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Recent advances in virus imprinted polymers

Mariana S. Amorim, M. Goreti F. Sales **, Manuela F. Frasco *

BioMark@UC/CEB - LABBELS, Department of Chemical Engineering, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

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ABSTRACT

Molecular imprinting is a mature and appealing technology to obtain highly selective recognition sites created within a polymeric network, having other important features such as robustness and cost effectiveness. The applications of molecularly imprinted polymers (MIPs) are vast, but there is a continuous particular interest in their integration into sensing devices. MIPs possess remarkable properties in terms of selectivity and low-cost of tailored biomimetic recognition elements. The need to improve disease diagnosis and prevention by detecting pathogenic viruses and other biological targets at the nanoscale has led to great advances in MIPs. Despite still facing many challenges, imprinting approaches can provide rapid and accurate virus recognition and thus be applied as new sensing materials. Following a general overview of MIP technology, key examples of virus imprinted polymers, the application of MIP-based materials to other nanoscale targets, and their detection are presented here. Perspectives and challenges are also highlighted foreseeing new future strategies and MIP designs.

1. Introduction

The ability to create biomimetic materials covers several fields of research. One of the most prolific examples is the artificial ligand-binding sites created within a polymeric matrix, known as molecularly imprinted polymers (MIPs). The inspiration comes from the natural molecular interactions resulting in very stable binding phenomena that occur in the biological systems. Thus, MIPs are described as plastic antibodies, and are robust and highly selective materials. The history of MIPs is linked to separation processes, *i.e.*, the necessity for enrichment or extraction of small molecules (Greber and Flatt, 2019). Nonetheless, the use of this technology has rapidly evolved and is used for the detection of a range of molecules, from small to larger targets (Refaat et al., 2019). Not surprisingly, MIPs have now been successfully applied to large and complex viruses, bacteria, and cells (Liu et al., 2020; Ren and Zare, 2012). Regarding viruses, the challenges posed by recurrent outbreaks of pathogenic viruses, and their impact on health and the economy, make them appealing targets for continuous improvement of detection methods.

In this review, a general overview of the MIP technology is first outlined to then highlight the most significant examples of MIP-based materials developed to recognize viruses and biological targets at the nanoscale. MIPs developed using whole or intact viruses can be more challenging, but at the same time offer greater insight on the synergy between the established multiple interactions and the shape-complementary cavities. Thus, virus imprinted polymers will be the focus of this review, hoping the examples of biomimetic materials presented will inspire the development of new strategies and MIP designs.

2. Molecular imprinting technology

MIPs are tailor-made synthetic materials with artificially created recognition sites that are able to selective rebind a target compound, instead of closely related compounds (Turiel and Esteban, 2019). MIPs are generated by polymerizing functional and crosslinking monomers around a template molecule, thus obtaining a crosslinked three-dimensional network polymer (Turiel and Esteban, 2019). Compared to other recognition systems, MIPs possess three major unique features: structure reliability, recognition specificity and universal application. Thus, their use is very significant in many fields, ranging from purification and separation, catalysis, chemo/biosensing, and drug delivery. The features of MIPs regarding high chemical and physical stability, straightforward preparation, remarkable robustness and low-cost are very attractive in these research fields (Cardoso et al., 2018; Chen et al., 2016). Among the many applications, the integration of MIPs into biosensors is one of the most creative due to the need to

* Corresponding author.
** Corresponding author.
E-mail addresses: goreti.sales@eq.uc.pt, goreti.sales@gmail.com (M.G.F. Sales), mffrasco@gmail.com (M.F. Frasco).

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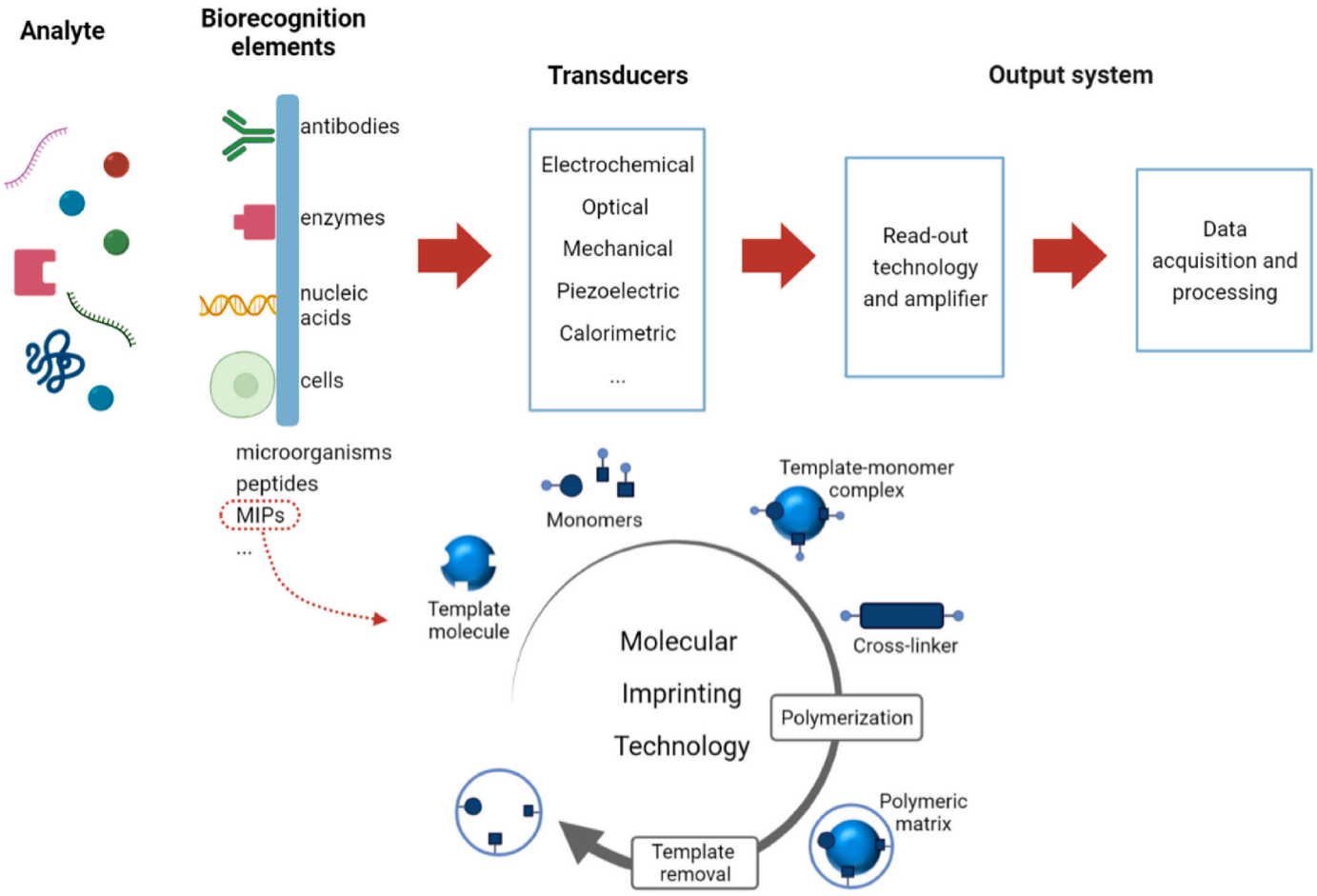

Fig. 1. Schematic representation of biosensors, alongside the scheme for producing MIPs.

improve the selectivity and sensitivity of detection or diagnostic methods through cost-effective and robust biomimetic strategies (Fig. 1).

2.1. MIP components

The effectiveness, affinity, and selectivity of the recognition sites of a MIP are greatly influenced by its components (template, monomers, crosslinker, polymerization initiator and solvent) and by the quality of the interactions between them. Thus, when designing the MIP, the selection of its components is a critical step to achieve the desired properties for a certain application (Suryana et al., 2021; Turiel and Esteban, 2019).

First, it is crucial to consider the molecular structure of the template that will determine the choice of the functional monomer(s). The structure and the number of binding sites in the imprinted polymer, *i.e.*, the molecular recognition, depends on the chemical bonds established between them. For this reason, the template and functional monomer(s) must complement each other to maximize the imprinting process (Cormack and Elorza, 2004; Suryana et al., 2021). Moreover, the template should not possess functionalities responsible for potentially inhibiting or retarding the polymerization and should be stable under the synthesis conditions (Cormack and Elorza, 2004). The crosslinker has also to be considered as a fundamental element of a MIP. Several crucial steps depend on a proper crosslinking of the polymer, such as: (a) formation of the imprinted pocket by freezing the template-monomer complex upon polymerization; (b) stabilizing the imprinting binding site; (c) ensuring polymer mechanical stability; (d) controlling the morphology of the polymer matrix. Thus, the reactivity of the crosslinker and the ratio monomer to crosslinker are very important to obtain functionalized sites that are spread uniformly throughout the polymeric network and to have properly spaced cavities with no constrains to the diffusion of the template (Bergmann and Peppas, 2008).

The polymerization begins with initiators as source of chemical species that react with the monomers to form an intermediate compound capable of linking successively with other monomers in a chain-growth polymerization until a polymeric compound is reached. They are often thermo-, photo- or redox-initiators, which have been extensively used in commonly free radical polymerizations. The solvent is the medium where the polymerization occurs and its molecules occupy space in the polymeric matrix, thus creating pores and being named as porogen. In this sense, the nature and volume of the solvent is also important to develop favorable pores in the matrix that will contribute to a proper diffusion of the template out of the network and its subsequent diffusion back into the polymer during recognition (Bergmann and Peppas, 2008; Vasapollo et al., 2011).

2.2. MIP synthesis and preparative approaches

The structure of the polymer matrix is macroporous with nanocavities complementary to that of the template molecule (Włoch and Datta, 2019). To achieve this organized network, the molecular imprinting process has several steps (Fig. 1): (1) dissolution of template, functional monomers, crosslinker, and initiator in the solvent; (2) interaction of the functional monomers with the template molecule, which results in the formation of a stable template-monomer complex; (3) fixing the functional monomers positioned around the template by copolymerization with crosslinkers; (4) removal of the template

molecules from the matrix, for example by extraction with a solvent; (5) the polymer matrix is left with binding sites complementary in shape, size and functionalities to the target compound (Turiel and Esteban, 2019). Therefore, the obtained polymer matrix recognizes and binds selectively the template molecules (Włoch and Datta, 2019).

The resulting imprinted polymers are stable, robust, and resistant to a wide range of pH, solvents, and temperature. Therefore, the behaviour of MIPs mimics the interactions established by natural receptors to selectively retain a target molecule but without the associated stability limitations. Besides, the synthesis of MIPs is relatively simple and inexpensive, providing a clear alternative to the use of natural receptors (Turiel and Esteban, 2019).

Based on the types of interactions between the target molecule and the functional monomers, three general approaches have been described for the synthesis of MIPs, namely, covalent, non-covalent, and semi-covalent approaches. The covalent approach involves the formation of reversible covalent bonds between the template molecule and monomers before polymerization. Then, the template is removed from the polymer by cleavage of the corresponding covalent bonds, which are reformed upon rebinding of the target compound (Turiel and Esteban, 2019). Since covalent imprinting is stoichiometric, functional monomer residues exist only in the imprinted cavities. Thus, it minimizes the presence of nonspecific sites due to the high stability of template-monomer interactions (Chen et al., 2016; Turiel and Esteban, 2019). However, the covalent approach is less flexible related to limited bond formation and cleavage readily reversible reactions under mild conditions and it is difficult to reach thermodynamic equilibrium due to slow binding and dissociation resulting from strong covalent interactions (Chen et al., 2016; Turiel and Esteban, 2019). The non-covalent approach is based on establishing non-covalent interactions between the template and the monomers, such as hydrogen bonds, ionic interactions, van der Waals forces and $\pi-\pi$ interactions. Usually, the major interaction is hydrogen bonding, and this approach is by far the most common because the preparation of MIPs is simple, there are many available monomers, and the binding and removal steps are rapid (Chen et al., 2016; Turiel and Esteban, 2019). Nonetheless, the non-covalent imprinting is less robust because the interactions holding the complex together can be more easily disrupted (Chen et al., 2016). An intermediate alternative is to combine the stability of covalent imprinting and the fast target uptake of non-covalent imprinting, by following a semi-covalent imprinting (Chen et al., 2016; Turiel and Esteban, 2019). In this case, the template is covalently bound to a functional monomer, but the rebinding is based only on non-covalent interactions (Chen et al., 2016).

There are also a variety of methods available for preparing the imprinted polymers, resulting in different formats, and the choice is usually intrinsic to the final application. MIPs can be prepared as bulk polymer monoliths, requiring subsequent mechanical griding or be obtained already in microsizes using methods such as precipitation and emulsion polymerizations or sol-gel processes. Other very interesting approaches have emerged with the possibility of surface imprinting, surface grafting, solid-phase synthesis, click-chemistry, electropolymerization, among plenty examples that can be found in the literature (Chen et al., 2016; Refaat et al., 2019; Vaneckova et al., 2020).

3. Applications

The first and most successful application field of MIPs is in separation technologies. The MIPs are used as sorbents in solid-phase extraction due to their structural predictability, thermal and chemical stability. These features are essential so that different types of composites can be formed, and thus applied to selectively concentrate samples in biological, pharmaceutical, and environmental fields. This procedure is directly coupled with specific analytical systems, such as high-performance liquid chromatography, minimizing sample manipulation, reducing the loss of analytes, the risk of contamination and the time of pre-treatment of the samples. The aim is to innovate the solid-phase extraction method towards fewer steps, that ultimately lead to simplicity, automation, and miniaturization. Additionally, when the sample is complex and the presence of interferents may prevent quantification by standard chromatographic techniques, a customized sample treatment step can be made with this technology prior to the final determination (Gao et al., 2020; Qiao et al., 2006; Tamayo et al., 2007; Vasapollo et al., 2011).

Catalysis is another interesting research field with many potentials for MIPs. Enzymes, naturally and efficiently, catalyze a large variety of chemical reactions, due to the specific interactions in the active site between key amino acid residues and substrates. However, there are some limitations to their wide applications usually connected to their low stability in organic solvents, as well as in extreme temperatures and pH. For this reason, when searching for artificial mimics that could overcome these constraints, but at the same time maintain the expected selectivity and specificity provided by natural enzymes, MIPs constitute an efficient technology. With MIPs, it is possible to obtain polymers that display enzyme-like catalytic activity by imprinting analogues of substrates or transition states. Therefore, these synthetic polymers are certainly of great interest when developing enzyme mimics, relying on their thermal and chemical stability, being easily produced for industrial applications. Nonetheless, the positive properties of MIPs as catalysts, might also represent a disadvantage. Namely, the tight substrate binding and the rigidity of the recognition pocket may result in low reactivity and product inhibition (Dramou and Tarannum, 2016; Mirata and Resmini, 2015; Vasapollo et al., 2011; Wulff, 2001).

In recent years, the application of MIPs in drug delivery systems has been growing rapidly. The research on stimuli-responsive MIPs and the use of biocompatible polymeric materials has many enthusiastic

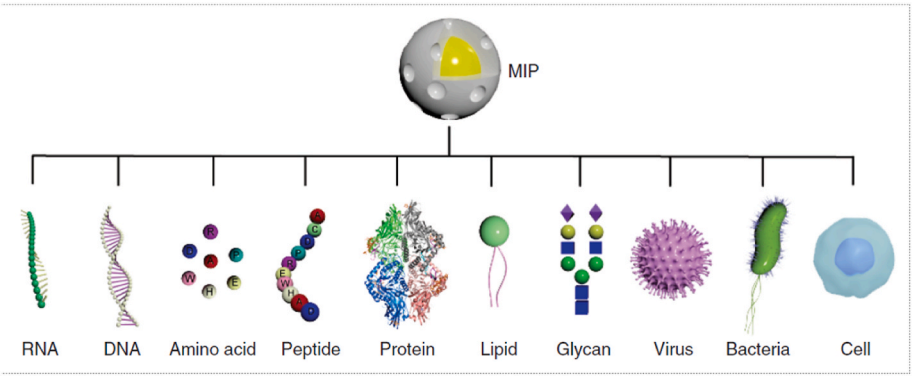

Fig. 2. Illustration of the numerous templates that have been used to develop MIPs. Copyright 2020, Reproduced under the terms and conditions of the Creative Commons Attribution 4.0 License (El-Schich et al., 2020).

prospects in hybrid biomedical *in vivo* approaches (Chen et al., 2015; El-Schich et al., 2020; Haupt, 2001; Iskierko et al., 2016; Saylan et al., 2017; Xu et al., 2011). The memory nanocavities that could be loaded with a myriad of molecular compounds and be off-loaded through various routes of administration (*e.g.*, ocular, dermal, intravenous, etc.) have enormous potential both for imaging and therapy (Vaneckova et al., 2020).

MIP-based biosensors, for sensitive, rapid, low-cost point-of-care diagnostics, is also a huge field of interest, as MIPs can be combined with several different transducer approaches in a wide variety of sensor platforms. Biosensors combine a bio-recognition element with a signal processor and a suitable transducer approach, which can be of electrochemical, mass, or optical nature, among many others, for detecting substances and monitoring biological interactions (Fig. 1) (Cardoso et al., 2018; Chen et al., 2020). The bio-based recognition element includes a capture compound that binds to/interact selectively with the target analyte. In this sense, MIPs can be used as excellent recognition elements due to their high selectivity, sensitivity, long-term stability, and chemical inertness (Vasapollo et al., 2011).

4. Virus imprinted polymers

Infectious diseases widely disseminated by pathogenic microorganisms, namely viruses, are of particular concern. However, despite huge progress in diagnostics, treatment and prevention, these diseases are still a serious global health risk. For this reason, early diagnosis of viruses is essential for clinical and point-of-care applications. The tools used for detection of viruses are mainly based on enzyme-linked immunosorbent assay and polymerase chain reaction (PCR) amplification (Boonham et al., 2014). These methods are recognized by their high sensitivity, but they are still time-consuming, with high production costs, and require operation by a specialist (Boonham et al., 2014; Cui et al., 2020).

Molecular imprinting can be applied to a wide range of target molecules, which is one of the many attractive features of this technique (Fig. 2). The imprinting of low molecular weight compounds as target analytes is well established and highly successful. When it comes to prepare selective recognition receptors for larger biological targets, using macromolecular templates, there are inherent challenges such as: (a) the bulkiness of the template molecules is connected to slow diffusion to the molecular cavities, making the response of the MIP sensor undesirably long; (b) charged functional monomers may be less efficient in the imprinting of high molecular weight compounds; (c) the step of template removal is harder, as it cannot be removed smoothly from the imprinted cavities, thus lowering the desorption efficiency of the template (El-Schich et al., 2020; Haupt, 2001; Iskierko et al., 2016; Saylan et al., 2017).

These difficulties are sometimes surpassed by epitope imprinting, *i.e.*, a small fragment of the macromolecule is imprinted aiming to create stronger interactions. Nonetheless, this approach also has intrinsic limitations that may lead to less specific interactions, *e.g.*, unsuitable selection of epitope length and structure (Dietl et al., 2021; Zhang et al., 2021). Attempting to grasp the complexity of macromolecules in a polymeric image of the whole, preserved in the imprinted substrates, can be particularly relevant to improve the recognition of virus and other biological targets with sizes at the nanoscale.

Particularly for the detection of intact viruses, the application of MIPs can ultimately be a way to overcome the limitations of current methodology, and interest in this field of research has been thriving (Afzal et al., 2017; Altintas, 2016; Cui et al., 2020; Jamalipour Soufi et al., 2021). Interesting examples of MIPs developed by templating whole viruses and integration of those materials within diverse transducer schemes are discussed in the following sections.

4.1. Tobacco mosaic virus

Tobacco mosaic virus (TMV) is the first observed and reported virus disease, which has been well-studied and characterized. This virus has the potential to damage the leaves of different plants, particularly tobacco, being responsible for tobacco mosaic. TMV is stable in various conditions of pH, temperature, solvents, and reducing agents. The TMV virion is a rodlike virus with a length of 300 nm and diameter of 18 nm, composed of 2130 protein subunits that form a helical structure (Hema et al., 2019; Souiri et al., 2019).

There are several works for TMV detection based on different imprinting methods. For example, a MIP approach relied on producing a flexible non-covalent TMV imprinted polymer hydrogel of polyallylamine crosslinked with ethylene glycol diglycidyl ether using TMV as template (Bolisay and Kofinas, 2010). Nonetheless, most studies found in the literature are based on surface imprinting. One MIP has been integrated on a microfluidic biochip and the detection relied on contact-less bioimpedance spectroscopy (Birnbaumer et al., 2009). The MIP was assembled by surface imprinting using a virus stamp that was pressed into a co-polymer of methacrylic acid and *N*-vinylpyrrolidone, spin-coated on the device. One of the advantages of combining microfluidics with the MIP had to do with the precise control over fluid dynamic shear forces. Thus, it was possible to study viral binding affinity and dissociation kinetics, and the developed chip presented fast response times and reusability (Birnbaumer et al., 2009). Interestingly, such stamping technique has been explored in other works together with mass-sensitive measurements using quartz crystal microbalances (QCM) (Dickert et al., 2003, 2004, 2003; Hayden et al., 2003, 2006). In such stamping method, the stamp and the polymer coating are prepared separately, and the final material is obtained by mechanically pressing the two substrates together (Hayden et al., 2006). TMV was successfully stamped on pre-polymerized mixtures of methacrylic acid, styrene and divinylbenzene, followed by UV curing and removal of viruses. The cavities on the imprinted polymer surface recognized the virus and the sensor effects were observed on QCM (Dickert et al., 2003). The resulting sensitive layer containing the surface patterning was generated directly on gold electrodes. Using this type of sensors and polymers based on acrylic acid and ethylene glycol dimethacrylate (EGDMA), TMV could be detected in a range of concentrations from 100 ng mL^{-1} to 1 mg mL^{-1}, within minutes (Dickert et al., 2004). When photoimprinting TMV on the surface of an azopolymer, the immobilization after photoirradition was studied by atomic force microscopy (AFM) and by an immunological enzyme luminescence method (Ikawa et al., 2010). The AFM analysis revealed that TMV gradually embeds into the azopolymer as a groove beneath the virus increases during irradiation, *i.e.*, as the azopolymer surface deforms complementary to the shape of TMV. Also, the luminescence assay revealed that the immobilization efficiency increased proportionally to the photoirradiation time. Thus, the photophysical induced change in the surface shape and the isomerization of the azo-dyes enables to imprint both the topographical feature and the surface characteristics of virus like TMV (Ikawa et al., 2010).

4.2. Tobacco necrosis virus

Tobacco necrosis virus (TNV) is a 26 nm diameter necrovirus, which is no longer associated with diseases of tobacco, but with bean stipple streak and tulip necrosis diseases. This worldwide spread virus is transmitted by the aquatic fungus *Olpidium brassicae* and often occurs in irrigated soils and greenhouses (Palukaitis, 2017; Tolin, 2008).

Wankar et al. (2016) formed molecular imprints of the TNV within polythiophene nanofilms of approximate 200 nm thickness, which have been electrochemically deposited onto conducting gold surfaces. Upon rebinding, the TNV polythiophene complex changes the fluorescence intensity of the nanofilm proportionally to the concentration of TNV. It was shown that the nanofilm responds to TNV within 2 min in the 0.1–10 ng L^{-1} range and with a limit of detection (LOD) of 2.29 ng L^{-1}. Moreover, the selectivity was tested using TMV, which is rod-shaped and bigger than TNV, and the sensor response showed to be selective to TNV. This work has demonstrated the potential of fluorescence for specific,

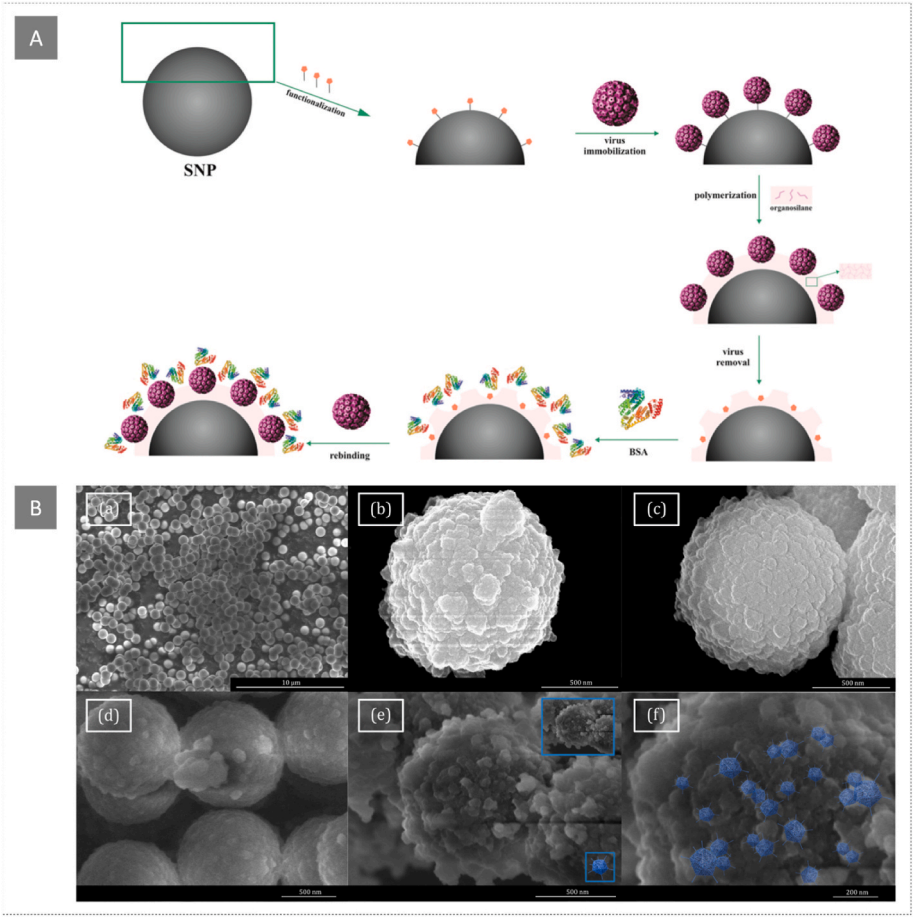

Fig. 3. Schematic illustration of surface-imprinting core-shell particles for AdV recognition (A), and images of scanning electron microscopy of bare silica particles (a), imprinted particles obtained by the co-polymerization of organosilanes (b) and after lysis (c), non-imprinted particles (d), and particles after virus rebound (e), with schematic insets of AdV (e, f) (B). Copyright 2018, American Chemical Society, Reproduced with permission (Gast et al., 2018).

label-free and rapid detection of TNV in water resources using nanofilm sensors (Wankar et al., 2016).

4.3. Adenovirus

Adenovirus (AdV) virion has a unique icosahedral shape with about 90 nm in diameter. AdVs cause respiratory, ocular, urinary tract and gastrointestinal, transmittable, and sometimes highly contagious, infections. These infections are generally self-limiting, but for unknown reasons, they can lead to local epidemics. However, there are currently no available therapies proved effective anti-AdV. For this reason, there is an urgency to have rapid detection and early diagnosis of this virus (Greber and Flatt, 2019).

Altintas et al. (2015) reported a novel MIP technology for specific and sensitive recognition of AdVs based on MIP nanoparticles coupled to surface plasmon resonance detection. In this work, MIP nanoparticles were produced by a solid-phase synthesis method where glass beads were used as solid support for the immobilization of the target AdVs, while a mixture of monomers, namely *N*-isopropylacrylamide, acrylic acid, *N,N′*-methylenebisacrylamide, *N-tert*-butylacrylamide, and *N*-(3-aminopropyl) methacrylamide hydrochloride, was used for polymerization. The recognition of AdVs was studied in a concentration range of 0.01–20 pmol L^{-1}, and a LOD of 0.02 pmol L^{-1} was obtained (Altintas et al., 2015). A different synthetic strategy for AdVs capture was proposed by using surface-imprinted core-shell particles. The material was obtained by immobilizing the viruses on the surface of micrometer silica particles, following by co-polymerization of selected organosilanes and removal of the template viruses (Gast et al., 2018) (Fig. 3). To prevent unspecific binding, a protein, bovine serum albumin, was used as blocking agent. This sol-gel imprinting method yielded excellent binding affinity, selectivity, and regeneration ability (Gast et al., 2018). Moreover, the amount of bound virus was determined by quantitative PCR, both during virus imprinting and rebinding experiments, revealing its value considering the absence of any prior DNA isolation steps (Gast et al., 2018). A later expansion of this MIP methodology was presented by Gast et al. (2020) by combining it with fluorescence labeling. In this work, it was possible to visualize individual viruses attached to the developed imprinted particles by super-resolution microscopies (Gast et al., 2020).

4.4. Japanese encephalitis virus

Japanese encephalitis virus (JEV) is a positive sense single-strand RNA mosquito-borne flavivirus covered with a viral capsid. This virus causes Japanese encephalitis, a viral encephalitis that affects thousands of people every year, mainly in the Asia Pacific region. Thus, there is a great need for quick and low-cost detection methods for this virus (Ganeshpurkar et al., 2018; Strikas et al., 2018).

In the work by Feng et al. (2018), fluorescence detection of JEV was demonstrated by resorting to surface molecular imprinting on silica microspheres modified with a fluorescent dye, dansyl chloride. The developed MIPs, prepared with (3-aminopropyl) triethoxysilane (APTES) and tetraethyl orthosilicate (TEOS), showed to selectively recognize JEV, by fluorescence quenching, in the presence of hepatitis A virus (HAV), simian virus 40 and rabies virus. Moreover, the method proved to be sensitive, giving a response within 55 min, and with a LOD

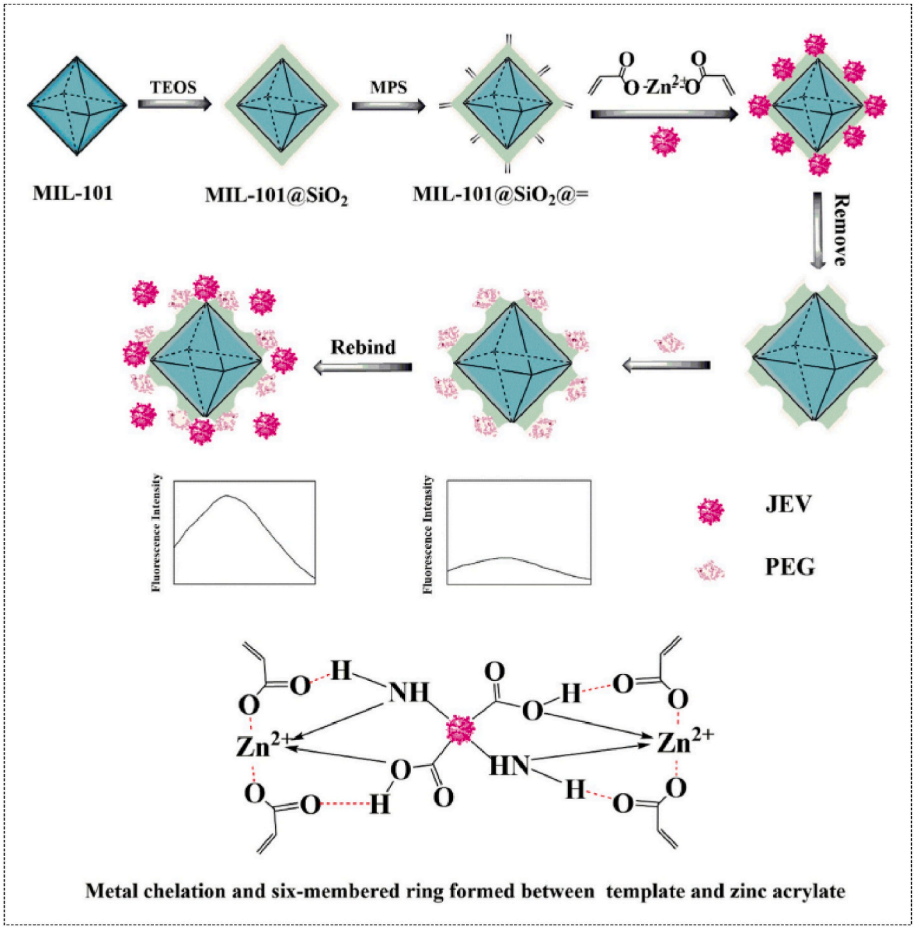

Fig. 4. Scheme of JEV imprinting on the surface of a MOF material, using zinc acrylate as functional monomer and PEG as passivating agent, and the detection principle. Copyright 2020, Elsevier, Reproduced with permission (Yang et al., 2020).

in the picomolar range (Feng et al., 2018). Similarly, Liang et al. (2016) developed a fluorescent sensor based on a MIP layer anchored on the surface of fluorescent silica microspheres (Liang et al., 2016). In this study, JEV was detected based on fluorescence resonance energy transfer between the virus as energy donor and the fluorescent dye (pyrene-1-carboxaldehyde) as energy acceptor. Thus, an enhancement of fluorescence intensity occurred proportionally to the concentration of the virus in the range of 24–960 pmol L^{-1}. The LOD was determined to be 9.6 pmol L^{-1}, and the selectivity was demonstrated when testing other viruses, namely HAV, leprosy virus and rabies virus (Liang et al., 2016). Taking advantage of silica microparticles with a magnetic core, Luo et al. (2019a) developed a magnetic surface molecularly imprinted-resonance light scattering (RLS) sensor for rapid and highly sensitive detection of JEV (Luo et al., 2019a). The capture of JEV by the imprinted Fe_3O_4@SiO_2 microspheres resulted in an increase of the RLS intensity, with a response time within 20 min, and LOD of 1.3 pmol L^{-1}, allowing rapid and sensitive detection of JEV in practical applications. Regarding the selectivity, the sensor demonstrated a selective response to JEV when other viruses were evaluated (HAV, dimensionally different rabies virus, simian vacuolating virus 40) (Luo et al., 2019a). A different fluorescent sensor based on a metal-organic framework (MOF) has also been proposed (Yang et al., 2020). The MOF material (MIL-101) was coated with silica and further vinyl-functionalized to enable the imprinting of JEV using zinc acrylate as functional monomer and EGDMA as crosslinker (Fig. 4). Moreover, polyethylene glycol (PEG) was used as blocking agent. In the presence of JEV the intensity of the fluorescence signal increased linearly in a wide range of concentrations (50 pmol L^{-1} to 1400 pmol L^{-1}) within 20 min, also presenting a good selectivity and a low LOD of 13 pmol L^{-1}. In addition, the MIP particles were selective to JEV when HAV, rabies, and leprosy viruses were tested (Yang et al., 2020).

4.5. *Influenza virus*

Influenza virus belongs to the *Orthomyxoviridae* family and can be divided in three subtypes A, B and C, which have similar structure but different antigenic properties. Influenza viruses have roughly a spherical shape with a size of about 80–120 nm and contain a single-stranded negative-sense segmented RNA genome. The infection by influenza A virus (IAV), which is generally found in humans, is most common and severe. This is a highly contagious airborne disease, and the symptoms range from mild fatigue to respiratory failure and death. This disease spreads rapidly and greatly affects the human population globally within a short period of time (Dangi and Jain, 2012).

In the work by Randriantsilefisoa et al. (2020), the optical properties of gold nanoparticles and the high swelling capacity of polyol-based hydrogels were used to form a nanocomposite of both that changes its colour and shrinkage in the presence of IAV (Randriantsilefisoa et al., 2020). The hydrogel was formed by click chemistry using functional dendritic polyglycerol cyclooctyne and polyethylene glycol diazide while sialic acids provided the specific and high binding affinity to the hemagglutinin on IAV. Thus, the template IAV (strain H3N2) mixed with gold nanoparticles functionalized with sialic acids were added to the hydrogel precursor mixture. The responsive imprinted hydrogel produced an optical and mechanical response upon removal and rebound of the IAV (Randriantsilefisoa et al., 2020). Different MIPs, based on

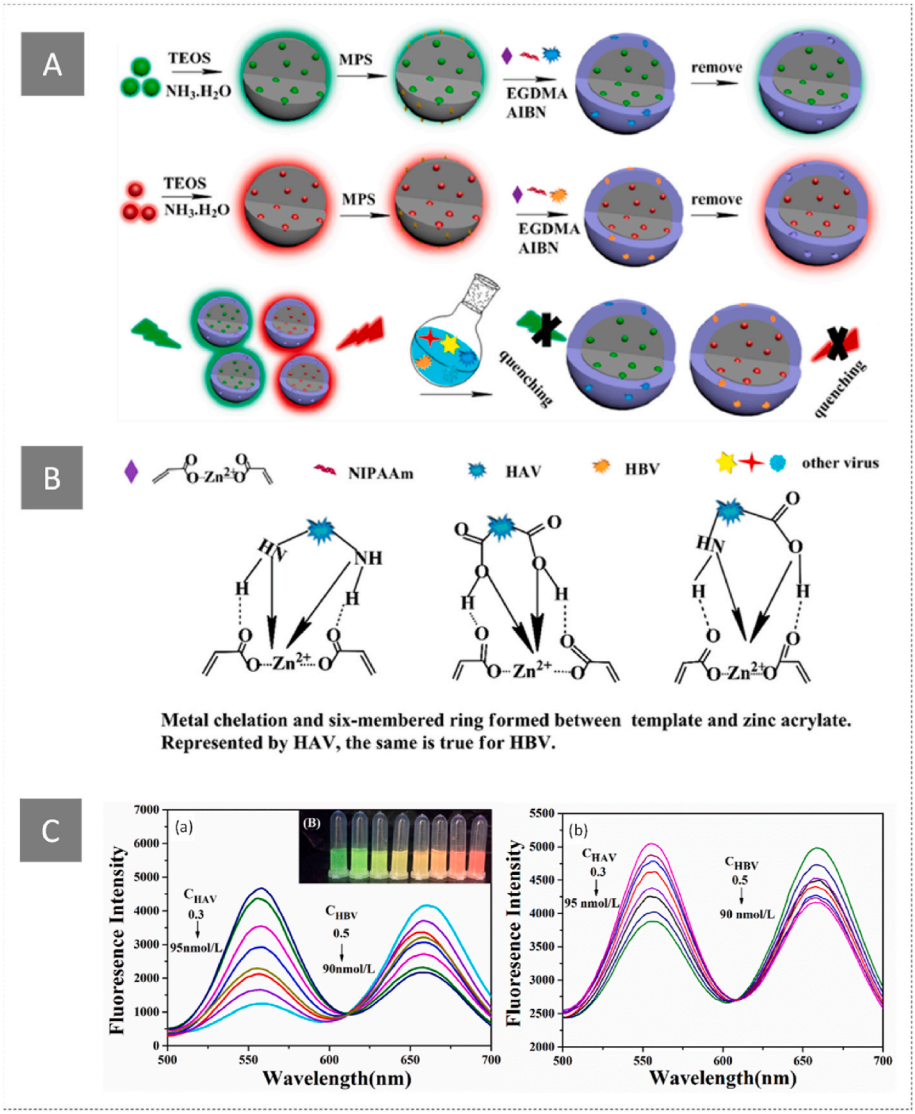

Fig. 5. Schematic illustration of MIPs fluorescence sensor (A), the metal chelation and six-membered ring formed between the template and zinc acrylate (B), effect of indicated concentrations of HAV and HBV on fluorescence intensity of MIPs (a) with an inset of fluorescence images of hybrid MIPs solutions under a 365 nm UV lamp, and the respective effect on the fluorescence intensity of NIPs (b) (C). Copyright 2019, American Chemical Society, Reproduced with permission (Luo et al., 2019b).

acrylamide, methyl methacrylate, methacrylic acid and *N*-vinylpyrrolidone, developed for the pathogenic strain H5N1 have been studied and optimized both by bulk imprinting suspension copolymerization to form polymer beads and by surface imprinting using a virus stamping and forming a thin-film. These MIPs recognize IAV, being, in this way, a viable method for the detection of the virus (Sangma et al., 2017). Another interesting work was developed by Wangchareansak et al. (2013), which applied a molecular imprinting strategy as a screening protocol for different influenza A subtypes (H5N1, H5N3, H1N1, H1N3 and H6N1), creating MIPs for each subtype and evaluating sensor characteristics on a QCM (Wangchareansak et al., 2013). The sensors were prepared on gold electrodes of a dual-electrode QCM by spin-coating the pre-polymer, consisting of acrylamide, methacrylic acid, methyl methacrylate and *N*-vinylpyrrolidone, and polymerizing the mixture in the presence of a stamp coated with the template virus. The sensor showed to be sensitive, leading to LODs as low as 10^5 particles mL^{-1}, and selective, allowing for virus subtype characterization and rapid screening (Wangchareansak et al., 2013). This imprinting methodology was later used to screen molecular probes, of different size, shape and binding affinities, which could bind to the virus (H5N1) and induce a conformational change (Wangchareansak et al., 2014). The method was successful in differentiating between induced conformational effects arising from high and low affinity ligands because the MIP binding was proportionally affected in comparison to the recognition of unmodified virus. These results suggest a very interesting application of MIPs to study novel inhibitors and their mode of action (Wangchareansak et al., 2014). Despite surface imprinting by stamping coupled to QCM has been highly explored for various IAV subtypes (Lieberzeit et al., 2011; Wangchareansak et al., 2013), another strategy has been also advanced through the synthesis of granular MIPs by precipitation polymerization (Sukjee et al., 2017). Four monomers, namely acrylamide, methacrylic acid, methyl methacrylate and *N*-vinylpyrrolidone, were used to prepare the MIP. The method was considered low-cost and easy as the MIPs can be produced in large quantities, while the recognition ability of the MIP was estimated based on indirect agglutination test and also on QCM, the latest demonstrating a better analytical performance (Sukjee et al., 2017).

4.6. Hepatitis virus

HAV is a non-enveloped single-stranded positive-sense RNA virus, with a size of 7.5 kb and a diameter of 27 nm, a member of the *Picornaviridae* family that is spread by the faecal-oral route, but the site of virus replication is the liver. HAV causes the hepatitis A disease, which is an acute inflammatory condition and is characterized by several symptoms, including anorexia, fatigue, weight loss and jaundice.

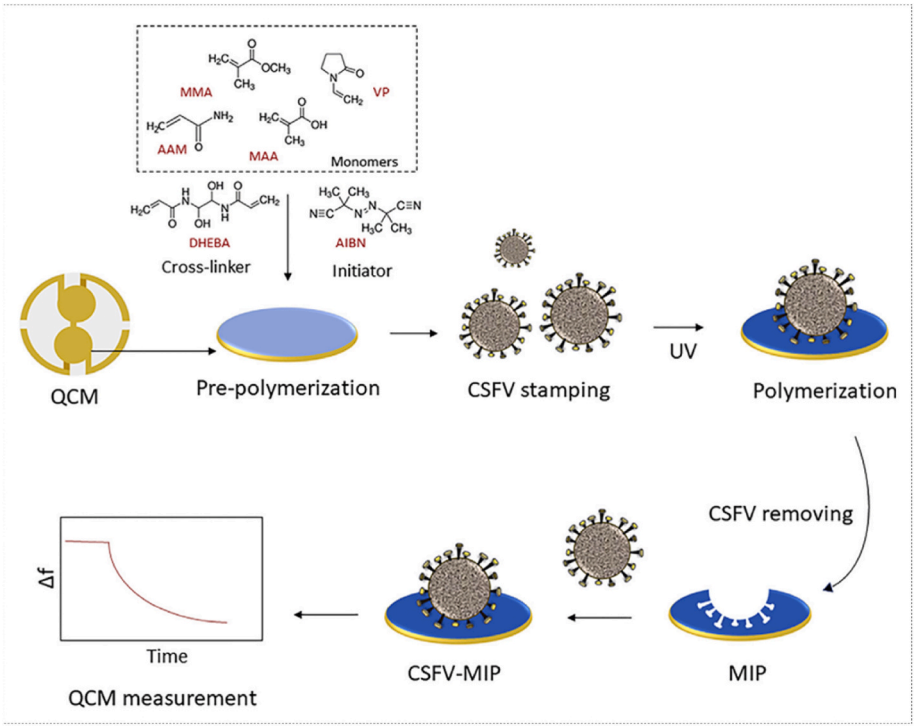

Fig. 6. Schematic representation of the MIP prepared for CSFV, using several monomers (acrylamide – AAM; methacrylic acid – MAA; methyl methacrylate – MMA; *N*-vinylpyrrolidone – VP), the crosslinker (dihydroxyethylene-bisacrylamide – DHEBA) and the initiator of the polymerization (2,2′-azobis(isobutyronitrile) – AIBN). The surface-imprinted polymer enabled to develop a QCM-based sensor. Copyright 2020, Reproduced under the terms and conditions of the Creative Commons Attribution BY-NC-ND 4.0 License (Klangprapan et al., 2020).

However, the symptoms of the disease are heterogeneous depending on the age of infection, ranging from silent inflections mainly in young children to classical hepatitis in older age groups, and fatal courses of the disease also occur (Averhoff et al., 2015; Dotzauer, 2008; Gupta, 2018).

The MIP technology has also been profiting from the use of stimuli-responsive polymers. In a work by Liu et al. (2017), the use of a thermosensitive polymer made of *N*-isopropylacrylamide enabled the development of an imprinted polymer on the surface of silica particles as support material for recognition of HAV (Liu et al., 2017). The specific capture of the virus occurred at 40 °C and the release at 20 °C, *i.e.*, at the lower temperature the polymer swelled and at the higher one the polymer shrinked. The sensor performance was followed by RLS intensity, which increased upon HAV detection. The sensor showed to be selective when tested in the presence of interfering viruses, namely hepatitis B virus (HBV), rabies virus and JEV, and demonstrated a very good LOD of 1.1 pmol L^{-1} (Liu et al., 2017). Likewise, Luo et al. (2020) applied MIP nanoprobes to the selective determination of HAV through RLS technique, obtaining a lower LOD of 0.1 pmol L^{-1} and a linear concentration range of 0.02–2.0 nmol L^{-1} (Luo et al., 2020). In this work, the pH-sensitive imprinted polymer dimethylaminoethyl methacrylate was prepared (swelling with pH decrease) on the surface of a MOF support. Moreover, the pH-responsive MOF nanocomposite presented a selective response, which indicated its potential ability to determine HAV in real applications (Luo et al., 2020). An enhancement of RLS intensity has been also employed in other works where MIPs were produced by surface imprinting of silica or magnetic particles (Yang et al., 2017; Zhang et al., 2018). In both these works, the self-polymerization ability of dopamine, creating a polydopamine-based MIP, was studied as a biomimetic mussel-inspired approach, resulting in selective and sensitive sensors with LODs in the picomolar range (Yang et al., 2017; Zhang et al., 2018). In a different type of strategy, Luo et al. (2018) created a fluorescence MIP made from CdTe/CdS quantum dot (QD)-based silica nanoparticles using a sol-gel process. The HAV was selectively captured by the imprinted polymer layer, and the fluorescent quenching of the QDs was analysed within 20 min. Moreover, a linear range between 0.2 and 1.4 nmol L^{-1} and a LOD of 88 pmol L^{-1} were obtained (Luo et al., 2018).

Regarding the HBV, its genome is a partially double-stranded circular DNA of about 3.2 kb pairs that belong to the *Hepadnaviridae* family (Averhoff et al., 2015; Dotzauer, 2008; Gupta, 2018). HBV is responsible for the potentially life-threatening liver infection: hepatitis B. Hepatitis B is a major global health problem that leads to a wide spectrum of liver diseases, ranging from acute to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (liver cancer) (Liang, 2009).

Some studies combine the use of MIPs with other recognition probes. An approach developed to detect HBV employed a dual-recognition method based on MIPs and aptamers in a sandwich RLS sensor (Chen et al., 2021). The MIP targeting HBV was achieved by surface imprinting made of TEOS on carbon spheres as carriers, while the other probe was obtained by modification of silicon spheres with aptamers. The use of a second probe was meant to improve the specific recognition of HBV and to provide a second enhancement of the RLS intensity. With this MIP-HBV-aptamer sandwich, the sensor response demonstrated high sensitivity and good selectivity, with a LOD of 0.011 nmol L^{-1} (Chen et al., 2021).

There are also attempts to pursue the simultaneous detection of multiple viruses. In the work of Luo et al. (2019) the use of green and red coloured QDs enabled such multiplex analysis to detect both HAV and HBV (Luo et al., 2019b). The MIPs were synthesized using zinc acrylate and *N*-isopropylacrylamide as monomers on the surface of green and red emitting QDs coated with shells of silica (Fig. 5). The fluorescence sensor enabled simultaneous detection of HAV (LOD of 3.4 pmol L^{-1}) and HBV (LOD of 5.3 pmol L^{-1}) by a decrease in both emission peaks (Luo et al., 2019b).

4.7. Zika virus

Zika virus (ZIKV) is a positive sense, single-strand RNA virus with a genome size of approximately 11 kb. ZIKV is an arthropod-born virus (arbovirus) belonging to the *Flaviviridae* family. The transmission of ZIKV typically occurs through the bite of an infected female mosquito during its blood feeding, leading to the appearance of the ZIKV disease. It is estimated that most cases of this disease are asymptomatic. However, it has recently caused outbreaks and epidemics, being associated

with severe clinical manifestations and congenital malformations (Zanluca and dos Santos, 2016).

Ricotta et al. (2019) developed a potential point-of-care diagnostic system using a chip-based potentiometric sensor incorporating the molecular imprinting technology (Ricotta et al., 2019). The imprinting process was designed by the co-adsorption of the ZIKV and a self-assembled monolayer (SAM) of hydroxyl-terminated alkanethiols on a gold-coated chip. This system was able to detect 10^{-1} PFU mL^{-1} ZIKV in a buffered solution, and 10 PFU mL^{-1} ZIKV in samples of human saliva containing clinical viral loads, thus with great prospects for rapid and accurate screening of ZIKV (Ricotta et al., 2019).

4.8. Poliovirus

Poliovirus is a non-enveloped, positive-sense, single-stranded RNA virus, member of the family *Picornaviridae*, which causes poliomyelitis. Poliovirus is mainly transmitted by the faecal-oral route and replicates in the pharynx and lower intestinal tract. Poliovirus is entirely asymptomatic in 90% of individuals, however, in fewer than 1% of cases the virus enters the central nervous system, infecting and destroying motor neurons, leading to muscle weakness and acute flaccid paralysis (Clatworthy, 2014; Garg and Karst, 2016; Troy and Maldonado, 2012).

Wang et al. (2010) applied surface molecular imprinting using SAMs on a gold-coated silicon chip (Wang et al., 2010). The design consisted in co-adsorbing the template with hydroxyl-terminated alkanethiol molecules on the metal surface allowing them to form the SAM. After template removal, the imprinted cavities are left behind on the sensor surface. As demonstrated in a similar approach for ZIKV (Ricotta et al., 2019), this strategy was successfully applied in the potentiometric detection of poliovirus, with no cross-reactivity to AdV (Wang et al., 2010).

4.9. Classical swine fever virus

Classical swine fever virus (CSFV) of 40-60 nm in diameter, are enveloped virus of icosahedral symmetry of the family *Flaviviridae,* and the viral genome is a single-stranded positive-sense RNA of approximately 12.3 kb. CSFV infection leads to a breakdown of the immune system accompanied by a pro-inflammatory response. This disease is associated with many symptoms, including severe lymphopenia and lymphocyte apoptosis, thrombocytopenia, platelet aggregation, bone marrow depletion, thymus atrophy, and thymocyte apoptosis. Since classical swine fever is considered one of the most relevant re-emergent fatal viral diseases in swine, there is an economic necessity that urges rapid early detection of this virus (Ganges et al., 2020). A MIP recognition element has been developed to detect this virus, relying on CSFV stamping that was pressed on a pre-polymer of acrylamide, methacrylic acid, methyl methacrylate and *N*-vinylpyrrolidone, spin-coated on QCM gold electrodes (Fig. 6). This sensor selectively binds CSFV, with a LOD of 1.7 μg mL^{-1} (Klangprapan et al., 2020).

4.10. Foot and mouth disease virus

Foot and mouth disease virus (FMDV) is a small, icosahedral, non-enveloped, single-stranded, positive-sense RNA virus of approximately 8.3 kb, that belongs to the family *Picornaviridae*. This highly infectious pathogen causes serious debilitating disease, the foot and mouth disease, in cattle and other livestock and wildlife, and is thus considered a relevant veterinary pathogen (Malik et al., 2017; Yan et al., 2017).

An electrochemical polymerization of the oxidized o-aminophenol film with FMDV serotype O on a gold screen-printed electrode deployed a new recognition system for this virus (Hussein et al., 2019). The biosensor showed a high selectivity to FMDV serotype O in comparison to serotype A, SAT-2, inactivated serotype O, and lumpy skin disease virus, and a LOD of around 2 ng mL^{-1}. Besides, the fast response (5 min) and the reusability of the biosensor, presents a promising, affordable, and portable tool that could be used in the field (Hussein et al., 2019).

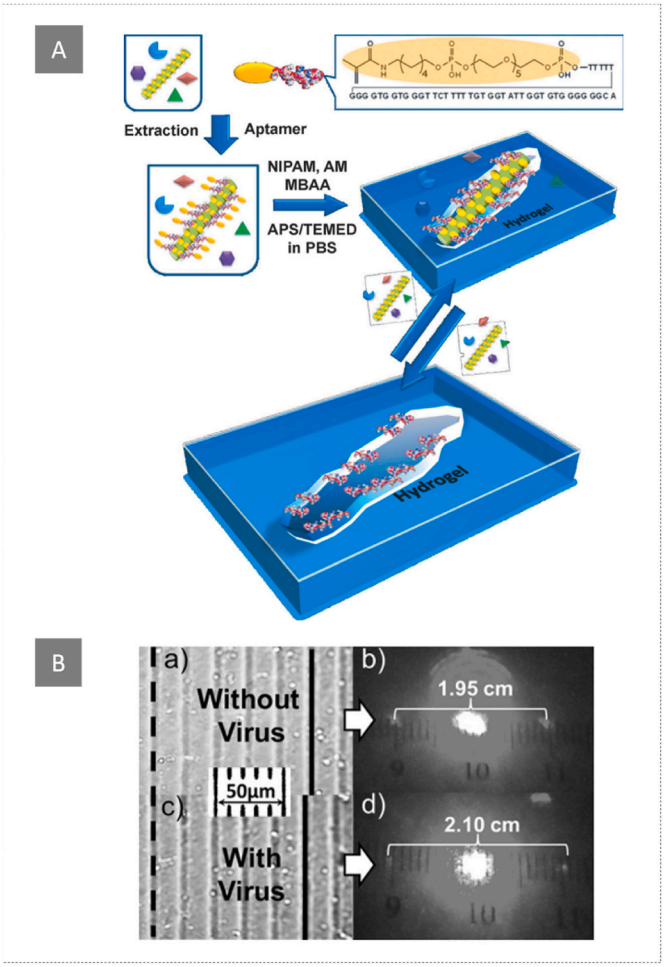

Fig. 7. Scheme of the bioimprinting process to obtain virus responsive super-aptamer hydrogels, using polymerizable specific aptamers copolymerized with *N*-isopropylacrylamide – NIPAM and acrylamide – AM as monomers, *N,N′*-methylenebisacrylamide – MBAA as crosslinker, and ammonium persulfate – APS and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) for initiation of the polymerization in phosphate-buffered saline – PBS (A); optical microscopy images of the responsive MIP for ASPV and the corresponding laser diffraction patterns without virus (a, b) and in the presence of ASPV (c, d) (B). Copyright 2014, Wiley-VCH, Reproduced with permission (Bai and Spivak 2014).

4.11. Other viruses

Cumbo et al. (2013) described a synthetic strategy to produce organic/inorganic nanoparticulate hybrids containing virus imprints on the surface. For that, virus-imprinted particles were produced for plant viruses as models, namely tomato bushy stunt virus (TBSV) and turnip yellow mosaic virus (TYMV) (Cumbo et al., 2013). The viruses were first bound on the surface of silica nanoparticles, followed by incubation with a mixture of organosilanes and subsequent polycondensation to grow an organosilica (silsesquioxane) recognition layer, which after virus removal display the free imprints. The TBSV and TYMV imprinted silica nanoparticles displayed a remarkable selectivity and affinity for both viruses, recognizing them in water at concentrations down to the picomolar range (Cumbo et al., 2013). A similar approach was pursued but using virus-like particles as a safe substitute for the imprinting of human pathogenic Noroviruses, and the MIP showed also an excellent affinity in the picomolar range (Sykora et al., 2015).

A novel double imprinting method has been proposed for the apple stem pitting virus (ASPV) (Bai and Spivak, 2014). The

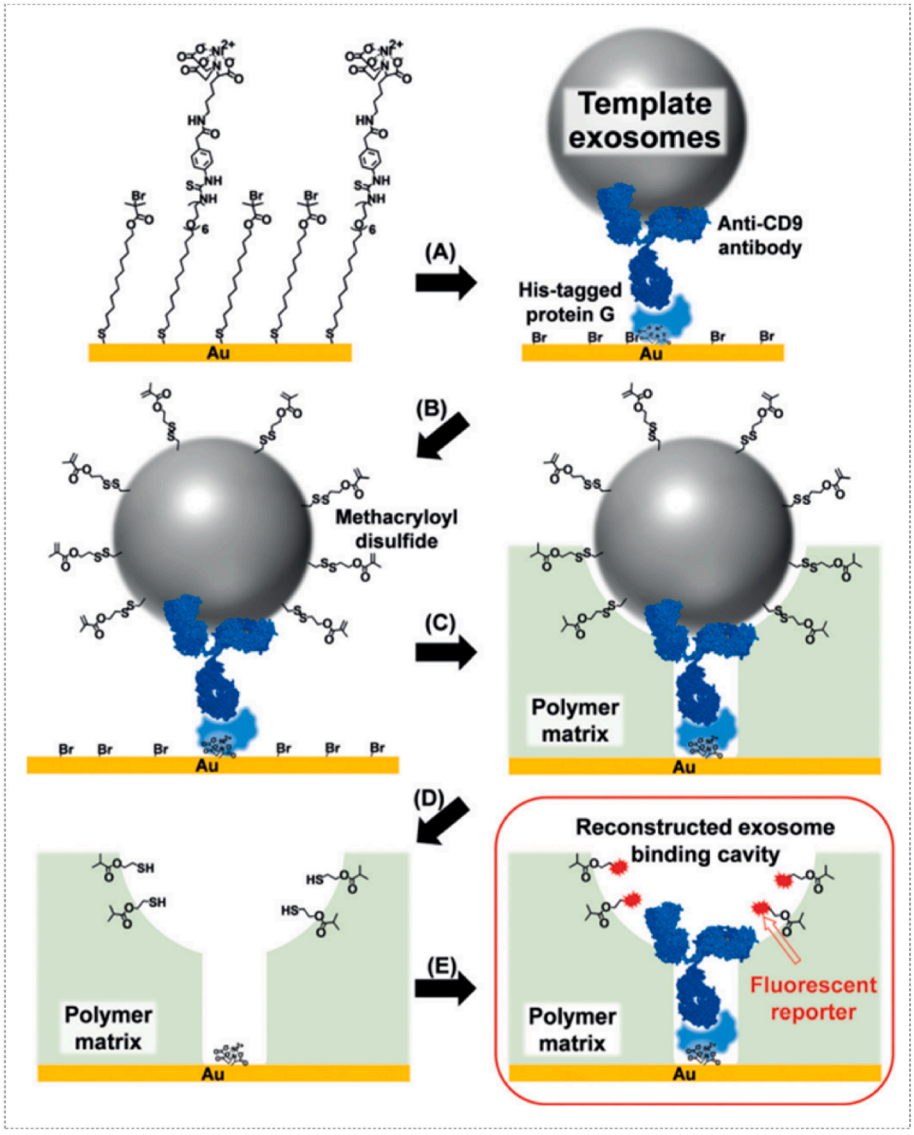

Fig. 8. Schematic illustration of the several steps required to prepare the binding cavities for EVs by imprinting and post-imprinting in-cavity modifications: (A) oriented immobilization of template EVs; (B) anchoring of methacryloyl disulfide groups on the template EVs; (C) surface-initiated atom transfer radical polymerization; (D) removal of the template and antibody; (E) reconstruction of the binding cavities and in-cavity specific introduction of fluorescent reporter molecules. Copyright 2019, Wiley-VCH, Reproduced with permission (Mori et al., 2019).

ASPV-bioimprinted hydrogel was micromoulded into a diffraction-grating sensor to give a MIP Gel Laser Diffraction Sensor (Fig. 7A). A change in diffraction upon virus recognition was accurately measured by a laser transmission apparatus. Owing to the multivalent interactions of polymer-bound aptamers with ASPV, a visible volume-shrinking occurred upon virus rebinding, while in the control hydrogels the aptamers were randomly placed and there was no response. The decrease of the hydrogel grating period expands the distance between the two projected laser points when evaluated by laser diffraction (Fig. 7B). Also, the incorporation of the polymerizable virus-specific aptamers in the bioimprinting process, enabled the use of an impure virus extract as the source of the template. The ASPV-specific response could be seen at concentrations as low as 10 ng mL^{-1} (Bai and Spivak, 2014).

5. Recognition of other nanoscale biological targets

Current research endeavors to understand how cells communicate through membrane vesicles, called extracellular vesicles (EVs), have been unravelling their crucial biological functions, including in disease. Moreover, there are a number of resemblances in both structural and functional aspects between EVs and viruses. Besides virus-infected cells continuously producing EVs that contain viral materials, there are many limitations is separating EVs from some virions (Hoen et al., 2016). EVs are nanovesicles produced by most cell types, which contain a specific composition of nucleic acids, proteins and lipids, mediating intercellular communication between different cell types in the body, and thus affecting normal and pathological conditions (Torrano et al., 2016). Because EVs have different cargo depending on the physiology and cell of origin, they have been extensively studied as novel biomarkers, therapeutic targets, and drug/gene delivery vectors. There is a huge potential in future clinical application of EVs as they may contribute to the development of minimally invasive diagnostics and advanced therapies (Tkach and Théry, 2016). However, current isolation and detection methods are time-consuming and require the use of sequential techniques because many of them enable only the enrichment and not the separation of a particular subtype of EV (Doyle and Wang, 2019).

An interesting work reported a novel designed molecular imprinting method for creating binding cavities capable of recognizing intact EVs, followed by post-imprinting in-cavity modifications to introduce antibodies and fluorescent reporter molecules inside the binding cavities (Fig. 8) (Mori et al., 2019). The template EVs were recognized by antibodies already immobilized on a gold-coated substrate, which was also modified with the 2-bromoisobutyril initiator for atom transfer radical polymerization (ATRP). Then, methacryloyl disulfide groups were anchored on the surface of the EVs and the surface-initiated ATRP was

conducted using 2-methacryloyloxyethyl phosphorylcholine as monomer (Mori et al., 2019). After removal of the template, the cavities underwent a post-chemical processing to introduce a thiol-reactive fluorescent dye as reporter and an antibody through oriented-conjugation. This platform demonstrated to provide a rapid and highly sensitive fluorescence detection of EVs, making possible the differentiation of cancer-secreted exosomes from normal ones without time-consuming pre-treatments (Mori et al., 2019). A similar strategy of using an antibody-conjugated signalling MIP nanocavity has been presented (Takeuchi et al., 2020). In this study, the nanocavities were fabricated by silica nanoparticles-based dynamic moulding. This method presents the advantage of controlling the homogeneity and the size of the nanocavity by using nanoparticles of specific sizes. Again, this method proved to be a powerful tool for rapidly sensing intact small EVs in biological fluids, as demonstrated by the successful analysis of tears to detect cancer-related EVs. Interestingly, the dynamic moulding can be sized according to the application, as well as the antibodies used (Takeuchi et al., 2020).

6. Conclusions and future perspectives

Improving the outcomes of diseases by making new diagnostic technologies available is crucial. Among them, more affordable methods allied to portability are of particular interest. In this context, MIPs are biomimetic recognition materials that present remarkable features with practical application value. Much progress has been achieved regarding the imprinting of whole or intact templates in the nanoscale size range. Most examples in the literature rely on surface imprinting to improve the binding kinetics and capacity. The summarized achievements, in terms of critical properties that define the success of future commercial diagnostics and point-of-care applications, *i.e.*, selectivity, sensitivity, robustness, low-cost and reusability, demonstrated by the developed MIP-based materials are very encouraging. Moreover, some developed MIPs already present high sensitivity and specificity, including the ability to discern virus subtypes and differentiate EVs from healthy and diseased conditions. Nonetheless, the field still faces many challenges related to the intrinsic heterogeneity of the EVs and mutability of viruses to be analysed. In addition, under real conditions, these MIP-based sensing devices have to show precise affinity and selectivity when applied in biofluids or other complex samples. Further potential limitations pertain to the need for up-scaling the processes and select more eco-friendly and biocompatible materials. In addition, the use of large biological targets for imprinting poses challenges related to their intrinsic heterogeneity, difficulties in obtaining pure templates, less specific recognition binding sites and low reproducibility for mass production. Particularly for viruses, routine handling of pathogenic species should be ideally avoidable. In the perspective of creating biomimetic products in this context, emerging MIPs can be boosted by using virus-like particles or synthetic polymer particles and lipid nanovesicles, considering the ability to tailor some of the diversity found in structural features like shapes, sizes, and functional surface groups of natural targets. The use of nanomaterials and nanocomposites has been also improving the analytical performance of MIP sensors. The future certainly holds very interesting opportunities to design novel MIPs both to gain insight on biomolecular interactions and expand the use of detection devices that can improve our ability to prevent diseases and improve global health.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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