Since their conception 50 years ago, molecularly imprinted polymers (MIPs) have seen extensive development both in terms of synthetic routes and applications. Cells are perhaps the most challenging target for molecular imprinting. Although early work was based almost entirely around microprinting methods, recent developments have shifted towards epitope imprinting to generate MIP nanoparticles (NPs). Simultaneously, the development of techniques such as solid phase MIP synthesis has solved many historic issues of MIP production. This review briefly describes various approaches used in cell imprinting with a focus on applications of the created materials in imaging, drug delivery, diagnostics, and tissue engineering.

The Drive To Recognize and Interact with Cells

The vital functions of organisms are governed by specific crosstalk between cells which ultimately relies on macromolecular interplay. Dysfunctional molecular interactions at the cellular level are often responsible for cell malfunctioning and consequent onset of disease [1]. Biomimetic tools that explore molecular interactions have been used for cell imaging, improving drug delivery, tissue engineering, and diagnostics [2]. However, the design of such tools is not easy because of the complex nature of molecular interactions and the lack of affordable generic protocols for the development of supramolecular receptors with ordered systems of functional groups that mimic natural molecules. The present review focuses on MIPs as an alternative to biomimetics and biosimilars. We discuss here the historical foundations and recent technological advances for the preparation of MIPs suitable for cell recognition, and frontier applications to cells and cell biology, highlighting the achievements, current limitations, and future trends.

MIPs: The Concept

MIPs are recognition materials prepared by a template-assisted synthesis [3,4]. The imprinting process, schematized in Figure 1, comprises polymerization of the monomers and the crosslinker in the presence of a target molecule that acts as a template. Driven by thermodynamics, the template interacts with the monomers to form a prepolymerization complex that is stabilized by molecular interactions, and that is later ‘frozen’ by polymerization. As a result, molecular impressions of the template are stamped into the formed polymeric material, thereby creating specific binding sites capable of recognizing the template and its analogs.

MIPs are robust and possess affinity and selectivity for the template comparable with 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]. Given recent progress in the development of MIP NPs (nanoMIPs) [8,9], this technology has become suitable for frontier applications in the domain of life science and medicine.

The Development of Whole-Cell Imprinted MIPs

Although the molecular imprinting of small molecules, peptides, and even proteins is well established, with many examples in the 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 culture.
Over the past two decades much effort has been expended 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]. This involved cell lithography for the preparation of polymers with affinity for bacteria. Since then, microcontact printing (see Glossary) has seen extensive development, as well as alternative strategies such as the preparation of MIPs from self-assembling silica NPs, and the use of cell epitopes in place of whole cells.

Microcontact Stamping

Microcontact stamping, otherwise referred to as microprinting, is the most frequently explored technique for generating MIPs using whole cells as templates [7]. It involves deposition of the target cells onto a flat solid support layer and then topping them with monomers or a soft polymer such as prepolymerized 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]. A key example of cell-recognition MIPs is the use of imprinted polyvinylpyrrolidone (PVP) for the selection of erythrocyte subtypes [13]. Developed MIPs have shown outstanding selectivity towards erythrocyte subgroups A1 and A2, even though both types expose the same antigens on the surface and differ solely in the density of glycolipids on the respective cells. From these results it was concluded 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, and show 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; key examples are reported in Table 1 [15,16].

Figure 1. Schematic of the Concept of 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.

Glossary

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

Electrochemical sensor: according to the International Union of Pure and Applied Chemistry (IUPAC) definition and classification, electrochemical sensors are a category of chemical sensors that couple the receptor part of the device to an electrochemical transducer. The transducer transforms the analytical information originating from the analyte–electrode electrochemical interaction into a measurable electrical signal.

Electrochemiluminescence biosensor: a biosensor that measures the emission of visible light as the result of an electrochemical reaction. Electrochemiluminescent 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 this can be correlated with the quantity of analyte in the sample.

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

Epitopes: also known as antigenic determinants, epitopes are the part of an antigen that are recognized by the immune system.

Idiotypic peptide: a molecular arrangement of amino acids that is unique to the antigen-binding site of a particular antibody. The molecular structure and conformation of an antibody confers its antigen specificity.
Microcontact stamping has been successfully exploited for cell recognition, cell selection, and sensing (Table 1). Microcontact stamping has also provided surfaces suitable for controlled cell growth. Interestingly, comparison of cells grown on flat and imprinted surfaces showed that MIP surfaces promoted higher expression levels of adhesion proteins, confirming that the MIP substrate elicits a 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), polycrylate, polyvinylpyrrolidone (PVP), polyacrylamide, PU, and Epon1002F have been used to generate imprinted surfaces for Bacillus cereus [20]. The best performance was achieved with PU and Epon1002F. This is an important result because it allows self-synthesized polymers to be replaced by well-characterized commercial materials, allowing this technique to be used by nonspecialists in polymer synthesis.

More recently, Dulay and colleagues assessed the ability of a polydimethylsiloxane (PDMS) layer created by microcontact stamping of bacterial cells to distinguish between living and inactivated cells [21]. These polymers showed significantly higher affinity for inactivated cells prepared using the same technique as that used for polymer imprinting [14,17]. Because of the synthetic limitations of PDMS, the authors moved to organosiloxane polymers generated by sol–gel chemistry. The broad selection of available silanes made it possible to benefit from a plethora of functionalities while retaining optical transparency and mechanical resistance [21]. Although the mechanical stability of inorganic materials is usually higher than that of their organic counterparts, it is important to consider that the mechanical stress which cells undergo during the stamping procedure might be damaging for 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 as a template instead of cells. This may improve the ease, reproducibility, and safety of making imprinted polymer layers because no living cells are needed after the first imprint [18].

Cell recognition can also be achieved by imprinting sections of the cell membrane. Charged proteins exposed on the cell membrane play a key role in cell adhesion, proliferation, interaction, and localization. Bao and colleagues reported a novel method to produce bacteria-imprinted polymers by exploiting bacterial surface-charge heterogeneity using charged methacrylate ethyl trimethyl ammonium chloride and 3-dimethyl (methacryloyloxyethyl) ammonium propane sulfonate fixed in a polymer network by surface-initiated atom transfer radical polymerization (ATRP) [22]. The charge distribution on the imprinted cavities complemented the charge distribution of the bacterial surface, allowing stronger electrostatic-mediated recognition. Borovicka and colleagues generated ‘colloid antibodies’ by coating microbial cells with a silica shell that was subsequently fragmented to create complementary shell fragments [23,24]. The authors demonstrated that recognition is mediated not only by the size and shape of the imprints but also by 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 Escherichia coli and Pseudomonas 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 onto the polymers allowing simultaneously binding and visualization of the pathogens (Figure 3).

Epitope Imprinting
The whole-cell imprinting approach produces a shape-recognition material that might not be optimal when the goal is to recognize a specific type of human cell, for example, to distinguish or locate...
cancer cells in a tissue or organ. Given the high plasticity of mammalian cells, shape recognition alone does not always offer the level of discrimination required for success. Moreover, MIPs intended for cell recognition in vivo should have the size of natural macromolecules (nanometers) to be suitable for circulation within vessels, within the lymphatic system, and for intracellular space diffusion, whereas the imprint of a whole cell inevitably results in a micrometer size. For all these reasons, alternative imprinting approaches needed to be proposed. In this case, attention should focus on particular molecular components on the cell surface such as proteins, lipids, saccharides, and their derivatives.

**Saccharides**

For targeting glycomoieties that are typically present at the cell surface, imprinting was performed by stamping portions of the glycoarchitecture in a process analogous to 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 created core-shell imprinted gold NPs bearing thermoresponsive N-isopropylacrylamide (NIPAm) imprinted with *E. coli* O157 lipopolysaccharide [34]. The target bacteria bound to the NPs with excellent selectivity (>15) relative to other types of *E. coli*. NanoMIPs prepared using a solid-phase approach with immobilized trisaccharide of the blood-type B antigen were able to distinguish between erythrocytes of different blood types [35]. Similarly, MIPs 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, whole proteins were imprinted in the preparation of a fibronectin (FN)-imprinted polysiloxane membrane in which silanes were used as functional monomers and a calcium alginate hydrogel membrane provided the substrate. The FN-imprinted polysiloxane membrane provided improved cell adhesion and favorable 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 to imprint a small peptide sequence, or...
epitope, that is characteristic of a particular protein and exposed on its surface. Owing to the extreme complexity of the proteome, finding such epitopes is a difficult task. A short summary provides an outline of the strategy currently used in the rational selection of epitopes for molecular imprinting (Boxes 1 and 2).
A recent example of epitope imprinting employed the peptide arginylglycylaspartic acid (RGD) that has a well-known function in cell adhesion. 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 for molecular imprinting of zeolite-chitosan-TiO₂ microspheres for dot-blot immunoassays with multiple native antigens for the 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 used lipophilic chain conjugation to ensure that the template was at the NP surface during the inverse microemulsion polymerization process.

Similarly, cancer cells overexpressing epidermal growth factor receptor (EGFR) have been successfully targeted by imprinting NIPAm-based MIPs with an EGFR epitope [43]. The resulting MIPs were able to discriminate between cells with different levels of EGFR expression. These MIPs were prepared by immobilizing the template peptide on glass beads before polymerization. Using this solid-phase approach, it was possible to remove low-affinity polymers and monomers via a low-temperature washing step, and high-affinity MIPs could be easily separated from template molecules (Figure 4).

To conclude, two approaches continue to dominate cell imprinting: microprinting and epitope imprinting. A range of organic and inorganic polymers have been employed to imprint bacterial
and mammal cells successfully. Although microprinting is perfectly suited for producing cell-specific surfaces, epitope imprinting can be used to produce nanoMIPs that are able to address cell targets in vivo.

Applications of Cell Imprinting

Cell Concentration and Separation

Most successful examples of the use of cell-imprinted MIPs in cell 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, and showed 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

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**Box 1. Rational Selection of Linear Epitope Templates**

**Which Bioinformatics Resources Are Available and When To Use Them**

A peptide can be a ‘signature’ for the whole protein. Such a peptide, that is an idiotypic peptide (i.e., is unique), is an ideal target for imprinting. In addition to cost-associated considerations, imprinting of only a small portion of the protein bypasses problems associated with unfolding during the imprinting process, and generates a material with imprinted stereochanical images of 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, namely websites where all the known protein sequences are stored (e.g., NCBI, UniProt) [123].

Finding a unique peptide sequence within the targeted protein is facilitated by sequence alignment and comparison tools provided by the databases. Following submission of the sequence comparison query, the program scores the goodness of the alignment between the compared sequences [124], and 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); peptides that are too small are discarded (matches to very small peptides can be found at high frequency and thus lack uniqueness), whereas peptides of significant length (8–15 amino acids) are aligned to the whole protein sequence database. The best peptide epitope is that which, when aligned towards the whole database of protein sequences, has the best match (highest score, S) with the parental protein, and has the lowest E value (value indicating the number of distinct alignments, with a score equivalent to S, that occur in the database by chance).

**How To Use Them**

(i) From the Website www.uniprot.org/

Search for the target protein sequence; copy the sequence in FASTA canonical format.

(ii) From the Website web.expasy.org/peptide_cutter/

Paste the FASTA sequence into 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).

(iii) From the Website http://blast.ncbi.nlm.nih.gov/Blast.cgi

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). Record the 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.

(iv) 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 (grand average of hydropathy) index.
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 to imprint poly(dimethylsiloxane) films with inactivated Mycobacterium smegmatis [14].

MIPs have also been used for spore capture and concentration in an integrated biological detection system for Bacillus anthracis [47]. The binding assay showed strong spore-binding and a robust imprinting effect that accounted for 25% additional binding over nonimprinted controls. This process was rapid, taking only 30 minutes.

In a different example, cell adhesion was improved by imprinting with FN and the cell adhesion peptide Arg-Gly-Asp-Ser [48,49]. Template-enhanced adhesion of fibroblasts, MC3T3-E1, and L929 cells was observed after 24 h (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 the rearrangement of adhesion proteins and the cytoskeleton, which in due course affect intracellular processes [52]. The predictive design of tissue scaffolds is difficult owing to limited understanding of the 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]. In addition to residual cellular fragments present on the template surface, the imprinted topography of the templates played a role in stem cell differentiation. In a similar study, mature human keratinocyte cells were used for imprinting of PDMS. Human adipose-derived stem cells (ADSCs) seeded onto 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. The authors speculated that mechanical deformation caused by cell–imprint interaction may induce signal transduction by affecting the arrangement of chromatin inside the stem cell nucleus. ADSCs, semifibroblasts, and tenocytes were differentiated, redifferentiated, and transdifferentiated, respectively, into chondrocytes after being cultured for 2 weeks on chondrocyte-imprinted PDMS substrates [55]. A similar effect was also observed when ADSCs were cultured on keratinocyte-imprinted substrates [54] or on chondrocyte- or fibroblast-imprinted substrates [53]. Although the aim of these studies 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. There is also an opportunity to use MIPs in the clinic to enrich for cell populations: for example, to separate leukocytes by aphaeresis, to enrich for hematopoietic stem cells, or to aid repopulation of the immune system 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 ability of nanoMIPs is at least comparable with that of antibodies [60]; in addition, the possibility to...
produce fluorescent nanoMIPs and/or core-shell magnetic nanoMIPs is well recorded [61], and MIP technology is therefore sufficiently mature for the challenge.

Drug Delivery

A current trend in pharmacology is evidenced by the increasing number of FDA-approved NP formulations, amounting to ~50 in 2017 [62]. Currently, several types of NP-based drug carriers are available on the market. These are based on solid dispersion (Gris-PEG, Sandimmune, Intellec, etc.), self-emulsifying drug delivery systems (Neoral®, Agenerase, Aptivus, etc.), or nanocrystals (NanoCrystal®, Rapamune, Megace® ES) [63]. The polymer architecture of NPs dictates drug loading efficacy, drug-release rate, and biodistribution [64]. NPs smaller than 8 nm are cleared rapidly from the bloodstream by the renal system, whereas 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 the half-life of a drug 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 NP transport is adsorptive endocytosis [68]. Summarizing numerous absorption studies, there seems to be agreement that the optimum size of NPs for drug delivery via the oral route is 10–100 nm [69]. The extent of systemic appearance of this type of NP after gastrointestinal absorption has been reported to be 10–15% [70]. NanoMIPs, in contrast to antibodies and aptamers, are capable of penetrating cell membranes by endocytosis, and can even reach nuclei [67,71]. The same mechanism is used for oral delivery of drugs assisted by nanoMIPs. In one such example, nanoMIPs were generated by precipitation polymerization and were used for the oral delivery of insulin via a transmucosal oral route (Figure 6) [72]. Ongoing work compares intravenous and oral delivery of nanoMIPs and their impact on the clearance of NPs through kidney and bile. The nanoMIPs were successfully excreted in both urine and feces (Figure 7).

Given 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 per mg of NPs, depending on the drug type and the synthetic protocol used for nanoMIP preparation [71,73–75]. The imprinting process ensures a 2–3-fold increase in the quantity of entrapped drugs compared with nonimprinted particles [75]. The half-time drug release in these experiments varied from 2–20 h based on the polarity of the drug and its affinity for 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 result obtained for nanoMIP circulation in the body is 7 days, which is an improvement compared with the circulation of small drug molecules [77].

Targeted drug delivery originates from the ability of MIPs to interact specifically with cell receptors. Most therapeutic agents (90% or more) will inevitably be concentrated in reticuloendothelial organs such as the liver and spleen as a result of 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 NPs 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 nonfolate NPs and free paclitaxel, with half-maximal inhibitory concentrations (IC50s) 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 an EGFR epitope [81]. In a similar way, senescent cells were targeted by dasatinib-bearing nanoMIPs imprinted with an epitope of the senescence marker B2M [82]. NanoMIPs loaded with drugs were able to specifically kill senescent cells, and showed a significantly greater level of binding within the organs of older animals. Targeted delivery can be achieved using external factors such as a magnetic field [77]. In this work, nanoMIPs with magnetic cores were prepared via coprecipitation polymerization in the presence of olanzapine as a template, and were used for magnetic field-guided drug delivery of olanzapine to rat brain.

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

In a rare example, nanoMIPs themselves were used as a drug [86]. In this work, nanoMIPs, imprinted with the quorum-signaling 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 *P. aeruginosa* were used as a template in the preparation of nanoMIPs by the inverse emulsion method [87]. Fluorescent nanoMIPs labeled 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 also used for *in vitro* photodynamic therapy. Compared with nonimprinted NPs, an almost two orders 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 6 months of storage.

There are several issues to be resolved and questions to answer before practical application of nanoMIPs in drug delivery can be considered: how safe are nanoMIPs? Should nanoMIPs be biodegradable? How do the properties of nanoMIPs 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 have been tested mainly *in vitro*. Cell viability tests using NIH-3T3 cells and human embryonic kidney cells (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 hepatocytes or the structure of the kidney. No
A sign of toxicity was found and 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, clearance of biodegradable nanoMIPs might be simplified clearance. However, the byproducts of polymer degradation might be more toxic than the NPs themselves. Monomers such as methacrylic acid, methyl methacrylate, and ethylene glycol dimethacrylate are biocompatible and nontoxic [89,90]. The same is not true for some other monomers such as acrylamide [91]. The examples reported here, as well as many other relevant examples from the literature, imply that nondegradable polymers might be safer for use in medical devices and drug delivery [92,93]. In addition to 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 nonpolymerized components from MIP formulations.

In addition to complications in the experimental design of NPs, there are multiple challenges for the manufacture, regulation, and approval of NPs 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 to combine nanoMIP synthesis with an affinity separation step in a single procedure, using an immobilized template for MIP formation [95]. The resulting process allowed the construction of the first prototype automatic nanoMIP synthesizer [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 1 h, but they also possess nanomolar dissociation constants for their respective targets, no residual template is present, and the immobilized template can be reused. This automated process overcomes all the historic drawbacks of bulk MIPs, and raises the exciting possibility of deploying nanoMIPs in therapeutic applications. Despite this success, bringing manufacturing protocol into 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 have been used in combination with nanoMIPs, including pyrene, fluorescein, and rhodamine derivatives [29,96], quantum dots (QDs) [97], and carbon dots [33]. In one study, two differently colored nanoMIPs were imprinted with D-glucuronic acid and N-acetylgalactosamine. Both MIPs were found to be
highly selective for their target monosaccharides because no crossreactivity was observed with other sugars 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). Monodisperse 400 nm-sized particles bound to extracellular targets, whereas 125 nm particles were also able to stain intracellular and pericellular regions.

In similar work, fluorescent nanoMIPs were imprinted with sialic acid and used for imaging cancer cells [31]. These nanoMIPs exhibited selective staining for DU145 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 fluorescence signal was detected from the MIPs in MDA-MB-468 cells that overexpress 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 vascular endothelial growth factor (hVEGF) and coupled with QDs for cancer imaging [98]. The composite NPs exhibited specific binding to human melanoma cell xenografts overexpressing hVEGF in zebrafish embryos. In another work, fluorescein-labeled 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 that can be considered for advancing imaging, in particular when antibodies are less desirable because of their immunogenicity or long production time. Moreover, one of the main limitations associated with the state of art in imaging techniques is the detection limit of fluorescent antibodies that is currently restricted to antigens expressed on the target at more than 1000 copies/cell, whereas key inflammatory and cancer markers such as interleukins are often present in only a few hundreds of copies on the cell membrane, therefore falling below current detection limits [99]. However, given their larger dimensions (10–400 nm), nanoMIPs that contain a significant number of fluorophores per NP can circumvent the aforementioned limitations,
without any need for secondary binding or catalytic amplification. Despite holding great promise, 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 magnetic resonance imaging (MRI) and positron emission tomography (PET) imaging. It is crucially important that safety issues are addressed and manufacturing problems solved before this technology can advance.

**Sensing**

In diagnostics, cell-imprinted MIPs are used almost entirely for the detection of microorganisms. Currently, laboratory-based biochemical methods for microorganism analysis employ standard antibody assays and PCR [100]. Cell culture remains a standard technique for identifying bacterial species; however, it usually requires 24–48 h, 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 quartz crystal microbalance (QCM) sensor platform was developed for the detection of *E. coli*, *B. cereus*, *P. aeruginosa*, *B. subtilis*, and *Staphylococcus aureus* [101–103]. Imprinted PPy and PU were generated directly onto the sensor surface. The QCM device allowed detection of microorganisms at concentrations of $1.4 \times 10^8$ cells/ml within 2–3 minutes. Overall, QCM sensors have shown similar sensitivity to surface plasmon resonance (SPR), allowed 10 regeneration cycles, and remained operational for at least 3 months [104]. In an example of practical application, a PU-based QCM sensor was used to follow the growth of *E. coli* and Saccharomyces cerevisiae in a bioreactor [105,106]. The sensor was able to identify different stages of the cell cycle, with a limit of detection (LoD) of $1.6 \times 10^8$ cells/ml.

Electrochemical sensors based on conducting MIP materials, such as electropolymerized 3-aminophenol and 3-aminophenylboronic acid, were used for the detection of *S. aureus* and *S. epidermidis* [107,108]. Cyclic voltammetry and electrochemical impedance spectroscopy in the presence of redox probes were explored for the specific detection of the target bacteria at $10^2$–$10^7$ colony-forming units (cfu)/ml concentrations. Imprinted PPy/poly(3-methylthiophene) was used in impedance detection of *B. subtilis* endospores at $10^5$–$10^7$ cfu/ml [109]. Surface-imprinted polydopamine was used for yeast sensing, allowing a LoD of 50 cfu/ml with excellent selectivity versus smaller *Vibrio alginolyticus*, *E. coli*, and *S. 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.
Electropolymerized 3-aminophenylboronic acid was used to create a sensor for *S. 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 $1 \times 10^4$–$1 \times 10^5$ cfu/ml [112]. NanoMIPs were synthesized using a sol–gel method with cerium dioxide NPs in the presence of an indium tin oxide [113]. This assay was used to detect *S. aureus* on the surface of an indium tin oxide [113]. This assay was used to detect *S. aureus* at $10^4$–$10^5$ cfu/ml.

An electrochemiluminescence biosensor was developed for the quantitative detection of *E. coli* O157:H7 based on a polydopamine-imprinted polymer [114]. However, in this work MIPs were only used for capturing bacteria, and electrochemiluminescent detection was achieved using a polyclonal antibody labeled with nitrogen-doped graphene QDs. 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 $3 \times 10^4$ cfu/ml in spiked urine.

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

In most of these examples, imprinted films were prepared by stamp imprinting or by electropolymerization. The main problem with these approaches lies in their poor reproducibility and the inefficiency of the mass manufacture of sensor devices owing to the use of live bacteria as templates. There is also danger in using pathogenic bacteria as a template for sensor production. A solution to these problems was found in anti-idiotype imprinting using PDMS master stamps with ‘plastic copies’ of natural cells [119]. Sensitive layers created this way were capable of the differentiation between *S. cerevisiae* and *S. bayanus*, and could 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.

### Concluding Remarks

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 range from whole-cell imprinting to targeting specific and distinctive cell-surface components. 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 provide a reliable supply of soluble synthetic NPs and polymer coatings with predetermined molecular recognition properties, subnanomolar affinities, and defined size and surface chemistry 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 help to revolutionize clinical practice and enable personalized medicine [33,40,98]. One challenge (see **Outstanding Questions**) is to produce nanoMIP architectures that are suitable for translating MIP-mediated cell recognition from passive binding to a defined target to active intervention in the cell biology process. To accomplish this important step, the integrated design of multifunctional MIPs is expected, gathering in a single nanoMIP particle the ability to activate or silence biochemical pathways [37,120]. Success in this area will result in new paradigms for MIP applications that both complement existing therapeutic and diagnostic techniques, and will open doors to in situ programmed nanomachines for precision medicine interventions and tissue regeneration.

### Outstanding Questions

- How can a consistent method to produce cell imprints be developed?
- Which materials are best to produce cell-imprinted materials?
- Can we devise general guidelines for cell imprinting to help to translate the process to large-scale and make it widely available?
- Which frontier applications require cell-imprinted materials?
- How safe are nanoMIPs?
- How do the properties of nanoMIPs 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?
- How can nanoMIPs be modified to interact with cellular process and become primary tools for managing misregulated cellular processes?

### References

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