidis* using electrochemical impedance spectroscopy [108]. The same type of transducer was exploited by Qi and colleagues to create an imprinted sensor for sulfate-reducing bacteria on chitosan doped with reduced graphene sheets. The sensor performed in the range of 1×10^4 - 1×10^8 cfu/mL [112]. NanoMIPs were synthesized using a sol–gel method with cerium dioxide nanoparticles in the presence of *Staphylococcus aureus* on the surface of an indium tin oxide [113]. This assay was used to detect *Staphylococcus aureus* at 10^4-10^5 cfu/mL concentrations.

An electrochemiluminescence biosensor was developed for the quantitative detection of *Escherichia coli* O157:H7 based on a polydopamine-imprinted polymer [114]. However, in this work MIPs were only used for capturing of bacteria, and the electrochemiluminiscent

detection was achieved using a polyclonal antibody labeled with nitrogen-doped graphene quantum dots. The LoD was very low, at 8 cfu/mL.

Thermal wave analysis was used for a bacterial identification assay involving PU imprinted with nine different bacterial targets [115]. The limit of selectivity of the sensor was tested in a mixed bacterial solution in the presence of a 99-fold excess of competitor species. This platform was able to detect bacteria at 3×10^4 cfu/mL in spiked urine.

In a rare example of a non-bacteria imprinting, the microprinting approach has been exploited to produce sensors capable of detecting breast-cancer cells (MCF-7 or ZR-75-1 cells), immortalised T-lymphocytes associated with leukaemia (Jurkat cells) and healthy peripheral blood mononuclear cells [116–118].

In most of these examples, imprinted films were prepared by stamp imprinting or by electropolymerisation. The main problem of these approaches lies in their poor reproducibility and inefficiency in mass manufacturing of sensor devices, due to the use of live bacteria as templates. There is also danger in using pathogenic bacteria as a template in sensor production. The solution to these problems was found in anti-idiotypic imprinting using PDMS master stamps with "plastic copies" of natural cells [119]. Sensitive layers created this way were capable of the differentiation between *Saccharomyces cerevisiae* and *Saccharomyces bayanus* and detect erythrocytes in ABO blood group typing [45]. In addition to the advantage of improved reproducibility and standardization, such layers on mass-sensitive devices featured the same selectivity and sensitivity as MIPs generated using native cells.

5 Conclusion

Molecular imprinting represents the most generic, versatile, scalable and cost-effective approach to the creation of synthetic molecular receptors for small molecules and cells to date. The approaches reported so far span from whole cell imprinting to targeting specific and distinctive cell-surface components and the many recent developments in the synthesis of MIPs, such as the use of a solid phase approach and contact printing permit, for the first time, a reliable supply of soluble synthetic nanoparticles and polymer coatings with pre-determined molecular recognition properties, sub-nanomolar affinities, defined size and surface chemistry available for life science applications, drug delivery, imaging and diagnostics. Indeed targeting specific cells, such as human cancer cells, or pathogenic bacteria, by utilizing nanoMIPs would contribute to revolutionize clinical practice enabling personalized medicine [33, 40, 98]. Summarized in the Outstanding Questions are many crucial open challenges that should be addressed. Worth mentioning is the challenge of producing nanoMIP architectures suitable to translating MIP-mediated cell-recognition from the passive stage of binding to its defined target, to the active intervention in the cell biology process. To accomplish this important step, the integrated design of MIPs with multi-functions is expected, gathering in a single nanoMIP particle ability to activate or silence biochemical pathways [37,120]. The success in this area will result and in new paradigms for MIP applications both complementing existing therapeutic and diagnostic techniques and opening doors to *in situ* programmed nanomachines for precision medicine interventions and tissue regeneration.

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Legend to Figures

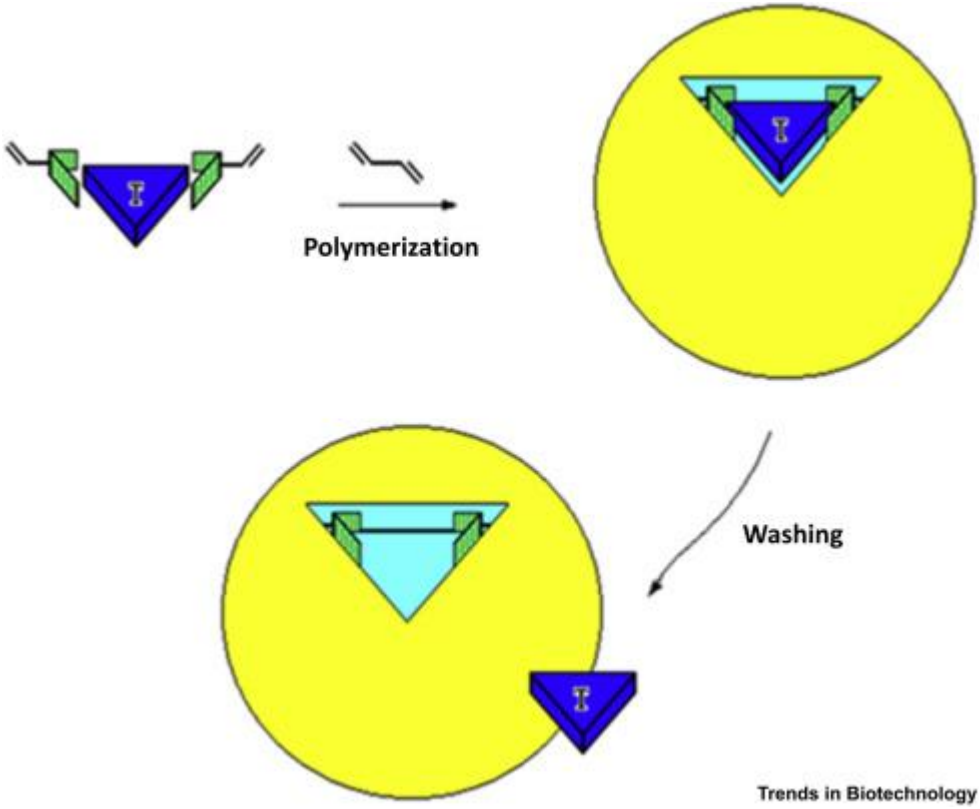
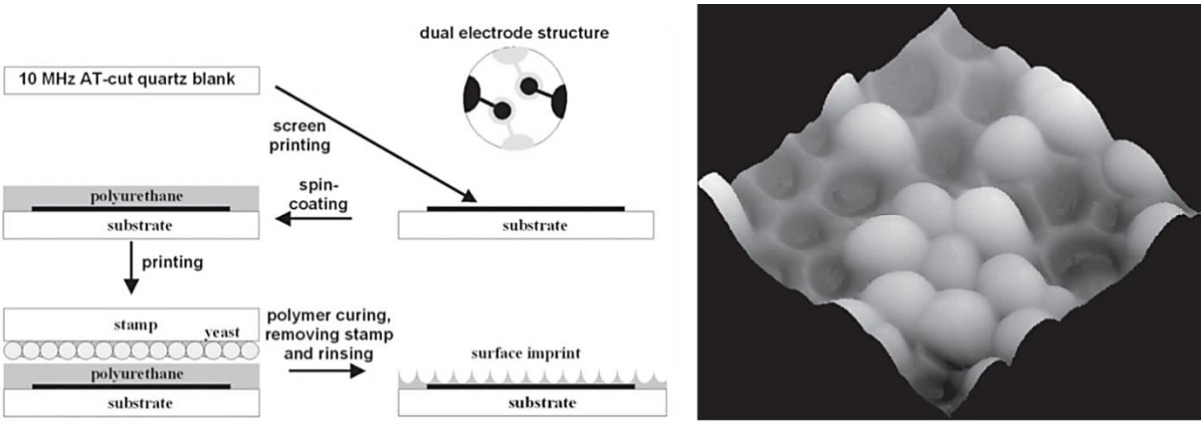


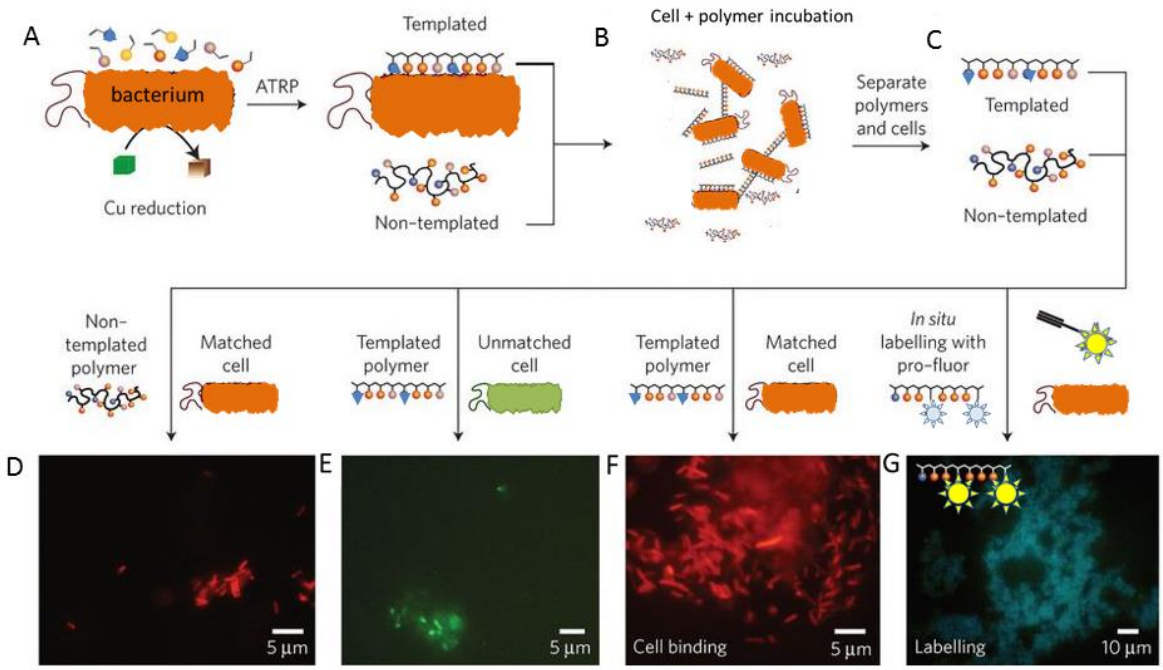
Figure 1. Schematic of the concept of the molecular imprinting. The template (blue triangle) and the functional monomers (green) interact in solution forming a pre-polymerization complex. The addition of the crosslinker and of the initiators yield to the synthesis of the molecularly imprinted polymer (MIP; yellow). At the completion of the process, the template is removed from the MIP by washing steps. The stamped recognition cavities are complementary to the template and ready for its binding.



878

879 Figure 2. Process of microprinting. As an example yeast cells were imprinted with PU matrix
880 [12,121]. As typical for micro-contact stamping, a stamp containing the microorganisms (made
881 by preparing a "sandwich" of cells between glass and Teflon) was pressed into a prepolymer
882 mixture which was then cured, with the resulting cavities exhibited hexagonal, honeycomb-
883 like packing [122]. Left - the surface of quartz crystal is coated with pre-polymerized
884 polyurethane and stamped with immobilised *S. cerevisiae*, creating imprints capable of re-
885 binding of template species. Right - a tapping mode AFM image of the imprinted polyurethane
886 layer after exposition to a *S. cerevisiae* solution. Reprinted with permission from [12].

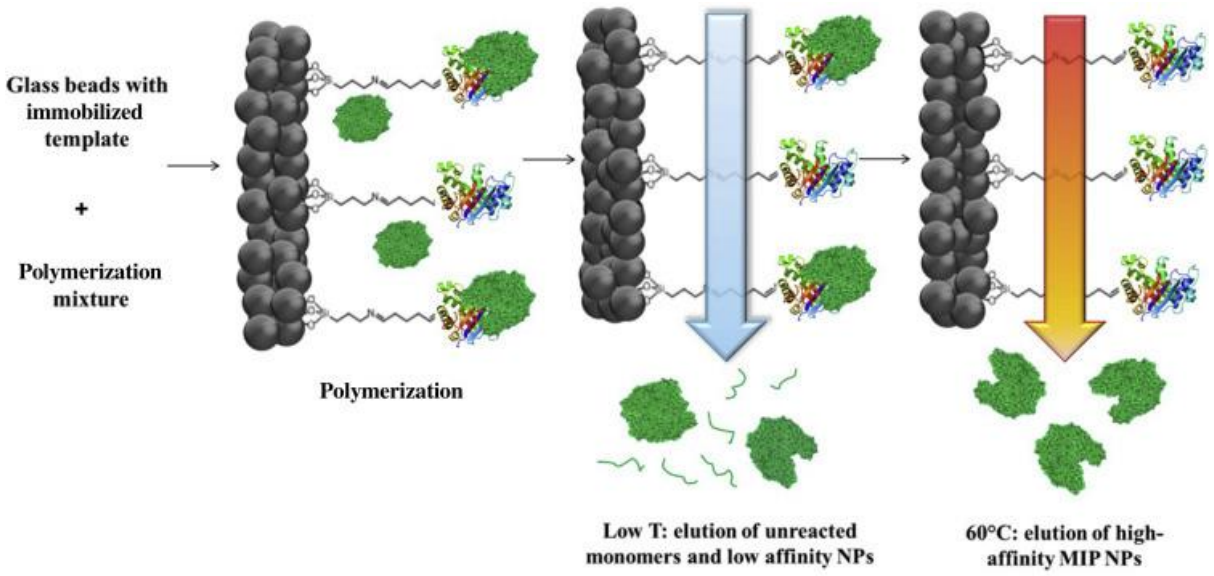
887



888

889 Figure 3. (A,B) Bacteria induce polymerization in monomer suspensions to generate MIPs. (C)
890 Polymers are recovered from the suspensions to generate templated and nontemplated
891 fractions. (D) Incubation of polymers with bacteria results in low binding of cells to
892 nontemplated MIPs or (E) where a polymer templated with one cell type (shown in orange) is
893 incubated with a cell (shown in green) of another type. (F) Addition of a polymer, templated
894 by one cell type, with its own 'matched' cell population results in the formation of large
895 polymer–cell clusters. (G) Labeling the cells *in situ* via pro-fluorescent markers, which react
896 with cell surface-bound polymers containing 'clickable' residues. Adapted, with permission,
897 from [25].

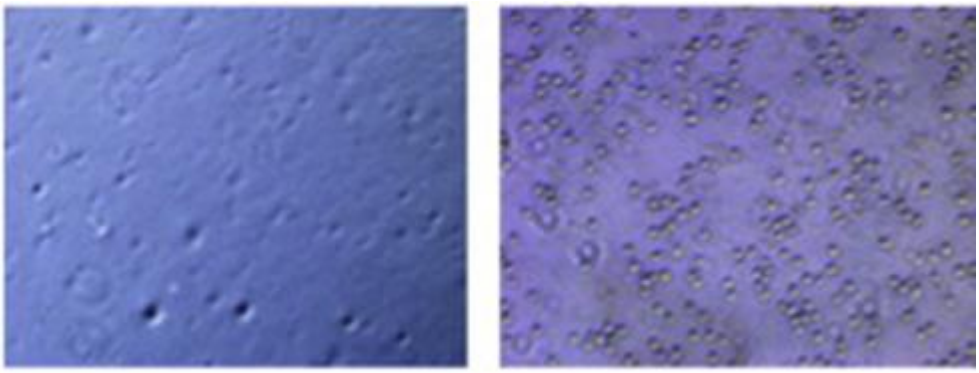
898



899

900 Figure 4. Schematic Representation of the Automated Synthesis of Nanoscale Molecularly
901 Imprinted Polymers (NanoMIPs) Using an Immobilized Template (Melamine) [8].
902 Abbreviation: NP, nanoparticle.

903



904

905 Figure 5. L929 cell adhesion on BSA (left) and FN-imprinted (right) substrates. Reprinted
906 with permission from [48].

907

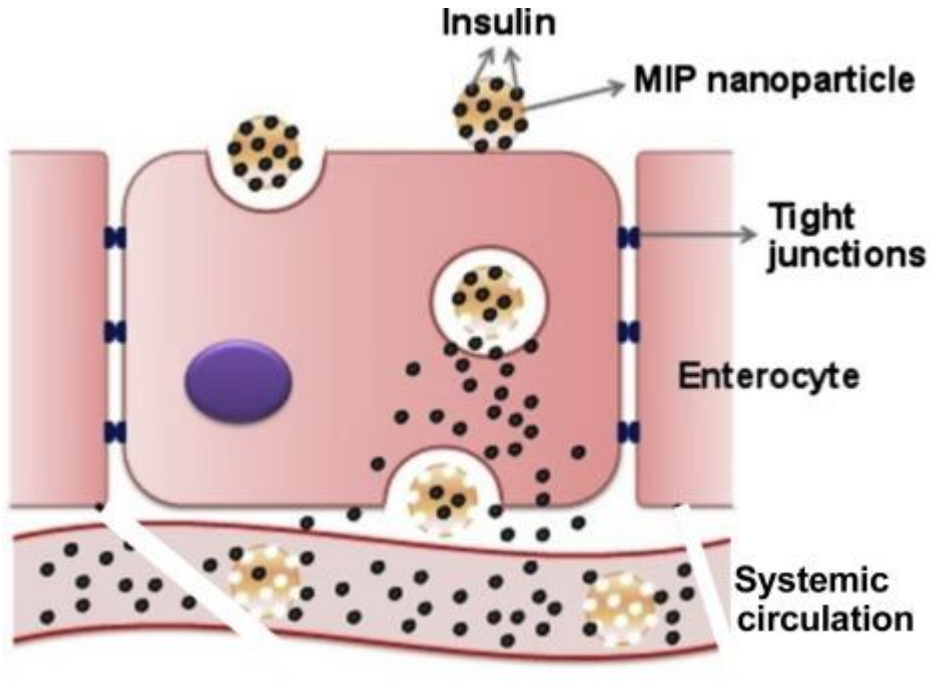
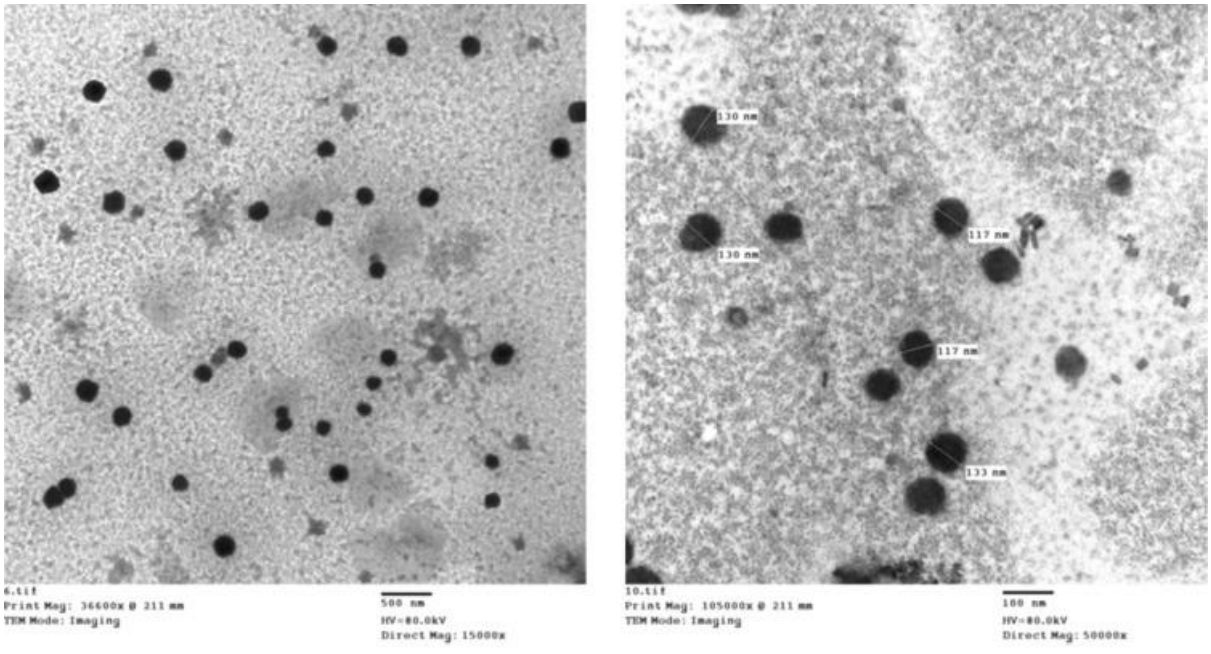
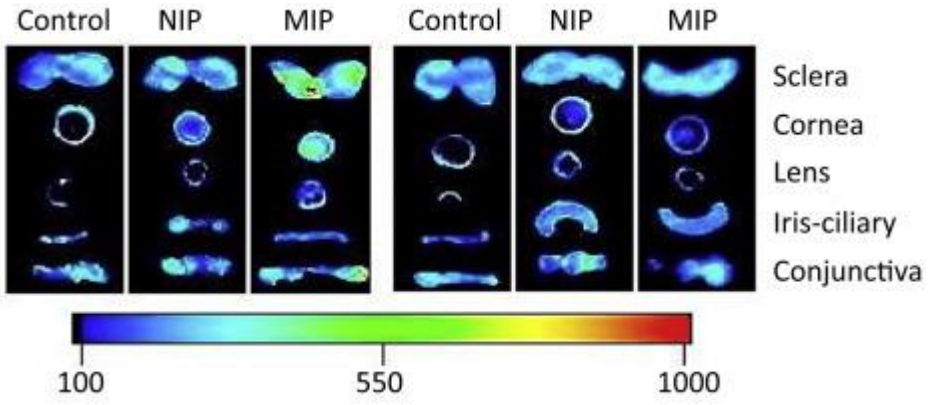


Figure 6. Schematic of the Transport of Insulin-Loaded Molecularly Imprinted Polymer (MIP) Nanoparticles across Intestinal Epithelial Cells Following Oral Administration and Insulin Release by Endocytosis and Transcytosis through Enterocytes. Reprinted with permission from [72].

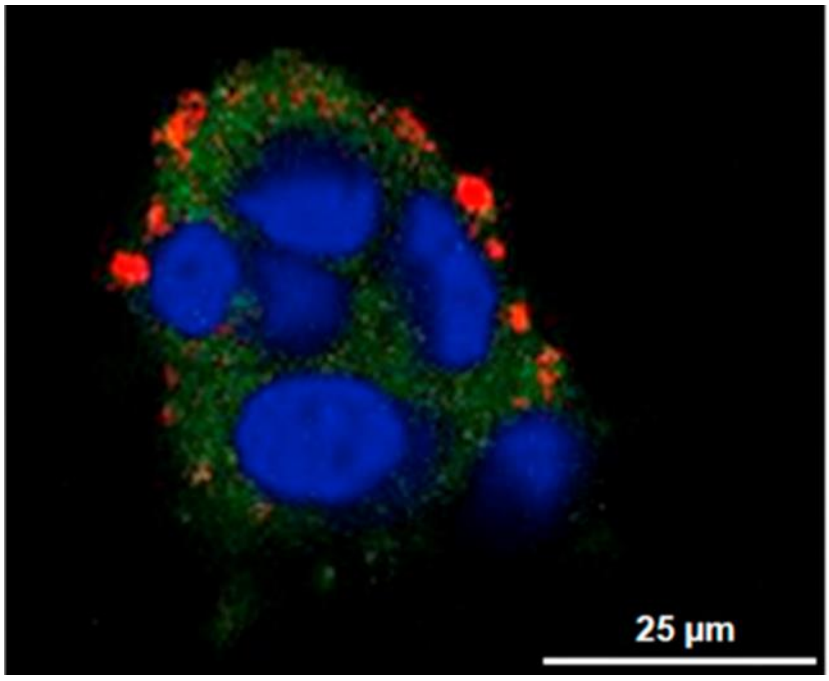


915 Figure 7 Representative Illustration of Nanoparticles (NPs) Detected in Urine by Scanning
916 Electron Microscopy (SEM). NPs were prepared by the solid phase approach using
917 vancomycin as a template..
918



919

920 Figure 8. Fluorescence imaging of rabbit eye tissues taken from the keratitis model rabbits
921 (left) or normal rabbits (right), following treatment with IR-783-loaded molecularly imprinted
922 (MIP) and non-imprinted (NIP) nanoparticles. The control was treated with IR-783 solution
923 alone. Reprinted with permission from [87].
924



925

Figure 9. Confocal Image Showing Simultaneous Multiplex Labeling of D-Glucuronic Acid (GlcA) and N-Acetylneuraminic Acid (NANA) on Fixed Human Keratinocytes by Molecularly Imprinted Polymer (MIP) GlcA Quantum Dots (QDs) (MIPGlcA-QDs, Green) and MIPNANA-QDs (red), respectively.. Reprinted with permission from [32].

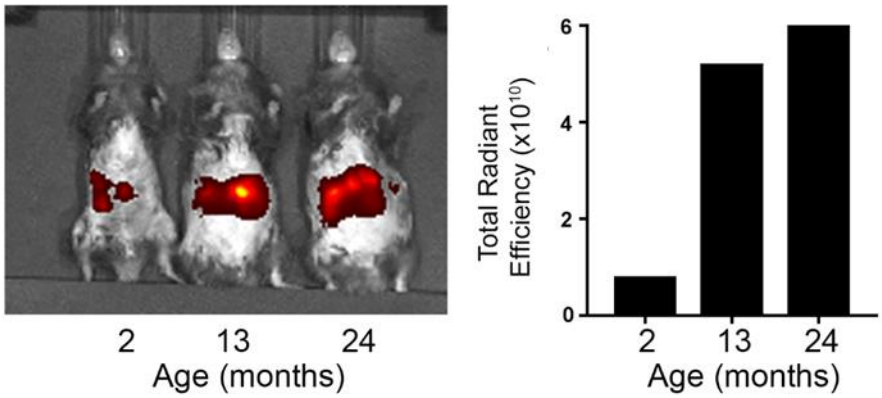


Figure 10. Representative images of group of mice of different ages, injected intravenously with Alexa Fluor 647-tagged B2M Nanoscale Molecularly Imprinted Polymers (nanoMIPs). Animals were imaged 2 h after injection. Total fluorescence signals were quantified and are shown in units of radiant efficiency. Reprinted, with permission, from [82].

BOX 1

Rational selection of linear epitope templates

When to use & which bioinformatics resources are available: A peptide can be a "signature" for the whole protein. Such a peptide, called idiotypic, or unique, is envisaged as ideal target for the imprinting. Beside the cost-associated considerations, the imprinting of a small portion of the protein bypasses all the problems associated with unfolding during the imprinting process, enabling to provide a material with imprinted stereochemical images for the target peptide. The selection of a signature peptide from a protein is enabled by the access to free web-curated repositories of proteomics information, i.e. the websites where all the known protein sequences are stored (e.g. NCBI, UniProt) [123].

The goal to find a unique peptide sequence within the targeted protein can be fulfilled thanks to the sequence alignment of comparison tools provided by the database. Once the sequence comparison query is submitted, the query is replied by a report in which a scoring system gives the measure to the goodness of the alignment between the compared sequences [124], consequently the selection of the unique peptide has been named "rational" to indicate that objective goodness criteria are applied in form of a score [28]. The steps for the identification of the epitope are: the target protein sequence is selected, cut *in silico* into peptides by choosing a suitable cutting agent (e.g. trypsin); too small peptides are discarded (these are considered too combinations that can be found with high frequencty, thus not good to mark uniqueness), whereas peptides of significant length (8-15 aminoacids) are aligned to the whole protein sequences database. The best peptide epitope is the one that aligned towards the whole database of protein sequences provides the best match (highest score; S) for the very parental protein, while having the lowest E-value (value indicating the number for distinct alignments, with a score equivalent to or better than S, but expected to occur in a database search by chance).

1.From the website http://www.uniprot.org/

- search for the target protein sequence ; copy the sequence in FASTA canonical format

2. From the website http://web.expasy.org/peptide_cutter/

- paste the FASTA sequence in the appropriate box ;
- select the desired cleavage method (enzymes, chemicals) ; select "Table of sites, sorted sequentially by amino acid number" and cleave the protein;
- select peptides not shorter than 8-10 (idiotypic sequences) and not longer than 15 residues (avoid secondary structures);

3. From the site http://blast.ncbi.nlm.nih.gov/Blast.cgi and select "protein blast"

- enter one by one the sequences of the selected peptides in the appropriate box and set the following parameters before running:

* Database: non-redundant protein sequences (nr) ; * Organism: e.g. Homo sapiens ; * Algorithm: blastp (protein-protein BLAST)

- report the found identity value, the total score and the E-value of each peptide; choose the peptide with the highest total score and the lowest E-value

4. From the website http://web.expasy.org/protparam/

- Enter the sequence of the selected peptide/peptides in the appropriate box to calculate the parameters: molecular weight, isoelectric point, number of negatively and positively charged residues, GRAVY index.

BOX 2

Rational selection of structured epitopes as template

When to use & which bioinformatics resources are available: When the target protein is exposed at the cell surface, the epitope might be protruding out of the membrane in fixed and defined orientation, or when peptides associated with high scores are not accessible for binding, because they are hidden in the protein core, masked by membrane or associated to other proteins and glycocomponents.

The positioning of the chosen epitope on the protein 3D structure is a prerequisite for successful imprint. Moreover, using directional peptides (e.g. circular peptides) instead of linear ones is another strategy to ultimately gain in MIP selectivity [37, 125]. Database's tools associated with 3D view of proteins permit to define the epitope localization in the protein structure. Experimental, literature and predicted information gathered by and available in protein-protein interaction databases (see for example: http://string-db.org; https://www.ebi.ac.uk/intact/) permit to finalize the selection of the epitope restricting to the one accessible to the surface (and prone for binding) and to these not involved in functional association to other proteins or glycol-partners.

1.From the website http://www.uniprot.org/

- Search the target protein sequence identification number (ID)

2. From the website http://uniprot.org/uniprot/ *Add appropriate ID* /protvista

- Enter the Uniprot ID of the target protein in the Uniprot database;

In the Display click on Feature viewer then select peptides by using the following two options:

(A) On Structural features; Turn

- Select a turn and click on it to view its location on the 3D structure
- Identify the tryptic peptide that contains the desired turn by clicking on Proteomics and on the Unique peptide sequence.

(B) On Antigenic sequences

- Select an antibody binding sequence among those marked on the sequence of the target protein
- Define a unique tryptic peptide within the antigenic sequence clicking on Proteomics and on the Unique peptide sequence.

1012 Table 1. List of materials stamped by microcontact printing technique and their uses

Type of cell	Biological imprinted target	Material used	Application	Key observations	Reference
Bacteria	E. Coli	Gold-coated microbeads with Nafion + polypyrrole (PPy) imprinted layer	Cell sorting; sensing	E. Coli-shaped cavity to be 1.1×10^5 M^{-1}; discrimination of *E. coli, Acinetobacter calcoaceticus* and *Serratia marcescens*	126, 127, 128
		Polypyrrole (PPY)	Sensor; Quartz crystal microbalance (QCM)	discrimination of *E. coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, A. calcoaceticus and S. marcescens*	101,129
		Polypyrrole (PPY)	Sensor; QCM for food poisoning detection	discrimination of *E. coli* O157:H7, *Salmonella enterica, Vibrio parahaemolyticus, S. aureus*	130
Mammalian cells	Cardiomyocytes	Polydimethylsiloxane (PDMS)	Cell differentiation	The MIP drives the differentiation of pluripotent cells into the desired specific subtypes	131
	Ishikawa endometrial adenocarcinoma cells	Polymethacrylate and Polystyrene (PS)	Cell culturing Cancer development mechanisms	Cells grown on imprinted surfaces expressed more adhesion proteins.	18,19,132, 133
Yeast	yeast cells	Polyurethane (PU)	Proof of principle QCM and optical sensors	PU MIPs proved sensitive coatings to planar waveguides and mass-sensitive devices for the selective detection of various microorganisms	12, 121
		Sol-gel	Proof of principle of cell discrimination	Discriminate between different strains of yeast; little or no non-specific binding taking place	134
Algae	algae	Poly(ethylene-co-vinyl alcohol)	Cell culturing Biofuel/cell	the imprinted matrix improved the overall energy production, proving the mechanical/physical effect of the topographical environment on the metabolism/growth of the cells	135, 136

1013

1014

Glossary

Atom transfer radical polymerisation (ATRP): it is a reversible-deactivation radical polymerization suitable for forming carbon-carbon bonds with a transition metal catalyst. ATRP permits a high degree of control of the composition and of the architecture of macromolecules, ultimately providing polymeric materials with highly specific and uniform characteristics.

Electrochemical sensor: according to IUPAC definition and classification, is a category of chemical sensors, designed by coupling the receptor part of the device to an electrochemical transducer. The transducer transforms the analytical information originating from the electrochemical interaction analyte-electrode into a measurable electrical signal.

Electrochemiluminescence biosensor: it a biosensor that measures the emission of visible light as the result of an electrochemical reaction. Electro-chemiluminescent molecules, after becoming electronically excited, release visible electromagnetic energy when returning to their relaxed state. In the biosensor, the light-emitting molecules that interact with the analyte of interest are introduced into the solution, the amount of emitted light is measured and correlated to the quantity of analyte present in the sample.

Electropolymerization: it is the polymerization of electroactive monomers under the influence of an electric current. The method is straightforward to obtain polymer films with a certain thickness by controlling the number of cycles or the current that is applied to the electrode.

Epitope: known as antigenic determinant, it is the part of an antigen that is recognized by the immune system.

Idiotypic peptide: is a molecular arrangement of amino acids unique to the antigen-binding site of a particular antibody. The molecular structure and conformation of an antibody that confers its antigenic specificity.

Microcontact printing: is a method of transferring patterns of various materials such as polymers, proteins, nanoparticles, etc., onto another surface. Typically a polydimethylsiloxane (PDMS) stamp is dipped in a solution of a material that has to be patterned and is brought into contact with the surface. Transfer of micrometer (μm)/nanometer (nm)-size patterns is possible by this technique.

Quartz crystal microbalance (QCM) sensor: called also acoustic sensor, or quartz crystal microbalance. It is based on a piezoelectric material, or quartz crystal resonator, to which a frequency is applied. Usually the receptor, i.e. the selective MIP, is deposited on the surface of the quartz crystal resonator. The QCM measures the mass variation per unit area by measuring the change in frequency of the quartz crystal resonator. The resonance is perturbed by the addition or removal of an analyte at the surface of the acoustic resonator.